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Microcystin accumulation in fish muscle tissue: Exploring the safety of fish consumption in several Michigan water bodies

Heather Snyder

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

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Dedication

I would like to dedicate my thesis to my advisor Megan Woller-Skar, my lab mate Jacob Gaskill, and my family, without whom this project would not have been possible.

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I would like to thank my advisor Dr. Megan Woller-Skar who has given me great career and research advice. I would also like to thank the rest of my committee, Dr. Mark Luttenton and Joseph Bohr for their contributions to my work. I would also like to thank Dr.Gregory Boyer for opening his lab to me for my research, as well as for his much needed chemistry advice. Additionally, I would like to thank Star Santiago, Diane Laughlin, and all the student workers in the prep area for their help in acquiring lab supplies.

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Abstract

A variety of anthropogenic factors, including global climate change and eutrophication, are causing increases in cyanobacterial abundance. Increased prevalence of cyanobacteria can be detrimental, as some genera of cyanobacteria have the ability to produce toxic secondary metabolites known as cyanotoxins. The cyanotoxin microcystin- a hepatotoxin, is the most ubiquitous and toxic cyanotoxin in freshwater ecosystems, and has been quantified in a number of aquatic organisms. Microcystin was quantified in 8 fish species from St. Mary's River in June, Saginaw Bay in September, and from Stony Creek Lake in October in 2014 by the Michigan Department of Environmental Quality and the Michigan Department of Natural Resources and findings were compared to current microcystin consumption advisories in order to determine what quantities of fish could be safely consumed. Total microcystin was determined in each sample with enzyme linked immuno sorbent assays and the total quantity of the microcystin variant microcystin-LR (the most toxic variant of microcystin) was determined with Liquid Chromatography coupled with tandem Mass Spectrometry. Results indicated that fish from these water bodies were well within established consumption limits. However, although results indicate that consumption need not be limited, findings from the present study should not be extrapolated beyond the specific time frames and locations studied, due to variability in environmental microcystin concentrations. Therefore, further research spanning long time frames and locations needs to be done in order to confidently determine safe fish consumption limits in regards to microcystin.

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Chapter 1: Introduction to Research Question

1.1 Introduction

Increasing ambient temperatures due to climate change, coupled with anthropogenic eutrophication of water bodies is causing an increase in cyanobacterial abundance. Cyanobacteria, a division of algae, can grow in rapid bursts, causing visible accumulation of algae known as "bloom events" (see Ch. 3.1 Cyanobacteria). This excessive growth of algae can have a suite of negative effects on both the ecosystem and on individual organismal health. Organismal health is negatively impacted by the ability of some cyanobacteria to produce secondary metabolites known as cyanotoxins. The most ubiquitous of these cyanotoxins is microcystin. Microcystin negatively affects organisms by binding to the enzymes PP1 and PP2A (see Ch 3. Microcystin inhibition of protein phosphatases). The irreversible binding of toxin to these enzymes can cause a wide variety of negative health effects including cancer and death (see Ch3. Adverse health effects due to MC exposure).

Fish can be exposed to microcystin either through exposure to water or consumption of aquatic organisms (see Ch 3. Accumulation of microcystin in fish). This exposure can lead to accumulation of microcystin in the organism, which then has the potential to be a vector of the toxin to humans who consume them (see Ch 3.Human Exposure to Microcystin). The World Health Organization (WHO) has adopted a consumption advisory level of 0.04 µg MC/ kg body weight in an effort to address these concerns. This advisory limit, although helpful from a scientific perspective, is not accessible to the general population who have no way to determine the quantity of this toxin in consumable fish. This disconnect has prompted a research effort aimed at quantifying cyanotoxins, microcystin, specifically, in various aquatic organisms around the globe. In particular, fish are often considered due to their dietary importance. Unfortunately,

this sizeable research effort has limited application outside the location and time of the current study because of the large variability of microcystin production and accumulation. For these reasons, additional research is paramount to confidently determine what quantities of certain fish species are safe for human consumption.

1.2 Purpose

The primary purpose of this thesis was to quantify microcystin concentrations in fish from Saginaw Bay, Stony Creek Lake, and St. Mary's River located in Michigan, in order to determine if concentrations were above advisable consumption limits (Ch. 2). In addition, an extensive literature review was done to assess factors contributing to the field of research regarding quantification of microcystin in aquatic organisms (Ch. 3). Specifically, an overview of current literature regarding accumulation of microcystin in various organisms, along with an overview of the perceived health risks posed due to microcystin are covered in detail (3.1 Extensive Literature Review). Furthermore, a critical comparison of the field's two leading chemical analyses is included in order to highlight current analytical strengths and weaknesses (Ch. 4 Methods for Quantifying Microcystin).

1.3 Scope

The scope of the present study is limited to the specific time frames and locations from which fish samples were collected. As iterated throughout the study, conclusions drawn from data cannot be confidently extrapolated outside the confines of the study due to spatial and temporal variability in microcystin production. Since samples were only collected on one date from each location, this study is restricted from determining definitive consumption advisories

for even the locations observed. Therefore, the results from this study are best suited as pilot data intended for determining the scale of necessary future research.

1.4 Assumptions

From a sampling perspective, this study assumes that fish populations were randomly sampled and by extension are reflective of the population as a whole. From an analytical perspective, this study assumes that microcystin accumulation -is homogenous throughout the fish muscle- because only a portion of the entire fish was analyzed. Finally, it was assumed that the concentrations of microcystin determined by chemical analysis were reflective of the samples' true microcystin concentration. This assumption is challenged by literature that states potentially high false positive rates for ELISA analyses due to cross reactivities with the antibody coating in each well (See 4.1.3 ELISA for detailed description). The present study addresses this concern of false positives by determining the threshold of false positives through analysis of negative controls with ELISA (see Ch 2.3.4 ELISA QA/QC for more details). Although clean fish were used as negative controls to determine the rate of false positives, only one species of fish (channel catfish) was used when assessing the negative control rate, and therefore, it was assumed that all species of fish had equal thresholds for false positives.

The assumption that the concentration of microcystin detected by the analyses is indicative of true microcystin concentration levels is further contested by microcystin's affinity to plastic. The unavoidable use of plastic in some lab equipment (primarily the use of plastic micropipette tips) suggests that some toxin present in fish does not get analyzed, (due to being bound to the lab equipment) and is subsequently not accounted for in chemical analyses. To address this concern, this study limits the use of pipette tips, thereby limiting the potential loss of toxin (See Step 16 in 4.2.1 Detailed ELISA Extraction Protocol). Ultimately, although long

standing technical concerns regarding the last study assumption are present, it is believed that the measures taken to address the concerns eliminate the risk of significant toxin loss.

1.5 Significance

The logistical constraints of this project limits the broad application of this project to aquatic systems; however the implications towards which this study builds are significant. Specifically, although this project lacks the data necessary to make definitive statements regarding the risk of microcystin consumption in fish, the larger body of work that this study prefaces will have important health implications. A long term study spanning more seasons and bodies of water, such as the one suggested as a conclusion of this body of work, would likely have the ability to determine the risk of fish consumption in regards to microcystin consumption. Considering the increase in cyanobacteria blooms, this study warrants execution. Therefore, although this study does not provide a definitive conclusion regarding safe fish consumption, it is a necessary catalyst for a larger research project capable of making definitive claims.

1.6 Definitions

Oligotrophic: Term used in reference to lakes with low levels of nutrients, and by extension lower levels of primary productivity.

Eutrophication: A term used to describe increasing nutrient levels in aquatic water bodies. Increased nutrient levels are often coupled with high primary productivity.

Bloom Event: In the context of this thesis, bloom event refers to explosive growth in the number (or density) of cyanobacterial cells. This often (but not always) results in visible accumulation of cyanobacteria on the surface of the water.

Hepatotoxin: A toxin that primarily impairs liver function

Biodilution: Refers to a toxin decreasing in concentration with increases in trophic level

Bioaccumulation: Refers to the measurable accumulation of a substance from the surrounding environment into an organism.

Congener: In context of this thesis, refers to a variation, or type, of microcystin.

Matrix Effect: Refers to the cross reactivity of molecules in fish tissue to the antibody coatings in ELISA plates which can result in false positives.

1.7 Abbreviations

BW = Body Weight

DNR= Department of Natural Resourcs

ELISA= Enzyme Linked Immuno Sorbent Assay

LC-MS= Liquid Chromatography -Mass Spectrometry

LC-MS/MS= Liquid Chromatography - Mass Spectrometry/ Mass Spectrometry

MC= Microcystin

MC-LA= Microcystin-LA (Leucine and Alanine)

MC-LR= Microcystin- LR (Leucine and Arginine)

MDEQ= Michigan Department of Environmental Quality

MeOH= Methanol

NOAEL= No Observed Adverse Effects Limit

TDI= Tolerable Daily Intake

TN=Total Nitrogen

TP=Total Phosphorous

WHO= World Health Organization

Chapter 2: Manuscript

Title: Quantification of microcystin among various fish species in Michigan: Are fish consumption guidelines needed?

Running Head: Quantification of microcystin among Michigan fish species

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Key Words: microcystin, cyanobacteria toxins, fish consumption

2.1 Abstract

The frequency and intensity of aquatic cyanobacterial blooms are increasing due to climate change and anthropogenic eutrophication, resulting in an increase in cyanotoxins such as microcystin. Microcystin (MC) is harmful if ingested and increasing levels of the toxin are a cause for concern from a human health perspective. A total of 56 fish comprised of 8 species were collected by the Department of Environmental Quality from St. Mary's River and Stony Creek Lake and by the Department of Natural Resources from Saginaw Bay in Michigan. Fish muscle tissue was tested for total MC using enzyme linked immunosorbent assay (ELISA) and the concentration of microcystin-LR (MC-LR) was quantified with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). ELISA readings ranged from below the limit of detection (0.187 μg MC kg^{-1} fish tissue muscle) to 0.757 μg MC kg^{-1} fish tissue and all LC-MS/MS readings came in below the limit of detection (11 μg MC kg^{-1}). Values of MC were well below the acceptable daily intake limits proposed by Mulvenna et al. (2012) and Ibelings and Chorus (2007) in all water bodies and across all species. Although toxin concentrations were within acceptable limits, high variability in toxin concentrations suggests that more sampling over larger time scales would be necessary before confidently determining no need for consumption advisories concerning MC.

2.2 Introduction

Global climate change coupled with anthropogenic eutrophication has resulted in more frequent cyanobacterial blooms, which has led to an increased observation of the negative effects that accompany high cyanobacterial abundance (Huisman et al., 2005). Cyanobacteria are photosynthetic bacteria characterized by rapid bursts of growth referred to as "bloom events." Increasing cyanobacterial abundance is becoming a cause for concern since some genera possess the ability to produce toxic secondary metabolites known as cyanotoxins. Although there are a variety of cyanotoxins, the cyanotoxin microcystin (MC) present in freshwater systems, is of highest concern due to its abundance and toxicity (Figueiredo et al., 2004).

MC is a monocyclic heptapeptide (Schmidt et al., 2013) that negatively impacts liver function by covalently binding to phosphatases PP1 and PP2A (Goldberg et al., 1995). There are roughly 80 structural variations of MC that are differentiated by the types of amino acids at position 2 and 4 as well as the methylation state of the amino acids at the positions 3 and 7 (Trinchet et al., 2011). Each variant, or congener, of MC is depicted by the letters of the amino acids occupying positions 2 and 4. The most toxic congener is Microcystin- LR (MC-LR), which is characterized by the amino acids leucine and arginine (Gupta et al., 2003; Trinchet et al., 2011). MC-LR is generally a large fraction of the MC produced in areas where MC is a concern, however, the abundance and frequency of congeners varies among water bodies. Although all variants of MC are harmful, MC-LR is considered to have the highest potency and is therefore of primary concern to aquatic biologists (Gupta et al., 2003).

MC can accumulate in aquatic organisms that either feed on organisms contaminated with MC, or by being exposed to the toxin in the water column. An extensive amount of research has identified MC accumulation in a variety of freshwater aquatic species such as freshwater

shrimp (Song et al., 2007), snails (Song et al., 2007; Ozawa et al., 2013), bivalves (Chen and Xie, 2005; Wood et al., 2006; Song et al., 2007), and eels (Amrani et al., 2014). In addition, a wide variety of fish such as channel catfish, tilapia, goldfish, yellow perch, and many more (see Schmidt et al., 2013 for review) have been found to contain detectable levels of MC. Similarly, a large sampling effort by Poste et al., (2011) resulted in documented concentrations of MC in a wide variety of fish (see Poste et al. 2011 for complete list), including some species examined in this study (northern pike, white perch, white bass, and walleye). These aquatic organisms have the ability to depurate ingested toxin hindering the toxin from magnifying up the food web (Ibelings and Chorus, 2007). Although MC is not believed to biomagnify up food webs the literature is inconclusive in regards to which fish tend to accumulate the largest quantity of MC. For instance, research by Zhang et al. (2009) found a decrease in MC concentration with every increase in trophic level. Conversely, Xie et al., (2005) found an increase in MC concentration with every increase in trophic level. Agreeability between studies focused on quantifying MC is difficult because MC concentrations vary significantly across time and space. This phenomenon ultimately makes extrapolating results from one waterbody to another difficult, and therefore highlights the need for future studies, particularly on fish and other aquatic organisms routinely consumed by humans.

In order to buffer the negative health impacts of high MC exposure, research has aimed at providing a safe consumption limit of MC for humans. A study by Fawell et al., (1999) observed a "No Observed Adverse Effect Level" (NOAEL) of 40 µg of MC-LR per kg of body weight in mice. This NOAEL was divided by an uncertainty factor (UF) of 1000 yielding a Tolerable Daily Intake (TDI) value of 0.04 µg of MC-LR per kg of body weight for lifetime consumption and a TDI of 0.4 µg of MC-LR per kg of body weight for seasonal consumption (Ibelings and Chorus ,

2007). Although, this TDI has been adopted by the World Health Organization, other researchers have applied different uncertainty factors to the 40 µg of MC-LR per kg of body weight NOAEL resulting in different TDIs. Mulvenna et al. (2012) divided the 40 µg of MC-LR per kg of body weight NOAEL by an uncertainty factor of 200 resulting in a TDI of 0.2 µg of MC-LR per kg of body weight. Both of these calculated TDIs can be multiplied by the weight of the individual consuming contaminated products as well as by an additional allocation factor. These calculations result in an acceptable daily limit of MC-LR consumption, which can be directly compared to the concentration of MC-LR in an aquatic organism multiplied by the serving size consumed. Consumption advisories are commonly only reported for MC-LR and therefore many researchers report the sum concentration of all detected variants as MC-LR. Since MC-LR is considered to be the most harmful congener, assuming the total of all variants is MC-LR is considered a conservative practice. Using this model, several studies have quantified levels of MC-LR in fish above the recommended TDIs, suggesting that excessive exposure to MC through fish consumption is a concern for human health (Magalhaes et al., 2003; Peng et al., 2010; Xie et al., 2005; Poste et al., 2011).

MC exposure maybe harmful to humans if consumed above established safe consumption advisories. Exposure at acute and chronic toxicity levels of MC, can cause negative health effects including liver necrosis, tumor promotion, and death (Ibelings and Chorus, 2007). To date, only one known case of acute MC-LR poisoning has been recorded. In 1996, a multitude of patients at a hemodialysis center in Brazil began experiencing gastrointestinal distress, which led to mass casualties. An extensive examination by the Center for Disease Control identified the center's water source, which was found to be contaminated with high levels of MC-LR, to be the source of the casualties (Jochimsen et al., 1998). Additionally, although consumption of contaminated

fish tissue has not been explicitly linked to any cases of human mortality, several studies suspect the heightened rates of liver cancer in several Chinese fishing villages are the result of exposure to high levels of MC (Zhang et al., 2009; Chen et al., 2009).

Currently, two chemical analyses- liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and enzyme linked immuno sorbent assays (ELISA) - are routinely used for detecting MC in fish tissue. LC-MS/MS allows for the quantification of individual congeners, however, it is slower and requires advanced chemical instrumentation in comparison with ELISA. Additionally, LC-MS/MS has a higher limit of detection (LOD) (11µg/kg in the present study), making it less effective when analyzing samples with low quantities of toxin (McElhiney and Lawton, 2005). Conversely, ELISA has a low LOD (0.187 µk/kg in the present study), is relatively fast, requires very basic lab instruments, and is cheaper to run if the advanced equipment needed for LC-MS/MS is not already available. However, although this test allows for quantification of total toxin (including many MC variants and nodularin), it does not allow quantification of individual congeners (Geis-Asteggiante et al., 2011). Additionally, ELISA tests are subject to matrix effects within the wells, resulting in false positives in samples with low levels of MC (Schmidt et al., 2014). For these reasons, both LC-MS/MS and ELISA analyses were utilized for this study.

The objective of this study was to quantify the concentration of total MC (all assumed to be MC-LR) in several fish species from several water bodies in Michigan. White bass (*Morone chrysops*), walleye (*Sander vitreus*), channel catfish (*Ictalurus punctatus*), rock bass (*Ambloplites rupestris*), carp (*Cyprinus carpio*), northern pike (*Esox lucius*), and white perch (*Morone americana*) were collected by the Michigan Department of Environmental Quality (MDEQ) from St. Mary's River and Stony Creek Lake and by the Michigan Department of

Natural Resources (MDNR) from Saginaw Bay. MC was quantified in the muscle tissue of fish using ELISA and LC-MS/MS. Results were compared to the acceptable daily intake limits proposed by Mulvenna et al. (2012) and Ibelings and Chorus (2007).

2.3 Methods

2.3.1 Sample Sites

Saginaw Bay is a 296,000 ha bay of Lake Huron, located along the mid-eastern portion of Michigan. The bay is routinely classified into two sections: the outer bay and the inner bay. The outer bay has a mean depth of 13.7 m, while the inner bay is significantly shallower with a mean depth of 5.1 m. The bay is primarily fed by the Saginaw River, which drains a large watershed comprised primarily of agricultural and residential land (Francis, 2012). Historically, the bay has experienced large cyanobacterial blooms, and high concentrations of MC have been observed (Fahnenstiel et al., 2008). Initially, high cyanobacterial biomass was attributed to increased levels of phosphorus in runoff from the surrounding watershed. However, current elevated levels of cyanobacteria are attributed to selective filter feeding by zebra mussels, which reduces cyanobacterial competition (Vanderploeg et al., 2001). Research to date has identified MC-LR and MC-LA to be the primary congeners of MC present in Saginaw Bay (Dyble et al., 2008).

The St. Mary's River is a 112 km river that connects Lake Superior and Lake Huron in Northern Michigan. The river is currently on the Environmental Protection Agency's (EPA) Area of Concern List (AOC) due primarily to impairments caused by legacy point source pollution and current nonpoint source pollution (EPA, 2015). These sources of pollution have resulted in the presence of harmful algae, suggesting that MC may be present in this water body.

Stony Creek Lake is a 199.4 ha eutrophic impoundment of Stony Creek in Macomb County, Michigan. The lake is bounded by an upper dam that was constructed in 1962 and a lower dam that was constructed in 1963. The impoundment reaches a maximum depth of 7 m, however, the majority of the lake has a mean depth of around 3 m. The surrounding watershed is comprised primarily of agricultural and residential land (MDNR). To date, the presence of MC has not been quantified in this water body.

2.3.2 Sample Collection and Extract Preparation

A total of 56 fish were collected by the MDEQ (following fish contaminant monitoring program fish collection procedures, #WRD-SWAS-004) from the three aforementioned freshwater bodies in Michigan. Specifically, 10 white bass, 9 walleye, and 7 channel catfish were collected from Saginaw Bay on September 8, 2014. Fish from Saginaw Bay were collected via two gill nets placed in the inner bay (43.783397,-83.850047; 43.783592,-83.833417) (Personal Communication, 2015) following DNR sampling protocol (Fielder and Thomas, 2014). A total of 5 rock bass, 4 carp, and 2 northern pike were collected from Stony Creek Lake on October 14, 2014 using a boom shocker around the entire perimeter of the lake. Finally, 9 white perch and 10 northern pike were collected from the St. Mary's River on June 14, 2014.Samples from St. Mary's River were collected along the shore of Munuscong Bay from the mouth of Munuscong River to Maple point with a boom-shocker.

In addition to collecting samples in the field, fish not exposed to MC were obtained to serve as negative controls. Four Channel catfish were obtained from the Upper Midwest Environmental Sciences Center in La Crosse, Wisconsin. At this facility, fish were housed in indoor tanks supplied with well water, absent of cyanobacteria.

Frozen muscle filets from both the MDEQ and the Midwest Environmental Sciences Center were shipped, wrapped in tin foil, on ice. To extract MC from the fish tissue, roughly 30 g of wet tissue was weighed and then placed in a 20-ml glass vial. Samples were lyophilized in a Labco lyopholizer for 72 hours at -53^{0}C (pressure= 0.002 mbar). Lyopholized tissue was weighed and then ground with a mortar and pestle. Ground muscle tissue was stored at -20^{0}C prior to being used for ELISA and LC-MS/MS analyses.

2.3.3 ELISA Protocol

Approximately 400 mg of ground tissue was placed in a 10 ml glass scintillation vial followed by 2 mL of 80% aqueous Methanol (MeOH). Scintillation vials were placed on ice and tissue was homogenized in MeOH for 1 minute using a hand held automated homogenizer (VWR Power Max AHS 200). Homogenized samples were then sonicated with a probe sonicator (Braun- Sonica U) at 400 watts for 1 minute on ice. Sonicated samples were kept in the darkness, at room temperature for 24 hours, after which time they were centrifuged for 10 minutes at 8,000 rpm. A 150 µL aliquot was taken from the MeOH supernatant and placed in a clean 20 ml glass scintillation vial. All aliquots were evaporated to dryness using a mixture of compressed nitrogen and vacuum chambers. Vacuum chambers were comprised of 400 mL glass beakers sealed with a rubber stopper, fitted with a tube, which was hooked up to a vacuum. To expedite drying glass beakers were placed on hot plates set to 60^0 C. Dried crystals were dissolved in 40 µL of 20% aqueous MeOH and placed on an orbital shaker for 2 minutes. Upon removal from the orbital shaker, 20 µL of the dissolved solution was added to an ELISA kit (Envirologix, Portland, ME, catalog number: EP 022). Microplates were read with a BioRAD iMark microplate reader and concentration readings were corrected to represent concentration of toxin per kilogram of fish.

All samples were run in duplicate (i.e. two 400 mg samples were run independent of one another), and average concentrations for each sample were used in statistical analyses.

2.3.4 ELISA QA/QC

The ELISA kit used in this study had a detection limit of 0.187 µg kg^{-1} fish tissue muscle (dw). The average absolute difference between duplicate samples was 0.279 µg MC kg^{-1} of tissue (dw) with an average percent difference 33.31%. Of the four negative control fish analyzed, one sample had both duplicates register below the ELISA detection limit. The ELISA readings from the remaining three negative control samples ranged from below the detection limit to 0.058 µg MC kg^{-1} fish muscle (ww), suggesting the occurrence of false positives.

2.3.5 LC-MS/MS Protocol

Five mL of 80% MeOH was added to 400 mg of ground, dry tissue. Samples were then sonicated (Misonix Sonicator 3000) for 30 seconds on ice at 400 watts, left to sit for 30 seconds, and then sonicated for 30 more seconds. Sonicated samples were placed into a -20℃ freezer for 30 minutes, after which time they were centrifuged with a Sorvall RC-5B refrigerated superspeed centrifuge at 14,000 rpm for 15 minutes at -5℃. Supernatant was then decanted into an 8 mL glass tube using a glass pasteur pipette. The supernatant was then evaporated to dryness using a Savant SC110 speed vac. Dried crystals were resuspended in 1 mL of 80% MeOH and mixed with a vortex shaker for 60 seconds. Homogenized samples were cooled for 30 minutes in a -20℃ freezer and were then centrifuged at 3,000 rpm for 10 minutes in a swinging bucket rotor. The resulting supernatant was decanted into a 1 mL glass autosampler vial and kept cold in a -20℃ freezer until analysis.

Prior to running the LC-MS/MS analysis, a 13 mm nylon chromatography filer (VWR Scientific, Randor PA) chromatography filter was extracted in 50% MeOH using ultrasound and clarified by centrifugation. Samples were examined for Microcystin-LR using the transitions m/z 995.2 -> 107.00 m/z; 134.5 m/z; 155.0 m/z. MC-LR was quantified with the 135 m/z ion and was confirmed with the 107 m/z and 155 m/z ion. Positive samples had to have the correct retention time (13.35 min ∓ 5 %) and the correct ratio between transitions (ratio 135/107= 0.5-0.75; ratio 135/155 = 0.15-0.5). Nodularin spiked samples were assessed using the transitions 825.2 => 135.4 m/z; 163.4 m/z; 226.4 m/z.

2.3.6 LC-MS/MS QA/QC

Response factors using standards run every 20 samples were used to determine the individual detection limit for each sample. The 100% recovery detection limit was 11 µg MC/ kg tissue. The instrument detection limit was determined using the standard deviation of 7 injections and was found to be 4.5 ng/ mL. Three of every species of fish from every location was spiked with 30 µL of 1.25 ppb nodularin in order to determine recovery rates. Recovery rates ranged from 50%- 187%, with an average recovery rate of 108%. Variation in run time over the 2 month period was 0.2% and within day precision was 11%.

2.3.7 Statistical Analysis

A two-way ANOVA taking into account both water body and species was not be used due to the presence of an unbalanced design, and therefore differences in MC concentrations were evaluated between water bodies and between species separately. A One-way ANOVA was used to determine significant differences in ELISA readings among species in the Saginaw Bay basin, while a two-tailed, two sample t-test was used to determine differences amongst species in both

the St. Mary's River and Stony Creek Lake. Data values from the two northern pike sampled from Stony Creek Lake were excluded from statistical analysis due to insufficient sample size. A One-way ANOVA to determine differences in average total microcystin ELISA concentrations among the three water basins was not interpreted due to significant differences detected between species.

The average ELISA MC concentrations were plotted against both mass (kg) and length (cm) in order to determine if there were any relationships among size and toxin concentration. We used Spearman Rank tests to evaluate the strength of relationships between total MC concentration and mass or length of fish.

The assumption of normality was assessed statistically with a Shapiro Wilk's test, while the assumption of equal variance was determined with a Bartlett's test. A square root transformation was able to normalize all data failing to meet the necessary assumptions, except for weight and length; therefore, a non-parametric test was utilized. A Holm's adjustment was used in all ANOVA post hoc tests. All analyses were run using R 3.1.1 (Vienna, Austria).

2.3.8 Safe Consumption Calculations

Dry weight (dw) of fish (kg) was converted to wet weight (ww) of fish (kg) prior to running all analyses. All samples were weighed prior to being lyophilized in order to obtain the sample wet weight. Samples were likewise weighed after being lyophilized in order to obtain the sample dry weight. The sample wet weight was divided by sample dry weight in order to determine the proportion of weight loss due to freeze drying. The average proportion of weight loss was calculated and used for determining toxin concentrations per gram wet weight, prior to executing consumption calculations.

The concentration of total MC (µg MC per kg fish tissue) was calculated for all species using both the average and maximum MC concentration (Table 1). The total concentration of MC was then compared to acceptable daily intake values found in the literature. Ultimately, the acceptable daily limit values cited by Mulvenna et al., 2007 and Ibelings and Chorus 2005, were divided by both the average MC concentration and maximum MC concentration for each species in order to yield the maximum amount of fish tissue (kg ww) that could be consumed per day without exceeding the acceptable daily limit value for each scenario.

2.4 Results

2.4.1 LC-MS/MS

All samples were below the method detection limit of 11 µg MC /kg tissue.

2.4.2 ELISA

Within Saginaw Bay, there was not a significant difference in MC concentrations among white bass (mean=0.255 µg kg ww^{-1}, std.=0.168 µg kg ww^{-1}), channel catfish (mean=0.179 µg kg ww^{-1}, std.= 0.103 µg kg ww^{-1}), and walleye (mean= 0.230 µg kg ww^{-1}, std=0.115 µg kg ww^{-1}) (F=0.4892, p-value=0.62). However, there were significant differences among species in both St. Mary's River and Stony Creek Lake. In the St. Mary's River, northern pike (mean=0.294 µg kg ww^{-1}, std= 0.213 µg kg ww^{-1}) had a significantly higher mean concentration of total MC in comparison to white perch (mean= 0.094, std= 0.067 µg kg ww^{-1}) (t=-2.905, p-value=0.010). In Stony Creek Lake, carp (mean= 0.185 µg kg ww^{-1}, std= 0.051 µg kg ww^{-1}) had significantly higher concentrations of total MC in comparison to rock bass (mean= 0.019 µg kg ww^{-1}, std=0.023 µg kg ww^{-1}) (t=5.545, p-value= 0.001; Figure 1).

Results from the Spearman Rank test indicated that there was a correlation between fish mass and the concentration of MC in the fish tissue, when considering all species together (S= 17667, p-value= 0.007, ρ=0.36). However, a significant linear model could not be fit to the data (F=3.865e-06, p-value=0.9984). Likewise, results from an additional Spearman Rank indicated a significant positive correlation between fish length and the concentration of MC in the fish tissue (S=15582, p-value= 0.001, ρ= 0.44), yet a significant linear model could not be fit to the data (F=2.962, p-value=0.0911). Spearman Rank correlations were also assessed within each species to determine whether the lack of a relationship when assessing all species together, was due to inter-species variability or truly due to a lack of a significant relationship. There were no significant correlations between fish mass and MC concentration (p-value >0.257) or between fish length and MC concentration (p-value>0.870), except for northern pike collected from the St. Mary's River (mass vs MC concentration: S=284, p-value= 0.024, ρ=-0.721; length vs MC: S= 276, p-value= 0.039, ρ= -0.673). A significant linear regression model was determined for mass and MC concentration (F=9.779, p-value= 0.014, R^2= 0.494; Figure 3) as well as for length and MC concentration (F=14.34, p-value= 0.005, R^2=0.597; Figure 2)

Calculated consumption limits within the acceptable daily limit proposed by both Mulvenna et al. (2012) and Ibelings and Chorus (2007) indicated that large quantities of fish could be safely consumed given the observed average and maximum MC concentrations (Table 2). Considering the acceptable daily intake limit proposed by Mulvenna et al. (2012), adults (weighing 74 kg; table2) could consume anywhere between 50.3 kg and 779.9 kg of fish muscle (ww) using average MC concentrations and anywhere between 19.6 kg and 268.1 kg of fish muscle using maximum MC concentrations observed per species. Conversely, children (weighing 38 kg; table 2) could consume only between 25.9 kg and 400.5 kg of fish muscle

using average MC concentrations and between 10 kg and 137.7 kg of fish muscle using maximum MC concentrations. Acceptable daily consumption levels were considerably lower using the acceptable daily intake level for daily lifetime consumption proposed by Ibelings and Chorous (2007), ranging between 10.2 kg and 158.1 kg fish muscle using average MC concentrations and between 4 kg and 54.3 kg fish muscle using maximum MC concentrations for adults (weighing 75 kg; table 2). Conversely, children (weight 10 kg; table 2) could consume less, between 1.4 kg and 21.1 kg of fish muscle using average MC concentrations and between 0.5 kg and 7.2 kg of fish muscle per day using maximum MC concentrations. Overall, these acceptable limits were considerably smaller than the portions allowed for both adults and children using a seasonal rather than lifetime TDI metrics (Table 2).

2.5 Discussion

ELISA MC concentrations detected in the present study were generally lower than those found by other research. Poste et al. (2011) measured northern pike, white perch, and walleye from Lake Ontario containing 25.9 µg MC kg^{-1} ww, 4.5 µg MC kg^{-1} ww, and 2.1 µg MC kg^{-1} ww respectively. This same research project measured white perch, white bass, and walleye in Lake Erie containing 5.6 µg MC kg^{-1} ww, 18.3 µg MC kg^{-1} ww, and 23.9 µg MC kg^{-1} ww respectively. These observed concentrations are significantly higher than those observed in this study. Research conducted on two eutrophic lakes in China also observed MC concentrations of 26 µg MC kg^{-1} ww (Xie et al., 2005) and 67 µg MC kg^{-1} dw (Zhang et al., 2009) in carp, both of which are orders of magnitude higher than the concentrations found in the present study (0.122-0.236 µg MC kg tissue ww^{-1}). Although a large portion of published literature cites MC concentrations higher than that found in the present study, other research projects have observed low occurrences of MC fish tissue. For instance, research conducted at shallow reservoirs in the

Czech Republic found northern pike and carp, both of which, had muscle MC concentrations below the level of detection (1.2-5.4 ng MC g^{-1})(Kopp et al., 2013). Additionally, Wilson et al. (2008) found yellow perch from Lake Erie that had a range of 0.12 to 4.02 ng MC g dw^{-1} in the muscle tissue similar to fish collected in the present study (range containing between .139 and 3.49 ng MC g dw^{-1}).

The relatively low ELISA concentrations of MC observed in this study, suggests consumption of greater fish biomass is well within safe daily consumption levels. The most conservative acceptable daily limit per day presented in this project, is for lifetime consumption of MC using a tolerable daily intake value of 0.04 µg MC kg body $weight^{-1}$ $per\ day^{-1}$ for children, which equates to an acceptable daily intake limit of 0.4 µg per day (Table 2). Using the highest observed MC concentration in this study, a northern pike from St. Mary's River containing 0.757 µg MC kg fish tissue ww^{-1}, a 10 kg child would have to consume roughly 0.5 kg of fish per day to be in danger of exceeding current advisories. This quantity of fish is well within daily average fish consumption, considering an average serving size of fish to be between .056 kg and .085 kg (American Heart Association, 2014). This acceptable limit is also higher than current advisory limits set by the Michigan Department of Community Health which recommends children (weighing 45 kg) eat no more than 0.057 to 0.113 kg of northern pike per day. Currently, the threshold of tissue containing MC that can be safely consumed per day is higher than the recommended level of fish consumption available to the public for contaminants other than MC. This suggests that current consumption advisories are acceptable and need not be changed in regards to MC exposure in the St. Mary's River, Saginaw Bay, and Stony Creek Lake.

ELISA results from the present study showed a significant negative relationship between size and MC concentration for northern pike collected from St. Mary's River (Figure 2 and Figure 3). The

discovery of a negative correlation between both length and MC concentration is in line with findings made by Zhang et al. (2013) and Papadimitiou et al. (2012), both of whom observed negative correlations between fish length and MC concentration in muscle tissue. From a consumption advisory standpoint, the current data suggests that individuals would receive less MC exposure when eating larger rather than smaller northern pike from St. Mary's river. This trend is opposite of other harmful substances that tend to biomagnify up the food web (i.e. mercury) and therefore developing consumption advisories encouraging consumption of larger fish would be in contradiction to current advisories. Additionally, the lack of a significant correlation in the additional species sampled, suggests that consumption advisories should not be tailored to either fish length or mass. This conclusion agrees with research conducted by Semyalo et al. (2010) who found no significant correlation between both length and MC concentration in fish muscle, or mass and MC concentrations. The inconsistent findings across literature and within the current study suggest that fish length and mass may not be reliable indicators of MC concentration, and therefore, should not be heavily considered when determining consumption advisories.

The positive ELISA readings observed among the negative control samples suggest that matrix effects are a source of error in this present study. Research by Moreno et al. (2005), found ELISA readings below 5.9 µg MC kg dw^{-1} of tissue to be confounded by matrix effects. This value is considerably higher than the highest concentration detected in the negative controls of this study, which was 3.49 µg MC kg dw^{-1}. All the samples in the present study that were within the test's standard curve had values less than the cutoff value suggested by Moreno et al. (2005). However, only about 11.5% of the values within the standard curve were below the matrix effects threshold determined in the present study.

Additionally, the LC-MS/MS findings from the present study suggest that LC-MS/MS may be an inadequate analysis for assessing MC in regards to consumption for human health. For instance, the lack of positive LC-MS/MS findings in this study was due to the test's higher detection limit (11 μg MC/ kg tissue), which was considerably higher than the proposed daily intake limits proposed by WHO for lifetime consumption (3 μg/ day for adults weight 75 kg and 0.4 μg/ day for adults weight 10 kg; Ibelings and Chorus, 2007). This high detection limit indicates that MC concentrations could not be measured by LC-MS/MS tests until fish MC concentrations reached levels that vastly exceeded current consumption advisories. Therefore, reliance may need to be placed more heavily on ELISA readings, however, the propensity for false positives suggests that results from ELISA readings may need to be interpreted cautiously.

Although the results from this study suggest that fish consumption in regards to MC need not be added to the list of regulated toxins, they should be interpreted as a snapshot of these basins' conditions rather than used as long term metric. Within this study alone, fish species could not be appropriately pooled and analyzed due to differences among basins. The large amount of variability in both toxin production and toxin accumulation prevent extrapolating results from this study to other areas or even to other seasons within the same basin (i.e. concentrations of toxin present during one season in a basin might not be indicative of toxin present in the same location on a different date). For instance, fish sampled from Saginaw Bay, an area with a history of persistent cyanobacteria blooms (Fahnenstiel et al., 2008; Vanderploeg et al., 2001), had concentrations of MC well within advisable consumption limits, suggesting even areas plagued with high cyanobacterial abundances have not reached limits high enough to limit fish consumption.

Although relatively low levels of MC were observed in this study, it is difficult to confidently conclude whether these results were due to the levels truly being within an acceptable range or if the observed low levels were due to the time of the study. For instance none of the fish collected for this study were collected during times when blooms would be expected. Fish from Saginaw Bay and Stony Creek Lake were collected after the bloom (September and October respectively), while fish from St. Mary's River were collected prior to that year's bloom (June). Samples collected from St. Mary's River are of particular interest because they contained some of the highest concentrations of MC in the present study, even though the fish had not been exposed to MC since the previous season. The presence of MC in these fish therefore questions the fate of MC once ingested by aquatic organisms. Controlled depuration studies suggest that aquatic organisms can quickly excrete MC from their digestive tract. Research by Adamovsky et al. (2007) found that both Silver and Common Carp were able to completely eliminate MC from both their liver and muscle tissue within 1 to 2 weeks. Likewise, mussels fed MC containing alga had no detectable levels of MC after 13 days (Vasconcelos, 1995). The findings from these studies suggest that the toxin found in the fish from St. Mary's River is due to a continuous low level exposure to MC (i.e. MC in the surrounding environment is not completely eliminated) rather than incomplete excretion of the toxin by the organism. Research supporting this hypothesis suggests that intact cyanobacterial cells (and the associated toxin) can persist in the sediments (Brunberg and Blomqvist, 2002) and be assimilated by benthic organisms such as chironomids (Toporowska et al., 2014), mayflies (Smith et al., 2008), and zebra mussels (Poste and Ozersky, 2013), which can then pass the toxin up through the food web. Conversely, research by Smith and Haney (2006) found that pumpkinseed sunfish fed MC excreted the majority of toxin within 9 days (in align with previous

research), however, detectable levels were found in the liver and muscle after 3 weeks of acclimation. The authors suggest that the pumpkinseed fish failed to eliminate all the toxin because they were fasted during the depuration period, slowing down toxin excretion. This observation suggests that fish in temperate climates entering seasons of low food consumption may be able to retain detectable levels of MC in their tissues into the following spring (Smith and Haney, 2006). This hypothesis could potentially explain the toxin present in the fish collected from St. Mary's River in the present study, however, more research is necessary in order to definitively determine the lagging source of toxin in fish.

Although MC concentrations detected in this this study were within an acceptable range, values will likely continue to rise as the intensity and frequency of cyanobacterial blooms continue to increase. Additionally, the concentrations of MC are likely much higher in these areas during the seasonal cyanobacteria bloom and therefore samples in these areas should be collected closer to the time of the bloom in order to get a better idea of maximum MC concentrations in these areas. Future studies should attempt to sample large numbers, of the same species, from multiple lakes. Obtaining a data set with a balanced design would allow researchers to simultaneously differentiate the effects of MC accumulation across locations and species as well as determine the interaction effect between these two variables which is a major limitation of this study. Ultimately, the large variability of this toxin across time and space would require significant sampling efforts over larger time frames in order to either confidently establish appropriate local consumption guidelines or to definitively determine the lack of necessity for MC consumption advisories in fish.

2.6 Acknowledgements

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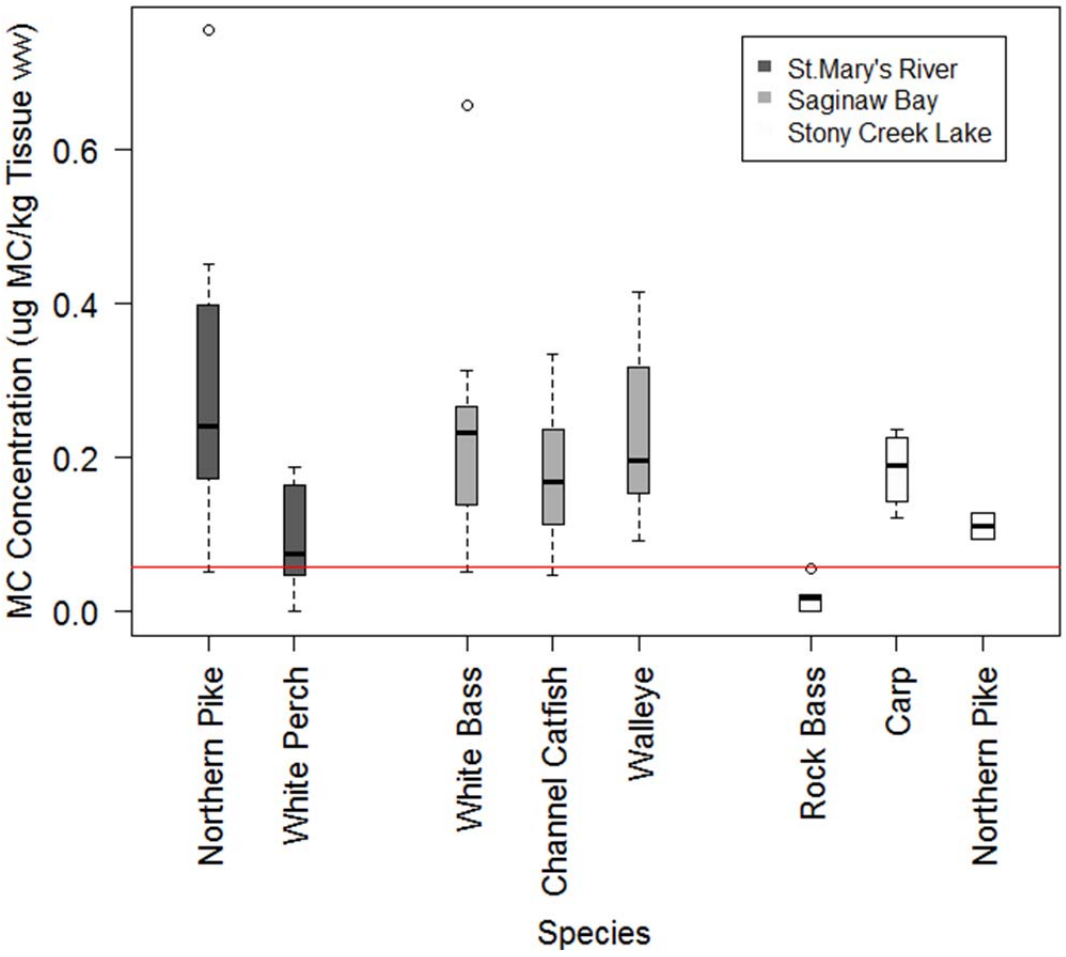


Figure 1: Microcystin concentrations determined with ELISA, compared among species collected from three Michigan water bodies. Microcystin concentrations of northern pike, white perch, white bass, channel catfish, walleye, rock bass, and carp muscle tissue (dry weight) from St. Mary's River, Saginaw Bay and Stony Creek Lake, MI were compared. Boxes in plot represent the interquartile range and the whiskers represent the 25th and 75th percentile. Bars located in box represent the median and outliers are defined as being greater than 1.5 times the interquartile range. Red line represents the false positive threshold determined with negative controls.

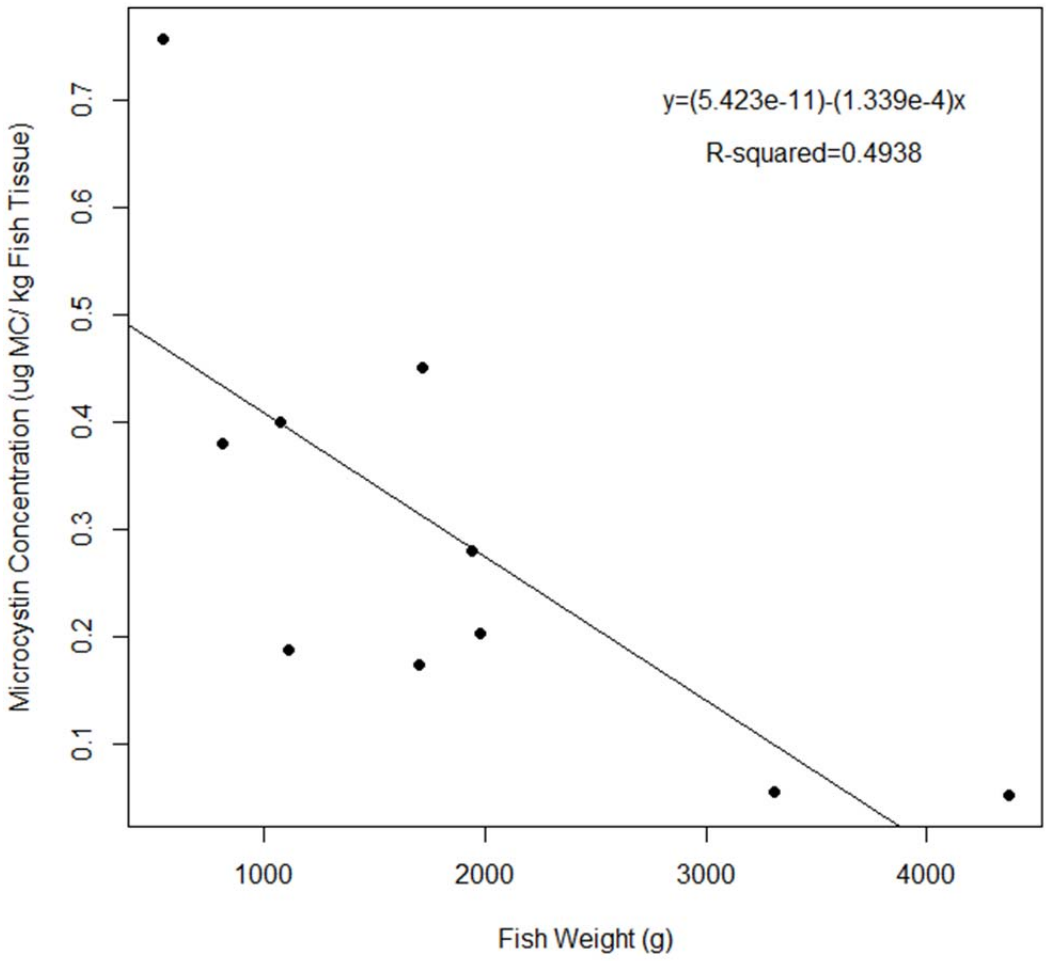


Figure 2: Relationship between fish mass and microcystin concentration for northern pike collected from the St. Mary's River, MI. A significant negative correlation was detected between total microcystin concentration within each northern pike and the total fish mass (S= 284, p-value= 0.024, ρ= -0.072). A significant linear regression model was fit to data in order to characterize the observed trends (F=9.352, p-value= 0.016).

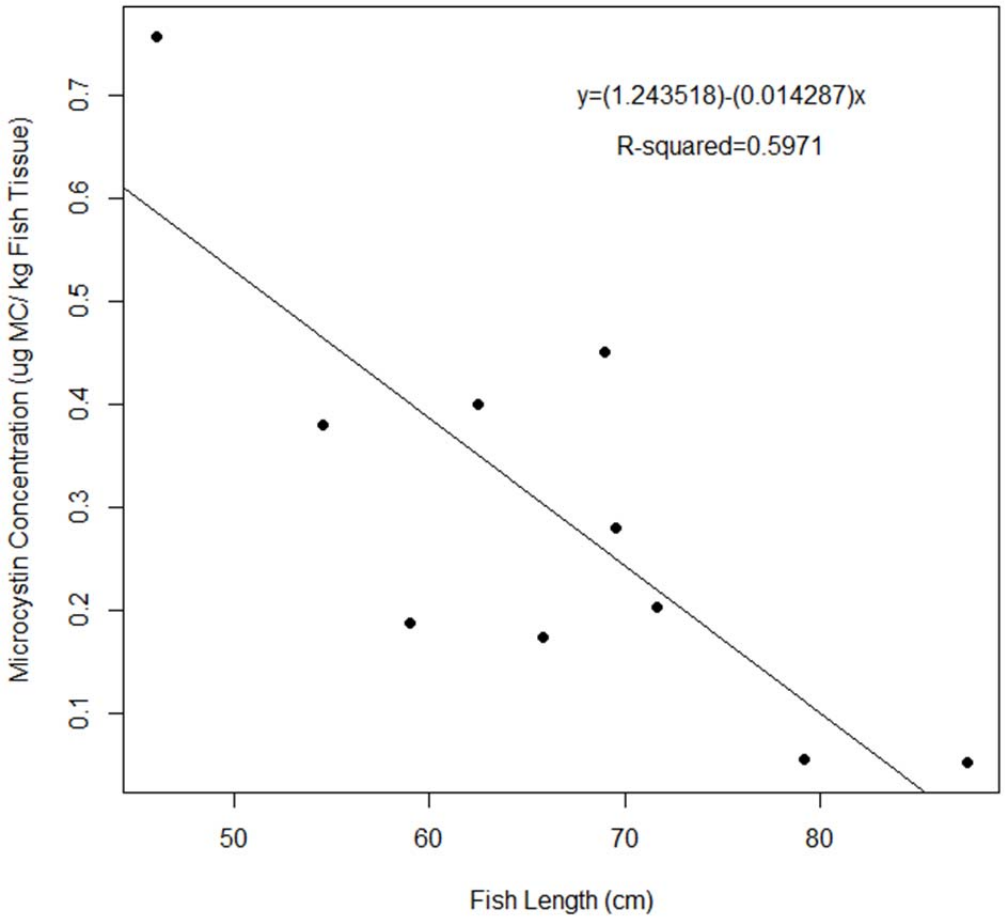


Figure 3: Relationship between fish length and microcystin concentration for northern pike collected from the St. Mary's River, MI. A significant negative correlation was detected between total microcystin concentration within each northern pike and the total fish length (S= 276, p-value= 0.039, ρ= -0.673). A significant linear regression model was fit to data in order to characterize the observed trends (F=13.51, p-value= 0.006).

Table 1. Average and maximum microcystin concentration (ww) in fish tissues of fish species collected from Saginaw Bay, Stony Creek Lake, and St. Mary's River located in Michigan. Microcystin concentrations (µg MC kg tissue $^{-1}$)were determined using average and maximum microcystin readings from Enzyme Linked Immuno Sorbent Assays for each species. Rockbass from Stony Creek Lake had the lowest concentrations of microcystin, while northern pike from St. Mary's River had the highest.

	Saginaw Bay			Stony Creek Lake			St. Mary's River		Neg. Control
	White bass	Walleye	Channel Catfish	Carp	Rock bass	Northern Pike	White Perch	Northern Pike	Channel Catfish
Average Microcytsin Concentration (µg MC per kg tissue)	0.255	0.230	0.179	0.185	0.019	0.111	0.094	0.294	0.003
Maximum Microcystin Concentration (µg MC per kg tissue)	0.658	0.417	0.336	0.236	0.055	0.129	0.188	0.757	0.005

Table 2: Range of consumption guidelines for fish muscle for eight species of fish collected from three Michigan water bodies.Fifty six fish comprised of eight different species were collected from the St. Mary's River, Saginaw Bay, and Stony Creek Lake, MI. Fish muscle tissue was analyzed for total microcystin concentrations (µg MC kg tissue^{-1}; Table 1) using Enzyme Linked Immuno Sorbent Assays (ELISA). The acceptable daily limits (µg day^{-1}) proposed in literature were divided by these ELISA microcystin concentrations in order to determine the maximum amount of fish muscle (kg) that could be consumed per day, without exceeding proposed consumption limits. A maximum consumption range was determined using both average toxin levels calculated per species, as well as, maximum observed toxin levels per

Acceptable Daily Intake Limits Cited in Literature				Range of Consumption Guidelines (kg fish tissue day-1) using Maximum and Average Toxin Levels (µg MC kg tissue wet weight-1)							
				Saginaw Bay			Stony Creek Lake			St. Mary's River	
Publication	**Advisory Time Frame**	**Average Weight (kg)**	**Acceptable Daily Limit (µg/day)**	White bass	Walleye	Channel Catfish	Carp	Rock bass	Northern Pike	White Perch	Northern Pike
Mulvenna et al., 2012	Life Time Consumption	74	14.8	22.5-58.0	35.5-64.4	44.1-82.8	62.6-80.1	268.1-779.9	114.9-133.2	78.6-157.2	19.6-50.3
		38	7.6	11.5-29.8	18.2-33.1	22.6-42.5	32.1-41.1	137.7-400.5	59.0-68.4	40.4-80.7	10.0-25.9
Ibelings and Chorus, 2007	Life Time Consumption	75	3	4.6-11.7	7.2-13.0	8.9-16.8	12.7-16.2	54.3-158.1	23.3-27.0	15.9-31.9	4.0-10.2
		10	0.4	0.6-1.6	1.0-1.7	1.2-2.2	1.7-2.2	7.2-21.1	3.1-3.6	2.1-4.2	0.5-1.4
	Seasonal Consumption	75	30	45.6-117.5	72.0-130.5	89.3-167.8	126.9-162.3	543.4-1581.0	233.0-270.0	159.4-318.6	39.6-102.0
		10	4	6.1-15.7	9.6-17.4	11.9-22.4	16.9-21.6	72.4-210.8	31.1-36.0	21.3-42.5	5.3-34.0

Chapter 3: Literature Review

3.1 Cyanobacteria

Cyanobacteria (blue green algae) are a division of photosynthetic algae characterized by the accessory pigment phycocyanin and are the only division of algae located in the bacteria kingdom. Therefore, although they are photosynthetic like other types of algae, they are also prokaryotic and have features characteristic of bacteria, such as peptidoglycan cell walls. In addition, some genera of cyanobacteria contain specialized structures such as akinetes, heterocysts, and gas vacuoles. Akinetes are specialized thick walled cells that allow some species to overwinter on the sediments, while heterocysts are structures that allow select genera to transform nitrogen from nitrate and nitrite (biologically unavailable forms) to ammonia (a biologically available form). Gas vacuoles are structures that cause some genera of cyanobacteria to be buoyant in the water column, allowing them to access more sunlight, ultimately maximizing photosynthesis. These gas vacuoles, coupled with periodic bursts of excessive cyanobacteria growth, result in dense formations of algae known as algae blooms. These algae blooms, although natural, have been progressively increasing in frequency, which is causing concern. Ultimately, the frequency and intensity of cyanobacterial blooms are influenced by a suite of factors, many of which had been anthropogenically altered.

3.2 Environmental Factors Influencing Cyanobacteria abundance

Temperature is one of the most important driving forces dictating the increased prevalence of cyanobacteria (Izydorczyk et al., 2008; Ni et al., 2012; Lu et al., 2013). Temperature directly influences phytoplankton composition because different divisions of phytoplankton have different temperature requirements. For instance, cyanobacteria tends to have optimal growth at higher ambient temperatures- generally above 25 ℃ (Paerl and Huisman,

2008), and therefore increases in average temperature are predicted to favor cyanobacterial proliferation (Klaus et al., 2008; O'Neil et al., 2012). Temperature indirectly influences cyanobacteria by increasing the stability of the water column. Specifically, increased temperatures cause water columns to stratify, which ultimately selects for genera of cyanobacteria capable of forming blooms by using their gas vacuoles to access both adequate light and nutrients amidst still stratified waters (Paerl and Paul, 2012). .

Increased nutrient loading (primarily phosphorous and nitrogen) due to anthropogenic eutrophication is also believed to be a driving force of increased cyanobacteria abundance (Heisler et al., 2008). The total nitrogen to total phosphorous ratio (TN: TP) was originally believed to be the primary indicator of cyanobacterial abundance. Preliminary research suggested that cyanobacteria blooms would flourish under low TN: TP conditions, due to a greater need of phosphorus than nitrogen (Smith, 1983). Additionally, Downing et al. (2001) found that the TN: TP ratio could influence the composition of cyanobacteria genera present. The TN: TP ratio specifically influences genera composition because some genera of algae are capable of transforming nitrite and nitrate (biologically unavailable forms) into ammonia (biologically available form), while others are incapable of this function (WHO, 1999). Although this line of research suggested that TN:TP was the most important factor (in terms of available nutrients) influencing cyanobacterial abundance, recent research has contradicted this prior understanding and has revealed that the overall abundance of TP and TN separately are better indicators of cyanobacteria abundance than TN:TP ratios alone (Downing et al., 2001).

Although temperature and nutrient concentrations are two primary forces influencing cyanobacteria abundance and composition, other factors also influence cyanobacteria prevalence. Specifically, the colonization of invasive zebra mussels has been identified as a factor causing

increased cyanobacteria abundance, particularly in oligotrophic waters (Knoll et al., 2008, Raikow et al., 2004). Research suggests that zebra mussels increase cyanobacteria abundance by selectively filtering out other algae genera, and ejecting "unpalable" cyanobacteria genera back into the water with psuedofeces (Vanderploeg et al., 2001; Fahnenstiel et al., 1994; Juhel et al., 2006). *Microcystis*, a bloom forming genus of cyanobacteria capable of producing toxins, is believed to be selectively filtered out by zebra mussels (Vanderploeg et al., 2001) allowing for increases in undesirable cyanobacteria by reducing competition for resources.

3.3 Negative Effects due to Cyanobacteria Blooms

Due to the physical and biological degradation of water bodies experiencing blooms, cyanobacteria are often considered nuisance organisms. The presence of blooms can significantly reduce dissolved oxygen levels which can result in fish kills as well as diminished lake aesthetics (Quibler et al., 2013). Although these negative impacts are of concern, the most severe consequence of abundant cyanobacteria blooms is the increasing prevalence of the toxic secondary metabolites that many species of cyanobacteria produce (Valerio et al., 2010).

Toxic secondary metabolites can be categorized as either hepatotoxins, neurotoxins, or dermatotoxins (Figure 1). Of these toxins, hepatotoxins are of primary research interest due to their high abundance and toxicity (Figueiredo et al., 2004). Hepatotoxins encompass several types of toxins all known to cause liver damage. The hepatotoxin variants include: nodularins, cylindrospermopsins, and microcystins (Huisman et al., 2005). Of these variants, microcystin is the most prevalent and harmful of the hepatotoxins and therefore it has been most heavily researched.

3.4 Microcystin Structure

Microcystin is a monocyclic peptide (Schmidt et al., 2013) comprised of a hydrophobic Adda chain, a Masp group, a D-Ala group, a D-Glu group, and a Mdha chain. There are roughly 80 structural variations of microcystin that are differentiated by the types of amino acids at position 2 and 4 as well as the methylation state of the amino acids at the positions 3 and 7 (Trinchet et al., 2011). The different variations or congeners of microcystin are depicted by the letters of the amino acids occupying positions 2 and 4.

The degree of microcystin toxicity is variable amongst different congeners. The various amino acids that dictate different microcystin congeners, give the overall molecule different polarities. The differences in polarity allow some variants of the toxin to be more easily transported through the bile duct, causing them to be more harmful to human health (Schmidt et al., 2014). Due to this phenomenon, Microcystin- LR has a lower LD_{50} (43 µg /kg body weight) than other common congeners such as Microcystin-RR (LD_{50} =235.5 µg/kg body weight) and Microcystin-YR (LD_{50} =110.6 µg/kg body weight) (Gupta et al., 2003). Therefore, due to its potent toxicity, microcystin-LR is of primary concern to biologists and consequently the most widely studied congener of Microcystin (Huisman et al., 2005).

3.5 Microcystin Inhibition of Protein Phosphatases

The potent toxicity of microcystin is primarily attributed to its inhibition of the protein phosphatases PP1 and PP2A. PP1 and PP2A are eukaryotic enzymes that encompass a wide variety of functions including: storing energy as glycogen, allowing actomyosin fibers to relax, enabling transcription factors to be reused, ion pump regulation, initiation of cell apoptosis, and regulation of cell cycle (Ceulemans and Bollen, 2004). These enzymes are rendered inactive when microcystin irreversibly binds to them. This is caused by the hydrophrophic Adda chain on

the microcystin molecules embedding in the hydrophobic groove on the protein phosphatase enzymes, enabling the leucine amino acid in microcystin to adhere to a Tyrosine amino acid located at the end of the c-terminal groove (Goldberg et al., 1995). Structural changes within the protein phosphatases occurs so that steric interference between the Tyrosine amino acid and the Mdha chain can be avoided (Bartford et al., 1998). This binding is accompanied by a carboxylate and carbonyl oxygen on microcystin binding with water molecules attached to metal (specific metal compounds unknown to date) ligands within the protein phosphatases (Goldberg et al., 1995). Finally, the end carbon in the microcystin Mdha chain binds to a cysteine in the protein phosphatase enzyme, however, this final binding process is not believed to inhibit enzyme function (Runnegar et al., 1995).Together these reactions - baring the end carbon in the MdHa chain binding to a cysteine- render the enzymes PP1 and PP2A inactive resulting in harm to the organism, due to the loss of functions performed by these enzymes.

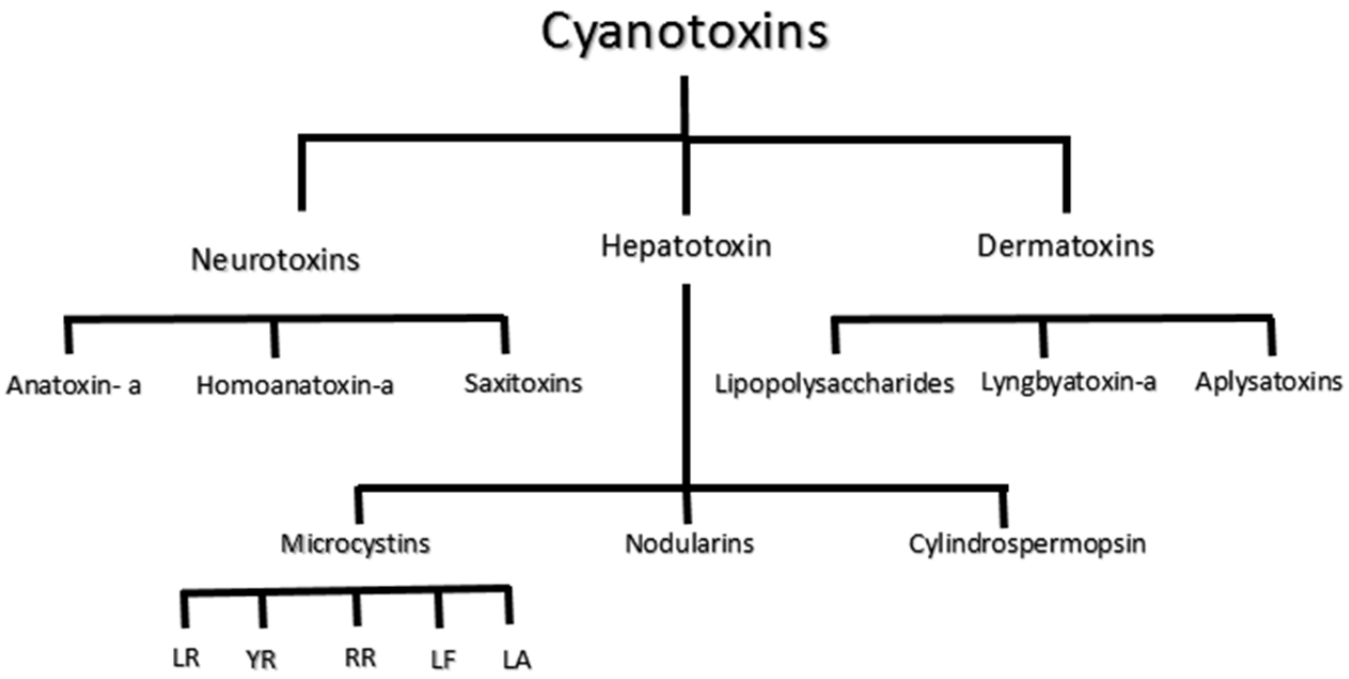


Figure 4: Summary chart of cyanotoxins. The three primary classes of cyanotoxins include: neurotoxins, hepatotoxins, and dermatoxins. There are currently over 80 structural variants of microcystin; the five most common variants have been listed in this figure.

3.6 Ecological Impacts of Microcystin

Negative impacts of microcystin exposure extend to all ecosystem levels spanning both aquatic and terrestrial environments. Literature reviews by Vasconcelos (2001) and Ferrao- Filho (2011) cite literature stating harmful effects of microcystin to bacteria, fungi, phytoplankton, invertebrates, fish, birds, and mammals. The impacts of microcystin on both aquatic and terrestrial plants has also been studied. Mitrovic et al. (2005) found decreased growth in the aquatic plants *Lemna minor* and *Wolffia arrhiza* when exposed to microcystin. Likewise, irrigation water containing microcystin was found to decrease the growth of both potato and mustard plants (McElhiney et al., 2001).

3.7 Adverse Health Effects due to Microcystin Exposure

Exposure at both acute and chronic toxicity levels of microcystin, can cause negative health effects in mammals. Acute exposure of mice injected with 23 μg/mL of microcystin- LR resulted in the death of all test subjects; Post-mortem examinations revealed the cause of death to be circulation failure as a result of acute liver damage (Falconer et al., 1981). Damage due to microcystin exposure is primarily concentrated in the liver because organic anion transporting polypeptides (OATP) located in the liver facilitate transport of microcystin molecules, allowing for increased cellular uptake of the toxic molecule (Fischer et al., 2005; Monks et al., 2007). Fawell et al. (1999) examined the effects of long term chronic exposure to microcystin-LR, in a 13 week long study, where mice were injected with varying concentrations of microcystin-LR that could be potentially observed in natural waters. Results identified 40 μg/ kg of body weight as an appropriate No Observed Adverse Effect Level (NOAEL) (Fawell et al., 1999). This NOAEL was then divided by an uncertainty factor of 1000 (10 for interspecies variation, 10 for intraspecies variation, 10 for limitations in data) to yield a Tolerable Daily Intake (TDI) limit of

0.04 μg MC / kg of body weight, which has been adopted by the World Health Organization (WHO, 1999). Application of knowledge from these studies has contributed to preventing the negative health impacts associated with microcystin exposure.

3.8 Human Exposure to Microcystin

Humans can be exposed to microcystin-LR through recreational activities by either accidental consumption during recreation, inhalation of aerosolized toxin, or through dermal contact (Figueiredo et al., 2004). By sampling a nasal swab from water recreationists pre- and post- bloom, Backer et al. (2010) determined that individuals had higher concentrations of microcystin in their nasal mucous post bloom than when compared with pre bloom. A case study conducted at George Elliot Hospital in Britain (Turner et al., 1990) identified exposure to microcystin as the cause of ailment for two soldiers who had been exposed to a bloom during a canoeing drill. Both patients, who recounted accidentally swallowing water during the exercise, presented with a sore throat, blistering near the mouth, vomiting, malaise, pleuritic pain on the left side, fever, and basal pneumonia (Turner et al., 1990). Exposure to cyanobacterial blooms via dermal contact can also result in an allergic reaction which presents as a rash in sensitive individuals (WHO, 2003).

Humans can also be exposed to microcystin through the intentional consumption of contaminated drinking water. To date, there are no federal guidelines regarding the regulation of microcystin in drinking water, however, the toxin has continued to be researched through the EPA's Unregulated Contaminant Monitoring Rule (UCMR) program (EPA, 2015). Fristachi et al. (2007), utilized a conceptual model using data collected from 45 different water facilities, to determine that an average American will be exposed to 1.7×10^{-3} μg MC/ kg bw over a time span of 75 years - a quantity below the WHO advisory limit. Although research by Fristachi et

al. (1990) suggests that the average American is safe from chronic exposure via drinking water, periodic blooms plague areas reliant on surface water. For example, recent large blooms in Lake Erie forced a ban on Toledo's public water supply, which resulted in Toledo residents being completely dependent on bottled water until the majority of toxins naturally degraded (Frankel, 2014).

Medical patients have the potential to be exposed to microcystin intravenously. Intravenous exposure to microcystin poses a high risk route that expedites the presentation of symptoms by allowing direct access of the toxin to the circulatory system (Pouria et al., 1998). This route of exposure led to the only known case of microcystin induced human mortality which occurred in 1996 when 131 patients in Brazil were exposed to microcystin via hemodialysis treatments. Of the 131 patients exposed, 52 expired due to acute liver failure (Azevedo et al., 2002; Jochimsen et al., 1998). Although intravenous exposure to microcystin has devastating health effects, the accidental poisoning in Brazil is the only known case of exposure via this route.

Finally, individuals have the potential to be exposed to microcystin via consumption of contaminated aquatic organisms. Research by Poste et al. (2011), spanning water bodies in Uganda, Canada, and the United States found microcystin accumulation in fish muscle from all sites - ranging from 0.5 to 1917 µg/ kg - suggesting hazardous exposure to microcystin via fish consumption. Research by Chen et al. (2009) and Uneno et al. (1996) suggests that microcystin exposure via heightened consumption of contaminated fish (coupled with consumption of contaminated water) might account for high incidences of liver cancer in areas experiencing high microcystin exposure, such as in China. Microcystin was detected in the blood serum of Chinese fishermen who were estimated to consume roughly 2.2- 3.9 µg MC-LReq per day (Chen et al.,

2009). Likewise, this chronic exposure to microcystin due to an increased dietary reliance on fish, has been identified as a possible source of the increased prevalence of primary liver cancer in China (Ueno et al., 1996). The risk of exposure to microcystin via consumption of fish is dependent on the accumulation of the toxin in fish muscle tissue.

3.9 Accumulation of Microcystin in Fish

Toxin uptake in fish is primarily attributed to ingestion of either contaminated water or other aquatic organisms containing microcystin. Tencalla et al. (1994) observed 100% mortality of fish galvaged with toxin concentrations equal to that in the surrounding environment, but saw no mortality in ungalvaged fish, suggesting negligible amounts of toxin are absorbed through the skin or gills. Additionally, Smith and Haney (2006) observed microcystin accumulation in pumpkinseed fish and found that microcystin was absorbed in greater quantities by the gastrointestinal tract when consumed through a vector (another aquatic organism containing microcystin) than through direct consumption of toxic cyanobacteria. Upon sampling macroinvertebrates and zooplankton from four freshwater lakes, Kotak (1996) found detectable limits of microcystin in gastropods and zooplankton, with zooplankton exhibiting the largest accumulation of microcystin. Data compiled from 55 separate studies found that, among invertebrates, zooplankton had the highest concentrations of microcystin, followed by gastropods, bivalves, and crustaceans respectively (Ferrao-Filho et al., 2011).

3.10 Differential Microcystin Accumulation in Fish

Upon being consumed, microcystin has differential accumulation in various fish tissues. Papadimitriou et al. (2009) saw the highest concentrations of microcystin in the liver followed by the intestines, kidneys, brain, ovaries, and muscle tissue. Conversely, Xie et al. (2005) found higher concentrations of microcystin in the intestines and blood than in the liver, bile, and kidneys; however, both studies detected the lowest concentration of microcystin in the muscle tissue. Additionally, Papadimitriou et al. (2012) observed detectable levels of microcystin in the brains of common carp, suggesting the toxin has the capability of penetrating the blood- brain barrier.

Uptake of microcystin by fish has the potential to negatively harm both juvenile and adult fish. Research by Jacquest et al. (2004) saw that Medaka eggs microinjected with 10 and 1 µg/ ml had between 78 to 88% reduction in survival rate coupled early hatching rates (1-2 days early). Likewise, adult rainbow trout galvaged with 6600 µg MC/ kg bw had 100% mortality due to liver necrosis, showing hepatocyte swelling after 3 hours of exposure and lethargy after 12 to 24 hours of exposure (Tencalla et al., 1994). Additionally, adult carp injected with 250 and 300 µg MC-LR/ kg had degenerated architecture of the parenchymal cells, necrosis, and swollen Bowman's capsule in the kidney (Rabergh et al., 1991). Finally, microcystin is also harmful to fish at environmentally relevant levels. Medaka fish exposed to 5 µg MC-LR/ ml showed cell lysis and overall loss of architecture in the liver, intestine, spleen, testis, and ovaries (Trinchet et al., 2011). Therefore, although microcystin accumulation in fish is concerning for human health, it is also a concern for the fish being exposed (see Malbrouck and Kestemont, 2006 for a full review of the effects of microcystin on fish).

3.11 Microcystin Accumulation and Depuration in Fish

Concentrations of microcystin have been observed in decreasing amounts with increasing trophic levels suggesting that microcystin tends to biodilute moving up the aquatic food web, as a result of toxin depuration (Ferrao-Filho et al., 2011; Ibelings et al., 2005). Adamovsky et al. (2007) found that carp with 3.3-19 ng/g of microcystin in their muscle tissue and up to 226 ng/g of microcystin in their hepatopancreas, were completely clean of microcystin within one to two weeks. Likewise, Smith and Haney (2006) observed that pumpkinseed fish continuously fed 50 ng MC/ kg / day, saw declines in microcystin accumulation after 9 days of exposure, however, detectable levels remained even after two weeks in a microcystin free environment. Additional studies also suggest that only a small proportion of toxin ingested was processed through the liver. Bieczynski et al. (2013) found that patagonian pejerrrey fed 1.3 ug MC/ g bm had roughly 32% of the toxin in the intestine and 2% in the liver after 3 hours and 7% in the intestine and 11% in the liver after 6 hours. Ultimately, organisms' ability to depurate microcystin results in biodilution rather than biomagnification as microcystin accumulates in aquatic food webs (Ibelings and Chorus, 2007).

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Chapter 4: Methods for Quantifying Microcystin

4.1 Literature Review of Chemical Analyses

4.1.1 Introduction

In addition to variability of microcystin concentrations across time and space, there is also considerable variability regarding analyses used for quantification. To date, there are two chemical analyses generally used for quantifying microcystin in fish tissue: liquid chromatography, coupled with mass spectrometry (LC-MS) or double mass spectrometry (LC-MS/MS) and enzyme linked immuno sorbent assay (ELISA). Although these two analyses are the current convention for microcystin quantification, there exist a multitude of extraction protocols. The lack of a standard analysis protocol further complicates research regarding microcystin quantification and limits the extent to which findings from one study can be compared to other studies.

4.1.2 Extraction Protocols

Prior to running chemical analyses, microcystin bound to solid fish tissue must be extracted in a liquid medium. There are a multitude of ways that microcystin can be extracted from fish tissue, and this variability is what contributes to the variability in testing across studies. However, although a wide variety of extraction techniques exist, all procedures have underlying similarities due to their common goal of extracting microcystin from a solid into an aqueous solution. Extraction protocols generally begin by freeze drying, most commonly through utilization of a lyopholizer (Peng et al., 2010; Smith and Boyer, 2009; Smith and Haney, 2006; Xie et al., 2005; Zhang et al., 2010). Tissue is then conventionally ground with either some form of automated homogenizer (meat grinder or hand held homogenization system) (Mekebri et al.,

2009; Moreno et al., 2005; Smith and Haney, 2006) or with a mortar and pestle (Smith and Boyer, 2009; Xie et al., 2005; Zhang et al., 2010).

Solvents used to extract microcystin from tissues vary substantially. All extraction solvents generally have some combination of methanol (MeOH), butanol (BuOH), acetic acid (AcOH), and water. Aqueous MeOH is the most commonly utilized solvent, due to its ability to extract microcystin variants of varying polarity (Adamovsky et al., 2007; Biecczynski et al., 2013; Magalhaes et al., 2001; Magalhaes et al., 2003; Mekebri et al., 2009; Moreno et al., 2005; Peng et al., 2010; Smith and Boyer, 2008; Smith and Haney, 2006; Williams et al., 1997; Wood et al., 2006). Ward et al. (1997) found maximum extraction efficiency when using between a 50% and 80% aqueous MeOH solvent, with lower concentrations failing to extract hydrophobic variants (MC-LW and MC-LF), and higher concentrations failing to extract less hydrophobic variants (MC-LR). Within this range of values, 75% aqueous MeOH has been determined to be the most efficient solvent by Fastner et al. (1998), although Smith and Boyer (2009) maintain that 80% MeOH is advantageous due to its ability to degrade proteins bound to microcystin. The efficiency of toxin extraction can additionally be increased by performing multiple extractions, utilizing different combination of solvents in order to increase recovery rates (see Sangolkar et al. (2006) for detailed review of solvents).

Cells containing microcystin are additionally broken up through some form of sonication, after being mixed in with the extraction solvent. Generally either a water bath (Adamovsky et al., 2007; Kohoutek et al., 2010; Kopp et al., 2013; Mekebri et al., 2009; Sipia et al., 2002) or probe sonicator (Smith and Boyer, 2009) is used to disrupt cell membranes, however repeated freezing and thawing can also be utilized (Smith and Haney, 2006). Of the methods used for disrupting

cell membranes, utilization of a probe sonicator yields the highest recovery rates (Rapala et al., 2002; Spoof et al., 2003).

Samples often need to be "cleaned" prior to running extraction solvents through chemical analyses, in order to remove compounds extracted from the fish that have the potential to cross react with chemical analyses. Clean up methods include either repeated mixtures of hexane to remove lipids from samples (Adamovsky et al., 2007; Geis- Asteggianted et al., 2011; Magalhaes et al., 2001; Magalhaes et al., 2003; Kohoutek et al., 2010; Kopp et al., 2003), running samples through a filter (Sipia et al., 2002; Smith and Haney, 2006), or running samples through a solid phase extraction (SPE) cartridge (Bieczynski et al., 2013; Ernst et al., 2004; Mekebri et al.,2009; Peng et al., 2010; Sipia et al., 2002; Xie et al., 2005; Zhang et al., 2010). When samples are run through SPE extraction cartridges, the microcystin in the sample binds to the substrate in the cartridge, while the other contaminants pass through without binding. The bound microcystin is then eluted off the cartridge with an organic solvent (usually MeOH) that has a higher affinity for microcystin than the cartridge substrate.

Toxin extracted per unit of fish tissue is often below the detection limit of the chemical analysis, therefore, concentration steps are often used. Concentration at this step of extraction is achieved by evaporating the extraction solvent and resuspending the dried crystals in a smaller volume of liquid than was used to extract the toxin. Various methods are used for evaporating the extraction solvent, including use of a rotary evaporator (Moreno et al., 2005; Peng et al., 2010), vacuum centrifuge (Williams et al., 1997), Turbovap (Poste et al., 2011), vacuum evaporation (Wilson et al., 2008), lyopholizer (Smith and Haney, 2006), as well as blowing samples dry with compressed Nitrogen (Bieczynski et al., 2013; Ernst et al., 2004; Sipia et al., 2002). The dried crystals are often resuspended in either water (Adamovsky et al., 2007;

Bieczynski et al., 2013; Moreno et al., 2005; Peng et al., 2010; Poste et al., 2011; Sipia et al., 2002; Smith and Haney, 2006; Wilson et al., 2008; Zhang et al., 2010) or aqueous MeOH (Magalhaes et al., 2001; Magalhaes et al., 2003; Moreno et al., 2005; Kohoutek et al., 2010; Kopp et al., 2013; Smith and Boyer, 2008; Wood et al., 2006; Xie et al., 2005). Upon resuspension, samples are ready to be processed via chemical analyses.

The two most common chemical analyses used for quantifying microcystin are enzyme linked immuno sorbent assays (ELISA) (Adamovsky et al., 2007; Geis-Asteggiated et al., 2011; Magalhaes et al., 2001; Magalhaes et al., 2003; Peng et al., 2010; Sipia et al., 2002; Smith and Haney, 2006; Wilson et al., 1997; Wood et al., 2006) and either liquid chromatography coupled with mass spectrometry (LC-MS) (Geis- Astegginated et al., 2011; Zhang et al., 2010), or liquid chromatography coupled with double mass spectrometry (LC-MS/MS)(Kohoutek et al., 2010; Kopp et al.,2013; Sipia et al., 2002).

Tissue Dried
Lyopholized
Peng et al., 2010
Smith and Boyer 2009
Smith and Haney, 2006
Xie et al., 2005
Zhang et al., 2010
Freeze Dried
Sipia et al., 2002
Tissue Ground
Mortar and Pestal
Smith and Boyer, 2009
Xie et al., 2005
Zhang et al., 2010
Automated Tissue Grinder
Mekebri et al., 2009
Moreno et al., 2005
Smith and Haney, 2006
Tissue Extracted
50-100% MeOH
Adamovsky et al., 2007
Bieczynski et al., 2013
Magalhaes et al., 2001/2003
Mekebri et al., 2009
Moreno et al., 2005
Peng et al., 2010
Smith and Boyer, 2008
Smith and Haney, 2006
Williams et al., 1997
Wood et al., 2006
MeOH:BuOH
Ernst et al., 2004
Xie et al., 2005
Zhang et al., 2010
MeOH+AcOH
Poste et al., 2011
Wilson et al., 2008
MeOH + FA
Geis-Asteggianted et al.,2011
Sonication/ Cell Rupture
Water Bath Sonication
Adamovsky et al., 2007
Kohoutek et al., 2010
Kopp et al., 2013
Mekebri et al., 2009
Sipia et al., 2002
Probe Sonicator
Smith and Boyer, 2009
Ultrasonication
Ernst et al., 2004
Freeze/ Thaw
Smith and Haney, 2006

Sample Clean- UP
Solid Phase Extraction
Bieczynski et al., 2013
Ernst et al., 2004
Magalhaes et al., 2001
Magalhaes et al., 2003
Mekebri et al., 2009
Peng et al., 2010
Sipia et al., 2002
Wood et al., 2006
Xie et al., 2005
Zhang et al., 2010
Mixed with Hexane
Adamovsky et al., 2007
Geis-Asteggianted et al., 2011
Magalhaes et al., 2001
Magalhaes et al., 2003
Kohoutek et al., 2010
Kopp et al., 2013
Wood et al., 2006
Filtered
Magalhaes et al., 2001
Magalhaes et al., 2003
Sipia et al., 2002
Smith and Haney, 2006
Wood et al., 2006
Solvent Evaporated
Compressed Nitrogen
Bieczynski et al., 2013
Ernst et al., 2004
Sipia et al., 2002
Lyopholized
Smith and Haney, 2006
Vacuum Centrifuge
Williams et al., 1997
Rotary Evaporation
Moreno et al., 2005
Peng et al., 2010
Smith and Boyer, 2008
Sample Rewetted
Water
Adamovsky et al., 2007
Bieczynski et al., 2013
Moreno et al., 2005
Peng et al., 2010
Poste et al., 2011
Sipia et al., 2002
Smith and Haney, 2006
Wilson et al., 2008
Zhang et al., 2010
MeOH
Magalhaes et al., 2001
Magalhaes et al., 2003
Moreno et al., 2005
Kohoutek et al., 2010
Kopp et al., 2013
Smith and Boyer, 2008
Wood et al., 2006
Xie et al., 2005
Chemical Analysis
ELISA
Adamovsky et al., 2007
Geis-Asteggianted et al., 2011
Magalhaes et al., 2001
Magalhaes et al., 2003
Peng et al., 2010
Sipia et al., 2002
Smith and Haney, 2006
Wilson et al., 1997
Wood et al., 2006
LC-MS
Geis-Asteggianted et al., 2011
Zhang et al., 2010
LC-MS/MS
Kohoutek et al., 2010
Kopp et al., 2013
Sipia et al., 2002
HPLC (& HPLC-MS)
Ernst et al., 2004
Peng et al., 2010
Smith and Boyer, 2008
Xie et al., 2005
PPIA & GC/MS
Bieczynski et al., 2013
Williams et al., 1997

Figure 5: Microcystin chemical analysis extraction protocols where each box represents a separate stage of the extraction protocol and different methods for each stage with the corresponding work utilizing that technique, listed in the corresponding box. Note that not all papers explicitly state the technique used for each stage, and therefore works utilizing each technique are only listed in the figure, if clearly stated in the research paper.

4.1.3 ELISA

ELISA is a chemical analysis that quantifies total microcystin via competitive binding to anti-body coated microplates. Microplates coated with antibodies conducive to microcystin binding are purchased from chemical suppliers (Envirologix, Portland, ME and Abraxis, Warminster, PA to name a few) along with a range of standards of known microcystin concentration. The analysis begins by placing the solvent containing the microcystin extracted from samples (See section 4.1.2 Extraction Protocols for additional information) into separate wells within the coated plate along with supplied standards containing known concentrations of microcystin. The microcystin in the solvent and standard binds to the antibodies lining the microplate well until either all the antibody binding locations are saturated or all the toxin in the solvent is bound. After a set time frame, a competitive enzyme is introduced into the well. The competitive enzyme binds to any antibody binding sites remaining. A color reagent that binds to the competitive enzyme is placed in each well, changing the solvent. After a stop solution is added to prevent further binding, plates are read on a microplate reader (See Figure 3 for additional details). The amount of light absorbance is recorded for all samples and standards from the microplate reader. The color reagent binds to the competitor, so darker colors indicate smaller concentrations of microcystin. A standard curve is determined from the standards (concentration versus absorbance) and unknown concentrations for each sample are determined from the standard curve.

Although ELISA can be an effective way to quantify microcystin, it has several disadvantages that must be considered.ELISA analyses do not have the ability to differentiate between the different variations of microcystin toxin (MC-LR, MC-RR, MC-LF, etc.), because the method indiscriminately quantifies anything that has the ability to bind to the antibodies

coating the microplate well (i.e. the antibodies targeting a particular section of the microcystin structure could bind to other compounds with a similar chemical composition). This observed indiscriminate quantification also lends the test vulnerable to error. ELISA tests are known to have high rates of false positives (Schmidt et al., 2013), due to interactive effects between non-microcystin compounds in fish tissue (such as humic material, protiens, and biological macromolecules; Preece et al., 2015) and the antibody coating on the microplate wells. These effects therefore limit the interpretation of low ELISA readings and it has been suggested that samples containing less than 5.9 µg/ kg dry weight are possibly due solely to interactive effects rather than presence of microcystin (Schmidt et al., 2014). Additionally, partially metabolized conjugates of the microcystin toxin (ie. Microcystin-LR-GSH, Microcystin-LR-cysteine-glycine, and Microcystin-LR cysteine) have the affinity to bind to the antibodies in the ELISA wells; however, these metabolites have lower toxicity rates than pure microcystin-LR, therefore, readings including metabolites give ELISA readings an overinflated toxicity level (Metcalf et al., 2000). Likewise, although most kits attempt to target microcystin-LR, they often bind with other microcystin congeners that are not as potent, again resulting in inflated toxicity readings (McElhiney and Lawton, 2005). The inability to differentiate between congeners and the propensity for false positives, although limiting, are coupled with benefits. ELISA is considered a rapid analysis that has a relatively low detection rate in comparison to other microcystin analyses(see Ch. 2 section 2.3.4 ELISA AQ/QC and section 2.3.6 LC-MS/MS QA/QC). Considering these limitations, ELISA is appropriate when analyzing samples with low concentrations of microcystin, and when quantifying specific congeners is not necessary.

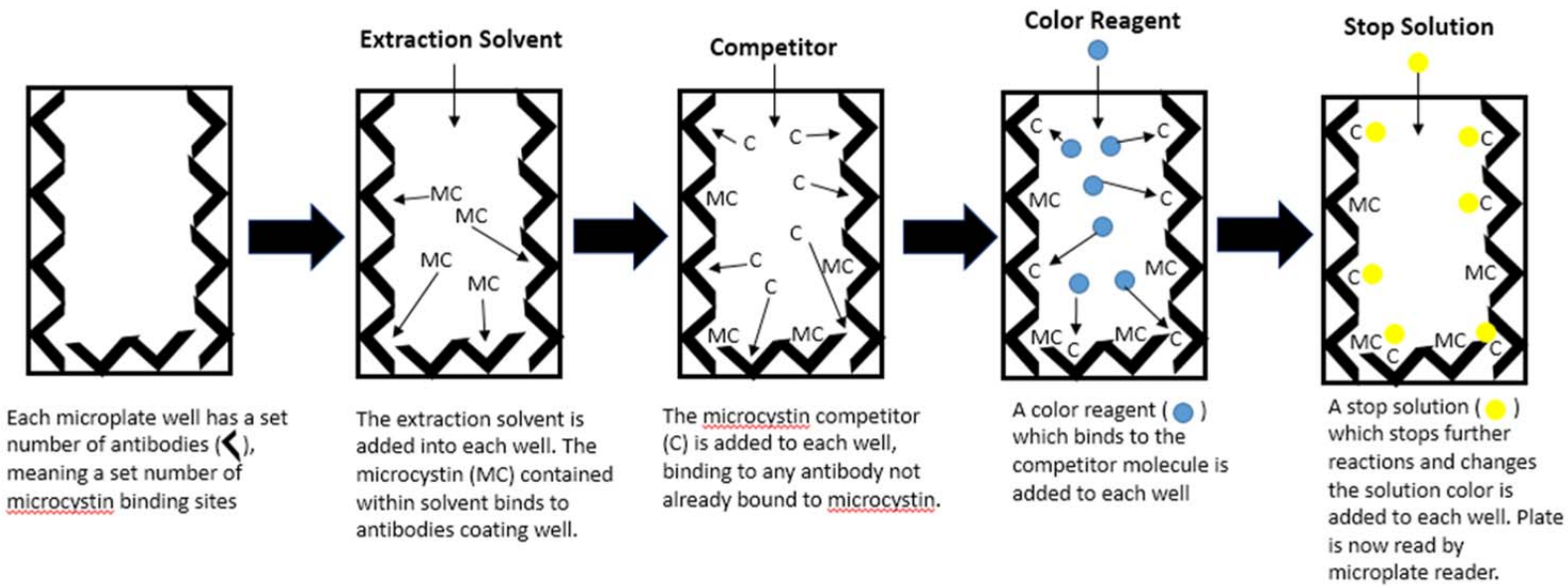


Figure 6: Schematic depiction of enzyme linked immune sorbent assay process for microcystin detection. Enzyme linked immune sorbent assay (ELISA) kits are microplate well kits, containing wells coated with antibodies capable of binding to features of the microcystin molecule. The rectangles pictured above represent a cross section of a single microplate well.

4.1.4 LC-MS

Liquid chromatography coupled with mass spectrometry (LC-MS) is a chemical analysis that quantifies the different congeners of microcystin through separating the congeners based on their different polarities. A steady stream of extracted sample (see section 4.2.1 Extraction protocols for additional details) is run through a chromatography mobile phase (the two most common being ammonium acetate: acetonitrile and trifluoroacetic acid:acetonitrile), which causes the various congeners of microcystin to separate based on polarity (Rapala et al., 2002). Congeners with higher polarities (ie. MC-RR, MC-LR) exit the column first, followed by less polar variants (ie. MC-LF, MC-LW). A sample exiting the mobile phase proceeds directly to the mass spectrometry machine for quantification via ionization. The mass spectrometer bombards the sample with electrons, causing individual molecules to become ions. The newly created ions are accelerated down a chamber and are deflected by a magnetic force before reaching the end of the acceleration chamber which houses electrons. Larger molecules (larger variants of microcystin) are deflected a shorter distance than smaller molecules. The distance of deflection is recorded by noting the location where electrons leave the end of the acceleration plate to join the positively charged ion.

Samples run through liquid chromatography coupled with one phase of mass spectrometry (LC-MS) have the propensity for false positives, because molecules other than microcystin can have similar molecular weights to microcystin, (Kohoutek et al., 2010) and therefore samples are often run through an additional mass spectrometry phase, resulting in liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Microcystin concentrations are calculated from LC-MS analyses by taking the area under the curve, generated as the toxin leaves the liquid chromatography machine. Upon leaving the first mass spectrometry phase, the sample is hit with

an ionization spray that causes the microcystin molecule to fracture in a predictable pattern. Several of the fractured pieces can be identified with the second mass spectrometry reading by their unique molecule weights. Ultimately, only molecules that exhibit all fractured pieces are counted as microcystin, ensuring that values read by the liquid chromatography are in fact microcystin and not tissue compounds with similar properties to microcystin (Boyer, Personal Communication). The ability for some data validation is one of the primary benefits of using LC-MS or LC-MS/MS over other chemical analyses.

Liquid chromatography coupled with mass spectrometry (LC-MS) is a chemical analysis that has complementary advantages and disadvantages to ELISA. Some of the negative attributes concerning LC-MS or LC-MS/MS involve the longer analysis time and the heightened level of expertise necessary to operate the chemical equipment for this test. Additionally, LC-MS/MS has a higher limit of detection than ELISA (0.187 $\mu g\ kg^{-1}$ versus 11 $\mu g\ kg^{-1}$ in the present study), restricting the quantification of microcystin in samples with low toxin concentrations. The primary advantage to running LC-MS or LC-MS/MS in addition to, or in lieu of, ELISA, is the ability to differentiate microcystin congeners. Any congener or metabolite that has an analytical pure standard can be quantified with LC-MS/MS. An additional benefit of LC-MS/MS is the ability to check samples against multiple ion channels for sample validation (ie. reduce risk of false positives).

4.1.5 Quantification of Bound versus Free Microcystin

A large quantity of the microcystin contained within an organism is irreversibly bound to protein phosphatases. Research conducted on snails estimated that on average, 44% of the total microcystin measured was bound (Lance et al., 2010). This fraction of microcystin is difficult because it cannot be extracted with organic solvents (such as the ones used in the methods

above). Methods for detecting bound microcystin have been developed (Williams et al., 1997a), and several research efforts have quantified bound microcystin (Lance et al., 2010; Pires et al., 2004; Williams et al., 1997 a,b,c), however, the vast majority of research utilizes methods that do not account for the bound microcystin fraction. This suggests that the total amount of microcystin quantified in aquatic organisms is significantly underestimated. This potential underestimation is believed to have little relevance to toxicology studies, because it is believed that the bound fraction (i.e. the fraction not measured by most tests) is not bioavailable and therefore not harmful to organisms ingesting it (Ibelings and Chorus, 2007).

4.2 Extended Methodology

4.2.1 Detailed ELISA Extraction Protocol

- Step 1: Excise a piece of fresh fish tissue, and place in a 20 mL wheaton glass scintillation vial
    - The exact amount of tissue used is not important, however, the vial should be close to 70% filled because multiple runs will need to be done on each sample. Leave air pockets in vial (~30% of space), open so that there is space for moisture to leave sample. Chunks of excised tissue should be thin and narrow in order to increase surface area, and subsequently decrease drying time. Fish skin should be removed prior to drying if the species being analyzed is generally consumed without skin.
- Step 2: Weigh Sample
    - Prior to putting sample in the wheaton glass vial, weigh the vial empty to determine vial weight. After fresh tissue has been placed in the vial, weigh again.

Subtract the weight of vial from the weight of vial plus tissue to determine starting wet weight.

- Step 3: Lyopholize tissue for approximately 72 hours
    - After determining wet weight, place a piece of tin foil (shiny side down) over the top of the un-covered glass scintillation vial. Poke several holes in the tin foil with a pin to allow moisture to escape from sample .
    - Samples should be lyopholized with a pressure of 0.0012 mbar and a temperature between -52℃ and -53℃.
    - Samples must be completely frozen prior to being lyopholized
- Step 4: Weigh Sample
    - Upon removing from the lyopholizer, weigh sample (without the tin foil cover). Subtract the vial weight from the resulting weight in order to determine dry weight.
    - The proportion of moisture lost can now be determined by dividing the sample's wet weight by dry weight. The proportion of moisture lost is important to know when back calculating the concentration of toxin in the dry matter to the concentration of toxin in wet tissue (which is the medium people generally consume).
- Step 5: Homogenize Tissue in Powder

- Place dried tissue into a mortar and use a pestal to grind the tissue into a fine powder. Note: an automated homogenizer can be used in place of a mortar and pestal if available.
- The mortar and pestal should be wiped clean and gently rinsed with at least 50% MeOH in between each sample.

- Step 6: Weigh out dry sample
  - Weigh roughly 400 mg of fish powder into a 5 mL glass scintillation vial.
  - The weight used in a given analysis is completely contingent on the concentration of toxin in the sample. Since ELISA kits tend to have a narrow range of detection (dictated by the standards supplied by the company), it is important to determine what weight of sample will allow a given sample to fall within the allotted curve. The appropriate weight can only be determined by referencing literature of similar study sites and pilot runs. Running a few samples of each species, from each location, at varying weights is a good way to establish appropriate sample weights. Samples with very low concentrations of toxin can also be manipulated into the standard curve by diluting the standards provided by the company (instructions for this process are included in ELISA instructions).
- Step 7: Add 2 mL of 80% aqueous MeOH to each sample
  - Varying levels of MeOH as well as other solvents can be used in this step (see Chapter 3, Extraction Protocols for more information).
- Step 8: Homogenize sample with automated homogenizing system for 1 minute on ice.

- This step is done to ensure that the fish tissue is completely homogenous and is not necessary if an automated homogenizer was used in place of a pestle and mortar. The automated homogenizing system available at the time of this research project was meant to homogenize substrate in solution, which is why this step was added as is.

- Step 9: Sonicate sample at 400 watts for 1 minute on ice
- Step 10: Allow samples to sit for 24 hours, in the dark, at room temperature
- Step 11: Centrifuge samples for 10 minutes
    - The centrifuge available at the time of this study only reached roughly 8,000 rpm. Ideally, a centrifuge with more power would be used. Literature cites usage ranging from 1300 rpm (Sipia et al., 2002) to 24,000 rpm (Smith and Boyer, 2008).
- Step 12: Decant 150 µL of the supernatant into a clean 5 mL glass scintillation vial
- Step 13: Evaporate solution to dryness
    - In the present study, samples were evaporated to dryness in order to concentrate the toxin, because very low levels of toxin were present. If higher concentrations of toxin are present then samples may need to be diluted with water prior to analysis. Whether to concentrate or dilute samples is determined with pilot analyses.
    - Samples can be evaporated to dryness in a variety of ways (See 4.1.2, Extraction Protocols for more details). In the present study, some samples were dried by

running compressed nitrogen through an evaporation manifold into each vial. The remaining samples were dried by placing scintillation vials into a 400 mL glass beaker fitted with a rubber stopper. A small hole was drilled into the top of the stopper and a rubber tube was snuggly fitted through the hole. The beaker was placed on a hot plate set to 60℃ and the tube was hooked to a vacuum.

- Step 14: Reconstitute dried crystals into 40 µL of 20% aqueous MeOH
    - 20% aqueous MeOH was purposively chosen, because MeOH has a high affinity for microcystin (due to its polarity) and is therefore a good solvent. However, MeOH solutions above 20% have not been tested for cross reactivity in ELISA kits. Since, 20% aqueous MeOH is the highest concentration deemed acceptable by EnvironLogix, it was the solvent used.
- Step 15: Place samples on orbital shaker for 2 minutes
    - This step ensures that the reconstituted crystals are homogenized in the aqueous MeOH.
- Step 16: Pipette Sample into microplate and follow ELISA instructions
    - The EnviroLogix instructions recommend using a multichannel pipettor to add samples into the microplate wells. Unfortunately, the spacing of the channels on the multichannel pipettor requires that samples be first pipetted from the scintillation vial to a microwell plate. Microcystin has a high affinity to plastic Pipette tips, which are made of plastic, and have been shown to reduce toxin recovery rates (determined to be 4.2% lost per pipette transfer) (Hyenstrand et al., 2001). For this reason, a single channel micropipette was used to transfer samples

one at a time into each well, in order to minimize the number of pipette exposures. This technique, although helpful at reducing adsorption to plastic, also limits the number of wells that could be run in one analysis. Since the Envirologix instructions indicate that the last sample must be added 10 minutes from the addition of the first sample, no more than 32 samples could be run in one analysis.

4.2.2 Detailed LC-MS/MS Extraction Protocol

The methodology presented below was created by Juliette Smith (2008) from Gregory Boyer's Lab at SUNY ESF. Minor amendments have been made to the procedure for the present study.

- Step 1-4: Same as ELISA extraction protocol
- Step 5: Add 400 mg of dry tissue to a plastic centrifuge tube along with 0.5 mL of milli-q water
    - The protocol originally called for 100 mg, however, this was changed to match what was used for ELISA analysis. At this stage of the protocol tissue could be added to a plastic container because a high percentage of MeOH was added immediately after addition of tissue and water. Although microcystin has a high affinity for plastic, it has a higher affinity for high percentages of MeOH. Sample containing microcystin will experience negligible losses with an aqueous MeOH solvent of at least 25% (Codd, G., Personal Communication).
- Step 6: Add 5 mL of 80% MeOH
    - 80% MeOH was used for the same reasons outlined in step 7 of detailed ELISA Extraction Protocol. Five mL is used in this protocol to accommodate potentially higher quantities of microcystin (i.e. to ensure that the solvent is not saturated). Considering the low concentrations of microcystin present in the present studies

sample, 2 mL (as used in the ELISA protocol) or 5 mL would be equally sufficient for extracting all the microcystin.

- Step 7: Sonicate for 1 minute on ice (30 seconds on, 30 seconds off, 30 second on), then rinse tip with MeOH into container.
    - Pausing sonication for 30 seconds midway through was done to keep the sample from getting too warm. Although this was not done in the ELISA extraction protocol, the heat incurred from continuous sonication would not affect the toxins being detected (Boyer, G., Personal Communication). The end of the sonication probe was rinsed into the centrifuge tube to ensure no toxin was lost. This step could feasibly be done in the LC-MS/MS extraction protocol because the entire volume of solvent was evaporated off. Evaporating off all the solvent meant that the solution being sonicated did not have to be a known volume, therefore allowing the unknown addition of MeOH through rinsing. Conversely, the ELISA extraction protocol evaporated off only a subsample of the solvent sonicated (due to equipment limitations), and therefore a known volume of solvent was needed.
- Step 8: Place samples in -20℃ freezer for 30 minutes
- Step 9: Centrifuge samples for 15 minutes at 14,000 rpm at -5℃
    - This step fulfills the same goal as step 11 of ELISA Extraction Protocol. High rpm and temperature control were utilized in this protocol due to availability of more advanced equipment.
- Step 10: Decant supernatant into 8 mL glass tube using glass pasteur pipette

    - Analogous to step 12 of the ELISA Extraction Protocol, except that all of the supernatant was decanted in the LC-MS/MS Extraction Protocol versus only a subset of supernatant being decanted in the ELISA Extraction Protocol.
- Step 11: Samples were placed in a speed vac (vacuum centrifuge) and evaporated to dryness
    - Analogous to step 13 of ELISA Extraction Protocol
- Step 12: Reconstitute microcystin crystals into 1 mL of 80% MeOH and vortex each sample for 20 secs.
    - Analogous to step 14 and 15 of ELISA Extraction Protocol, except different percentages of MeOH and equipment were used. High percentages of MeOH were restricted from use in the ELISA Extraction Protocol due to the ELISA kit's inability to handle such solvents. This same limitation is not present in the LC-MS/MS protocol and therefore higher percentages of aqueous MeOH were used.
    - A vortex shaker was used in the LC-MS/MS Extraction Protocol because the sample containers used had caps to prevent splashing. Conversely, the scintillation vials used did not have caps, and therefore the orbital shaker was used in place of the vortex shaker to prevent spilling.
- Step 13: Place samples in -20℃ freezer for 30 minutes
- Step 14: Centrifuge samples for 10 minutes at 3,000 rpm at room temperature in a swinging bucket rotor.
    - Samples were centrifuged again to remove any particulates from the overlying supernatant. This extra centrifuge step is necessary for LC-MS/MS analyses which are very sensitive to particulate matter.

- Step 15: Transfer supernatant with a glass pasteur pipette, avoiding precipitate into a 1 mL autosampler vial and place into LC-MS/MS autosampler
    - An additional centrifuge step may be required if additional particulate matter is visible in the solvent.
    - Samples should be stored at -20℃ until ready for analysis

4.3 References

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Appendix

A.1 Sampling Maps

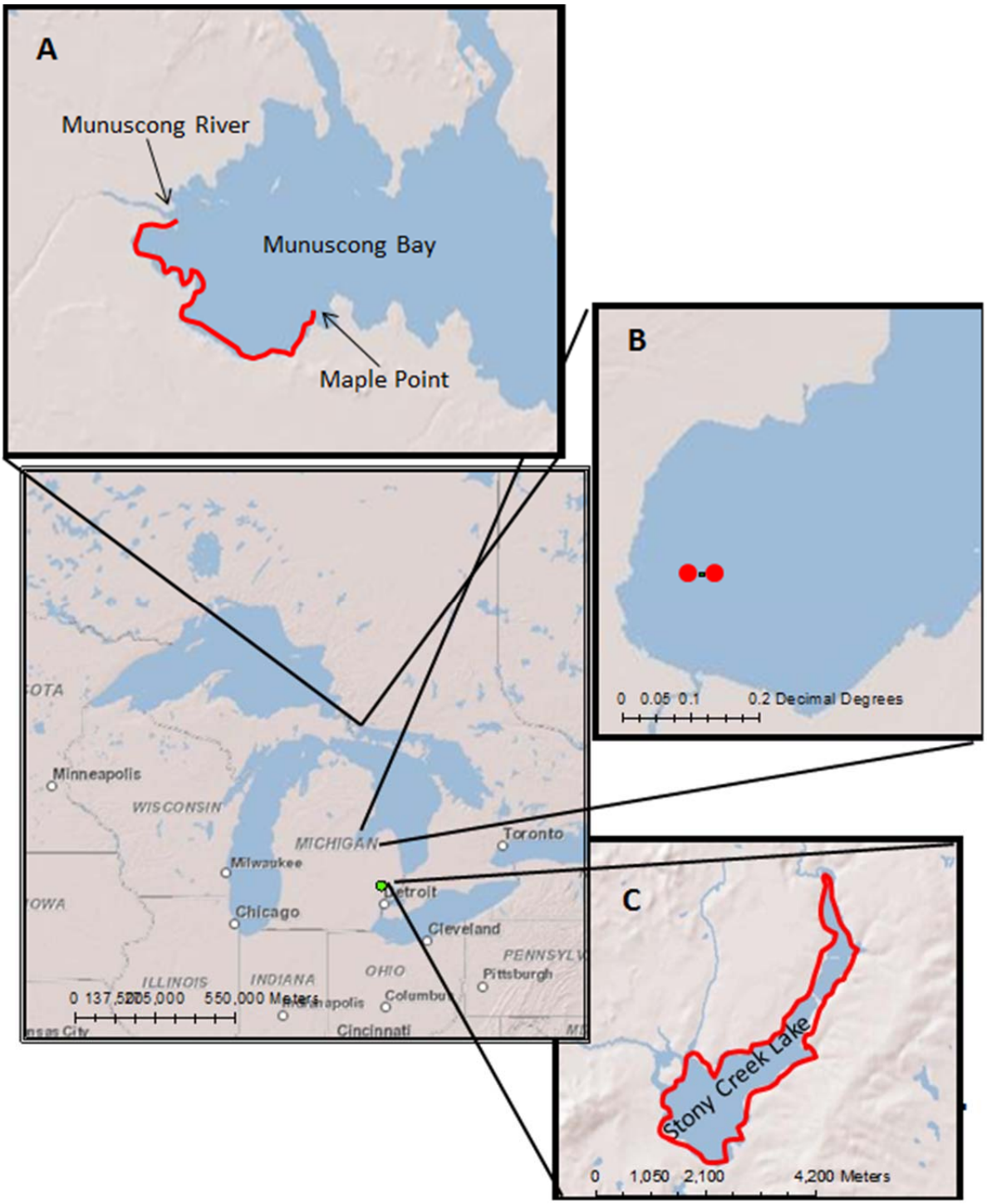


Figure 7: St. Mary's River, Stony Creek Lake, and Saginaw Bay Fish Sampling map for 2014. Sampling locations from 2014 depicted in red, including St. Mary's River (A): white perch and northern pike, Saginaw Bay (B): channel catfish, walleye and white bass, and Stony Creek Lake (C): carp, rock bass, and northern pike.

Table 3: Microcystin concentrations quantified using ELISA in white bass (n=10), walleye (n=9), and channel catfish (n=7) collected from Saginaw Bay on September 8, 2014 by the DNR. . True duplicate runs were performed on all samples except those with Run 2 listed as NA. Cells highlighted in yellow had ELISA readings below the tests detection limit.

ID	Species	Length (cm)	Weight (g)	Run 1 (µg MC/ g tissue)	Run 2 (µg MC/ g tissue)	Average (µg MC/ g tissue)
11-211	White bass	28.6	320	0.000230227	0.000210596	0.000220412
12-211	White bass	29.4	310	0.000638333	0.000677877	0.000658105
13-211	White bass	30.8	390	0.000147385	0.000129417	0.000138401
14-211	White bass	30.6	390	5.26946E-05	NA	5.26946E-05
15-211	White bass	29	340	9.24363E-05	0.000184321	0.000138379
16-211	White bass	29.6	340	0.000290572	0.000195982	0.000243277
17-211	White bass	32.8	520	0.000295647	0.000238417	0.000267032
18-211	White bass	NA	NA	0.00033334	0.000294311	0.000313826
19-211	White bass	36.7	620	0.000313931	0.000217725	0.000265828
20-211	White bass	37	680	0.000109902	0.000104888	0.000107395
22-211	Walleye	41.5	560	0.000110574	NA	0.000110574
23-211	Walleye	42.2	590	0.000195938	NA	0.000195938
24-211	Walleye	39.6	570	9.17065E-05	NA	9.17065E-05
25-211	Walleye	44.5	740	0.000242557	0.00011499	0.000178774
26-211	Walleye	47.5	890	0.000113252	0.000193235	0.000153244
27-211	Walleye	44.3	970	0.0002464	0.000213304	0.000229852
28-211	Walleye	44.5	700	0.000387178	0.000446253	0.000416716
29-211	Walleye	48.1	700	0.00030235	0.000331513	0.000316932
30-211	Walleye	46	840	0.000420517	0.000330687	0.000375602
31-211	Channel Catfish	37.5	430	0.000280262	0.000285485	0.000282874
32-211	Channel Catfish	40.2	540	9.41562E-05	0	4.70781E-05
33-211	Channel Catfish	39	610	0.000146923	0.000152221	0.000149572
35-211	Channel Catfish	41	600	0.000327246	0.000344302	0.000335774
36-211	Channel Catfish	44	800	0.000171848	0.000164013	0.000167931
38-211	Channel Catfish	53	1330	0.000190457	NA	0.000190457
39-211	Channel Catfish	55	1850	0	0.000155913	7.79565E-05

Table 4: Microcystin concentrations quantified using ELISA in carp (n=4), rockbass (n=5), and northern pike (n=2) collected from Stony Creek Lake on October 14, 2014 by the MDEQ. Cells highlighted in yellow had ELISA readings below the tests detection limit.

ID	Species	Length (cm)	Weight (g)	Run 1 (µg MC/ g tissue)	Run 2 (µg MC/ g tissue)	Average (µg MC/ g tissue)
12-212	Carp	55	2560	0.00010799	0.000137002	0.000122496
16-212	Carp	56.05	2580	0.000249316	0.000223524	0.00023642
17-212	Carp	61	3670	0.00020314	0.00022914	0.00021614
19-212	Carp	64.9	3850	0.000142421	0.000186271	0.000164346
3-212	Rock bass	22.2	220	0	0	0
4-212	Rock bass	22.5	250	5.52121E-05	NA	5.52121E-05
5-212	Rock bass	22.6	270	3.63679E-05	0	1.8184E-05
6-212	Rock bass	23	290	4.29648E-05	0	2.14824E-05
8-212	Rock bass	23.5	300	0	0	0
41-212	Northern Pike	61.3	1160	8.80929E-05	9.88749E-05	9.34839E-05
42-212	Northern Pike	55.5	1020	0.000148452	0.000109095	0.000128774

Table 5: Microcystin concentrations quantified using ELISA in northern pike (n=10) and white perch (n=8) collected from St. Mary's River on June 6, 2014 by the MDEQ. True duplicate runs were performed on all samples except those with Run 2 listed as NA. Cells highlighted in yellow had ELISA readings below the tests detection limit

ID	Species	Length (cm)	Weight (g)	Run 1 (µg MC/ g tissue)	Run 2 (µg MC/ g tissue)	Average (µg MC/ g tissue)
10-270	White Perch	29	430	5.61404E-05	3.92768E-05	4.77086E-05
1-270	White Perch	21.5	120	0	0	0
21-270	Northern Pike	62.5	1070	0.000399513	NA	0.000399513
22-270	Northern Pike	59	1110	0.000199843	0.000176704	0.000188274
23-270	Northern Pike	65.8	1700	0.000193186	0.000153127	0.000173157
24-270	Northern Pike	69.5	1940	0.000296732	0.000262573	0.000279653
25-270	Northern Pike	71.6	1980	0.000202566	NA	0.000202566
26-270	Northern Pike	46	540	0.000757031	NA	0.000757031
27-270	Northern Pike	54.5	810	5.06761E-05	0.000710313	0.000380495
28-270	Northern Pike	69	1720	0.00025213	0.000650097	0.000451114
29-270	Northern Pike	79.2	3310	6.39665E-05	4.77857E-05	5.58761E-05
30-270	Northern Pike	87.5	4370	4.70834E-05	5.71066E-05	0.000052095
3-270	White Perch	23.6	210	0	7.83081E-05	3.91541E-05
4-270	White Perch	23	190	0.000141379	6.23195E-05	0.000101849
5-270	White Perch	25.2	250	0.000200146	0.000144464	0.000172305
6-270	White Perch	23.3	200	4.35809E-05	7.41581E-05	5.88695E-05
7-270	White Perch	25.7	290	0.000226279	0.000101255	0.000163767
8-270	White Perch	27	310	6.07434E-05	9.02378E-05	7.54906E-05
9-270	White Perch	28.4	340	0.000330047	4.64104E-05	0.000188229

Table 6: Microcystin concentrations quantified using ELISA in captive raised channel catfish (n= 4) collected from the Upper Midwest Environmental Science Center. Cells highlighted in yellow had ELISA readings below the tests detection limit.

Negative Controls (µg MC/ kg tissue)		
Sample	Run 1	Run 2
NC CCF 8	5.81E-05	0
NC CCF 10	4.88E-05	0
NC CCF 2	0	0
NC CCF 3	4.46E-05	0