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QUANTIFICATION AND DETECTION OF THE ALGAL TOXIN MICROCYSTIN IN WATER, SEDIMENT AND TISSUE

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QUANTIFICATION AND DETECTION OF THE ALGAL TOXIN MICROCYSTIN IN WATER, SEDIMENT AND TISSUE

BY

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ABSTRACT

QUANTIFICATION AND DETECTION OF THE ALGAL TOXIN MICROCYSTIN IN WATER, SEDIMENT AND TISSUE

By

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Microcystins are hepatotoxins produced by *Microcystis spp.* and some other species of cyanobacteria. The World Health Organizations has set a guideline of 1 μg/L for microcystin-LR in drinking water and cyanobacteria has been placed on the EPA's contaminant candidate list. The first portion of this research aimed to identify and quantify *Microcystis* and the associated toxins in source waters in Florida and Michigan. The second portion of this research evaluated existing methods and new techniques for toxin detection, including Enzyme-Linked ImmunoSorbent Assay (ELISA) and Polymerase Chain Reaction (PCR). This research also analyzed methods to effectively extract toxins from sediments and tissues. Cyanobacteria and the toxin microcystin were both present in Florida waters, with *Microcystis* and *Anabaena* as the dominant cyanobacteria. Toxin levels remained steadily low throughout the sample period, with the exception of Lake Monroe (max. concentration = 2176 ng/L). Evaluation of Michigan Lakes found approximately three times higher concentration of microcystin in lakes containing zebra mussels, than lakes without zebra mussels. Laboratory studies identified ELISA and PCR as good analytical tools for the evaluation of toxins levels and the presence of genes indicating possible toxin production. Preliminary studies of toxin extraction methods from tissue and sediments gave a basis for further evaluation of procedures to improve recovery efficiencies.

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"Images in this thesis are presented in color."

Literature Review

History and Prevalence of Cyanobacteria and Their Toxins in Surface Waters

Cyanobacteria evolved during the early Precambrian Period (~ 570 million years ago) and are classified within Eubacteria (Wehr and Sheath 2003). Cyanobacteria consist of 124 genera, 53 of which are unicellular or colonial and 71 of which are filamentous (Wehr and Sheath 2003). As a general description, cyanobacteria are prokaryotes with gram-negative cell walls and contain four photosynthetic pigments: chlorophyll, phycoerythrin, phycocyanin, and allophycocyanin. Many species contain gas vacuoles, which aid in buoyancy during changing environmental conditions (Whitton and Potts 2000). They generally range in individual cell size from 1 to 20 μm, however a cluster or colony may exceed 100 μm. Cells divide by simple binary fission through simultaneous invagination (pinching) of all layers of the cell wall (Wehr and Sheath 2003). Cyanobacteria have unique properties as well. Many types of cyanobacteria possess nitrogenase and have the ability to fix atmospheric nitrogen. Nitrogen fixation generally takes place inside the heterocyst where nitrogenase is contained and thus protected from the outside environment (Whitton and Potts 2000). Cyanobacteria are tolerant organisms, able to withstand low oxygen conditions and have a high tolerance for ultraviolet-B and –C radiation. Cyanobacteria have been recorded at a maximum temperature of 73°C in thermal springs of western North America; however, this is rare and only occurs with selective species (Whitton and Potts 2000).

Cyanobacterial species of Public Health Concern

Several species of cyanobacteria have shown to be toxigenic to both humans and animals. The presence of these organisms in surface and source waters have been

suggested as a risk to public health. The main toxin-forming genera are *Microcystis*, *Anabaena, Aphanizomenon, Oscillatoria* and *Cylindrospermopsis*. *Microcystis* is a colonial, free-floating type of cyanobacteria with an individual cell size ranging from 0.8 to 6.0 μm in diameter. *Microcystis* is commonly found in both eutrophic and hypereutrophic water bodies; in many cases *Microcystis* can be the dominant phytoplankton species (Watanabe et al. 1996). *Microcystis* may form blooms and surface scums. Blooms typically occur in temperate zones during the spring and summer, most often in eutrophic lakes. *Microcystis viridis*, *Microcystis aeruginosa*, and *Microcystis ichthyoblade* are three toxic species that have been found in freshwaters of North America (Wehr and Sheath 2003). One hundred and ten species of *Anabaena* of have been described. A large majority of these are planktonic and can form dense surface blooms. *Cylindrospermopsis* is commonly found in tropic and sub-tropic eutrophic waters. Nine species of *Cylindrospermopsis* have been described, all of which are planktonic and bloom forming (Wehr and Sheath 2003).

Cyanobacterial toxin formation

Three main types of toxins have been identified: neurotoxins, hepatotoxins and contact irritants (Repavich et al. 1990). Toxin production can vary between species within a single genus or even between particular species (Codd et al. 1999). Mode of toxicity, chemical composition and structure are the main classification criteria for known toxins (Codd et al. 1999). Neurotoxins (Table 1) are produced by *Anabaena*, *Oscillatoria* and *Aphanizomenon* and interfere with the nervous system (Pitois et al. 2000). Neurotoxins may act as depolarizing neuromuscular blockers or anticholinesterase inhibitors, having effects similar to organophosphate insecticides. Types of neurotoxins

include anatoxin-a, anatoxin-a(s), and saxitoxin (Whitton and Potts 2000). Anatoxin-a, a structural analog of cocaine and anatoxin-a(s) appear to be unique to cyanobacteria (Pitois et al. 2000). Signs of poisonings by these toxins include staggering, muscle fasciculation, gasping, salivation and convulsions (Whitton and Potts 2000; Pitois et al. 2000).

Hepatotoxins (Table 1) inhibit protein phosphatase enzymes, thus damaging the liver and in severe cases can lead to liver failure. There is some evidence that the hepatotoxin microcystin may be a tumor promoter (Falconer et al. 1996). The most common heptatoxin is microcystin, a cyclic heptapeptide. Microcystin is produced by different genera of cyanobacteria including *Microcystis*, *Anabaena*, and *Oscillatoria* (Haider et al. 2003). Microcystin has at least 65 known variants, which differ by the two variable protein amino acids (Codd 2000). Microcystin-LR is the most common variant found in the environment.

Cylindrospermopsin (a cyclic guanine alkaloid) is also a hepatotoxin as well as a protein synthesis inhibitor (Codd 2000). Cylindrospermopsin can cause damage to the kidneys, spleen, intestine, thymus, heart and liver (Codd et al. 1999). Evidence suggests that chromosome breakage and loss, in vitro, may be attributed to cylindrospermopsin (Backer 2002). Symptoms of hepatotoxic poisonings include anorexia, diarrhea, vomiting and weakness (Whitton and Potts 2000).

Table 1: Cyanobacterial Toxin Sources and Mechanisms

Toxin	Toxin Variant	Genera	Water Type	Mechanism of Toxicity	Symptoms	Reference
Neurotoxins	Anatoxin-a Anatoxin-a(s) Saxitoxin	Anabaena Oscillatoria Aphanizomenon	Fresh	Neuromuscular blocker Anticholinesterase inhibitor	Staggering, Muscle fasciculation, Gasping, Salivation, Convulsions	Whitton and Potts, 2000 Pitis et al, 2000
Hepatotoxins	Microcystin Cylindro-spermopsin	Microcystis Anabaena Oscillatoria Cylindro-spermopsis	Fresh	Inhibits Protein Phosphatase Enzymes	Abdominal pain, diarrhea, vomiting, blistered mouths, dry coughs, headaches, painful breathing, pneumonia, liver damage	Haider et al, 2003 Falconer 1996 Codd 2000

Factors Effecting Cyanobacteria Growth and the Promotion of Bloom Formation

Cyanobacteria are a large group of organisms found virtually throughout the world, having a wide range of biological abilities. The prevalence of bloom-forming cyanobacteria is typically dependent on the overall quality of a water body, specifically the level of nutrients present in the water. Factors critical to cyanobacterial growth include: stable water column (low turbulence/low mixing), warm water temperature (15-30°C), high nutrient concentrations (may arise from community, industrial, or agricultural waste), low nitrogen: phosphorus ratios, high pH (pH >8), low CO_2 concentrations, ample sunlight and reduced grazing by large zooplankton (Zurawell 2000; Haider et al. 2003). Phosphorus (and in some cases nitrogen) is the main nutrient influencing biomass of cyanobacteria, with high phosphorus levels promoting increased algal growth (Backer 2002). Past reviews have identified nine mechanisms that influence and promote the dominance of cyanobacteria in freshwaters. These factors include resource ratio competition, differential light requirements, CO_2 competition, buoyancy, high temperature tolerance, avoidance by herbivores, superior cellular nutrient storage,

ammonium-N exploitation and trace element competition (Downing et al. 2001). Eutrophication, the excess input of nutrients to a waterbody is generally recognized as a result of human activities (Codd 2000 and Skulberg et al. 1984). These and other influential factors may, at times, cause massive levels of algal cells, resulting in blooms and surface scums.

Various types of cyanobacteria are classified as bloom forming. The term "bloom" is used to describe levels of biomass that are significantly higher than a lake's average, cause aesthetic degradation and pose a risk to human health (Whitton and Potts 2000). Bloom intensity is influenced by several interacting factors such as extent and duration of calm conditions, size of buoyant population, average potential flotation rate and extent of vertical distribution prior to calm condition (Reynolds and Walsby 1975). Parameters such as chlorophyll, primary productivity, hypolimnetic oxygen depletion, water color and turbidity have been used as criteria for determining bloom conditions (Paerl 1988). Major bloom-forming species are from ~11 genera, both filamentous and non-filamentous and are all gas-vacuole species. Sudden massive occurrences of cyanobacteria at the surface are not a result of rapid cell growth, but rather an upward migration of a dispersed population (Whitton and Potts 2000). Blooms can range in duration from a few hours or in extreme situations last up to a few months (Whitton and Potts 2000). Paerl (1988) identified three nuisance categories attributable to some bloom-forming phytoplankton including: water quality deterioration, chronic or intermittent health hazards and loss of aesthetic and recreational values of affected waters. Nuisance blooms may be enhanced and maintained in part by biotic reactions such as algal-bacterial synergism, algal-micrograzer synergism and absence or reduced

activity of macrograzers (Paerl 1988). Blooms are typically comprised of only one or two species and occur during periods of calm weather conditions and low water turbulence. The bloom is identified by the dominant species (e.g. *Microcystis* bloom, etc.) (Whitton and Potts 2000).

Harmful algal blooms (HABs) are classified as blooms that adversely affect ecosystem, plant or animal health. The fact that algal blooms are not always visible is of importance, but at a biomass of greater than 10,000 cells/ml of water; the water clarity likely decreases (Backer 2002). Large blooms likely only occur when algae are unable to correct over buoyancy. This inability may occur for four possible reasons: algae pass from light-limiting to light-inhibiting intensities before it can react, algae float to the surface at night, algae are senescent and algae photosynthesis is limited by the rate of carbon dioxide diffusion in still water (Reynolds and Walsby 1975). Also noteworthy, is that not all blooms will be made up of a single type of blue-green algae, nor will they necessarily contain toxins (Backer 2002). Oh et al. (2001) suggests that the production of toxins, specifically microcystin, is a result of cyanobacterial blooms caused by favorable environmental conditions and that microcystin concentrations will be affected by changing environmental factors, such as varying levels of nitrogen and phosphorus. Carmichael (2001) suggested several reasons why cyanobacterial blooms may not appear toxic to animals. These reasons include: low concentration of toxin within species or strains comprising the bloom, low biomass concentration of the bloom, variation in sensitivity from animal to animal, age and sex of the animal and amount of food in the animal's gut.

A study by Kotak et al. (1993) evaluating the occurrence of cyanobacterial toxins in lakes and farm dugouts, found that thirty-seven of thirty-nine bloom samples analyzed contained microcystin-LR. Thirty-four out of the thirty-seven combined samples contained *M. aeruginosa.* In these cases the presence of M. aeruginosa is a consistent indicator of microcystin-LR presence (Kotak et al. 1993).

Effects of Exposure

Historically, blue-green algae have been responsible for taste and odor problems in drinking water. In recent years, however, the adverse health effects caused by algal toxins have become a major concern for both drinking water utilities, their customers and to a lesser degree, recreational water users. Several incidences of exposure to microcystin have occurred throughout the world in both animals and humans. These exposures resulted in varying symptoms and degrees of toxicity.

Several cases of blue-green algae toxicosis in domestic animals have been recorded and there have been reports of toxicosis in fish and other wild animals (Sahin et al. 1995) (Table 1). In July of 1997, twenty-four out of a herd of 175 Hereford Angus cattle in Burlington, Colorado died over a three day period from ingestion of water experiencing an algal bloom (Puschner et al. 1998) (Table 2). Through high-performance liquid chromatography (HPLC), the drinking water source containing algae was found to have a microcystin-LR concentration of 148 μg/g of cell biomass (Puschner et al. 1998).

Exposure due to recreational activities was observed when a group of army trainees were swimming in a lake, in England, experiencing a bloom of *Microcystis aeruginosa* (Table 2). Some of the "scum" was swallowed and possibly inhaled by the trainees. The clinical signs reported, include: abdominal pain, diarrhea, vomiting,

blistered mouths, dry coughs and headaches. Two of the trainees were reported to have pleuritic pain (painful breathing), pneumonia and liver damage. The obvious ingestion of a blue-green algae bloom followed by the above clinical signs was a clear indication that *Microcystis* are able to cause adverse health effects (Falconer 1996).

Human fatalities have also occurred as a result of algal toxins (Table 2). In February 1996, at a dialysis center in Caruaru, Brazil, 52 patients died from a syndrome, now known as Caruaru Syndrome, in which high concentrations of microcystin toxins were detected in the water used for treatment. A total of 116 patients, including those who died, experienced visual disturbances, nausea and vomiting after dialysis treatments. One hundred patients developed acute liver failure (Carmichael et al. 2001). Liver and serum samples from 39 of the 52 patients who died were examined for microcystin levels. The mean blood serum levels for the patients were 2.2 ng/ml. Caruaru Syndrome is currently characterized by a large number of symptoms including jaundice, liver cell deformity, necrosis and apoptosis (Carmichael et al. 2001).

Table 2: Examples of Animal and Human Exposure Events to Microcystin

Date/Ref.	Water	Concentration	Location	Organisms Affected	Number Ill	Symptoms
July 1997 Puschner et al. 1998	Pond	148 µg/g of cell biomass	Burlington, CO	Hereford Angus cattle	24 deaths	Nervousness, weak, anorectic, and hypersensitive to noise
1989 Falconer 1996	Lake	N/A	England	Army Trainees	2	Abdominal pain, diarrhea, vomiting, blistered mouths, dry coughs, headaches, painful breathing, pneumonia, liver damage
February 1996 Carmichael et al. 2001	Tap Water	2.2 ng/ml in liver serum	Caruaru, Brazil	Dialysis Patients	116 ill, 52 deaths	Visual disturbances, nausea, vomiting, jaundice, liver cell deformities, necrosis

As previously mentioned, microcystin has been implicated as a possible tumor promoter. Studies have determined that microcystin affects liver cells by mechanisms similar to that of okadaic acid, which has also been liked to the promotion of tumor production (Nishiwaki-Matsushima et al. 1992). In studies by Nishiwaki-Matsushima et al. (1992) the conclusion was that microcystin is one of the strongest liver tumor promoters found to date. Epidemiological data are beginning to emerge in support for these findings, which enhances the concern of human exposure to microcystin. In China's Quidong County, where ponds and ditches (with average microcystin concentrations of 101 pg/ml) are used for drinking water sources, the incidence of liver cancer is approximately eight times higher than in counties using well water (Watanabe

et al. 1996). When the water source was switched from ditches to wells, human liver cancer incidence was decreased (Watanabe et al. 1996).

Microcystis may also have effects on ecosystem health. There is some indication that the formation of blooms may inhibit other primary producers by shading and competitive exclusion, as well as influence total primary production and oxygen production in lakes. Cyanobacteria may attribute to the poor taste of some aquatic species such as crayfish and may be responsible for fish and bird kills. Decaying blooms of cyanobacteria may cause degradation of water quality and be associated with potentially toxic or pathogenic bacteria and other microorganisms. Although the full spectrum of their effects is not clear, it appears that cyanobacteria can affect organisms, communities and entire ecosystems (Lindholm 1992).

Toxin Accumulation

Several recent studies have focused on the possible accumulation of cyanobacteria in sediments and methods of extraction of cyanobacterial toxins from sediment samples specifically for quantification of microcystins (Kankaanpaa et al. 2001; Tsuji et al. 2000). Cyanobacterial cells have been found in surface sediments, along with trace amounts of toxins (Kankaanpaa et al. 2001). Results of studies by Tsuji et al. (2000) determined that cyanobacterial toxins adsorb to sediments and this likely contributes to detoxification of microcystins under natural environmental conditions. Bacteria found in lake sediments appear to be capable of degrading cyanobacterial toxins and viruses may be linked to lysis of cyanobacteria (Kankaanpaa et al. 2001). Various methods of extraction have been used to recover cyanobacterial toxins from sediment samples for quantification of

microcystins, each appeared to have some degree of success (Kankaanpaa et al. 2001; Tsuji et al. 2000).

Bioaccumulation of cyanobacterial toxins in aquatic organisms is another topic of concern. Researchers have documented the presence of microcystins and other toxins in fish and crustaceans (Magalhães et al. 2001, 2003; Kankaanpaa et al. 2001). A study, to collectively determine the presence of microcystins in aquatic biota and to evaluate the human health risk via consumption of aquatic animals, revealed the presence of microcystins in fish and crustaceans (Magalhães et al. 2003). As with sediment evaluation, tissue extraction procedures differ from study to study, with positive results for multiple procedures.

Toxicological Studies

Toxicity testing has been performed on both mice and pigs to determine the effects of acute, short and long-term exposure, reproductive and developmental toxicity, mutagenicity and carcinogenicity. Data from these tests have helped in proposing provisional guideline values for the microcystin-LR variant. Microcystin-LR occurs most frequently and is responsible for more toxic effects than other strains, although it is believed that other variants of microcystin may exhibit toxic effects at similar levels.

Acute toxicity testing provided LD_{50} (dose at which death occurs in 50% of organisms tested) values for both the intraperitoneal and oral routes of exposure. Doses of 25-150 and 5000 μg/kg of body weight (bw) in mice respectively, were shown to produce the LD_{50} value. Acute exposure to microcystin caused severe liver damage, with disruption of liver cell structure, loss of sinusoidal structure, increase in liver weight,

haemodynamic shock, heart failure and death in tested hosts. Adverse effects were also found in the kidneys and lungs at the same dose levels (WHO 1998).

Effects of short-term exposure were evaluated through two different studies. The first used 30 mice (15 male and 15 female), which were exposed orally to varying doses of microcystin-LR ranging from 0 to 1000 μg/kg body weight (bw) per day. The testing took place over a 13 week time period and adverse effects were seen at 200 μg/kg bw or higher. Severe liver damage was seen and a No Observable Adverse Effect Level (NOAEL) was determined to be 40μg/kg of body weight per day. A study conducted with pigs over a 44 day period with doses of 280, 800, and 1310 μg/kg bw per day produced comparable results and established the Lowest Observable Adverse Effect Level (LOAEL) of 280μg/kg of body weight per day (WHO 1998).

Reproductive and developmental toxicity, mutagenicity and carcinogenicity were each evaluated to determine any health effects caused by microcystins. There was no evidence showing effects on reproduction and development, although some maternal toxic effects (liver damage) were observed at doses of 2000 μg/kg bw per day or higher. Due to this fact, a developmental toxicity NOAEL of 600 μg/kg bw per day was determined. Microcystins produced no mutagenic response in the Ames *Salmonella* assay.

Currently there are no official guidelines to control the amount of *Microcystis* a human can be exposed to on a daily basis. The World Health Organization, based on toxicity testing, has proposed that drinking water guidelines be established for microcystin-LR (the only variant as of yet to be studied extensively). The World Health Organization proposes a guideline value of 1μg/l for finished drinking water. This value

was determined by using the NOAEL value of 40 μg/kg bw per day, established by the toxicity testing on mice. A total daily intake (TDI) level of 0.04 μg/kg bw per day (for an adult) was determined by applying an uncertainty factor of 100 for intra- to interspecies variation, 10 for limitations in the database, and 0.80 for the proportion of daily exposure arising from drinking water, thus resulting in a 1μg/l guideline for finished drinking water (WHO 1998). Cyanobacterial source characterization, hazard assessment, human and animal exposure and health consequence assessments are needed to determine effective guidelines and standards (Codd et al. 1999).

Methods of Detection

Several different methods have been developed for the detection of cyanobacteria and toxins (Table 3). Chlorophyll *a* can be a good indication of the amount of cyanobacteria present in a water body (Chorus and Bartram 1999). Microscopic enumeration is the best way to determine types and quantities of cyanobacteria. Polymerase Chain Reaction (PCR) can be used to determine the presence of both cyanobacterial species and toxin genes (Neilan et al. 1997). High-Performance Liquid Chromatography, Protein Phosphatase Inhibition Assay, mouse bioassay and Enzyme-Linked ImmunoSorbent Assay (ELISA) can all be used for the detection of cyanobacterial toxins (Chorus and Bartram 1999).

Table 3: Methods of Detection for Cyanobacteria and Their Toxins

Method	Type of Analysis	Reference
Chlorophyll *a*/Fluorometry	Measures primary photosynthetic pigment	Chorus and Bartram, 1999 Soranno and Knight, 1992 Sartory and Grobbelaar, 1984
Microscopic enumeration	Determines biomass and genera	Chorus and Bartram, 1999 APHA, 1998
Polymerase Chain Reaction	Amplifies DNA sequences to identify genes of species and toxin	Wilson et al, 2000 Neilan et al, 1997 Tillett et al, 2001
High Performance Liquid Chromatography	Determines toxin concentrations	Chorus and Bartram, 1999 Lawton et al, 1994 Welker et al, 2002
Protein phosphatase inhibition assay	Measures biochemical activity of toxins	Chorus and Bartram, 1999
Enzyme-Linked ImmunoSorbent Assay	Determines toxin concentrations	Chu et al, 1989

Chlorophyll *a* is an indirect method of determining the biomass of phytoplankton present in a water body (Chorus and Bartram 1999) and is measured as a result of pigment absorption of wavelengths using fluorometry. Chlorophyll *a* is the primary photosynthetic pigment and light receptor in algae and higher plants (Wehr and Sheath 2003). Quantifying this target is a quick and simple way to suggest a range of cyanobacteria that may be present. The drawback to this method is that chlorophyll *a* for other phytoplankton will also be detected, thus this method can rarely be directly correlated to cyanobacteria levels, unless cyanobacteria are the dominant organisms (Chorus and Bartram 1999).

Microscopic enumeration allows for direct assessment of the presence of various genera of cyanobacteria. Counting the cells can be used to determine the biomass of cyanobacteria present in water samples and the amount of potentially toxic organisms can be determined (Chorus and Bartram 1999). Identification is made by morphometrics

after settling samples in a settling chamber and viewing non-overlapping fields with an inverted microscope and phase contrast microscopy (APHA 1998).

Identification of cyanobacteria and toxic species on a molecular level can be achieved through the use of Polymerase Chain Reaction (PCR). PCR allows for the amplification of specific DNA sequences, which can subsequently be used to characterize samples both taxonomically and phylogenetically (Wilson et al. 2000). The 16S rRNA gene is used most often, in the analysis of cyanobacterial taxonomy, due to its ubiquitous distribution throughout prokaryotic phylogentic groups (Wilson et al. 2000). However, primers have been developed to target genes more specific to individual species and toxins. A DNA dependent RNA polymerase (*rpoC1*) gene unique to *Cylindrospermopsis raciborskii* offers a species specific identification of this type of cyanobacteria (Wilson et al. 2000). Neilan et al. (1997) have identified primers that are specific to the genera *Microcystis*, allowing for the detection of that group of organisms. Still other primers have been developed to target the microcystin synthetase gene *mcyA*, which allows for the detection of the toxin and thus confirmation of the presence of a toxic species (Tillett et al. 2001).

HPLC is another method used to determine the quantity of toxin present in a water sample. It is the most widely used analytical technique for the determination of toxin concentrations (Chorus and Bartram 1999). HPLC combined with UV detection relies on retention time for identification and thus requires standards (Chorus and Bartram 1999). The detection of microcystins is carried out using a C18 silica column and data is gathered at an absorbance of 238 nm (Chorus and Bartram 1999). HPLC provides a determination of quantity within about 24 hours and allows for the accurate

analysis of both intra- and extracellular toxins (Lawton et al. 1994). Similar procedures can be carried out for the quantification of cylindrospermopsin in environmental samples. Detection of cylindrospermopsin by UV-scanning is conducted at an absorbance of 262 nm (Welker et al. 2002).

The protein phosphatase inhibition assay is a screening method that measures the biochemical activity of the toxins (Chorus and Bartram 1999). Two different versions of this method are used. The first uses ^{32}P-phosphate released from a radiolabelled substrate by the activity of the protein phosphatase enzyme and the second uses a colorimetric assay. Based on biological activity, the toxicity of an environmental sample can be determined (Chorus and Bartram 1999).

A mouse bioassay is an additional method to detect the presence of microcystins and other cyanobacterial toxins. In these assays, mice (most often Male Swiss Albino) are administered microcystins interperitoneally. Mice are generally observed for at least twenty-four hours then euthanised and examined for tissue injury to aid in determination of which cyanotoxin is present. In addition to tissue injury, mouse bioassays allow for determination of LD_{50} values. Toxicity levels are expressed as LD_{50} mg cell dry weight per kg mouse body weight, with >1000 being classified as non-toxic and <100 highly toxic (Chorus and Bartram 1999).

Enzyme-Linked ImmunoSorbent Assay (ELISA) is a newer method for the detection of cyanobacterial toxins. Currently, assays are available only for the detection of microcystins and saxitoxins. ELISA is a rapid, specific and easy to use method for analysis. Chu et al. (1989) developed the procedure based on polyclonal antisera raised in rabbits against bovine serum albumin conjugated to microcystin-LR (Chorus and

Bartram 1999). Kits (polyclonal) for the analysis of microcystins can be purchased commercially and results measured colorimetrically using an ELISA plate reader. Microcystin concentrations are inversely proportional to color intensity, which is produced by addition of a substrate (Chorus and Bartram, 1999).

Studies by Rivasseau et al. (1999) evaluated the use of commercially available ELISA kits for monitoring environmental samples for the presence of microcystins. The kits were compared to results obtained from solid phase extraction followed by liquid chromatography. The studies evaluated dose-response curves, reproducibility, accuracy in various aqueous mediums and cross-reactivity. The kits proved capable of displaying reproducibility and accuracy in analyzing environmental samples, although some cross-reactivity between microcystin variants was seen due to the similarity in structure of microcystin variants. The conclusions of these studies were that commercially available ELISA kits are good screening tools for environmental samples (Rivasseau et al. 1999).

Studies by Metcalf et al. (2003) examined the use of ELISA for evaluation of microcystins in water samples. Mean recovery efficiencies between 99% and 101% were found for the commercial kits evaluated. False positive results were produced in samples spiked with sodium chloride. This finding is critical to consider when conducting analysis of microcystin concentrations in brackish waters (Metcalf et al. 2003).

Methods for Removal of Toxins

Large concentrations of cyanobacteria (blooms) are often caused by and are indicators of nutrient enriched waters, thus the best practice would be to reduce nutrient loading by point and non-point source pollutants. Levich (1996) suggest two ecological approaches for controlling cyanobacterial growth. The first is nutrient manipulation,

which increases the nitrogen-to-phosphorus ratio and decreases one of the optimum growth conditions for cyanobacteria. The second is through biomanipulation by introducing planktivorous fish into the water body (Levich 1996).

Water treatment studies have also been conducted in order to reduce any risk to drinking waters (Table 4). The use of activated carbon, both powdered and granular, appears to be effective in the removal of microcystins from raw water. Laboratory testing through the use of isotherms and full-scale water treatment using carbon filtration produced results indicating more than an 80% reduction in microcystin concentrations (Lambert et al. 1996). Ozone, in laboratory tests, has shown to be extremely effective and even more so when used in conjunction with hydrogen peroxide. The only factor that appears to affect its treatment capabilities is pH; destruction of toxins is reduced under alkaline conditions (Rositano et al. 1998). Studies also indicate that higher doses of ozone are needed to destroy microcystins within cells due to cellular material (Rositano et al. 1998). In another study by Rositano et al. (2001), ozonation of four treated bodies of water, prior to chlorination, resulted in 100% destruction of microcystins (0.5-1.1 mg/L) at ozone doses of 0.5 to 1.1 mg/L. The exact dose needed for destruction was found to be dependent on water quality (Rositano et al. 2001). Other studies have also found ozone to be an effective technology for the removal of cyanobacterial toxins, but point out that TOC/DOC influence the effects of ozone and may cause a rapid depletion (Hoeger et al. 2002).

Table 4: Water Treatment Methods For Removal of Cyanobacterial Toxins from Drinking Water Source

Reference	Seeded/Method	Removal	Doses/Parameters	Points/Conclusions
Hoeger, S. J. et al. (2002)	Artificial Lake Water Seeded with: 10 µg/L MC-LR in milli-Q water M. aeruginosa 10^5 cells/ml (12 µg MC-LR equiv/L) M. aeruginosa 5 x 10^5 cells/ml (60 µg MC-LR equiv/L) Method to Evaluate: Protein Phosphatase Inhibition Assay	Removal given as increase in Protein Phosphatase activity (%) 10 µg/l MC-LR: 0.5mg/L ozone-62.5% to 103.2 % 1.0mg/L ozone-69.3% to 94% 1.5mg/L ozone-62.7% to 103.4% 10^5 cells/ml: 0.5mg/l ozone-35.7% to 88.9% 1.0mg/L ozone-36.8% to 101.9% 1.5mg/L ozone-34.6% to 92.3% 5 x10^5 cells/ml: 1.0mg/L ozone-0.9% to 77.9% 1.5mg/L- 0.5% to 92.6 %	Ozone: 0.5, 1.0, 1.5 mg/L MC-LR: 10 µg/L M. aeruginosa: 1 x 10^5, 5 x 10^5 Contact time: 9 min Reaction time: 60 min	Toxicity Analysis: Protein Phosphatase Inhibition Assay Competition between organic material & toxins may rapidly deplete ozone; TOC/DOC influence effects of ozone Filtration using 3 types of filter beds also briefly discussed in article Recommends pre- and intermediate ozonation, filtration steps, monitoring of TOC/DOC & cell densities
Rositano, J. et al. (2001)	Four treated waters prior to chlorination were used: Hope Valley Reservoir, Myponga Reservoir, Morgan (River Murray), & Edenhope (Lake Wallace & well water 70:30) Waters dosed with 40 µg/L MC-LR and/or MC-LA	100% destruction of microcystins: 0.5-1.1 mg/L ozone(dependent on water quality)	Ozone: 0.5-4.0 mg/L Range of alkalinities, DOC, & NOM Temperature: 20 +/-2°C Reaction Time: 5 minutes	Analysis: UV absorbance Ozonation reactions influenced by NOM, pH, alkalinity & DOC Doses needed for 100% destruction & residuals vary depending on water quality

Table 4: Water Treatment Methods For Removal of Cyanobacterial Toxins from Drinking Water Source Continued				
Rositano, J. et al. (1998)	High purity water (Milli-Q) was seeded with MC-LR or extract from M. aeruginosa at concentrations given Analysis by HPLC	166 µg/L MC-LR oxidized to below detection limit by a dose < 0.2 mg/L ozone in 4 minutes 220 µg/L MC (algal extract) was almost completely removed by 1.0 mg/L ozone in 5 minutes	Ozone: 0-1.12 mg/L, 0-2.1 mg/L MC-LR: 166 µg/L M. aeruginosa MC: 220 µg/L DOC: 8.5 mg/L TOC: 8.0 mg/L pH~7.0	Analysis of pure toxin and organism cultures Less effective oxidation at higher pH Higher doses needed to destroy microcystins with intact cells due to cellular material
Lambert, T. M. et al. (1996)	Milli-Q water spiked with MC-LR @ 1,5,10 µg/L Analysis by Protein Phosphatase Inhibition Assay and HPLC	~80% removal of MC	3 commercial types of activated carbon evaluated Equilibrium time assessed MC-LR: 1.0, 5.0, 10.0 µg/L	Equilibrium reached after 1 day Concentration of 0.1-0.5 µg/L are achievable ~80% removal of MC
Newcombe, G (2002)	Ozone/GAC pilot plant 2 treated waters prior to chlorination seeded with up to 25 µg/L MC-LR & MC-LA mixture Analysis by HPLC	Toxins in both waters removed to below detection with virgin carbon Breakthrough of toxins at spiking trials @ 1, 3.5, & 6 months	Ozone contact time: 15 min Ozone residual: 0.3 mg/L GAC EBCT: 15 min	Myponga source waters filters showed signs of biodegradation at 6 months

Scope of Research and Purpose of the Current Study

Identification, quantification and control of cyanobacteria and their toxins are of increasing importance to the drinking water community, especially for consumers using surface waters as drinking sources. Cyanobacteria are on the Environmental Protection Agency's contaminant candidate list (CCL) as established under the 1996 Safe Drinking Water Act (SDWA) amendments. This list focuses on contaminants that require research

and may need regulation in the future. Cyanobacteria and other microbials were selected for the CCL based on the following criteria: (1) public health significance, (2) documentation of occurrence in source water, (3) effectiveness of current water treatment, and (4) adequacy of analytical methods to detect the organism (Balbus 2002). Characterization of the geographic and seasonal distribution of cyanobacteria and the presence of toxins will aid in understanding the ecology of blue-green algae, their toxins in surface waters and potential health risks. This survey work was focused on two distinct geographic areas and water types in Florida and Michigan. Classification and quantification of algal types was determined using microscopic techniques. While some studies have examined both occurrence of toxic genera and toxins, new methods (ELISA) make it possible to undertake more detailed assessments of the relationships between biomass, species and toxin presence. However, application of better and more specific methods is needed to understand what populations carry the genetic potential for toxin production and the possibilities of toxin accumulation in sediments and tissues. This research explored new methods, along with conventional methods and their application in a comparative study of oligotrophic and eutrophic waters in the United States.

The specific goals of this research were to examine distribution and occurrence of cyanobacteria and specifically their toxins in natural waters, including:

1. Determination of the various genera present and the biomass fluctuations through seasonal changes
2. Quantification of toxin concentrations in surface waters
3. Evaluation of existing and new methods for toxin detection
4. Analysis of methods to effectively extract toxins from sediments and tissues

Evaluation of Florida and Michigan Surface Water for the presence of Cyanobacteria, Toxins and Microbial Impacts

Introduction

The availability of groundwater in Florida is insufficient to meet the growing population demand for drinking water, thus communities are becoming more dependent on surface waters as drinking water sources. With increased surface water use comes the possibility for increased contact with cyanobacterial toxins such as microcystin. The goal of this portion of the research was to identify and quantify the algal species *Microcystis*, other toxic species and the toxin microcystin in source waters. Three water bodies along the St. Johns River in Florida, which are used for drinking water, served as sample locations for this study. The St. Johns River flows northward and empties into the Atlantic Ocean in Jacksonville, FL. In addition, 39 inland lakes in Michigan had been previously sampled for the occurrence and identification of cyanobacteria. These samples were processed for the determination of toxin levels.

Materials and Methods

Study Sites in Florida

Results of this eight-month study are to be used to determine if blue-green algae toxin levels pose a health threat to populations using these sources for drinking water supplies and recreation.

The sites are shown on Figure 1 and include:

1. Intake to the water plant at Lake Washington, Melbourne, Florida
2. Intake to the water plant at Lake Monroe, Orlando, Florida

3. Intake to the water plant at Taylor Creek, Cocoa, Florida (surface sample and sample from pump station)

These sites were sampled twice per month (in approximately two week intervals) for eight months from March 2003 to October 2003.

Figure 1: Surface Water Sampling Sites Along the St. Johns River, Florida

Sample Collection and Shipping

Coolers containing sterilized sample bottles were shipped from the Michigan State University Water Quality and Health Laboratory to Florida for each sampling event. Each cooler contained one 250 ml glass bottle containing Lugol's preservative, one 500 ml dark nalgene bottle, and three 1 L clear nalgene bottles for each sample site. Each container was labeled with the site identification, sample number and sample date. A

total of 3.75L of water was collected for each sample site. Each cooler also included a chain of custody, field data sheet and ice packs. All samples were grab samples. Each bottle was filled completely and placed on ice. Coolers were shipped priority overnight to Michigan State University the same day that samples were taken.

Bacterial and Viral Indicator Analysis

Bacterial indicators were evaluated for each sample and included total coliforms, fecal coliforms, *E. coli*, enterococci, *Clostridium perfringens*, as well as the viral indicator, coliphage.

Total Coliforms

Total coliforms were analyzed using Standard Methods 9222B membrane filtration method in accordance with Standard Methods for the Examination of Water and Wastewater (AWWA, 1995). Total coliforms were analyzed by the membrane filter technique using m-Endo media. After water samples were passed through 0.45 μm membrane filters, each filter was placed on an m-Endo media plate and incubated for 24 +/- 2 hours at 37° ± 0.5 C. Total coliform colonies were pink to dark red with a metallic sheen. Light pink, blue or white colonies were considered non-coliforms.

Fecal Coliforms

Fecal coliforms were evaluated using Standard Methods 9222D membrane filtration method in accordance with Standard Methods for the Examination of Water and Wastewater (AWWA, 1995). Fecal coliforms were analyzed by the membrane filter technique using membrane fecal coliform (mFC) media. For analysis, after water samples were passed through 0.45 μm membrane filters, each filter was placed on an mFC media plate. These plates were then placed into whirl-pack bags with waterproof enclosures and incubated submerged in a water bath at 44.5 ± 0.2° C for 24 +/- 2 hours.

Blue to blue-gray colonies were counted as fecal coliforms. Pink, cream, gray or other non-blue colored colonies were not considered fecal coliforms.

Escherichia coli

Escherichia coli was analyzed using Standard Method 9222G (AWWA, 1995). Samples were enumerated as fecal coliforms according to method 9222D as described above. *E. coli* bacteria were analyzed by membrane filtration using EC agar plates supplemented with defined MUG (4-methylumnelliferyl-β-glucuronide). After incubation, colonies were verified as *E. coli* by viewing the plate under a shortwave UV lamp. Colonies that fluoresce with a blue glow were counted as *E. coli*.

Enterococci

Enterococcus spp were evaluated using EPA 1600 membrane filtration method. Samples were filtered as described above. The filters were then placed on *Enterococcus* agar (mE, Difco) supplemented with indoxyl β-D-glucoside substrate (mEI) and incubated at 41°C for 24 hours as outlined in EPA Method 1600 (EPA, 1997). Enterococci are small, gray colonies with a blue fringe. Only colonies with this appearance were counted as enterococci.

Clostridium perfringens

Clostridium perfringens were analyzed by membrane filtration and enumeration on mCP agar as described by Bisson and Cabelli (1979). Plates were incubated in an anaerobic chamber at 45 $\pm$ 0.2°C for 24 +/- 2 hours (BBL GasPak). After incubation, plates with colonies were exposed to ammonium hydroxide fumes by holding the plate inverted close to an open dish of ammonium hydroxide for 10 seconds. After exposure,

straw colored colonies, which turn pink to red were counted as *C. perfringens*. All other colonies were not considered C. *perfringens*.

Coliphage

Coliphage was determined using the double-agar overlay method as described by Sobsey, *et al.* (1995) (EPA Method 1602). Samples were assayed by adding the water sample and 1 ml of a log-phase growth culture of bacterial host (*E. coli* C-3000) to melted Tryptic Soy Agar. Samples were thoroughly mixed and poured onto Tryptic Soy Agar plates. After solidification, the plates were then incubated at 37°C for 24 +/- 2 hours. After incubation, circular lysis zones (plaques) in the lawn of bacterial cells were counted. The total number of Coliphage was expressed as plaque forming units (PFU)/100 ml.

Chlorophyll *a*

Samples for chlorophyll were filtered onto Whatman GF/C filters. The filters were placed into dark containers and the containers were placed in the freezer for at least 24 hours. After the filters were frozen, 25 ml of ethanol was added to each canister (extract volume). Filters were placed in the refrigerator and allowed to extract for 24 hours. Samples were then analyzed using a TURNER 10-AU-005 Fluorometer (F_b). After analysis of all samples 50 µL of 1N hydrochloric acid was added to each sample and each sample was run again (F_a). Extract concentration (EC) was calculated for each sample using the following equation: EC (µg/L) = 516.14 (F_b - F_a), where 516.14 is based on the calibration curve for the fluorometer. Actual chlorophyll *a* concentrations were calculated by inserting EC into the following equation:

Lake Chlorophyll Concentration (µg/L) = EC (µg/L) x Extract Volume (ml)

Volume Filtered (ml)

Determination of chlorophyll *a* concentrations were based on the work by Nusch (1980).

Microscopy for Identification and Enumeration of Algae

Samples were identified and enumerated by microscopy using protocols from the 20th edition of Standard Methods for the Examination of Water and Wastewater (APHA 1998). Samples to be analyzed for phytoplankton counts were collected in 250 ml glass bottles and preserved with 1% Lugol's solution. Samples can be stored at room temperature for several years. More preservative may be added if needed. The preserved samples were settled in glass-graduated cylinders for a specified period of time based on chlorophyll *a* concentrations. Samples were then transferred to a 10 ml plankton chamber (Hydrobios, Germany) and settled for an additional 48 hours. Phytoplankton were identified and enumerated by total cell counts using an Olympus inverted microscope with phase-contrast objectives (10X, 20X, 40X, 1000X). Counts were made by viewing 50 non-overlapping fields or until 100 cells of the most dominant species were seen. Identification was made at 1000X magnification, with the assistance of *Identification of Algae in Water Supplies* cd-rom published by the American Water Works Association (AWWA, 2002). After identification, dry biomass (µg/L) was calculated for both cells and filaments using the following steps of equations:

For Cells:

Dry Biomass (µg/L) = (((((inner cells counted/fields viewed)*0.517 + (outer cells counted/fields viewed)*0.483)*202800)* mean cell volume)/1000)*0.1)

This is determined though the following process:

1. Mean Cells/Field =

(inner cells counted/fields viewed)*0.517 + (outer cells counted/fields viewed)*0.483 where 0.517 and 0.483 are constants based on inner and outer area percentages.

2. Cells/ml = Mean cells/field * 202800 (a conversion factor based on chamber area and micrometer calibrations)
3. Biovolume (μm^3/ml)– cells/ml*mean cell volume (calculated based on the organisms shape)
4. Wet Biomass (µg/L) = Biovolume/1000
5. Dry biomass (µg/L) = Wet Biomass*0.1

For Filaments:

Dry Biomass (µg/L) = (((((inner intersections counted/fields viewed)*0.517 + (outer intersections counted/fields viewed)*0.483)* 1594008)* mean cell volume)/1000)*0.1)

This is determined though the following process:

1. Mean intersections/Field =

(inner intersections counted/fields viewed)*0.517 + (outer intersections counted/fields viewed)*0.483 where 0.517 and 0.483 are constants based on inner and outer area percentages.

2. Intersections/ml = Mean intersections/field * 1594008 (a conversion factor based on chamber area and micrometer calibrations)
3. Biovolume (μm^3/ml)– intersections/ml*mean filament diameter
4. Wet Biomass (µg/L) = Biovolume/1000
5. Dry biomass (µg/L) = Wet Biomass*0.1

Enzyme-Linked ImmunoSorbent Assay (ELISA) for Microcystins

ELISAs were performed in 96 well plates using a commercially available kit according to manufacturer's instructions (Envirologix, Inc., Portland, Maine). Assay parameters were set for increased sensitivity (limit of quantification = 0.06 ppb, limit of detection = 0.03 ppb) in order to detect microcystin at levels of 50 ng/L or higher. Microcystin Assay Diluent (50 µL) was added to each well that was to be used. Immediately, 50 µL of negative control, 50 µL of each microcystin-LR standard (0.05, 0.20, 0.83 ppb) and 50 µL of each sample were added to their respective wells. Contents of the wells was mixed thoroughly and incubated at ambient temperature for 30 minutes. After incubation microcystin-enzyme (horseradish peroxidase) Conjugate (100 µL) was added to each well. Contents were again mixed and incubated at ambient temperature for 30 minutes. Following incubation, well contents were emptied and each well was washed four times with a wash solution (phosphate-buffered saline) to remove any unbound microcystin. A substrate (3,3,5,5' tetramethylbenzidine (TMB)) (100 µL) was then added to the wells, the contents was mixed thoroughly, and incubated at ambient temperature for 30 minutes. Finally, 100 µL of stop solution (1.0 N Hydrochloric acid) was added to each well and mixed thoroughly. Standard curves were established using known concentrations of microcystin followed by colorimetric analysis using dual wavelengths of 450 and 620 nm on Labsystems Mulitskan RC manufacturer plate reader. Microcystin concentrations from surface water samples were compared to standards and estimated by extrapolation using Genesis Lite software. All samples were analyzed in duplicate and duplicate samples having a coefficient of variation >20% were rerun. All concentrations are reported as ng/L.

Microcystin Analysis via ELISA in Michigan Lakes

Samples collected by Dr. Orlando Sarnelle's Laboratory at Michigan State University from Michigan inland lakes in summer of 2002 and 2003 were examined for the presence of microcystin. Thirty-nine lakes were evaluated in 2002 and thirty-four lakes in 2003. The lakes that were evaluated are listed in Appendix A (page 75). A known volume of lake water was filtered for each sample taken. Volumes filtered varied from lake to lake. Filters were extracted using a simple methanol extraction method. Filters were extracted three times in 10 ml of 75% methanol for forty-five minutes while shaking. The methanol extracts (30 ml total volume) were combined and analyzed using ELISA per procedures listed above. After analysis by ELISA the environmental toxin concentration was calculated using the following equation:

$$\text{Env. Toxin Conc. (ng/L)} = \frac{\text{Toxin Extract Conc. (µg/L)*Toxin Extract Volume (ml)}}{\text{Volume Filtered (ml)/1000}}$$

Statistical Analysis

Statistical analysis of all experimental data was performed using Microsoft Excel (Microsoft Office XP). Basic statistical parameters for both environmental samples and laboratory experiments are reported in this research. Statistical procedures used include calculation of arithmetic means, standard deviations, correlations and T-tests. The calculation of a one-way ANOVA for comparison of multiple data sets was carried out using SPSS 11.5 for Windows. These parameters were evaluated to better understand the relationships between the various types of experimental data obtained in this research.

Results

Physical/Chemical Water Quality in Florida Waters

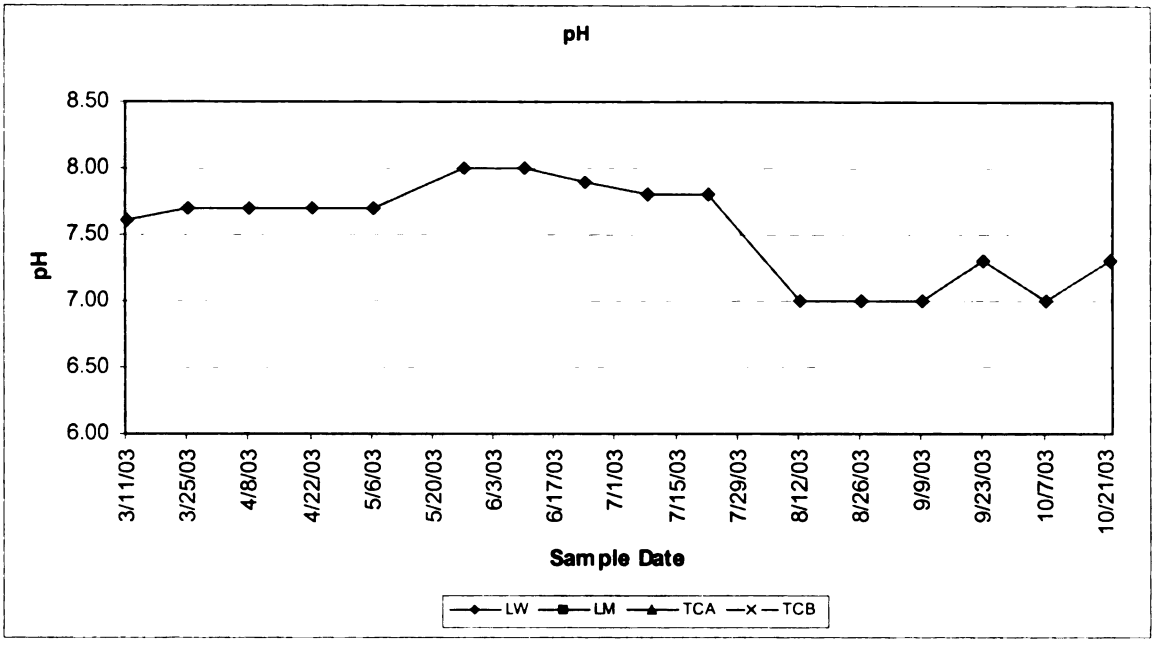
Average physical and chemical data are presented in Table 5. pH, dissolved oxygen and water temperature readings were taken at the time of sample collection for each sampling event. pH results (Table 5, Figure 2) for each sampling site remained stable throughout the sampling period. Levels in Lake Washington (LW) ranged from 7.0 to 8.0, with an average pH of 7.53 (SD= 0.37). pH in Lake Monroe (LM) ranged from 6.82 to 7.89, with an average pH of 7.22 (SD= 0.29). TCA (surface of Taylor Creek) pH levels ranged from 6.1 to 7.2, with an average pH of 6.67 (SD= 0.35). TCB (Taylor Creek at a depth of 15 to 20 feet) pH levels ranged from 6.3 to 7.2, with an average of 6.63 (SD= 0.28) for TCB. LW was slightly alkaline, while Taylor Creek (at both sites) was slightly acidic.

Table 5: Arithmetic Averages and Ranges of Physical/Chemical Parameters, Chlorophyll a and Microcystin Levels in Florida Surface Waters

Parameter	N=16	LW	LM	TCA	TCB
pH	Average	7.53	7.22	6.67	6.63
	Range	7.0-8.0	6.82-7.89	6.10-7.20	6.30-7.20
	SD	0.37	0.29	0.35	0.28
Temperature °C	Average	27.16	27.30	25.88	24.77
	Range	23.50-31.00	23.60-31.20	18.00-30.00	19.00-28.00
	SD	1.89	2.25	3.09	2.70
Dissolved Oxygen (mg/L)	Average	4.89	ND	1.56	8.92
	Range	3.21-6.40	ND	0.60-10.56	2.59-8.92
	SD	0.95	ND	2.81	1.83
Chlorophyll *a* (µg/L)	Average	13.44	15.72	7.55	2.84
	Range	0.95-55.49	1.21-48.00	0.88-18.74	0.77-7.74
	SD	17.69	16.74	5.15	2.31
Microcystin (ng/L)	Average	90	322	71	78
	Range	<50-350	<50-2176	<50-130	<50-196
	SD	78	550	25	41

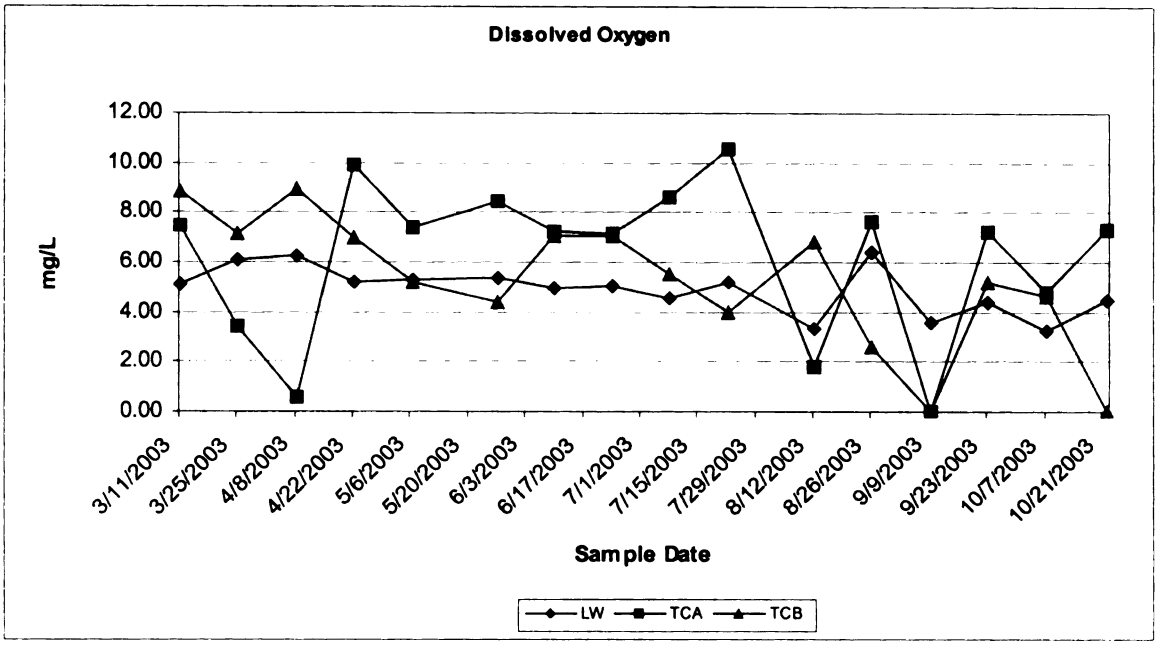
SD = Standard Deviation ND = Not Determined

Figure 2: pH levels in Florida Surface Waters



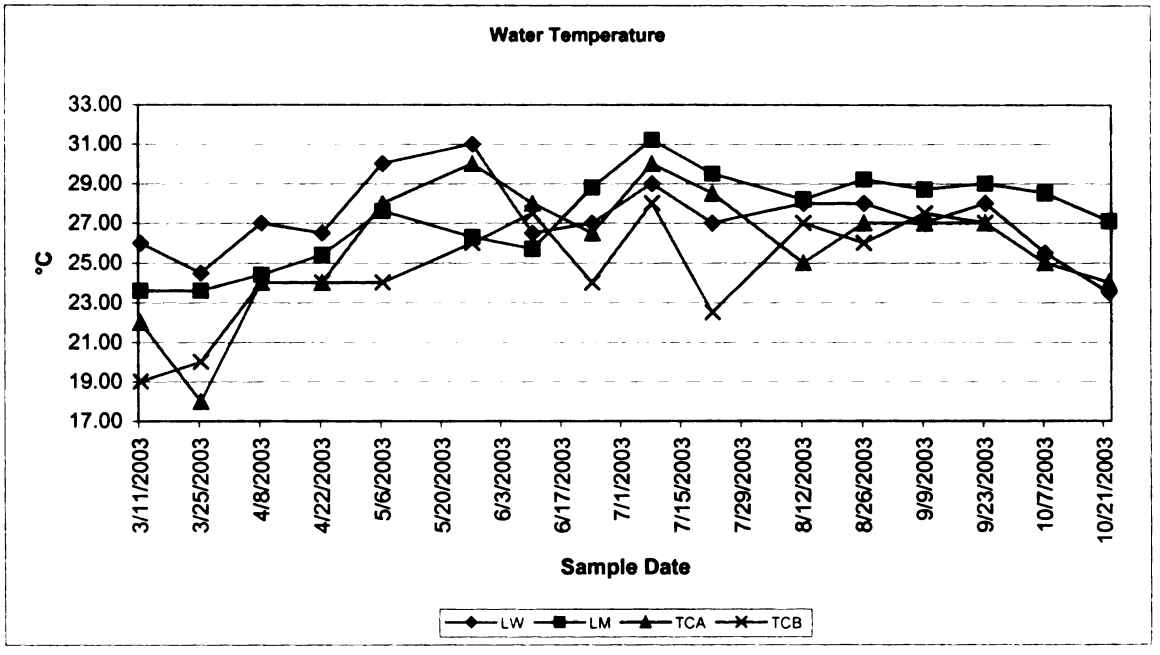
Dissolved oxygen levels (Table 5, Figure 3) were taken at LW, TCA and TCB, but were not available for LM. No major fluctuations were seen in LW, however the concentration of dissolved oxygen in TCA and TCB did fluctuate over the sample period. Dissolved oxygen concentrations drastically decreased on two sampling dates (4/8/2003 and 9/9/2003). Dissolved oxygen levels ranged from 3.21 to 6.4 mg/L at LW (average of 4.89 mg/L (SD= 0.95)). Dissolved oxygen levels at TCA ranged from 0.6 to 10.56 mg/L (average of 6.63 mg/L (SD= 2.81)). Dissolved oxygen concentrations at TCB ranged from 2.59 to 8.92 mg/L (average of 6.03 mg/L (SD= 1.83)).

Figure 3: Dissolved Oxygen Levels for Florida Water Samples



Water temperatures (Table 5, Figure 4) also remained mostly steady throughout the sample period. Seasonal changes were observed with water temperatures highest in the summer months. Water temperatures averaged 27.16° C (SD=1.89), with a high of 31.0° C in May and a low of 23.5° C in October for LW. In LM, the low temperature was 23.6° C in October and the high was 31.2° C in July, with an average of 27.3° C (SD=2.25). Water temperatures in Taylor Creek averaged 25.88°C (SD=3.09) for TCA and 24.77° C (SD= 2.70) for TCB, with ranges from 18.0° C to 30.0° C.

Figure 4: Water Temperature in Florida Water Samples



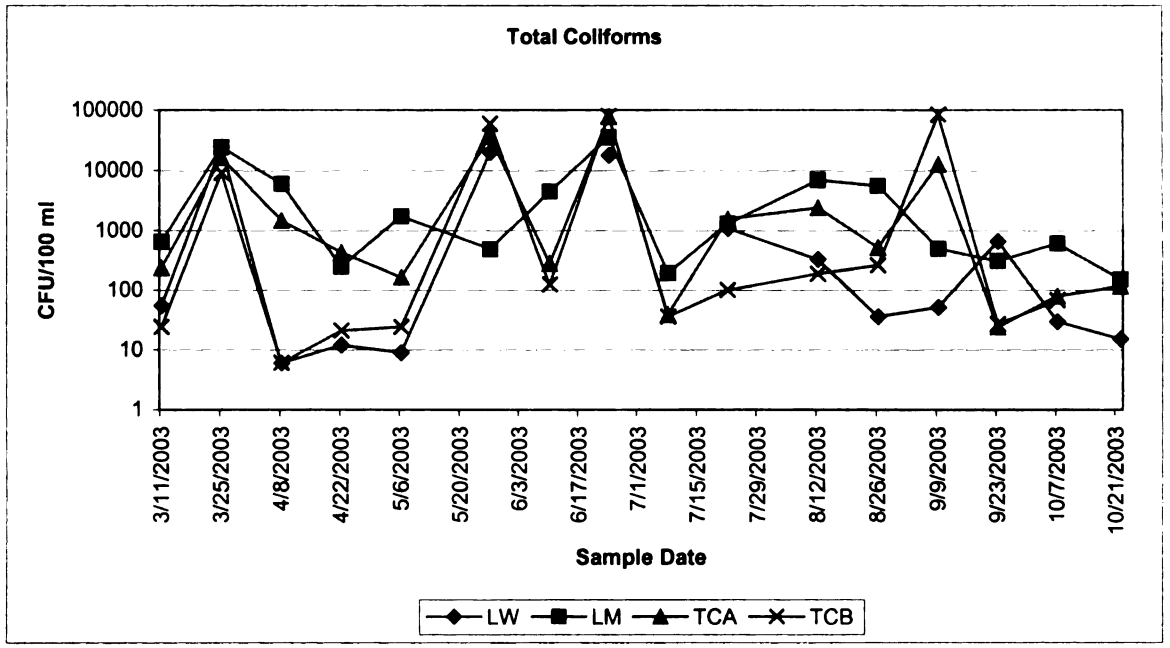
Bacterial Indicator and Coliphage Results in Florida Waters

Microbial quality of the Florida surface waters is shown in Table 6. All sample sites were positive for the general indicators (total coliforms, fecal coliforms and Enterococci) 100% of the time. The microbial data shows that Lake Monroe is the most contaminated, with bacterial levels consistently high. Coliform levels in Taylor Creek fluctuated greatly at both sites sampled, possibly due to spikes in contamination during rainfall events. Average total coliform levels (Table 6, Figure 5) for the four sites were 4578 CFU/100 ml (SD= 8790), 5548 CFU/100 ml (SD= 9996), 9361 CFU/100 ml (SD=2.06E+04), and 1.54E+04 CFU/100 ml (SD=3.07E+04) for LW, LM, TCA and TCB respectively. The arithmetic averages at each site and the graphical representation show that the lakes are highly impacted by fecal pollution. Major fluctuations in total coliform levels indicate summer peaks in contamination as well as peaks possibly brought on by rainfall events.

Table 6: Microbial Quality of Florida Surface Waters (Averages are Arithmetic)

Parameter	N=16	LW	LM	TCA	TCB
Total coliforms (CFU/100ml)	Average	4578	5548	9361	1.54E+04
	Range	6-2.43E+04	152-3.55E+04	24-7.74E+04	6-8.39E+04
	SD	8790	9996	2.06E+04	3.07E+04
Fecal Coliforms (CFU/100ml)	Average	1652	4944	6580	9719
	Range	<1-7933	103-2.88E+04	12-6.40E+04	11-6.24E+04
	SD	2994	8124	1.70E+04	2.12E+04
E. coli (CFU/100ml)	Average	1095	1173	1313	3511
	Range	2-4866	21-6233	12-7933	5-3.73E+04
	SD	1720	1968	2459	9629
Enterococci (CFU/100ml)	Average	46	901	163	14
	Range	<1-240	61-3600	2-1320	2-47
	SD	73	1094	320	15
Clostridium perfringens (CFU/100ml)	Average	6	21	24	4
	Range	<1-20	<1-77	<1-143	<1-9
	SD	6	20	53	3
Coliphage (PFU/100ml)	Average	32	268	18	24
	Range	<10-80	<10-750	<10-50	<10-25
	SD	32	268	18	24

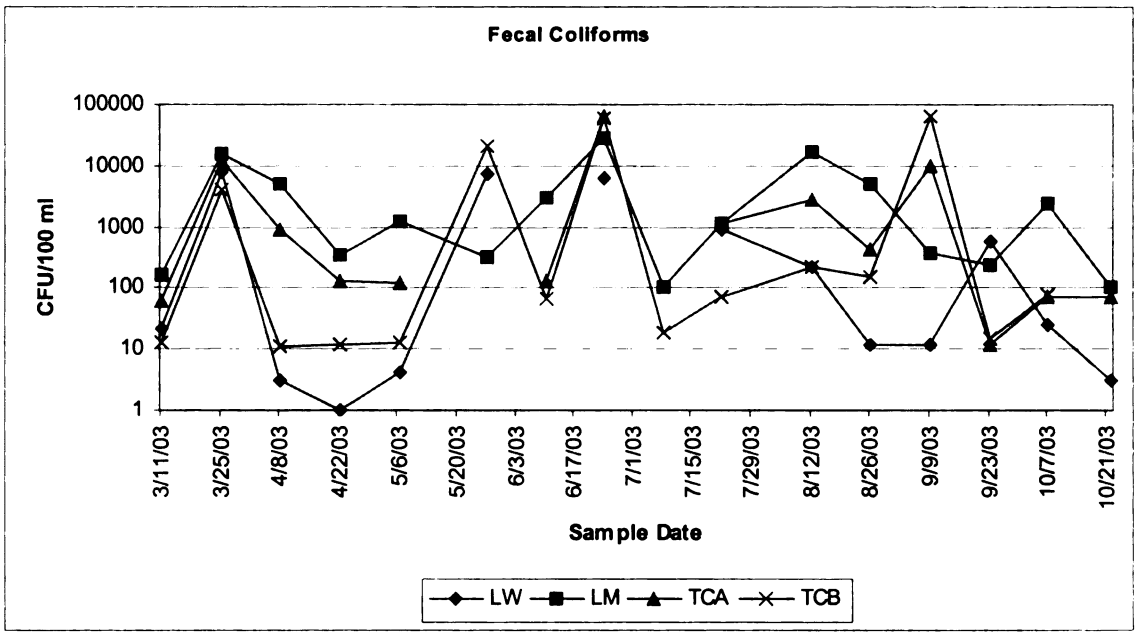
Figure 5: Total Coliform Levels in Florida Water Samples



Fecal coliform standards for recreational water use in Florida are <200 CFU/100 ml (based on a geometric mean) for primary contact. The geometric means were 73, 1220, 509 and 194 CFU/100ml for LW, LM, TCA and TCB, respectively. Both LM and

TCA were above the recreational use standard. Fecal coliform arithmetic averages (Table 6 and Figure 6) were 1652 CFU/100 ml (SD= 2994), 4944 CFU/100 ml (SD= 8124), 6580 CFU/100 ml (SD= 1.70E+04), 9719 CFU/100 ml (SD= 2.12E+04) for LW, LM, TCA and TCB, respectively. Taylor Creek fecal coliforms levels varied greatly throughout the sample period. Four of the seventeen samples contained extremely high levels of fecal coliforms. This may be due to the fact that a cattle ranch surrounds the Taylor Creek Reservoir.

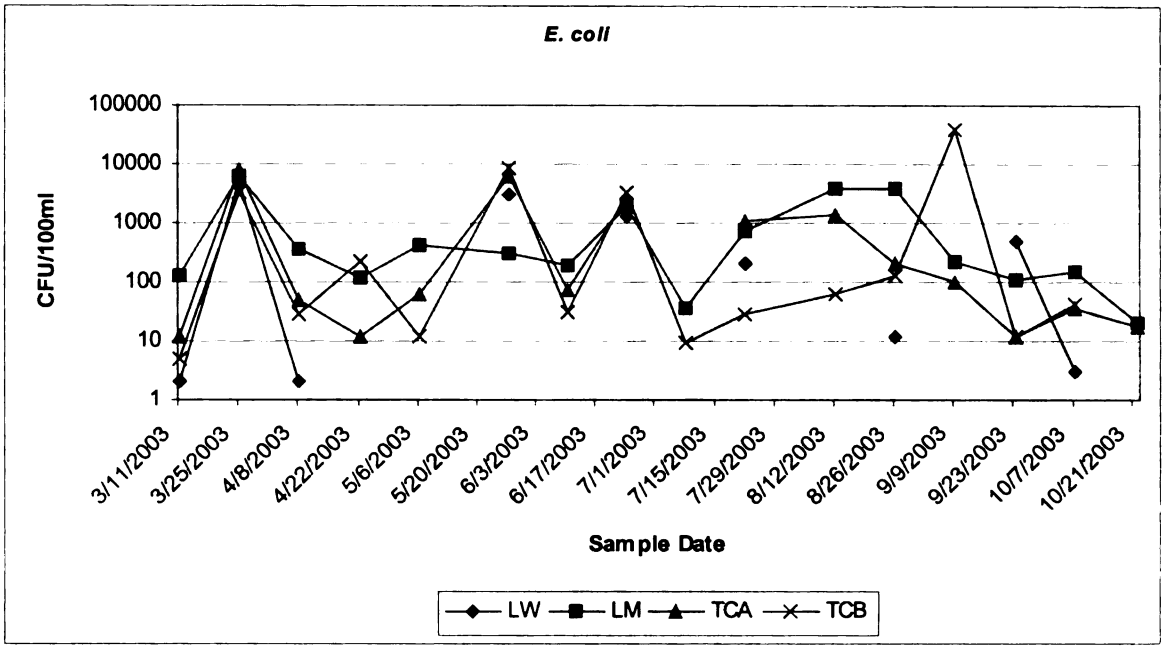
Figure 6: Fecal Coliform Levels for Florida Water Samples



The EPA has set a guideline for *E. coli* for waters used for recreational purposes of <126 CFU/100 ml (based on a geometric mean). The geometric means were 86, 349, 157 and 126 CFU/100 ml for LW, LM, TCA and TCB, respectively. The levels of *E. coli* at LM and TCA were greater than the recreational standard for *E. coli*, while TCB was at the standard. Arithmetic average *E. coli* concentrations (Table 6, Figure 7) were 1095 CFU/100 ml (SD= 1720), 1173 CFU/100 ml (SD= 1868), 1313 CFU/100 ml (SD= 2459) and 3511 CFU/100 ml (SD= 9629) for LW, LM, TCA and TCB, respectively. Levels

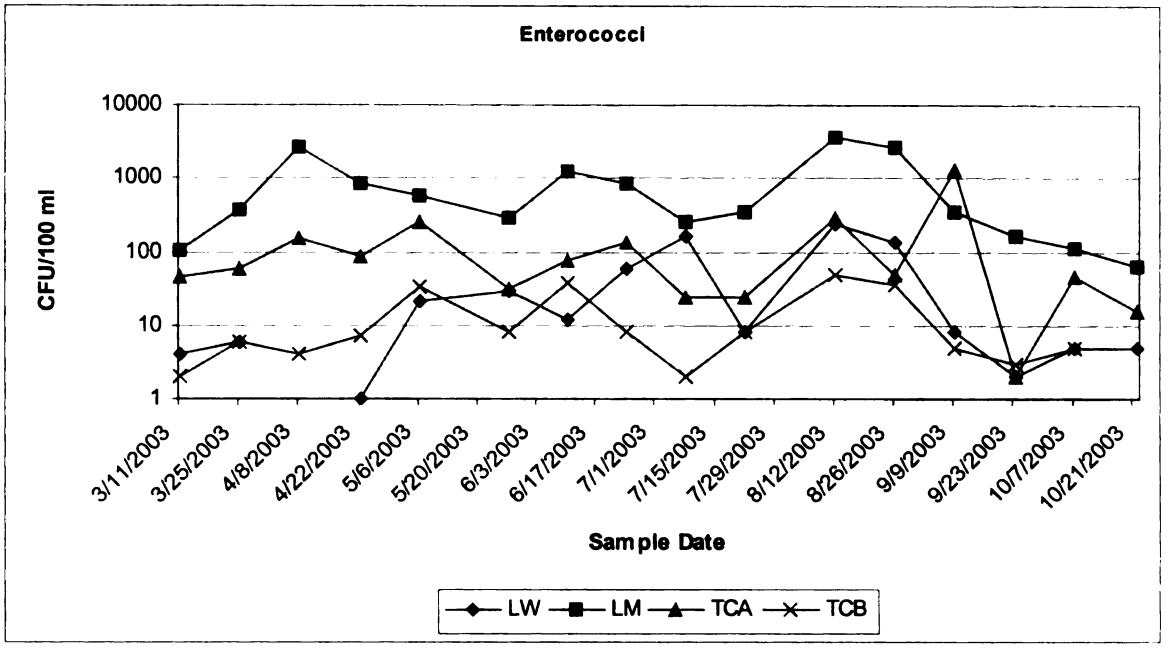
ranged from <1 CFU/100 ml in both Lake Washington and Taylor Creek to above nearly 40,000 CFU/100 ml in Taylor Creek.

Figure 7: *E. coli* levels for Florida Water Samples



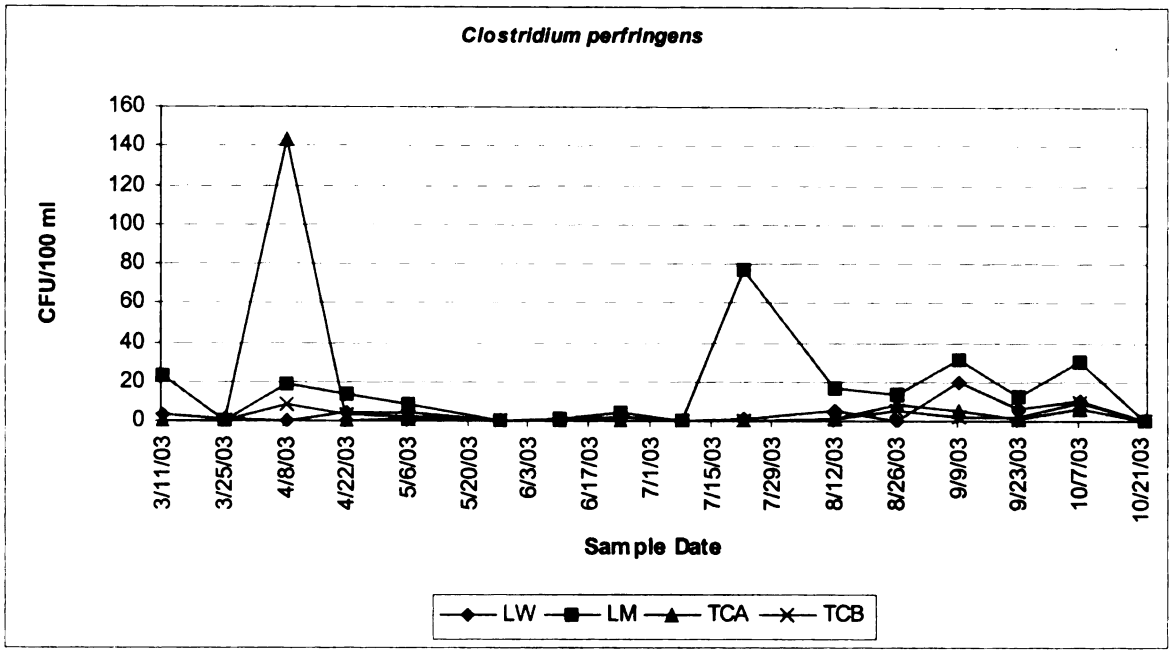
Primary contact recreational EPA guidelines for *Enterococcus* are set at <33 CFU/100 ml (based on a geometric mean). Geometric means were 14, 463, 61 and 8 CFU/100ml for LW, LM, TCA and TCB, respectively. The levels in samples taken from LW and TCB were below the recreational standard. Levels of *Enterococcus* (Table 6, Figure 8) were consistently highest in Lake Monroe where levels peaked at 3600 CFU/100 ml. Low levels were observed in Taylor Creek with only one sampling event that levels were above 1000 CFU/100 ml. Average (arithmetic) *Enterococcus* levels for the samples sites were 46 CFU/100 ml (SD= 73), 901 CFU/100 ml (SD=1094), 163 CFU/100 ml (SD= 320) and 14 CFU/100 ml (SD= 15) for LW, LM, TCA, and TCB respectively.

Figure 8: Enterococci Levels for Florida Water Samples



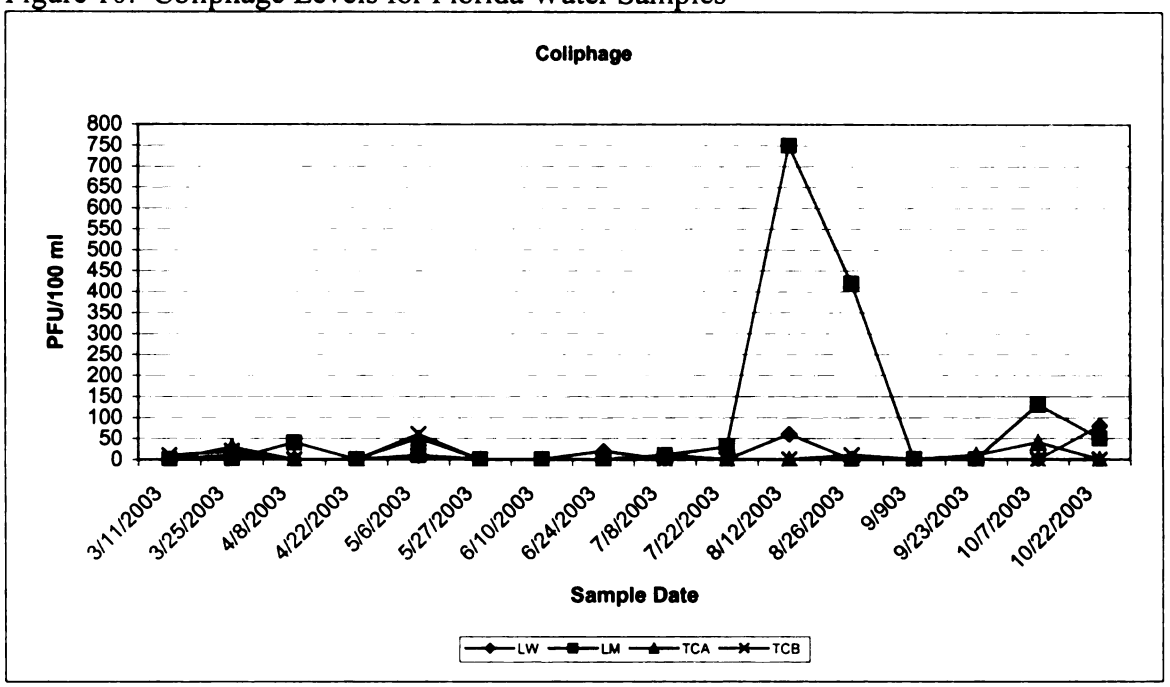
Clostridium perfringens, a spore forming bacteria, are used for recreational guidelines in Hawaii, with standards of <50 CFU/100 ml in marine waters and <5 CFU/100 ml in fresh waters (Fujioka et al, 1985). Average levels for *Clostridium perfringens* (Table 6, Figure 9) were 6 CFU/100 ml (SD= 6), 21 CFU/100 ml (SD= 20), 24 CFU/100 ml (SD=53) and 4 CFU/100 ml (SD= 3) for LW, LM, TCA, and TCB respectively. Spikes in *C. perfringens* levels occurred in samples taken from TCA on 4/8/2003 and LM on 7/22/2003. The presence of elevated levels of *C. perfringens* indicates fecal contamination, but also may indicate resuspension of sediments as the spores accumulate and survive in the sediments.

Figure 9: *Clostridium perfringens* levels for Florida Water Samples



No federal standards for coliphage in drinking waters or in waters for recreational use have been set. The highest coliphage levels (Table 6, Figure 10) were observed in Lake Monroe (LM) in August (8/12/2003). High levels of coliphage may indicate a recent fecal pollution event as coliphage have limited survival rates in warm waters. LW coliphage levels ranged from <10 to 80 PFU/100 ml, with an arithmetic average of 36 PFU/100 ml (SD= 32). LM coliphage levels ranged from <10 to 750 PFU/100 ml, with an arithmetic average of 180 PFU/100 ml (SD= 268). Coliphage levels in Taylor Creek ranged from <10 to 60 PFU/100 ml. TCA had an arithmetic average coliphage concentration of 28 PFU/100 ml (SD= 18), while TCB had an arithmetic average of 25 PFU/100 ml (SD= 24).

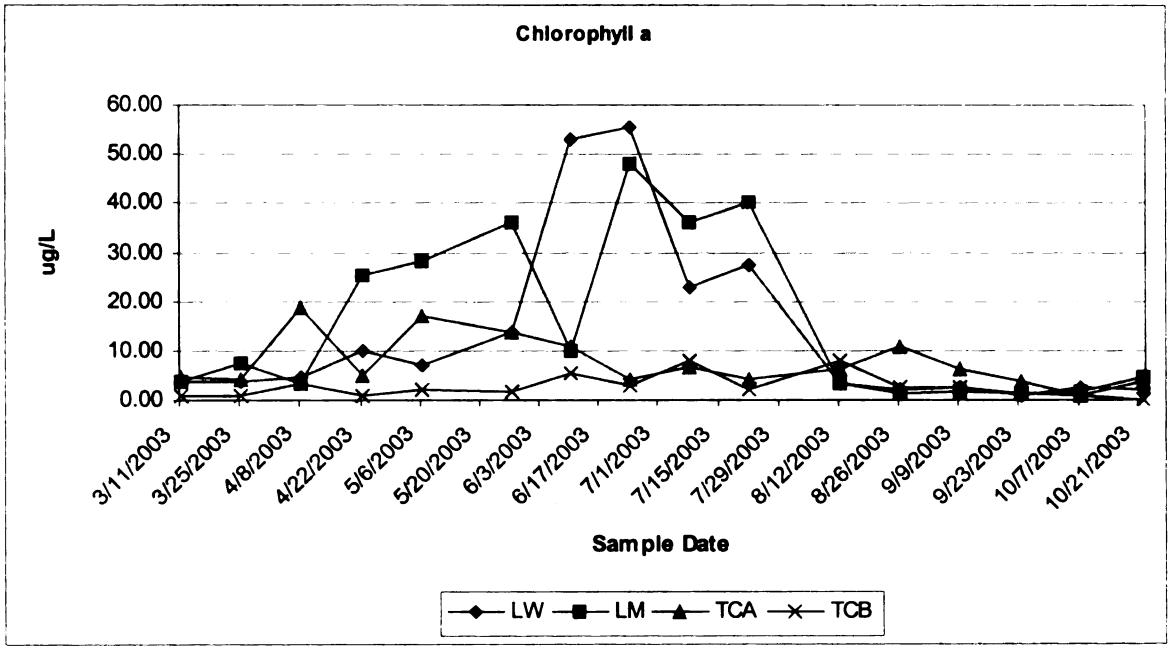
Figure 10: Coliphage Levels for Florida Water Samples



Chlorophyll *a* in Florida Surface Waters

Chlorophyll *a* levels (Table 5, Figure 11) varied from <1 to >55 µg/L throughout the sample period both between lakes and within each sample site. Seasonal trends were seen in LW and LM, both of which are highly eutrophic lakes. LW levels varied from 0.95 µg/L in September to 55.49 µg/L in June, with an average concentration of 13.44 µg/L (SD= 17.69). Levels in LM varied from 1.21 µg/L in September to 48.00 µg/L in June, with an average concentration of 15.73 µg/L (SD=16.74). Chlorophyll *a* concentrations in Taylor Creek remained fairly steady throughout the sample period, with average concentrations of 7.55 µg/L (SD=5.15) and 2.85 µg/L (SD= 2.31) for TCA and TCB respectively. TCA levels ranged from 0.88 µg/L to 18.74 µg/L while TCB ranged from 0.77 µg/L to 7.74 µg/L.

Figure 11: Chlorophyll *a* Levels for Florida Surface Waters



Identification of Phytoplankton Genera in Florida Waters

Diverse phytoplankton genera were detected in each of the three water bodies throughout the sampling period. In LW, twelve groups of algae (Figure 12) were present including cyanobacteria such as *Microcystis* and *Anabaena*. These toxin forming algae were not the dominant species in LW. However, the exception was with samples collected on May 6, 2003 and May 27, 2003 in which *Anabaena* was the dominant organism. In addition, *Microcystis* was the dominant organism during the June 10, 2003 sampling event. In the phytoplankton sample collected on June 10, 2003, 99% of the total biomass of phytoplankton was toxin-forming species of cyanobacteria (Figure 14). Other cyanobacteria comprised <1% to ~25% of the biomass in samples collected (Figure 13). Various diatoms and the genera *Phacus* were the dominant phytoplankton types in ten of the sixteen samples.

Figure 12: Lake Washington (LW) Phytoplankton Diversity

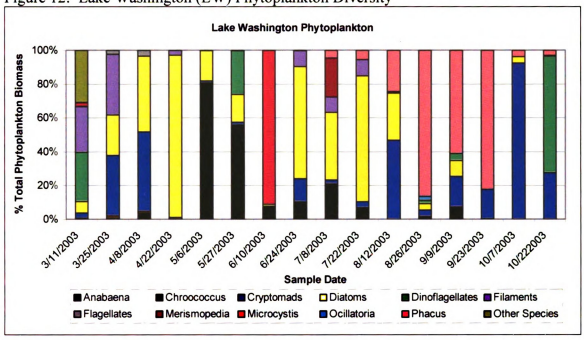


Figure 13: Relative Abundance of Cyanobacteria in Lake Washington (LW)

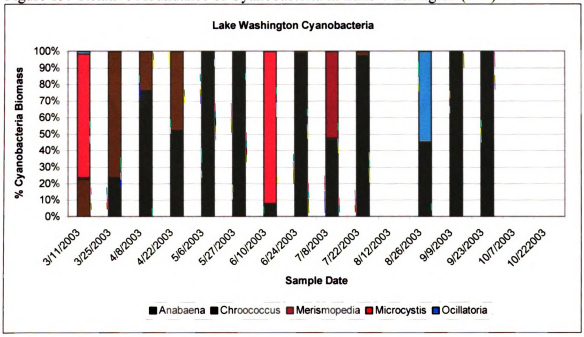
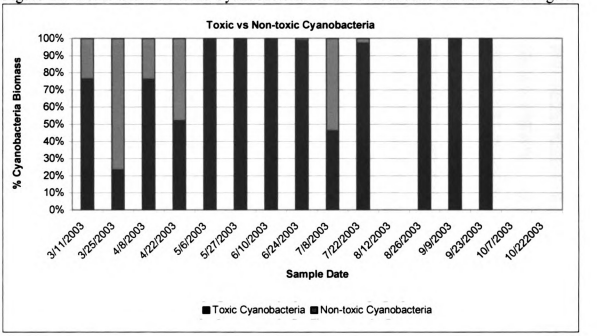


Figure 14: Toxic and Non-toxic Cyanobacteria Relative Abundance in Lake Washington



Lake Monroe (Figure 15) also contained both *Anabaena* and *Microcystis*, but neither was found to be dominant, with the exception of *Microcystis* in the sample collected on May 27, 2003. In the sample collected during the March 11, 2003 sampling event *Microcystis* and *Anabaena* comprised ~50% of the total phytoplankton biomass. Other cyanobacteria comprised between <1% and 35% of the organisms present (Figure 16). In Lake Monroe, toxic cyanobacteria comprise >25% of the phytoplankton population in eight of the fifteen samples evaluated (Figure 17).

Figure 15: Lake Monroe Phytoplankton Diversity

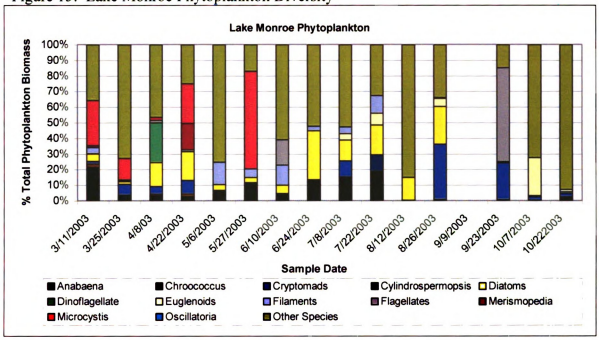


Figure 16: Relative Abundance of Cyanobacteria in Lake Monroe (LM)

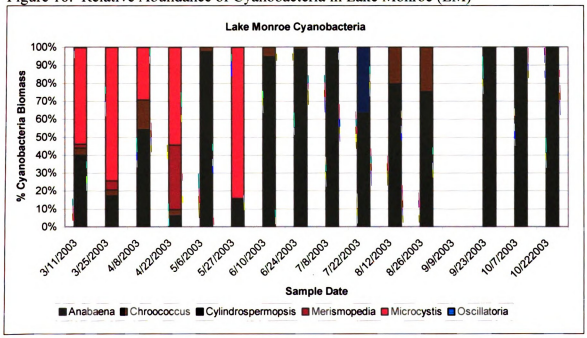
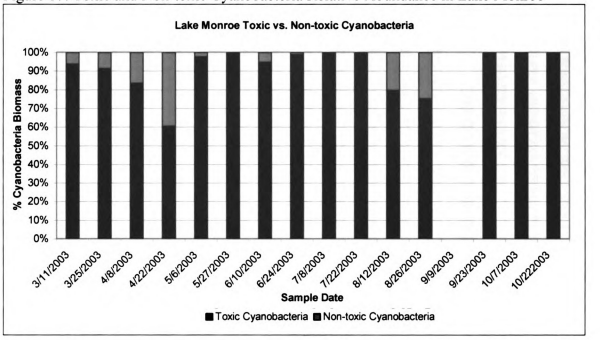


Figure 17: Toxic and Non-toxic Cyanobacteria Relative Abundance in Lake Monroe



In Taylor Creek at sample site TCA (Figure 18), toxin forming cyanobacteria were present, but in low quantities. The exception was the sample event on July 22, 2003 in which *Anabaena* made up ~40% (dry biomass) of the total organisms. During the sixteen sampling events toxic cyanobacteria ranged from <1% to ~45% of total phytoplankton biomass (Figure 19 and 20).

Figure 18: Taylor Creek-A Phytoplankton Diversity

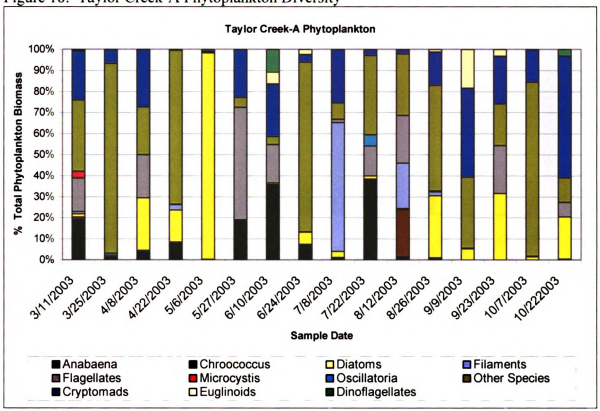


Figure 19: Relative Abundance of Cyanobacteria in Taylor Creek-A (TCA)

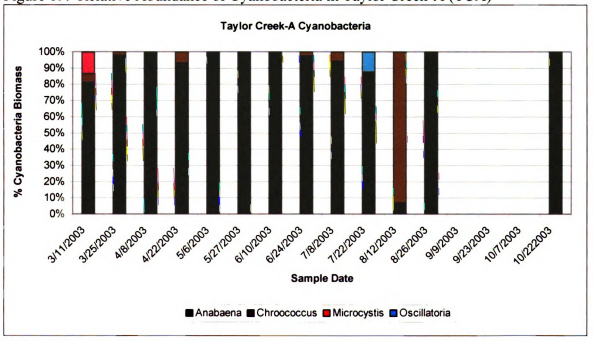
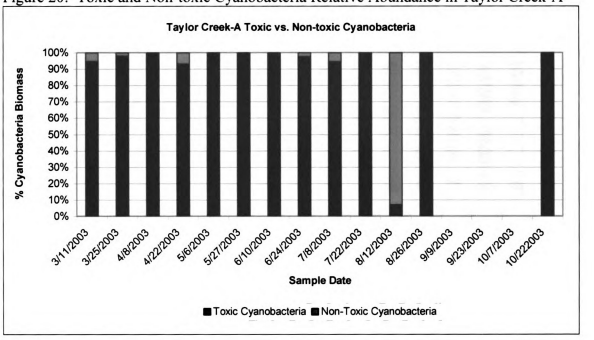


Figure 20: Toxic and Non-toxic Cyanobacteria Relative Abundance in Taylor Creek-A



At TCB (Figure 21) *Anabaena* was dominant (~60%) during the sampling event on August 12, 2003, but accounted for only <1% to 11% of the total phytoplankton biomass in the other sampling events. *Microcystis* was the dominant organism present in the sample collected during the June 10, 2003 sampling event, comprising ~65% of the total phytoplankton biomass. Other cyanobacteria accounted for <1% to 11% of the total phytoplankton biomass present (Figure 22 and 23).

Figure 21: Taylor Creek-B Phytoplankton Diversity

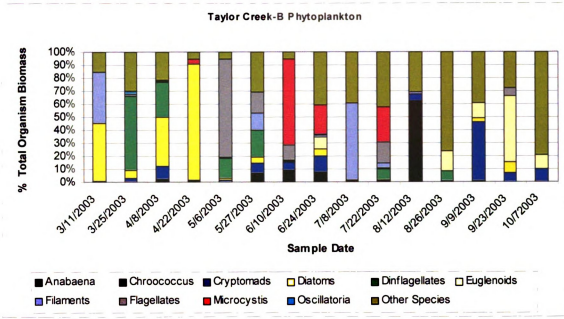


Figure 22: Relative Abundance of Cyanobacteria in Taylor Creek-B (TCB)

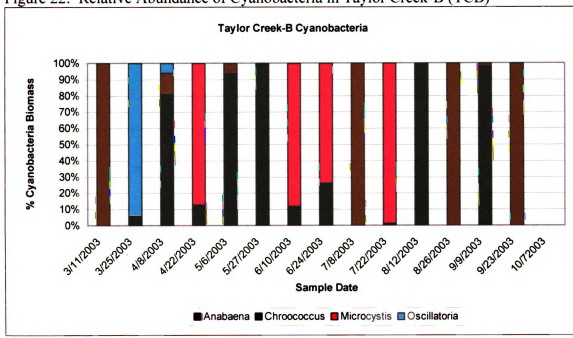
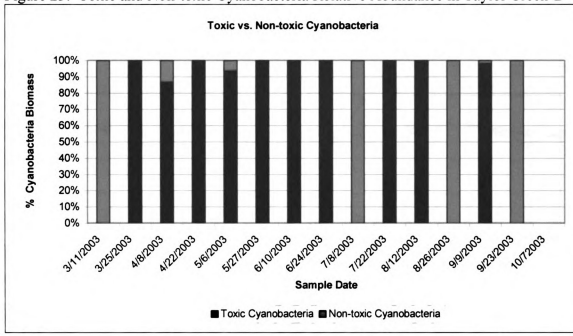


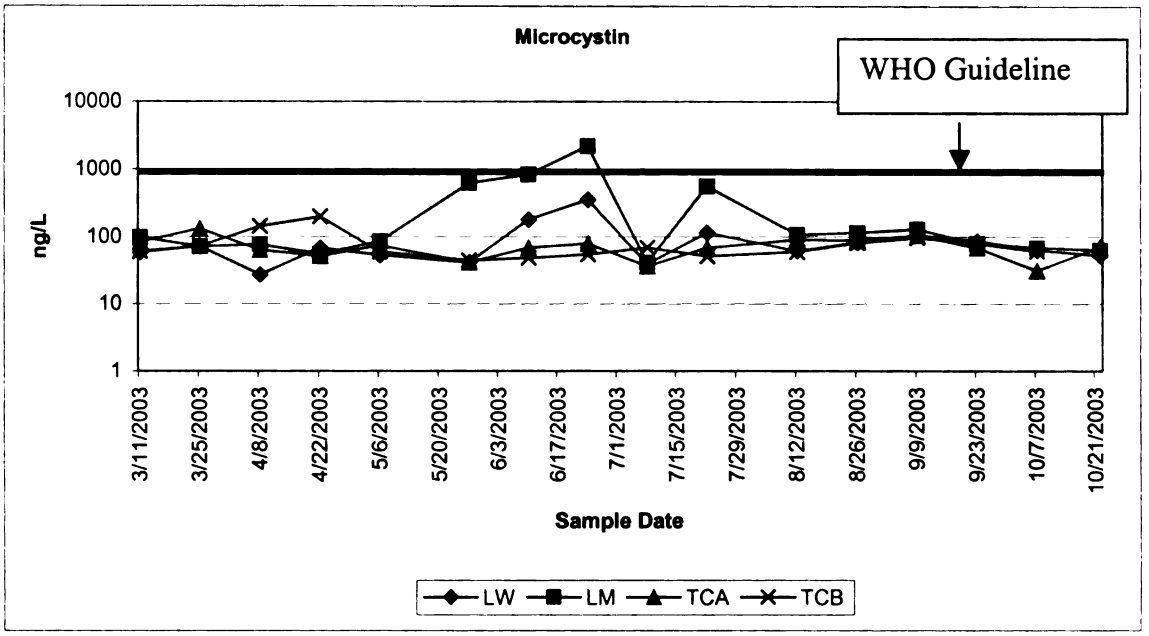
Figure 23: Toxic and Non-toxic Cyanobacteria Relative Abundance in Taylor Creek-B



Microcystin Results by ELISA in Florida Surface Waters

Microcystin levels for total water samples (Table 5, Figure 24) were low in most of the samples throughout the sample period, compared to the World Health Organizations (WHO) proposed standard of 1 μg/L (1000 ng/L). In LW toxin levels ranged from below the detection limit of 50 ng/L to 350 ng/L, with an average concentration of 90 ng/L (SD=78). LM toxin concentrations peaked in June at 2176 ng/L, with an average concentration of 322 ng/L (SD= 550). Taylor Creek levels remained low for all sampling events with an average concentration of 71 ng/L (SD= 25) for TCA and 78 ng/L (SD= 41) for TCB.

Figure 24: Microcystin Levels in Florida Surface Waters



Results of Microcystin Concentrations in Michigan Lakes

During the summers of 2002 and 2003 a total of seventy-three samples were collected and analyzed for microcystin (Appendix A). This was part of a larger study conducted by Dr. Orlando Sarnelle at Michigan State University. Toxin concentrations in particulates during summer 2002 ranged from 2.0 ng/L to 303.4 ng/L (Figure 25). Samples from summer 2003 (only six lakes were sampled in both 2002 and 2003) ranged from 1.4 ng/L to 137.8 ng/L (Figure 26). Of the lakes examined 47% (34 of 73) were positive for the presence of zebra mussels. Comparison of chlorophyll and total phytoplankton biomass levels shows that levels were similar in lakes with and without zebra mussels. Average chlorophyll concentrations were 5.05 and 7.84 μg/L for lakes with and without zebra mussels, respectively. Total phytoplankton biomass averages were 79.73 and 103.32 μg/L for lakes with and without zebra mussels, respectively. As expected, chlorophyll levels and total phytoplankton biomass were strongly correlated, r = 0.980 and 0.994 for lakes with and without zebra mussels, respectively. In the lakes

containing zebra mussels the average microcystin concentration was 46.6 ng/l, while lakes with no zebra mussels had an average concentration of only 17.0 ng/L (Table 7). Microcystin concentrations in lakes with zebra mussels was significantly different than lakes without zebra mussels at the 95th percentile ($p = 0.008$). Lakes that were positive for the presence of zebra mussels had approximately three times higher concentration of microcystin.

Table 7: Arithmetic Averages and Ranges of Chlorophyll, Phytoplankton Biomass and Microcystin Levels in Michigan Lakes, Both Positive and Negative for Zebra Mussel Presence (Data Provided by Dr. Orlando Sarnelle's Laboratory)

Zebra Mussel	Chl (ug/L)		Total Phytoplankton Biomass (ug/L)		Microcystin (ng/L)	
	Range	Average	Range	Average	Range	Average
Positive	0.9-38.6	5.1	17-740	80	2-131	47
Negative	1.1-80.2	7.8	19-849	103	2-81	17

Figure 25: Michigan Lake Microcystin Concentrations – 2002

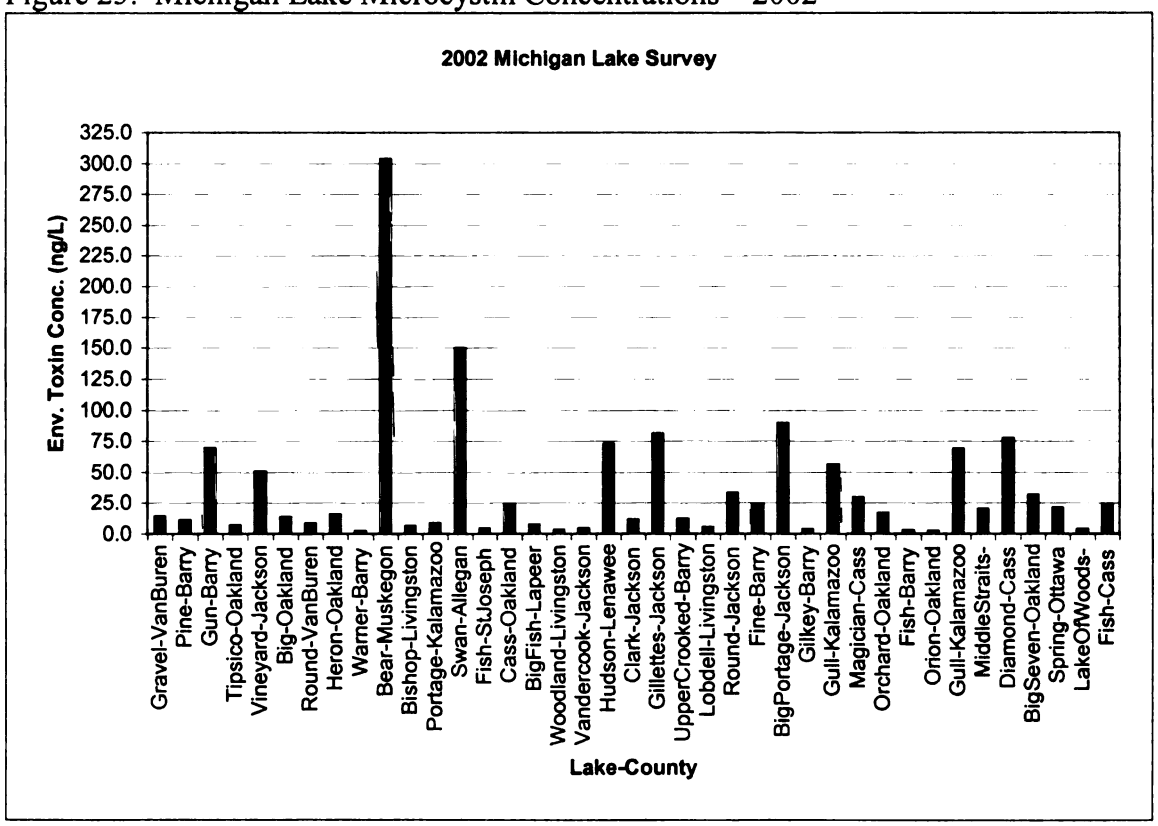
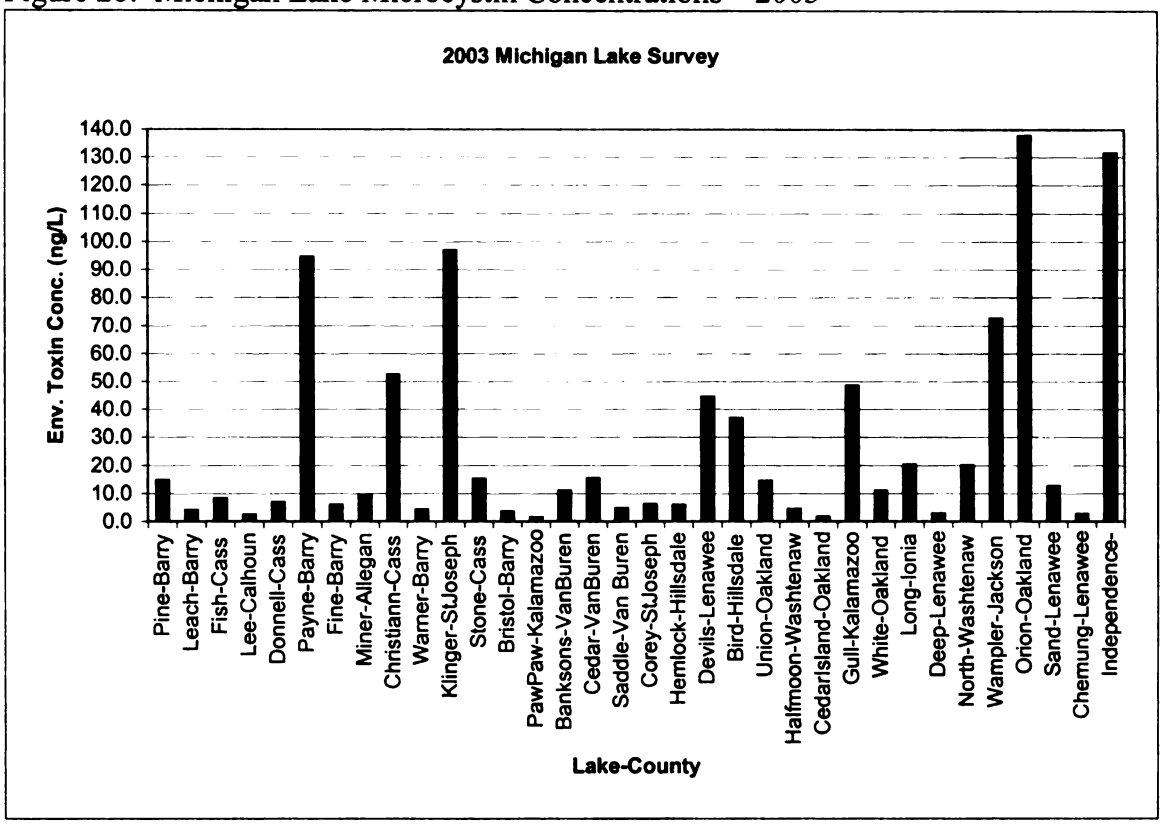


Figure 26: Michigan Lake Microcystin Concentrations – 2003



Florida Lake Monitoring Discussion

Microcystin levels show little correlation with physical, chemical or pollution levels (as measured by the fecal indicators) (Table 8). No significant correlations were observed between temperature and the other parameters examined, including bacterial indicators, toxin levels, chlorophyll *a* and toxic cyanobacterial biomass. There was a positive statistically significant correlation between chlorophyll *a* and microcystin in Lake Washington ($r = 0.805$). However, this correlation may be specific to the water body, as no correlation was found for Taylor Creek and the correlation for Lake Monroe was $r = 0.606$. Microcystin concentrations were significantly different between the four sample sites at the 95th percentile ($p = 0.039$). The concentrations in samples taken from Lake Monroe, which had the highest average toxin levels (322 ng/L), were significantly

different from the concentrations in samples at the other three samples sites. Lake Monroe microcystin concentrations were relatively high over a six-week period (May 27, 2003 to June 24, 2003). When microcystin concentrations in samples collected throughout the sample period were compared to chlorophyll *a* levels (Figure 27) and biomass of toxin producing cyanobacteria (Figure 28) similar trends can be seen. As toxin levels peak (June 24, 2003), so do both chlorophyll *a* levels and biomass of toxin producing cyanobacteria. However, as can be seen in Figure 34, the presence of toxin forming cyanobacteria does not always indicate the presence of high levels of toxin. During the March 25, 2003 sampling event, toxic cyanobacteria were present in high levels, however toxin concentrations were low (71 ng/L). No correlation was seen between toxic cyanobacteria biomass and microcystin levels in the three water bodies. This further emphasizes that toxin forming cyanobacteria may be present without producing toxins.

Similar relationships were observed in samples collected from LW when comparing toxin concentrations to both chlorophyll *a* (Figure 29) and toxin forming cyanobacteria biomass (Figure 30). Elevated microcystin and chlorophyll *a* levels in LW and LM were seen in summer months.

Table 8: Correlations between microcystin levels in Florida waters and physical, chemical and biological parameters

Parameter	LW	LM	TCA	TCB
pH	0.271	0.529	0.328	-0.295
Temperature	-0.105	0.133	-0.565	-0.043
Dissolved Oxygen	-0.003	NA	-0.343	0.320
Chlorophyll a	0.805	0.606	-0.075	-0.219
Total Coliforms	0.477	0.498	0.430	-0.316
Fecal Coliforms	0.445	0.435	0.587	-0.291
E. coli	0.372	0.260	0.337	0.048
Enterococci	0.142	0.170	0.350	-0.203
Clostridium perfringens	-0.244	-0.489	-0.208	0.228
Coliphage	-0.150	0.086	0.218	-0.498
Toxic Cyanobacteria	0.335	0.694	0.243	-0.118

NA = Data not available

Figure 27: Chlorophyll *a* and Microcystin Trends in Lake Monroe

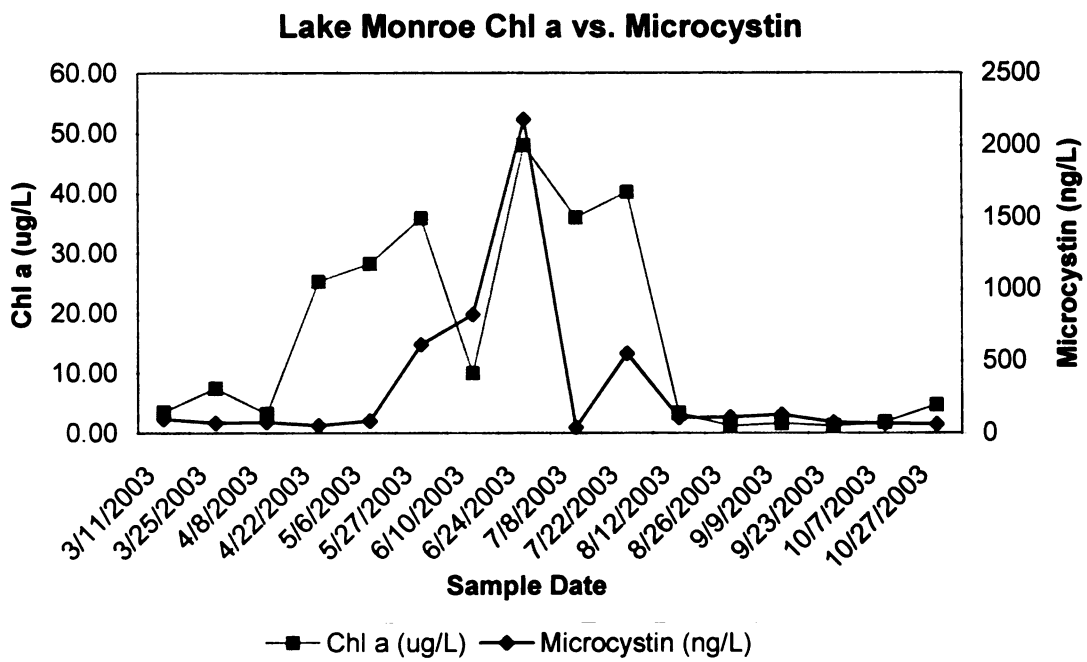


Figure 28: Microcystin Concentrations and Biomass of Toxin Producing Cyanobacteria in Lake Monroe

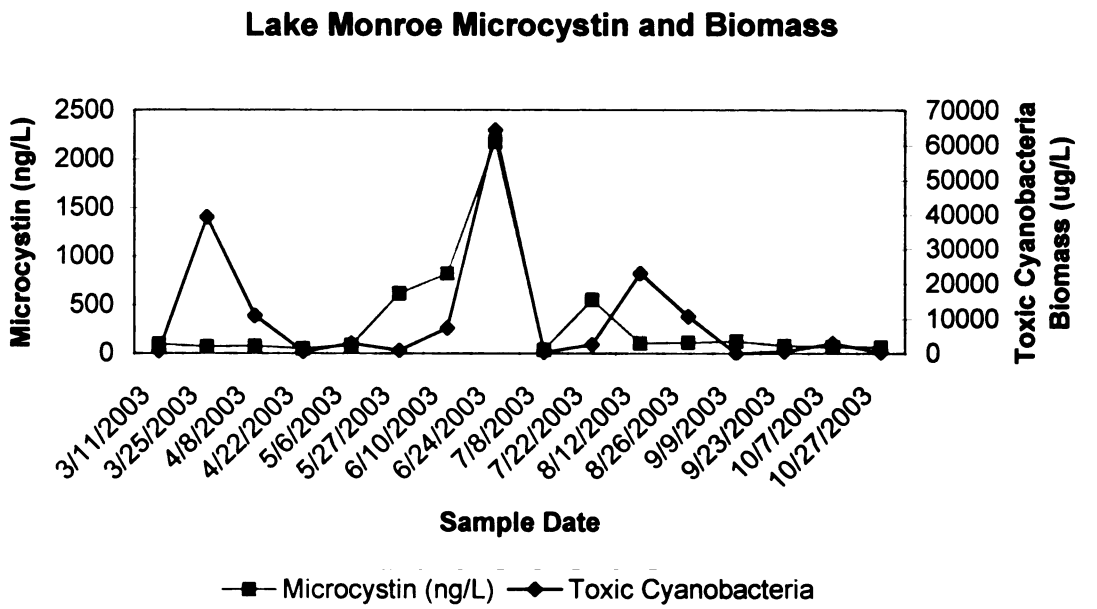


Figure 29: Chlorophyll *a* and Microcystin Trends in Lake Washington

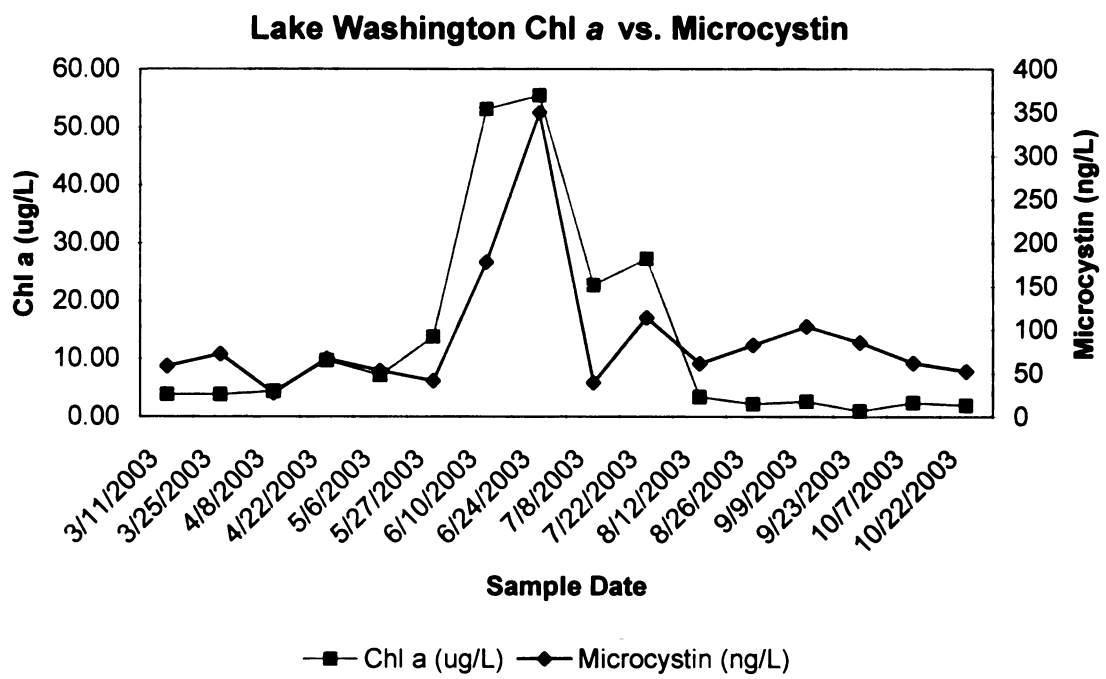
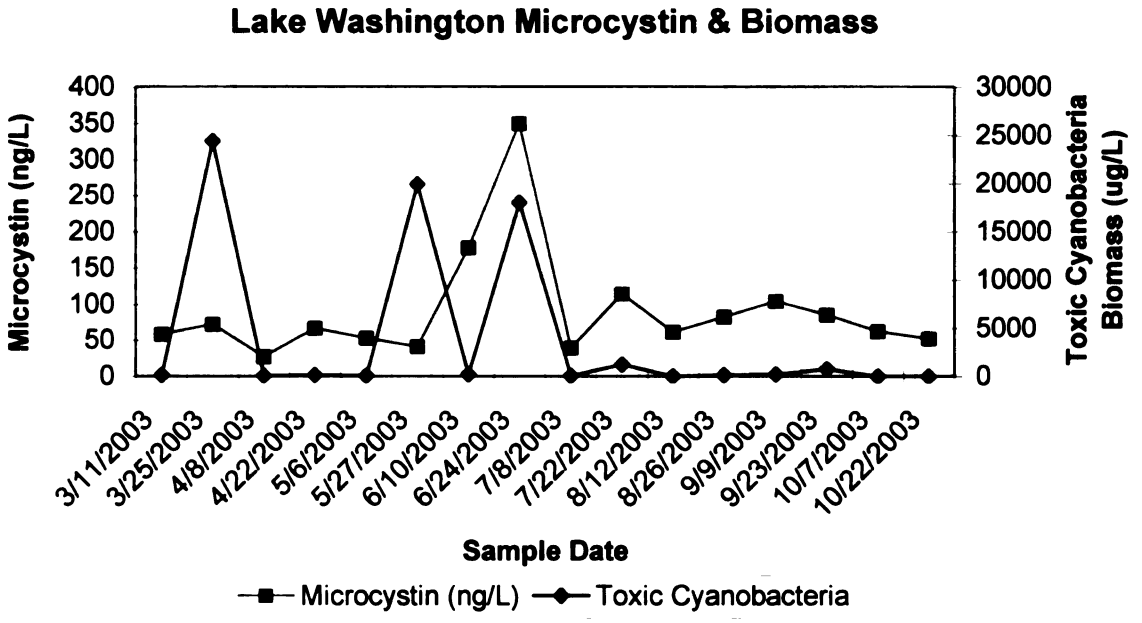


Figure 30: Microcystin Concentrations and Biomass of Toxin Producing Cyanobacteria in Lake Washington



The taxonomic diversity at each sample site was greatly different, with some toxin forming cyanobacteria present at each site. *Anabaena* and *Microcystis* were the two dominant forms of toxin producing cyanobacteria present at the four sample sites. During the eight months of monitoring these water bodies, cyanobacteria domination fluctuated, with increased *Microcystis* and *Anabaena* levels generally occurring in the months of May, June and July. This is thought to be in part due to increased temperatures and ample sunlight during summer months coupled with a stable water column.

Bacterial indicator data fluctuated greatly in samples collected from each sample site with levels of each indicator exceeding recreational standards at some point during the sampling period. An expected and statistically significant positive correlation was seen between total coliforms and fecal coliforms for each sample site. Correlations (based on log 10 data transformations) were r= 0.980, 0.945, 0.988 and 0.992 for Lake

Washington, Lake Monroe, TCA, and TCB respectively. Other indicator bacteria showed little or no correlation. The exception to this being a strong negative correlations between *E. coli* and bacteriophage (r = -1.0) in Lake Washington and between *Clostridium perfringens* and bacteriophage (r = -1.0) at TCB. These correlations, however, may be site specific since such correlations were not seen at the other sampling sights.

The three bodies of water examined in this research are clearly impacted by fecal pollution, with the seasons influencing bacteria levels. The lakes are also clearly eutrophic (with the possible exception of Taylor Creek) based on chlorophyll *a* levels, which increased greatly during the summer months, displaying expected seasonal trends. Toxin levels in the three lakes were consistently around 100 ng/L in samples obtained during most of the sampling events, thus adverse human health effects from acute exposure events might not be expected. However, since samples were only taken every two weeks the exact fluctuations in toxin concentrations throughout the sample period cannot be fully determined. Also, since there are few published reports detailing the adverse health effects for low-level chronic exposures to microcystin, the true risk of human exposure cannot be determined.

Discussion of Toxin Levels in Michigan Lakes

Zebra mussels were first introduced into the United States in the mid-1980's and have since become prevalent in the Great Lakes and Mississippi River. Zebra mussels are also rapidly invading inland lakes (Raikow et al. 2004). Current trends in Michigan lakes indicate that lakes containing the invasive species *Dreissena polymorpha* (Zebra Mussel) have higher levels of the cyanobacterial toxin microcystin than lakes not

containing zebra mussels. Vanderploeg et al. (2001) developed a strong hypothesis concerning the relationship between zebra mussels and *Microcystis* blooms in Saginaw Bay and Lake Erie. The hypothesis stated that mussels continually feed in the presence of *Microcystis*, that mussels would ingest all algae except *Microcystis* and that mussels would produce loosely consolidated pseudofeces that would be injected back into the water column (Vanderploeg et al 2001). Studies by Raikow et al. (2004) surprisingly reports that zebra mussel presence promotes the dominance of *Microcystis* in waters having a Total Phosphorus (TP) concentration of ≤25 µg/L. The results of this study support the previous hypothesis as well as the study hypothesis (Orlando Sarnelle, Michigan State University) that lakes invaded by zebra mussels contain higher levels of microcystin. This is the first research reporting increased levels of microcystin in lakes containing zebra mussels. Along with nutrient levels, zebra mussel presence is another factor that appears to contribute to the occurrence of cyanobacteria and cyanotoxins in Michigan Lakes.

Discussion of Monitoring Study

Samples from lakes in Florida and in Michigan both tested positive for the presence of the algal toxin microcystin. Florida samples were evaluated for the presence of total toxin (both intra- and extracellular). Michigan particulate samples were evaluated to determine the intracellular toxin levels. However, the average levels and possible reasons for cyanobacteria and toxin occurrence are different. The four sample sites examined in the state of Florida had an overall microcystin average concentration of 140.25 ng/L (SD= 121.42). Michigan lakes (both with and without zebra mussels) had an overall microcystin average concentration of 31.8 ng/L (SD= 20.93). The three Florida

water bodies examined in this study contained an approximate four times greater overall average concentration of microcystin than the overall average concentration found in Michigan lakes. Presence of cyanobacteria and the potential for toxin production in Florida water is likely due to the appropriate environmental conditions that promote growth, such as ample sunlight and warm water temperatures (15 to 30° C). Increased occurrence of cyanobacteria and toxin levels in Michigan lakes seems more likely attributable to the ever multiplying levels of the invasive species *Dreissena polymorpha* (Zebra Mussel). One way in which the zebra mussel may be promoting increases in cyanobacterial toxins is through selective feeding. By grazing on non-toxin forming species of phytoplankton, the abundance of cyanobacterial may be increased. This is despite the relatively oligotrophic nature of most Michigan lakes.

Previous monitoring studies conducted in the United States and through out the world reported various cyanobacteria and algal toxin results. A study by Karner et al. (2001) examined five drinking water facilities in Wisconsin for three months in 1998. Samples were collected from a mesotrophic and a eutrophic lake and were analyzed via ELISA. For the first four weeks of the study toxin levels in samples taken were steadily increasing, but remained below the WHO recommended standard (1000 ng/L). During week five of the study samples taken at intakes to three of the five plants exceeded the WHO standard and by week seven samples taken at all five plants exceeded the standard. A total of 289 samples were taken and 37% of those exceeded the 1000 ng/L recommended standard. Average concentrations for the five drinking water plants ranged from 585 to 1216 ng/L, with the highest toxin levels (1900-6100 ng/L) detected in the last week of sampling. In 1999, three of the five facilities were further evaluated in an

eleven-week study. Average concentrations were reported as 123-993 ng/L (n = 448). The decrease in toxin concentrations from 1998 to 1999 were attributed to year-to-year variations in temperature, rainfall and nutrient loading, all of which influence cyanobacterial growth (Karner et al 2001).

An earlier study by McDermott et al (1995) in Northeast Wisconsin examined lakes and rivers during cyanobacterial blooms in August through October 1993. In this study Microcystis was dominant and microcystin was present in 40 of 46 sites when analyzed via ELISA. Toxin concentrations ranged from just a trace to 200,000 ng/L, with concentrations being the greatest in samples obtained immediately along the shoreline (McDermott et al 1995).

Kotak et al. (1993) conducted studies of cyanobacterial toxin occurrence in eight lakes and six farm dugouts in Alberta, Canada. This study examined microcystin (via HPLC) and anatoxin-a (via GC-MS) concentrations. Microcystin-LR was detectable in 95% of the samples taken and concentrations ranged from non-detect to 605 μg/g (MC-LR/biomass of cyanobacteria). Anatoxin-a was not found in any of the lake samples (Kotak et al. 1993).

Outside of North America several studies have been conducted to analyze cyanobacteria and toxin occurrence. Oh et al. (2001) studied the levels of microcystin in a South Korean reservoir subject to agricultural runoff. Twenty-five samples were analyzed via HPLC and PPIA. Microcystin concentrations ranged from ~20 ng/L to ~250 ng/L in both dissolved and particulate forms (Oh et al. 2001). Researchers in Turkey examined three freshwater lakes in 1998 (Albay et al. 2003). Samples were analyzed using HPLC, PPIA and ELISA. Cyanobacteria were present in 61 of 73

samples and the presence (%) of microcystin-LR generally increased with depth (Albay et al. 2003).

Still other studies have focused on less common cyanobacteria such as the subtropical species, *Cylindrospermopsis racborskii* (Istvanovics et al. 2002 and Briand et al. 2002). *Cylindrospermopsis raciborskii* emergence and growth in Hungarian Lakes seems to be attributable to external nutrient loading (mainly from sewage) and the species ability to generate an internal phosphorus load (Istvanovics et al. 2002). In ponds in France, *Cylindrospermopsis raciborskii* growth was attributable to high temperature, low nutrient concentrations and high and constant sulfate concentrations. *Cylindrospermopsis raciborskii* was characterized as having good adaptability and low competitiveness with other phytoplankton species (Briand et al. 2002).

Results from the studies of Florida and Michigan lakes show both similarities and differences from previous published studies. Similar methodologies (ELISA) were used in our studies as was used in studies by Karner et al. (2001), McDermott et al. (1995) and Albay et al. (2003). Microcystin levels varied between the previously published studies, with ranges from non-detect up to >6000 ng/L. Like all of the previous studies, the Florida water body toxin concentrations fluctuated and each water body contained different ranges and averages of toxin concentrations. Our studies and previous studies indicate that a wide range of factors play a role in the proliferation of cyanobacteria and in algal toxin production. Like the waters evaluated in Florida the waters from the previous studies were classified as mesotrophic, eutrophic or hypereutrophic. The results of the Florida monitoring study are more comparable to previous studies because of there eutrophic classification. The majority of the lakes evaluated in Michigan were

oligotrophic, therefore the emergence of cyanobacteria and the increased levels of toxins seem to be related to factors (zebra mussels) not common in previously reported studies.

The main objectives of these studies were achieved through the performed research. Cyanobacterial genera, as well as total phytoplankton biomass, were identified for the three water bodies in Florida. Seasonal fluctuations in toxin forming cyanobacterial biomass were observed in the Florida lakes, with the highest levels seen in the summer months, when conditions are most favorable. Quantification of levels of microcystin were also determined for both Florida and Michigan waters, allowing for a better interpretation of toxin production and occurrence in both eutrophic and oliotrpohic lakes in the United States.

Due to the potential for cyanobacterial toxins to cause adverse health effects as has been seen in cases throughout the world, the continuing push to better understand these compounds should be a major priority for regulators and drinking water utilities. The World Health Organization's recommended drinking water guidelines for microcystin-LR and the EPA's placement of cyanobacterial on the Contaminant Candidate List implies the continued need to identify occurrence and health significance of these organisms. In conjunction, the best available analytical techniques and treatment processes need to be determined. Only when we better understand these organisms and their toxins will we be able to protect against adverse health effects.

Use of Enzyme-Linked ImmunoSorbent Assay (ELISA) for the Detection of Microcystins in Sediments and Tissues

Introduction

Microcystins are a group of biotoxins known as hepatotoxins. Microcystins are most commonly produced by the cyanobacteria *Microcystis*, however other species of cyanobacteria can produce the toxin (Watanabe et al. 1996). Microcystins are water-soluble heptapeptides containing seven amino acids (Karner et al. 2001). To date approximately fifty variants of microcystin have been isolated from *Microcystis* (Watanabe et al. 1996). *Microcystis* is commonly found in both eutrophic and hypereutrophic water bodies; in many cases *Microcystis* can be the dominant phytoplankton species (Watanabe et al. 1996). As mentioned in Chapter 1 (page 9), accumulation of toxins in sediments and tissues is of concern. However, inadequate assessment of the methods for recovery of the toxin and subsequent quantification with ELISA methods has not been undertaken.

Objectives

The objectives of this portion of the research are the evaluation of: i. extraction procedures for analyzing microcystins found in sediments, ii. an extraction method for the analysis of concentrations of microcystin found in Zebra Mussel (*Dreissena polymorpha*) tissue and iii. analysis of these extracts via ELISA.

Materials and Methods

Enzyme-Linked ImmunoSorbent Assay (ELISA) for Microcystins

ELISAs were performed in ninety-six well plates, using a commercially available kit, according to manufacturer's instructions (Envirologix, Inc., Portland, Maine) as described in Chapter 2 (page 29).

Preparation of Microcystin Culture Stock

Pure non-axenic *Microcystis aeruginosa* (UTEX 2667) culture, grown in BG-11 media, was provided by Dr. Orlando Sarnelle at Michigan State University. UTEX 2667, a toxin producing strain of cyanobacteria, was diluted by factors between ten and one thousand. Fifty milliliters of each dilution was filtered in duplicate onto Whatman GF/C 0.45 μm filters and both filters were frozen at -20° C until analysis. Before extraction filters were thawed at room temperature. Toxins were extracted using a simple methanol extraction method. Filters were extracted three times in 10 ml of 75% methanol for forty-five minutes in a 50 ml centrifuge tubes while shaking. The methanol extracts were combined and analyzed using ELISA per procedures described in Chapter 2 (page 28).

Examination of Sediments

Six soil types (Table 9) were obtained from the Department of Crop and Soil Sciences at Michigan State University. Each soil type contained varying percentages of organic matter, sand, silt and clay. Each soil type was seeded with microcystin and extracted. Four grams of each soil type was mixed in weigh boats using scoopulas with 1 ml of a ~1000 ng/L water solution of microcystin-LR (Alexis Corporation, San Diego, CA) resulting in a starting toxin concentration of ~0.250 μg/g. Each soil type was split into two equal portions by weighing the soil on a Mettler analytical balance and placed

into cryovials. The soil samples were incubated at ambient temperature for approximately forty-eight hours.

Table 9: Soil Types Used in Sediment Evaluation

Soil	pH	Organic Matter %	Sand %	Silt %	Clay %
Capac (Cp)	6.5	2.9	32	37	31
Parkhill (Pr)	6.8	5.2	32	39	29
Colwood 1 (Co1)	6.0	5.4	42	36	22
Colwood 2 (Co2)	5.6	6.1	48	40	12
Oshtemo (Os)	5.6	2.5	68	23	9
Montcalm (Mt)	5.4	4.5	76	19	5

In Extraction Method 1, (Kankaanpaa et al. 2001) one set of the six soil types was placed into centrifuge tubes with an equal volume (2 ml) of 100% methanol. Samples were vortexed for five minutes, followed by centrifugation for ten minutes at 1000 RPM (International Equipment Company). Supernatants were gently poured off the sediment into separate centrifuge tubes and filtered through 0.45 μm syringe filters. Filtrates were analyzed in duplicate via ELISA.

The second set of samples were extracted by Extraction Method 2 (Tsuji et al. 2001) in which soils were placed in centrifuge tubes with 40 ml of 5% acetic acid in 0.1% TFA-methanol. Samples were ultrasonicated for five minutes at 60 Hertz in a waterbath sonicator (Fisher Scientific) then supernatants were poured off the sediment and filtered through 0.45 μm syringe filters. The pH of each filtrate was adjusted to 6.0 ± 0.2 with 1N HCL and filtrates were analyzed in duplicate using ELISA procedures.

Extraction and Examination of Tissue

Zebra mussels collected from two lakes in Northern Michigan were evaluated for naturally occurring microcystin concentrations. Samples from one lake were collected at three different depths (3, 9, and 20 feet), resulting in zebra mussels from a total of four

locations within two lakes. Zebra mussel shells were opened and the tissue was removed from the shell using forceps. Zebra mussel tissues from each site were pooled to achieve a composite sample of 0.25 grams wet weight from each site (approximately 10-12 zebra mussels). Two different tissue extraction procedures were evaluated.

In Tissue Extraction Method 1 (Magalhães et al. 2001; 2003) whole tissue samples (0.25 grams) were covered with 1.0 ml of 100% methanol and extracted while shaking for forty-five minutes. Supernatant was removed from the tissue using a pipet and placed in a culture tube. This step was performed three times for a total extract volume of 3.0 ml. Sample extracts were then mixed with equal parts hexane (3 ml to 3 ml) by inverting the culture tube several times, the hexane portion separated from the methanol portion and the hexane portion was pipeted off the top of the methanol and discarded. The methanol fraction was dried using a freeze drier, then redissolved in 1 ml of 100% methanol and analyzed via ELISA.

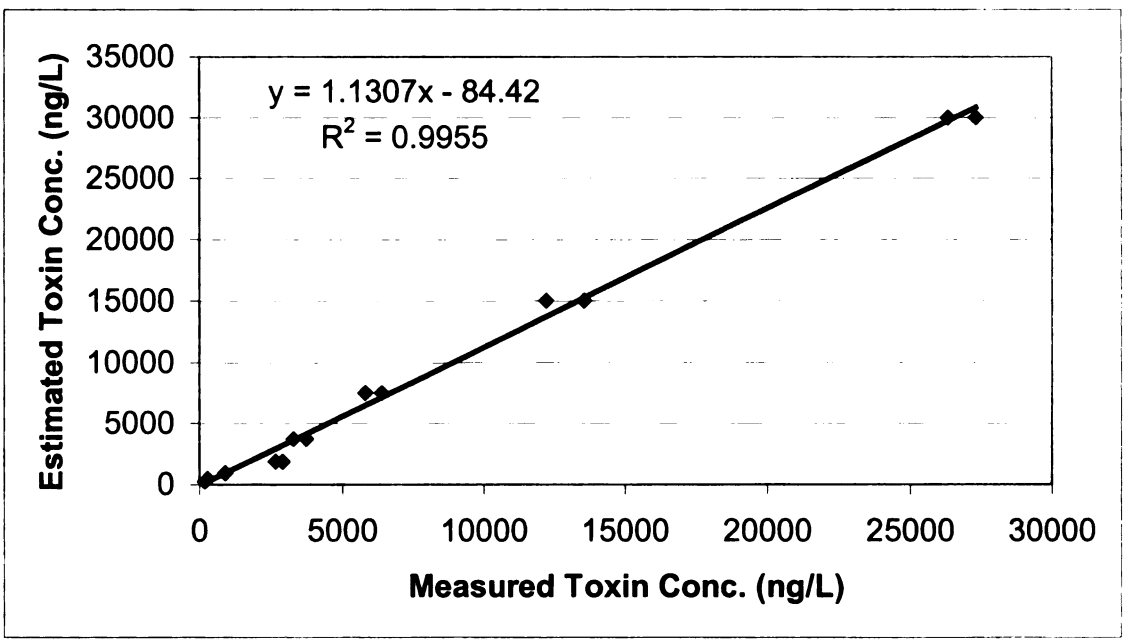
In the second extraction procedure (Tissue Extraction Method 2) (Kankaanpaa et al. 2001) the 0.25 g composite tissue samples were placed in culture tubes, lyophilized in a freeze drier for approximately three hours (or until tissue was completely dry), homogenized using disposable pellet pestles and stored at -20°C. Before extraction, samples were brought to room temperature. Samples in culture tubes were then extracted with 4 ml of 100% methanol in an ultrasonic waterbath at 60 Hertz (Fisher Scientific). Supernatants were collected and samples were extracted for forty minutes and methanol extract was evaluated using ELISA.

Results

Evaluation of Culture Samples

Microcystis aeruginosa culture samples diluted by factors from ten to one thousand were filtered, extracted using methanol and analyzed via ELISA. Measured toxin concentrations were compared with the estimated toxin concentrations for the same samples. Estimated toxin concentrations were estimated based on a concentration at the lowest dilution of 30,000 ng/L. Estimated concentrations were then calculated for each dilution. The diluted samples had measured toxin concentrations ranging from 199 ng/L to 27,310 ng/L. Comparing the estimated concentrations (234 to 30,000 ng/L) and the measured concentrations, the data provided an r^2=0.9955 over the range of dilution factors (Figure 31).

Figure 31: Comparison of Estimated Microcystin Concentrations and Measured Microcystin Concentrations in a *Microcystis aeruginosa* Culture (UTEX 2667)



Sediment Samples

Soil sample extraction methods were evaluated in four replicates with separate experiments. In the first experiment (extraction 1) samples were only extracted once,

using previously described methods, then analyzed by ELISA. In experiment 2 the samples from experiment 1 were extracted fully a second time (extraction 2) and evaluated using ELISA to determine if more toxin could be recovered from the sediments (these concentrations were added to concentrations from the first extraction to determine the total amount of toxin recovered). In the third experiment (extraction 3) and fourth experiment (extraction 4), each soil type was extracted three times and the extract was combined and analyzed via ELISA. Sediment toxin concentrations were determined by the following equation:

$$\textbf{Sediment Toxin Conc. (µg/g)} = \frac{\textbf{Extract Conc. (ng/L)}}{\textbf{1000}} * \frac{\textbf{Extract Vol. (ml)/1000}}{\textbf{Sediment (g)}}$$

Sediment extraction method 1 resulted in various percentage recoveries, depending on the number of times the extraction was performed (which influenced the total extract volume). Recovery efficiencies were determined by the following equation:

Recovery Efficiency(%) = (Sediment Conc. (µg/g) / Starting Toxin Conc. (µg/g))*100

Experiment 1 in which samples where only extracted once had an extract volume of 2 ml recovery efficiencies ranged between 0.05% and 0.07% with average concentrations between 0.0001 and 0.0002 µg/g (Figure 32 and Figure 33). Extraction 2 resulted in recovery efficiencies between 0.06% and 0.23% (average ranges of 0.0002 and 0.0006 µg/g). Extractions 3 (extract volume of 6 ml) and 4 (extract volume of 6 ml) resulted in recovery efficiencies ranging from 0.17% to 0.29% for the various soil types (average ranges of 0.0004 and 0.0007 µg/g).

Figure 32: Recovery Efficiencies for Sediment Extraction Method 1 using Six Soil Types

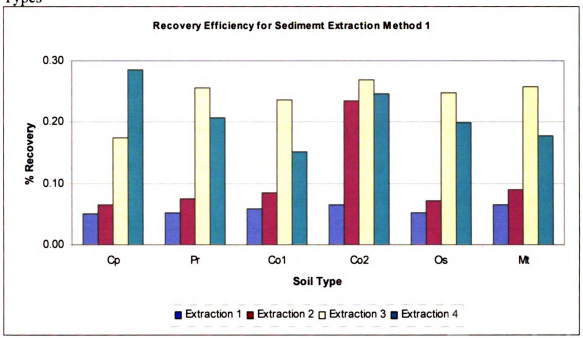
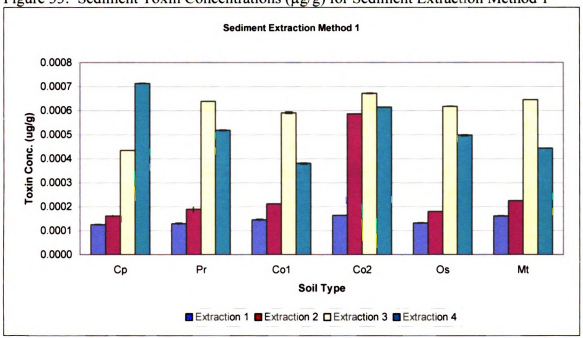


Figure 33: Sediment Toxin Concentrations (µg/g) for Sediment Extraction Method 1



Sediment toxin concentrations and recovery efficiencies for sediment extraction method 2 were calculated in the same manner as sediment extraction method 1.

Experiment 1, in which samples where only extracted once had an extract volume of 2 ml recovery efficiencies ranged between 1.10% and 1.98% with average concentrations between 0.0027 and 0.0049 μg/g depending on the soil type (Figure 34 and Figure 35). Extraction 2 resulted in recovery efficiencies between 1.11% and 2.92% (average ranges of 0.0028 and 0.0073 μg/g). Extractions 3 (extract volume of 6 ml) and 4 (extract volume of 6 ml) resulted in recovery efficiencies ranging from 3.31% to 12.82% for the various soil types (average ranges of 0.0083 and 0.0320 μg/g).

Figure 34: Recovery Efficiencies for Sediment Extraction Method 2 using Six Soil Types

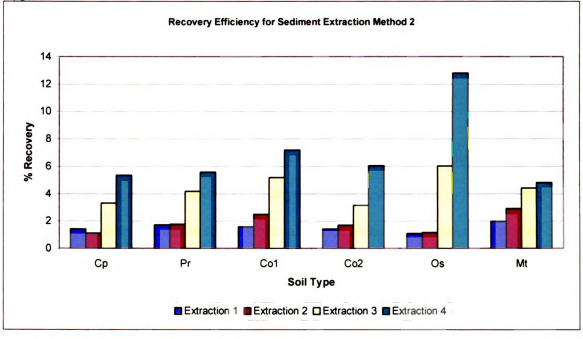
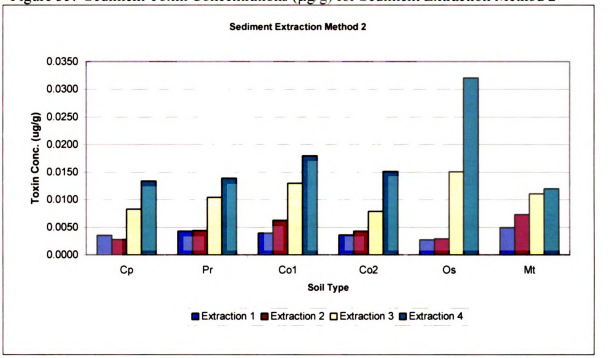


Figure 35: Sediment Toxin Concentrations (μg/g) for Sediment Extraction Method 2



Tissue Samples

The four sets of tissue samples were evaluated using ELISA after extraction per the procedures listed above. Extract microcystin concentrations (ng/L) were determined and the toxin concentrations in tissue were calculated using the following equation:

$$\textbf{Tissue Toxin Conc. (μg/g)} = \frac{\textbf{Extract Conc. (ng/L)}}{\textbf{1000}} * \frac{\textbf{Extract Vol. (ml)/1000}}{\textbf{Tissue (g)}}$$

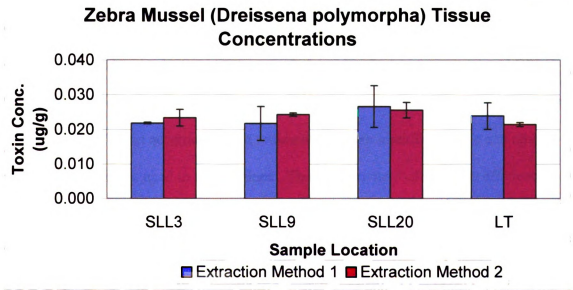
An average toxin concentration for the four tissue samples using tissue extraction method 1 was 0.024 μg/g. An average tissue concentration using tissue extraction method 2 was also 0.024 μg/g. Table 10 and Figure 36 show tissue toxin concentrations for each tissue sample. Since beginning concentrations of the toxins are not known, recovery efficiency could not be determined. A T-test demonstrated no statistical difference ($p = 0.90$)

between the two extraction methods at the 95th percentile. Tissue Extraction Method 2, based on methods by Kankaanpaa et al. 2001, is easier to use, requiring only the use of methanol or the extraction procedure, takes less time to perform and after lyophilization samples can be stored at -20° C for extended periods of time prior to analysis.

Table 10: Tissue Extraction Method Toxin Concentrations for Zebra Mussels in Michigan Lakes (SLL3=Lake 1-3', SLL9=Lake 1-9', SLL20=Lake 1-20' and LT=Lake 2)

Sample	Method	Tissue (g)	Extract (ml)	Conc. (ug/g)	SD
SLL3	1	0.25	3	0.022	0.0002
SLL9	1	0.25	3	0.022	0.0049
SLL20	1	0.25	3	0.027	0.0060
LT	1	0.25	3	0.024	0.0038
SLL3	2	0.25	4	0.023	0.0024
SLL9	2	0.25	4	0.024	0.0005
SLL20	2	0.25	4	0.026	0.0022
LT	2	0.25	4	0.021	0.0006

Figure 36: Comparison of Tissue Extraction Methods for Evaluation of Microcystin in Zebra Mussels (SLL3=Lake 1-3', SLL9=Lake 1-9', SLL20=Lake 1-20' and LT=Lake 2)



Discussion

Both the sediment and tissue extraction methods were developed based on previous studies in published literature, which examined sediments and tissues (fish, crab and shrimp). The methods for both the media were similar in the use of methanol as a base solvent, but procedures for toxin extraction are very different for the two media.

Low recovery efficiencies were seen with both sediment extraction methods. Low recovery efficiencies using the solvent methanol have been reported in the literature, with reports of increased efficiency when TFA was added to the solvent mixture (Tsuji et al. 2001). The same pattern was observed in the extraction methods observed in this research. Sediment extraction method 2, which used a 5% acetic acid in 0.1% TFA-methanol solution, resulted in up to ~12% better recovery for the same soil types. However, this recovery was still greatly lower than that reported by Tsuji et al (2001), in which the extraction method was based. Tsuji et al (2001) reported recovery efficiencies up to 60%. The methods tested in this research used ELISA to analyze the toxin concentrations, whereas the literature in which the methods were based on used HPLC for analyses. This could also contribute to the differences in recoveries reported. Since the possibility of toxin accumulation in sediment has been identified, these and other extraction procedures need to be examined. The improvement of recovery efficiencies as well as the determination of the best analytical technique (ELISA vs. HPLC) needs to be established.

As presented in the literature ELISA was used to analyze the toxin levels in the zebra mussels. The tissue extraction procedures need to be further evaluated using controlled laboratory studies in an attempt to determine recovery efficiencies. By

seeding tanks containing zebra mussels with microcystin, approximate uptake rates and recovery efficiencies can be estimated. This will provide further support that the two extraction procedures evaluated are useful in studies focusing on bioaccumulation of cyanobacterial toxins in aquatic organisms.

Detection of Microcystin Synthetase Gene (*mcyA*) of *Microcystis* in Culture and Environmental Samples

Introduction

Cyanobacteria (blue-green algae) contain toxins that can adversely affect human health and have been included on the EPA's Contaminant Candidate List (CCL). The CCL is designed to address future regulations concerning waterborne microbial contaminants by improving the understanding of both occurrence in water and health risk. Previous problems associated with algae have been aesthetic, specifically odor and taste, however, the production of hepatotoxins and neurotoxins in certain species of algae has prompted researchers to focus on the health hazards of cyanobacteria and their toxins. Microcystin is the predominant toxin produced by the genera *Microcystis*, *Anabaena* and *Oscillatoria*. Microcystin toxicity is mediated by active transport of toxins into hepatocytes via the bile avid transport system, followed by inhibition of protein phosphatases 1 and 2A (Tillett et al. 2001).

Identification of those cyanobacteria capable of producing toxins on a molecular level can be achieved through the use of Polymerase Chain Reaction (PCR). PCR allows for the amplification of specific DNA sequences, which can subsequently be used to taxonomically and phylogenetically characterize samples (Wilson et al. 2000). The 16S rRNA gene is generally used in the analysis of cyanobacterial taxonomy due to ubiquitous distribution throughout prokaryotic phylogentic groups (Wilson et al. 2000). However, primers have been developed to target genes more specific to individual species and toxins. Neilan et al. (1997) have identified primers specific to the genera *Microcystis*, allowing the detection of organisms relative to that particular group. Other primers have been developed to target the microcystin synthetase gene *mcyA*. This

allows detection of the presence of a toxic species (Tillett et al. 2001). The *mcy* (microcystin synthetase) gene cluster encodes peptide synthetases which nonribosomally synthesize microcystins. Genes for the enzymes involved in microcystin biosynthesis are found only in toxic isolates. (Pan et al. 2002). The goal of this portion of the research is to evaluate new techniques for the genetic detection of toxin-producing cyanobacteria. Through the development of PCR primers that target genes associated with toxin production, the potential for a cyanobacterial population to produce toxins can be determined.

Materials and Methods

Cyanobacterial Strains

A total of six *Microcystis* isolates were screened for the presence of the *mcyA* gene. Three were from culture collections and known to produce toxins (PCC 7820, UTEX 2664 and UTEX 2667). The other three were isolated from Gull Lake in Michigan (EE, II, and KK).

Culturing of Cyanobacteria

All six cyanobacteria isolates were cultured in screwcap test tubes containing approximately 20 ml of BG-11 medium. All isolates were incubated at 23° C under cool white fluorescent lighting (light:dark cycles).

DNA Extraction

DNA extraction was performed using Qiagen QIAamp DNA mini kit. Approximately twenty milliliters of each sample (*Microcystis* in BG-11 medium) was filtered through 0.45 μm Millipore MF membrane filters, then the filter was suspended in 180 μL of 20 mg/ml lysozyme and incubated at 37°C for thirty minutes. Proteinase K (20

μL) and Buffer AL (200 μL) were then added and the solution was incubated at 56°C for thirty minutes, followed by incubation at 95°C for fifteen minutes. The supernatant was pipeted into a microcentrifuge tube. Ethanol (200 μL) was added to the microcentrifuge tube, mixed and centrifuged. Buffer AW1 (500 μL) was then added and centrifuged, followed by addition of Buffer AW2 (500 μL), which was centrifuged as well. Finally, the sample was resuspended in 200 μL of a Buffer AE.

Polymerase Chain Reaction

The presence of the gene for the cyanobacterial toxin, microcystin, was assessed through PCR. Primers were developed to target the microcystin *mcyA* gene. These primers included the Forward Primer, AACAGGAATTAGGCGATATTC and the Reverse Primer, AAGGTTAATTTCTCCCTCCAG. PCR was performed using HotStarTaq DNA polymerase (Qiagen, Inc.). Thermal cycling conditions consisted of an initial step at 95°C for fifteen minutes (to activate Hot Start Polymerase), followed by thirty-three cycles of 94°C-1 minute, 55°C-1 minute and 72°C-1 minute. Each 50 μL reaction contained 5 μL of 10X PCR Buffer, 1 μL of 10 mM nucleotide mix, 5 μL of each primer, 0.25 μL of *Taq* DNA polymerase, 5 μL of template DNA and water to reach final volume. The presence or absence of the gene was confirmed by agarose gel electrophoresis and UV trans-illumination. The DNA product was 208 bp in size.

Cloning

Isolates testing positive for the presence of *mcyA* gene were cloned using Invitrogen TOPO TA Cloning Kit (Carlsbad, CA). Transforming One Shot TOP10F' Competent Cells Method was used per manufacturer's instructions. Each transformation (50 μL) was spread onto LB agar plates with 50 μg/ml ampicillin containing 40 mg/ml

X-gal and 100 mM IPTG. These plates were incubated at 37°C overnight. White colonies were picked and grown in LB broth containing 50 μg/ml ampicillin at 37°C overnight. Samples were then extracted using Qiagen Plasmid Extraction Kit. Plasmid samples were confirmed by agarose gel electrophoresis and UV trans-illumination. Michigan State University's Genomics Technology Support Facility sequenced plasmid samples.

Results

Six *Microcystis* isolates were examined; four tested positive for the presence of the *mcyA* gene (Table 11). Each isolate was examined twice using PCR with the same results for both experiments. All three culture strains (PCC 7820, UTEX 2664 and UTEX 2667) were positive. One environmental isolate (II, Gull Lake) also contained the *mcyA* gene. Three of the four sequences (UTEX 2667, UTEX 2664 and II) were cloned and sequenced. Cloning of PCC 7820 was attempted, but did not work. The three sequences were aligned and compared for similarity. Sequences for cloned isolates UTEX 2667, UTEX 2664 and Gull Lake II are listed in Appendix B (page 84). UTEX 2664 and UTEX 2667 aligned to 99%, UTEX 2664 and Gull Lake sample II aligned to 97% and UTEX 2667 and Gull Lake sample II aligned to 96%. The primers developed in this study showed positive results in detecting the mcy gene of *Microcystis* species in culture and environmental isolates.

Table 11: Results of PCR on *Microcystis* isolates

Sample	Primer Target	Result
PCC 7820	*mcyA* gene	+
UTEX 2667	*mcyA* gene	+
UTEX 2664	*mcyA* gene	+
Gull Lake Isolate EE	*mcyA* gene	-
Gull Lake Isolate II	*mcyA* gene	+
Gull Lake Isolate KK	*mcyA* gene	-

Discussion

Researchers have used PCR as a way to detect *Microcystis* strains from both culture and environmental samples. Pan et al. (2002) conducted whole-cell PCR on 38 cyanobacterial culture strains of different species and on 200 water samples that tested positive for microcystin via ELISA. Primers used in that study targeted the *mcyB* gene (Pan et al. 2002). Eighteen out of the thirty culture strains examined, previously known to be hepatotoxic, tested positive via PCR for the presence of the *mcy gene*. None of the eight filamentous strains tested gave a detectable signal. All 200 of the environmental samples tested positive for the presence of the *mcyB* gene (Pan et al. 2002).

Primers in this study were designed to target the *mcyA* gene, which plays a role in microcystin synthesis. The *mcyA* gene, like the *mcyB* gene is part of the *mcy* gene cluster, is always present in toxic isolates, thus is a good target for the detection of a cyanobacterial strain, which has the capability of producing toxins (Pan et al. 2002).

Analysis on a larger catalog of *Microcystis* strains (both toxic and non-toxic) is required to further confirm the utility of these primers. Toxin producing species of cyanobacteria, such as *Anabaena* and *Oscillatoria*, need to undergo evaluation to determine if these primers work towards the detection of the *mcyA* gene in species other than *Microcystis*. These primers will also need to be tested using environmental samples, to determine the detection ability in samples that are not pure culture. Also a study of the method sensitivity needs to be conducted in order to determine the detection limits for this procedure.

The three Gull Lake isolates evaluated in this study were isolated on the same day and from the same bloom. One of the three *Microcystis* isolates (II) tested positive for

presence of the *mcyA* gene. This initial work indicates that cyanobacterial blooms contain various genotypes of cyanobacteria, both toxin forming and non-toxic forming. In the future, surveys of toxic-cyanobacteria genera, presence of the toxic gene and the toxin in populations and blooms will be possible.

Appendix A

Michigan Lakes Survey in 2002 and 2003

Location (Lake-County)	**Mussel Status (+/-)**	**Location (Lake-County)**	**Mussel Status (+/)**
Gravel-VanBuren	neg	LakeOfWoods-VanBuren	pos
Pine-Barry	neg	Fish-Cass	pos
Gun-Barry	neg	Pine-Barry	pos
Tipsico-Oakland	neg	Leach-Barry	pos
Vineyard-Jackson	neg	Fish-Cass	pos
Big-Oakland	neg	Lee-Calhoun	pos
Round-VanBuren	neg	Donnell-Cass	pos
Heron-Oakland	neg	Payne-Barry	pos
Warner-Barry	neg	Fine-Barry	pos
Bear-Muskegon	neg	Miner-Allegan	pos
Bishop-Livingston	neg	Christiann-Cass	pos
Portage-Kalamazoo	neg	Warner-Barry	pos
Swan-Allegan	neg	Klinger-StJoseph	pos
Fish-StJoseph	neg	Stone-Cass	pos
Cass-Oakland	neg	Bristol-Barry	pos
BigFish-Lapeer	neg	PawPaw-Kalamazoo	pos
Woodland-Livingston	neg	Banksons-VanBuren	pos
Vandercook-Jackson	neg	Cedar-VanBuren	pos
Hudson-Lenawee	neg	Saddle-Van Buren	pos
Clark-Jackson	neg	Corey-StJoseph	pos
Gillettes-Jackson	neg	Hemlock-Hillsdale	pos
UpperCrooked-Barry	neg	Devils-Lenawee	pos
Lobdell-Livingston	neg	Bird-Hillsdale	pos
Round-Jackson	neg	Union-Oakland	pos
Fine-Barry	neg	Halfmoon-Washtenaw	pos
BigPortage-Jackson	neg	CedarIsland-Oakland	pos
Gilkey-Barry	neg	Gull-Kalamazoo	pos
Gull-Kalamazoo	neg	White-Oakland	pos
Magician-Cass	neg	Long-Ionia	pos
Orchard-Oakland	neg	Deep-Lenawee	pos
Fish-Barry	neg	North-Washtenaw	pos
Orion-Oakland	neg	Wampler-Jackson	pos
Gull-Kalamazoo	neg	Orion-Oakland	pos
MiddleStraits-Oakland	neg	Sand-Lenawee	pos
Diamond-Cass	neg	Chemung-Lenawee	pos

BigSeven-Oakland	neg	Independence-Washtenaw	pos
Spring-Ottawa	neg		

Appendix B

Gull Lake Isolate II Sequence

gtgtggtttctagatccgatcaagagcggccgcttctgtgatggaggtaggcagaattcn
eccttaacaggaattagacgatattctggcagaaattgactaataaggagaatctttcat
ggcagacacccttctcaacccgccaaaaatgtggagtctatttatcctctttcccccatg
caggaagggatgctctttcatagtctttatactcctgattcagggatttattgtagccaa
actctaattactctggaggagaaattaaccttaagggcgaattccagcacactggcggcc
gttactagtggatccgagctcggtaccaagcttggcgtaatcatggtcatacctgtttcc
tgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcataaagtg
taaagcctggggtgcctaatgagtgagctaactcacattaactgcgttgcgctccactgc
ccgctttctattcgggaaactcgcctgtcctttgcattcatgaatccgccaaccctctgg
gtagaggcggttgcgaattgggcgctcttccggttccttcctcctcgatcccctgcgccc
cgtcccttcgcgctcccgaggggtatccgctcccteacaggcgcaaatctgttatccacg
aatccgggggataatgcagttataactcctctcgcaaaagcctgctacagggccggaacc
gctaaaacgccccttgctcggatttctccctatgctccgccccctcgacaggctcacca
aaattctcccccccaactcaacgtgccctat

UTEX 2664 Sequence

eggagtatgnatcctatagggcgaattggggtttctagatggagactcgagcggccgcta
ctgtgatggatatctgcagaattcgcccttaacaggaattagacgatattctggcagaaa
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UTEX 2667 Sequence

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