CYANOTOXINS IN FISH AT VOYAGEURS NATIONAL PARK

by

SETH MCWHORTER

(Under the Direction of Susan Wilde)

ABSTRACT

Eutrophication leads to the occurrence of harmful cyanobacterial algal blooms at Lake Kabetogama in Voyageurs National Park that release cyanotoxins. We measured toxins accumulated in fish from Lake Kabetogama. We extracted tissues from whole young of year fish as well as the liver and muscle from adult fish. Tissue concentrations were measured using enzyme-linked immunosorbent assays and liquid chromatography tandem mass spectrometry. Most enzyme-linked immunosorbent assay toxin results were above the upper limit of detection of 5.0ppb, and all but six liquid chromatography tandem mass spectrometry microcystin-LR samples were below the lower limits of detection, which varied per sample. Five samples were Smallmouth Bass, five samples were at sites near deep areas that internally load phosphorus, and six samples were in livers. Levels of microcystin and anatoxin-a in fish were low enough as to not be of risk to humans, but wildlife could be at risk.

INDEX WORDS: Anatoxin-a; cyanobacteria; cyanotoxins; fish tissue extraction; harmful algal blooms; Lake Kabetogama; microcystin; Rainy Lake; saxitoxin; Voyageurs National Park

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DEDICATION

I hereby dedicate this labor to my lovely wife Elizabeth French for her undying support throughout my adventure in graduate school. Through her constant motivation, I forged opportunities that furthered my education and career. I would also like to dedicate this thesis to my loving and supportive parents and grandparents, who instilled in me a love and respect for the natural world during my developmental years. Finally, I dedicate this to my closest friend Carlos Santiago, as he wants to read this for some reason, and I don't want him to feel left out. Without him, I might have had straight A's in my undergraduate schooling.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction

Increased nutrient loading— from anthropogenic and environmental sources— causes higher productivity and the eutrophication of lake systems. Eutrophication increases occurrence of cyanobacterial algal blooms. Some cyanobacterial algal blooms become cyanobacterial harmful algal blooms (cHABs) that release cyanotoxins into the water. Harmful blooms are forecasted to increase in severity due to climate change stressors and eutrophication (Chapra et al. 2017; O'Neil et al. 2012). An increase in the occurrence of cHABs could negatively impact the health of organisms, including humans, living near blooms sites. Humans may encounter cHABs through recreational aquatic activities such as swimming, kayaking, or angling. Some cyanotoxins accumulate in the tissues of fish (Hardy et al. 2015), exposing anglers and wildlife that consume contaminated organisms to the toxins.

One area impacted by cHABs is Voyageurs National Park (VOYA). The two largest bodies of water at Voyageurs National Park are Rainy Lake and Namakan Reservoir. Namakan Reservoir is comprised of Namakan, Sand Point, Crane, Kabetogama, and Little Vermilion Lakes. Little Vermilion lies outside VOYA boundaries while Crane Lake is partially outside VOYA boundaries. Rainy, Namakan, and Sand Point Lakes have portions inside US and Canadian borders. The two systems were natural lakes before being dammed in the early 1900s (Kallemeyn et al. 2003). Most people visit VOYA for angling, as the lakes contain a variety of fish species, including Northern Pike, Smallmouth Bass, and Walleye. Rainy, Namakan, and

Sand Point Lakes are oligotrophic, although Lake Kabetogama is borderline eutrophic, as is Black Bay on Rainy Lake (Christensen and Maki 2015).

Internal loading of phosphorus from iron-poor soil in deep regions of Lake Kabetogama leads to nitrogen limitation (Christensen et al. 2013), which can increase occurrences of cHABs. The toxin producing genes *mcyE*, *sxtA*, and *anaC*, which correspond with the toxins microcystin, saxitoxin, and anatoxin-a respectively, were identified in species of cyanobacteria at VOYA, and all three toxins have been detected in water samples (Christensen 2019). These three toxins all accumulate in fish tissues (Magalhães et al. 2003; Testai et al. 2016; Pawlik-Skowrońska et al. 2013). If humans or wildlife eat contaminated fish, their health may be at risk. We measured cyanotoxin levels in fish muscle and liver tissues of adult fish and whole young of year (YOY) fish and determined whether there is a risk to human and wildlife health from fish consumption based on the cyanotoxin concentrations. The further understanding of microcystin, saxitoxin, and anatoxin-a is a high priority in aquatic resource management at VOYA.

Background

Chemistry and Toxicology

The three toxins found at VOYA present different health risks stemming from differences in their chemistry. The structure of microcystin is a monocyclic heptapeptide, and toxicity occurs due to an unusual amino acid in position 5: (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Dawson 1998). A difference in stereochemistry of the conjugated double bond in Adda determines the level of microcystin toxicity (Trogen et al. 1996). Microcystin functions as a hepatotoxin (targeting the liver) and is taken into hepatocytes via a carrier-mediated transport system, where it inhibits the production of eukaryotic protein phosphatase types 1 and 2A, causing excessive phosphorylation of cytoskeletal filaments

(Honkanen et al. 1990). The imbalance in protein phosphorylation leads to hepatic hemorrhage and sometimes death. Microcystin may also cause gastroenteritis, allergic reactions, and tumor promotion (Codd and Bell 1999). The lethal dose from microcystin varies greatly among structures, but the most common isoform, microcystin-LR, causes death in mice at 50 μg/kg (Krishnamurthy et al. 1986).

Saxitoxin is a trialkyl tetrahydropurine with variations in structure at four positions where it may be hydroxylated, sulfated, or carbamoylated (Llewellyn et al. 2006). Toxicity of the varying structures differ—for example, carbamate structure is 10-100 times more potent than the *N*-sulfo-carbamoyl structure (Anderson et al. 1990). However, *N*-sulfo-carbamoyl is highly labile and may be converted to a toxic structure (Cembella et al. 1994). Saxitoxin is a neurotoxin that blocks voltage gated sodium channels in neuronal cell membranes (Kao et al. 1986). Symptoms of saxitoxin poisoning start with the numbing of the face and escalate to perspiration, vomiting, and diarrhea (Llewellyn et al. 2006). Acute poisoning can lead to paralysis. Lethal levels of saxitoxin cause cardiovascular failure from respiratory muscle paralysis (Llewellyn et al. 2006). Human deaths associated with saxitoxin have occurred from quantities as small as 1 mg (Evans 1969).

Anatoxin-a is another neurotoxin. It is a homotropane alkaloid derivative with the structural name 2-acetyl- 9-azabicyclo[4.2.1.]non-2-ene (Devlin et al. 1977). Once anatoxin-a is consumed, it functions as a potent cholinergic agonist that competes for acetylcholine receptors, causing the overstimulation of muscles (Campos et al. 2006; Carmichael 1994). Muscle overstimulation can cause respiratory arrest, leading to death (Spivak et al. 1980). Lethal doses in rats are as low as 0.2 mg/kg, and death may occur as fast as two minutes (Stolerman et al. 1992).

Occurrences in Freshwater Ecosystems

The presence of these toxins can cause negative effects in the freshwater ecosystems they contaminate. Microcystin is produced by the genera of cyanobacteria *Microcystis, Anabaena, Oscillatoria,* and *Nostoc,* though some species in these genera are unable to produce microcystin. Microcystin production in freshwater is influenced by abundance or biomass of toxin-producing species, presence of non-toxin-producing cyanobacteria, phosphorous and nitrogen content and ratios, light availability, and other environmental variables (Zurawell et al. 2005; Chaffin et al. 2018). Occurrences of microcystin in freshwater are thought to be the cause of several mass casualty events at severely impacted sites in organisms from different trophic levels including macroinvertebrates and fish (Zurawell et al. 2005).

Hepatotoxic blooms are more common than neurotoxic blooms in freshwater (Rapala and Sivonen 1998). There are few reported cases of neurotoxic blooms causing negative effects in freshwater ecosystems. Freshwater occurrences of saxitoxins are generally linked with the cyanobacteria *Anabaena circinalis, Aphanizomenon sp., Aphanizomenon gracile, Cylindrospermopsis raciborskii* and *Lyngbya wollei* (Carmichael et al. 1994). Saxitoxin production is linked with several environmental factors including alkalinity, high ammonia content, and high conductivity (Brentano et al. 2016). Laboratory studies indicated that temperature, light, pH, salinity, and nutrient concentrations may also affect saxitoxin production (Sivonen and Jones 1999). Negative impacts in marine environments suggest saxitoxin may negatively impact wildlife through all trophic levels in freshwater ecosystems. Massive fish casualty events have been associated with saxitoxin in saltwater ecosystems (Fire et al. 2012), and recent studies suggest freshwater fish casualty events could be from saxitoxin exposure as

well (Moustaka-Gouni et al. 2017). Saxitoxin in freshwater needs further research to determine their presence and impacts on ecosystems.

Anatoxin-a is produced by the cyanobacteria genera *Anabaena, Aphanizomenon, Cylindrospermum, Oscillatoria, Microcystis, Raphidiopsis, Planktothrix, Arthrospira, Nostoc* and *Phormidium.* Due to the quick degradation of anatoxin-a, limited field research is available on the toxin. However, anatoxin-a may lead to reduced reproduction in zooplankton species, which could affect all trophic levels (Christoffersen 1996). Anatoxin-a has been linked with mass casualty events involving fish, waterfowl, cats, cattle, and dogs (Vehovszky et al. 2015; Moustaka-Gouni et al. 2009; Onodera et al. 1997; Vehovszky et al. 2015; Edwards et al. 1992; Devlin et al. 1977). Further studies are needed to determine the role of anatoxin-a in ecosystems (Testai et al. 2016).

Toxin in Animal Tissues

Humans and wildlife could potentially intake lethal doses of cyanotoxins from fish tissues if the toxins are accumulating at high rates. The ability of microcystin to accumulate has been studied extensively in lab settings, but further documentation of accumulation in the field is needed. Microcystin accumulates in the tissues of fish (Magalhães et al. 2003), shrimps (Chen and Xie 2005), gastropods (Zhang et al. 2012), and bivalves (Williams et al. 1997). Most studies measure microcystin accumulation in muscle and liver (Jia et al. 2014) because humans consume the muscle, and microcystin accumulates at high rates in the liver. Different fish species accumulate microcystin at varying levels (Xie et al. 2005).

While freshwater accumulation of saxitoxin has not been studied extensively, saxitoxin accumulates in marine food webs, especially in shellfish (Testai et al. 2016). Though little information exists on saxitoxin accumulation in freshwater, the potential for saxitoxin presence

in freshwater biota is high. *Daphnia magna* exposed to saxitoxin accumulated the toxin, which suggests saxitoxin has the potential to transfer throughout freshwater food webs (Nogueira et al. 2004). Further study on the accumulation of saxitoxin in freshwater fish is needed.

Anatoxin-a quickly degrades, lowering the potential for accumulation in animals (Funari and Testai 2008). The few studies that documented accumulation of anatoxin-a show low concentrations in fish tissues (Testai et al. 2016). Levels of accumulation vary with concentrations of toxins and algal cell density. Accumulation is usually studied in the liver and muscle (Pawlik-Skowrońska et al. 2013). While anatoxin-a toxin concentrations are low in the literature, this finding could be from the toxin degrading quickly. Further study of the accumulation of anatoxin-a from field studies is necessary moving forward.

Cyanotoxins in Fish at VOYA

Since saxitoxin, anatoxin-a, and microcystin accumulate in fish and are present in the water at VOYA, measurements of toxins in fish tissues are important to assess the risk of fish consumption to human and wildlife health. The fish selected in this study are Yellow Perch (*Perca flavescens*), Walleye (*Stizostedon vitreum*), Smallmouth Bass (*Micropterus dolomieu*), and White Sucker (*Catostomus commersoni*). Cyanotoxins have been detected in Yellow Perch, Walleye, and Smallmouth Bass (Wilson et al. 2008; Poste et al. 2011), but there are no documented cases of toxins in White Sucker. Walleye and Smallmouth Bass are important game fish at VOYA and are frequently eaten by visitors, so the possible accumulation of cyanotoxins in their tissues is of high importance. Yellow Perch are the most ubiquitous fish at VOYA (Kallemeyn et al. 2003), and both Smallmouth Bass and Walleye predate on Yellow Perch frequently. Walleye diets at VOYA consist mainly of YOY smallmouth bass, YOY yellow perch, and darters (Kallemeyn et al. 2003). An analysis of Yellow Perch, Walleye, Smallmouth

Bass, and White Sucker will provide information on the ability of cyanotoxins to move through the aquatic food web. Little is known about the accumulation of multiple cyanotoxins in a single system, but laboratory studies suggest that some cyanotoxins act synergistically and lower the dose needed to cause death in mammals (Fitzgeorge et al. 1994). The simultaneous measurement of all three toxins found at VOYA is important in completing a risk assessment.

Our study was the first examining presence of cyanotoxins in fish at VOYA. The fish were collected near annual water quality monitoring sites on Lake Kabetogama, which experiences severe annual blooms (Figure 1.4). We analyzed microcystin and anatoxin-a concentrations in the muscle and liver of adult fish and whole YOY fish at VOYA, measured the concentration of toxins at multiple trophic levels, and completed a risk assessment on the risk of cyanotoxins at VOYA. In comparing toxin levels among site locations, we determined whether contaminated fish were found near deep P-loading sites that fuel nearby littoral cHABs or if there were contaminated fish throughout impacted water systems. We used enzyme-linked immunosorbent assays (ELISA) and liquid chromatography with tandem mass spectroscopy (LC-MS/MS) to detect toxins from fish tissue extractions (Geis-Asteggiante et al. 2012). Since ELISA may produce false positives (Guo et al. 2017; Ernst et al. 2005), we compared ELISA readings with LC-MS/MS to see if the measurements agreed. As the risk of cHABs increases, we must continue monitoring impacted sites to increase the efficacy of our extraction and analysis methods and evaluate the risk of toxins to humans and wildlife.

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CHAPTER2: MICROCYSTIN AND ANATOXIN-A IN FISH TISSUE FROM LAKE KABETOGAMA, VOYAGEURS NATIONAL PARK[1]

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Abstract

Cyanobacterial harmful algal blooms occur annually at Lake Kabetogama in Voyageurs National Park. Microcystin, anatoxin-a, and saxitoxin have previously been detected in water samples from the lake. In this study, we captured young of year and adult fish from seining and gillnetting sites in July to September of 2017 to 2018. We attempted to extract anatoxin-a and microcystin from adult fish liver, adult fish muscle, and whole young of year fish utilizing a heptane lipid cleanup technique. While we did not detect anatoxin-a, micrcocystin-LR was present in 6 out of 77 samples. All the detections were from adult fish liver, and most were from Smallmouth Bass. Five of the samples were collected at sites near deep locations that release phosphorus from soil during stratification events. Since our data were zero-inflated, resulting in large confidence intervals, further research is necessary to greater understand risk of cyanotoxins in fish tissues at Voyageurs National Park. Future studies should focus on sampling at sites near deep areas with iron-poor sediments. Our data suggests that there is no risk to human health currently from exposure via fish tissues at Voyageurs National Park, but wildlife could be at risk. We recommend continued monitoring of cyanotoxins in water and fish.

Introduction

Cyanobacterial harmful algal blooms (cHABS) are an increasing cause for concern in aquatic resource management. As annual temperatures increase worldwide, occurrences and intensity of cHABs are also increasing (O'Neil et al. 2012). Land misuse and nutrient pollution contribute to intense cHABs (Rigosi et al. 2014). Intense cHABs can become ubiquitous in water bodies, especially during summer and fall months (Wynne and Stumpf 2015). Once a bloom becomes intense, photosynthetic activity from cyanobacteria can decrease free CO_2 and increase pH, allowing for the cyanobacteria to outcompete other primary producers (Havens 2008).

Intense blooms can create anoxic conditions, potentially resulting in lower survivability of oxygen-dependent organisms (Carmichael and Boyer 2016). Depending on the species of cyanobacteria in the blooms, cHABs release potent toxins known as cyanotoxins into the water. Cyanotoxins pose health risks to humans and other organisms that encounter contaminated water (Funari and Testai 2008).

Multiple species of cyanobacteria can release multiple toxins simultaneously, and some single species dominated blooms release multiple toxins, creating potential synergistic effects on human and ecosystem health (Freitas et al. 2014). Cyanotoxin release is expected to increase in the coming years, causing economic and environmental impacts throughout the United States (Chapra et al. 2017). Cyanotoxins caused large casualty events for fish and waterfowl—and caused death of livestock and pets— in areas impacted by cHABs (Hilborn and Beasley 2015). Human lives are also at risk, as failure to effectively remove cyanotoxins from drinking water sources resulted in human casualties (Azevedo et al. 2002). Humans may encounter cyanotoxins during recreational water activities such as kayaking, boating, swimming, and fishing (Koreivienė et al. 2014). Consumption of fish caught from cHABs poses a potential health threat. Cyanotoxins accumulate in fish inhabiting cHABs (Pham and Utsumi 2018). Microcystin, anatoxin-a, and saxitoxin are three cyanotoxins previously measured in fish tissues at levels threatening to fish predators, including humans (Haddad et al. 2019).

Several genera of cyanobacteria produce hepatotoxins, neurotoxins, or dermatoxins (Carmichael 1997). Microcystin is a hepatotoxin commonly found in freshwater cHABs that creates an imbalance in protein phosphorylation, leading to hepatic hemorrhage and possible death (Campos and Vasconcelos 2010). *Microcystis, Planktothrix, Anabaena, Oscillatoria,* and *Nostoc* are the genera containing species of cyanobacteria that produce microcystin (de

Figueiredo et al. 2004). Occurrences of microcystin in freshwater resulted in mass casualty events— most notably of macroinvertebrates and fish— at some intensely impacted sites (Zurawell et al. 2005).

The neurotoxin anatoxin-a is more difficult to detect than microcystin, as anatoxin-a quickly degrades (Funari and Testai 2008). However, anatoxin-a was detected in blooms associated with bird, cat, dog, and cattle fatalities (Onodera et al. 1997; Vehovszky et al. 2015; Edwards et al. 1992; Devlin et al. 1977). Anatoxin-a disrupts acetylcholine receptors, leading to the overstimulation of muscles and potentially death (Campos et al. 2006). Anatoxin-a is produced by *Anabaena, Aphanizomenon, Cylindrospermum, Oscillatoria, Microcystis, Raphidiopsis, Planktothrix, Arthrospira, Nostoc,* and *Phormidium* (Osswald et al. 2007).

Saxitoxin, another neurotoxin, is less common in literature focusing on freshwater cyanotoxins than microcystin or anatoxin-a. As a paralytic shellfish toxin, saxitoxin is studied more in the marine environment (Testai et al. 2016). While saxitoxin has long been linked with massive casualty events in marine environments, growing evidence suggests saxitoxin threatens freshwater ecosystems with large fish kills as well (Moustaka-Gouni et al. 2017). Saxitoxin blocks voltage gated sodium channels, which at lethal levels, paralyzes respiratory muscles and causes death (Kao et al. 1986). In freshwater, saxitoxins are produced by *Anabaena circinalis, Aphanizomenon sp., Aphanizomenon gracile, Cylindrospermopsis raciborskii* and *Lyngbya wollei* (Carmichael et al. 1994).

Microcystin, anatoxin-a, and saxitoxin have occurred simultaneously in water and fish tissue (Haddad et al. 2019), though all three occurring simultaneously is rare. Lake Kabetogama at Voyageurs National Park (VOYA) experiences annual cHABs containing the species of cyanobacteria capable of producing microcystin, anatoxin-a, and saxitoxin (Christensen et al.

2019). Located in northern Minnesota on the USA-Canadian border, VOYA is an interconnected series of lakes containing two large waterbodies: Rainy Lake and Namakan Reservoir. Internal loading from deeper areas of Lake Kabetogama, one of five Namakan Reservoir lakes, could be leading to the increased occurrence of cHABs during stratification events, as these areas of this polymictic lake have high nutrient levels in sediment and water samples (Christensen et al. 2013). Quantitative polymerase chain reaction analysis (qPCR) of bloom water from Lake Kabetogama revealed cyanobacteria genomes containing genetic code for producing all three toxins (Christensen et al. 2019). Microcystins, anatoxin-a, and saxitoxins have been detected in the water throughout the bloom season using enzyme-linked immunosorbent assays (ELISA). The presence of the three toxins at VOYA could pose a threat to park visitors engaging in recreational activities such as camping, canoeing, swimming, and angling.

Angling is the most popular recreational activity at VOYA. Walleye (*Sander vitreus*), Smallmouth Bass (*Micropterus dolomieu*), and Yellow Perch (*Perca flavescens*) are targeted fish at VOYA, and the three species, along with White Sucker (*Catostomus commersonii*) play important roles in trophic interactions in the park ecosystem. Cyanotoxins have been measured in Walleye, Yellow Perch, and Smallmouth Bass in other lake systems (Wilson et al. 2008; Poste et al. 2011), but no studies examined toxins in White Suckers. Young of year (YOY) of the four species are common prey items for adult Walleye, Smallmouth Bass, and Yellow perch, while White Suckers are primarily insectivores (Kallemeyn et al. 2003). No evidence suggests microcystin, anatoxin-a, or saxitoxin biomagnify, though trophic transfer of the toxins could be possible.

In this study, we measured microcystin and anatoxin-a levels in liver and muscle tissues of adult Walleye, Yellow Perch, Smallmouth Bass, and White Suckers as well as YOY fish of

Smallmouth Bass and Yellow Perch from Lake Kabetogama. We investigated whether the toxin concentrations differed from lower trophic levels (YOY) to higher trophic levels (adults) and whether toxin concentrations differed between muscles, liver, and YOY. We explored potential variance in toxin levels among fish species and site location. Knowledge of toxin levels in fish aid park management in understanding the threat of microcystin and anatoxin-a at VOYA to human and ecosystem health.

Methods

Study Site

The study was conducted at Lake Kabetogama in Voyageurs National Park (VOYA) in northern Minnesota (Figure 2.1). Lake Kabetogama is polymictic and borderline eutrophic (Christensen and Maki 2015). We sampled from July to September in 2017 and 2018. In 2017, we sampled four seine and ten gillnet sites but did not target active algal blooms. In 2018, we sampled three seine and three gillnet sites while targeting active algal blooms.

Fish Sampling

Fish species targeted in this study were Yellow Perch, Walleye, White Sucker, and Smallmouth Bass. Adult fish were captured using gillnetting at annual Minnesota Department of Natural Resources (MNDNR) gillnetting sites while YOY fish were captured using seining at annual MNDNR seining sites or targeted bloom sites. Site names were abbreviated to GN for gillnetting sites and SN for seining sites. Numbers associated with sites are for identification purposes only. Fish were shipped to the University of Georgia on dry ice and stored in darkness at -18 C°. Fish were thawed from three to five hours at room temperature (20 C°).

Tissue Preparation for Toxin Extraction

After the specimens thawed, we obtained the wet weight from each fish. The liver and muscle were removed from each adult fish. Dissection tools were rinsed with DI water and methanol between each fish sample to avoid cross-contamination. Organs and YOY were placed in Whirlipak bags and stored in the dark at -18 C°.

Toxin Extraction

Toxins were extracted utilizing published extraction methods (Smith and Boyer 2009; Adamovský and Bláha 2016; Pawlik-Skowrońska et al. 2013). We pooled samples from one to five fish in each extraction, aiming for five fish per sample when possible. Samples were weighed at a standard mass of ~2.0g, though some samples had higher mass. After we confirmed the weight, the tissue samples were placed in 50 mL centrifuge tubes with acidified (0.002 M Acetic Acid) 100% methanol at 6 mL/g. We homogenized the samples with a FisherBrand Powergen 700 homogenizer. We rinsed the homogenizer with water and methanol between samples to avoid cross contamination. Centrifuge tubes were placed in an ultrasonic ice water bath (-10 C°) and were sonicated at 320 W for 30 minutes. After storing in a dark environment at 4 C° for 24 hours, samples were centrifuged (-5 C°) at 2900 x g for 10 minutes. We transferred the supernatant to sterile 50 mL centrifuge tubes using micropipettes. N-heptane (1 mL/g) was added to the 50 mL tubes, and the samples were shaken vigorously for one minute. The shaken samples were centrifuged (-5 C°) at 2900 rounds per minute for 10 minutes. We removed the upper lipid (heptane) layer with micropipettes. The solution was evaporated using a stream of N gas at room temperature in darkness for 6-8 hours. We transferred the remaining solution to amber microcentrifuge tubes and evaporated the solution at 35 C° in a vacuum rotary evaporator. The residue was dissolved in 300 µL of DI water and stored at -18 C° until analysis.

Extraction Analysis

Control samples were spiked with standards purchased from Abraxis. YOY and adult tilapia obtained from UGA fisheries lab were used as control fish. Control samples were spiked with 10 ng of anatoxin-a and 23 ng of microcystin. Controls included samples spiked before extraction methods, samples spiked after extraction methods, blank samples containing fish tissue with no spikes, and spiked DI water samples. We measured toxins in fish tissues using LC-MS/MS. We shipped samples on ice to SUNY College of Environmental Science and Forestry, where samples were reconstituted in 1 mL of DI water for LC-MS/MS. To obtain a measurement for µg/kg, readings were converted from µg/L to µg/mL and multiplied by the volume in mL. We then divided the resulting number by fish weight from each sample in kg to obtain µg/kg.

Statistical Analysis

Use of MANOVA allowed us to determine if the toxins accumulated at different levels from each other, among organs and YOY, among sites, among fish species, and between years. The MANOVA alpha value was 0.05, and analyses were completed using R software (v4.0.0). The MANOVA was completed with the "manova()" script from the R base package, and variables were ordered as follows: fish plus organs plus site locations plus year. For readings below the limit of detection, we divided the lower limit of detection (LLOD) by the square root of two to account for variability in statistical tests (Croghan and Egeghy 2003). We transformed the data by the natural log to normalize the datasets. Use of ANOVA, with the same variable order as MANOVA, coupled with Tukey's test revealed multiple comparisons within the independent variables.

Results

Water quality

Water quality data was not measured at fish collection sites in 2017. In 2018, site averages for Secchi depth was 1.82 m, temperature was 22.4 C°, pH was 8.15, specific conductance was 102 µS/cm, and DO% was 116% (Table 2.1).

In 2018, we ran LC-MS/MS on water samples from seining and gillnet sites. Anatoxin-a was found in all the seine sites but none of the gillnet sites. Microcystin was at two gillnet sites but absent in all seine sites (Table 2.2). Saxitoxin was absent in all sites.

Fish captures

In 2017, we captured 15 adult Yellow Perch and 15 YOY Yellow Perch, 10 adult Walleye and 0 YOY Walleye, 11 adult Smallmouth Bass and 15 YOY Smallmouth Bass, and 10 adult White Sucker and 0 YOY White Sucker. In total, we captured 76 fish in 2017 (Table 2.3).

In 2018, we captured 14 adult Yellow Perch and 45 YOY Yellow Perch, 15 adult Walleye and 0 YOY Walleye, 6 adult Smallmouth Bass and 21 YOY Smallmouth Bass, and 11 adult White Sucker and 0 YOY White Sucker. In total, we captured 112 fish in 2018 and 188 fish over both years.

Control Samples

Control samples for microcystin were below the LLOD, including the spiked DI water sample. The spiked DI water sample for anatoxin-a was 3.52 µg/L, but all other control samples for anatoxin-a were below the LLOD (Table 2.4).

Cyanotoxin measurements and statistics

We did not screen for saxitoxin with LC-MS/MS due to machine complications. Microcystin-LR was the only identified form of microcystin in the LC-MS/MS tests, though we

screened for microcystin-RR, -NOD, -dRR, -dLR, -CsLR, -GSH_LR and -CysGlyLR as well. From LC-MS/MS results, anatoxin-a levels differed significantly from microcystin-LR among fish species ($F = 2.23$, $df = 3$, $p = 0.045$), among organs and YOY ($F = 2.71$, $df = 2$, $p = 0.03$), and between years ($F = 1.08$, $df = 1$, $p = 0.02$) (Table 3.5). All anatoxin-a levels were below the LLOD.

Microcystin-LR varied among organs and YOY ($F = 5.25$, $df = 2$, $p = 0.01$) according to LC-MS/MS. Liver levels of microcystin-LR differed from muscle ($p = 0.01$) and YOY ($p = 0.045$). Microcystin-LR levels averaged 19.48 µg/kg in liver ($n = 29$, $SE = 10.34$), >0.42 µg/kg in muscle ($n = 30$, $SE = 0.01$), and >0.48 µg/kg in YOY ($n = 18$, $SE = 0.031$) (Figure 3.3). In all tissue samples, microcystin-LR levels at sites near Grave Island averaged 16.2 µg/kg ($n = 27$, $SE = 11.00$), near Gappas Landing averaged 11.47 µg/kg ($n = 6$, $SE = 10.89$), and in Lost Bay averaged 1.82 µg/kg ($n = 33$, $SE = 0.80$) (Figure 3.4). All tissues from Smallmouth Bass averaged 18.20 µg/kg ($n = 30$, $SE = 10.04$) of microcystin-LR (Figure 3.5).

Anatoxin-a LLOD varied among fish species ($F = 3.66$, $df = 3$, $p = 0.02$) and between years ($F = 8.42$, $df = 1$, $p = 0.005$) from LC-MS/MS data. Tukey's test showed a significant interaction between Yellow Perch and Smallmouth Bass ($p = 0.01$).

Discussion

The average of positive microcystin-LR concentrations was relatively high (91.95 µg/kg, $n = 6$), causing a significant difference in concentrations from anatoxin-a— mainly due to the absence of anatoxin-a detection in all samples. All statistics involving anatoxin-a refer to the LLOD divided by the square root of two to account for variation in potential readings below the LLOD. Many microcystin-LR readings were below the LLOD as well, which complicated the statistics. The number of samples with no detections compromised the ability to rely on a general

linear model to extrapolate the data below the LLOD, though the method we utilized introduced an upwards bias to the statistics. Dividing the LLOD by the square root of two biases the data less than replacing with zero, but both methods increase in bias with a higher amount of non-detects (Croghan and Egeghy 2003). An alternative to both methods is to treat a non-detect as nominal data— however, this method greatly reduces the power of statistical tests. There is currently no optimal system to adjust for zero-inflated data, and the potential presence of false zeroes in our data amplifies uncertainty (Ciannelli et al. 2008). In the case of our study, we must rely on raw observational data to infer relationships regardless of statistical significance, as zero-inflation accompanied with Poisson noise (from two exceedingly high microcystin-LR readings) confounded the statistics (Harrison 2014).

We detected a large phenylalanine peak that masked our ability to detect anatoxin-a, further reducing not only the certainty of our detections below the LLOD being zero, but also of our samples containing low amounts of anatoxin-a (Figure 2.5). Phenylalanine, an amino acid ubiquitous in animal tissue, most likely is present due to inadequate cleaning of fish proteins from the sample, though it is possible to separate the phenylalanine peak from the anatoxin-a peak— both of which occur at *m/z* 166 (Bogialli et al. 2006). The retention time of anatoxin-a in control water samples was 10.63 minutes, where the retention time of a peak from our spiked organ samples was 10.91 minutes, indicating matrix suppression from phenylalanine.

While the results are potentially false negatives, anatoxin-a has a half-life of roughly 14 days (Kaminski et al. 2013)— though degradation can be faster— and transportation among labs during this project might have allowed anatoxin-a to degrade below the LLOD. However, the most likely explanation of this finding is that our fish samples simply did not contain anatoxin-a. Nebraska reservoirs contained cHABs associated with anatoxin-a as high as 35 µg/L compared

to our highest reading of 0.463 µg/L, yet still no anatoxin-a was detected in fish from blooms in the reservoirs (Al-Sammak et al. 2014). Conversely, Lake Syczyńskie in Poland measured anatoxin-a at levels in water more comparable to our study, yet fish tissues from the lake (extracted using methods nearly identical to our own that involved heptane cleanup) contained levels of anatoxin-a as high as 19 µg/g in livers and 25 ng/g in muscles (Pawlik-Skowrońska et al. 2012). Lake Syczyńskie and the Nebraska reservoirs, however, suffered from intense cHABs reoccurring from spring until fall, while Lake Kabetogama experiences cHABs sporadically from mid/late-summer through mid-fall, potentially catalyzed through rare stratification events (Christensen et al. 2013). The infrequent and erratic nature of blooms on Lake Kabetogama is reflected in our toxin data, as most of our samples contain no toxins, but our positive samples do have high concentrations.

Due to our reliance on MNDNR capture sites, we were limited in our ability to choose date and location of samples, though sites in 2018 had blooms present. We captured YOY fish in mid-July and adult fish from early/mid-September, potentially at too early of times for significant accumulation. Anatoxin-a was detected in small amounts at seining sites from 2018 during active blooms, but cyanotoxins sometimes release into water at higher levels once cyanobacteria cells lyse (Campinas and Rosa 2010). Sampling in the periods during and directly after algal blooms might have increased chances of accumulation in fish tissue compared to sampling in active blooms alone, but sample collection for this project was limited to a specific timeframe of MNDNR sampling for a long term fisheries monitoring program.

In 2017, the MNDNR randomly selected fish from any of their gillnetting sites rather than focusing on three sites that had active blooms. We did not collect water toxin data in 2017, but the lack of control over sites with active or dissipating blooms most likely increased zero-

inflation, especially in anatoxin-a samples. The rapid degradation of anatoxin-a in the environment makes sampling for the toxin difficult (Kaminski et al. 2013), and a more flexible sampling regime could increase confidence in obtaining results representative of anatoxin-a fish accumulation in Lake Kabetogama.

The zero-inflation and Poisson noise in microcystin-LR levels might result from the timing of sampling as well, as the gillnetting sites in 2018 were during active algal blooms, and 2017 occurred at random sampling sites without regard for timing of bloom activity (Figure 2.6A, B). Our highest microcystin-LR reading (255 µg/kg) occurred during the day of the most intense bloom of a cHAB that lasted several days on Lake Kabetogama. We observed a sample with low microcystin-LR (16 µg/kg) the following day when no bloom was visible. Since the highest result occurred during the most intense and final day of the bloom, the cells may have been lysing and releasing more cyanotoxins into the water. We detected microcystin-LR at GN09 (0.60 µg/L) and GN10 (0.51 µg/L), both of which were sampled on the final day of the bloom. No cyanotoxins were detected in water at GN14 the day after the bloom dissipated.

All positive samples in both years were in the liver (Figure 2.6C), in accordance with previous studies showing high liver cyanotoxin values compared to other tissues (Mohamed et al. 2003), as fish effectively break down microcystin-LR and anatoxin-a in their livers (Malbrouck and Kestemont 2006; Osswald et al. 2013). Liver microcystin-LR values also differed significantly from YOY samples since there were no YOY positive samples, suggesting that accumulation is higher in adult fish than in younger fish. However, younger fish should be at risk to accumulate higher cyanotoxins due to an underdeveloped immune system (Lance et al. 2006). Limited seining site selection focused mainly in July and August of 2017 and 2018 confounded statistics involving YOY fish by ignoring temporal variation of microcystin-LR in the

midwestern United States (Makarewicz et al. 2009). Microcystin-LR usually peaks in late-August to early-September at Lake Kabetogama (Christensen et al. 2019). Seining at the same time as gillnetting, which was during early to late September, would have improved chances of confirming microcystin-LR in YOY tissues. Two of the seining sites from 2018 at the mouth of Lost Bay, a location of Lake Kabetogama with relatively high nutrient concentrations (Christensen et al. 2013), were intensely blooming during 2018 gillnetting sample dates. However, Walleye YOY move from littoral areas after July, making seining for Walleye and other YOY suboptimal. Electrofishing could be used to capture YOY later in the season. Future YOY sampling efforts should focus on sampling later in the season at areas with high nutrient concentrations.

While Lake Kabetogama is a polymictic lake, several areas are deep enough to stratify for brief periods of up to a few days in summer months, including in Lost Bay (GN 13 and GN14; SN101, SN102, and SN16), near Gappas Landing (GN19; SN103) and near Grave Island (GN09 and GN10; SN13). During stratification events, the hypolimnion in the deepest areas of Lake Kabetogama become anoxic, and bacteria in anoxic sediments reduce sulfate (SO_4), which results in dissolved ferrous-phosphate minerals (Orihel et al. 2015). The free sulfur (S) then bonds with the free iron (Fe) into insoluble minerals, creating an iron-poor environment and leaving excess P in the anoxic zone. Polymixis during high wind events— common on Lake Kabetogama— internally loads P into littoral zones (Cyr et al. 2009), leading to low nitrogen (N):P ratios and N-limitation. N-limitation, especially when total N to total P (TN:TP) ratios are intermediate (Scott et al. 2013), drives production of microcystin and anatoxin-a (Gagnon and Pick 2012). Fluctuations of TN:TP ratios from internal loading by deep sites, such as Lost Bay, Grave Island, and Gappas Landing, may result in the toxic cHABs on the surface of Lake

Kabetogama, potentially increasing cyanotoxins in fish tissues at those sites. Gillnetting sites at the mouth of Lost Bay contained two positive microcystin-LR at GN13 (22.3 µg/kg) and GN14 (16.4 µg/kg) (Figure 2.8). Near Grave Island, single samples from GN10 (255 µg/kg) and GN09 (164 µg/kg) had the highest microcystin-LR in the study (Figure 2.9). Near Gappas Landing, GN19 had a high average microcystin-LR, though sampling there was low (33.23 µg/kg, N=2) (Figure 2.10). Site GN04 near the shallower Cemetery Island— which released P from sediments in laboratory incubations but had lower TP field values compared to Lost Bay, Grave Island, and Gappas Landing (Christensen et al. 2013)— also had a positive sample (28 µg/kg); however, all other sites near Cemetery Island (GN02, GN03, and GN05) had toxin measurements below the LLOD. Variation within sites in the study resulted in a lack of statistically significant differences, but observation suggests that there is a higher risk at sites associated with Fe-poor sediments and P internal loading. Future sampling efforts should focus efforts at littoral zones near these sites.

Five out of six samples in which microcystin was detected were Smallmouth Bass and the other was a Walleye, meaning only top-level predators were represented in the positive samples (Figure 2.6D). Smallmouth Bass and Walleye have been documented as accumulating higher levels of microcystin compared to other fish species before (Poste et al. 2011), and our data, though not statistically significant, seems to further support this notion. While we did not detect microcystin-LR in YOY Yellow Perch, which is the preferred prey item of both Smallmouth Bass and Walleye at Voyageurs National Park (Kallemeyn et al. 2003), we did not collect YOY with adult fish, leaving an unanswered question as to what levels would be in prey items compared to predators on the same dates of intense cHABs.

Due to this limitation in our design and a lack of statistical significance, we cannot make sound inferences on whether trophic transfer occurs to higher level organisms, especially since there is no previous evidence for biomagnification (Ibelings et al. 2005). However, the two outlier samples of high microcystin-LR indicate potential magnification in Smallmouth Bass, at least on a short-term scale. While fish metabolize cyanotoxins effectively long term, cyanotoxins might increase as they move up trophic levels during and shortly after blooms before the fish metabolizes the toxins. The diet of top aquatic predators at VOYA could increase cyanotoxin exposure.

We did not conduct stable isotope studies, but stomach dissections revealed crayfish remains in Smallmouth Bass (Figure 2.11). Crustaceans are effective at transferring cyanotoxins to fish (Kotak et al. 1996), and crayfish have been measured to accumulate high levels of cyanotoxins (Wood et al. 2012). The rusty crayfish invaded waters at VOYA, and their presence increases crayfish abundance in invaded waters significantly (Wilson et al. 2004). As efficient littoral zone grazers (Roth et al. 2006), rusty crayfish could accumulate high levels of microcystin-LR, anatoxin-a, or saxitoxin during intense bloom events. Their high abundance at VOYA could lead to an increase in top predators consuming them, which could account for the spikes of high microcystin-LR readings in our data if the crayfish consumed cyanobacteria. Smallmouth Bass in particular prefer the warmer waters in shallow littoral zones, and they consume crayfish from littoral zones more often than other fish species in this study, potentially exposing them to cyanotoxins in crayfish as well as higher cyanotoxin concentrations in water from littoral zones. Further research at VOYA is necessary to understand the role of cyanotoxins in trophic interactions.

Risk Assessment

We did not observe any cyanotoxins in muscle, and humans do not frequently consume Smallmouth Bass or Walleye livers, if ever. The lack of toxin observations in muscle tissue suggest that there is little to no threat to humans of effectual microcystin-LR exposure at Voyageurs National Park. While our study failed to observe anatoxin-a in fish tissues, we found varying levels of microcystin-LR levels in the target angling species Walleye and Smallmouth Bass. The average concentration of all tissue samples, 7.799 µg/kg (n=77), falls far below the LD_{50} of microcystin-LR, which is roughly 11 mg/kg (Yoshida et al. 1997). Even our highest samples fell far below the LD_{50}. Our positive sample average neared the NOAEL of microcystin-LR at 40 $\mu g/kg^{-1}$ bodyweight per day, and our three highest samples exceeded that limit (Fawell et al. 1999), potentially putting wildlife that consume fish livers at risk.

Wildlife at the park has a higher threat of exposure to microcystin-LR, but the risk of negative effects from microcystin-LR are low according to our current dataset. Larger mammals that would consume fish livers have too large of masses to be at great risk. Waterfowl and birds of prey could be at risk if they consume large quantities of whole fish. Levels of cyanotoxins in the fish tissues are not high enough to cause lethal or chronic effects in fish. Overall, the risk of cyanotoxins producing negative effects from exposure in fish tissues is low.

However, continued monitoring and further research at the park is necessary to fully evaluate the risk. Limitations of sampling dates and location contributed to statistical uncertainty. More fish should be examined at sites where P is released from Fe-poor sediments. Sampling of YOY and adult fish, alongside water toxin monitoring, throughout the bloom season would increase understanding of temporal alterations of cyanotoxins in fish and water tissues. Stable-isotope tests or gut analysis coupled with cyanotoxin fish tissue measurement would increase

understanding of cyanotoxin trophic transfer at VOYA, as would examination of organisms other than fish, such as crayfish. Continued monitoring of cyanotoxins at VOYA will expand our knowledge on cyanotoxins while ensuring park managers take proper actions to protect humans and wildlife if cyanotoxin levels at the park reach dangerous levels.

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CHAPTER 3: A COMPARISON OF CYANOTOXIN ANALYTICAL TECHNIQUES AT VOYAGEURS NATIONAL PARK: ELISA AND LC-MS/MS[2]

[2]McWhorter, Seth. To be submitted to *Harmful Algae*.

Abstract

Optimizing cyanotoxin detection methods for fish tissues will aid managers in protecting aquatic resources. Two common methods of cyanotoxin measurement include enzyme-linked immunosorbent assays and liquid chromatography tandem mass spectrometry. We tested the efficacy of both methods for measuring fish tissue extractions of microcystin and anatoxin-a. We also aimed to compare concentrations of cyanotoxins between each other, among fish tissues and whole YOY fish, among site locations, among fish species, and between years. We caught fish using gillnetting and seining and removed their liver and muscles. Liver and muscle measurements were compared to young of year whole fish. Most measurements from liquid chromatography tandem mass spectrometry were below the lower limit of detection, while most enzyme-linked immunosorbent assays were above the upper limit of detection. These readings could be either false negatives in liquid chromatography tandem mass spectrometry, lying between the limits of detection for both methods, or false positives in enzyme-linked immunosorbent assays. Enzyme-linked immunosorbent assays allow for measurements too low for liquid chromatography tandem mass spectrometry to pick up. However, ecological significance of miniscule toxin measurements should be considered when determining which methods are best for a study.

Introduction

Detection of cyanotoxins released from harmful cyanobacterial algal blooms (cHABs) is quintessential to protecting human and environmental health. Cyanotoxins, such as microcystin, anatoxin-a, and saxitoxin, are released into the water during intense cHAB events and present issues to ecosystems throughout trophic levels. The toxins exhibit allelopathic effects on competitors to cyanobacteria, such as macrophytes including submerged aquatic vegetation

(Pflugmacher 2002). Macrophytes impacted by allelopathic cyanotoxins suffer from growth inhibition, reduction in photosynthetic oxygen production, and changes in pigment pattern, potentially resulting in a bottom-up trophic cascade. Cyanotoxins threaten organisms higher in trophic level as well, as long term exposure to cyanotoxins are fatal to *Daphnia spp.* (Chen et al. 2005), and cyanotoxins damage fish gills, digestive tract, and liver, which can lead to massive fish casualty events and potential top-down trophic cascade (Zanchett and Oliveira-Filho 2013).

Cyanotoxin levels become highest immediately after blooms dissipate as cells begin to lyse and release stored toxins (Zastepa et al. 2014). The toxins can persist in the water from days to weeks, even when blooms are not visible. Understanding when cyanotoxins are at their most toxic levels are important in protecting human and environmental health in impacted water bodies.

Several detection methods have been utilized to determine toxicity of cyanotoxin impacted systems. Cyanotoxin test strips provide concerned citizens with a unique opportunity to test water samples for cyanotoxins in the field (Humpage et al. 2012), yet the methods are still slightly complicated for citizen science, and results are unreliable. Quantitative polymerase chain reaction (qPCR) allows the detection of genes responsible for producing cyanotoxins but does not detect toxin levels (Pacheco et al. 2016). While useful as an initial screening, the presence of toxin producing genes occurs even in water samples that contain no toxins.

A common detection method for cyanotoxin in water samples is enzyme linked immunosorbent assays (ELISA). In ELISA, cyanotoxins bind to wells along with detection enzymes (Carmichael and An 1999). A color agent is added to produce a measurable result. ELISA binding sites allow for detection of specific cyanotoxins (Graham et al. 2010). However,

if a water sample contains excessive particulate matter and complex matrices, ELISA can produce false positives (Antoniou et al. 2005).

When testing samples with complex matrices, many researchers prefer liquid chromatography tandem mass spectroscopy (LC-MS/MS) over ELISA and qPCR. The LC-MS/MS utilizes electromagnets to charge ions on molecules, allowing for the detection of compounds of different masses at separate times (Li et al. 2001). Since compounds enter the detection device at intervals, LC-MS/MS can measure analytes in complex solutions. The LC-MS/MS produces fewer false positives than ELISA, though lower limits of detection (LLOD) are generally higher for LC-MS/MS, potentially leading to false negatives. There is no "golden standard" when accounting for cost, practicality, and reliability (Gaget et al. 2017).

While methods for water sample detection of cyanotoxins are well-established, methodology for extracting and detecting cyanotoxins from fish tissues is still in development. Many cyanotoxins, such as microcystin, anatoxin-a, and saxitoxin, have been detected in fish tissues (Haddad et al. 2019), mainly the muscle and liver. Further detection of toxin levels in fish tissues will help resource managers in areas impacted by cHABs to preserve human and environmental health. The ELISA has been used to detect cyanotoxins in fish tissues with mixed results. While ELISA indicates toxin presence (Freitas de Magalhães et al. 2001), some of the readings could be false positives due to the complexity of fish tissue matrices (Geis-Asteggiante et al. 2011). The LC-MS/MS might provide a more reliable alternative to ELISA for detection of cyanotoxins in fish due to the ability to detect compounds in intervals.

Voyageurs National Park, an interconnected area containing two major lake systems and many smaller lakes protected from major anthropogenic impacts, experiences annual cHABs on Lake Kabetogama and Black Bay of Rainy Lake (Christensen and Maki 2015). Genes capable of

producing microcystin, anatoxin-a, and saxitoxin are present in cyanobacteria at the park (Christensen et al. 2019). Microcystin, anatoxin-a, and saxitoxin were detected throughout the bloom season (June to October) at Lake Kabetogama using ELISA, though there are no examinations to determine whether fish in the park contain cyanotoxins during and after cHABs. Angling is the primary activity of visitors to the park, so detecting toxins in fish tissues will allow us to determine if cyanotoxins are at dangerous levels in fish eaten by visitors.

In this study, we aimed to detect toxins in young of year (YOY) and adult Walleye (*Sander vitreus*), Smallmouth Bass (*Micropterus dolomieu*), Yellow Perch (*Perca flavescens*), and White Suckers (*Catostomus commersonii*) utilizing ELISA and LC-MS/MS and compared the efficacy of the two methods. We determined whether ELISA is a viable option for complex fish matrix solutions or if future research should focus on using only LC-MS/MS. We also aimed to compare concentrations of anatoxin-a and microcystin between each other, among fish tissues and whole YOY fish, among site locations, among fish species, and between years. Determining best practices for detecting cyanotoxins in fish tissues will aid researchers in further understanding the threat of cyanotoxins in fish to humans and the environment.

Methods

Study Site

The study was conducted at Lake Kabetogama in Voyageurs National Park (VOYA) in northern Minnesota (Figure 3.1). Lake Kabetogama is polymictic and borderline eutrophic (Christensen and Maki 2015). In 2017, we sampled four seine and ten gillnet sites but did not target active algal blooms. In 2018, we sampled three seine and three gillnet sites while targeting active algal blooms.

Fish Sampling

Fish species targeted in this study were Yellow Perch, Walleye, White Sucker, and Smallmouth Bass. Adult fish were captured using gillnetting at annual Minnesota Department of Natural Resources (MNDNR) gillnetting sites while YOY fish were captured using seining at annual MNDNR seining sites or targeted bloom sites. Site names were abbreviated to GN for gillnetting sites and SN for seining sites. Numbers associated with sites are for identification purposes only. Fish were shipped to the University of Georgia on dry ice and stored in darkness at -18 C°. Fish were thawed from three to five hours at room temperature (20 C°).

Tissue Preparation for Toxin Extraction

After the specimens thawed, we obtained the wet weight from each fish. The liver and muscle were removed from each adult fish. Dissection tools were rinsed with DI water and methanol between each fish sample to avoid cross-contamination. Organs and YOY were placed in Whirlipak bags and stored in the dark at -18 C°.

Toxin Extraction

Toxins were extracted utilizing several published extraction methods (Smith and Boyer 2009; Adamovský and Bláha 2017; Pawlik-Skowrońska et al. 2013). We pooled samples from one to five fish in each extraction, aiming for five per sample when possible. Samples were weighed at a standard mass of ~2.0g, though some samples had higher mass. After we confirmed the weight, the tissue samples were placed in 50 mL centrifuge tubes with acidified (0.002 M Acetic Acid) 100% methanol at 6 mL/g. We homogenized the samples with a FisherBrand Powergen 700 homogenizer. We rinsed the homogenizer with water and methanol between samples to avoid cross contamination. Centrifuge tubes were placed in an ultrasonic ice water bath (-10 C°) and were sonicated at 320 W for 30 minutes. After storing in a dark environment at

4 C° for 24 hours, samples were centrifuged (-5 C°) at 2900 x g for 10 minutes. We transferred the supernatant to sterile 50 mL centrifuge tubes using micropipettes. N-heptane (1 mL/g) was added to the 50 mL tubes, and the samples were shaken vigorously for one minute. The shaken samples were centrifuged (-5 C°) at 2900 rounds per minute for 10 minutes. We removed the upper lipid (heptane) layer with micropipettes. The solution was evaporated using a stream of N gas at room temperature in darkness for 6-8 hours. The remaining solution was transferred to amber microcentrifuge tubes and evaporated at 35 C° in a vacuum rotary evaporator. Residue was dissolved in 300 µL of DI water and stored at -18 C° until analysis.

Extraction Analysis

The LC-MS/MS detected microcystin-LR while ELISA tested for multiple microcystins that contained the amino acid ADDA, henceforth referred to as microcystin-ADDA. Control samples were spiked with standards purchased from Abraxis. The YOY and adult tilapia obtained from UGA fisheries lab were used as control fish. Samples were spiked with 10 ng of anatoxin-a and 23 ng of microcystin. No saxitoxin standard was used in this study. Controls included samples spiked before the extraction methods, samples spiked after extraction methods, blank samples containing fish tissue with no spikes, and DI water samples spiked with toxins. We measured toxins in fish tissues using ELISA and confirmed with LC-MS/MS. After ELISA testing, we shipped samples on ice to SUNY College of Environmental Science and Forestry, where we reconstituted in 1 mL of DI water for LC-MS/MS. To obtain a measurement for µg/kg for LC-MS/MS, readings were converted from µg/L to µg/mL and multiplied by the volume in mL. For ELISA, we converted ppb (µg/L) to µg/µL and multiplied by the volume in the ELISA well of 150 µL. We then divided the resulting number by the weight of fish used in the sample in kg to obtain µg/kg.

Statistical Analysis

Use of MANOVA allowed us to determine if the toxins accumulated at different levels from each other, among organs and YOY, among site locations, among fish species, and between years. The MANOVA alpha value was 0.05, and all analyses were completed using R software (v4.0.0). The MANOVA was completed with the "manova()" script from the R base package, and variables were ordered as follows: fish plus organs plus site locations plus year. For readings below the limit of detection, we divided the lower limit of detection (LLOD) by the square root of two to account for variability in statistical tests (Croghan and Egeghy 2003). We transformed the data by the natural log to normalize the datasets and to account for upper limit of detection (ULOD) in ELISA. Use of ANOVA, with the same variable order as MANOVA, coupled with Tukey's test revealed multiple comparisons within the independent variables.

Results

Water quality

Water-quality data was not measured at fish collection sites in 2017. In 2018, site averages for Secchi depth was 1.82 m, temperature was 22.41 C°, pH was 8.15, specific conductance was 102.17 µS/cm, and DO% was 116.32% (Table 3.1).

In 2018, we ran LC-MS/MS on seining and gillnet sites. Anatoxin-a was found in all the seine sites, but none of the gillnet sites. Microcystin-LR was at two gillnet sites, but absent in one gillnet site and all seine sites (Table 3.2). Saxitoxin was absent in all sites. We ran ELISA only on gillnet sites, where we found anatoxin-a in one gillnet site and microcystin-ADDA in all gillnet sites.

Fish captures

In 2017, we captured 15 adult Yellow Perch and 15 YOY Yellow Perch, 10 adult Walleye and 0 YOY Walleye, 11 adult Smallmouth Bass and 15 YOY Smallmouth Bass, and 10 adult White Sucker and 0 YOY White Sucker. In total, we captured 76 fish in 2017 (Table 3.3).

In 2018, we captured 14 adult Yellow Perch and 45 YOY Yellow Perch, 15 adult Walleye and 0 YOY Walleye, 6 adult Smallmouth Bass and 21 YOY Smallmouth Bass, and 11 adult White Sucker and 0 YOY White Sucker. In total, we captured 112 fish in 2018 and 188 fish over both years.

Control Samples

Control samples for microcystin-LR were below the LLOD in LC-MS/MS, including the spiked DI water sample. The spiked DI water sample for anatoxin-a was 3.52 µg/L in LC-MS/MS, but all other control samples for anatoxin-a were below the LLOD (Table 3.4). Contrary to the LC-MS/MS results, ELISA found all control samples of both toxins above the ULOD, including blank fish tissue samples. Standard curves allowed for determination of ELISA results inside a range of 0.1 µg/L to 5.0 µg/L.

Cyanotoxin measurements and statistics

We did not screen for saxitoxin with LC-MS/MS due to machine complications. Microcystin-LR was the only identified form of microcystin in the LC-MS/MS tests, though we screened for microcystin-RR, -NOD, -dRR, -dLR, -CsLR, -GSH_LR and -CysGlyLR as well. From LC-MS/MS results, Anatoxin-a levels differed significantly from microcystin-LR among fish species ($F = 2.23$, $df = 3$, $p = 0.045$), among organs and YOY ($F = 2.71$, $df = 2$, $p = 0.03$), and between years ($F = 1.08$, $df = 1$, $p = 0.02$) (Table 3.5). All anatoxin-a levels were below the LLOD.

Microcystin-LR varied among organs and YOY ($F = 5.25$, $df = 2$, $p = 0.01$) according to LC-MS/MS. Liver levels of microcystin-LR differed from muscle ($p = 0.01$) and YOY ($p = 0.045$). Microcystin-LR levels averaged 19.48 µg/kg in liver ($n = 29$, $SE = 10.34$), >0.42 µg/kg in muscle ($n = 30$, $SE = 0.01$), and >0.48 µg/kg in YOY ($n = 18$, $SE = 0.031$) (Figure 3.2). In all tissue samples, microcystin-LR levels at sites near Grave Island averaged 16.2 µg/kg ($n = 27$, $SE = 11.00$), near Gappas Landing averaged 11.47 µg/kg ($n = 6$, $SE = 10.89$), and in Lost Bay averaged 1.82 µg/kg ($n = 33$, $SE = 0.80$) (Figure 3.3). All tissues from Smallmouth Bass averaged 18.20 µg/kg ($n = 30$, $SE = 10.04$) of microcystin-LR (Figure 3.4).

Anatoxin-a LLOD varied among fish species ($F = 3.66$, $df = 3$, $p = 0.02$) and between years ($F = 8.42$, $df = 1$, $p = 0.005$) from LC-MS/MS data. Tukey's test showed a significant interaction between Yellow Perch and Smallmouth Bass ($p = 0.01$).

According to ELISA, microcystin-ADDA differed from anatoxin-a among fish species ($F = 3.68$, $df = 3$, $p = 0.002$), among sites ($F = 2.91$, $df = 13$, $p = 0.00005$), and between years ($F = 5.42$, $df = 1$, $p = 0.007$). Most microcystin-ADDA samples indicated values above the ULOD, while all but one anatoxin-a value were above the ULOD (Table 3.6).

From ELISA data, microcystin-ADDA differed among site locations ($F = 4.27$, $df = 3$, $p = 0.01$). Lost Bay sites differed significantly from sites near Cemetery Island ($p = 0.01$) (Figure 3.5). Microcystin-ADDA levels near Grave Island averaged 0.42 µg/kg ($n = 27$, $SE = 0.08$), near Gappas Landing averaged 0.32 µg/kg ($n = 6$, $SE = 0.04$), near Cemetery Island averaged 0.24 µg/kg ($n = 11$, $SE = 0.03$), and in Lost Bay averaged 0.41 µg/kg ($n = 33$, $SE = 0.03$).

Anatoxin-a ELISA data showed differences among fish ($F = 3.7$, $df = 3$, $p = 0.02$) and between years ($F = 11.02$, $df = 1$, $p = 0.002$). Yellow Perch differed significantly from Smallmouth Bass ($p = 0.01$).

Microcystin-LR differed from microcystin-ADDA among tissues and YOY ($F = 3.26$, $df = 2$, $p = 0.01$) and among site locations ($F = 2.40$, $df = 3$, $p = 0.03$). Anatoxin-a differed between LC-MS/MS and ELISA methods among fish ($F = 3.01$, $df = 3$, $p = 0.009$) and between years ($F = 6.07$, $df = 1$, $p = 0.004$).

Discussion

Though most of our LC-MS/MS readings were below LLOD, most of our ELISA readings were unexpectedly above the ULOD. Complicated fish tissue matrices can create false positives in ELISA testing (Geis-Asteggiante et al. 2011), especially if proteins from fish bind to ELISA well walls. Our anatoxin-a LC-MS/MS readings showed a large phenylalanine peak burying the anatoxin-a peak. Phenylalanine is an amino acid ubiquitous in animal tissues and has a structure and molecular weight similar to anatoxin-a (Figure 3.6), and their structures appear at similar frequencies in LC-MS/MS (*m/z* 166) (Faassen et al. 2012). In ELISA, phenylalanine could be binding with well walls and indicating anatoxin-a presence, resulting in false positives.

Compared to anatoxin-a, microcystin has a large molecular weight, with anatoxin-a having a weight of 165 g/mol and microcystin-LR having a weight of 995 g/mol (Huang et al. 2007). Molecules as large as microcystin in nature are rare, which makes the peak of microcystin potentially easier to isolate than other cyanotoxin peaks (McElhiney and Lawton 2005). In our study, we struggled to isolate anatoxin-a from fish proteins for LC-MS/MS— even though anatoxin-a was present in water— but we found microcystin readings at relatively high concentrations. Anatoxin-a has a short half-life of fourteen days in natural photoperiods (Kaminski et al. 2013), making the handling of anatoxin-a from water and fish tissue extractions difficult. Antithetical to the ease of microcystin detection, difficulty of anatoxin-a detection could bias research away from focusing on anatoxin-a, even if it is present.

Though microcystins are easier to detect, we still seemed to have possible false positives in our ELISA microcystin readings. Fish proteins interfering with microcystin LC-MS/MS readings, such as phenylalanine interfering with anatoxin-a, is relatively easy to avoid, but complex fish matrices could have still interfered with ELISA binding sites, leading to false positives (Bláha et al. 2004). Even in our blank tissues where no positive should have been recorded if matrix interference was not present, all ELISA samples for both toxins indicated levels above ULOD. Most LC-MS/MS samples indicated levels below LLOD.

In our LC-MS/MS readings, our blank microcystin-LR water sample indicated values below the LLOD. However, our ELISA readings showed values above the ULOD for blank water samples, potentially indicating degradation of microcystin during travel to SUNY labs for LC-MS/MS analysis. Degradation of microcystin usually occurs two to eight days after addition to water (Jones 1994), and we shipped the samples overnight on ice. Anatoxin-a water samples were still positive in LC-MS/MS readings. Taking into consideration the quick shipping of samples and the presence of the highly labile anatoxin-a in the blank water sample, we do not believe that microcystin degraded in the water samples. More probably, the spikes to fish tissues and blank water of microcystin were not high enough to be read by LC-MS/MS, resulting in false negatives. After being reconstituted at 1 mL for LC-MS/MS, remaining the remaining microcystin-LR concentration would have been 0.97 µg/L, which was below the LLOD in LC-MS/MS (1.23 µg/L). For ELISA, reconstituting at 300 µL left samples too concentrated, where with LC-MS/MS, reconstituting the samples at 1 mL made samples too diluted. Microcystin can also bind to plastic, which we stored the samples in, potentially lowering microcystin concentrations (G. Boyer, personal communication).

Since the microcystin levels in blank water were probably between the ULOD of ELISA and LLOD of LC-MS/MS, it is possible that other fish samples were between the LOD as well in both microcystin and anatoxin-a. Though the positive blank tissue readings in ELISA greatly increase uncertainty, the presence of positive microcystin and anatoxin-a readings below the ULOD of ELISA potentially validates the assertion of samples containing low levels of cyanotoxins, but these readings could also indicate lower levels of fish matrix interferences (Figure 3.7). Two of the positive microcystin samples below the ULOD were YOY and two were muscle—neither YOY nor muscle samples showed positive readings in LC-MS/MS. The low samples in YOY could lead to introduction of cyanotoxins to predators of YOY fish; however, levels in both muscle and YOY at that miniscule a level pose no threat to predators, including humans.

Unfortunately, readings of blank fish tissue over the ULOD drastically lowers confidence in our fish samples over the ULOD containing toxins. More efficient lipid and protein cleanup methods could alleviate this issue in the future. Recently, researchers are beginning to utilize solid phase extraction (SPE) to remove lipids and proteins from samples, resulting in cleaner samples and higher recovery rates from control samples (Mashile and Nomngongo 2017). We did not budget for the increased expenses of SPE and instead focused on quantity of fish samples. If cyanotoxin studies in fish is repeated at VOYA, the project budget should allocate funding for SPE rather than increased sample numbers. Improving quality of samples over quantity of samples would relieve budgeting constraints and preserve statistical power (Field et al. 2005)— as well as greatly increase our ability to adequately measure the risk of cyanotoxins at VOYA.

Conclusions

Readings of cyanotoxins differed significantly between LC-MS/MS and ELISA detection methods. While ELISA tests have provided false positives in fish tissue— mainly due to their production for rapid water toxin testing rather than fish tissue extraction testing— LC-MS/MS risks providing false negatives in fish tissue and water tissue if the concentration values are low. Both methods would benefit from the implementation of SPE methods rather than heptane lipid removal. Coupled with SPE, LC-MS/MS is probably the preferred method to ELISA for measuring toxins in fish tissues, due to the ability to measure specific components of a complex matrix individually by charging their ions and fragmenting the ions. Still, the applicability of ELISA in reading lower concentration samples could provide useful information on whether values are below the LLOD of LC-MS/MS. Running ELISA and LC-MS/MS together optimizes the value range of detection. However, the ecological significance of values below the LLOD of LC-MS/MS is probably low. Ecological significance should be taken into consideration when allocating funding for projects, and a finetuned and fiscally conscious approach could improve available budget and ecological applicability of studies.

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APPENDICES

Tables

Table 2.1:
Water quality measurements on Lake Kabetogama at gillnet and seine sites in 2018

	Date	Conditions	Algae	Secchi (m)	Temp (C°)	pH	SPC (µS/cm)	DO%	DO (mg/L)
SN101	8/17/2018	Clear	Moderate	3.2	27.0	9.3	105.0	150.0	12
SN102	8/17/2018	Clear	Moderate	1.0	26.9	9.3	105.0	148.5	11.8
SN103	8/22/2018	P. Cloudy	Moderate	0.5	23.8	8.6	101.0	112.8	9.5
GN09	9/19/2018	Overcast	Moderate	1.2	18.6	7.7	103.0	101.1	9.2
GN10	9/19/2018	P. Cloudy	Serious	1.5	19.2	7.5	101.0	100.5	9.0
GN14	9/20/2018	Overcast	None	3.5	19.0	6.6	98.0	85.0	7.9
Average				**1.8**	**22.4**	**8.2**	**102.2**	**116.3**	**9.9**

Table 2.2:
Cyanotoxin measurements in water at gillnet and seine sites on Lake Kabetogama, LC-MS/MS

	Microcystin-LR (µg/L)	Anatoxin-a (µg/L)	Paralytic Shellfish Toxins (µg/L)
SN101	ND	0.436	ND
SN102	ND	0.463	ND
SN103	ND	0.051	ND
GN09	0.600	ND	ND
GN10	0.510	ND	ND
GN14	ND	ND	ND
Average	**0.185**	**0.158**	**ND**

Table 2.3:
Fish captured Lake Kabetogama gillnet and seine sites

		Year		
		2017	**2018**	**Total**
Adult				
	Yellow Perch	15	14	29
	Walleye	10	15	25
	Smallmouth Bass	11	6	17
	White Sucker	10	11	21
Young	**Of Year**			
	Yellow Perch	15	45	60
	Walleye	0	0	0
	Smallmouth Bass	15	21	36
	White Sucker	0	0	0
	Total	**76**	**112**	**188**

Table 2.4:
Cyanotoxin concentrations of blanks and spiked controls, LC-MS/MS

	Microcystin-LR (µg/kg)	**Anatoxin-a (µg/kg)**
Spiked DI water	< 1.23 µg/L	3.52 µg/L
Blank Muscle	< 1.23	< 0.06
Blank YOY	< 0.70	< 0.03
Pre-spike Extraction YOY1	< 0.90	< 0.04
Pre-extraction spike YOY2	< 0.79	< 0.04
Pre-extraction spike Liver	< 2.51	< 0.11
Pre-extraction spike Muscle	< 1.16	< 0.05
Post-extraction spike YOY1	< 0.68	< 0.03
Post-extraction spike YOY2	< 1.18	< 0.05
Average	**< 1.14**	**< 0.05**

Table 2.5:
Microcystin-LR and Anatoxin-a LC-MS/MS MANOVA

	Df	Pillai	F	numDf	denDf	p
Fish	3	0.17	2.07	6	134	0.06.
Organ	2	0.15	2.77	4	134	0.03*
Location	3	0.08	1.00	6	134	0.43
Year	1	0.01	0.28	2	66	0.75
Residuals	57					

*** = 0.001 ** =0.01 *0.05 . = 0.1

Table 3.1:
Water quality measurements on Lake Kabetogama at gillnet and seine sites in 2018

	Date	Conditions	Algae	Secchi (m)	Temp (C°)	pH	SPC (µS/cm)	DO%	DO (mg/L)
SN101	8/17/2018	Clear	Moderate	3.2	27.0	9.3	105.0	150.0	12
SN102	8/17/2018	Clear	Moderate	1.0	26.9	9.3	105.0	148.5	11.8
SN103	8/22/2018	P. Cloudy	Moderate	0.5	23.8	8.6	101.0	112.8	9.5
GN09	9/19/2018	Overcast	Moderate	1.2	18.6	7.7	103.0	101.1	9.2
GN10	9/19/2018	P. Cloudy	Serious	1.5	19.2	7.5	101.0	100.5	9.0
GN14	9/20/2018	Overcast	None	3.5	19.0	6.6	98.0	85.0	7.9
Average				**1.8**	**22.4**	**8.2**	**102.2**	**116.3**	**9.9**

Table 3.2:
Cyanotoxin measurements in water samples at gillnet and seine sites on Lake Kabetogama, LC-MS/MS and ELISA

	Anatoxin-a ELISA (µg/L)	Anatoxin-a LC-MS/MS(µg/L)	Microcystin ELISA (µg/L)	Microcystin LC-MS/MS (µg/L)
SN101	NA	0.436	NA	ND
SN102	NA	0.463	NA	ND
SN103	NA	0.051	NA	ND
GN09	0.6	ND	3.380	0.6
GN10	ND	ND	3.660	0.51
GN14	ND	ND	2.460	ND
Average	**0.200**	**0.158**	**3.167**	**0.185**

Table 3.3:
Fish captured Lake Kabetogama gillnet and seine sites

		Year		
		2017	**2018**	**Total**
Adult				
	Yellow Perch	15	14	29
	Walleye	10	15	25
	Smallmouth Bass	11	6	17
	White Sucker	10	11	21
Young	**Of Year**			
	Yellow Perch	15	45	60
	Walleye	0	0	0
	Smallmouth Bass	15	21	36
	White Sucker	0	0	0
	Total	**76**	**112**	**188**

Table 3.4:
Cyanotoxin concentrations of blanks and spiked controls, LC-MS/MS and ELISA

	Microcystin LC-MS/MS (µg/kg)	**Anatoxin-a LC-MS/MS (µg/kg)**	**Microcystin ELISA (µg/kg)**	**Anatoxin-a ELISA (µg/kg)**
Spiked DI water	< 1.23 (µg/L)	3.52 (µg/L)	> 5.00 (µg/L)	> 05.00 (µg/L)
Blank Muscle	< 1.23	< 0.06	> 0.38	> 0.38
Blank YOY	< 0.70	< 0.03	> 0.38	> 0.38
Pre-spike Extraction YOY1	< 0.90	< 0.04	> 0.38	> 0.38
Pre-extraction spike YOY2	< 0.79	< 0.04	> 0.38	> 0.38
Pre-extraction spike Liver	< 2.51	< 0.11	> 0.38	> 0.38
Pre-extraction spike Muscle	< 1.16	< 0.05	> 0.38	> 0.38
Post-extraction spike YOY1	< 0.68	< 0.03	> 0.38	> 0.38
Post-extraction spike YOY2	< 1.18	< 0.05	> 0.38	> 0.38
Average	**< 1.14**	**< 0.05**	**> 0.38**	**> 0.38**

Table 3.5:
Microcystin-LR and Anatoxin-a LC-MS/MS MANOVA

	Df	**Pillai**	**F**	**numDf**	**denDf**	**p**
Fish	3	0.17	2.07	6	134	0.06.
Organ	2	0.15	2.77	4	134	0.03*
Location	3	0.08	1.00	6	134	0.43
Year	1	0.01	0.28	2	66	0.75
Residuals	57					

*** = 0.001 ** =0.01 *0.05 . = 0.1

Table 3.6A: Results of ELISA and LC-MS/MS testing on cyanotoxins in fish tissue extractions from Voyageurs National Park

Fish	Tissue	Site	Year	n	Mass (g)	MCY LC-MS/MS (µg/kg)	ATX-A LC-MS/MS (µg/kg)	MCY ELISA (µg/kg)	ATX-A ELISA (µg/kg)
SMB	Liver	GN10	2017	1	5.830	<0.210	<0.009	>0.129	>0.129
SMB	Liver	GN13	2017	1	3.920	**22.300**	<0.014	>0.191	>0.191
SMB	Liver	GN13	2017	1	5.470	<0.220	<0.010	>0.137	>0.137
SMB	Liver	GN15	2017	1	1.160	<1.060	<0.048	>0.647	>0.647
SMB	Liver	GN19	2017	2	6.310	**65.900**	<0.009	>0.119	>0.119
SMB	Liver	GN4	2017	1	4.330	**28.000**	<0.013	>0.173	>0.173
SMB	Liver	GN5	2017	1	3.780	<0.330	<0.015	>0.198	>0.198
SMB	Liver	GN5	2017	1	5.060	<0.240	<0.011	>0.148	>0.148
SMB	Liver	GN9	2017	1	5.560	**164.000**	<0.010	>0.135	>0.135
SMB	Muscle	GN10	2017	1	2.460	<0.500	<0.022	>0.305	>0.305
SMB	Muscle	GN13	2017	2	2.060	<0.600	<0.027	>0.364	>0.364
SMB	Muscle	GN15	2017	1	2.120	<0.580	<0.026	>0.354	>0.354
SMB	Muscle	GN19	2017	2	2.210	<0.560	<0.025	>0.339	>0.339
SMB	Muscle	GN4	2017	1	2.020	<0.610	<0.027	>0.371	>0.371
SMB	Muscle	GN5	2017	3	2.050	<0.600	<0.027	>0.366	>0.366
SMB	Muscle	GN9	2017	1	2.350	<0.520	<0.024	>0.319	>0.319
SMB	YOY	SN13	2017	4	1.030	<1.190	<0.054	**0.405**	>0.728
SMB	YOY	SN16	2017	5	1.280	<0.960	<0.043	>0.586	>0.586
SMB	YOY	SN16	2017	6	1.200	<1.020	<0.046	**0.448**	>0.625
WAE	Liver	GN10	2017	5	1.710	<0.720	<0.032	>0.439	**0.188**
WAE	Muscle	GN10	2017	5	1.980	<0.620	<0.028	>0.379	>0.379
WAE	Muscle	GN10	2017	5	2.220	<0.550	<0.025	>0.338	>0.338
WTS	Liver	GN10	2017	3	1.970	<0.620	<0.028	>0.381	>0.381
WTS	Liver	GN10	2017	5	2.850	<0.430	<0.019	**0.181**	>0.263
WTS	Muscle	GN10	2017	5	2.020	<0.610	<0.027	>0.371	>0.371
WTS	Muscle	GN10	2017	3	2.040	<0.600	<0.027	>0.368	>0.368
WTS	Muscle	GN13	2017	1	2.110	<0.580	<0.026	>0.355	>0.355
WTS	Muscle	GN13	2017	1	1.750	<0.700	<0.032	>0.429	>0.429
YP	Liver	GN2	2017	5	1.870	<0.660	<0.030	**0.322**	>0.401
YP	Liver	GN2	2017	5	2.050	<0.600	<0.027	**0.147**	>0.366
YP	Liver	GN3	2017	5	0.960	<1.280	<0.058	**0.370**	>0.781
YP	Muscle	GN2	2017	5	2.070	<0.590	<0.027	**0.074**	>0.362
YP	Muscle	GN2	2017	5	2.230	<0.550	<0.025	**0.165**	>0.336

Table 3.6B: Results of ELISA and LC-MS/MS testing on cyanotoxins in fish tissue extractions from Voyageurs National Park

Fish	Tissue	Site	Year	n	Mass (g)	MCY LC-MS/MS (µg/kg)	ATX-A LC-MS/MS (µg/kg)	MCY ELISA (µg/kg)	ATX-A ELISA (µg/kg)
YP	Muscle	GN3	2017	5	2.160	<0.570	<0.026	>0.347	>0.347
YP	YOY	SN13	2017	15	1.790	<0.690	<0.031	>0.419	>0.419
SMB	Liver	GN10	2018	3	2.080	**255.100**	<0.027	>0.361	>0.361
SMB	Liver	GN13	2018	2	1.980	<0.620	<0.028	>0.379	>0.379
SMB	Liver	GN14	2018	1	1.990	<0.620	<0.028	>0.377	>0.377
SMB	Muscle	GN10	2018	3	2.060	<0.600	<0.027	>0.364	>0.364
SMB	Muscle	GN13	2018	2	2.020	<0.610	<0.027	>0.371	>0.371
SMB	Muscle	GN14	2018	1	2.090	<0.590	<0.026	>0.359	>0.359
SMB	YOY	SN102	2018	5	2.070	<0.590	<0.027	>0.362	>0.362
SMB	YOY	SN102	2018	5	2.280	<0.540	<0.024	>0.329	>0.329
SMB	YOY	SN102	2018	5	2.140	<0.570	<0.026	>0.350	>0.350
SMB	YOY	SN101	2018	2	2.010	<0.610	<0.027	>0.373	>0.373
SMB	YOY	SN103	2018	4	2.060	<0.600	<0.027	>0.364	>0.364
WAE	Liver	GN10	2018	5	2.240	<0.550	<0.025	>0.335	>0.335
WAE	Liver	GN14	2018	5	2.030	**16.400**	<0.027	>0.369	>0.369
WAE	Liver	GN9	2018	5	1.900	<0.650	<0.029	>0.395	>0.395
WAE	Muscle	GN10	2018	5	2.040	<0.600	<0.027	>0.368	>0.368
WAE	Muscle	GN14	2018	5	1.970	<0.620	<0.028	>0.381	>0.381
WAE	Muscle	GN9	2018	5	2.050	<0.600	<0.027	>0.366	>0.366
WTS	Liver	GN10	2018	4	2.200	<0.560	<0.025	>0.341	>0.341
WTS	Liver	GN13	2018	3	1.580	<0.780	<0.035	>0.475	>0.475
WTS	Liver	GN14	2018	2	1.000	<1.230	<0.055	>0.750	>0.750
WTS	Liver	GN9	2018	5	2.040	<0.600	<0.027	>0.368	>0.368
WTS	Muscle	GN10	2018	4	1.890	<0.650	<0.029	>0.397	>0.397
WTS	Muscle	GN13	2018	3	1.970	<0.620	<0.028	>0.381	>0.381
WTS	Muscle	GN14	2018	2	2.090	<0.590	<0.026	>0.359	>0.359
WTS	Muscle	GN9	2018	5	2.360	<0.520	<0.023	>0.318	>0.318
YP	Liver	GN10	2018	2	0.310	<3.960	<0.178	>2.419	>2.419
YP	Liver	GN13	2018	2	0.710	<1.730	<0.078	>1.056	>1.056
YP	Liver	GN14	2018	5	2.080	<0.590	<0.027	>0.361	>0.361
YP	Liver	GN9	2018	5	2.010	<0.610	<0.027	>0.373	>0.373
YP	Muscle	GN10	2018	2	2.100	<0.590	<0.026	>0.357	>0.357
YP	Muscle	GN13	2018	2	1.950	<0.630	<0.028	>0.385	>0.385

Table 3.6C: Results of ELISA and LC-MS/MS testing on cyanotoxins in fish tissue extractions from Voyageurs National Park

Fish	Tissue	Site	Year	n	Mass (g)	MCY LC-MS/MS (µg/kg)	ATX-A LC-MS/MS (µg/kg)	MCY ELISA (µg/kg)	ATX-A ELISA (µg/kg)
YP	Muscle	GN14	2018	5	2.010	<0.610	<0.027	>0.373	>0.373
YP	Muscle	GN9	2018	5	1.930	<0.640	<0.029	>0.389	>0.389
YP	YOY	SN102	2018	5	2.050	<0.600	<0.027	>0.366	>0.366
YP	YOY	SN102	2018	5	2.070	<0.590	<0.027	>0.362	>0.362
YP	YOY	SN102	2018	5	2.080	<0.590	<0.027	>0.361	>0.361
YP	YOY	SN101	2018	5	2.160	<0.570	<0.026	>0.347	>0.347
YP	YOY	SN101	2018	5	1.960	<0.630	<0.028	>0.383	>0.383
YP	YOY	SN101	2018	5	2.000	<0.610	<0.028	>0.375	>0.375
YP	YOY	SN103	2018	5	2.140	<0.570	<0.026	>0.350	>0.350
YP	YOY	SN103	2018	5	2.010	<0.610	<0.027	>0.373	>0.373
YP	YOY	SN103	2018	5	2.110	<0.580	<0.026	>0.355	>0.355
Means				**278**	**2.257**	**7.799**	**0.030**	**0.382**	**0.401**

Figures

Figure 1.1: Lake Kabetogama gillnetting (GN) and seining (SN) sites from 2017 and 2018

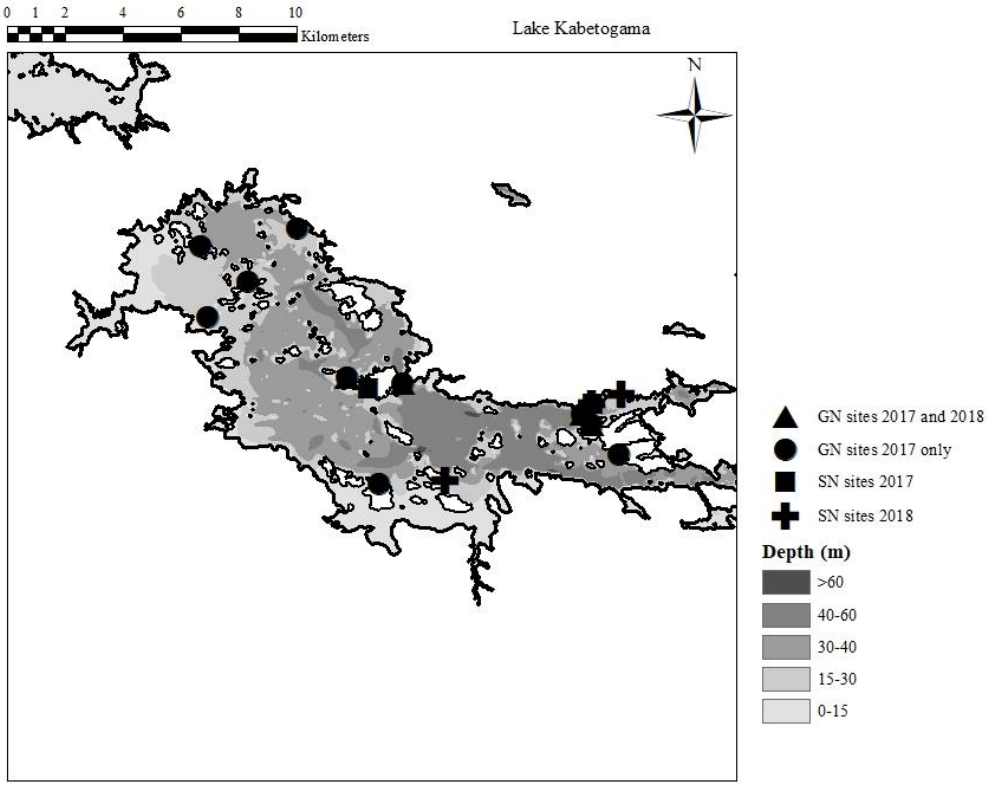


Figure 2.1: Lake Kabetogama gillnetting (GN) and seining (SN) sites from 2017 and 2018

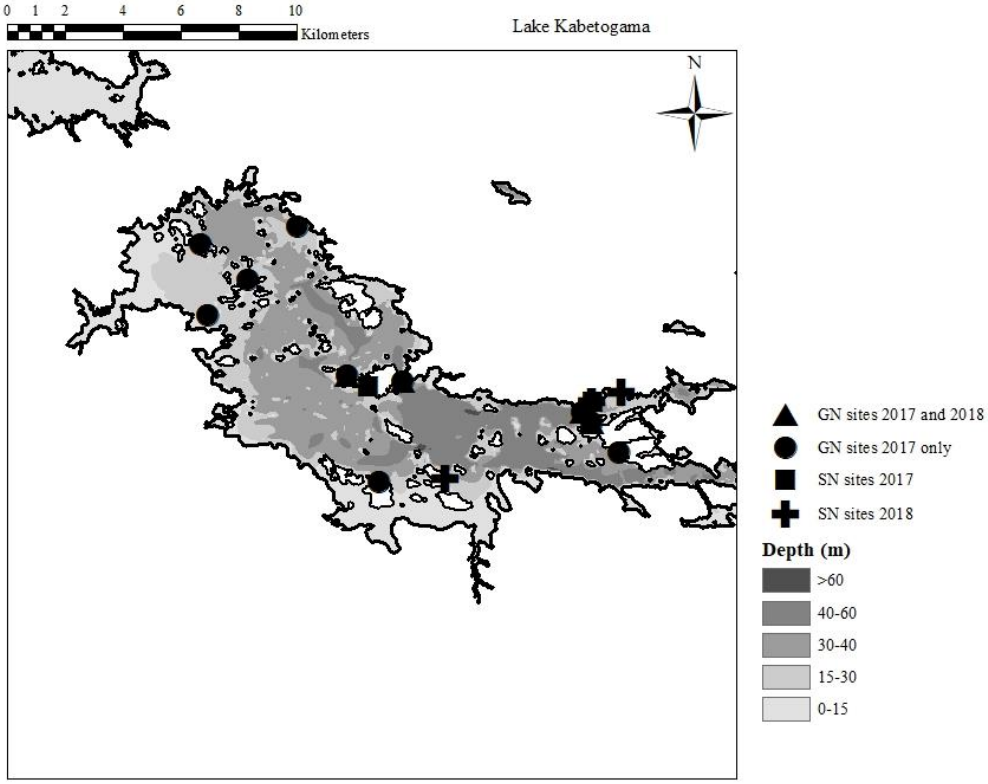


Figure 2.2

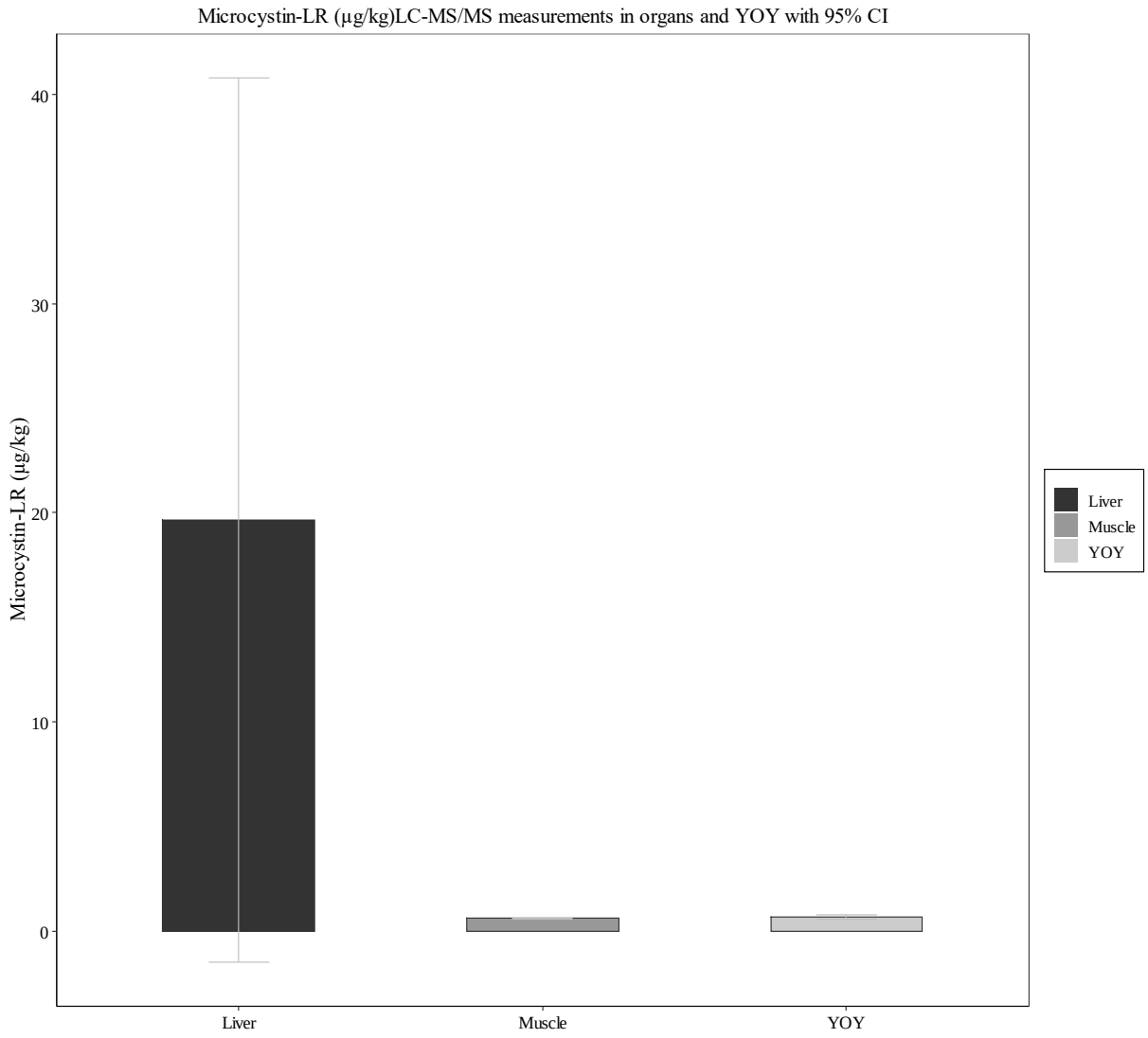

Microcystin-LR (µg/kg)LC-MS/MS measurements in organs and YOY with 95% CI
Microcystin-LR (µg/kg)
40
30
20
10
0
Liver
Muscle
YOY
Liver
Muscle
YOY

Figure 2.3

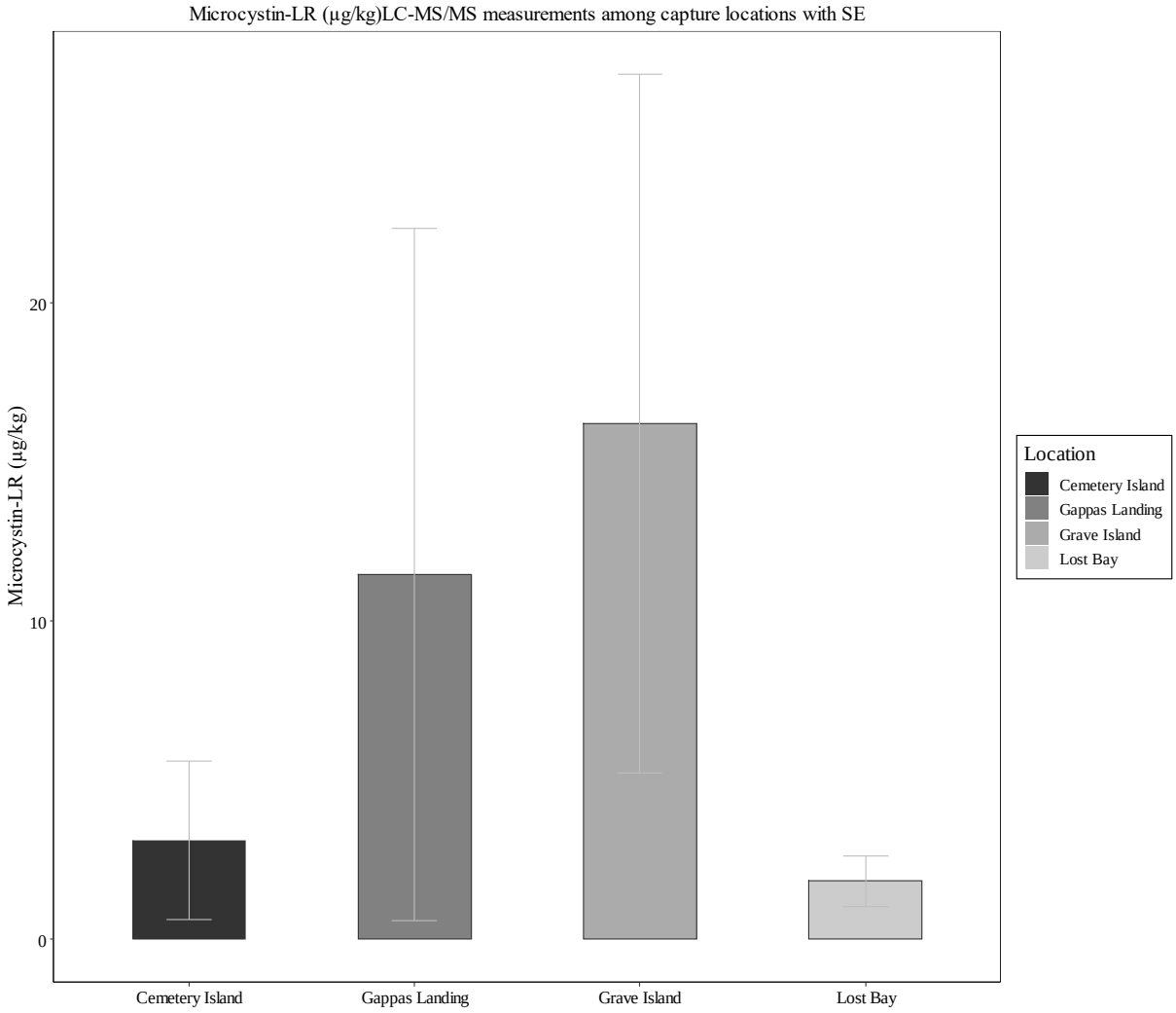

Microcystin-LR (µg/kg)LC-MS/MS measurements among capture locations with SE
Microcystin-LR (µg/kg)
20
10
0
Cemetery Island
Gappas Landing
Grave Island
Lost Bay
Location
Cemetery Island
Gappas Landing
Grave Island
Lost Bay

Figure 2.4

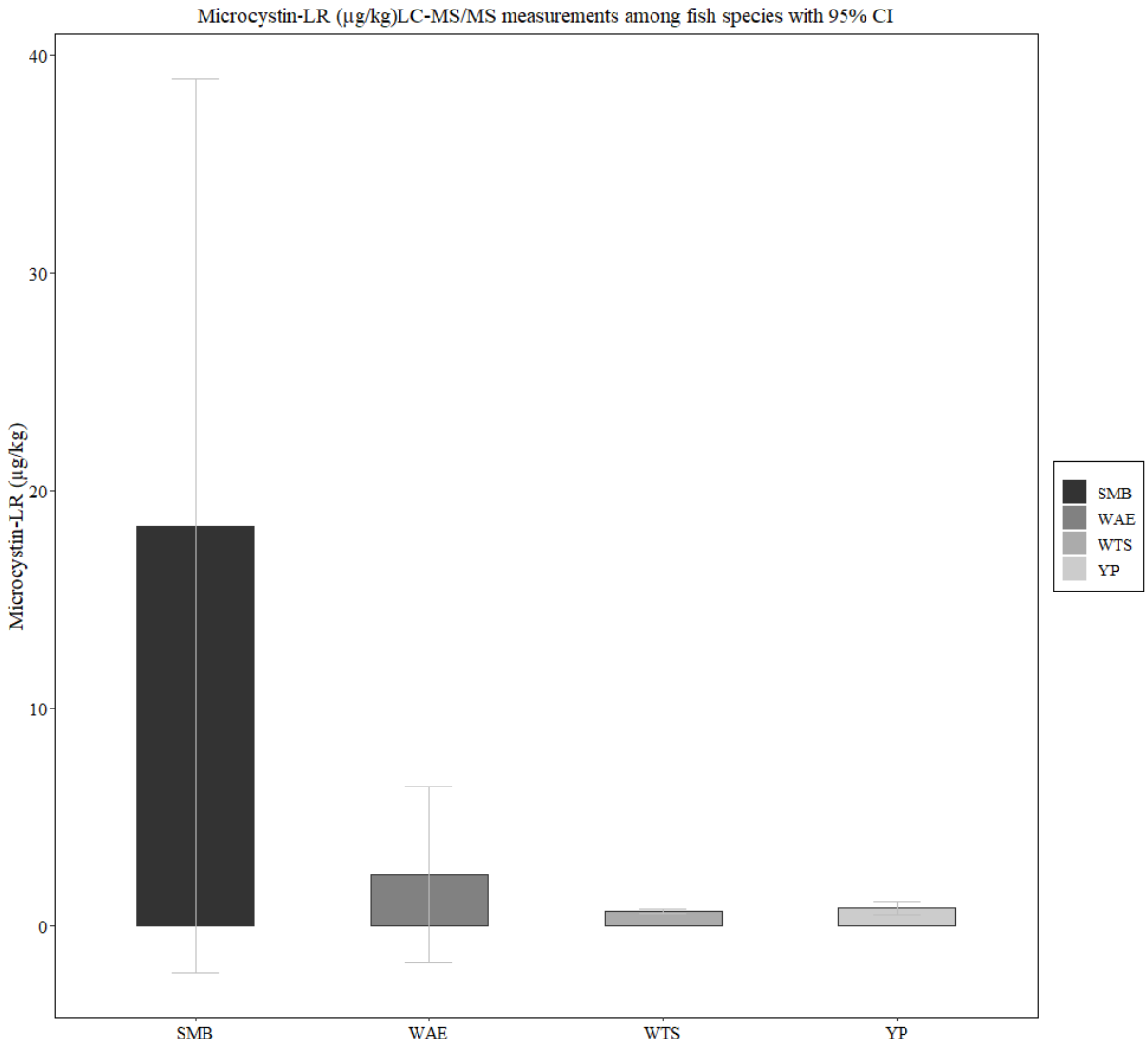
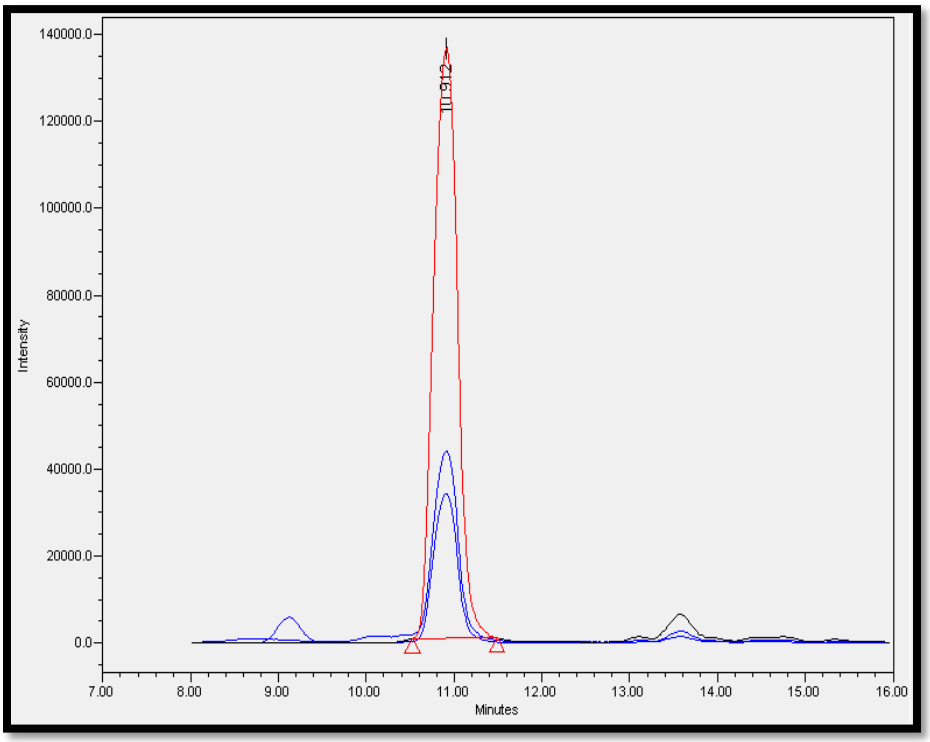

Microcystin-LR (µg/kg)LC-MS/MS measurements among fish species with 95% CI
Microcystin-LR (µg/kg)
40
30
20
10
0
SMB
WAE
WTS
YP
SMB
WAE
WTS
YP

Figure 2.5 A) LC-MS/MS chromatogram showing phenylalanine peak; B) zoomed image of phenylalanine peak showing matrix suppression of anatoxin-a

A)



B)

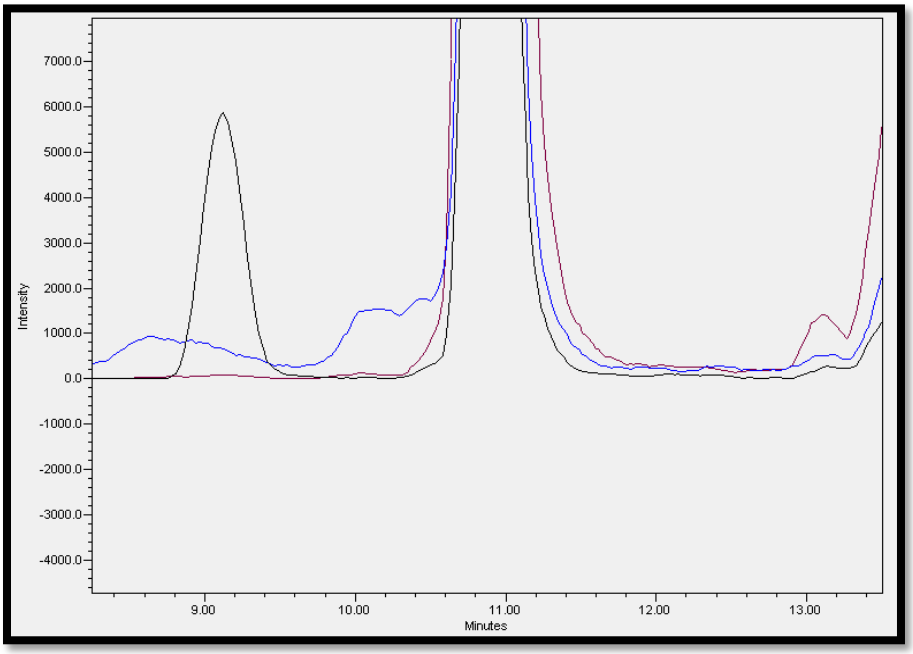
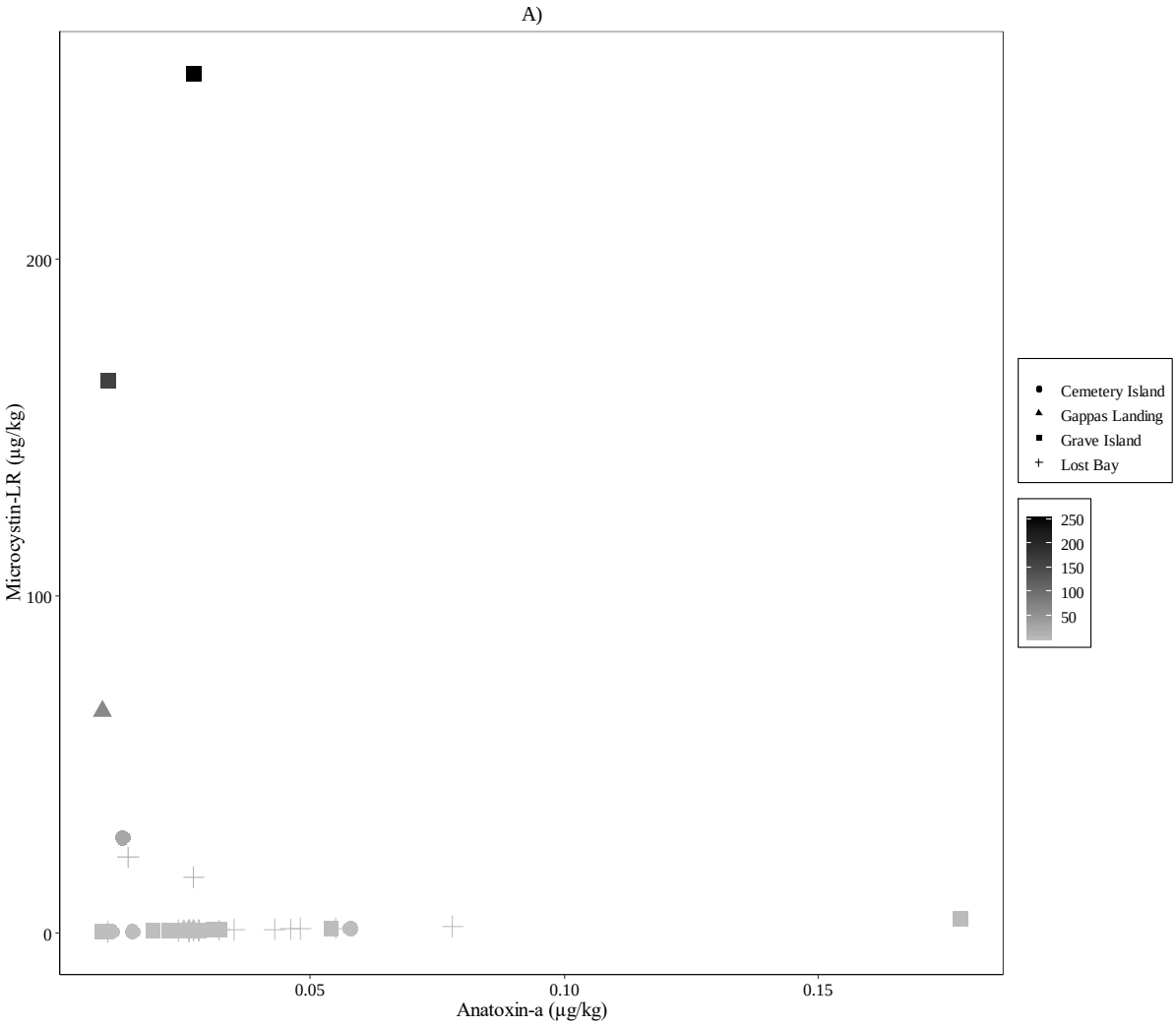
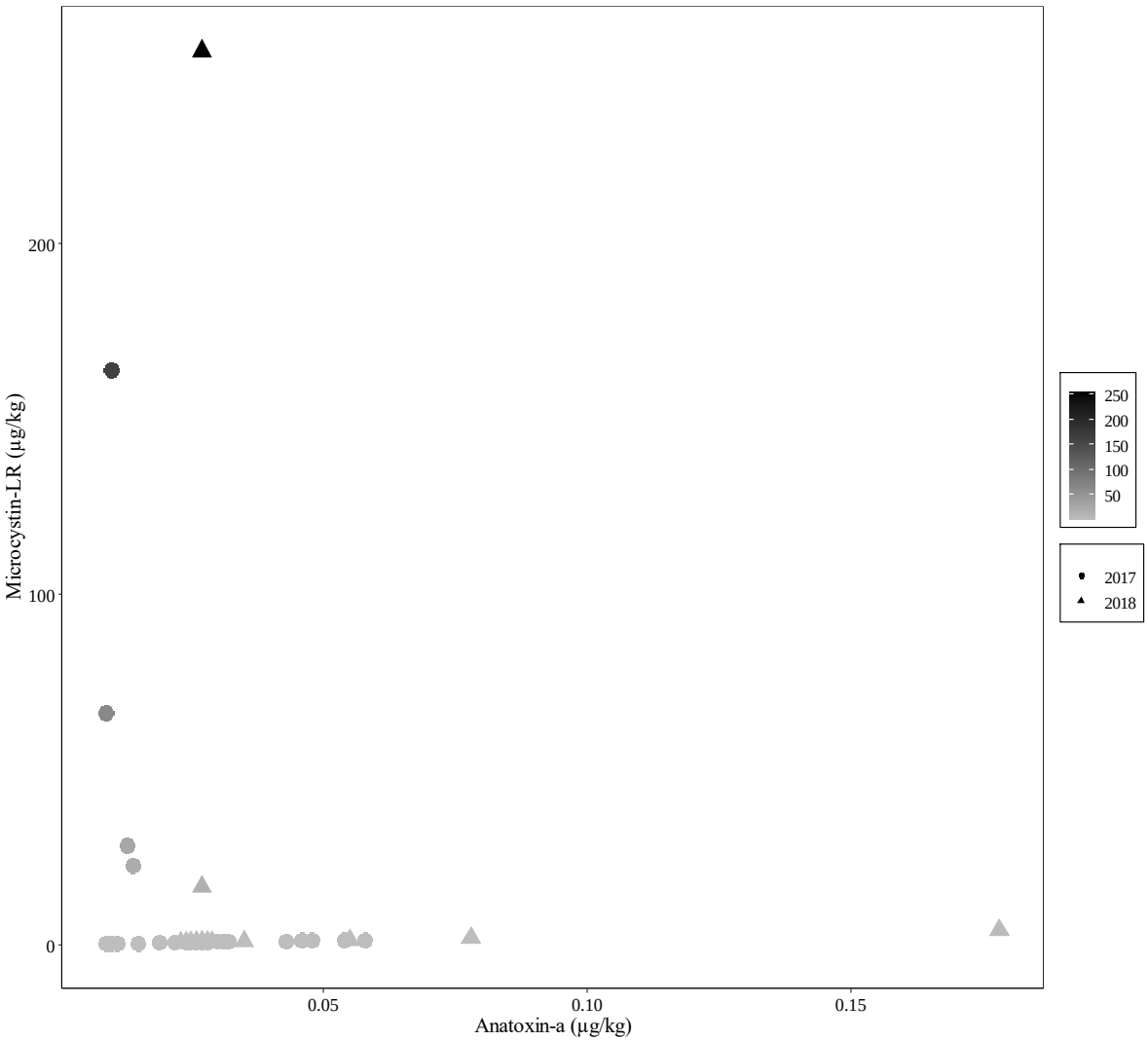


Figure 2.6

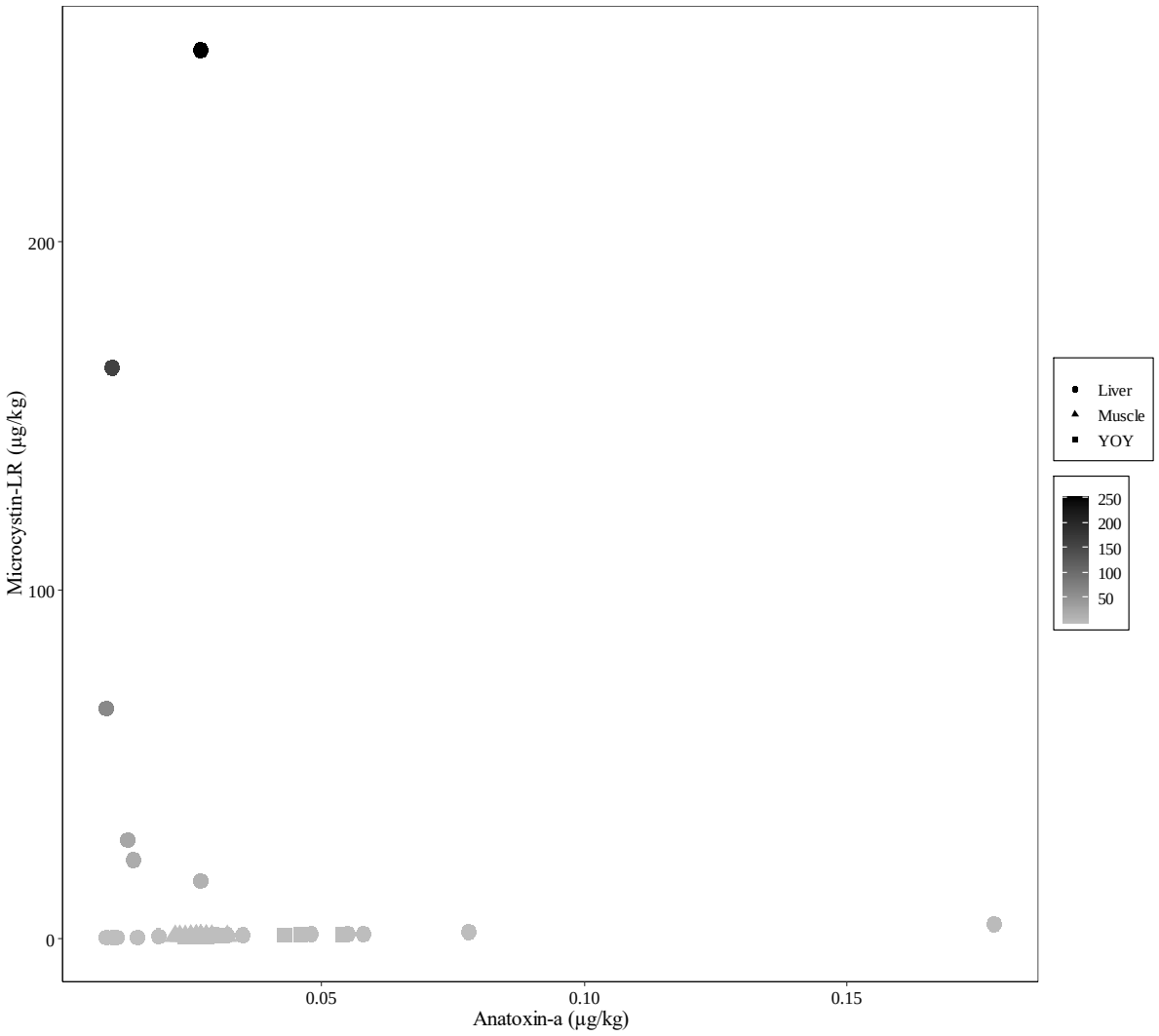
Microcystin-LR (µg/kg)LC-MS/MS plotted against anatoxin-a (µg/kg) LC-MS/MS



B)


Microcystin-LR (µg/kg)
200
100
0
0.05
0.10
0.15
Anatoxin-a (µg/kg)
250
200
150
100
50
2017
2018

C)


Microcystin-LR (µg/kg)
200
100
0
0.05
0.10
0.15
Anatoxin-a (µg/kg)
Liver
Muscle
YOY
250
200
150
100
50

D)

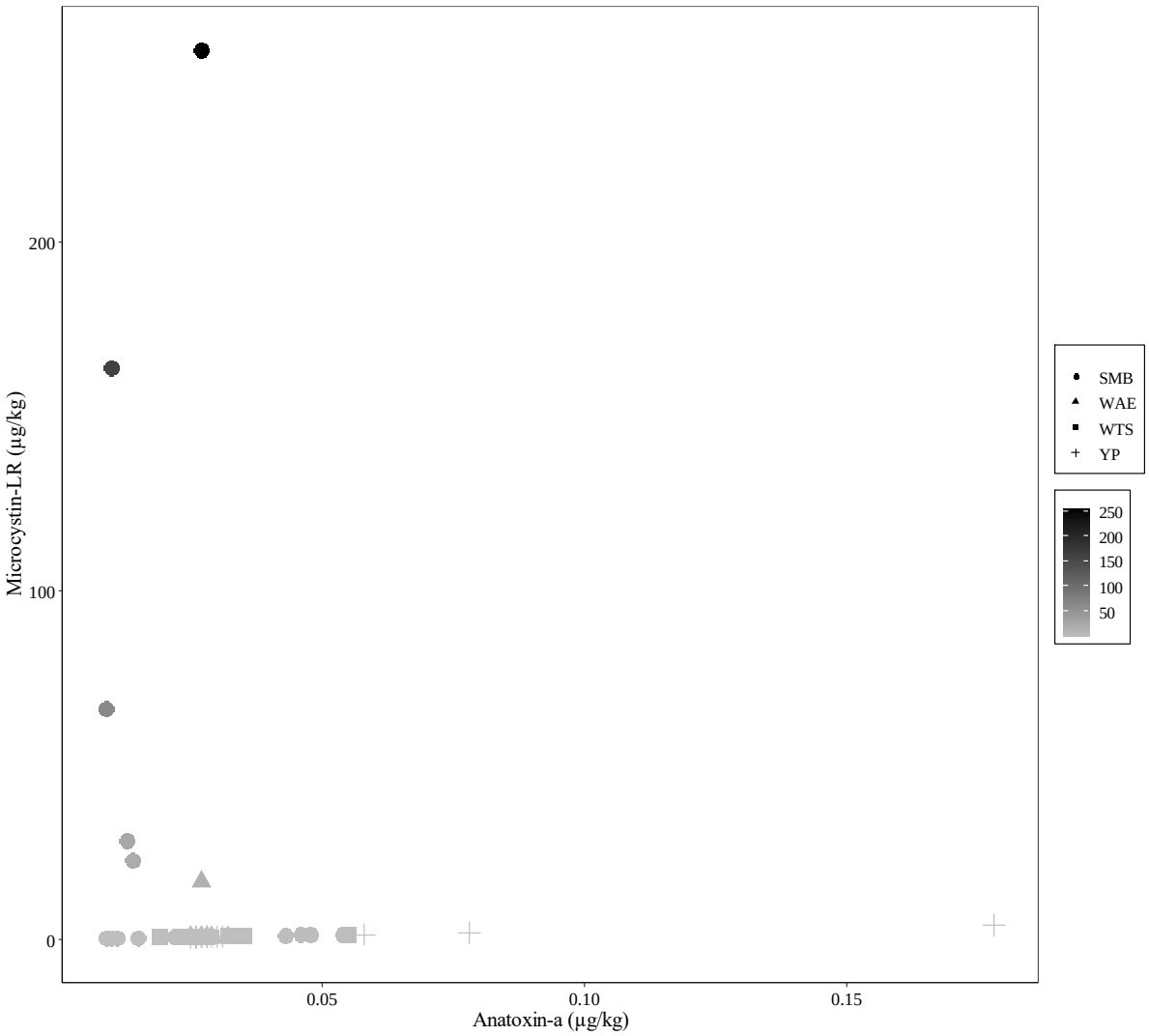

Microcystin-LR (µg/kg)
200
100
0
0.05
0.10
0.15
Anatoxin-a (µg/kg)
SMB
WAE
WTS
YP
250
200
150
100
50

Figure 2.7 Map of Lake Kabetogama

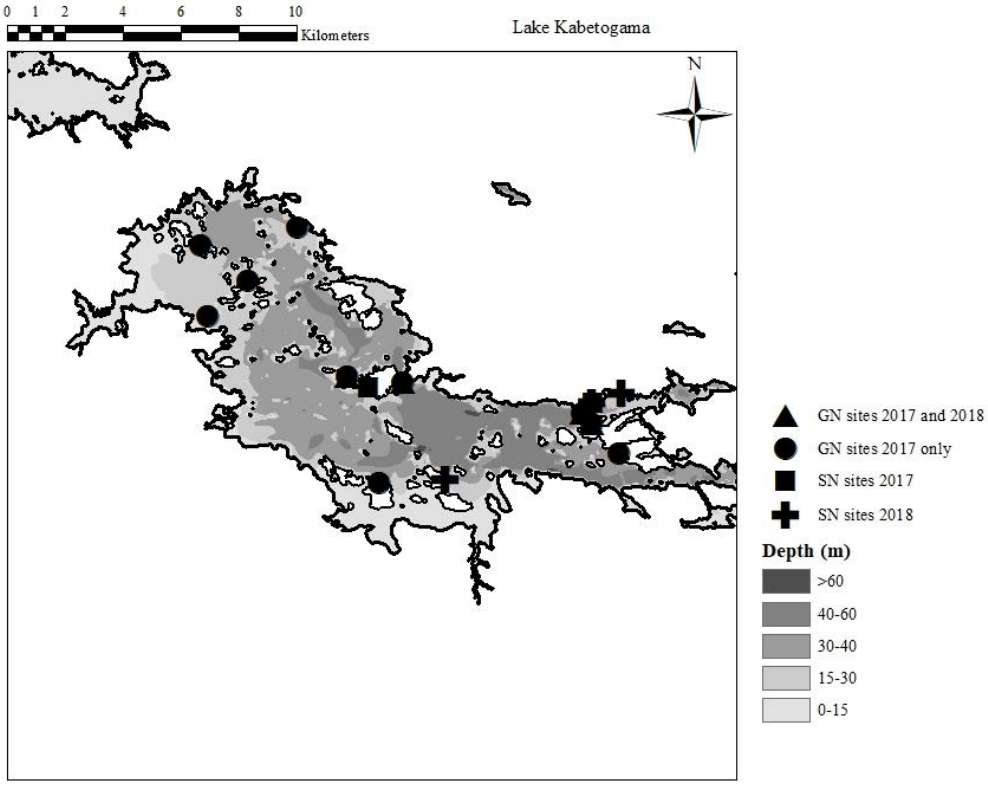


Figure 2.8 Map of Lost Bay location on Lake Kabetogama

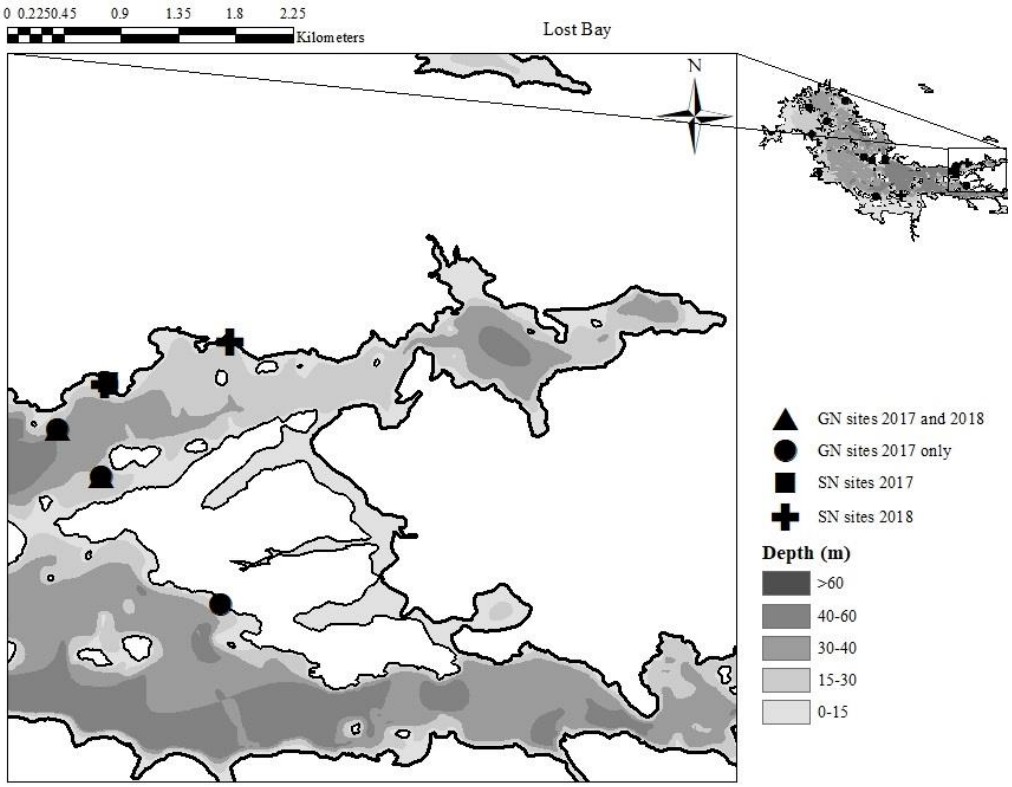


Figure 2.9 Map of near Grave Island location on Lake Kabetogama

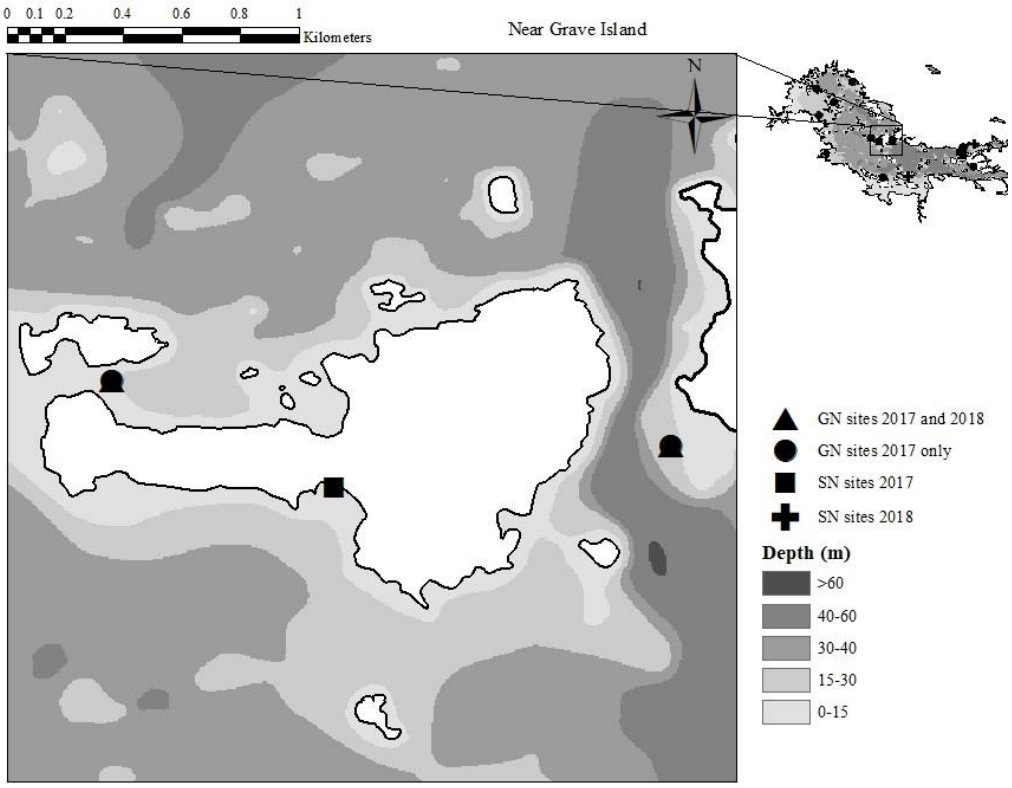


Figure 2.10 Map of near Gappas Landing location on Lake Kabetogama

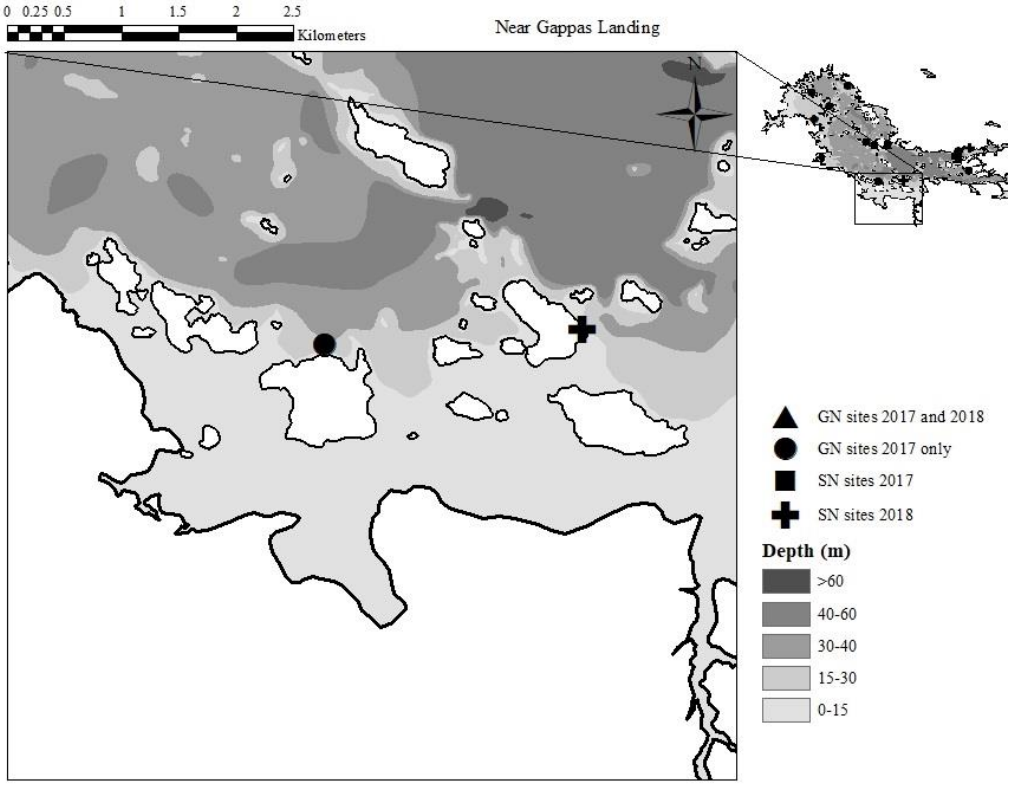


Figure 2.11 Smallmouth Bass stomach contents including crawfish remains

Figure 3.1 Lake Kabetogama gillnetting (GN) and seining (SN) sites from 2017 and 2018

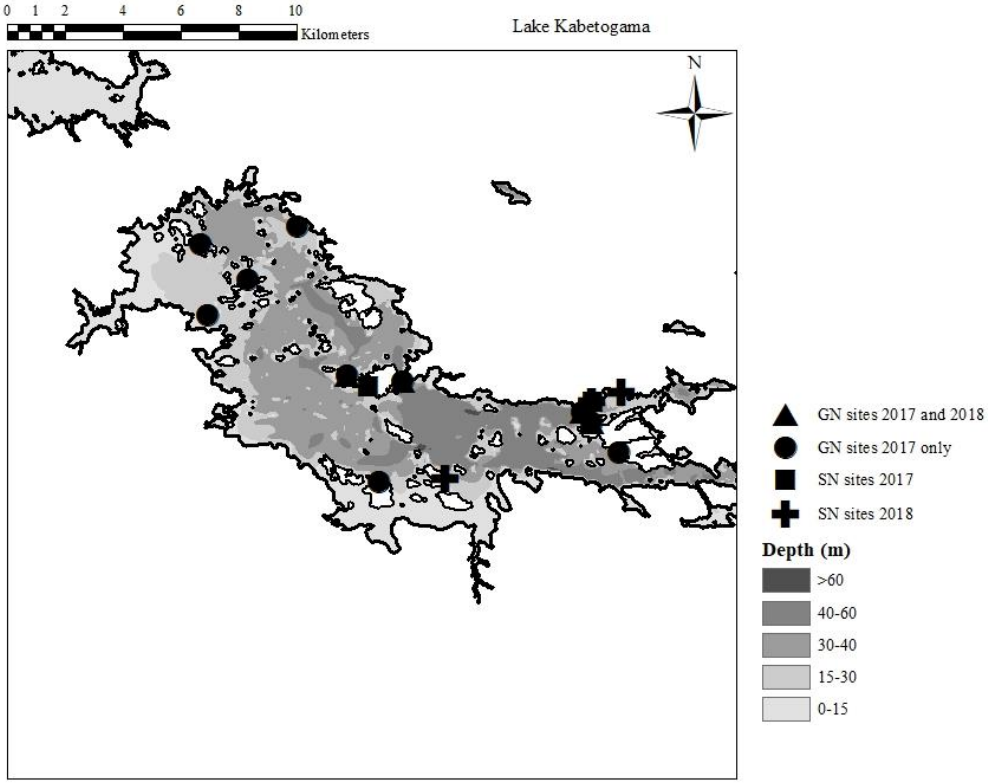


Figure 3.2

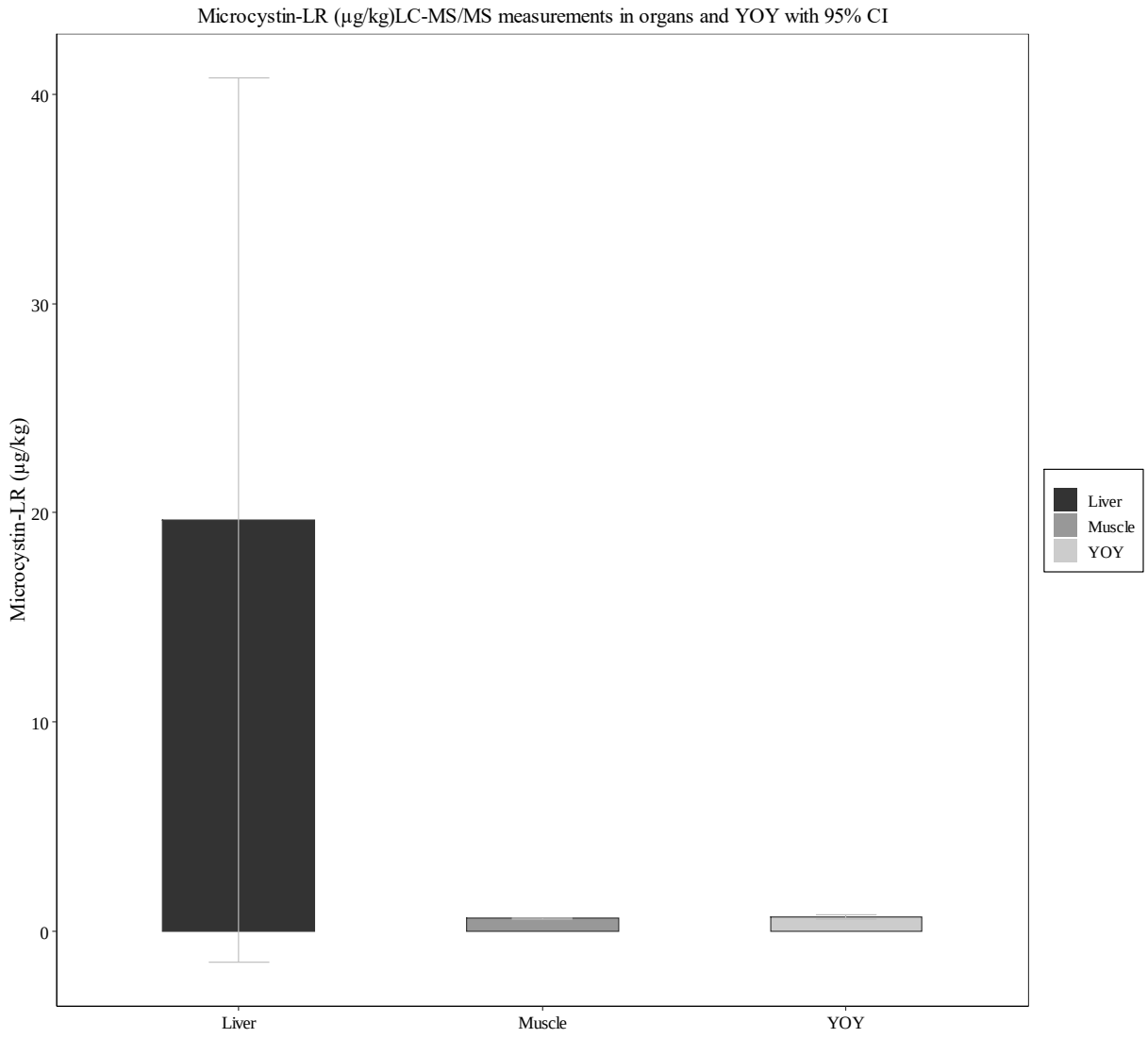

Microcystin-LR (µg/kg)LC-MS/MS measurements in organs and YOY with 95% CI
Microcystin-LR (µg/kg)
40
30
20
10
0
Liver
Muscle
YOY
Liver
Muscle
YOY

Figure 3.3

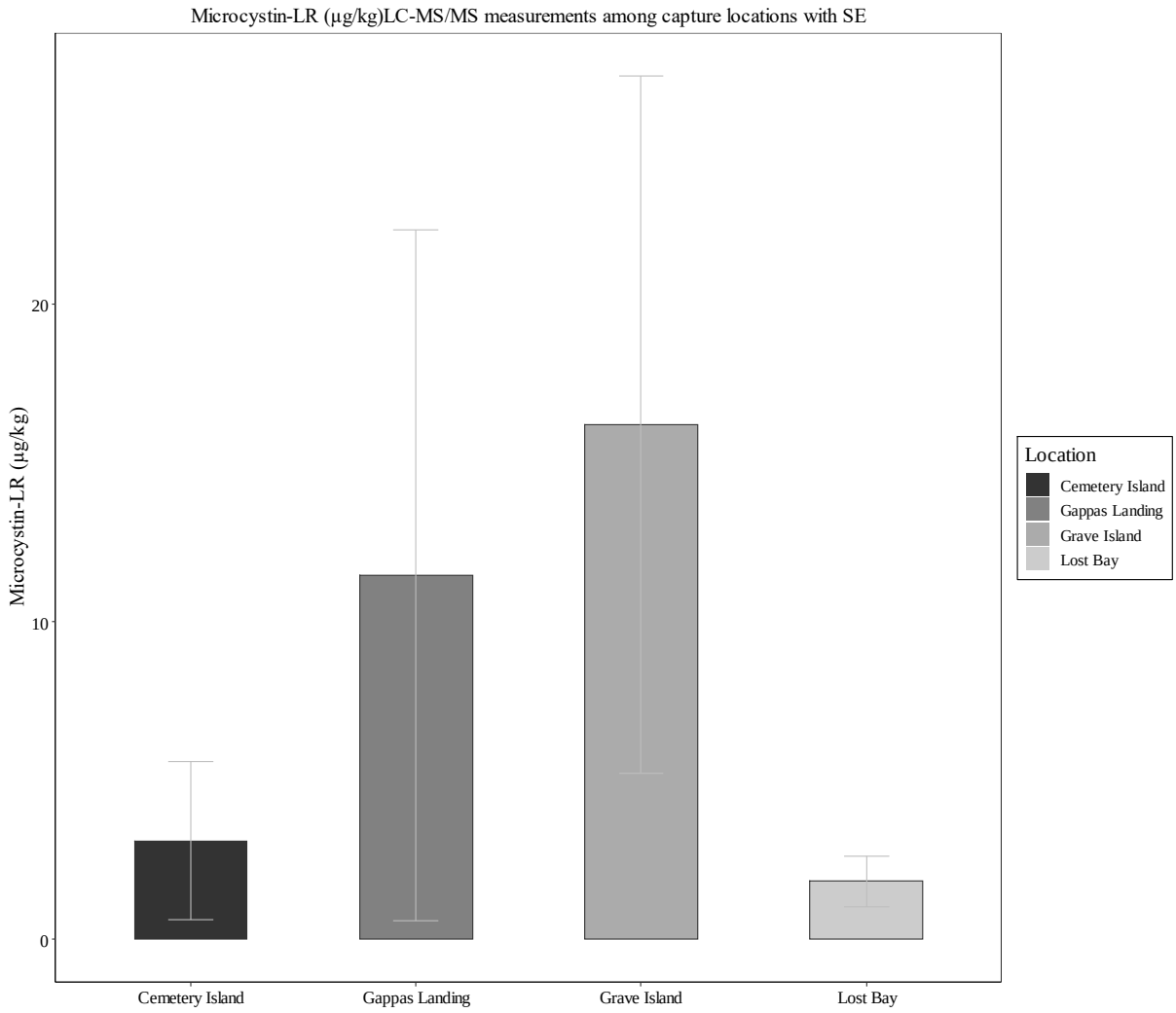

Microcystin-LR (µg/kg)LC-MS/MS measurements among capture locations with SE
Microcystin-LR (µg/kg)
20
10
0
Cemetery Island
Gappas Landing
Grave Island
Lost Bay
Location
Cemetery Island
Gappas Landing
Grave Island
Lost Bay

Figure 3.4

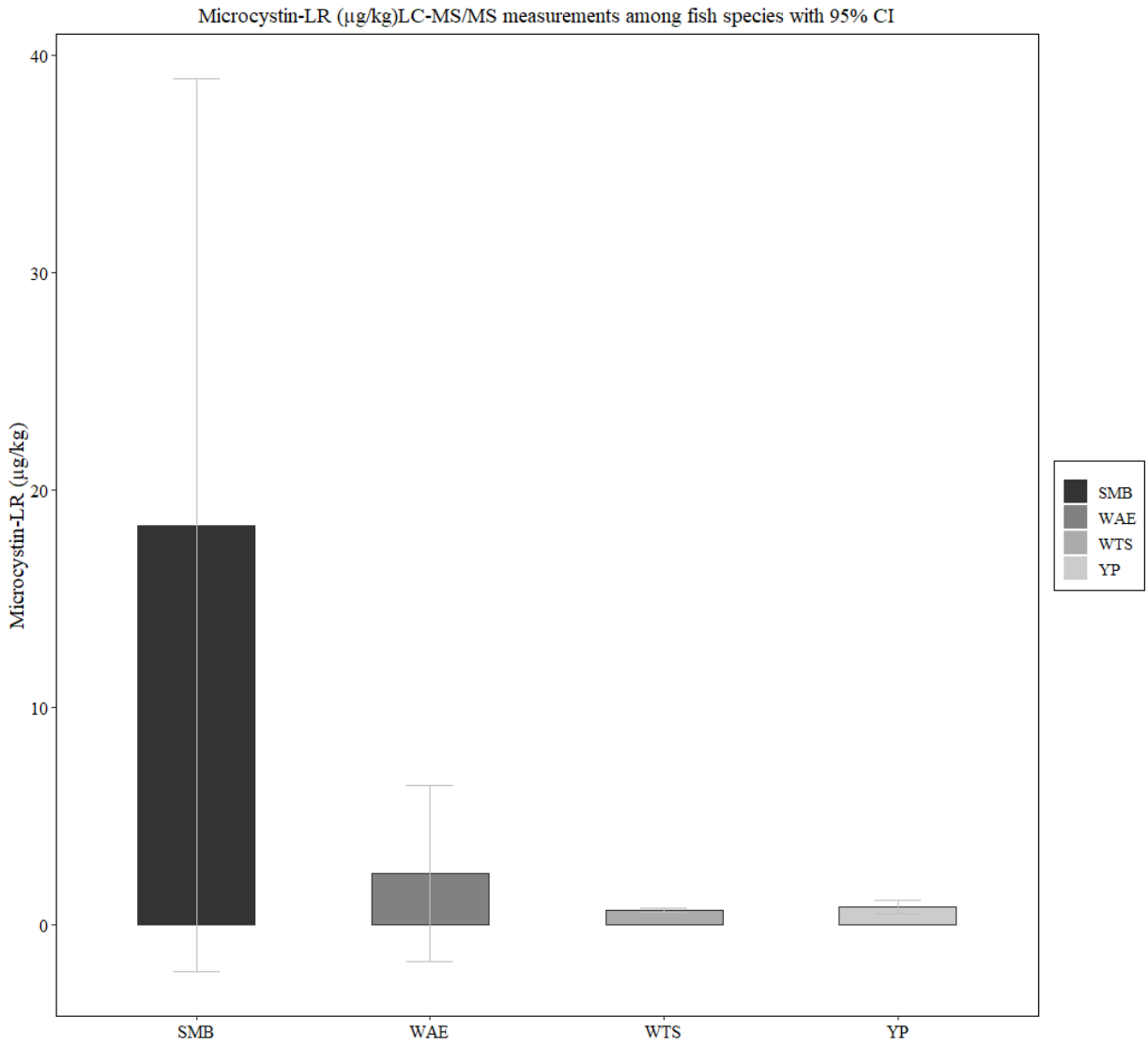

Microcystin-LR (µg/kg)LC-MS/MS measurements among fish species with 95% CI
Microcystin-LR (µg/kg)
40
30
20
10
0
SMB
WAE
WTS
YP
SMB
WAE
WTS
YP

Figure 3.5

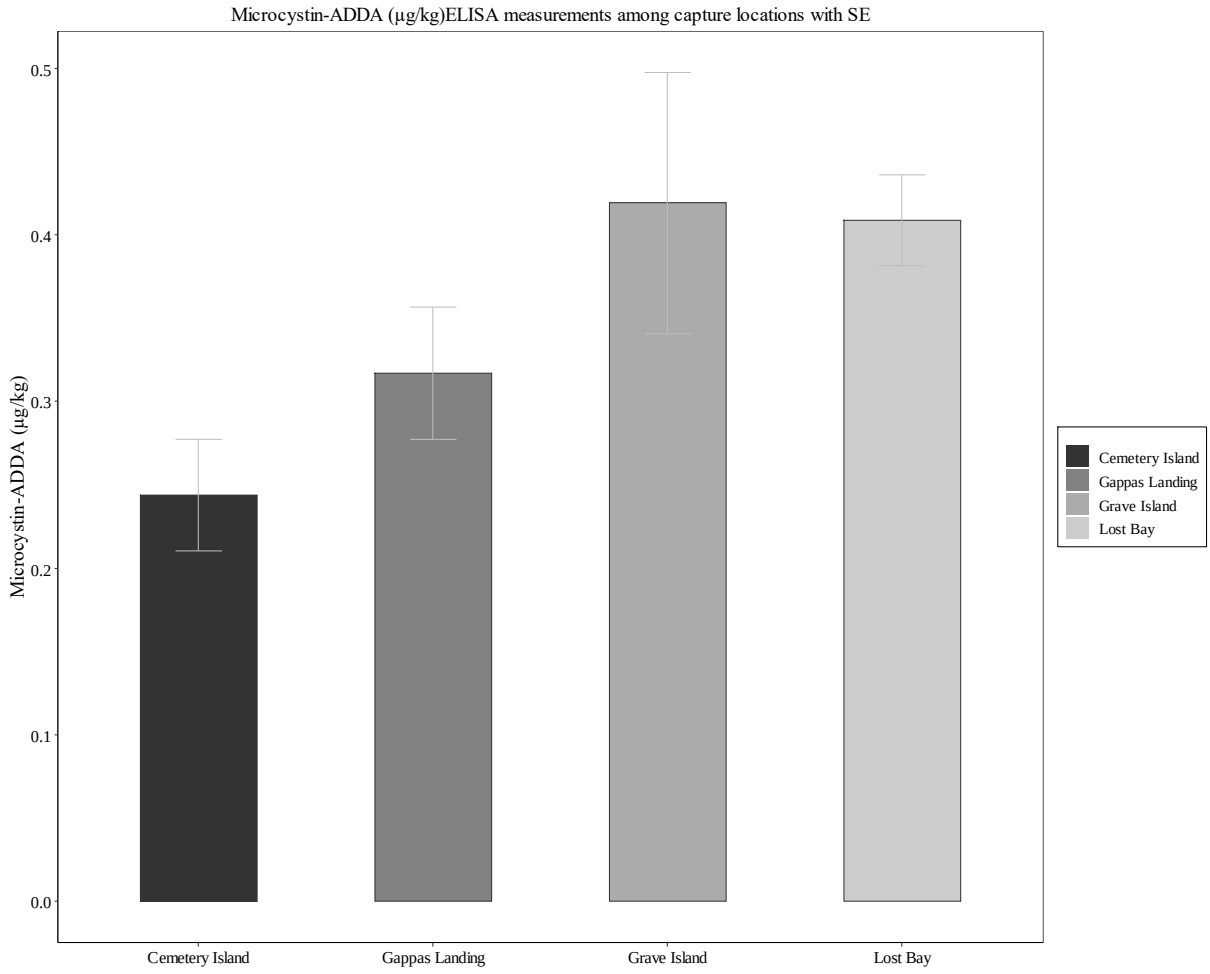
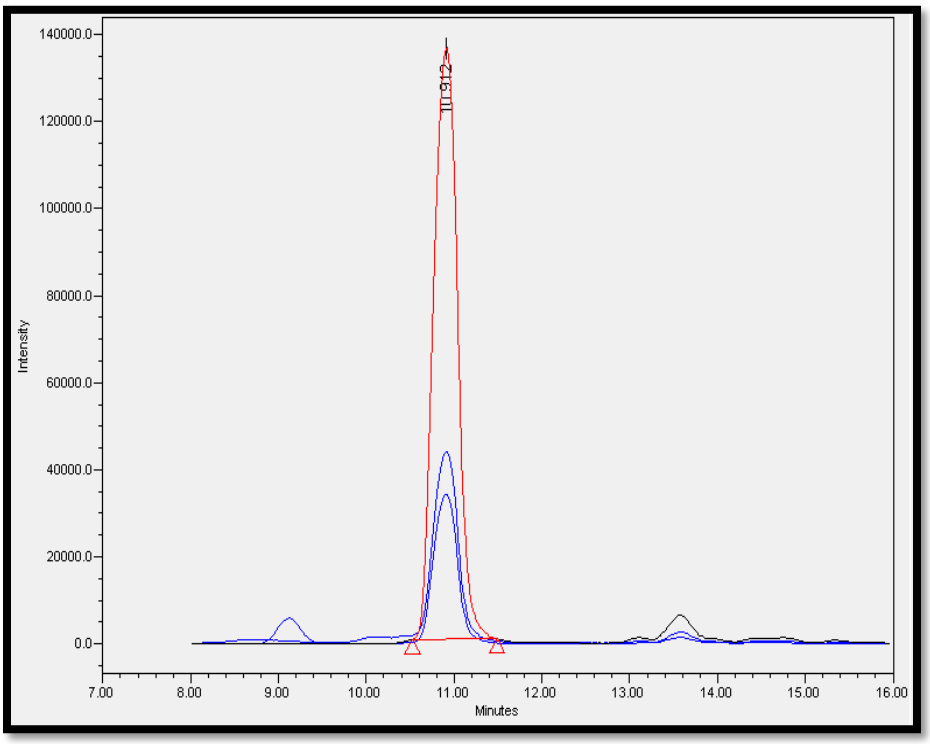

Microcystin-ADDA (µg/kg)ELISA measurements among capture locations with SE
Microcystin-ADDA (µg/kg)
0.0
0.1
0.2
0.3
0.4
0.5
Cemetery Island
Gappas Landing
Grave Island
Lost Bay
Cemetery Island
Gappas Landing
Grave Island
Lost Bay

Figure 3.6 A) LC-MS/MS chromatogram showing phenylalanine peak; B) zoomed image of phenylalanine peak showing matrix suppression of anatoxin-a

A)



B)

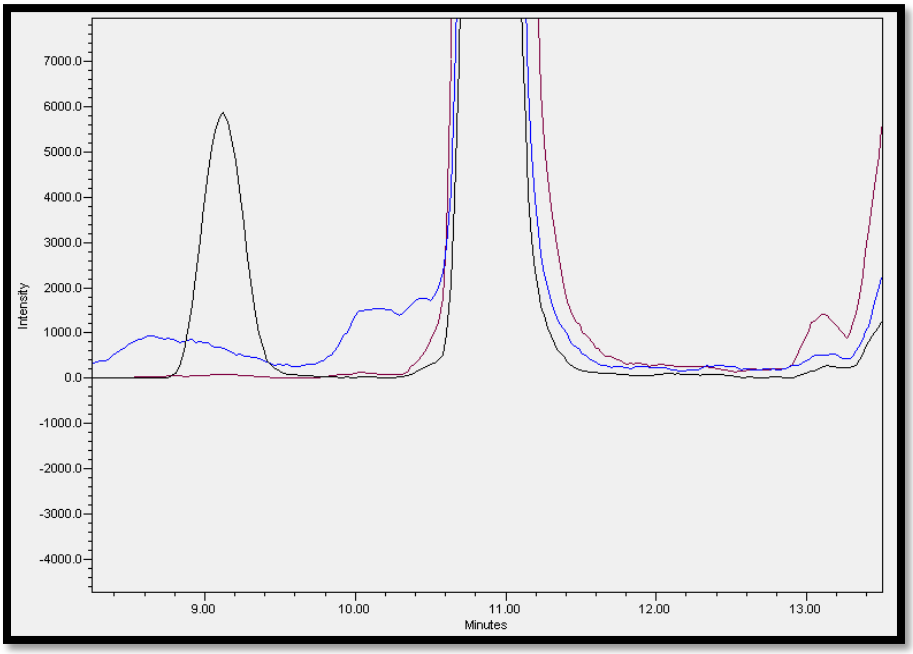
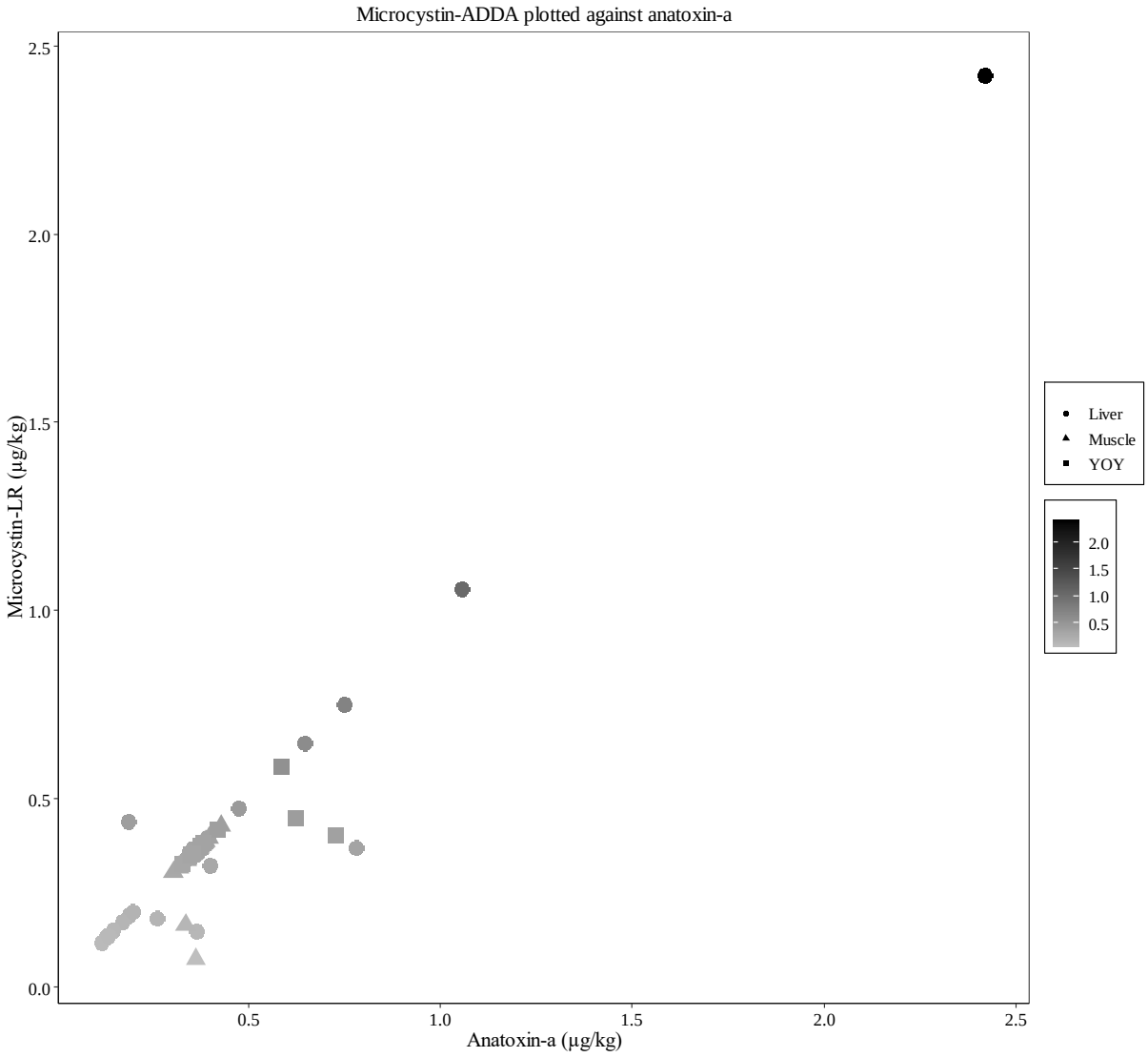


Figure 3.7


Microcystin-ADDA plotted against anatoxin-a
Microcystin-LR (µg/kg)
Anatoxin-a (µg/kg)
0.0
0.5
1.0
1.5
2.0
2.5
Liver
Muscle
YOY
2.0
1.5
1.0
0.5