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Sublethal Toxicity of Microcystis and Microcystin-LR in Fish

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To the Graduate Council:

I am submitting herewith a dissertation written by Emily Dawn Rogers entitled "Sublethal Toxicity of Microcystis and Microcystin-LR in Fish." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Natural Resources.

Richard J. Strange, Major Professor

We have read this dissertation and recommend its acceptance:

Theodore B. Henry, Steven W. Wilhelm, Gary S. Sayler

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Sublethal Toxicity of *Microcystis* and Microcystin-LR in Fish

A Dissertation Presented for
the Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Emily Dawn Rogers
December 2010

Dedication

In loving acknowledgement of my parents for their endless encouragement and support

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Abstract

The occurrence of blooms of toxic cyanobacteria in freshwater environments is a global ecological and public health concern. Species of *Microcystis* are of particular importance because blooms occur in many freshwater environments throughout the world and microcystin toxin concentrations can exceed World Health Organization advisory levels. While microcystin has been associated with fish kills, sublethal effects of chronic exposure at environmentally relevant concentrations are relatively unknown. The objective of this research was to evaluate toxicity of microcystin and *Microcystis* in fish during all life history stages. We evaluated global gene expression response in larval zebrafish (*Danio rerio*), and a sub-set of biomarker genes indicative of microcystin exposure were identified. In addition, vitellogenin genes were highly up-regulated in zebrafish exposed to *Microcystis* but not the microcystin toxin, indicating potential endocrine disrupting effects of *Microcystis* blooms. Effects on reproduction were evaluated in adult zebrafish exposed to *Microcystis*. There was a significant decrease in the percentage of adults that spawned, however fecundity and larval survival were not affected. Laboratory mesocosm experiments with channel catfish (*Ictalurus punctatus*) were also conducted to determine the importance of dietary and aqueous exposure in microcystin bioaccumulation and assess histopathological lesions. Tissue toxin concentrations and histopathological lesions were also evaluated in channel catfish collected from Lake Erie and Waterville Reservoir, North Carolina to monitor fish living in environments affected by *Microcystis* blooms and relate responses to those observed in laboratory exposures.

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SECTION I

Literature Review

The occurrence of toxic algal blooms in freshwater and marine environments has recently become a global ecological and public health concern (Codd et al. 2005; Luckas et al. 2005). In addition to affecting recreational activities, algal toxins have been detected in municipal drinking water supplies (Codd et al. 2005), have been associated with fish kills (Chorus et al. 2000), and may bioaccumulate in fish species that are consumed by humans (Xie et al. 2005). The occurrence of toxic algal blooms is projected to increase with changes in climate and increased anthropogenic enrichment of surface waters (Carmichael 2001; Paerl and Huisman 2008). Because toxic algal blooms have the potential to negatively affect both human and ecosystem health, understanding processes influencing toxicity of algal toxins is important.

While there are many different types of harmful algae, blooms that produce microcystin toxins are especially relevant because their occurrence is widespread and toxin concentrations can exceed levels considered safe for humans and wildlife. Microcystins are a diverse group of toxins produced primarily by cyanobacteria of the genus *Microcystis*. Microcystins are cyclic heptapeptides, and more than 80 forms have been described, each differing slightly in chemical structure (Dittmann and Wiegand 2006; Tanabe et al. 2004). Microcystin-LR, mainly produced by *Microcystis aeruginosa*, is recognized as being the most toxic microcystin and is globally distributed in freshwater environments (Codd et al. 2005; Luckas et al. 2005). Blooms producing microcystin-LR have been reported yearly in Lake Erie since 1995, and concentrations often exceed the World Health Organization drinking water advisory level of 1 µg/L (Chorus et al. 2000).

Of particular relevance is microcystin exposure in fish, because fish are important components of aquatic ecosystems and bioaccumulation in fish may affect human health through consumption. In fish, microcystin can be hepatotoxic, but effects on ion regulation have also been reported (Zambrano and Canelo 1996). Microcystin acts by inhibiting protein phosphatases 1 and 2A, resulting in increased phosphorylation of cellular proteins. The microcystin–LR molecule contains an adda moiety, which binds noncovalently to the catalytic site of the protein phosphatase enzyme and renders it inactive (Dittmann and Wiegand 2006). This step is responsible for initial toxicity and is reversible (Fischer et al. 2000). Delayed non-reversible covalent binding of microcystin-LR to a cysteine residue of the protein phosphatase enzyme gives stability to the complex but is not required for toxicity (Dittmann and Wiegand 2006; Fischer et al. 2000). In liver tissue, inhibition of protein phosphatases results in over-phosphorylation of cytokeratins (Eriksson et al. 1990; Ohta et al. 1992), resulting in cytoskeletal rearrangement and compromised liver function, including necrosis, apoptosis and intrahepatic hemorrhage (Fischer et al. 2000). In gill tissue, protein phosphatase inhibition results in the inactivation of Na^+/K^+ ATPase, an enzyme that regulates ion transport across the gills (Zambrano and Canelo 1996). The degree to which microcystin disrupts ion homeostasis in fish is not well understood and may depend upon exposure route (Malbrouck and Kestemont 2006).

Fish living in environments affected by microcystin may be exposed through two major exposure routes. In the environment, fish may encounter the toxin through ingestion of whole cells or passively by contact with surrounding water (respiration), or a combination of both (Malbrouck and Kestemont 2006). Aqueous exposure to microcystin and *Microcystis* cells in fish has been reviewed in the literature; however, the methods used, in most cases, were not comparable to an environmental exposure scenario. For example, the majority of studies

conducted with aqueous microcystin have been acute exposures during early developmental stages (Best et al. 2002; Best et al. 2003; Liu et al. 2002; Oberemm et al. 1997; Oberemm et al. 1999; Wiegand et al. 1999), and fewer studies of chronic exposure have been conducted with adult fish (Adamovsky et al. 2007; Carbis et al. 1996a; Carbis et al. 1996b; Li et al. 2007; Mares et al. 2009; Qiu et al. 2009). Another type of acute exposure found in the microcystin literature is microinjection (Huynh-Delerme et al. 2005; Jacquet et al. 2004; Wang et al. 2005). The purpose of this method is to mimic uptake of the toxin from the surrounding water by the embryo or transfer of microcystin from females to eggs; however, the occurrence of maternal transfer has not been established for microcystin, and this type of exposure is somewhat presumptive (Malbrouck and Kestemont 2006). Finally, in studies addressing effects of microcystin via ingestion, microcystin has been administered through oral gavage (Carbis et al. 1996a; Fischer and Dietrich 2000; Fischer et al. 2000; Tencalla and Dietrich 1997) and intraperitoneal injection (Carbis et al. 1996a; Fournie and Courtney 2002; Malbrouck et al. 2003); however some studies have incorporated *Microcystis* into fish feed (Dong et al. 2009; El Ghazali et al. 2010; Li et al. 2004; XY Li et al. 2005; Soares et al. 2004; Zhao et al. 2006a; Zhao et al. 2006b), which is more comparable to a natural feeding situation. While chronic exposures to *Microcystis* and/or MC-LR with environmentally relevant exposure scenarios are generally lacking, the existing studies offer some results that should be reviewed further.

The majority of studies with *Microcystis* and MC-LR have focused on early life stages of fish. Exposure to purified MC-LR seems to interfere with hatching, however results are conflicting. In a study with rainbow trout, advanced hatching occurred at 50 µg/L MC-LR, but hatching was unaffected in zebrafish (*Danio rerio*) at similar concentrations (Oberemm et al. 1999). In chub (*Leuciscus cephalus*) and loach (*Misguruns mizolepis*), decreased growth and

survival was observed during exposure to MC-LR at concentrations ranging from 0.5-500 µg/L (Liu et al. 2002; Oberemm et al. 1999). Interestingly, in zebrafish, these effects did not occur during exposure (0.5-50 µg/L MC-LR), but only after the exposure was terminated and larvae were reared in clean water (Oberemm et al. 1997; Oberemm et al. 1999). In another study with zebrafish, increased activity of detoxification enzymes (glutathione S-transferase and glutathione peroxidase) was observed, which suggests that larval fish may be able to metabolize MC-LR and thus avoid toxicity (Wiegand et al. 1999) and could explain why mortality was not observed during exposure (as in Oberemm et al. 1997; Oberemm et al. 1999). Other sublethal effects of aqueous exposure to purified MC-LR during early development include edema, reduced head size, curved body and tail, enlarged heart, increased heart rate and damaged hepatocytes (Jacquet et al. 2004; Liu et al. 2002; Oberemm et al. 1999). Exposure to MC-LR via microinjection into eggs gave similar results. Hatching was accelerated (Jacquet et al. 2004), survival decreased (Jacquet et al. 2004; Wang et al. 2005), and skeletal malformations (Wang et al. 2005) as well as damage to the liver (Huynh-Delerme et al. 2005; Jacquet et al. 2004) and digestive tract (Huynh-Delerme et al. 2005) were observed.

When early life stages of fish were exposed to aqueous *Microcystis*, as opposed to purified MC-LR, effects were similar and in some cases more pronounced. Exposure of carp (*Cyprinus carpio*) and southern catfish (*Silurus meridionalis*) embryos to crude extracts of *Microcystis* resulted in high mortality rates, delayed hatching, malformations, and lesions in the liver detected by histopathology (Palikova et al. 2003; Palikova et al. 2004; Zhang et al. 2008). When zebrafish embryos were exposed to crude *Microcystis* extract and an equivalent dose of purified MC-LR, effects on antioxidant enzymes were 2-fold greater in the group exposed to *Microcystis* (Pietsch et al. 2001). Similarly in several different species including zebrafish,

rainbow trout (*Oncorhyncus mykiss*), roach (*Rutilis rutilis*), bream (*Abramis brama*), white asp (*Leucaspius delineatus*) and stone loach (*Cobitus taenia*), effects on hatching, survival and malformations were more pronounced when embryos were exposed to *Microcystis*, rather than purified MC-LR (Oberemm et al. 1999). It has been hypothesized that the presence of compounds within *Microcystis* cells not related to the toxin, such as lipopolysaccharides, may be able to increase effects of MC-LR by interfering with its detoxification pathway (Best et al. 2002; Best et al. 2003). Further research is needed to characterize and distinguish effects of non-toxin related *Microcystis* compounds from effects of MC-LR.

In contrast to early life stages of fish, much less is known about aqueous exposure of adult fish to *Microcystis* and MC-LR. No exposures of adult fish to purified MC-LR were found in the literature, presumably due to the high cost of purified MC-LR (1mg retails for $330 USD (http://www.axxora.com/)) and amount of compound needed to achieve relevant concentrations in the volume of water required for exposure of larger fish. Aqueous exposure data is therefore limited to studies that have tested *Microcystis* blooms or extracts. Carp were exposed to a *Microcystis* extract for 7 days and elevation of stress-related enzymes and cellular degeneration and necrosis were observed in the liver (Carbis et al. 1996a; Carbis et al. 1996b). Carp were also exposed to a natural *Microcystis* bloom in Meiliang Bay, China and monitored over the course of one year. MC-LR was detected in liver and kidney, with concentrations generally higher in liver tissue. Elevation of stress hormones was also correlated with microcystin concentrations as well as ultrastructural damage to the liver (Li et al. 2007). In the only chronic laboratory exposure of adult fish to aqueous *Microcystis*, carp were exposed to a *Microcystis* bloom containing 182-539 µg total MC/g dry mass for 9 weeks, followed by an 8 week elimination period where they were moved to clean water. There was no mortality during the exposure; however, microcystins

accumulated in liver and muscle, with concentrations10-fold higher in liver. Microcystin concentrations reached a maximum at 4 weeks and elimination proceeded rapidly once exposure was terminated. The authors performed risk calculations and concluded that a total microcystin concentration in muscle of 29.3 ng/g would not pose a significant risk to humans (Adamovsky et al. 2007). These studies form a good foundation for examination of exposure of adult fish to *Microcystis* via aqueous exposure; however, it is difficult to make comparisons among studies because bloom compositions vary and methods used to measure microcystin accumulation in tissues (total MCs versus MC-LR) are not consistent across experiments.

Studies examining dietary exposure to MC-LR and *Microcystis* in adult fish are more plentiful. These studies employ a variety of exposure methods including incorporation of *Microcystis* or MC-LR into fish feed, which mimics natural ingestion, as well as artificial methods such as intraperitoneal (i.p.) injection and oral gavage. While intraperitoneal injection and oral gavage methods are not representative of the way that fish would ingest *Microcystis* in the environment, they nevertheless provide additional data on toxin accumulation and sublethal effects.

Administration of MC-LR or *Microcystis* via i.p. injection generally supports effects observed during aqueous exposure. In most studies, fish were given a single high-dose injection of MC-LR ranging from 50 to 600 µg/kg bw (Carbis et al. 1996a; DP Li et al. 2009; Zhang et al. 2009) and samples were collected within a short period of time, usually within hours to a few days post-injection. These doses generally resulted in high mortality exceeding that of comparable doses administered through oral or aqueous routes (Carbis et al. 1996a; Carbis et al. 1996b), which calls into question the usefulness of the results. Nevertheless, i.p. injection studies have confirmed basic effects of MC-LR in fish found in aqueous exposure studies, such

as protein phosphatase inhibition (Malbrouck et al. 2004), accumulation of MC-LR in liver tissue (Lei et al. 2008; L Li et al. 2005; Malbrouck et al. 2003; Williams et al. 1997), and liver tissue damage (mainly necrosis) (Carbis et al. 1996b; Fournie and Courtney 2002; L Li et al. 2005; Malbrouck et al. 2003). Additional effects included decline in heart rate and blood pressure (DP Li et al. 2009), as well as anemia (Zhang et al. 2007).

The effects of oral gavage studies were generally not as severe as those obtained with i.p. injection. Again, doses administered were high, ranging from 2.5 - 5,700 µg/kg bw (Carbis et al. 1996a; Carbis et al. 1996b; Fischer et al. 2000; Tencalla and Dietrich 1997), but did not result in acute toxicity (Carbis et al. 1996a; Carbis et al. 1996b). Accumulation of MC-LR in the liver occurred (Djediat et al. 2010; Fischer and Dietrich 2000; Fischer et al. 2000; Mezhoud et al. 2008; Tencalla and Dietrich 1997) and lesions in liver were described; however, effects were less severe than those reported in i.p. injection studies (Carbis et al. 1996b; Djediat et al. 2010; Fischer and Dietrich 2000; Fischer et al. 2000; Mezhoud et al. 2008; Tencalla and Dietrich 1997).

In contrast to i.p. injection and oral gavage, the most realistic type of dietary exposure involves incorporation of *Microcystis* or MC-LR into fish food. With aqueous exposure, both direct ingestion of *Microcystis* and entry via respiration and drinking are possible, whereas feeding studies examine ingestion only and do so by allowing fish to feed naturally. The majority of dietary studies used food spiked with *Microcystis*- either unaltered culture (Li et al. 2004), sun dried culture (Dong et al. 2009; Zhao et al. 2006a; Zhao et al. 2006b), filtered cells (Soares et al. 2004), or lyophilized cells (El Ghazali et al. 2010; XY Li et al. 2005). In only one study was purified MC-LR administered to fish via the diet (Deng et al. 2010). Most studies measured toxin accumulation in liver and muscle and were semi-chronic to chronic in duration ranging

from 15 days (Soares et al. 2004) to 12 weeks of exposure time (Zhao et al. 2006a; Zhao et al. 2006b), and in a few studies data was collected for a period of time post-exposure to determine elimination rates (Dong et al. 2009; Soares et al. 2004). Both exposure and tissue concentrations varied, as well as reporting methods. Doses were difficult to interpret. In two studies feeding rate was not controlled and fish were fed to satiation, making it impossible to determine the amount of toxin ingested (Zhao et al. 2006a; Zhao et al. 2006b). In many cases, because *Microcystis* blooms were used, exposure concentrations were reported in terms of total MCs, rather than MC-LR (Dong et al. 2009; Soares et al. 2004; Zhao et al. 2006a; Zhao et al. 2006b). To further complicate matters, bloom composition varied greatly across studies. Some blooms contained predominately MC-LR (Soares et al. 2004; Zhao et al. 2006b) while others were dominated by MC-RR (Li et al. 2004; XY Li et al. 2005; Zhao et al. 2006a), such that studies having similar total MC exposure concentrations could not be compared. Likewise, tissue concentrations were reported as total MCs (Dong et al. 2009; Soares et al. 2004; Zhao et al. 2006a; Zhao et al. 2006b), and in some cases within the same study, MC-LR was measured in tissues while exposure concentrations were reported as total MCs, making results difficult to interpret (El Ghazali et al. 2010; Li et al. 2004).

Despite the variations in experimental design and methods of reporting exposure and tissue concentrations, results of dietary accumulation studies showed a few general trends. Microcystin concentrations were higher in liver than in muscle (Dong et al. 2009; Li et al. 2004; Soares et al. 2004; Zhao et al. 2006a; Zhao et al. 2006b), and in a few cases muscle concentrations exceeded the WHO tolerable daily intake (TDI) value for human consumption of 0.04 µg/kg body weight/day (Dong et al. 2009; Li et al. 2004; Soares et al. 2004; Zhao et al. 2006b). Elimination of microcystin in one study was rapid (Soares et al. 2004), however in

Dong et al. (2009) muscle concentrations still exceeded the TDI 55 days after exposure ended. Because the WHO TDI is based on MC-LR concentration and not total MCs, these studies may overestimate risk. More studies that monitor MC-LR specifically, in both food and tissue samples, are needed in order to determine if bioaccumulation of MC-LR in fish presents a significant risk to human populations.

Another area in need of further study is the potential for microcystin to affect fish reproduction. Maternal transfer has been studied by injecting eggs with MC-LR (results previously discussed in this chapter in section describing effects on early developmental stages of fish); however no study has actually documented accumulation of MC-LR in eggs as a result of parental exposure. Maternal transfer experiments are therefore presumptive and have limited value as a means of assessing reproductive effects. Only one study (Deng et al. 2010) exists where adult fish were exposed to MC-LR, allowed to reproduce, and effects on progeny were assessed. In that study MC-LR was incorporated into fish food and administered to Japanese medaka at a controlled feeding rate for 8 weeks. After 4 weeks of exposure, male and female fish from corresponding treatments were allowed to spawn. Embryos were transferred to clean water where they were monitored for survival and adults were returned to exposure tanks. Embryo survival was significantly reduced at the highest concentration tested (3.93 µg MC-LR/g diet), and effects on adults included reduced growth following spawning, inhibition of protein phosphatases in the liver, and hepatic lesions (Deng et al. 2010). This study indicates negative effects on reproduction in fish as a result of dietary exposure to MC-LR; however, reproductive effects following aqueous exposure to MC-LR and *Microcystis* blooms have not yet been evaluated.

Previous research has not adequately addressed sublethal chronic exposure or effects on reproduction, development, or bioaccumulation (Malbrouck and Kestemont 2006). Because fish that live in environments affected by blooms of *Microcystis* can be exposed to microcystin toxins at low concentrations for long periods of time, there is potential that all life history stages of fish are affected. Reproductive effects need further evaluation, and controlled laboratory studies to confirm bioaccumulation via aqueous and dietary exposure are inconclusive (Malbrouck and Kestemont 2006). More research on reproduction, development, and bioaccumulation will improve our understanding of microcystin toxicity in fish.

While uncertainties exist regarding toxicity of microcystin, the use of new technologies in gene expression analysis has the potential to further our understanding of the effects of this toxin. Gene expression analyses such as microarray and quantitative real-time PCR can give insight into toxicology mechanisms and identify genes that can be used as biomarkers of exposure. By coupling these analyses with traditional endpoints including mortality, reproduction, and histopathology, the differential expression of certain genes can be associated with specific negative reproductive or physiological outcomes in fish (Miracle and Ankley 2005). Using this approach, biomarker genes identified by gene expression analysis can be used to evaluate the condition of fish living in environments affected by toxic algal blooms.

Due to the relative novelty of the application of gene expression analysis in toxicology, information is limited and has only addressed MC-LR exposure. Microarray investigations with adult zebrafish injected (i.p.) with MC-LR revealed that numerous immune-related genes, in addition to genes involved in tumorigenesis and cell cycling, were differentially regulated in liver tissue (Wei et al. 2008). In larval zebrafish, immune related genes and heat shock proteins were also differentially expressed in targeted analyses using quantitative PCR (YA Li et al.

2009). These studies have just begun detailing the sub-lethal effects of MC-LR in fish and effects of *Microcystis* exposure also need to be addressed.

Objectives

The primary objective of this research was to investigate the effects of chronic exposure to environmentally relevant concentrations of microcystin during all life history stages of fish. Biomarker genes were identified that may have the potential to be used in the future as a field monitoring tool to make predictions about the condition of fish living in environments where *Microcystis* blooms are present. Bioaccumulation of microcystin in fish was also evaluated and related to the potential for adverse human health effects through fish consumption.

Hypotheses

1. Specific genes respond to microcystin-LR and *Microcystis* exposure in larval zebrafish and these genes can be identified as potential biomarkers of exposure (Section II).

2. Chronic exposure to *Microcystis* decreases zebrafish fitness and reproductive success, and induces lesions in liver tissue detectable by histopathology (Section III).

3. Route of exposure will influence bioaccumulation of microcystin-LR and *Microcystis* in channel catfish, and differences in response due to exposure route can be evaluated by liver histopathology and tissue toxin burden (Section IV).

4. Tissue toxin burden and histopathological effects observed in fish collected from lakes affected by *Microcystis* can be related to responses of channel catfish exposed to *Microcystis* and MC-LR under laboratory conditions in order to better understand toxic effects occurring in wild fish and the potential for human exposure through fish consumption (Section IV).

5. Primer sets for biomarker genes identified in zebrafish can be designed for channel catfish for future assessment of expression of these genes by quantitative PCR in laboratory exposures and samples collected from channel catfish living in areas where *Microcystis* is present (Section IV).

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SECTION II

Global gene expression profiling in larval zebrafish exposed to microcystin-LR and *Microcystis* reveals endocrine disrupting effects of cyanobacteria

This section is a version of a journal article that is under review in *Environmental Science and Technology*. My contribution to the paper was conducting the exposure of zebrafish to MC-LR and *Microcystis*, extracting RNA, analyzing microarray data under the guidance of Michael Twiner, and compiling background information and writing the paper.

Rogers, Emily D., Theodore B. Henry, Michael J. Twiner, Julia S. Gouffon, Jackson T. McPherson, Gregory L. Boyer, Gary S. Sayler, and Steven W. Wilhelm. In review. Global gene expression profiling in larval zebrafish exposed to microcystin-LR and *Microcystis* reveals endocrine disrupting effects of cyanobacteria. *Environmental Science and Technology*.

Abstract

Microcystis blooms occur worldwide and threaten aquatic ecosystems and human health. Sublethal effects on early developmental stages of fish are largely unknown and research has mainly focused on microcystin (MC) toxins, rather than *Microcystis* cells. We exposed (96 h) zebrafish larvae to purified MC-LR (0-1,000 µg/L) or lyophilized *M. aeruginosa* containing 4.5 µg/L MC-LR and evaluated changes in global gene expression (Affymetrix GeneChip® Zebrafish Genome Arrays). Significant changes in gene expression (≥ 1.7-fold change, $p<0.0001$) were determined with Rosetta Resolver® 7.0, and ontology analysis was conducted with the DAVID bioinformatics tool. The number of differentially expressed genes relative to control increased with MC-LR concentration and included genes related to known mechanisms of action for MC-LR in mammals and older life stages of fish, as well as genes unique to larval zebrafish. Up-regulation of vitellogenin genes (vtg) (19.2 to >100-fold on arrays; 619.3-fold confirmed by qPCR) was observed in *Microcystis*-exposed larvae but not in larvae exposed to MC-LR. Up-regulation of vtg indicates exposure to estrogenic substance(s) and suggests that *Microcystis* may be a natural source of environmental estrogens. Concerns about the effects of *Microcystis* blooms may extend beyond those associated with the microcystin toxin.

Introduction

Toxin-producing harmful algal blooms occur throughout the world and are a major public health and ecological concern. While there are many different types of harmful algal blooms, cyanobacterial blooms are especially important because their occurrence is widespread and toxin concentrations regularly exceed levels considered safe for humans and wildlife (Carmichael 2001). Microcystins are a diverse group of toxins mainly produced by cyanobacteria of the genus *Microcystis*, and are cyclic heptapeptides with more than 80 forms described (Dittmann and Wiegand 2006; Tanabe et al. 2004). Microcystin-LR (MC-LR) is generally recognized as being the most toxic microcystin variant, and concentrations in surface waters often exceed the World Health Organization advisory level of 1 µg/L (Chorus et al. 2000).

The mechanisms of MC toxicity and detoxification in fish are believed to be similar to those reported in mammals. The liver is the major target organ (Fischer et al. 2000), and a primary mechanism of MC toxicity is inhibition of protein phosphatases (PPs) 1 and 2A (Fujiki and Suganuma 1993). Inhibition of PPs results in over-phosphorylation of cytokeratins (Eriksson et al. 1990; Ohta et al. 1992), resulting in cytoskeletal rearrangement and compromised liver function, including necrosis, apoptosis and intrahepatic hemorrhage (Fischer et al. 2000). The binding affinity of the various MC variants to PPs is believed to govern variant potency (Matsushima et al. 1990). Disruption of Na+/K+ ATPase pumps and subsequent dysfunction of ion regulation has been reported to be a consequence of inhibition of PPs by MC and a secondary mechanism of MC-induced toxicity in fish (Gaete et al. 1994; Zambrano and Canelo 1996). The degree to which MC disrupts ion homeostasis *in situ* is not well understood and may be an effect

of fatty acids associated with *Microcystis* rather than the microcystin toxins themselves (Bury et al. 1996; Bury et al. 1998). Detoxification of MC occurs in the liver via glutathione conjugation catalyzed by glutathione-S-transferase (Pflugmacher et al. 1998), subsequently resulting in biliary excretion (Sahin et al. 1996).

To date the major focus of research on MC effects in fish early life stages has been directed towards developing an understanding of toxicity during the embryo stage (before hatching), and in most cases where effects on larvae have been assessed, the exposure was terminated before hatching and downstream effects were observed while larvae were grown in clean water (Malbrouck and Kestemont 2006). In experiments where larvae were assessed following acute embryonic exposure to microcystin, effects included decreased survival (Oberemm et al. 1997; Oberemm et al. 1999; Jacquet et al. 2004; Wang et al. 2005), severe skeletal malformations (Wang et al. 2005), and hepatobiliary hypertrophy (Jacquet et al. 2004). In the few cases where fish were exposed to microcystin during the larval stage, effects were more severe than embryo exposure. Wiegand et al. (1999) demonstrated that uptake of MC-LR in larval zebrafish was greater than that of embryos. Loach larvae exposed to MC-LR had significantly lower survival rates than embryos and malformations, including alterations of hepatocytic organelles, heart muscle and erythrocytes, were also more severe (Liu et al. 2002). This information suggests that larval fish may be especially susceptible to MC-LR exposure, and a better understanding of mechanisms of toxicity during this stage is needed.

Recently, other investigators have applied gene expression analyses in an effort to better characterize the biochemical pathways influenced by MC-LR in fish. Microarray investigations with adult zebrafish injected (intraperitoneal) with MC-LR revealed that numerous immune-

related genes, in addition to genes involved in tumorigenesis and cell cycling, were differentially regulated in liver tissue (Wei et al. 2008). In larval zebrafish, immune related genes and heat shock proteins were also differentially expressed in targeted analyses using quantitative PCR (Li et al. 2009). These studies have just begun detailing the sub-lethal effects of MC-LR in fish. Exposure of larval fish to *Microcystis* blooms in the environment is a complex issue because larval fish are not exposed to MC-LR alone, but rather *Microcystis* cells and lysates that contain other substances in addition to MC-LR. *Microcystis* can produce numerous peptides classified as aeruginosins (Ishida et al. 1999), micropeptins (Yamaki et al. 2005), and microviridins (Rohrlack et al. 2003) that putatively have some type of biological function (Harada 2004; Smith et al. 2008). In addition, *Microcystis* cell walls contain lipopolysaccharides that can be toxic (Raziuddin et al. 1983). In several cases where fish were exposed to *Microcystis* and MC-LR during early development, the toxicity of *Microcystis* was greater than that of purified MC-LR (Oberemm et al. 1997; Oberemm et al. 1999; Best et al. 2001; Palikova et al. 2007). As such, it is important to consider both the effects of *Microcystis* and MC-LR in larval fish.

The objective of this study was to compare the response of larval zebrafish exposed to the purified MC-LR toxin with larval zebrafish exposed to *Microcystis*. A global gene expression approach was used to distinguish the biochemical pathways affected by MC-LR with those pathways influenced by exposure to *Microcystis*, which contains MC-LR and numerous other bioactive compounds. We predicted that MC-LR and *Microcystis* exposure to larval fish would result in distinctive sets of differentially expressed genes related to toxicity mechanisms and pathways, potentially impacting larval development and survival.

Materials and Methods

Experimental Fish

Zebrafish (*Danio rerio*) were obtained from the Zebrafish Research Facility in the Center for Environmental Biotechnology at the University of Tennessee. Fish husbandry, spawning, and experimental procedures were conducted with approval from the University of Tennessee Institutional Animal Care and Use Committee (Protocol #1690-1007). Water for holding fish and conducting experiments (hereafter referred to as fish water) consisted of MilliQ water (Millipore, Bedford, MA) with ions added: 19 mg/L $NaHCO_3$, 1 mg/L sea salt (Instant Ocean Synthetic Sea Salt, Mentor, OH), 10 mg/L $CaSO_4$, 10 mg/L $MgSO_4$, 2 mg/L KCl. Embryos were obtained by spawning adult fish with no history of contaminant exposure. Fertilization of embryos took place at the same time (± 15 min.), such that larvae used in experiments were of similar age at the time of exposure. All activities (maintenance of adult fish, spawning, and experiments) were conducted in an environmental chamber with a temperature of 27± 1 ºC and 14:10h light:dark photoperiod.

Preparation of Exposure Solutions

The *Microcystis* treatment was prepared using lyophilized cells of *Microcystis aeruginosa*. *M. aeruginosa* PCC-7806 was obtained from the Pasteur Culture Collection of Cyanobacteria and cultured in BG-11 media according to the same methods described for *M. aeurginosa* LE-3 isolates in Rinta-Kanto and Wilhelm (Rinta-Kanto et al. 2006). Live cultures of *M. aeruginosa* were centrifuged in 250 mL batches at 3,500 rpm for one hour to concentrate

cells into a pellet, combined, and pellets obtained after centrifuging 6 L of culture were combined. Cells were lyophilized for 48 hours using a freeze-dry system (Labconco, Kansas City, MO) and the total dry weight mass of algal cells obtained was 300 mg. For exposure of larval zebrafish, lyophilized *Microcystis* was reconstituted back to its original nominal concentration of 50 mg lyophilized cells/L.

Solutions for MC-LR treatments were prepared by dissolving 1 mg of purified microcystin-LR (Alexis Biochemicals, San Diego, CA) in 0.5 mL ethanol and diluting to 100 and 1,000 µg/L using fish water. The concentration of ethanol in all treatments was ≤ 0.05%, and a treatment of 0.05% ethanol was used as a vehicle control. Fish water served as the negative control.

Experimental Design

At 72 h post-fertilization, larvae were exposed to lyophilized *Microcystis* and purified MC-LR at concentrations of 100 and 1,000 µg/L. Controls consisted of zebrafish system water (negative control) and zebrafish system water containing 0.05% ethanol (vehicle control). Larvae from both control groups as well as 100 µg/L MC-LR, 1,000 µg/L MC-LR, and lyophilized *Microcystis* were exposed in groups of 50 with three replicates per treatment and were sacrificed after 96 hours for RNA extraction and subsequent microarray analysis. All larvae were exposed in beakers containing 100 ml of solution. Water samples for water quality measurements and microcystin analysis were taken during the experiment, and mortality and behavioral observations were recorded at 24-hour intervals.

Water Quality and Chemical Analyses

Water quality parameters measured following 96-hour exposure included dissolved oxygen (6.7 mg/L), pH (6.9), total alkalinity (36 mg/L as $CaCO_3$), total hardness (18 mg/L as $CaCO_3$), and ammonia (<0.2 mg/L). Analysis of MC-LR in samples was conducted at the State University of New York College of Environmental Science and Forestry (Syracuse, NY) by protein phosphatase inhibition assay following the methods of Carmichael and An (Carmichael and An 1999). Lyophilized *M. aeruginosa* pellets were extracted in 50% acidified methanol using ultrasound, (25 watts; three 20 sec bursts with 20 sec cooling on ice between bursts), and water samples were analyzed directly without concentration. Measured MC-LR concentrations (mean ± SD) were: 140 ± 12 µg/L (in the 100 µg/L MC-LR solution), 1,703 ± 71 µg/L (in the 1,000 µg/L MC-LR solution), and 4.5 µg MC-LR equivalents/L (in the lyophilized *Microcystis*). LC-MS analysis (Boyer 2007) of the MC-LR standards and PCC7806 *Microcystis* cell material indicated that microcystin-LR was the only toxin variant present in these samples.

Total RNA Extraction

Larvae were centrifuged for 10 min at 13,000 rpm to separate larvae from exposure water, and pellets containing larvae were stored at –80ºC until RNA extraction was performed the following week. Total larval RNA was extracted using the RNeasy mini extraction kit for animal tissues (Qiagen, Valencia, CA) and quantified using a UV-spectrophotometer (Nanodrop, Wilmington, DE) as previously described (Henry et al. 2009).

Microarray Methods

Microarray analysis was conducted at the Affymetrix Core Facility located on the University of Tennessee campus, with which our lab has previously conducted microarray experiments with zebrafish (Henry et al. 2007). Equal amounts of RNA from controls and treatments were used for cDNA synthesis and subsequent biotin labeling for microarray analysis (Message Amp™ II- Biotin Enhanced Kit, Ambion, Austin, TX). Samples were then applied to GeneChip® Zebrafish Genome Arrays (≈15,000 gene transcripts), and hybridization and scanning procedures were conducted according to Affymetrix GeneChip® Expression Analysis Technical Manual (Affymetrix).

Statistical Analysis of Microarray Data

Analysis of array data was conducted with Rosetta Resolver® 7.0 Gene Expression Data Analysis System (Rosetta Informatics, Seattle, WA, USA) using methods similar to Twiner et al. (2008). Using a rank consistency filter, features were subjected to a combination linear and lowness normalization algorithm. Based on the Rosetta error model, a composite array was generated for each treatment and control, in which the data underwent a weighted averaging based on feature quality in the triplicate arrays making up the composite. A list of "signature" gene features was then generated for each time point from the composite array by p value sorting and absolute differential expression ($\geq$1.7-fold, $p<0.0001$). The software does not assign an absolute value to expression ratios >100-fold or to p values <10-45. Signature gene lists for each treatment were further characterized by ontology using the Database for Annotation Visualization and Integrated Discovery (DAVID) (Dennis et al. 2003; Huang et al. 2009).

Quantitative Reverse Transcriptase PCR

Aliquots of the same RNA samples used for arrays were analyzed to confirm expression of vitellogenin type 1 (*vtg1A/B*) by quantitative reverse transcriptase PCR (qRT-PCR). The qRT-PCR protocol and primer/probe sets are described in Henry et al. (2009), and zebrafish β-actin gene was used as the internal control. Amplicons from zebrafish *vtg1A/B* and ß-actin were generated using Taq DNA Polymerase (Fisher Scientific, Pittsburg, PA, USA) and T/A cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA) for propagation and generation of template for RT-PCR. For reactions, plasmid DNA was linearized with BamH1 (Promega), enzyme removed with QIAquick PCR Purification Kit (Qiagen), and DNA quantified by NanoDrop measurement. Reverse transcription was performed with T7 RiboMAX Express Large Scale RNA Production System (Promega) and qRT-PCR with a QuantiTect Probe RT-PCR Kit (Qiagen). Each reaction contained 1µg of total RNA, 7.5 pM primers and 5 pM TaqMan probe, and each sample was run in triplicate.

Results

Differential Gene Expression

The selected doses of MC-LR and *Microcystis* caused no significant mortality (< 2%), and no observable behavioral changes in larval zebrafish during the 96-h exposure. However, changes in gene expression were observed after fish were exposed to MC-LR and *Microcystis*. Out of ~15,000 gene transcripts assessed on the zebrafish arrays, there was greater than 99.9% similarity in gene expression between the two controls (ethanol vs. fish water). As such, all treatment comparisons were made relative to the ethanol control. The total number of genes with significant changes in expression (≥1.7-fold change, $p<0.0001$) relative to the control increased with concentration of MC-LR, with 167 significant genes in fish exposed to 100 µg/L MC-LR and 916 significant genes in the 1,000 µg/L MC-LR treatment. Of the 916 differentially regulated genes in the 1,000 µg/L MC-LR treatment, 69 were also differentially expressed in the 100 µg/L MC-LR treatment (Figure 2.1). In larval fish exposed to *Microcystis*, 371 genes were significantly altered compared to control, and 79 of these genes were also differentially regulated in the MC-LR treatments. Of the 371 genes identified in the *Microcystis* treatment, 126 were not differentially expressed in either MC-LR treatment. All data are publicly available at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12214).

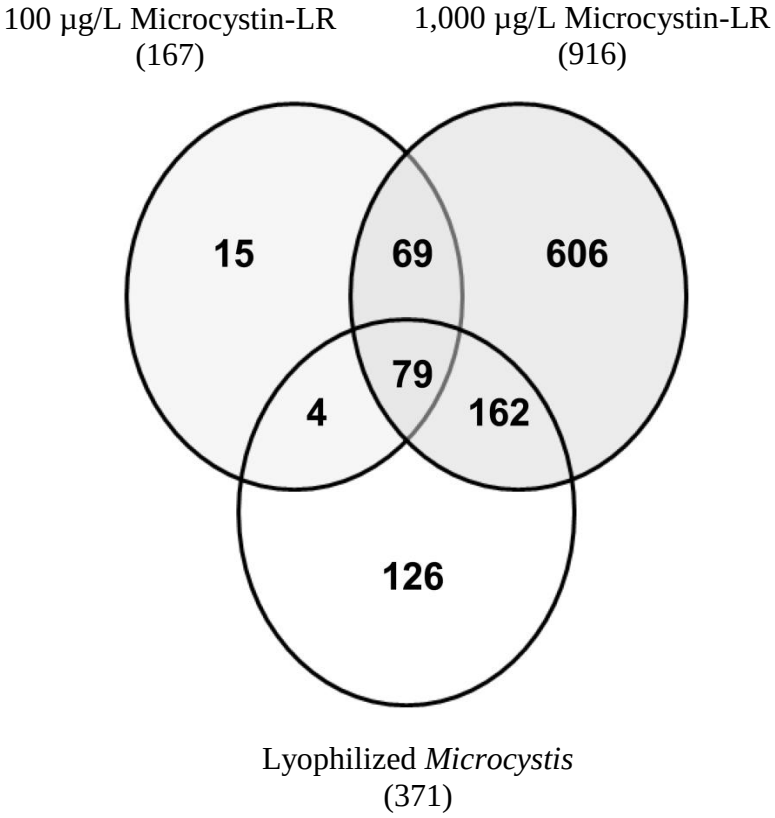

Figure 2.1. Numbers of genes in each treatment that differ significantly from controls (≥1.7-fold change, $p<0.0001$), where numbers in overlapping regions represent genes common to multiple treatments and in non-overlapping regions, genes expressed only in that treatment.

Functional Classification of Genes

Differentially expressed genes were functionally categorized using DAVID ontology analysis. Genes responding to exposure to MC-LR at 100 and 1,000 µg/L (n=69) (Figure 2.1) were related to detoxification and metabolism, lipid binding and transport, cell signaling and development, blood clotting and oxygen transport, ion transport, liver function, maintenance of tight junctions and thermoregulation (Table 2.1). Almost all genes in this group were down-regulated, and fold change values of fatty acid binding protein 1b (-19.9), type iv antifreeze (-14.6), phospholipase a2 (-11.2), fibrinogen b (-6.1), serpin peptidase inhibitor a7 (-4.8), and selenoprotein p1b (-4.5), showed the greatest decrease in expression in the 1,000 µg/L treatment compared to control.

The majority of differentially expressed genes common to all treatments (n=79) (Figure 2.1) were cell signaling and developmental genes including calpain genes, CD9 and CD81 antigens, and an apoptosis inducing factor (Table 2.2). Changes in expression of these genes may indicate altered cell cycle progression, carcinogenesis, cell death, or disruption of cellular differentiation during development. Genes showing the greatest degree of fold change were nephrosin (-4.4 fold change, $p = 2.0 \times 10^{-5}$) and mucin 2 (-4.7 fold change, $p = 2.0 \times 10^{-5}$). Immune-related genes were also affected, as well as those associated with cytoskeletal disruption, ion regulation, oxidative stress, and maintenance of tight junctions. Because these genes were affected by exposure to purified MC-LR and by the cyanobacterium responsible for production of this toxin, this group of genes may represent a response that more closely mimics a natural exposure situation than genes differentially expressed upon exposure to purified MC-LR alone.

Table 2.1. Genes common to 100 and 1,000 µg/L MC-LR treatments relative to control[a]

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Detoxification/ metabolism					
Dr.11596.1.S1_at	AI545537	tryptophan 2,3-dioxygenase	tdo2a	-3.4	-3.4
Dr.3498.1.S1_at	BC045343.1	methionine adenosyltransferase I, alpha	mat1a	-2.4	-3.6
Dr.18453.1.S1_at	BC044525.1	uridine phosphorylase 2	upp2	-2.4	-2.6
Dr.9478.1.S1_at	AB078927.1	cytochrome P450 1A	cyp1a	-2.4	-3.0
Dr.14058.1.A1_at	CD015351	glutathione s-transferase, theta 1A[c]	gstt1a	-2.3	-2.4
Dr.11729.1.S1_at	NM_152954.1	cytochrome P450 2J28	cyp2j28	-2.2	-2.2
Dr.2132.1.A1_at	BQ262149	hydroxyacid oxidase 1	hao1	-2.1	-3.4
Dr.4189.1.S1_at	BI891596	nad(P)H dehydrogenase quinone 1	nqo1	-1.9	-2.6
Dr.7977.1.S1_at	AW232474	glutathione peroxidase 1A	gpx1a	-1.9	-2.6
Dr.25191.1.S1_at	BC046894.1	isocitrate dehyrodgenase 1	idh1	-1.8	-2.5
Dr.7520.1.A1_at	AW019023	aconitase 1	aco1	-1.8	-2.3

Table 2.1. Continued.

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Detoxification/ metabolism					
Dr.1041.1.S1_at	BC050158.1	alpha-L-fucosidase 1	fuca1	-1.7	-2.6
Lipid binding/transport					
Dr.24953.1.S1_at	CD014348	apolipoprotein C-II	apoc2	-4.2	-7.8
Dr.24261.1.S1_at	BM182911	fatty acid-binding protein 1	fabp1b	-3.9	-19.9
Dr.13681.1.S1_at	BI867505	apolipoprotein M	apom	-3.6	-3.6
Dr.1323.1.S1_at	NM_131128.1	apolipoprotein A-I	apoa1	-3.2	-6.0
Dr.4002.1.A1_at	BG884597	apolipoprotein B	apob	-3.1	-9.6
Dr.5488.1.S1_at	AI477980	apolipoprotein A-IV	apoa4	-2.5	-4.7
Dr.5674.2.S1_at	BM186239	apolipoprotein C-I precursor[c]	apoc1l	-1.8	-2.7
Dr.59.1.S1_at	AY178793.1	annexin A1A	anxa1a	-1.8	-2.1
Cell signaling and development					
Dr.20054.1.S1_at	NM_131335.1	gastrulation specific protein	g12	-2.6	-3.3

Table 2.1. Continued.

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Cell signaling and development					
Dr.25140.7.A1_a_at	BQ262802	epithelial cell adhesion molecule	epcam	-1.9	-2.5
Dr.9122.1.S1_at	BM102177	CC chemokine SCYA103[c]	LOC795788	-1.8	-2.4
Dr.7503.1.A1_a_at	AW421072	pituitary tumor-transforming 1 interacting protein[b]	---	-1.8	-2.3
Dr.8149.1.A1_at	NM_131458.1	insulin-like growth factor binding protein 2B	igfbp2b	-1.8	-1.8
Dr.12557.1.A1_at	AW077290	calmodulin-binding transcription activator 1[c]	LOC797322	1.7	1.9
Dr.11457.1.S1_at	BC046887.1	zinc finger and BTB domain containing 16	zbtb16	1.8	2.1
Dr.5462.1.S1_at	BI878927	fibrinogen B	fgb	-2.9	-6.1
Dr.1450.1.S1_s_at	BI896310	hemoglobin alpha embryonic-3	hbae3	-1.9	-2.9
Dr.845.1.A1_at	BG729013	fibrinogen alpha chain	fga	-2.1	-2.7
Liver function					
Dr.8516.1.S1_at	NM_178298.2	selenoprotein P, plasma, 1B	sepp1b	-2.3	-4.5

Table 2.1. Continued.

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Tight junction					
Dr.994.1.S1_at	NM_131763.1	claudin B	cldnb	-1.9	-3.0
Thermoregulation					
Dr.696.1.S1_at	AI496864	type IV antifreeze protein	zgc:161979	-4.8	-14.6
Cytoskeletal function					
Dr.9252.1.A1_at	BE605502	sciellin	scel	-1.8	-2.0
Inflammation					
Dr.15332.1.S1_at	AL917567	phospholipase A2, group IB	pla2g1b	-11.1	-11.2
Muscle contraction					
Dr.20153.1.S1_a_at	AF210639.1	myosin light polypeptide 9 like	myl9l	-1.9	-2.2
Dr.23357.1.A1_at	BE201798	transmembrane protein 90A[c]	LOC569467	-2.2	-4.1
RNA processing					
Dr.24764.1.S1_at	AL727764	cleavage and polyadenylation specific factor 3[d]	hbae1	-1.7	-2.1

Table 2.1. Continued.

Sequence code	Accession number	Description	Gene symbol	Fold change MC-LR vs. control[a] 100 µg/L	1,000 µg/L
Actin binding					
Dr.20115.1.S1_at	BC049463.1	cofilin 1 (non-muscle)	cfl1	-1.8	-2.1
Dr.17687.1.A1_at	BQ078227	IQ motif containing GTPase activating protein 1	iqgap1	-1.8	-2.4
Translation					
Dr.20386.3.S1_at	BM141602	eukaryotic translation initiation factor 1A, X-linked, B	zgc:110087	-1.7	-1.8

[a] ≥ 1.7-fold change, *p<0.0001*

[b]weak similarity to gene indicated

[c]similar to gene indicated

[d]strongly similar to gene indicated

Table 2.2.. Genes common to all treatments relative to control.

Sequence code	Accession number	Description	Gene symbol	Fold Change vs. Control[a] *Microcystis*	MC-LR (µg/L) 100	1,000
Cell signaling and development						
Dr.17470.1.S1_at	AF498291.1	nephrosin	npsn	-4.6	-4.4	-7.4
Dr.914.1.A1_a_at	BE556864	WH2 domain-containing protein 1[d]		-1.8	-2.6	-5.0
Dr.18186.1.S1_at	BQ093694	S100 calcium-binding protein A1[d]	s100a1	-2.1	-2.5	-3.7
Dr.4236.1.S1_at	BQ092511	calpain 9	capn9	-2.2	-2.4	-3.6
Dr.13076.1.S1_at	BC053138.1	pleckstrin homology containing, family F, member 1	plekhf1	-3.0	-2.2	-5.3
Dr.17116.1.S1_at	AF282675.1	calpain 1	capn1	-2.5	-2.2	-3.0
Dr.11420.1.S1_at	BC050238.1	BAI1-associated protein 2-like 1A	baiap2l1a	-1.9	-2.0	-2.6
Dr.2251.1.A1_at	AI793815	golgi integral membrane protein 4A	golim4a	-2.5	-2.0	-3.7
Dr.1945.1.A1_at	CD015541	calpain 2, large subunit like	capn2l	-2.3	-2.0	-3.7
Dr.1116.1.S1_at	BQ092087	S100 calcium binding protein V2	s100v2	-1.8	-1.9	-2.1
Dr.4409.1.S1_at	BC049036.1	CD9 antigen like	cd9l	-1.9	-1.8	-3.1
Dr.11692.1.S1_at	BG727434	vasodilator-stimulated phosphoprotein[b]	vasp	-2.0	-1.8	-2.4
Dr.23066.1.S1_at	AW019779	apoptosis-inducing factor, mitochondrion-associated 2	aifm2	-1.8	-1.7	-2.6
Dr.10664.1.S1_at	NM_131518.1	cd81 antigen	cd81	-2.2	-1.7	-2.3

Table 2.2. Continued.

				Fold Change vs. Control[a]		
				Microcystis	MC-LR (µg/L)	
Sequence code	**Accession number**	**Description**	**Gene symbol**		**100**	**1,000**
Cell signaling and development						
Dr.18888.1.A1_at	BI842184	calcium/calmodulin-dependent protein kinase II delta 2	camk2d2	2.1	1.9	2.4
DrAffx.2.49.A1_at	AW116899	bromodomain containing 4	brd4	2.3	1.9	2.2
Dr.25935.1.A1_at	CD605501	zinc finger, CCHC domain containing 12[c]	zcchc12	1.9	2.0	2.1
Cytoskeleton						
Dr.7105.1.S1_at	BC053229.1	actin related protein 2/3 complex, subunit 1B	arpc1b	-2.5	-2.3	-4.9
Dr.13076.1.S1_at	BC053138.1	pleckstrin homology containing, family F, member 1	plekhf1	-3.0	-2.2	-5.3
Dr.9531.1.A1_at	BQ074417	myosin, heavy chain 9, non-muscle, like-2	myh9l2	-2.0	-2.0	-3.4
Dr.3432.1.S1_at	BC049461.1	capping protein (actin filament), gelsolin-like	capg	-2.2	-1.8	-4.3
Dr.14768.1.A1_at	BI983132	flavoprotein oxidoreductase mical3	mical3	2.8	2.4	2.7
Immune function, haematopoiesis						
Dr.25714.1.A1_at	AW232464	cathepsin S, B.2	ctssb.2	-1.9	-1.9	-4.5
Dr.4409.1.S1_at	BC049036.1	CD9 antigen like	cd9l	-1.9	-1.8	-3.1
Dr.11692.1.S1_at	BG727434	vasodilator-stimulated phosphoprotein[b]	vasp	-2.0	-1.8	-2.4

Table 2.2. Continued.

				Fold Change vs. Control[a]		
				Microcystis	MC-LR (µg/L)	
Sequence code	**Accession number**	**Description**	**Gene symbol**		**100**	**1,000**
Immune function, haematopoiesis						
Dr.10664.1.S1_at	NM_131518.1	cd81 antigen	cd81	-2.2	-1.7	-2.3
Ion regulation, membrane stability						
Dr.922.1.S1_at	BC044188.1	aquaporin 3	aqp3	-2.0	-1.8	-2.5
Dr.1735.1.A1_at	AI721648	chloride intracellular channel 1	clic1	-2.4	-1.8	-3.2
Dr.10467.1.S1_at	NM_131628.1	sodium channel, voltage-gated, type VIII, alpha A	scn8aa	1.7	1.7	2.0
Oxidative stress						
DrAffx.1.74.S1_at	AY216583.1	selenoprotein W2B	sepw2b	-3.0	-2.5	-5.8
Dr.7379.1.A1_at	AW232459	selenoprotein W2B	sepw2b	-1.8	-1.8	-2.6
Dr.17468.1.A1_at	BM956969	glutathione reductase[d]	gsr	-1.7	-1.7	-3.1
Tight junction						
Dr.7692.1.A1_at	BC049304.1	occludin	ocln	-2.2	-1.9	-3.3
Dr. 20610.1.S1_at	NM_131637.1	claudin 7	cldn7	-2.0	-1.9	-2.9
Liver effects						
Dr.8947.2.S1_at	CD594735	Kunitz-type serine protease inhibitor 2[d]	spint2	-3.1	-2.9	-5.6

Table 2.2. Continued.

				Fold Change vs. Control[a]		
				Microcystis	MC-LR (µg/L)	
Sequence code	**Accession number**	**Description**	**Gene symbol**		**100**	**1,000**
Liver effects						
Dr.2408.2.S1_at	BM571242	matrix metalloproteinase 2[c]	mmp2	-2.4	-1.9	-2.6
Endopeptidase inhibitor						
Dr.8947.2.S1_at	CD594735	Kunitz-type protease inhibitor 2[d]	spint2	-3.1	-2.9	-5.6
Dr.3073.1.A1_at	AI585030	serpin peptidase inhibitor clade A, member 7	serpina7	-2.0	-2.5	-6.5
Detoxification						
Dr.16014.1.S1_at	BM024109	glutathione transferase omega 1[b]	gsto1	-2.6	-2.4	-4.9
Thyroid hormone availability						
Dr.3073.1.A1_at	AI585030	serpin peptidase inhibitor, clade A, member 7	serpina7	-2.0	-2.5	-6.5
Steroid hormone synthesis						
Dr. 10671.1.S1_at	NM_131663.1	steroidogenic acute regulatory protein	star	-3.8	-3.3	-6.9
Endosome formation						
Dr.16802.1.S1_at	BC049333.1	vesicle-associated membrane protein 8	vamp8	-2.2	-1.8	-3.1
Intestinal effects						
Dr.14396.2.A1_at	BI673162	mucin 2[d]	muc2	-4.9	-4.7	-6.7

Table 2.2. Continued.

					Fold Change vs. Control[a]		
					Microcystis	MC-LR (µg/L)	
Sequence code	**Accession number**	**Description**		**Gene symbol**		**100**	**1,000**
Bacterial pathogenesis							
Dr.1991.1.A1_at	BM529391	globoside 1, like 4	alpha-1,3-N-acetylgalactosaminyltransferase	gbgt1l4	-2.2	-1.7	-2.5

[a] ≥ 1.7-fold change, *p<0.0001*

[b]weak similarity to gene indicated;

[c]moderate similarity to gene indicated;

[d]similar to gene indicated

Also of interest were genes that responded when larval zebrafish were exposed to *Microcystis* but not the purified MC-LR treatments. The majority of genes expressed only in the *Microcystis* treatment (n=126) (Figure 2.1) were genes involved in cell signaling and development (28%), neurological function (7%), visual perception (6%), and endocrine activity (6%) (Table 2.3). Other genes included those affecting ion regulation, apoptosis, glucose and amino acid transport, muscle contraction, DNA synthesis and degradation, intestinal function, metabolism, and protein targeting. The majority of genes were down-regulated, some genes related to cell signaling development, neurological function and visual perception were up regulated.

Vitellogenin Expression and Validation by qRT-PCR

Affymetrix probe sets designed for zebrafish vitellogenin genes indicated high up-regulation (19 to >100-fold change) of these genes in larval zebrafish exposed to *Microcystis* (Table 2.4). In larval zebrafish exposed to MC-LR, there was no significant effect on expression of any of the vitellogenin genes relative to the control. Up-regulation of vitellogenin was confirmed by qRT-PCR of *vtg1A/B* (mean fold-change ±SD = 619.3±130.2 (n=9))

Table 2.3. Genes unique to *Microcystis* treatment[a]

Sequence code	Accession number	Description	Gene symbol	[a]Fold change	p-Value
Cell signaling and development					
Dr.15260.1.S1_at	BI429195	nuclear receptor subfamily 1, group D, member 2B	nr1d2b	-3.4	6.03E-07
Dr.20567.1.S1_at	AW567115	calcium binding protein 39-like	cab39l	-2.9	1.48E-06
Dr.26399.1.A1_at	AL719041	PAR-6 gamma protein[b]	pard6gb	-2.8	4.43E-06
Dr.25140.8.A1_at	AL730238	CD81 antigen	cd81	-2.8	1.36E-09
Dr.12138.2.A1_at	AI957874	exostoses 1A	ext1a	-2.7	2.00E-05
Dr.18414.1.S1_at	AW165356	programmed cell death 4A	pdcd4a	-2.7	2.00E-05
Dr.19421.1.A1_at	AL725987	PHD finger protein 8	phf8	-2.4	6.54E-06
Dr.11726.1.S1_at	NM_131877.1	caspase 3	casp3	-2.4	3.00E-05
Dr.7424.2.S1_at	BI839632	COP9 constitutive photomorphogenic homolog subunit 5	cops5	-2.3	4.44E-06
Dr.3282.1.S1_at	NM_131691.1	endothelial differentiation sphingolipid G-protein-coupled receptor 1	edg1	-2.3	8.63E-06
Dr.25322.1.S1_at	AL726472	lin-7 homolog C	lin7c	-2.2	2.71E-08
Dr.13009.2.S1_at	BE556991	sprouty homolog 2	spry2	-2.2	4.48E-06
Dr.1710.2.S1_at	AI794095	eukaryotic translation initiation factor 4e family 3	eif4e3	-2.1	7.67E-08
Dr.7804.1.S1_at	AB069858.1	antizyme inhibitor 1	azin1	-2.1	3.00E-05
Dr.6932.3.S1_at	AL730217	high-mobility group box 3A	hmgb3a	-2.0	2.07E-11

Table 2.3. Continued.

Sequence code	Accession number	Description	Gene symbol	[a]Fold change	p-Value
Cell signaling and development					
Dr.15361.1.S1_at	AL926178	moderate similarity to ras-related protein rab-22A	rab22a	-2.0	2.60E-06
Dr.11764.1.S1_at	NM_131774.1	annexin A13	anxa13	-2.0	1.00E-05
Dr.7100.1.A1_at	BQ091992	immediate early response 2[b]	ier2	-1.9	9.07E-10
Dr.11698.1.S1_at	AL725759	transcription factor AP-2 gamma	tfap2c	-1.9	1.51E-09
Dr.16550.1.A1_at	BI709565	TM2 domain-containing 2	tm2d2	-1.9	1.82E-09
Dr.24242.2.S1_at	BQ131454	mitochondrial processing peptidase alpha[c]	pmpca	-1.9	3.11E-08
Dr.578.2.S1_a_at	U96848.1	thyrotroph embryonic factor	tef	-1.9	7.04E-08
Dr.5572.1.S1_at	NM_131101.1	homeo box B5A	hoxb5a	-1.8	6.73E-10
Dr.3238.1.A1_at	AI793363	Kruppel-like factor 11A	klf11a	-1.8	9.45E-09
Dr.7225.1.S1_at	BC045952.1	MOB1, Mps one binder kinase activator-like 1A (yeast)	mobkl1a	-1.7	4.88E-06
Dr.23293.1.A1_at	BE016153	tubulin polymerization-promoting protein family member 3	tppp3	-1.7	4.00E-05
Dr.26344.2.S1_a_at	AL717083	cell division cycle 42	cdc42	-1.7	6.00E-05
Dr.3472.1.A1_at	AI545021	spectrin, beta, non-erythrocytic 1	sptbn1	1.7	7.95E-13
Dr.15630.1.S1_at	CD594794	similar to tubulin folding cofactor E-like	tbcel	1.7	2.77E-06
DrAffx.1.10.S1_at	AY151045.1	cysteine rich transmembrane BMP regulator 1	crim1	1.8	1.82E-07
Dr.3421.1.A1_at	AW342746	ribosome binding protein 1 homolog (dog)	rrbp1	1.8	6.00E-05

Table 2.3. Continued.

Sequence code	Accession number	Description	Gene symbol	[a]Fold change	p-Value
Cell signaling and development					
Dr.12403.1.S1_at	NM_131633.1	roundabout homolog 2	robo2	1.9	2.46E-07
Dr.6210.1.S1_at	BM184670	cartilage acidic protein 1[b]	crtac1	2.0	1.91E-11
Dr.15261.1.A1_at	BI710394	cytoplasmic polyadenylation element binding protein 4	cpeb4	2.0	6.00E-05
Dr.52.1.A1_at	AA495026	phosphatidylinositol binding clathrin assembly protein, like	picalml	2.2	3.51E-06
Neurological function					
Dr.17557.1.S1_at	AL730871	neurocalcin delta[d]	ncald	-2.7	1.53E-14
Dr.24196.1.S1_at	NM_131452.1	embryonic lethal abnormal vision-like 1	elavl1	-2.1	4.11E-10
Dr.4230.1.S1_a_at	NM_130909.1	HuG	hug	-2.0	5.79E-17
Dr.1968.1.S1_at	BC049308.1	fusion involved in malignant liposarcoma	fus	-1.8	6.11E-09
Dr.25322.2.S1_at	BI888421	lin-7 homolog C	lin7c	-1.7	1.94E-13
DrAffx.1.10.S1_at	AY151045.1	cysteine rich transmembrane BMP regulator 1	crim1	1.8	1.82E-07
Dr.12617.1.A1_at	NM_131806.1	ephrin B3	efnb3	1.8	1.00E-05
Dr.12403.1.S1_at	NM_131633.1	roundabout homolog 2	robo2	1.9	2.46E-07
Dr.23350.1.S1_at	AF425739.1	parvalbumin 8	pvalb8	4.7	2.00E-05
Visual perception					
AFFX-Dr-NM_131175-1_s_at	NM_131175-1	opsin 1, long-wavelength-sensitive 1	opn1lw1	-3.0	4.92E-13

Table 2.3. Continued.

Sequence code	Accession number	Description	Gene symbol	[a]Fold change	p-Value
Visual perception					
Dr.26436.1.S1_at	AF210644.1	retinal degradation slow 4	rds4	-2.6	1.83E-11
Dr.12204.1.S1_at	BI706778	retinal degradation slow 4	rds4	-2.3	3.00E-05
Dr.8102.1.S1_at	NM_131253.1	opsin, medium-wavelength-sensitive 1	opn1mw1	-2.0	1.38E-08
Dr.9899.1.S1_at	NM_131868.1	guanine nucleotide binding protein, alpha transducing activity 1	gnat1	-1.9	1.08E-09
Dr.10433.1.S1_at	NM_131791.1	cryptochrome 2A	cry2a	-1.9	5.00E-05
Dr.8194.1.S1_at	BI879950	opsin, short-wavelength-sensitive 1	opn1sw1	-1.8	1.20E-08
Dr.8097.1.S1_at	NM_131192.1	opsin, short-wavelength-sensitive 2	opn1sw2	-1.7	1.65E-07
Endocrine activity					
Dr.10788.1.S1_at	NM_131804.1	nothepsin	nots	4.0	1.02E-18
Dr.10461.1.S1_at	NM_131642.1	cytochrome P450, family 19, subfamily A, polypeptide 1B	cyp19a1b	4.0	6.68E-08
Dr.25009.6.A1_at	BI878405	vitellogenin[d]	vtg	>100	<1.00E-45
Dr.25009.1.S1_a_at	NM_170767.1	vitellogenin 1	vtg1	>100	<1.00E-45
Dr.25009.6.A1_a_at	BI878405	vitellogenin 1	vtg1	>100	<1.00E-45
Dr.25009.4.A1_at	BG303658	vitellogenin 2	vtg2	>100	<1.00E-45
Dr.2978.1.S1_at	AI477604	vitellogenin 3	vtg3	19.2	<1.00E-45

Table 2.3. Continued.

Sequence code	Accession number	Description	Gene symbol	[a]Fold change	p-Value
Ion regulation					
Dr.3.1.S1_at	AF469651.1	ATPase, Na+/K+ transporting, beta 3A	atp1b3a	-2.2	4.44E-11
Dr.26437.1.S1_at	NM_131669.1	ATPase, Na+/K+ transporting, beta 2A	atp1b2a	-2.0	5.40E-07
Dr.3613.1.S1_at	BC048037.1	ceruloplasmin	cp	2.4	1.73E-09
Apoptosis					
Dr.18414.1.S1_at	AW165356	programmed cell death 4A	pdcd4a	-2.7	2.00E-05
Dr.11726.1.S1_at	NM_131877.1	caspase 3	casp3	-2.4	3.00E-05
Glucose transport					
Dr.17415.1.S1_at	BC050518.1	insulin receptor substrate 1B[d]	irs1b	-2.4	1.86E-07
Amino acid transport					
Dr.7516.1.A1_at	BM095174	solute carrier family 38, member 4	slc38a4	2.1	3.45E-12
Muscle contraction					
Dr.23350.1.S1_at	AF425739.1	parvalbumin 8	pvalb8	4.7	2.00E-05
DNA synthesis/degradation					
Dr.1691.1.S1_at	NM_131450.1	ribonucleotide reductase protein R2 class I	rrm2	-2.3	1.98E-06
Dr.1668.1.S1_at	BC046003.1	hypoxanthine phosphoribosyltransferase 1	hprt1	-1.8	2.90E-06

Table 2.3. Continued.

Sequence code	Accession number	Description	Gene symbol	[a]Fold change	p-Value
Intestinal function					
Dr.18599.1.S1_at	BQ479899	fatty acid binding protein 6	fabp6	1.9	3.81E-06
Metabolism					
Dr.14021.3.A1_at	BM095392	3-hydroxyisobutyrate dehydrogenase B	hibadhb	-2.0	2.55E-16
Protein targeting					
Dr.3075.1.S1_at	BC049337.1	translocase of outer mitochondrial membrane 34	tomm34	-1.8	1.00E-05

[a] $\geq$ 1.7-fold change, $p<0.0001$

[b]weak similarity to gene indicated

[c]moderate similarity to gene indicated

[d]similar to gene indicated

Table 2.4. Expression of vitellogenin genes relative to control and validation by qRT-PCR

				100 µg/L MC-LR		1,000 µg/L MC-LR		*Microcystis*	
				Fold change	p-Value	Fold change	p-Value	Fold change	p-Value
Microarray									
Dr.2978.1.S1_at	AI477604	vitellogenin 3	*vtg3*	1.1	0.51	1.3	0.05	19.2*	<1.00E-45*
Dr.25009.6.A1_at	BI878405	similar to vitellogenin	*vtg*	1.2	0.80	1.1	0.91	>100*	<1.00E-45*
Dr.25009.6.A1_a_at	BI878405	vitellogenin 1	*vtg1*	-1.2	0.80	1.5	0.38	>100*	<1.00E-45*
Dr.25009.4.A1_at	BG303658	vitellogenin 2	*vtg2*	-1.5	0.48	-1.6	0.30	>100*	<1.00E-45*
Dr.25009.1.S1_a_at	NM_170767.1	vitellogenin 1	*vtg1*	-1.5	0.34	-1.3	0.63	>100*	<1.00E-45*
qRT-PCR									
	NM_170767.1	vitellogenin type 1	*Vtg1A/B*					619.3±130.2[a]	

*significance determined by ≥ 1.7-fold change, $p<0.0001$

[a] mean ±SD (n=9); primer/probe sets for vitellogenin type 1 as described in Henry et al. 2009

Discussion

Gene expression results confirmed known mechanisms of action for MC-LR and also identified effects not previously associated with this toxin. When larval fish were exposed to both MC-LR and *Microcystis* treatments, the top functional categories affected (cell signaling and development, cytoskeleton, immune function, ion regulation, oxidative stress, tight junction, liver effects) were associated with effects shown previously for purified MC-LR exposure in fish and mammals (Fishcer et al. 2000; Gaete et al. 1994; Zambrano and Canelo 1996; Malbrouck and Kestemont 2006; Wang et al. 2005; Guzman and Solter 1999; Janssens and Goris 2001; Rymuszka et al. 2008; Toivola and Eriksson 1999) (Table 2.2). Because these genes were also affected in the *Microcystis* treatment in the present study, this is consistent with the presence of MC-LR toxin in the *Microcystis* treatment (i.e., MC-LR was generated by the *Microcystis* cells). MC-LR is known to affect immune response; however, there were few similarities between our work and previous gene expression studies that mainly focused on the response of immune-related genes in zebrafish exposed to purified MC-LR. Of the eleven genes tested by qPCR and found to be up-regulated in zebrafish by Li et al. (Li et al. 2009), none were differentially expressed in our 100 µg/L MC-LR or *Microcystis* treatments, but three heat shock protein genes were significantly down-regulated (fold change -2.2 to -1.9) at 1,000 µg/L MC-LR. When compared to the results of a microarray study by Wei et al. (2008), in which adult zebrafish were exposed to MC-LR by intraperitoneal injection, five genes reported by Wei et al. to be up-regulated were significantly down-regulated (fold change -4.2 to -1.8) in the 1,000 µg/L MC-LR treatment, and three of these same five genes were down-regulated (fold change -2.2 to -1.9) in

the *Microcystis* treatment. Variation between our work and these studies may be attributed to differences in life stage of the fish, MC-LR concentration, and/or exposure route.

Also when examining genes differentially expressed in both 100 and 1,000 µg/L MC-LR (but not the *Microcystis* treatment), different effects emerge other than those typically associated with MC-LR. There were additional cell signaling effects not affected by *Microcystis* (5 genes) (Table 2.1), however the majority of genes affected by both concentrations of MC-LR tested were related to detoxification/metabolism and lipid binding/transport. Detoxification/metabolism genes included glutathione-s-transferase, an enzyme involved in detoxification of MC-LR (Pflugmacher et al. 1998), as well as cytochrome p450 enzymes which are typically not associated with microcystin exposure. Lipid transport metabolism genes mainly included apolipoproteins, which are known to influence nutrition and immune response in fish (Concha et al. 2004). In addition, strong down-regulation of type IV antifreeze protein (-14.6 fold) may indicate that microcystin-LR may inhibit thermoregulation in fish, which is an effect that has not previously been associated with this compound. It is also interesting that significant expression of PP genes only occurred in exposures of 1,000 µg/L and not 100 µg/L, and these genes were also not differentially expressed in larval zebrafish exposed to *Microcystis*. This implies that larval fish were either able to compensate when exposed to low concentrations of MC-LR, or that PP enzymes may not be as responsive at this stage in development and a higher dose may be required for inhibition to occur. The absence of this major biomarker of MC-LR toxicity in larval fish exposed to *Microcystis* is also interesting, because it implies that MC-LR may not be the most important compound affecting fish when they are exposed to the cyanobacteria.

In addition to understanding how effects of the microcystin toxin differ when larval fish are exposed to the purified compound versus toxin contained in *Microcystis* cells, it was also our objective to clarify effects of *Microcystis* exposure in addition to those associated with the toxin. Although an investigation has been conducted with adult zebrafish exposed to MC-LR (Wei et al. 2008), the present study is the first to examine global gene expression changes in fish exposed to *Microcystis* cells. Similar to MC-LR treatment, cell signaling genes were affected by exposure to *Microcystis*; however the set of cell signaling genes differentially expressed were unique to the *Microcystis* exposure. This suggests that there are additional cell signaling effects that occur when fish are exposed to *Microcystis* other than those associated with the toxin. Most of the other gene function categories affected exclusively by *Microcystis* exposure were different from those associated with MC-LR. Several genes related to neurological development and/or function were differentially expressed, including ephrin b3 (fold change 1.8), a gene in which overexpression has been associated with notochord defects in zebrafish (Chan et al. 2001) and neurocalcin delta (-2.7 fold change), a neuronal calcium binding protein involved in visual transduction in the retina (Krishnan et al. 2004). Alteration in the regulation of these genes suggests that *Microcystis* may interfere with proper nervous system development in larval fish. Similarly, several genes involved in visual perception, including opsin genes were down-regulated, suggesting that development of eye structures may not proceed correctly- an effect that may affect fish at later life stages and interfere with the ability to regulate circadian rhythm, locate food, and avoid predation.

The most highly up-regulated genes were those coding for vitellogenin and this change in gene expression was observed only after zebrafish were exposed to the *Microcystis* treatment.

Vitellogenins are a group of lipoproteins produced in the liver in response to estrogen and are transported through the blood and deposited in the developing oocytes of female fish (Routledge et al. 1998). These vitellogenin genes are present in adult and larval fish, but are expressed only at low levels unless exposure to exogenous estrogen has occurred, and thus up-regulation of *vtg* in male and immature fish has become a biomarker of exposure to environmental estrogens (Sumpter and Jobling 1995). The induction of vitellogenin in fish or other effects associated with exposure to environmental estrogens in any organism have not been reported previously in the context of exposure to *Microcystis*. Recently, a low-level estrogenic response was observed in a human breast carcinoma cell line when cells were exposed to purified MC-LR (Oziol and Bouaïcha 2010). Our results, conversely, did not show vitellogenin induction in fish exposed to purified MC-LR, which indicates that the estrogen receptor-mediated induction of *vtg* in zebrafish was not activated by this toxin and that the estrogenic response observed in the human cell line may act by a different mechanism. Further evaluation of the human cell line with other substances (i.e., other than MC-LR) generated by *Microcystis* is warranted to determine the relative estrogenic potential of these substances and validate their estrogenicity in a model separate from zebrafish. Phytoestrogens are compounds identified in plants that can induce vitellogenin (Latonnelle et al. 2002; Inudo et al. 2004); and it is possible that the substances(s) produced by *Microcystis* that caused induction of *vtg* in this study are similar to phytoestrogens.

The possibility that *Microcystis* blooms may release estrogenic substances (aka "phycoestrogens") that can interfere with reproduction is of considerable environmental interest. Because compounds that induce vitellogenin in fish are generally able to do so in other species (Sumpter and Jobling 1995), endocrine disruption from *Microcystis* could extend throughout

aquatic ecosystems and also impact the terrestrial environment, including birds and mammals. Human exposure to *Microcystis* has long been a concern due to the microcystin toxin, and most monitoring programs are designed to evaluate presence of this toxin; however, the potential for endocrine disruption to occur that is unrelated to the presence of the toxin suggests that monitoring programs may need to be re-evaluated. Projected global increase in frequency of *Microcystis* blooms (Paerl and Huisman 2008) and the potential for estrogenic effects adds to the environmental and public health concerns related to bloom events.

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SECTION III

Reproductive effects of *Microcystis* exposure in zebrafish (*Danio rerio*)

This section is a version of a journal article for planned submission to *Environmental Toxicology and Chemistry*. My contribution to the paper was designing the experiment and supervising the exposure of zebrafish to *Microcystis*. I was also responsible for reviewing histology slides, analyzing data, and writing the paper.

Rogers, Emily D., Ann N. Wells, June-Woo Park, Theodore B. Henry, Gary S. Sayler, and Steven W. Wilhelm. In preparation. Reproductive effects of *Microcystis* exposure in zebrafish (*Danio rerio*).

Introduction

Microcystis blooms occur throughout the world and are a major ecological and public health concern (Codd et al. 2005; Luckas et al. 2005). *Microcystis* is a genus of cyanobacteria that produces a diverse group of toxins called microcystins, each differing slightly in chemical structure. Of the >80 forms of microcystins that have been identified, microcystin-LR (MC-LR) is generally considered to be most toxic (Dittmann and Wiegand 2006; Tanabe et al. 2004) and concentrations in surface waters frequently exceed the World Health Organization advisory level of 1 µg/L (Chorus et al. 2000). Concerns have been raised about the potential for MC-LR to bioaccumulate in fish tissues (Xie et al. 2005), and fish kills have been observed in areas where *Microcystis* was present (Chorus et al. 2000). Because fish are vital components of aquatic ecosystems and have considerable economic value in terms of commercial and recreational fishing, it is important to better understand the effects of *Microcystis* blooms on fish populations.

The mechanisms of MC-LR toxicity and detoxification in fish are believed to be similar to those reported in mammals. The liver is the primary organ affected (Fischer et al. 2000), and a primary mechanism of MC-LR toxicity is inhibition of protein phosphatases (PPs) 1 and 2A (Fujiki and Suganuma 1993). Inhibition of PPs results in over-phosphorylation of cytokeratins (Eriksson et al. 1990; Ohta et al. 1992), resulting in cytoskeletal rearrangement and compromised liver function, including necrosis, apoptosis and intrahepatic hemorrhage (Fischer and Dietrich 2000; Fischer et al. 2000). Detoxification of MC occurs in the liver via glutathione conjugation catalyzed by glutathione-S-transferase (Pflugmacher et al. 1998), subsequently resulting in biliary excretion (Sahin et al. 1996).

Because fish living in environments affected by *Microcystis* blooms are exposed throughout the life cycle, there is potential for *Microcystis* to affect fish reproduction and larval survival. Information about potential reproductive effects of microcystin exposure in fish is mainly limited to microinjection studies. When embryos were exposed to MC-LR by microinjection, hatching was accelerated (Jacquet et al. 2004), survival decreased (Jacquet et al. 2004; Wang et al. 2005), and skeletal malformations (Wang et al. 2005) as well as damage to the liver (Huynh-Delerme et al. 2005; Jacquet et al. 2004) were observed. Injection of microcystin into fish embryos was conducted to simulate maternal transfer; however adult exposure to microcystin and subsequent accumulation of the toxin in eggs has not been documented, which makes it difficult to draw conclusions about the relevance of results of microcystin exposure *in situ* based on microinjection studies.

The only assessment of reproductive effects in fish as a result of adult exposure to microcystin was conducted by Deng et al. (2010). In that study MC-LR was incorporated into fish food at concentrations ranging from 0.46-3.93 µg/g and administered to Japanese medaka at 5% body weight twice per day for 8 weeks. After 4 weeks of exposure, male and female fish from corresponding treatments were allowed to spawn. Embryos were transferred to clean water where they were monitored for survival and adults were returned to exposure tanks. Embryo survival was significantly reduced at the highest concentration tested, and effects on adults included reduced growth following spawning, inhibition of protein phosphatases in the liver, and hepatic lesions (Deng et al. 2010). The effects of MC-LR or *Microcystis* on reproduction after aqueous exposure in fish have not been evaluated.

The objective of this study was to evaluate reproductive effects as a result of aqueous exposure of adult fish to *Microcystis* at environmentally relevant concentrations. We predicted that adult fitness and reproductive ability, as well as survival of offspring would be negatively affected by *Microcystis* exposure.

Materials and Methods

Experimental Fish

Adult zebrafish (*Danio rerio*) were obtained from the Zebrafish Research Facility in the Center for Environmental Biotechnology at the University of Tennessee. Fish husbandry, spawning, and experimental procedures were conducted with approval from the University of Tennessee Institutional Animal Care and Use Committee (Protocol #1690-1007). Water for holding fish and conducting experiments (hereafter referred to as "fish water") consisted of MilliQ water (Millipore, Bedford, MA) with ions added: 19 mg/L $NaHCO_3$, 1 mg/L sea salt (Instant Ocean Synthetic Sea Salt, Mentor, OH), 10 mg/L $CaSO_4$, 10 mg/L $MgSO_4$, 2 mg/L KCl. Water quality measurements were recorded daily (dissolved oxygen, pH, ammonia) and weekly (conductivity, hardness, alkalinity) prior and during exposure periods and water had the following characteristics: dissolved oxygen (5.6-6.5 mg/L), pH (6.5-6.9), total alkalinity (34-42 mg/L as $CaCO_3$), total hardness (17-21 mg/L as $CaCO_3$), and ammonia (<0.2 mg/L). All activities (maintenance of adult fish, spawning, and experiments) were conducted in an environmental chamber with a temperature of 27± 1 ºC and 14:10 h light:dark photoperiod.

Spawning Procedure During Baseline and Exposure Periods

Adult zebrafish were separated by sex and housed in 7-L tanks containing 6 L of fish water. There were six tanks per treatment (three tanks per sex), each containing 5 fish (Fig. 3.1). For six weeks prior to *Microcystis* exposure (baseline period) both treatment and control fish were housed in fish water and weekly pair spawning was conducted in order to establish

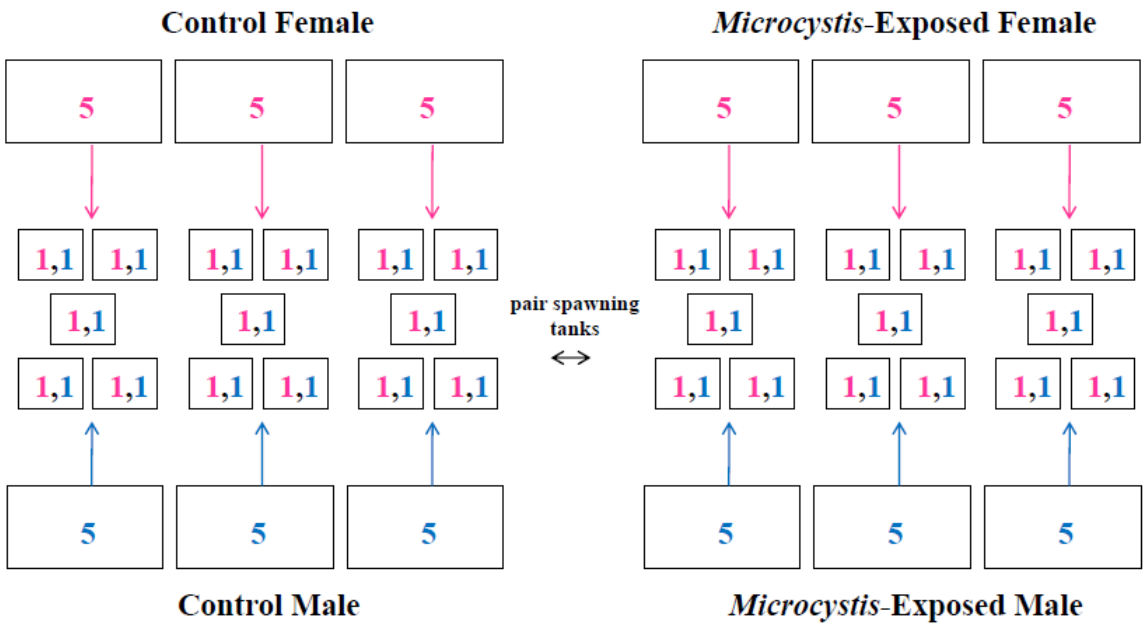

Figure 3.1. Set-up for weekly pair spawning during baseline and exposure periods. Numbers in blue and pink represent male and female fish, respectively. Numbers in large boxes represent numbers of fish in each treatment tank, while numbers in small boxes represent numbers of fish in each spawning tank.

consistent egg production and determine baseline fecundity. Spawning pairs were created by selecting at random one female and one male from replicate tanks having the same tank number (i.e.. female from control tank 1 + male from control tank 1), and fish were not paired across treatments at any time. Fish were paired in this fashion until there were no fish remaining (n = 15 spawning pairs/treatment). Fish were paired the evening before by placing a male and a female fish in purpose built spawning chambers (polycarbonate tanks with a false bottom for egg collection and a partition to separate male and female fish). The partition separating male from female fish was removed just before onset of the photoperiod the following morning and paired fish were given the opportunity to spawn for one hour before adults were placed back in their respective tanks.

Egg Collection and Quantification Procedure During Baseline and Exposure Periods

Clutches from individual spawning pairs were collected after each weekly spawning event during baseline and exposure periods and placed in separate petri dishes containing egg water (60 µg/mL sea salt solution). Unfertilized embryos were removed and each clutch was photographed with a digital camera for embryo quantification. Solutions were changed daily and dead embryos removed and noted. At 120 h (after hatching) photographs were taken to quantify the number of surviving larvae. Numbers of embryos and surviving larvae for each spawning pair were determined by counting the number of individuals in each digital image.

Preparation of Lyophilized Microcystis

The *Microcystis* treatment was prepared using lyophilized cells of *Microcystis aeruginosa*. *M. aeruginosa* PCC-7806 was obtained from the Pasteur Culture Collection of Cyanobacteria and cultured in BG-11 media according to the same methods described for *M. aeurginosa* LE-3 isolates in Rinta-Kanto and Wilhelm (2006). Live cultures of *M. aeruginosa* grown over the course of one year were centrifuged in 250 mL batches at 3,500 rpm for one hour to concentrate cells into a pellet, and pellets were immediately lyophilized for 48 hours using a freeze-dry system (Labconco, Kansas City, MO). After 55 L of *Microcystis* cultures had been lyophilized, individual pellets were combined, and the total mass of lyophilized algal cells was 1096 mg (20 mg lyophilized cells/L).

Exposure

Following the 6-wk baseline spawning period, all fish were anaesthetized in MS-222, weighed and measured, returned to tanks, and lyophilized *Microcystis* (20 mg/L) was added to treatment tanks. Fish were exposed for three weeks (exposure period), and control and *Microcystis* solutions were renewed weekly. Pair spawning and egg collection and quantification occurred weekly as described above (Fig 3.1). Adult fish were observed daily for mortalities and abnormal behavior. At the end of the 3-wk exposure period, adult fish were euthanized by overdose of MS-222 and weight and length were recorded. Liver tissue was collected from two fish per tank (n = 12/treatment) and preserved in Bouin's fixative for histopathological analysis (Figure 3.2).

Histopathology

After tissue fixation in Bouin's fixative (24-36 h) liver samples were transferred into 70% EtOH and subsequently processed and embedded in paraffin for routine histology. Sections (0.6 µm thick) were stained with hematoxylin and eosin and all sections were examined by light microscopy.

Statistical Analyses

Significant differences between control and treatment groups were determined by 1-way ANOVA. Percentage data (spawning success, % survival) was arcsine square root transformed prior to analysis. All statistical analyses were conducted with ToxStat 3.4© (Cheyenne, Wyoming) and a probability level of $P<0.05$ was used as the level for statistical significance.

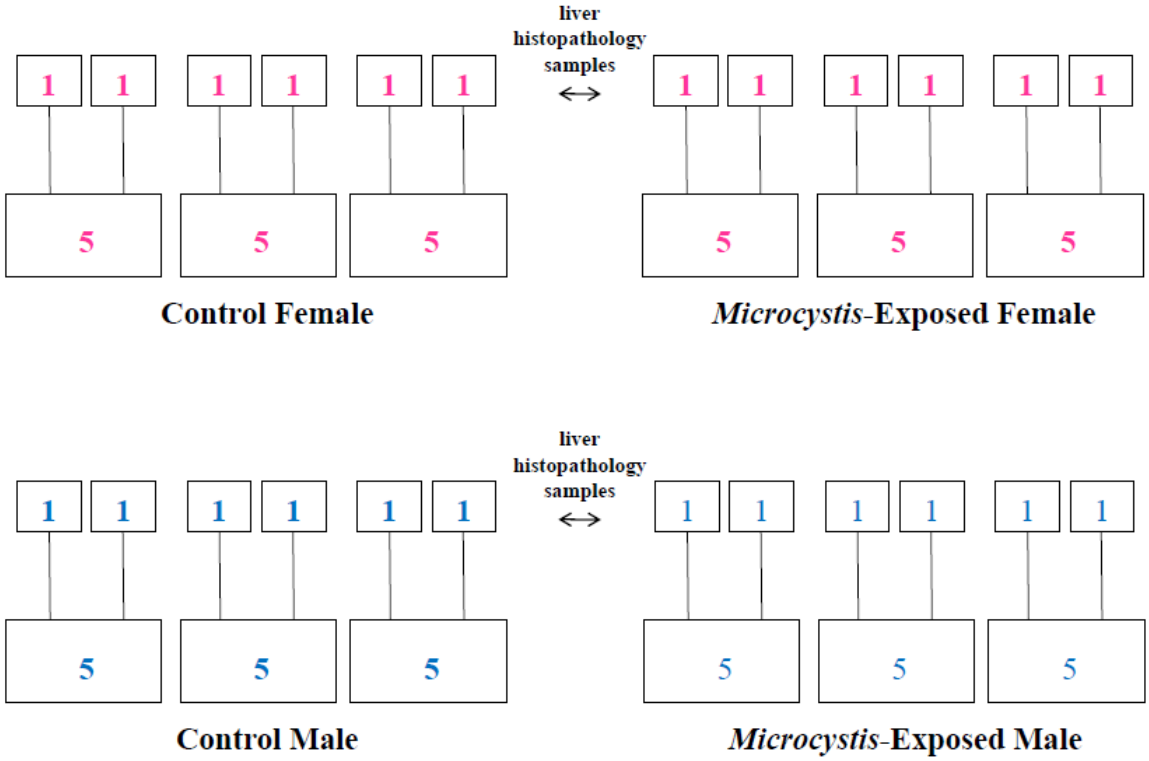

Figure 3.2. Sampling scheme for liver histopathology samples collected at the end of the exposure period. Numbers in blue and pink represent male and female fish, respectively. Numbers in large boxes represent numbers of fish in each treatment tank, while numbers in small boxes represent numbers of fish sacrificed for liver histopathology.

Results

Spawning was monitored for 6 weeks prior to exposure to give fish the opportunity to acclimate and to establish spawning consistency. During this baseline period, spawning success (percentage of pairs that spawned each week) and fecundity (number of embryos produced by each spawning pair) increased over time and appeared to stabilize during weeks 4 through 6 (Tables 3.1 and 3.2). Survival of larvae after spawning was greater than 80% by week 3 and was maintained throughout the baseline period (Table 3.3). There were also no significant differences in spawning success, fecundity or larval survival among control tanks and those that would ultimately receive *Microcystis*.

The same data were collected during the three week exposure to *Microcystis*. Spawning success of zebrafish exposed to *Microcystis* was significantly lower than controls and this effect was consistent throughout the exposure period (Table 3.4). There was no significant difference between treatments in the number of eggs produced per spawning pair (Table 3.2). The survival of larvae at 120-h post-fertilization was high (80-90%) in offspring of both control and *Microcystis*-exposed fish and there was no significant difference between treatments.

There were no significant effects observed in adult zebrafish exposed to *Microcystis*. Weight and length measurements recorded at the beginning and end of the exposure period showed no significant difference between control and treatment groups (Figs. 3.3 and 3.4). One female fish in the *Microcystis* treatment died during the first week of exposure; no other mortalities occurred. Liver histopathology was similar between control and *Microcystis*-exposed

Table 3.1. Spawning success during the baseline period.

Week	Control	*Microcystis*
1	46.7	46.7
2	53.3	40.0
3	53.3	20.0
4	60	53.3
5	73.3	53.3
6	66.7	53.3
Mean	58.9	44.4
SD	9.8	13.1
F	4.57	
p	0.06	

Data are weekly percentages calculated as the number of successful spawning pairs in relation to the total number of spawning pairs (n = 15). SD = standard deviation; F = 1-way ANOVA F statistic; p = p value. Statistics presented were performed on arcsine square root-transformed data.

Table 3.2. Fecundity during baseline and exposure periods.

	Baseline Period						Exposure Period		
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 1**	**Week 2**	**Week 3**
Control[a]	57 ± 72	95 ± 134	117 ± 169	119 ± 134	164 ± 150	142 ± 128	228 ± 128	230 ± 131	186 ± 142
Microcystis[b]	65 ± 94	76 ± 138	61 ± 128	152 ± 166	142 ± 175	147 ± 171	196 ± 176	175 ± 142	287 ± 270
F	0.08	0.15	1.07	0.36	0.14	0.01	0.30	1.18	1.63
p	0.78	0.70	0.31	0.55	0.71	0.92	0.59	0.29	0.21

Fecundity is defined as the mean number of embryos produced per spawning pair and only includes data for pairs that spawned successfully. Data are means ± standard deviation. F = 1-way ANOVA F statistic; p = p value; [a]n = 15 during baseline and exposure periods; [b]n = 15 during baseline period, n = 14 during exposure period.

Table 3.3. Larval survival at 120-h during baseline and exposure periods.

	Baseline Period												Exposure Period					
	Week 1	**n**	**Week 2**	**n**	**Week 3**	**n**	**Week 4**	**n**	**Week 5**	**n**	**Week 6**	**n**	**Week 1**	**n**	**Week 2**	**n**	**Week 3**	**n**
Control	49.2 ± 33	7	93.1 ± 9	8	79.7 ± 30	8	79.6 ± 37	9	85.7 ± 17	11	88.2 ± 11	10	85.0 ± 16	13	88.5 ± 19	13	90.4 ± 18	11
Microcystis	77.1 ± 31	7	73.5 ± 26	6	86.9 ± 7	3	86.7 ± 19	8	83.6 ± 12	8	78.3 ± 12	8	94.6 ± 5	9	88.6 ± 8	9	80.3 ± 17	9
F	3.30		2.37		0.01		0.02		0.15		4.26		2.95		0.20		2.87	
p	0.09		0.15		0.91		0.88		0.70		0.06		0.10		0.66		0.11	

Data are means ± standard deviation of individual percentages calculated as % of larvae alive at 120-h in relation to the number of eggs collected at spawning. F = 1-way ANOVA F statistic; p = p value. Statistics presented were performed on arcsine square root-transformed data.

Table 3.4. The number (percent) of paired fish that spawned at each opportunity during the exposure period.

Week	Control	*Microcystis*
1	86.7	64.3
2	86.7	64.3
3	73.3	64.3
Mean	82.2	64.3*
SD	7.7	0.0
F	20.47	
p	0.01	

Data are weekly percentages calculated as the number of successful spawning pairs in relation to the total number of spawning pairs (n = 15 for Control, n = 14 for *Microcystis*). SD = standard deviation; F = 1-way ANOVA F statistic; p = p value; * = significant at $p<0.05$. Statistics presented were performed on arcsine square root-transformed data.

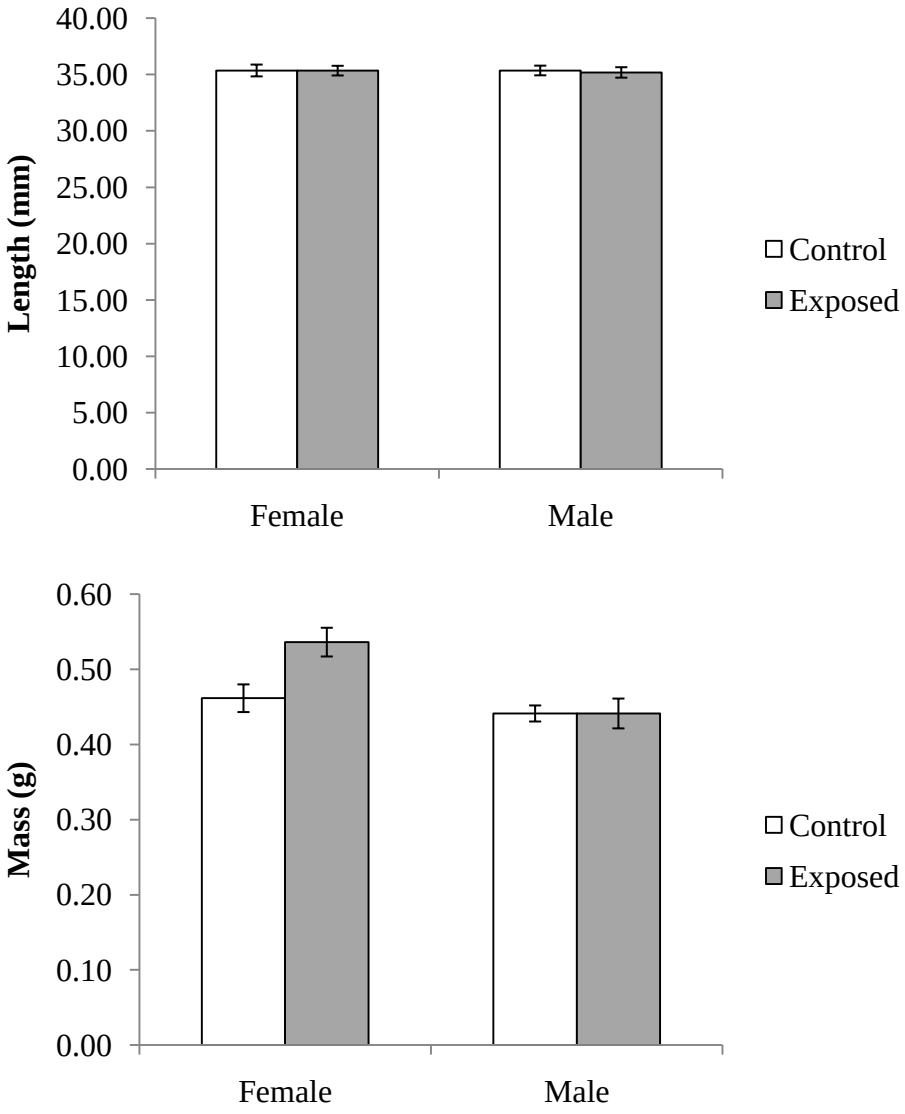

Figure 3.3. Length and weight of adult zebrafish at the start of the exposure period. Data are means ± standard error (n = 30). There were no significant differences between treatments ($p <$ 0.01).

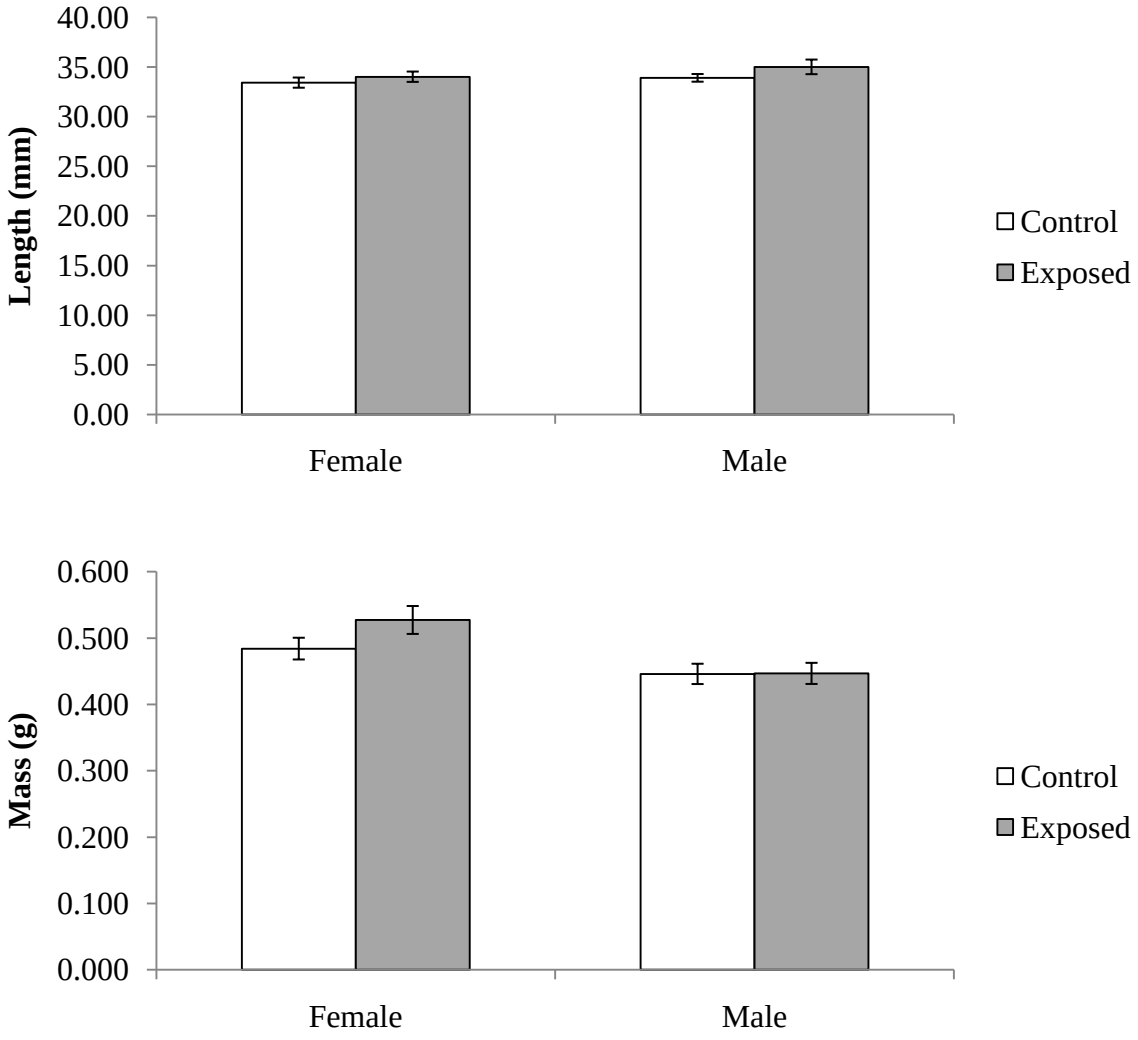

Figure 3.4. Length and weight of adult zebrafish at the end of the exposure period. Data are means ± standard error (n = 30 in control, n = 29 in *Microcystis* treatment).There were no significant differences between treatments ($p < 0.01$).

fish. There was very little vacuolization and no lesions were observed in control or treatment samples.

Discussion

Exposure to *Microcystis* had no effect on survival, growth or liver histopathology of adult zebrafish. Reduced growth of adult fish was observed in a previous study with Japanese medaka exposed to MC-LR via the diet, but effects were not observed until the fourth week of exposure (Deng et al. 2010). The absence of an effect on growth in zebrafish in the present study may have been due to the shorter exposure period used in this study or because the exposure route was different (aqueous vs. dietary). Mortality did not occur however, when carp were exposed to *Microcystis* for nine weeks (Adamovsky et al. 2007), which is consistent with the observation of no mortality of zebrafish in the present study. While effects on liver histopathology were not observed in this study, Carbis et al. (Carbis et al. 1996) reported cellular degeneration and necrosis of the liver in carp after 7 days of exposure to *Microcystis* and Deng et al. also observed single cell necrosis and lipidosis in livers of medaka exposed to MC-LR. Histopathological responses to *Microcystis* may vary between species, and it is possible that differences in MC-LR concentration or bloom composition may explain why effects did not occur in this study.

Microcystis exposure did, however, affect the reproductive capability of adult zebrafish. The percentage of pairs that spawned was significantly reduced in fish exposed to *Microcystis*. Only one other study has examined reproductive effects resulting from exposure in adult fish. In Deng et al. (2010), dietary exposure of medaka to MC-LR did not affect spawning. The medaka study used a batch spawning approach, where 20 males and 30 females per treatment were combined into a single tank to spawn. Because there was no replication of spawning events within treatments, spawning success could not be determined statistically and reproductive

success was assessed by fecundity. Deng et al. (2010) found no difference in fecundity between treatments and control, which is consistent with the present study. Because a pair spawning approach was used in the present study, we were able to detect a significant reduction in spawning success in zebrafish exposed to *Microcystis*.

While spawning success was reduced in zebrafish exposed to *Microcystis*, fecundity and survival of offspring were not affected. Each week the number of eggs produced by each spawning pair was quite variable, with standard deviation approaching and sometimes even exceeding the mean. If there had been a difference in fecundity between treatments, it would have been difficult to detect, given the variation in the data. Fecundity was also assessed by Deng et al. (2010), and they also found no significant difference in the number of eggs at spawning between treatments and control. In contrast to the present study where *Microcystis* exposure did not have a significant effect on larval survival, Deng et al. (2010) observed a significant decrease in survival of larvae, but only at the highest concentration of MC-LR tested (3.93 µg/g diet). This concentration was administered to medaka through the diet, whereas zebrafish were exposed to aqueous *Microcystis*, and it is difficult to make direct comparisons between the two studies, since exposure routes and substances under investigation (MC-LR vs. *Microcystis*) were different.

Additional information regarding *Microcystis* effects on larval survivorship in zebrafish, specifically, is available; however the comparative value of these studies to the present one is somewhat limited due to differences in experimental design. Larval survivorship in zebrafish has been previously examined in the context of embryo exposure. Oberemm et al. (1997) exposed zebrafish embryos to *Microcystis* extracts from fertilization through late embryonic

development, at which time they were transferred to clean water. All of the larvae died, but mortality did not occur until after exposure was terminated. In the present study, zebrafish larvae were also reared in clean water, however embryos were never exposed to *Microcystis* and any effects on larval survival would have been due to parental exposure only. Our findings suggest that adult exposure does not affect embryo survival per se, but effects may have occurred if embryos were also exposed to *Microcystis*. Since both adult fish and their offspring would be exposed to *Microcystis in situ*, future research that exposes adult fish to *Microcystis* before and during spawning with continuation of *Microcystis* administration to developing embryos, would more accurately simulate reproductive effects on fish living in environments affected by *Microcystis* blooms.

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SECTION IV

Bioaccumulation and toxicity of the cyanobacterium *Microcystis aeruginosa* and toxin microcystin-LR in field and laboratory exposed channel catfish (*Ictalurus punctatus*)

This section is a version of a journal article for planned submission to *Environmental Toxicology and Chemistry*. My contribution to the paper was conducting laboratory exposures with catfish, collecting catfish from Waterville Reservoir and Lake Erie, conducting molecular work to validate primer sets, interpreting histology slides, analyzing data, and writing the paper

Rogers, Emily D., Theodore B. Henry, June-Woo Park, Richard J. Strange, Gary S. Sayler, and Steven W. Wilhelm. Bioaccumulation and toxicity of the cyanobacterium *Microcystis aeruginosa* and toxin microcystin-LR in field and laboratory exposed channel catfish (*Ictalurus punctatus*). In preparation.

Introduction

Blooms of toxin-producing *Microcystis* occur throughout the world and are a growing public health concern. *Microcystis* is a genus of cyanobacteria that produces microcystin toxins, of which over 80 forms have been described, each differing slightly in chemical structure (Dittmann and Wiegand 2006; Tanabe et al. 2004). Microcystin-LR (MC-LR) is recognized as being the most toxic of the microcystins (Codd et al. 2005). Toxic *Microcystis* blooms can occur in many freshwater environments and have generally received the most attention in the United States because of the large-scale seasonal blooms that have formed annually since 1995 in the Western Basin of Lake Erie (Dyble et al. 2008). *Microcystis* blooms can also occur in smaller lakes. Our research group has documented the presence of *Microcystis* for the last three summers in Waterville Reservoir, a 1,178 km^2 mountain lake on the border of Tennessee and North Carolina (EPA 1975). In both of these lakes, MC-LR concentrations have exceeded the World Health Organization (WHO) advisory of 1 µg/L (Chorus et al. 2000). Because toxic *Microcystis* blooms are found in diverse habitats where recreational and commercial fishing takes place, there is concern about the potential for toxic effects in fish and in human populations as a result of fish consumption.

Fish live in waters where *Microcystis* blooms occur and are important components of the ecosystem that can be negatively affected by algal toxins. Microcystins (e.g., MC-LR) are hepatotoxins that act by inhibiting protein phosphatases 1 and 2A. In the liver, protein phosphatase inhibition is manifested by increased phosphorylation of cellular proteins, which results in cytoskeletal rearrangement and compromised liver function, including necrosis,

apoptosis and intrahepatic hemorrhage (Fischer and Dietrich 2000; Fischer et al. 2000). MC-LR mainly accumulates in the liver, but has also been detected in other organs including muscle in fish (Soares et al. 2004; Zhao et al. 2006a; Zhao et al. 2006b).

One of the biggest concerns about *Microcystis* blooms with respect to human health is the potential for MC-LR to bioaccumulate in fish tissues and be transferred to humans by consumption of fish. In fish, there are two potential exposure routes for *Microcystis*- 1. aqueous exposure through respiration and or/drinking 2. dietary exposure via ingestion of *Microcystis* cells and/or prey items that may have accumulated MC-LR. Aqueous exposure to *Microcystis* has been studied in both field enclosure and controlled laboratory situations (Adamovsky et al. 2007; Carbis et al. 1996a; Carbis et al. 1996b; L Li et al. 2007; Mares et al. 2009; Zhang et al. 2008a); however, tissue accumulation data from aqueous exposure is quite limited (but see (Adamovsky et al. 2007; L Li et al. 2007), and it is difficult to compare results across studies due to variations in bloom composition and microcystin concentrations. Dietary exposure has been simulated by oral gavage (Carbis et al. 1996a; Carbis et al. 1996b; Djediat et al. 2010; Fischer and Dietrich 2000; Fischer et al. 2000; Mezhoud et al. 2008; Tencalla and Dietrich 1997) or intraperitoneal injection (Andrinolo et al. 2008; Carbis et al. 1996a; Carbis et al. 1996b; Fournie and Courtney 2002; Lei et al. 2008; Li et al. 2009; L Li et al. 2005; S Li et al. 2007; Malbrouck et al. 2003, 2004; Williams et al. 1997; Zhang et al. 2009; Zhang et al. 2007; Zhang et al. 2008b) of MC-LR, but these types of exposures do not represent natural feeding and therefore have limited environmental relevancy. Several studies have been conducted, however, in which *Microcystis* cells or extracts were incorporated into artificial diets and fed to fish in the laboratory (Dong et al. 2009; El Ghazali et al. 2010; Li et al. 2004; XY Li et al. 2005; Soares et

al. 2004; Zhao et al. 2006a; Zhao et al. 2006b). Like aqueous exposures, MC composition of blooms incorporated into fish diets varies across studies and many measure accumulation of total microcystins in tissues, such that the specific accumulation of MC-LR (the MC toxin on which WHO guidelines are based) cannot be distinguished. Nevertheless, several of these studies indicate accumulation of total MCs in fish muscle at concentrations that exceed the WHO recommended tolerable daily intake (TDI) for humans of 0.04 µg/kg MC-LR/body weight/day (Chorus et al. 2000). In only one study, purified MC-LR was administered to Japanese medaka through the diet (Deng et al. 2010); however tissue concentrations of MC-LR were not measured, presumably due to size limitations of the species tested. Evidence for bioaccumulation of MC-LR from aqueous or dietary exposure routes is largely inconclusive, and further studies are needed that use environmentally-relevant exposure scenarios where fish are exposed to purified MC-LR or well-characterized *Microcystis* blooms with known MC-LR concentrations.

In the case of MC-LR, development of biomarker genes whose expression in fish can be measured using qPCR would be useful as a field biomonitoring tool, however no such genes have been identified in an environmentally relevant fish species. Channel catfish (*Ictalurus punctatus*) would be an ideal candidate species, because it is present in freshwater environments where *Microcystis* occurs and is targeted for consumption by commercial fishing operations and recreational anglers. Identifying which genes are likely to be important indicators of toxicity in catfish is challenging because effects of *Microcystis* and MC-LR on gene expression in catfish are unknown.

A model organism, such as zebrafish (*Danio rerio*), can be used to identify genes affected by MC-LR and *Microcystis*. In a recent study conducted by our laboratory (Rogers et al. in

review (Section II of this dissertation)), larval zebrafish were exposed to purified MC-LR (100 and 1,000 µg/L) and lyophilized *Microcystis* (4.5 µg/L MC-LR) and effects on global gene expression were determined using Affymetrix arrays. Biomarker genes were selected from a list of 79 genes differentially expressed in all treatments (i.e. toxin-related genes induced by *Microcystis*). From this list, 6 genes having the highest absolute fold change were identified as biomarker genes (Table. 4.1). In addition, vitellogenin was highly up-regulated only in zebrafish exposed to *Microcystis* (i.e. gene induced by *Microcystis* but not toxin-related) and was also included as a potential biomarker of *Microcystis* exposure. These biomarker genes differentially expressed in zebrafish exposed to MC-LR and *Microcystis* have the potential to be adapted for use in qPCR assays with channel catfish.

The objectives of this research were to: 1. Assess bioaccumulation of MC-LR and effects on liver histopathology in catfish exposed to MC-LR and *Microcystis* in the laboratory through dietary and aqueous administration. 2. Compare histological lesions in the liver with those observed in fish collected from lakes where *Microcystis* blooms were present. 3. Develop functional primer sets for channel catfish based on biomarker genes previously identified in zebrafish that can be used for future monitoring of gene expression in channel catfish by qPCR.

Table 4.1. Biomarker genes of interest.

								Relative expression in larval zebrafish					
								100 µg/L MC-LR		1,000 µg/L MC-LR		*Microcystis*	
Name	**Symbol**	**GenBank accession number**		**Primer sequence (5' - 3')**	**Product size**	**Primer works (y/n)**	**Annealing temperature (°C)**	**Fold change**	**p-value**	**Fold change**	**p-value**	**Fold change**	**p-value**
18S rRNA[a]	*18S*	AF021880	for	TGGTTAATTCCGATAACGAACGA	94	y	57	1.0	0.63	1.0	0.06	1.0	0.49
			rev	CGCCACTTGTCCCTCTAAGAA									
v-fos FBJ murine osteosarcoma viral oncogene	*fos*	FD105437.1	for	GCCCGGACCTGCAGTGGATG	145	n	n/a	-1.4	1.08E-01	-3.0	4.00E-05	-2.8	2.00E-05
			rev	GTGCTGCCGGGAGCCTTGTT									
hepatocyte growth factor activator inhibitor 2	*spint2*	CK416322.1	for	GGTTTGCTGTGCTAGAGA	165	y	57	-2.9	2.71E-08	-5.6	4.50E-14	-3.1	2.81E-09
			rev	CGGTCAGAGACCTGAATC									
mucin 2	*muc2*	FD129369.1	for	AGAGACGACTGATGCTGA	180	n	n/a	-4.7	2.00E-05	-6.7	1.67E-06	-4.9	1.00E-05
			rev	ACCGTGGTGTTCGTATGG									

Table 4.1. Continued.

								Relative expression in larval zebrafish					
								100 µg/L MC-LR		1,000 µg/L MC-LR		*Microcystis*	
Name	**Symbol**	**GenBank accession number**		**Primer sequence (5' - 3')**	**Product size**	**Primer works (y/n)**	**Annealing temperature (°C)**	**Fold change**	**p-value**	**Fold change**	**p-value**	**Fold change**	**p-value**
steroidogenic acute regulatory protein	*star*	FD186366.1	for	GCATGTGGAGCCCC TGCGTT	167	n	n/a	-3.3	1.00E -05	-6.9	1.92E -09	-3.8	2.31E -06
			rev	AGCGCATGGGCGAG TGGAAC									
nephrosin	*npsn*	BM438630.1	for	TGCGGACGGCGAGA GAAACG	181	y	57	-4.4	2.00E -05	-7.4	2.40E -07	-4.6	1.00E -05
			rev	TGGCGTGAAGCGGA TGCAGG									
vitellogenin	*vtg*	FD373053.1	for	TGCACTTCCTGTTGT GGGTA	226	y	64	-1.5	0.34	-1.3	0.63	>100	<1.00 E-45
			rev	CATAGAACCACAGC CAAGCA									
flavoprotein oxidoreductase mical3	*mical3*	FD351034.1	for	CAAACTCCTGCCATG AGGCATA	172	n	n/a	2.4	6.00E -05	2.7	8.59E -07	2.8	8.00E -05
			rev	GGAGCTTTGCCCTGA GTCCA									

[a]Housekeeping gene.

Materials and Methods

Experimental Fish

Juvenile (1 yr. old) channel catfish (*Ictalurus punctatus*) were obtained from Southland Fisheries (Hopkins, SC), transported to the Johnson Animal Research and Teaching Unit at the University of Tennessee, and kept in two 1,000 L holding tanks on a flow through system receiving de-chlorinated municipal water (hereafter referred to as system water) at 57 L/min until fish were allocated to experimental tanks. Prior to experiments fish were fed commercial trout pellets (control food) (Melick Aquafeed, Catawissa, PA- 45% protein, 15% fat, 3% fiber) daily at 2% body weight. Water quality measurements were recorded for system water daily (dissolved oxygen, pH, ammonia) and weekly (conductivity, hardness, alkalinity) throughout acclimation and exposure periods, and the system water had the following characteristics: dissolved oxygen (4.7-6.4 mg/L), pH (6.5-7.8), total alkalinity (186-204 mg/L as $CaCO_3$), total hardness (84-90 mg/L as $CaCO_3$), and ammonia (<0.2 mg/L). A photoperiod of 14:10h light:dark was also maintained throughout acclimation and exposure periods. Fish husbandry and experimental procedures were conducted with approval from the University of Tennessee Institutional Animal Care and Use Committee (Protocol # 1690-1007). For experiments, fish were randomly allocated to 890-L round tanks with continuous flow-through plumbing containing 500-L heated (28°C) system water with a flow rate of 1.5 L/min. Before each experiment fish were allowed to acclimate to experiment tanks for 7 d, during which time they were fed the control food.

Laboratory Experiments

Four laboratory experiments were conducted to assess bioaccumulation of MC-LR and *Microcystis* in channel catfish via dietary and aqueous exposure (Table 4.2). The objective of Experiment 1 was to assess chronic, low-dose accumulation of MC-LR and *Microcystis* via dietary exposure. Experiment 2 tested higher dose, semi-chronic dietary exposure to MC-LR and *Microcystis*. In order to compare dietary and aqueous exposure routes, catfish were exposed to aqueous *Microcystis* in Experiment 3. An additional acute waterborne exposure to the synthetic estrogen 17-alpha-ethinylestradiol (EE2) (Experiment 4) was also conducted to induce vitellogenin genes (*vtg*) in catfish and serve as a positive control for *vtg* primer development.

Experiment 1

Experimental diets were prepared using the same commercial pellets that fish were fed from the time that they arrived in our laboratory (described above). First, a stock solution was made by dissolving 1.25 mg purified MC-LR (Alexis Biochemical) in 1 mL ethanol. For the 1 µg MC-LR/g food treatment, 0.8 mL of stock solution was diluted to 200mL with Milli-Q water and for the 0.1 µg MC-LR/g food treatment, 0.08 mL of stock solution was diluted to 200mL with Milli-Q water. For each treatment, the entire 200mL of working solution was sprayed evenly over 1 kg of trout pellets. *Microcystis* diet was prepared by dissolving 50 mg lyophilized *M. aeruginosa* PCC-7806 culture in 200 mL Milli-Q water and spraying evenly over 1kg of trout pellets (0.05 mg lyophilized *Microcystis*/g food), and for control food 200 mL of Milli-Q water was sprayed over 1kg food. After air drying overnight, a gelatin coating was added to each diet

Table 4.2. Overview of experiments.

Experiment	Exposure route	Treatments	Concentrations in food and/or water	Feeding rate	MC-LR dose in fish	Duration	Sampling times
1	Dietary	MC-LR *Microcystis*	0, 0.1, 1 µg MC-LR/g food 0.05 mg lyophilized *Microcystis*/g food	1% bw/d	1, 10 µg/kg bw/d	30 d uptake 10 d depuration	0, 8, 15, 30d 6, 12, 24h 2,5, 10d
2	Dietary	MC-LR *Microcystis*	0, 100 µg MC-LR/g food 0.28 mL *Microcystis* culture/g food	2% bw/d	200 µg/kg bw/d	14 d uptake	0, 1, 3, 7, 14d
3	Aqueous	*Microcystis*	100% water control 20% BG-11 media control 20% *Microcystis* culture	n/a	n/a	7 d uptake	0 , 1, 3, 5, 7d
4	Aqueous	17-alpha ethinylestradiol (EE2)	0, 0.02, 0.2, 2 µg/L EE2	n/a	n/a	4 d uptake	4d

by spraying 200 mL of gelatin solution (33g/L) over food. All diets were air-dried overnight once more and stored in airtight containers for the duration of the experiment.

On day zero, one fish from each tank (n=12) was sampled before feeding to obtain a starting mass from which the initial amount of food required for the feeding rate of 1% body weight was determined. Treatment tanks were assigned (n=3/treatment) and 39 fish were allocated to each tank. Fish were fed experimental diets at 1% body weight daily for 30 days (uptake period) and for 10 days thereafter (depuration period); all tanks received control food at 1% body weight.

Three fish per tank (n=9) were euthanized by overdose of MS-222 on days 8, 15, and 30 (uptake) and at 12h, 24h, 2d, 5d, and 10d during the depuration period. Small pieces of liver tissue from individual fish were preserved in liquid nitrogen and Bouin's fixative for RNA extraction (n=9) and histopathology (n=9), respectively. Remaining liver and muscle tissue was also collected and frozen at -20°C for MC-LR analysis (n=9). Length and weight of each fish sampled was recorded, and feeding rates were adjusted at each sampling time based on average mass.

Experiment 2

A similar procedure was used to prepare solutions for Experiment 2. For the MC-LR treatment, 18 mg purified microcystin-LR (Alexis Biochemical) was first dissolved in 0.5 mL ethanol, diluted to 50 mL with Milli-Q water and sprayed evenly over 180 g of commercial trout pellets (100 µg MC-LR/g food). *Microcystis* diet was prepared by spraying 50 mL of *M. aeruginosa* PCC-7806 culture evenly over 180 g of commercial trout pellets (0.28 mL

Microcystis culture/g food), and for control food 50 mL of Milli-Q water was sprayed over 180 g food. After air drying overnight, a gelatin coating was added to each diet by spraying 50 mL of gelatin solution (33g/L) over food. All diets were air-dried overnight once more and stored in airtight containers during the experiment.

On day zero, one fish from each tank (n=12) was sampled before feeding to obtain a starting mass from which the initial amount of food required for the feeding rate of 2% body weight was determined. Treatment tanks were assigned (n=4/treatment) and fish were fed experimental diets at 2% body weight daily for 14 days.

Four fish per tank (n=16) were euthanized by overdose of MS-222 on days 1, 3, 7, and 14. Small pieces of liver tissue from individual fish were preserved in RNAlater (Qiagen, Valencia, CA) and Bouin's fixative for RNA extraction (n=16) and histopathology (n=16), respectively, and remaining liver tissue from 2 fish per tank was pooled in order to obtain >1g required for MC-LR analysis (n=8). Muscle tissue was also collected from individual fish for MC-LR analysis (n=16). Length and weight of each fish sampled was recorded, and feeding rates were adjusted at each sampling time based on average mass.

Experiment 3

M. aeruginosa was cultured in large quantities in our laboratory using BG-11 media as described in Rinta-Kanto et al. (2005), and 20 L of *Microcystis* culture was added to each tank (n=3) and then diluted to 100 L with system water (20% *Microcystis*). Media control tanks (n=3) were prepared by adding 20 mL BG-11media to each tank and diluting to 100 L with system

water (20% BG-11 media). Control tanks (n=3) contained 100 L system water. All tanks were stocked at a density of 18 fish/tank.

On days 1, 3, 5, and 7 four fish per tank (n=12/treatment) were euthanized by overdose of MS-222. Fork length and weight were recorded prior to dissection. Small pieces of liver tissue from individual fish were preserved in RNAlater (Qiagen, Valencia, CA) and Bouin's fixative for total RNA extraction (n=12) and histopathology (n=12), respectively, and remaining liver tissue from 2 fish per tank was pooled in order to obtain >1g required for MC-LR analysis (n=6). Muscle tissue was also collected from individual fish for MC-LR analysis (n=12). Because a limited quantity of *Microcystis* culture was available and solution renewals were not possible, fish were not fed during the exposure to prevent ammonia toxicity.

Experiment 4

Catfish were exposed to the synthetic estrogen 17-alpha ethinylestradiol (EE2). The purpose of the exposure was to induce vitellogenin gene expression in catfish and collect liver samples for RNA extraction to be used as positive controls for development of qPCR primers for *vtg*.

Catfish were allocated to glass aquaria containing 15 L of solution at a density of three fish per tank. Treatments consisted of control (0 µg/L EE2), 0.02, 0.2, and 2 µg/L and there were 3 replicate tanks per treatment. Fish were exposed for 4 days and were not fed in order to prevent ammonia toxicity. At the end of the exposure, fish were euthanized by overdose of MS-222 and liver samples were collected and preserved in RNAlater (Qiagen, Valencia, CA).

Field Sampling

Channel catfish were collected from two lakes where *Microcystis* blooms were present. In August 2008, channel catfish were collected by E. Rogers from Sandusky Bay, Lake Erie in collaboration with the Ohio Department of Natural Resources and again in October 2009 by the Ontario Ministry of Natural Resources. In August 2009, channel catfish were also collected by E. Rogers from Waterville Lake, NC. Collection procedures were identical for Lake Erie and Waterville Reservoir locations, except that liver samples for RNA extraction were not collected from Lake Erie in 2008. Channel catfish (n=10/ lake) were euthanized, weight and length recorded, and dissected on site. Two small pieces of liver from each fish were preserved in RNAlater (Qiagen, Valencia, CA) and Bouin's fixative for RNA extraction and histology, respectively. A portion of remaining liver tissue and muscle tissue from each fish (>1g) was collected for MC-LR analysis. Water samples were also collected from sites where fish were sampled for MC-LR analysis.

Toxin Analysis

Analysis of MC-LR in water and tissue samples was conducted at the State University of New York College of Environmental Science and Forestry (Syracuse, NY). Water samples were analyzed by protein phosphatase inhibition assay and tissue samples by ELISA following the methods of Carmichael and An (Carmichael and An 1999).

Histopathology

After tissue fixation in Bouin's fixative (24-36 h) liver samples were transferred into 70% EtOH and subsequently processed and embedded in paraffin for routine histology. Sections (0.6 µm thick) were stained with hematoxylin and eosin (Humason 1979) and all sections were examined by light microscopy.

RNA Extraction

Liver samples preserved in liquid nitrogen were transferred to a -80°C freezer and samples preserved in RNAlater were stored according to the manufacturer's instructions at -20°C until extraction. RNA was extracted using the RNeasy mini extraction kit for animal tissues (Qiagen, Valencia, CA) and quantified using a UV-spectrophotometer (Nanodrop, Wilmington, DE).

Biomarker Gene Selection and Primer Development

Genes of interest were selected based on a previous microarray study with zebrafish (Rogers et al. in press, Section II of this dissertation) and summarized in Table 4.1. These biomarker genes were selected from a list of genes differentially expressed in both *Microcystis* and purified MC-LR treatments and therefore most closely represent effects of the microcystin toxin as produced by *Microcystis*. Vitellogenin was also selected for monitoring because it was highly up-regulated only in fish exposed to *Microcystis* (not toxin-related) and represents a gene that may be used as a biomarker of effect of *Microcystis* exposure unrelated to MC-LR. Because

there was no significant up regulation of 18S rRNA among treatments, this gene was selected for use as an internal control.

Primer sets specific to channel catfish were developed for each of the genes listed in Table 4.1. Sequence information for primer design was obtained from expressed sequence tags available in GenBank. Primers were designed using the Primer-Blast tool (NCBI), and melting temperatures and potential for self-complementarity were determined using the oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) and Oligo Analyzer 3.1 (Integrated DNA Technologies, Coralville, IA), respectively. Primer sets were purchased from Biosearch Technologies (Novato, CA). The *18S* primer set for channel catfish was previously developed and provided by June-Woo Park.

Selection of Samples for Primer Validation

Appropriate RNA samples from catfish were selected to experimentally validate that the above primer sets amplified their intended gene targets and to determine optimal annealing temperatures. For all primer sets except vitellogenin, control RNA samples were used. For the vitellogenin primer set, control RNA samples were used as well as 2 samples from fish exposed to 2 µg/L EE2 (Experiment 4), which served as positive controls.

Reverse Transcription

Following RNA extraction, cDNA was generated from the above RNA samples using a Superscript® III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). Samples containing 1,000 ng of total RNA were used to synthesize single-strand cDNA in accordance with the

manufacturer's directions. Briefly, prior to reverse transcription, 1,000 ng total RNA in 8 µL RNAse free water was treated with 1 µL DNAse I (1U/µL) and 1 µL DNase I reaction buffer for 15 min. at room temperature in order to remove potential chromosomal DNA. Then, 1 µL of 25mM EDTA was added to each sample and incubated at 65°C for 10 min. to inactivate the reaction. Reaction mixture (13.2 µL) containing 11 µL 2X RT Reaction Mix (RTase, RNAse Out) and 2.2 µL RT enzyme mix (Oligo dT, dNTP, 25mM $MgCl_2$, random hexamers), was added to each RNA sample and incubated at 25°C for 10 min., 50°C for 30 min., and terminated at 85°C for 5 min. To improve sensitivity, the RNA template from the cDNA:RNA hybrid molecule was removed from each sample by digestion with 1 µL *Escherichia coli* RNase H (2U/µL) at 37°C for 20 min. Also, a negative control (sample without reverse transcriptase) was run in parallel, which resulted in no amplification of the PCR product.

Primer Validation

Primer sets were then validated using traditional PCR (GoTaq PCR Core System I, Promega, Madison, WI). Validation was conducted using control cDNA as a template for all primer sets, with the exception of vitellogenin (*vtg*), where both control cDNA and 2 cDNA samples from fish exposed to 2 µg/L EE2 (Experiment 4) were used as positive controls. Each 50 µL PCR reaction contained the following: 5X GoTaq® Flexi buffer (10 µL), 25mM $MgCl_2$ (6µL), 10 mM PCR nucleotide mix (1.5 µL), forward primer (3µL), reverse primer (3µL), GoTaq® DNA polymerase (0.5µL), 1/200 diluted cDNA (5µL), nuclease-free water (21µL). The PCR reaction mix was denatured at 94°C for 3 min. before the first cycle. Thermocycler conditions were as follows: 94°C for 45 sec., annealing at 55 °C for 40 sec., and extension at

72°C for 1.5 min. The process was repeated for a total of 40 cycles and then incubated at 72°C or 10 min. before cooling to 4°C. PCR products were run on 2.5% agarose gels at 100V for 1 hr. Only primer sets with PCR products resulting in bands matching the expected product size of the primer set being tested were determined to be working correctly.

Determination of Optimal Annealing Temperatures for qPCR Assays

Primer sets whose gene products matched expected product size via traditional PCR assays were subjected to further testing to determine optimal annealing temperatures for qPCR. All qPCR assays were performed using a SYBR® Green PCR Core Reagents kit (Applied Biosystems, Carlsbad, CA) and amplification was determined using a DNA Engine Opticon continuous fluorescence detection system (MJ Research, Waltham, MA). Primer sets determined to be working with non-quantitative PCR were tested by qPCR reactions using a 55-65 °C annealing temperature gradient with melting curve in order to determine the optimal annealing temperature for each primer set. Each 25 µL qPCR reaction contained the following: 10× SYBR® Green buffer (2.5 µL), 25 mM $MgCl_2$ (3 µL), 12.5 mM dNTP mix, 10 pM/µL forward primer (0.5 µL), 10 pM/µL reverse primer (0.5 µL), 5U/µL AmpliTaq Gold™ DNA polymerase (0.13 µL) 1U/µL AmpErase (0.25 µL), 1/200 diluted cDNA (5 µL), and nuclease-free water (12.62 µL). The PCR reaction mix was denatured at 94°C for 3 min. before the first PCR cycle. PCR cycles were as follows: denaturation for 20s at 94°C, 55-65°C annealing temperature gradient for 1 min., and extension for 1 min. at 72°C. The process was repeated for a total of 45 cycles followed by a melting curve from 50-95°C with plate reads every 0.2 sec and a hold time of 1 sec.

Statistical Analyses

For weight, length, and toxin concentration data, differences among treatment groups were determined by 1-way ANOVA followed by Duncan's multiple range test. All statistical analyses were conducted with ToxStat 3.4© (Cheyenne, Wyoming) and a probability level of $P<0.05$ was used as the level for statistical significance.

Results

Experiment 1

During both the uptake and depuration periods, fish showed no visible signs of stress, and mortality did not occur. There were no significant differences in growth among treatment groups during the exposure, except at the 12h time point during depuration, where average mass and length were significantly greater in fish fed the *Microcystis* diet (Tables 4.3 and 4.4). The MC-LR toxin was not found in liver or muscle tissue at concentrations above the limit of detection. Histological analysis showed no significant difference between controls and fish exposed to MC-LR or *Microcystis.*

Experiment 2

In Experiment 2, catfish were exposed to a higher dose of MC-LR for a shorter period of time. Again, there were no signs of mortality or stress during the exposure, and growth was not affected by treatments (Tables 4.5 and 4.6). Toxin analysis is underway. Examination of liver tissue for histological lesions showed no significant differences between control and treatment groups.

Experiment 3

In Experiment 3, catfish were exposed to aqueous *Microcystis.* No mortality occurred during the 7-d exposure, and there were no significant differences in growth among treatments

(Tables 4.7 and 4.8). Toxin analysis is underway. Examination of liver tissue for histological lesions showed no significant differences between control and treatment groups.

Table 4.3. Experiment 1 fish weights.

Treatment	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD
Uptake Time	**Day 0**		**Day 8**		**Day 15**		**Day 30**					
Control	19.6	5.0	21.8	5.4	19.7	6.2	20.6	5.8				
100 ng/g MC-LR	21.4	2.6	19.6	2.4	23.2	5.6	22.3	5.5				
1,000 ng/g MC-LR	22.0	6.1	26.1	5.8	20.6	5.5	25.7	6.9				
Microcystis	15.0	2.3	20.0	7.3	22.8	6.8	21.7	5.5				
F	1.63		2.73		0.80		1.23					
p	0.26		0.06		0.50		0.31					
Deputation Time	**6 h**		**12 h**		**24 h**		**Day 2**		**Day 5**		**Day 10**	
Control	22.3	6.4	23.0	4.6	22.3	5.5	22.3	5.5	22.3	5.5	22.3	5.5
100 ng/g MC-LR	22.4	4.2	21.0	6.9	25.7	6.9	25.7	6.9	25.7	6.9	25.7	6.9
1,000 ng/g MC-LR	22.4	4.4	22.2	4.9	21.7	5.5	21.7	5.5	21.7	5.5	21.7	5.5
Microcystis	26.6	6.0	28.3*	5.2	22.6	5.0	23.1	4.1	29.6	4.7	25.2	6.2
F	1.5		3.1		1.6		0.7		1.3		0.9	
p	0.25		0.04		0.21		0.54		0.29		0.45	

Data are means. Day 0 n = 3; all other time points n = 9; SD = standard deviation; F = 1-way ANOVA F statistic; p = p value; * significant at $p < 0.05$

Table 4.4. Experiment 1 fish lengths.

Treatment	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD
Uptake Time	**Day 0**		**Day 8**		**Day 15**		**Day 30**					
Control	148.3	13.2	151.3	11.5	144.8	14.6	146.0	13.5				
100 ng/g MC-LR	151.7	2.5	146.7	9.9	153.8	9.1	145.4	14.2				
1,000 ng/g MC-LR	152.3	11.5	157.8	12.0	145.4	15.5	149.8	9.8				
Microcystis	133.3	3.1	146.7	17.6	152.1	12.4	155.2	16.8				
F	2.95		1.62		1.13		0.96					
p	0.10		0.20		0.35		0.42					
Deputation Time	**6 h**		**12 h**		**24 h**		**Day 2**		**Day 5**		**Day 10**	
Control	145.9	15.5	149.7	12.4	160.0	12.2	151.4	12.9	158.1	7.8	161.4	13.0
100 ng/g MC-LR	147.3	11.8	146.2	13.2	159.4	10.3	158.1	13.2	161.9	16.6	162.3	8.6
1,000 ng/g MC-LR	150.6	7.5	149.4	12.0	155.8	11.5	158.9	20.4	157.0	8.9	163.8	12.8
Microcystis	155.4	13.9	163.2*	10.1	157.0	11.8	152.8	8.8	162.8	5.0	156.3	11.5
F	1.03		3.57		0.27		0.60		0.65		0.70	
p	0.39		0.02		0.84		0.62		0.59		0.56	

Data are means. Day 0 n = 3; all other time points n = 9; SD = standard deviation; F = 1-way ANOVA F statistic; p = p value; * significant at $p < 0.05$

Table 4.5. Experiment 2 fish weights.

Treatment	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD
Uptake Time	**Day 0**		**Day 1**		**Day 3**		**Day 7**		**Day 14**	
Control	27.6	7.4	28.9	15.8	26.7	5.9	25.3	6.8	24.7	7.3
100 µg/g MC-LR *Microcystis*	24.3	4.4	26.7	12.7	28.0	5.7	26.5	11.5	24.4	10.9
	18.3	3.5	29.6	11.1	28.2	8.6	26.6	7.7	26.7	9.9
F	3.02		0.21		0.21		0.11		0.28	
p	0.10		0.81		0.81		0.89		0.76	

Data are means. Day 0 n = 4; all other time points n = 16; SD = standard deviation; F = 1-way ANOVA F statistic; p = p value

Table 4.6. Experiment 2 fish lengths.

Treatment	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD
Uptake Time	**Day 0**		**Day 1**		**Day 3**		**Day 7**		**Day 14**	
Control	123.0	10.1	127.1	20.6	124.1	10.5	120.8	11.7	121.8	11.7
100 µg/g MC-LR	121.0	7.3	124.2	15.5	124.5	9.6	122.6	16.4	119.3	15.2
Microcystis	108.5	7.2	128.8	17.2	125.8	11.8	124.3	12.2	121.1	15.8
F	3.55		0.27		0.11		0.27		0.14	
p	0.07		0.76		0.90		0.76		0.87	

Data are means. Day 0 n = 4; all other time points n = 16; SD = standard deviation; F = 1-way ANOVA F statistic; p = p value

Table 4.7. Experiment 3 fish weights.

Treatment	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD	Weight (g)	SD
Uptake Time	**Day 1**		**Day 3**		**Day 5**		**Day 7**	
Control	38.2	10.1	37.9	11.6	34.8	16.4	31.7	13.3
BG-11 Control	37.7	12.8	38.5	19.1	32.2	10.5	33.3	11.9
Microcystis	33.1	11.2	36.8	13.1	34.8	12.3	35.1	13.8
F	0.73		0.04		0.16		0.21	
p	0.49		0.96		0.85		0.81	

Data are means. n = 12 for all time points; SD = standard deviation; F = 1-way ANOVA F statistic; p = p value

Table 4.8. Experiment 3 fish lengths.

Treatment	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD	Length (mm)	SD
Uptake Time	**Day 1**		**Day 3**		**Day 5**		**Day 7**	
Control	140.6	12.8	137.8	12.9	131.2	19.3	129.4	16.5
BG-11 Control	138.9	15.5	137.0	21.1	130.5	15.3	132.2	13.6
Microcystis	135.0	15.7	134.3	15.1	132.6	16.7	133.8	17.2
F	0.46		0.15		0.05		0.23	
p	0.64		0.87		0.96		0.80	

Data are means. n= 12 for all time points; SD = standard deviation; F = 1-way ANOVA F statistic; p = p value

Validation of Primers for Biomarker Genes

A summary of primers and their respective gene product sizes is given in Table 4.1. Primers whose PCR products gave single bands within expected size range using control cDNA template at an annealing temperature of 55°C included 18S rRNA (*18S*), hepatocyte growth factor activator inhibitor 2 (*spint2*), and nephrosin (*npsn*). All other primer sets amplified unintended gene products. Steroidogenic acute regulatory protein (*star*) and flavoprotein oxidoreductase mical3 (*mical3*) each gave a single band at <100 bp, and mucin 2 (*muc2*) and v-fos FBJ murine osteosarcoma viral oncogene (*v-fos*) each gave multiple bands that were >200 bp. A single band within expected size range was detected in both PCR products that used *vtg* primers and cDNA template from fish exposed to 2 µg/L EE2, whereas no band was detected for the PCR product containing *vtg* primers and control cDNA template (Fig. 4.1). Optimal annealing temperature was determined for all working primer sets (*18S*, *spint2*, *npsn*, *vtg*) using gradient qPCR. The optimal annealing temperature for *18S*, *spint2*, and *npsn* primer sets was 57°C and 64°C for *vtg*.

Field Samples

Toxin analysis of field samples is underway. MC-LR concentration (mean ± SD) in water samples collected from Waterville Reservoir was 862 ± 162 µg/L. A summary of numbers of species and sizes of fish collected from each site is given in Tables 4.9-4.11.

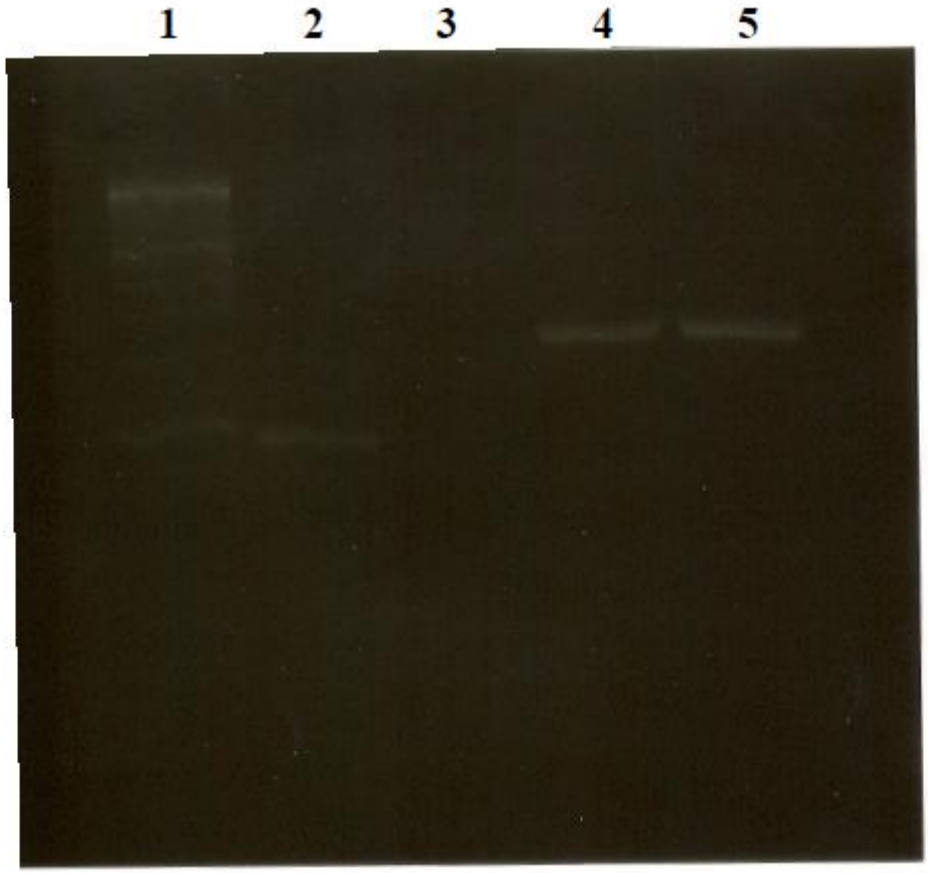

Figure 4.1 Gel image of vitellogenin amplification in channel catfish exposed to EE2. Lane 1= 100 bp DNA ladder, 2 = control cDNA + *18S* primer, 3 = control cDNA + *vtg* primer, 4 = cDNA from EE2-exposed fish #1 + *vtg* primer, 5 = cDNA from EE2- exposed fish #2 + *vtg* primer.

Table 4.9. Weights and lengths of fish sampled from Lake Erie in 2008.

Species	Weight (g)	Length (mm)
channel catfish	850	450
channel catfish	110	234
channel catfish	113	248
channel catfish	108	222
channel catfish	121	174
mean	**260.4**	**265.6**
SD	**329.6**	**106.8**
yellow perch	126	185
yellow perch	101	153
yellow perch	89	154
mean	**105.3**	**164.0**
SD	**18.9**	**18.2**
walleye	279	323
walleye	234	282
walleye	244	287
mean	**252.3**	**297.3**
SD	**23.6**	**22.4**

SD = standard deviation

Table 4.10. Weights and lengths of fish sampled from Lake Erie in 2009.

Species	Weight (g)	Length (mm)
channel catfish	1410	518
channel catfish	1894	542
channel catfish	801	455
channel catfish	702	425
channel catfish	2508	584
channel catfish	1750	585
channel catfish	1509	521
channel catfish	1039	444
channel catfish	795	428
channel catfish	1635	533
mean	**1404.3**	**503.5**
SD	**577.7**	**61.2**

SD = standard deviation

Table 4.11. Weights and lengths of fish sampled from Waterville Reservoir in 2009.

Species	Weight (g)	Length (mm)
channel catfish	320	332
channel catfish	290	318
channel catfish	4615	725
channel catfish	350	358
channel catfish	2530	614
channel catfish	3175	630
channel catfish	945	457
channel catfish	620	384
channel catfish	840	645
channel catfish	680	421
channel catfish	340	340
channel catfish	740	420
channel catfish	1020	468
mean	**1266.5**	**470.2**
SD	**1334.9**	**137.2**
yellow perch	760	359
yellow perch	360	292
mean	**560.0**	**325.5**
SD	**282.8**	**47.4**

SD = standard deviation

Discussion

Laboratory exposure to MC-LR and *Microcystis* had no effect on mortality of channel catfish. All fish survived in both the dietary and aqueous experiments. In Li et al. (2004), common carp that were fed *Microcystis* containing MC-LR for 4 weeks at a dose (50 µg/kg bw/day) similar to those administered in our experiments also exhibited no signs of mortality. In contrast, mortality was observed in dietary exposures of Nile tilapia (*Oreochromis niloticus*) and gibel carp (*Carassius gibelio*) to *Microcystis* where fish were fed diets containing concentrations as high as 5.5 µg MC/g diet to satiation twice daily (Zhao et al. 2006a; Zhao et al. 2006b). This concentration in the diet is higher than those used in Experiment 1 but lower than that of Experiment 2; however, tilapia and carp were fed to satiation twice daily and the amount of toxin ingested in terms of body weight or per fish was not specified. Despite this limitation, it is likely that these fish received a higher dose of MC than in our study, which could explain why mortality may have occurred, since these fish were fed an unlimited amount twice per day, whereas in our experiments channel catfish were fed a 1-2% bw (depending on experiment) once per day. The absence of effects on mortality of channel catfish when exposed to an aqueous *Microcystis* (Experiment 3) also seems to be supported in the literature. Adamovsky et al. (2007) exposed common carp to a *Microcystis* bloom for 9 weeks and no significant effects on mortality were observed. While our results suggest that chronic mortality of channel catfish as a result of *Microcystis* and MC-LR exposure, whether by dietary or aqueous means is unlikely, other species of fish may be more sensitive.

Similarly, exposure to *Microcystis* and MC-LR did not have significant effects on growth of channel catfish. Mean weights and lengths were not significantly different from controls in Experiments 2 and 3, however in Experiment 1 fish exposed to *Microcystis* via the diet were significantly larger than controls at 12 hours post-exposure. This observation was likely the result of sampling larger fish at the end of the experiment that were previously able to escape capture and was probably not an effect of treatment.

Development of primer sequences for biomarker genes previously identified in zebrafish was successful for only some of the candidate genes. PCR amplification within the size range of the target gene was observed for *18S, spint2, npsn* and *vtg* primer sets, while the remaining 4 primer sets did not work. Only expressed sequence tags were available for catfish in GenBank, and designing primer sets with low a probability of forming primer-dimers and hairpins was difficult when working with such short sequences (569-884 bp). Species differences may also explain why some primer sets did not work. While primer sets for all of the genes of interest were not validated, working primer sets for *spint2, npsn* and *vtg* were achieved, and these genes have the potential to serve as biomarkers of exposure whose expression can be monitored in future experiments and field monitoring of channel catfish exposed to *Microcystis*.

For *vtg*, bands were observed in PCR products containing positive control cDNA from fish exposed to EE2, but no band was observed when control cDNA was used. The absence of amplification in the sample containing control cDNA is consistent with expectations, because vitellogenin is not normally produced in juvenile fish, such as those used in this study. Amplification of *vtg1* in EE2 exposed samples was expected and confirms the functionality of this primer set for use in channel catfish, since EE2 is an estrogen mimic with documented

ability induce vitellogenin expression in juvenile fish (Henry et al. 2009). The absence of vitellogenin gene expression in control samples may also help explain why primer sets for *fos*, *muc2*, *star* and *mical3* did not work. It is possible that these genes too were weakly induced or not induced at all in control samples, but may have been differentially expressed in fish exposed to MC-LR and *Microcystis*. Additional testing of these primer sets with cDNA from fish exposed to MC-LR and *Microcystis* should help resolve this question.

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SECTION V

Conclusion

Toxin-producing *Microcystis* blooms are prevalent in many aquatic ecosystems throughout the world. Fish play an important role in maintaining the stability of these ecosystems while serving as a food source for human populations through commercial and recreational fishing. The objective of this research was to gain a better understanding of the sublethal toxicity of these blooms in fish during all life stages in order to make inferences about how *Microcystis* exposure may impact fish populations in the wild and to evaluate MC-LR bioaccumulation in fish tissues. A further objective was to identify genes that could be used as biomarkers of microcystin exposure in fish and design primer sequences for assessing expression of these genes in channel catfish.

First, toxicity was evaluated in early life stages of fish by exposing larval zebrafish to purified MC-LR and *Microcystis*. Significant mortality and developmental effects did not occur; however, changes in global gene expression were observed and indicated differences in toxicity between the MC-LR toxin and *Microcystis*. Of particular interest among genes responding only to *Microcystis* were vitellogenin genes (*vtg*), which are indicators of exposure to estrogens in fish. Induction of *vtg* in larval zebrafish was unexpected and indicated that *Microcystis* blooms may produce substances that mimic estrogen. These findings indicate the need for a shift in bloom management strategy away from measuring toxin concentrations to *in vivo* effects-based monitoring in fish that takes into account both toxin-associated effects as well as responses attributed to other compounds produced by *Microcystis*, such as vitellogenin induction. To this end, vitellogenin genes, as well as toxin-related genes induced by *Microcystis*, were identified in

zebrafish as potential biomarkers of exposure and were later adapted and validated for use in an environmentally relevant species (channel catfish).

Effects of chronic *Microcystis* exposure were evaluated in adult fish and reproductive effects were also assessed. *Microcystis* did not affect mortality of adult zebrafish, and histological lesions in the liver typically associated with *Microcystis* exposure were not observed. Differences in toxin concentration and composition between lyophilized *M. aeruginosa* and *Microcystis* blooms used in previous studies may account for the absence of histological effects. A significant decrease in the percent of pairs that spawned was noted, indicating potential for *Microcystis* to interfere with reproductive ability in adult fish. Impairment of spawning did not translate into effects on fecundity or embryo survival, however. Further reproductive testing with environmentally relevant species is necessary. If decreased spawning activity is confirmed, this finding could have serious implications for the stability of fish populations in aquatic ecosystems affected by *Microcystis* blooms.

Channel catfish were exposed to MC-LR and *Microcystis* through the diet and to *Microcystis* by aqueous administration. These exposures did not result in mortality or hepatic lesions. Mortality was not expected, however other investigators have found necrosis and lipidosis in other species of fish exposed to *Microcystis* via the diet. Microcystin concentrations in these studies were higher than those administered to catfish and may explain why hepatic lesions were not observed. Catfish were also collected from Waterville Reservoir and Lake Erie, and histopathology indicated lesions in the liver, but none that could be attributed to microcystin. Results of preliminary toxin analysis of muscle and liver tissues from laboratory and field-collected catfish are inconclusive at this time. Primer sets for biomarker genes identified in

zebrafish were designed for channel catfish. Out of seven genes attempted, primer sets for three of these genes (vitellogenin, nephrosin, hepatocyte growth factor activator inhibitor 2) were validated for use in quantitative PCR assays. Future development of these biomarker gene expression assays for channel catfish has the potential to serve as a biomonitoring tool to assess the health of channel catfish living in areas affected by *Microcystis* blooms.

Vita

Emily Dawn Rogers was born on March 10, 1980 in Pinehurst, North Carolina. She grew up in Clinton, South Carolina and attended Erskine College where she received a Bachelor of Science degree in Biology in 2002. She earned a Masters degree in Toxicology from the University of Georgia in 2004 and a Ph.D. in Natural Resources from the University of Tennessee in 2010.