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Cardiotoxicity associated with exposure to *Microcystis* in early life stage fishes

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Abstract of the Thesis

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Microcystis is one of the most common and frequently occurring cyanobacteria that cause harmful algal blooms in the world. Incidences and toxicity of blooms have been increasing in recent decades, a trend that is likely to continue due to anthropogenic actions, thus making characterization of effects important for predicting outcomes. Aquatic organisms are at a high risk for toxic effects, and early life stage (ELS) fishes are likely to represent a very susceptible member of this community. My thesis evaluated the effects of *Microcystis* on embryos of the Japanese medaka (*Oryzias latipes*), a common model fish, using bath exposures to field collected *Microcystis* blooms, cultured *Microcystis*, and isolated microcystin-LR (MC-LR) to provide a comparative assessment and give insight into possible mechanisms of bloom toxicity. This approach provides more environmentally relevant exposure conditions as compared to previous studies where effects were evaluated using pure MC-LR that was often introduced by microinjection. My research identified heart rate depression as the most sensitive toxic endpoint in ELS medaka exposed to *Microcystis*. In addition, field collected bloom samples were found to

cause the most severe effects, and exposures to whole cell algae (from either lab cultures or field collected blooms) depressed heart rate more than exposures to MC-LR alone when normalized to MC content. Further investigation of the cardiotoxicity resulting from exposure to algal cultures indicated that depressed heart rate was associated with the occurrence of apoptosis in the heart region. Apoptosis appeared to be a transitory phenomenon and generally was not observed several days after treated embryos were removed from *Microcystis* exposure. In contrast, elevated oxygen consumption rates and decreased body lengths appeared to be more lasting effects associated with *Microcystis* exposure. Exposure at later developmental stages (6 vs. 1 days post fertilization) had more lasting effects than early exposures. Future studies are needed to: 1) determine the mechanisms associated with increased apoptosis and reduced heart rate after exposure to *Microcystis*; 2) examine what factors associated with natural blooms and algal cultures contribute to toxicity other than the presence of MC; and 3) evaluate how these ELS effects may influence further development, growth, and survival at later life stages.

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List of Abbreviations

BA: bulbus arteriosus

CyanoHABs: Cyanobacterial harmful algal blooms

dph: days post hatch

dpf: days post fertilization

ELS: early life stage (e.g. embryo and larva)

MC: Microcystin

MC-LR: Microcystin-LR

SV: sinus venosus

Acknowledgements

Chapter 1

Introduction

Cyanobacteria have a long evolutionary history that have allowed for the development of diverse adaptations and strategies that make them successful in a wide range of ecosystems throughout the world (Paerl and Paul 2012). These adaptations allow for cyanobacterial species to outcompete other algal species and dominate phytoplankton assemblages under certain conditions, resulting in Cyanobacterial Harmful Algal Blooms (CyanoHABs). CyanoHABs have been increasing in occurrence in recent decades, thought to be largely due to anthropogenic actions that increase temperature and add limiting nutrients to bodies of water (Carmichael et al. 2001, O'Neil et al. 2012, Paerl and Huisman 2008, Paerl and Paul 2012). CyanoHABs are likely to continue increasing in severity and occurrence in coming years as climate change and eutrophication continue. Therefore, determining the effects of blooms on other aquatic species is important for developing management efforts and for making predictions of ecosystem outcomes in future climate scenarios.

Microcystis is one of the most frequently occurring species causing CyanoHABs worldwide (Fristachi et al. 2008). *Microcystis* has been previously described as a group of species, but recent work has shown that intraspecies variability exceeds interspecies variability in the previously defined groups, indicating it is more conservative to collectively refer to all species as *Microcystis* (Harke et al. 2016). *Microcystis* blooms often reach densities exceeding 100,000 cells mL^{-1}, which is a commonly used regulatory threshold that triggers closure of the water body for drinking water and recreational use (WHO 2003). The primary risk associated with *Microcystis* blooms is tied to their production of a group of heptacyclic peptides called microcystins (MCs). Evaluating the risk of *Microcystis* blooms is complicated because blooms

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are comprised of varying proportions of toxic and nontoxic strains (Carmichael 1994) and the

primary purpose of MC production to *Microcystis* is not yet known, although several theories

exist: iron acquisition, protection from light irradiation, colony formation, competition, and

grazing deterrence (Holland and Kinnear 2013). Estimating concentrations of MC in a bloom can

be difficult to predict. Blooms can have harmful effects in addition to MC production because of

the rapid accumulation of biomass at the surface of the water which can reduce penetration of

light to the benthos, decrease dissolved oxygen levels during decomposition, remove nutrients

for other beneficial phytoplankton species, and reduce the nutritional quality of food source for

higher organisms (Ger et al. 2010). MC is thought to be the predominant cause of *Microcystis*

bloom toxicity though, which has made it the target for toxicological studies.

There are over 80 known congeners of MC, all with the same base structure and a unique

-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) side chain (Rinehart

et al. 1988). Congeners differ due to two variable amino acids at positions X and Z in Figure 1

(Butler et al. 2009).
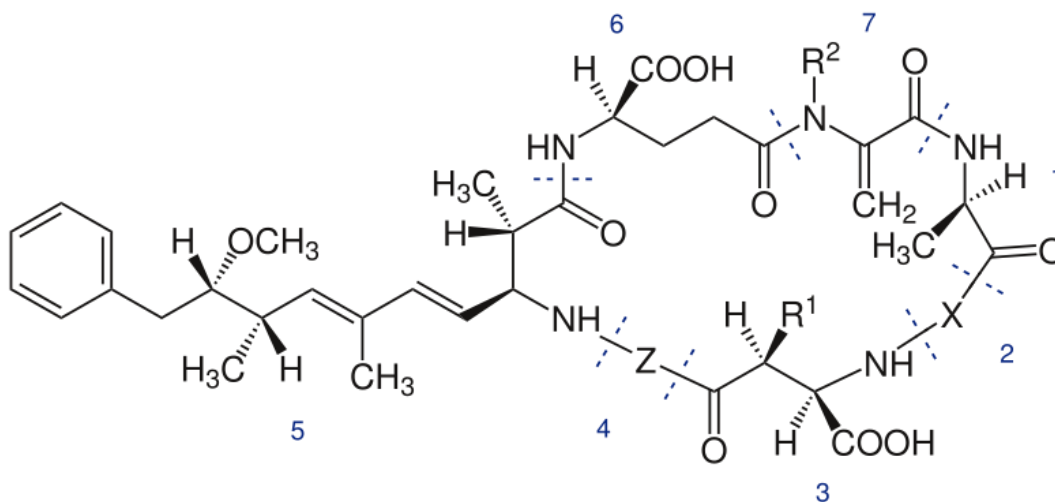
Figure 1: Structure of MC with variable X and Z amino acids positions

The three most common congeners of MC are MC-RR, MC-YR, and MC-LR, with their respective amino acids indicated in Table 1. MC congeners differ in their degree of toxicity, (also described in Table 1), which further complicates defining the toxicity of *Microcystis* blooms since each bloom has a unique proportion of MC congeners present. MC-LR is the most toxic congener identified thus far and generally, is the most frequently measured congener. Therefore, MC-LR is often the focus of toxicological studies on *Microcystis* blooms and is used by the World Health Organization to set drinking limits of 1 µg/L (Hotto et al. 2007, WHO 1999).

Table 1: Common MC Congeners: Structure and toxicity

Congener	Amino Acid at position X	Amino Acid at position Y	LD_{50} (mice studies) (Gupta et al. 2003)
MC-LR	Leucine (L)	Arginine (R)	43.0 µg/kg
MC-RR	Arginine (R)	Arginine (R)	235.5 µg/kg
MC-YR	Tyrosine (Y)	Arginine (R)	110.6 µg/kg

MC is an established hepatotoxin in vertebrates, including humans (Carmichael 1994). The predominant mechanism of MC toxicity has been determined using terrestrial organisms. When MC enters the body through consumption of algal cells or prey items containing MC, it is released in the stomach or intestinal region, and then is transported by bile carriers to the liver where it accumulates (Runnegar et al. 1981). MC causes hyperphosphorylation in liver cells by inhibiting protein phosphatases 1 and 2A, resulting in apoptosis, loss of liver structure,

hemorrhaging, and, at high exposures, death (Honkanen et al. 1990). MC likely has greater

toxicity in aquatic organisms however, due to their chronic exposure and multiple routes of entry

for the toxin (e.g. integument, ingestion, and gills).

Many toxic effects have been reported in response to MC exposure in adult and juvenile

fish. Fish exposed to MC-LR had altered antioxidant systems (Li et al. 2003), abnormal behavior

(Baganz et al. 1997), and dose dependent mortality (Jang et al. 2011). More severe effects were

observed in fish exposed to *Microcystis* blooms, including high incidences of histopathological

changes in the liver and kidneys (Fischer et al. 1999, Mitsoura et al. 2013), compromised

immune response (Liu et al. 2014), disrupted osmoregulation (Best et al. 2003, Bury et al. 1995),

and reduced growth (Acuna et al. 2012, Bury et al. 1995). These effects not only impact survival

of the affected fish, but have also been shown to have the potential to affect future generations of

fish by affecting the quality of embryos produced by exposed adults (Liu et al. 2014).

Fish directly exposed to *Microcystis* blooms during development or early life stages

(ELSs) are likely more susceptible due to their greater surface area to volume ratios, decreased

immune response capabilities, higher metabolic rate, and limited mobility. Studies on the effects

of blooms on ELS fish have predominately used MC-LR, reporting fish with irregular hatch

times (Jacquet et al. 2004), gross morphological deformities (i.e. curved body and tail) (Wang et

al. 2004), inhibition of critical regulators of development (Wang et al. 2004), absent or decreased

size of digestive organs (Huynh-Delerme et al. 2005), and tubular heart, poor yolk sac resorption,

and small head (Liu et al. 2002). These studies suggest that *Microcystis* blooms will have severe

impacts on developing fish. However, the results of these studies are not directly applicable to

the field since pure toxin was used rather than whole algal cells and microinjection was often the

route of exposure as opposed to bath culture, thereby discounting limitations due to

bioavailablity associated with more natural exposures. Two studies have shown the enhanced toxicity of developing fish exposed to cyanobacterial extracts compared to MC-LR alone (Ghazali et al. 2009, Oberemm et al. 1997). Another study showed the differential transcriptomic responses of zebrafish larvae exposed to high or low MC-LR as compared to exposure to *Microcystis* cells (Rogers et al. 2009). Collectively these studies suggest that whole cell cultures will likely have more severe impacts on the development and survival of ELS fish than MC-LR treatments alone. These effects are important to determine since toxic effects from algal cells could impact success of fish at population levels as well, which could have implications for ecosystems as a whole.

Recruitment of ELS fish to the population is predominately based on mortality and growth of developing fish, rates of which are both high and variable at early life stages. Cushing (1975) found a link between mortality and growth rates in early life stage fish and Peterson and Wroblewski (1984) later determined the numerical relationship between body weight (W) and mortality rate (M) in developing fish, $M = W^{-0.25}$. The negative relationship between body size and mortality has been observed in experiments with ELS teleosts (Bailey and Houde 1989), although others have shown that the relationship originally determined by Peterson and Wroblewski varies from species to species and that power coefficients are generally much greater than -0.25 (Houde 1997). This suggests that size plays a much greater role in mortality than previously thought. Therefore, reductions in body size or longer time spent in the larval stage could cause mortality greater than theory or experimental survival would suggest during bloom events.

The goal of my thesis research was to characterize effects of *Microcystis* blooms on the development of fish using more environmentally relevant exposure methods to make results

more applicable to the field. My experiments used embryos of the model freshwater fish species, the Japanese medaka (*Oryzias latipes*) and bath cultures as the route of exposure. Medaka were selected because of their past success in studies on developing vertebrate systems (Shima and Mitani 2004, Wittbrodt et al. 2002). In addition, their transparent chorions facilitates tracking stages throughout development, and longer embryonic stages compared to other model species (9-11 days for medaka versus 3 days for zebrafish) make timing of experiments more flexible. Medaka embryos were submerged in either field collected blooms from New York City lakes or *Microcystis* cultures grown in the lab as well as MC-LR solutions to independently evaluate the effects of the toxin alone, *Microcystis* cells, and natural blooms. Natural blooms represent a complex toxicant mixture, since multiple species of phytoplankton are present (although *Microcystis* was the dominant species in all samples used) that could produce additional toxins, and other toxic substances could also be present in natural waters. To determine effects of *Microcystis* alone, cultures of the algae were isolated and cultured from phytoplankton assemblages in Lake Erie for exposures. Exposures to MC-LR solutions allowed the effects from algal cells and extracts to be separated from the effects of MC-LR exposure. Collectively, these three exposure types allowed for the characterization of effects from *Microcystis* blooms to be determined as well as see if MC-LR is the primary toxin exhibiting effects on developing fish or if other bloom components also play a role. I hypothesized that if MC-LR was the primary cause of effects observed, then MC-LR exposures would be sufficient to describe outcomes of a bloom event but, if the algal cells also played a role, then exposures should be done with whole cell algae in order to determine environmentally relevant effects.

In addition to this introductory chapter, my thesis contains two data chapters to be submitted for publication and one final summary chapter that discusses the overall significance

of my work and logical next steps. The first manuscript compares the effects of natural blooms, *Microcystis* cultures, and MC-LR toxin exposures on developing medaka embryos. The second manuscript begins to look at the specific changes that *Microcystis* cultures have on the heart by examining heart looping, apoptosis in cardiomyocytes, and heart rate. Oxygen consumption rates and body length were also quantified in fish exposed to blooms to see if *Microcystis* exposures had the potential to affect bioenergetics of fish. The capacity for fish to recover from *Microcystis* exposures was also investigated by submerging fish in cultures during early and late development and then allowing embryos to recover in clean solution for several days. This work shows how developing fish are affected by exposure to natural *Microcystis* blooms as well as demonstrating the extent to which MC exposures are effective in describing the full extent of effects on developing fish. My studies also identify early endpoints of exposure and begin to explore possible causes and effects of these endpoints.

Effects of *Microcystis* on development of early life stage Japanese medaka (*Oryzias latipes*): I. Comparative toxicity of natural blooms, cultured *Microcystis* and microcystin-LR

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Abstract

Freshwater cyanobacterial harmful algal blooms (CyanoHABs) caused by algae in the genus *Microcystis* have been increasing in frequency and severity in recent decades. *Microcystis* blooms threaten aquatic organisms through impacts associated with the effects of the rapid increase of biomass (light attenuation, subsequent oxygen depletion due to decomposition of algae, reduced food quality, and competition for nutrients with more beneficial algae) and the production of the hepatotoxin microcystin (MC) by toxic strains. Among fish, effects of blooms are likely to be more severe for early life stages, and physiological impacts on this life stage could significantly impact recruitment and fish populations. This study explores effects of *Microcystis* blooms on the development of fish using the model organism, the Japanese medaka

(*Oryzias latipes*) under realistic exposure conditions. Medaka embryos were exposed to natural

blooms collected from New York City (USA) lakes, *Microcystis* lab cultures, and MC-LR

solutions. Field collected samples were more toxic than lab cultures (even when compared at the

same algal density or MC concentration), causing decreased survival, premature time to hatch,

reduced body length, yolk sac edema, and decreased heart rate while lab culture exposures only

resulted in bradycardia. Heart rate was the most sensitive endpoint measured, being depressed in

embryos exposed to both lab cultures and field collected blooms. Generalized linear model

analysis indicated bradycardia was statistically associated with both cell densities of blooms and

MC concentrations, while single factor analysis showed that MC concentrations had a stronger

correlation compared to cell densities. However, MC exposure could not fully explain the effects

observed, as exposures to MC-LR alone were not able to reduce heart rate as severely as algal

exposures. Collectively these experiments indicate that factors beyond exposure to MC or even

pure *Microcystis* lab cultures influence heart rate. Enhanced mortality and negative effects on

heart rate and development observed in response to environmentally realistic exposures of

Microcystis blooms could affect success of fish at individual or population levels.

Keywords: *Microcystis*, medaka, microcystin, natural bloom, embryo, development

1. Introduction

Cyanobacterial harmful algal blooms (CyanoHABs) are a public health and ecological

threat that has intensified in recent decades due to rising temperatures and increased nutrient

inputs, processes expected to worsen with climate change and eutrophication (O'Neil et al. 2012,

Paerl et al. 2011, Paerl and Huisman 2009). One of the most common freshwater CyanoHAB

species is *Microcystis aeruginosa*, which is found worldwide (Fristachi et al. 2008, Harke et al.

2016). Throughout this paper we will refer to this CyanoHAB as *Microcystis* since recent work

recognized that intraspecies variability was higher than the variability measured across *Microcystis* species, suggesting that all previously named *Microcystis* species be grouped in the same species (Harke et al. 2016). *Microcystis* blooms are problematic due to the rapid increase of algal biomass at the surface of the water limiting sunlight and with their decomposition potentially reducing dissolved oxygen concentrations. Cyanobacteria are not known to represent a high quality food source for higher organisms (Ger et al. 2010), and their use of available nutrients can limit growth of more beneficial algae. In addition, toxic strains of *Microcystis* produce a suite of monocyclic heptapeptides called microcystins (MCs) (Carmichael 1994). MCs are endotoxins, meaning that they are released upon cell lysis or cell death, making end of bloom exposures potentially more toxic. Specifically with *Microcystis*, bloom growth and toxin production are promoted by elevated temperature (Conradie and Barnard 2012, Davis et al. 2009) and nutrient concentrations (Davis et al. 2009, Harke and Gobler 2013, Horst et al. 2014), meaning that future climate conditions and eutrophication could promote both growth and toxicity of blooms (O'Neil et al. 2012).

Toxicity of *Microcystis* blooms has predominately been evaluated by examining the effects of just one of its toxins, MC-LR, considered the most toxic and frequently encountered congeners of MC (Van de Waal et al. 2009). MCs have been established as hepatotoxins in vertebrates, including humans (Butler et al. 2009). Studies primarily conducted on terrestrial vertebrates have demonstrated that MC inhibits protein phosphatases 1 and 2A, causing hyperphosphorylation in cells of exposed tissue that can lead to apoptosis, hemorrhaging, and in severe cases, death (Honkanen et al. 1990, Runnegar et al. 1981). Aquatic organisms are likely to suffer additional, and possibly more severe, effects due to their chronic exposure as well as the multiple routes of entry possible for the toxin (e.g. ingestion of cells or infected prey, direct

uptake by gills, or through their integument). Some studies have examined the effects of MC-LR on adult and juvenile fishes, showing altered antioxidant systems (Li et al. 2003), dose dependent mortality, and MC accumulation throughout the body (Jang et al. 2011). Effects can be more severe when fish are exposed to toxic strains of *Microcystis*, leading to increased incidences of histopathological changes in the liver and kidneys (Fischer et al. 1999, Malbrouck and Kestemont 2006, Mitsoura et al. 2013), compromised immune function (Liu et al. 2014), disrupted osmoregulation (Best et al. 2003, Bury et al. 1995), and reduced growth (Acuña et al. 2012, Bury et al. 1995). Toxic effects are likely to be more severe in early life stage (ELS) fish (i.e. embryos and larvae) due to their less developed immune systems, larger surface area to volume ratios, increased metabolism, and limited ability to control their position in water column (Pavagadhi and Balasubramanian 2013).

Several studies have begun to examine the effects of *Microcystis* blooms on development of ELS fish and have found more dramatic effects than were seen in adult and juvenile fish including imbalances in critical regulators of development (e.g. PP1 and 2A, β-catenin, and cadherins) (Wang et al. 2004), absent or decreased size of digestive organs (Huynh-Delerme et al. 2005), gross morphological deformities including curved body and tail (Wang et al. 2004), and tubular heart, poor yolk sac resorption, and small head (Liu et al. 2002). These effects were determined by exposing ELS fishes to isolated MC-LR toxin, often with microinjection as the route of exposure. There have been limited studies examining effects of cyanobacterial extracts on ELS fish, both showing greater toxicity than MC-LR exposures alone (Ghazali et al. 2009, Oberemm et al. 1997). A transcriptomic study using zebrafish larvae revealed that fish had differential responses when exposed to *Microcystis* blooms or high or low concentrations of MC-LR toxin (Rogers et al. 2011). Therefore, studies using isolated MC-LR toxin can suggest

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possible effects of *Microcystis* blooms and mechanisms of toxicity, but results may not necessarily reflect natural exposures.

This study examines effects of *Microcystis* blooms on the development of ELS fish using submersion as the route of exposure. We feel this approach provides an exposure pathway that is more environmentally realistic as compared to dosing regimens used in the preponderance of previous studies which examined effects on ELS fish resulting from exposure to pure MC toxin and/or dosing through microinjection. Japanese medaka (*Oryzias latipes*) were used for experiments due to their history as a model organism for vertebrate systems (Shima and Mitani 2004, Wittbrodt et al. 2002). Medaka also have transparent chorions that facilitate tracking developmental progress and longer development time compared to other fish models (9-11 days for medaka vs. 3 days for zebrafish) that allows for flexibility in timing of experiments. Embryos submerged in *Microcystis* lab cultures or field collected blooms allow for natural interactions of the algae with the chorion of embryos, better mimicking what developing fish are exposed to in an ecosystem setting. *Microcystis* lab cultures and field samples from natural *Microcystis* blooms were examined to isolate possible effects due to the *Microcystis* alone as well as determine impacts from whole blooms. Finally embryos were also exposed to isolated MC-LR solutions to determine if effects could be fully attributed to the presence of the toxin or if other algal components may contribute to toxicity.

2. Material and Methods

2.1 Experimental fish

Medaka were maintained in tanks at 28°C and 14:10 hr light: dark cycles during spawning periods. Fish were fed Aquatox flakes (Pentair Aquatic Eco-Systems, Apopka, FL)

twice per day, and hatched brine shrimp (Brine Shrimp Direct, Odgen, UT) once in the morning. Embryos were collected 1-2 hours after the brine feeding, and cultured in embryo rearing medium (ERM - 17 mM NaCl, 0.40 µM KCl, 0.36 mM $CaCl_2$, 0.66 mM $MgSO_4$-$7H_2O$) at 25°C. ERM was amended with methylene blue (0.0002%) for the first 24 hours to minimize fungal growth. At 1 day post fertilization (1 dpf), embryos were rinsed three times in normal ERM (with no methylene blue) and kept in normal ERM until the start of an experiment. Maintenance of medaka brood stock was carried out under Stony Brook University's IACUC approved protocol 1470 to A. McElroy.

2.2 Experimental design

Healthy fertilized embryos were selected at 1 dpf for experiments. Single embryos were individually exposed to 2 mL of their treatment solution in 4 mL glass vials and maintained in an incubator at 25°C and 12 hr:12 hr light dark cycle. Each experiment had a control group where embryos were individually exposed to 2 mL of ERM in 4 mL glass vials and maintained in the same incubator with treatment embryos. Experiments had 10-15 embryos per treatment as specified in Table 1 for each experiment. Embryos that were assessed for all endpoints (survival, time to hatch, body length, edemas, gross morphological abnormalities, and heart rate) were exposed to solutions for 15 days (1-16 dpf). Treatment solutions were not renewed throughout the experiments and larvae were not fed post hatch since experiments were terminated before or within a day of complete yolk absorption. Heart rate was measured at 6 dpf, as this is a critical stage of cardiac development in medaka. Heart rate was the only endpoint assessed in MC-LR exposures, so these experiments lasted only 5 days (1-6 dpf). Heart rate of each embryo was recorded for 15 seconds in duplicate using light microscopy. Survival was assessed for the duration of the experiment, 1-16 dpf for *Microcystis* exposures and 1-6 dpf for MC-LR

exposures. Body length, gross morphological abnormalities, and edema occurrence were assessed at 1 day post hatch (1 dph) using light microscopy.

2.3 Microcystis and MC analysis

Lab culture experiments used a *Microcystis* clone (LE-3) collected and isolated from Lake Erie (Brittain et al. 2000) and maintained at 21°C in BG-11 medium. Preliminary experiments revealed that BG-11 growth media for algae was toxic to developing fish unless diluted at least ten-fold (data not shown). Since dilutions would limit the density of algae able to be tested, BG-11 was removed by centrifuging lab cultures at 3300 rpm for 15 minutes using a Clay Adams Dynac centrifuge (Becton, Dickinson and Company, Parsippany, NY), and the algal cells were resuspended in ERM (Harke and Gobler, 2013). Dilutions of the resuspended cells were used for lab culture experiments. *Microcystis* densities were quantified on a sample of each resuspended culture after fixation with Lugol's Solution using a Beckman Coulter Multisizer 3 Coulter Counter, with algal counts verified in random samples using a gridded Sedgewick-Rafter. MC concentrations were determined using a commercial Microcystins (Adda specific) Enzyme-Linked Immunosorbent Assay (ELISA – Abraxis, Warminster, PE), which quantifies all MC congeners present in the sample. MC was evaluated only once per samples as preliminary work demonstrated minimal variability among replicate analyses, with an average percent standard deviation of 3.5% between replicates (Table S-1).

Water samples for natural bloom experiments were collected from the edge, and in some cases the middle of the Lake in Central Park in Manhattan and Prospect Park Lake in Brooklyn in NY, USA, by the New York City Parks Department. Samples were analyzed using a FluoroProbe (bbe Moldaenke GmbH, Schwentinental, Schleswig-Holstein, Germany), which differentiates groups of algae (blue-green algae, green algae, brown algae) based on

photosynthetic pigment fluorescence. FluoroProbe readings provided concentrations of total

blue-green algae and microscopic evaluation indicated *Microcystis* was the dominant

cyanobacteria present in samples unless otherwise indicated. The concentration of microcystins

where quantified as above. Samples used for medaka exposure experiments were stored at room

temperature and used without further treatment within 72 h of collection. In some experiments, a

simulated end of bloom sample was also prepared by lysing algal cells by rapid mixing bloom

water for 5 minutes with a Vortex Genie (Fisher Scientific, Bohemia, NY), and then

homogenizing using a rotor/stator tissue homogenizer (The Virtis Company, Gardiner, NY).

Estimated densities of natural blooms were converted from Fluoroprobe measurements to cells

mL^{-1} using a conversion factor determined from preliminary experiments where lab culture

samples were measured with the Coulter Counter and FluoroProbe and plotted to find the

relationship between the two quantitative measurements (Figure S-1).

For experiments with pure toxin, MC-LR was obtained from Cayman Chemical

Company (Ann Arbor, Michigan). Stock solutions were maintained in ethanol at -20ºC. For

each experiment an aliquot of the stock was taken to dryness and resuspended in ERM. MC-LR

concentrations of each test solution were determined using the Microcystins ELISA described

above.

2.4 Imaging

Images for body length, gross morphological abnormalities, and edema occurrence were

taken using a Nikon i80 microscope (Morrell Instruments, Melville, NY). Body length was

measured using images on the Photo Measure app for Windows (Braddock Software Ltd.) while

gross morphological abnormalities and edema occurrence were qualitatively recorded.

2.5 Statistics

All statistics were performed using R statistical project (R Core Team 2015). Heart rate and body length data were tested for normality and equal variances and then were analyzed using ANOVA and Tukey's post hoc analysis. Survival and time to hatch differences were examined using Kaplan Meier analysis with statistical differences determined with a log rank test. Yolk sac edema and gross morphological deformity occurrences were analyzed using logistic regressions. Generalized linear models (GLMs) were fit to the raw heart rate data as a function of MC concentration, *Microcystis* density, and bloom type (lab culture or natural bloom) of experiments. Models were compared using corrected Akaike Information Criteria (AIC$_C$). Data were considered statistically significant at $p < 0.05$. Significance was lowered to $p < 0.017$ in cases of multiple comparisons, for survival and time to hatch data where natural blooms and simulated end of blooms were being compared. Significance cutoffs are indicated in figure legends.

3. Results

3.1 Properties of Microcystis laboratory cultures and natural blooms

Cell densities of *Microcystis* in experiments with laboratory cultures ranged from 13 to 46 x 10^6 cells mL^{-1} with MC concentrations ranging from 690 to 7800 µg L^{-1} (Table 1). MC per cell differed in the two experiments (0.05 vs. 0.2 ng MC/cell in culture experiments 1 and 2 respectively). Field collected *Microcystis* samples from natural blooms in the Lake in Central Park and Prospect Park Lake in New York City were generally more dense than laboratory cultures (Table 1). *Microcystis* was the dominant species in all samples except for one, PPL2, where another toxic CyanoHAB, *Anabaena spp.*, was also found in high abundance (Table 1).

Cyanobacterial cell densities ranged from 1.0 to 244 x 10^6 cells mL^{-1} with MC concentrations ranging from 120 to 7000 µg L^{-1} (Table 1). Blooms from the Lake in Central Park were, on average, more toxic (higher MC per cell ratios) than blooms collected from Prospect Park Lake; averaging 0.09 ± 0.04 vs 0.02 ± 0.02 ng MC per cell for the Lake in Central Park and Prospect Park Lake respectively (Table 1).

3.2 Exposures to Microcystis laboratory cultures

Exposures to isolated *Microcystis* lab cultures did not affect medaka embryo survival. Survival to 16 dpf was always greater than 87% and averaged 91.5% for both lab culture experiments (Table S-2). Time to hatch and body length were only assessed in first culture experiment. Embryos had similar hatching times across treatments, with hatching occurring between 11-14 dpf (Figure S-2). No statistical differences in body length were observed across treatments, with length averaging 4.6 mm at 1 dph (Table S-3). Hatched embryos also showed little to no incidence of gross morphological abnormalities or yolk sac edema. Gross morphological abnormalities (i.e. body curvature, bent tail) only occurred in 3 of 120 embryos tested in culture experiments and there was no incidence of yolk sac edemas (data not shown).

In contrast to the parameters described above, embryos exposed to *Microcystis* cultures displayed a dose dependent decrease in heart rate compared to control embryos (Figure 1A and B). The second culture experiment was conducted at higher MC concentrations than the first culture and elicited a stronger response (Figure 1). Where MC concentrations between the two experiments were similar (2300 and 2400 µg L^{-1} MC for the first and second experiment respectively), heart rates for embryos in the first experiment were lower than heart rates of embryos in second experiment. This is likely due to the difference in algal density, as at similar MC concentrations algal cell density was approximately 3.5 times greater in the first experiment.

Thus, heart rate depression appears to be associated with a factor tied to the *Microcystis* cells beyond that captured by the MC concentration alone. There was no evidence of delayed development in any embryos exposed to cultures that would account for bradycardia observed.

3.3 Exposures to natural blooms

Natural bloom experiments with water from the Lake in Central Park (CPL) and Prospect Park Lake (PPL) were more toxic to embryos than algal cultures. Exposure to blooms with MC concentrations as low as 250 µg L^{-1} or blue-green algae concentrations as low as 56.3 ± 0.3 x10^6 cells mL^{-1} resulted in complete, and very rapid (sometimes within 24 hrs), mortality of all exposed embryos. Therefore, survival was the only endpoint that could be assessed for experiments CPL1, CPL3, and PPL2 (Figure 2A, C, D). Exposures with less concentrated bloom densities or lower MC concentrations, CPL2 and CPL4, had statistically significant decreases in survival for embryos exposed to blooms compared to control embryos (Figure 2B, E). In contrast to the other natural bloom samples, survival in embryos exposed to PP1 blooms appeared to be reduced as compared to survival in ERM, but this was not statistically significant different (Figure S-3).

MC is an endotoxin, therefore we hypothesized that toxicity might be enhanced by mechanically disrupting natural bloom samples to simulate end of bloom conditions in three of the six natural bloom experiments, CPL1, CPL2, and PPL1. Survival appeared to be reduced in embryos exposed to simulated end of bloom samples compared to unaltered natural bloom samples, however this reduction was not significantly different from that observed in the unaltered natural bloom samples (Figure 2D, E and S.2).

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Embryos exposed to natural blooms had statistically significant differences in hatching times in experiments CPL2 and CPL4 relative to their controls (Figure 3A&B). Hatching times were not statistically significant between embryos exposed to unaltered bloom samples and simulated end of bloom samples (Figure 3A). Prematurely hatched larvae showed no obvious signs of delayed or accelerated development relative to control embryos that could account for early hatching. Embryos in experiment PPL1 all had relatively similar hatching times despite treatment (Figure S-4). Control embryos were the only group to hatch in experiments CPL1, CPL3, and PPL2, thus time to hatch could not be analyzed for these experiments; however, controls in these experiments hatched in the same time range as was observed in control fish from other experiments (Figure S-5, S-6).

Body length at 1 dph was reduced in hatched embryos that were exposed to natural or simulated end of blooms compared to controls for two experiments, CPL2 and PPL1. However body lengths in natural or simulated end of bloom samples were not significantly different from each other (Table 2). In contrast, exposure to natural blooms from CPL4 had no effect on body length (Table S-4). Model analysis using GLMs did not reveal significant relationships between either MC concentration or *Microcystis* density, while MC per algal cell was found to correlated best with the reduced body length (data not shown).

Yolk sac edemas were observed in all three natural bloom experiments where treatment embryos hatched (CPL2, CPL4, and PPL1 – Table 3). Edema occurrence was quantified at 1 dph for each embryo, making sample sizes for treatment groups much smaller than control groups due to reduced survival. To account for this, edema occurrence from natural and simulated end of blooms was combined to generate a sufficient sample size to support statistical analysis. Edema occurrence was significantly increased after exposure to bloom samples, being observed

in only 0 to 8% of control embryos as compared to 48 to 86% of embryos exposed to algal

blooms. Although edemas were commonly observed in bloom-exposed embryos, other gross

morphological abnormalities were rare. Only one bent tail and one curved body were observed

in the 195 embryos examined in the six field sample experiments.

Bradycardia was observed in all bloom-exposed embryos surviving past 6 dpf when heart

rate was quantified (CPL2, PPL1, and CPL4 – Figure 4). There were no significant differences in

heart rates of medaka exposed to natural blooms compared to those exposed to simulated end of

blooms. Heart rate reductions from all experiments were correlated (as determined by the GLM

described below) with the concentration of MC and *Microcystis*. Control embryos from all six

experiments had heart rates that were not statistically different from one another.

3.4 Bradycardia across experiments

Since heart rate was the most sensitive endpoint assessed, having decreased in embryos

exposed to both culture and field sample blooms, GLMs were used to determine the parameters

that best predicted this trend. Models were fit to the raw heart rate data of all experiments,

considered the influence of all measured components (MC concentration, *Microcystis* density,

and bloom type (culture or field sample)) and were compared using AIC values (Table 4). For

field collected bloom experiments, only hearts rates of unaltered natural blooms were considered.

As expected, the full model with all three components fit the data best, showing that all three

variables are important to consider when predicting bradycardia in exposed embryos. Two

models, one with the two factors, *Microcystis* density and MC concentration, and another with

MC concentration as a single factor, ranked a distant second to the full, three parameter model,

but fit equally well to each other. This supports the hypothesis that cell density and MC

concentration are both important factors in bloom toxicity, but that MC alone can predict the heart rate decrease adequately.

3.5 The role of MC in bradycardia

AIC analysis indicated that MC alone could predict the decrease in heart rate as well as the model considering both MC and *Microcystis* densities, suggesting that the presence of MC in blooms was the primary cause of the bradycardia observed. To test this hypothesis, experiments were conducted to assess heart rate in embryos exposed to MC-LR solutions to see if cardiotoxicity was predominately induced by MC or if the presence of algal cells or other lake components also contributed to the effect. Embryos were exposed to MC-LR in ERM solutions at concentrations in the range of those seen in culture and field collected bloom experiments (600 - 6300 µg L^{-1} MC) in two different experiments. Heart rates of embryos exposed to MC-LR solutions were statistically lower than their respective controls, but the treatments were not statistically different from one another, indicating a lack of dose response (Figure 6). A linear model was fit to MC-LR experiments to assess the trend in heart rate decrease across treatments and was compared to linear models for heart rate data from culture and field experiments with MC as a single factor (Figure 6). The linear models had the same intercept but slopes were significantly different, with steeper slopes observed in the culture and natural bloom model, suggesting that MC depresses heart rate but is not the sole cause of bradycardia. The significantly steeper slope associated with whole cell algae model indicates that additional factors associated with the presence of *Microcystis* cells also contribute to the decrease in heart rate in developing medaka exposed to blooms.

3.6 Algal cell – chorion interactions during exposures

Exposure type, either natural bloom or lab cultures, resulted in different interactions of the algal cells with the embryo chorions. Culture experiments had few algal cells attach to the chorions of exposed embryos (Figure 7B), while the higher cell densities and greater proportion of cells in the colonies of field collected bloom samples often coated the chorions turning them visibly green (Figure 7C). The chorions of the embryos exposed to natural blooms were weakened and broke open easily, whereas the embryos exposed to lab cultures were only slightly weakened by the algal cells. Control embryos remained spherical and hard throughout the experiments (Figure 7A).

4. Discussion

The experiments described directly compare effects of natural blooms to laboratory *Microcystis* cultures and isolated MC-LR in fish embryos exposed through bath culture, thus providing a comprehensive and environmentally realistic assessment of what embryonic and larval fish may encounter in the wild. Algal and MC concentrations examined in these field bloom experiments represent intense bloom conditions, although these concentrations are not outside the range of those reported in water bodies throughout the world. Similar cell counts have been documented for *Microcystis* blooms in lakes in New York State (Davis et al. 2009) and in water bodies in several countries including South Africa (Conradie and Barnard 2012), Brazil (Ferrão-Filho et al. 2002), China (Hu et al. 2016), and France (Vezie et al. 1998), among others (Harke et al. 2016). Thus, the effects identified in this study, reduced survival, premature hatching, reduced body length, edemas, bradycardia, are likely to be occurring in ELS fishes that are naturally exposed.

Natural bloom exposures resulted in reduced larval survival, premature hatching, yolk sac edema, and stunted growth, all of which could affect success of fish at the individual and

population level. Reduced survival was observed in experiments, with some embryos ceasing

development within the first day of exposure. *Microcystis* blooms within temperate latitudes can

be present from spring through fall, with cell densities and MC concentrations varying

throughout the season. The results from this study indicate that even exposure to dense blooms

for a few days is enough to delay development and result in mortality of developing fish. Years

with especially severe blooms, due to high temperature and/or increased nutrient loading (O'Neil

et al. 2012), could negatively impact fish recruitment, as discussed below.

Natural bloom exposures also resulted in early hatching of embryos. Premature hatching

has been reported in other work where medaka embryos were microinjected with isolated MC-

LR (Jacquet et al. 2004) at concentrations much lower than those assessed in this study. Early

hatching could occur because of stress on the fish or because of impacts on structures that control

hatching. Other ELS fish studies have reported hatching gland edemas in zebrafish microinjected

with MC-LR (0-900 nM) (Wang et al. 2004) that could cause irregular hatching times. Other

work found that microinjections of MC-LR (of 0.2 pg/vitellus) cause yolk sac edemas along with

inhibition of yolk sac resorption in injected embryos (Huynh-Delerme et al. 2005), which would

limit access to nutrients and could also contribute to early hatching. Yolk sac edemas were

caused by exposure to natural *Microcystis* blooms in this study, suggesting that limited access to

nutrients could be the cause of early hatching seen with bloom exposed embryos. Reduced

nutrient availability could also explain the reduced body length seen in fish exposed to blooms.

Smaller body sizes were seen in other studies where ELS fish were exposed by bath culture to

MC-LR (0-5.0 mg L^{-1}) (Zeng et al. 2014) as well as in adult and juvenile fish exposed to

Microcystis blooms (Acuña et al. 2012, Bury et al. 1995). Reduced body size could alternatively

be explained by reallocation of energy from somatic growth to another vital life process. Yolk

sac edemas could be an indication of inhibited osmoregulation, since fish are hyperosmotic compared to their freshwater environment and difficulty controlling influx of water would cause fluid to accumulate, potentially causing an edema (Hill et al. 2004). Inhibited osmoregulation was reported in adult fish exposed to *Microcystis* extracts or cells (Best et al. 2003, Bury et al. 1995). In the experiments described here, the timing of the measurement may have also contributed to the smaller body length observed, since this measurement was taken at 1 dph and some bloom-exposed embryos hatched days earlier than controls. The smaller size at hatching would put fish at a greater risk in the natural environment, making them more susceptible to predation (Ware 1975).

Microcystis cultures were less toxic to developing fish than field collected bloom samples at similar *Microcystis* cell densities and MC concentrations. Field samples were collected from natural blooms occurring in eutrophic lakes, where excess nutrients in the water, especially nitrogen, could enhance *Microcystis* toxicity by promoting production of MC as has been observed under high nitrogen conditions (Horst et al. 2014, Harke and Gobler 2015). The behavior of *Microcystis* cells in natural bloom conditions could also have contributed to the enhanced toxicity observed in embryos exposed to natural blooms as compared to laboratory algal cultures. In the field, *Microcystis* forms colonies, while in lab cultures cells tend to remain as single cells (Bolch and Blackburn 1996). The colonies of cells may make the cells more likely to attach to the chorions and could cause deterioration of the chorion structure from their presence alone or from another mechanism like bacterial growth. The weakening of the chorion due to MC-LR exposure alone has been previously reported (Song et al. 2011) and the additional stress caused by other factors associated with algal cells in the current study likely exacerbates this effect. Attachment of cells to the chorion could further weaken the protective barrier, which

may contribute to the early hatching seen in some high density natural bloom exposures. Embryo chorions were greatly compromised by exposure to natural blooms while those exposed to lab cultures were only slightly affected. Other causes of enhanced toxicity from field collected blooms could be the presence of other components in the lake sample that were not quantified in this experiment including other CyanoHAB species, pollutants present in lake water, or other harmful substances produced by *Microcystis* or other algal cells.

Reduced heart rate was observed in embryos exposed to both field collected blooms and cultures, making this the most sensitive endpoint assessed in this study. Significant reductions in heart rate were seen at 1,400 µg L^{-1} MC and could contribute to the other effects reported by reducing blood flow, thus inhibiting transport of nutrients or oxygen to developing regions of the body. Cardiotoxicity was observed in other experiments with ELS zebrafish exposed to MC-LR solutions (Zeng et al. 2014) or crude *Microcystis* extracts (Oberemm et al. 1997). Altered heart rates were also seen in studies examining adult and juvenile fish exposed to *Microcystis* blooms, as reviewed by Malbrouck and Kestemont (2006), as well as in some rat and mice studies examining effects of MC-LR (Leclaire et al. 1995, Qiu et al. 2009). Cardiotoxicity could cause downstream implications in the development of the fish, as was seen with zebrafish embryos exposed to polycyclic aromatic hydrocarbons where cardiac dysfunction led to a suite of morphological abnormalities (Incardona et al. 2004).

Simulated end of bloom treatments were hypothesized to cause more severe effects since lysing of the cells should release more MC and other toxic components. Cells in the simulated end of bloom studies were notably affected by lysing steps, all algae sunk in vials while algal cells from unaltered bloom samples floated on top of the water in the vial. Yet no statistically significant differences in ELS medaka responses were observed between those exposed to

natural bloom and simulated end of bloom samples in the three experiments where this endpoint was evaluated (CPL1, CPL2, PPL1). Apparently sufficient MC release occurred from the apparently intact cells in our experiments to elicit the toxic responses observed.

The ability of isolated MC-LR toxin to produce bradycardia was not dose dependent, appearing to level off at higher doses, with no statistically significant differences observed among toxin doses. Based on the GLM models, MC likely contributes to the bradycardia observed in both natural bloom and algal culture experiments, but additional factors are likely also contributing, particularly at higher algal concentrations. The observation that field collected blooms caused a greater decrease in heart rate and mortality and other developmental toxicities at lower MC concentrations than the algal cultures also supports this hypothesis. Other potential factors include the presence of other algal or bacterial species, other algal toxins from *Microcystis* or other toxic species, different congeners of MC, other algal or bacterial exudates, or additional chemical or biological properties of the lake water or other contaminants. Clearly more work should be done to identify other factors beyond MC concentrations influence ELS toxicity in organisms exposed to natural *Microcystis* blooms. At the very least, algal densities should be evaluated in addition to MC when assessing risks associated with *Microcystis* blooms to the aquatic community.

Effects of blooms on development of individual fish observed in this study are likely to impact fish at the population level as well. Japanese medaka are a model organism that show many similarities to higher vertebrate organisms as well as other fish species (Furutani-Seiki and Wittbrodt 2004, Shima and Mitani 2004, Wittbrodt et al. 2002). The process of heart development is driven by networks of transcription factors that are evolutionarily conserved (Olson 2006), so perturbations to heart development are likely to be observed in other species.

Reduced cardiac function in ELS fish is expected to affect success of individual fish in the environment by reducing blood flow that could alter further development. Populations of fish are also likely to be affected, since year classes are predominately controlled by mortality and growth rate and it has been shown that even small changes in these factors cause disproportionate changes in recruitment that can be orders of magnitude in effect (Houde 1997). Size and growth rate of fish strongly influence mortality rates (Ware 1975), making premature hatching and reduced body length seen in these experiments a problem. Other developmental abnormalities, like edemas or heart dysfunction, could impact success in the environment.

5. Conclusions

This study is the first to our knowledge to directly compare the toxicity of natural blooms and algal cultures of *Microcystis* and exposure to pure MC-LR exposure through environmentally realistic bath cultures. Identifying cardiotoxic effects as the most sensitive endpoint indicates that MC and algal cells could impact the heart before the more well recognized mechanism of hepatotoxicity, an effect derived primarily from research on terrestrial organisms, is activated. Poor heart development is problematic to ELS fish and could lead to downstream problems as development continues. Effects like premature hatching or small body length after hatch could cause problems in success of fish in either foraging for prey or escaping predation (Ware 1975). Impacts on ELS fish could be bottlenecks for recruitment success with decreased performance upon hatch being problematic for population maintenance. This study shows that field collected blooms cause more severe developmental effects and can yield complete mortality and more extreme decreases in heart rate at MC concentrations and algal cells densities less than or similar to those in culture experiments, and emphasizes the importance of

considering whole algae samples rather than isolated MC-LR when deducing implications of a *Microcystis* blooms.

Acknowledgements

Figure Legends

Figure 1: Effect of *Microcystis* culture exposures on medaka embryo heart rate for culture 1 (A) and 2 (B) experiments. Letters represent statistically significant differences in heart rate of each treatment group, determined with nested ANOVA followed by Tukey's post hoc test.

Figure 2: Effect of *Microcystis* field collected blooms on medaka embryo survival in experiments where embryos were just exposed to natural blooms (A-C) and natural and simulated end of blooms (D & E). Kaplan-Meier analysis and log rank tests were used for analysis. A Bonferroni correction was used to correct for multiple comparisons in experiments testing the effects of both natural and simulated end of blooms (D&E), lowering the *p*-value threshold to 0.017.

Figure 3: Cumulative embryo hatch as a function of time in two experiments, CPL2 (A) and CPL4 (B). Kaplan-Meier analysis and log tank test were used to determine significance of hatching times, with a Bonferroni correction to correct for multiple comparisons in CPL2 experiments with both natural and simulated end of blooms, lowering the *p*-value to 0.0017.

Figure 4: Heart rate changes in 6 dpf medaka exposed to natural and simulated end of blooms. Statistical significance was determined using nested ANOVA and Tukey's post hoc analysis.

Figure 5: Model comparison of fit for individual parameters, MC concentration in $\mu g\ L^{-1}$ (A) and *Microcystis* density in cells/mL (B). Plotted points and error bars are the mean heart rate for each MC concentration with standard deviation. Mean linear fits and 95% confidence intervals are illustrated by the black lines and shaded gray area, respectively.

Figure 6: Model fits for heart rate decrease comparing MC concentrations ($\mu g\ L^{-1}$) from algal experiments and from isolated MC-LR experiments. Plotted points and error bars are the mean heart rate for each MC concentration with standard deviation. Model fits are shown by the solid lines, with 95% confidence intervals illustrated by the shaded area.

Figure 7: Representative embryos showing chorion structure after exposure to ERM (A), high density culture (B), and high density natural bloom (C). All images were taken at the same magnification, the scale bar in panel C represents 250 µm. Embryos are at different orientations, the control is from a head on angle (A), while the two treatment embryos are at side angles (B, C). Large dark spots are the embryo eyes.

Figure S-1: Relationship of Fluoroprobe (µg L^{-1}) to Coulter Counter (cells mL^{-1}).

Figure S-2: Cumulative time to hatch for culture 1 experiment. Kaplan-Meier and log rank analyses were used to determine statistically significant groups.

Figure S-3: Survival curves for PPL1 experiment. Kaplan-Meier and log rank analyses were used to determine statistically significant differences in treatments.

Figure S-4: Cumulative number of fish hatched over time for PPL1 experiment. Kaplan-Meier and log rank analyses were used to find statistically significant changes in hatching time.

Figure S-5: Cumulative time to hatch for CPL1 experiment. No treatment fish hatched during the experiment, although control fish still hatched within the same time period as controls from other experiments.

Figure S-6: Cumulative time to hatch for experiments CPL3 and PPL2. No treatment fish hatched during this experiment but control fish hatched within the same time period as control fish from other experiments.

Figures and Tables – Chapter 2

Table 1: Summary of algal density and total MC concentration for experiments. Each experiment had a respective control (0 cells/mL algae, 0 µg/L MC), not shown here.

	Experiment	Dilutions	Total MC (µg/L)	*Microcystis* (10^6 cells/mL)
Culture Experiments	Culture 1	1x	690	14 ± 2
		2x	1400	28 ± 3
		3x	2300	46 ± 5
	Culture 2	1x	2400	13 ± 1
		2x	4700	24 ± 3
		3x	7800	40 ± 5
	Experiment	**Date collected**	**Total MC (µg/L)**	***Microcystis* (10^6 cells/mL)**
Field Experiments	CPL1 (E)	30-Jun-15	3400	56.3 ± 0.3
	CPL2 (E)	7-Jul-15	120	1.3 ± 0.1
	CPL3 (E)	21-Jul-15	7000	132.5 ± 0.7
	CPL4 (M)	21-Jul-15	140	1.0 ± 0.1
	PPL1 (E)*	6-Jul-15	250	6.7 ± 0.1
	PPL2 (E)	20-Jul-15	2500	244 ± 1
	Experiment	**Dilutions**	**Total MC (µg/L)**	***Microcystis* (10^6 cells/mL)**
MC-LR Experiments	Culture 1	1x	600	NA
		2x	1200	NA
		3x	1800	NA
		4x	2400	NA
	Culture 2	1x	800	NA
		2x	1900	NA
		3x	6300	NA

CPL = Central Park Lake, PPL = Prospect Park Lake
E = collected from edge of lake, M = collected from middle of lake
*Presence of another dominant blue-green species (*Anabaena spp.*) in addition to *Microcystis*

Figure 1

(A)

Culture 1

(B)

Culture 2

Figure 2

(A) CPL3

Surviving Embryos / Days Post Fertilization

(B) CPL4

Surviving Embryos / Days Post Fertilization

(C) PPL2

Surviving Embryos / Days Post Fertilization

(D) CPL1

Surviving Embryos / Days Post Fertilization

(E) CPL2

Surviving Embryos / Days Post Fertilization

Treatments

— Control
- - Natural Bloom
····· Simulated End of Bloom

n = 15 each treatment
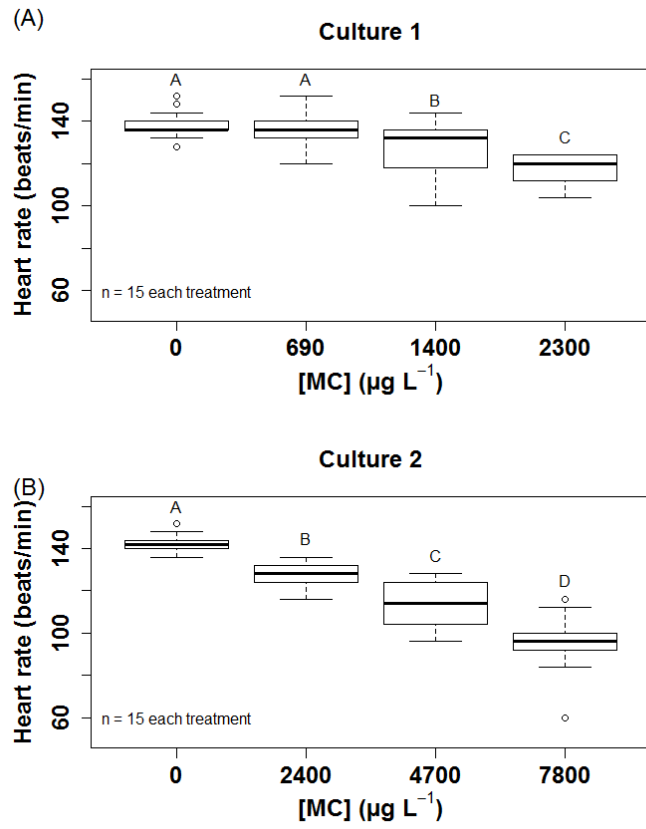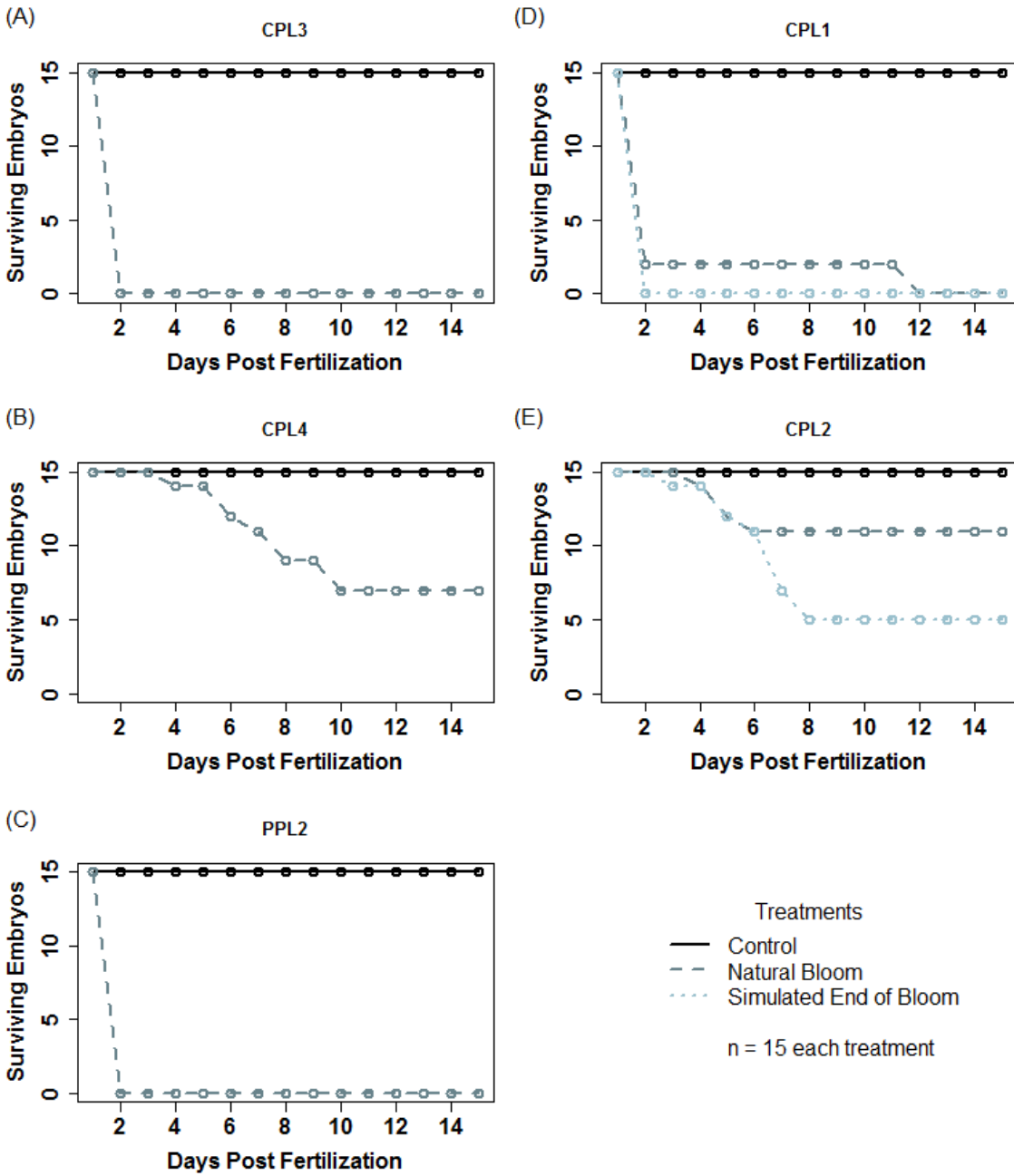
Figure 3
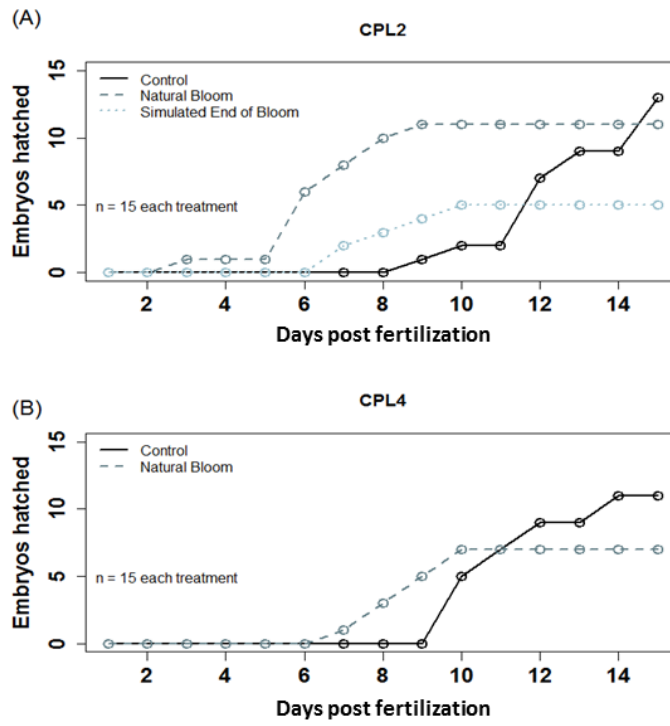
Table 2: Effect of exposures on body length of 1 dph medaka in experiments CPL2 and PPL1. Statistical significance was determined using nested ANOVA followed by Tukey's post hoc test.

Experiment	Treatment	Body length ± SD (mm)	n
CPL2	Control	4.18 ± 0.78	12
	Natural Bloom	3.83 ± 0.78 *	9
	End of Bloom	3.82 ± 0.80 *	7
PPL1	Control	4.45 ± 0.45	13
	Natural Bloom	4.13 ± 0.56 *	11
	End of Bloom	4.16 ± 0.64 *	9

* Statistically different from respective control ($p < 0.05$, nested ANOVA)

Table 3: Incidence of yolk sac edemas in 1 dph embryos. Statistical significance was determined using binomial logistic regression analysis.

Experiment	Treatment	Edema occurrence	n
CPL2	Control	7.7%	13
	Bloom	81.3% *	16
CPL4	Control	0.0%	11
	Bloom	85.7% *	7
PPL1	Control	0.0%	13
	Bloom	47.6% *	21

* Statistically different from respective control ($p < 0.05$, nested ANOVA)

Figure 4
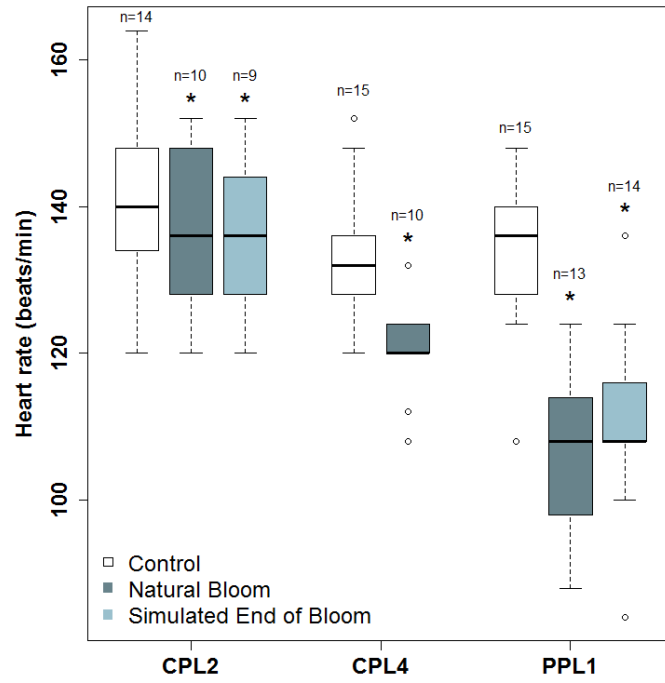
Table 4: Model comparison for heart rate decreases and measured algae components.

Model	AICc	ΔAICc	Model Rank
Heart rate ~ *Microcystis* + MC + Bloom type	3246	0	1
Heart rate ~ *Microcystis* + MC	3324	78	2
Heart rate ~ MC	3326	80	2
Heart rate ~ *Microcystis*	3355	109	4
Heart rate ~ Bloom type	3505	259	5

Bloom type = natural bloom or culture

Figure 5

(A)

MC Model Fit - Algae Data

(B)

***Microcystis* Fit - Algae Data**

Figure 6

Heart rate (beats/min) vs log([MC])

- All algae experiments (NB+CUL)
- Pure MC-LR experiments
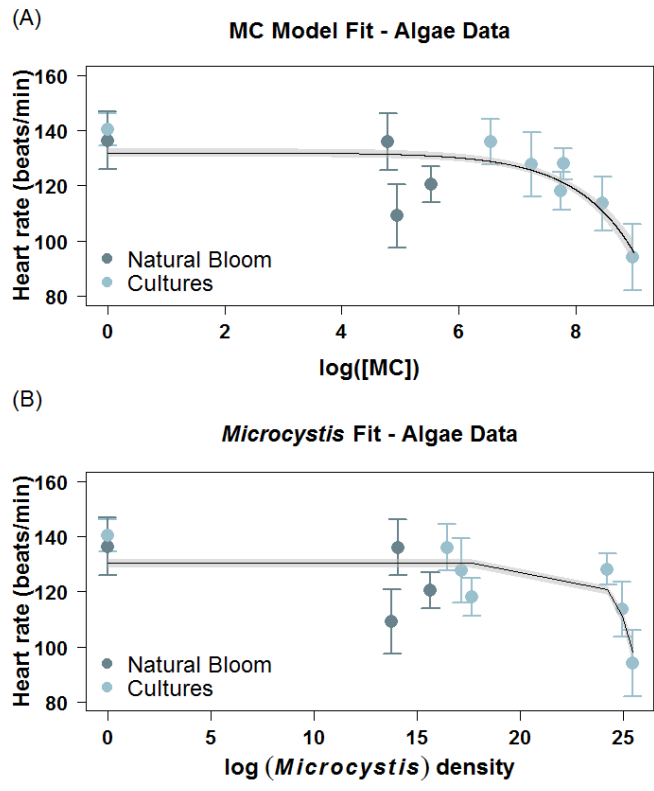
Algae lm fit: -0.0045 (± 0.0003) x [MC] + 132.0 (± 0.8)
MC-LR lm fit: -0.0032 (± 0.0003) x [MC] + 131.5 (± 0.6)
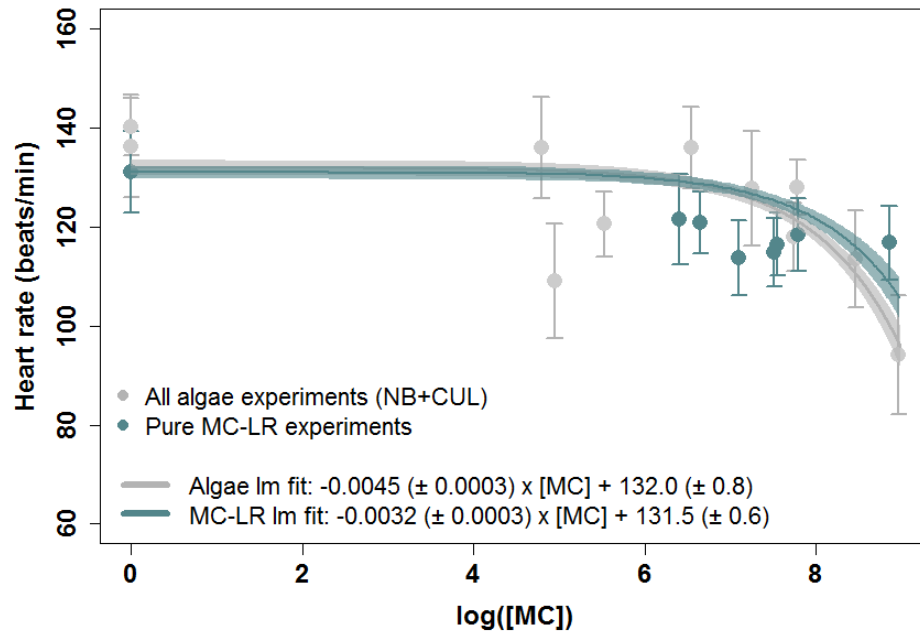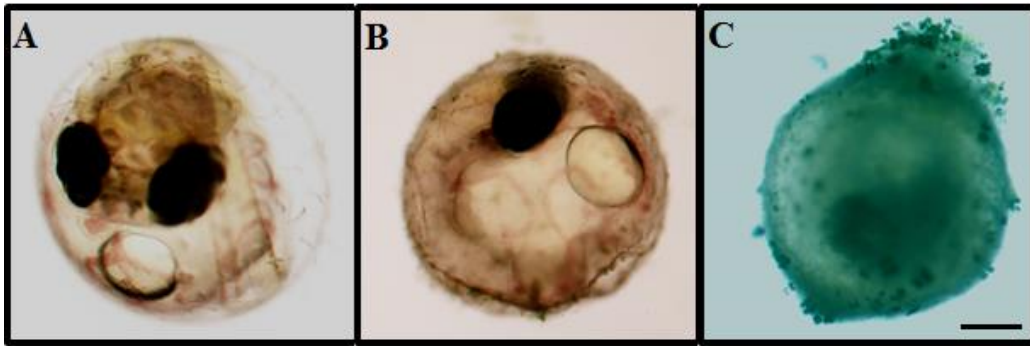
Figure 7

Supplemental Figures and Tables – Chapter 2

Figure S – 1

Fluoroprobe to cells mL^{-1} Relationship

$y = 2764.4x - 5077.8$
$R^2 = 0.9954$

x-axis: Fluoroprobe count (μg L^{-1})
y-axis: cells mL^{-1} count
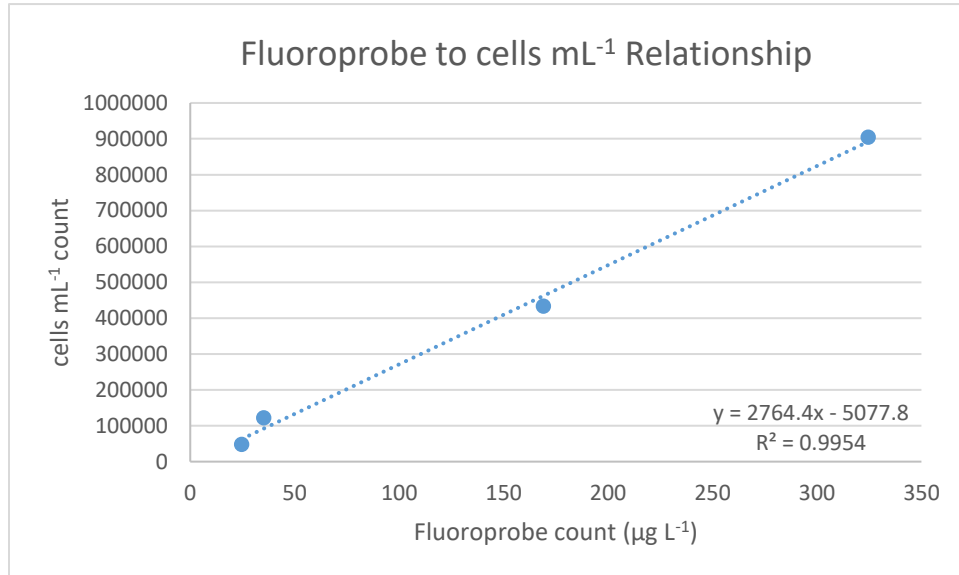
Table S-1: MC quantification with ELISA assay showing the consistency of the samples run on ELISA. Data are from an earlier experiment for preliminary work not included in this study.

Sample	Run	MC (μg L^{-1})
1	1	941.
	2	834.
	3	836.
2	1	1070
	2	1180
	3	1170
3	1	718.
	2	756.
	3	748.
4	1	384.
	2	394.
	3	398.
5	1	192.
	2	193.
	3	195.

Table S-2: Culture 1 and 2 survival for exposures. Kaplan-Meier and log rank tests were used to indicate statistical differences between treatments.

Experiment	~ Dilutions	% Survival (of 15 embryos)
Culture 1	Control	93.3%
	1x	86.6%
	2x	93.3%
	3x	93.3%
Culture 2	Control	100%
	1x	100%
	2x	93.3%
	3x	93.3%

Figure S-2
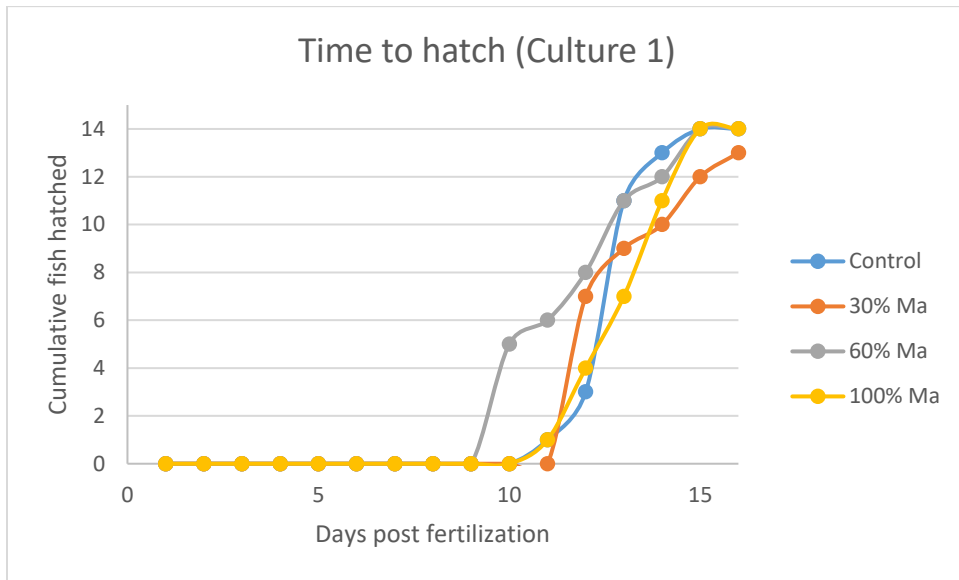
Table S-3: Culture 1 body length for exposures. ANOVA analysis was used to determine statistically significant differences between groups.

Treatment	Body length ± SD (mm)	n
Control	4.61 ± 0.12	14
1x *Microcystis*	4.58 ± 0.18	12
2x *Microcystis*	4.64 ± 0.23	12
3.3x *Microcystis*	4.53 ± 0.15	14

Figure S-3

PPL2

Surviving Embryos

Days Post Fertilization

Control
Natural Bloom
Simulated End of Bloom

Time to hatch (PPL1)

Time to Hatch (CPL1)

Figure S-6

Time to hatch (PPL2, CPL3)
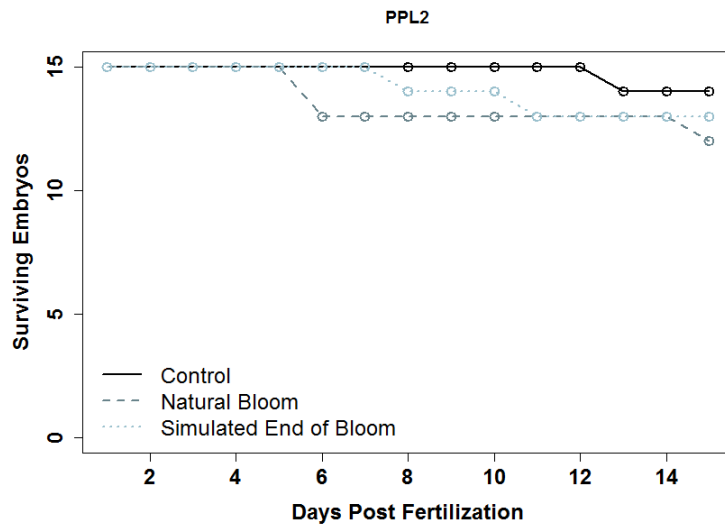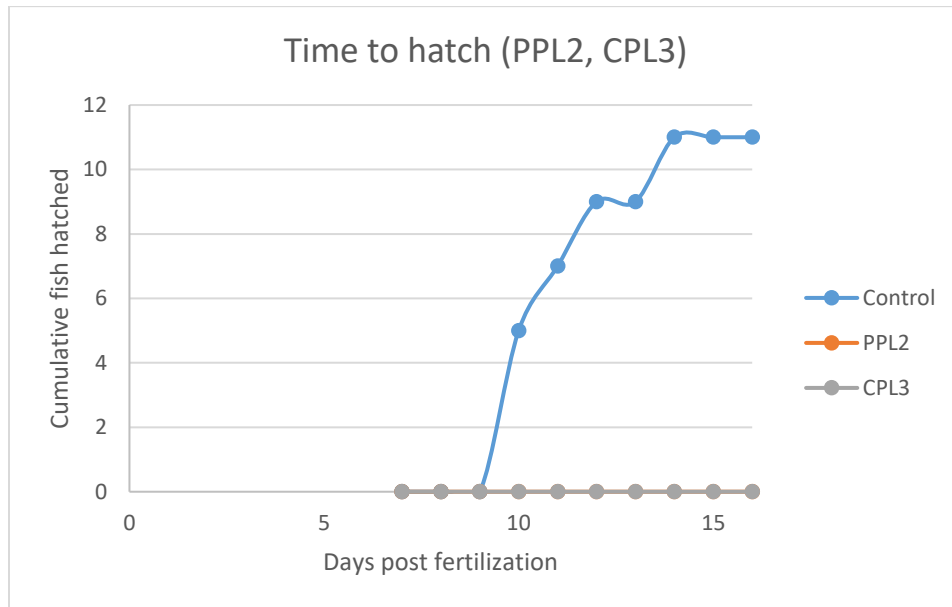
Table S-4: Body lengths of hatched embryos exposed to CPL4 bloom. ANOVA analysis indicated there was no statistical difference between treatments.

Treatment	Body length ± SD (mm)	n
Control	4.53 ± 0.70	14
CPL4 algae	3.90 ± 0.48	12

Chapter 3

Effects of *Microcystis* on development of early life stage Japanese medaka (*Oryzias latipes*):

II. Effects associated with cardiotoxicity and recovery

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Abstract

Microcystis is a common freshwater cyanobacterial harmful algal bloom (CyanoHAB) that is becoming more toxic and pervasive due to intensifying eutrophication and increasing temperature. *Microcystis* blooms are problematic because of the rapid increase in biomass of a single algal species as well as the production of the toxin microcystin (MC). ELS fish are generally more susceptible to effects of toxins, and effects on these stages will likely have negative impacts on recruitment of fish populations. While MC is an establ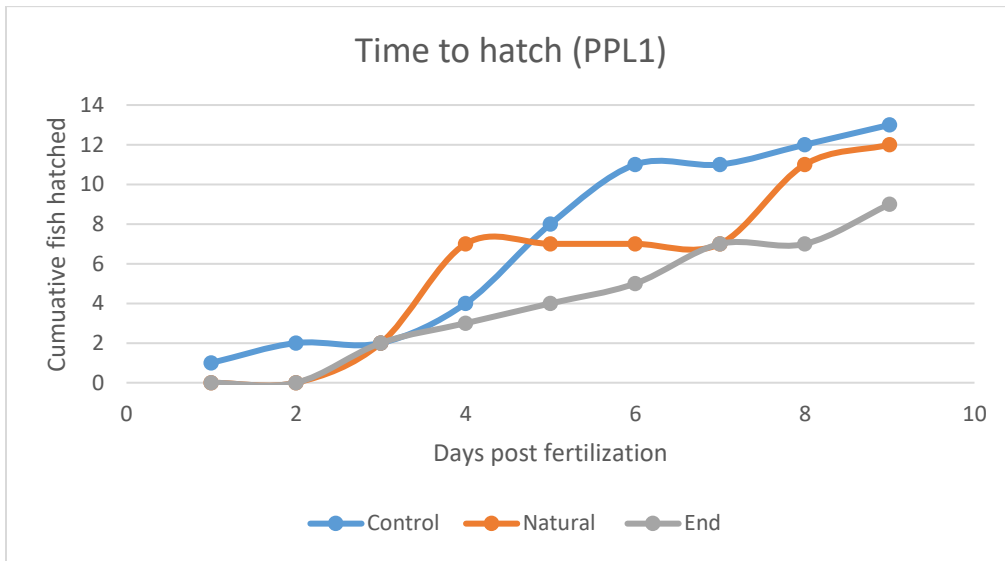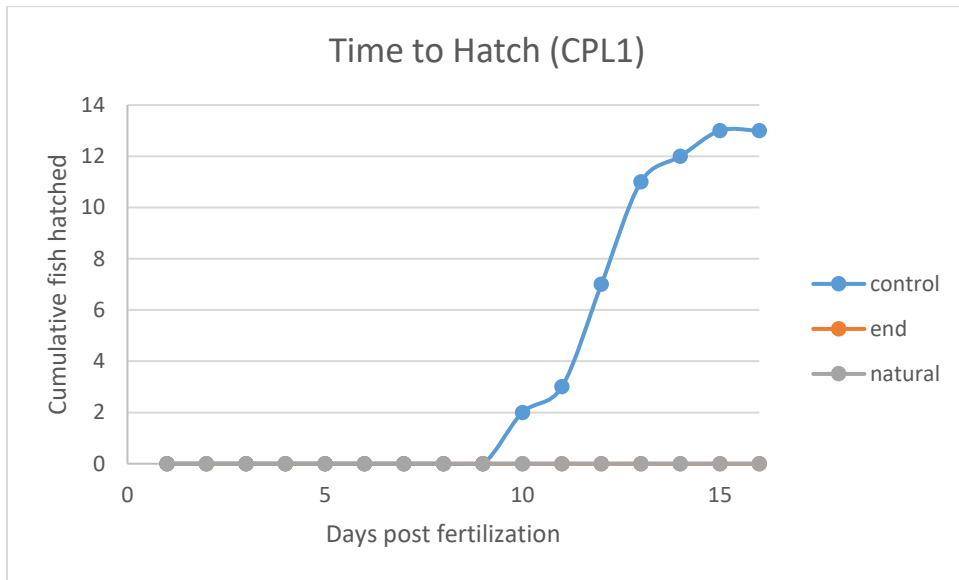ished hepatotoxin in vertebrate species, studies on ELS fish have indicated that cardiac effects (i.e. bradycardia) is a sensitive and early response to *Microcystis* exposure. This study further examines cardiac effects of *Microcystis* cultures on the developing heart of Japanese medaka. Apoptosis was found to be associated with bradycardia in embryos exposed to *Microcystis*, indicating its involvement in the

decreased heart performance seen in exposed embryos. Patterns of elevated oxygen consumption and reduced body length were also observed, indicating that exposure to *Microcystis* may negatively affect bioenergetics of exposed fish. Experiments examining recovery after removal of *Microcystis* and comparing responses and recovery after early (1-6 days post fertilization-dpf) and late (6-10 dpf) exposure times, demonstrated that heart rate and the presence of apoptotic cells returned to unexposed levels rapidly, indicating these responses are transient effects associated with *Microcystis* exposure. In contrast, later exposures were associated with more persistent effects on heart and oxygen consumption rates and body length even 5 days after exposure ceased, indicating effects that could carry on to later life stages. This study identifies apoptosis as a component in early onset cardiotoxicity associated with *Microcystis* exposure and indicates these effects may be associated with more persistent physiological effects on energetics and growth of larval fish.

1. Introduction

Microcystis is one of the most common freshwater cyanobacterial species, with blooms occurring worldwide (Harke et al. 2016). Distributions and severity of blooms are expected to increase with anthropogenic activity in coming years (O'Neil et al. 2012, Paerl et al. 2011), making it important to understand the potential effects of bloom events on ecosystems and human health. *Microcystis* blooms can have negative effects on aquatic species due to the rapid accumulation of biomass in the water, increased light attenuation, decreased oxygen concentrations from algal decomposition, and decreased abundance of more beneficial species in phytoplankton assemblages. In addition, toxic strains of *Microcystis* produce the heptacyclic peptide microcystin (MC - Carmichael 1994). Over 80 congeners of MC have been identified thus far with MC-LR being the most toxic congener, making it the most well studied. MC is a

known hepatotoxin in terrestrial vertebrates (Butler et al. 2009), although exposures are likely to have more severe and extensive effects on aquatic organisms due to their chronic exposure and multiple routes of exposure (e.g. integument, gills, ingestion). Adult and juvenile fish exposed to *Microcystis* exhibit histopathological changes in the liver and kidneys (Fischer et al. 1999), reduced growth (Acuna et al. 2012, Bury et al. 1995), altered immune function (Liu et al. 2014), and disrupted osmoregulation (Best et al. 2003, Bury et al. 1995). Early life stage (ELS) fish are known to be more susceptible than older organisms with more severe effects like decreased size or absent digestive organs (Huynh-Delerme et al. 2005), imbalanced developmental regulators (e.g. PP1 and 2A, β-catenin, and cadherins) (Wang et al. 2004), and tubular heart, poor yolk sac resorption, and abnormal morphological development (Liu et al. 2004) resulting from MC-LR exposure. Bradycardia in ELS zebrafish in response to exposures to MC-LR (Zheng et al. 2014) or cyanobacterial extracts (Oberemm et al. 1997) has been reported, and more recently observed in embryos of the Japanese medaka (*Orysias latipes*) in response to exposure to MC-LR, cultured *Microcystis*, or field collected blooms of *Microcystis* in our laboratory (Saraf et al. in review). More importantly, heart rate depression was observed in response to more environmentally relevant exposures (emersion in field collected natural blooms of algal cultures as opposed to injection studies with MC toxin), and cardiac effects have been observed without any gross morphological incidences or other deleterious effects described in other ELS studies using MC-LR (Oberemm et al. 1997, Saraf et al. in review). Therefore, cardiac effects now appear to be one of the earliest endpoints associated with exposure to *Microcystis* in ELS fish.

The mechanism of MC cardiotoxicity in ELS fish has not yet been fully determined. MC-LR is known as a hepatotoxin, acting through inhibition of protein phosphatase 1 and 2A (PP1 and 2A) (Hokanen et al. 1990, Runnegar et al. 1981). However, it is not likely that this

mechanism is the cause of depressed heart rate since inhibition of PP1 and 2A has been shown to enhance rather than depress pacemaker activity in guinea pig cardiomyocytes exposed to another known inhibitor, okadaic acid (Neumann et al. 1993). Other possible mechanisms have been proposed to explain MC-LR toxicity on the heart. Mitochondrial dysfunction in cardiac cells was reported in rats injected with MC-LR (Qui et al. 2009) and decreased heart rate in zebrafish larvae exposed to MC-LR was associated with the accumulation of reactive oxygen species and apoptosis in the heart (Zheng et al. (2014). While these studies provide possible explanations for the cardiotoxicity of MC-LR, other work has shown differential transcriptomic responses between fish larvae exposed to MC-LR or *Microcystis* extracts (Rogers et al. 2011). This makes it possible that other toxic mechanisms are associated with exposure to *Microcystis* and justifies the need to look beyond exposure to pure toxin when evaluating risks associated with exposures to *Microcystis* blooms in the environment.

This study begins to characterize the cellular physiological response associated with cardiotoxicity observed in ELS fish exposed to *Microcystis* algae. Japanese medaka were used in this study because of their history as a model organism for vertebrate systems (Shima and Mitani 2004, Wittbrodt et al. 2002) as well as their transparent chorions that facilitate viewing of developmental stages throughout the experiment, and their longer embryonic stage (10-12 days vs. 3 days in zebrafish) that provide more flexibility in experimental timing. Furthermore, previous studies in our laboratory had identified bradycardia as a sensitive endpoint in response of Japanese medaka to exposure to natural blooms and cultures of *Microcystis* as well as pure MC-LR (Saraf et al. in review). As was done on our previous study, responses of fish were assessed in embryos that were submerged in algal cultures to allow for natural interactions of the algae and toxin with the chorion, providing results that are more readily applicable to field

situations. Heart rate, heart length, apoptosis in heart tissue, body length, and respiratory rates were assessed in the present study. Heart rate was measured to verify that exposures were influencing cardiac function. Heart length—the distance from the sinus venosus (SV) to the bulbus arteriosus (BA)—was measured to indicate the extent of looping (i.e. natural transformation from the tubular to two-chambered heart) during development; inhibited looping would result in an elongated SV-BA length. Extent of apoptosis in the heart region, was also evaluated to see if enhanced programmed cell death is associated with decreased heart performance, as was suggested in previous studies with MC-LR (Zheng et al. 2014). Finally, oxygen consumption rate and body length were measured to determine if cardiac effects could cause alterations in whole larval bioenergetics. Oxygen consumption rates can be used as a proxy for metabolic costs (Wootton 1999), while reduced growth rate can indicate reallocation of energy from growth to metabolism or other processes. Body length is also important since reductions in size have been shown to significantly increase mortality rates (Houde 1997) and therefore, affect recruitment of a population. Endpoints were evaluated at different time points during exposure and after a recovery period to determine if there are developmental windows of altered sensitivity to *Microcystis* and to determine if effects observed are due to development or functional changes.

2. Materials and Methods

2.1 Experimental fish

Japanese medaka were maintained at 28°C and 14 hr: 10 hr light: dark cycles in tanks during the spawning period. Adult fish were fed three times per day: one morning and one afternoon feeding of Aquatox flakes (Pentair Aquatic Eco-Systems, Apopka, FL) and one morning feeding of hatched brine shrimp (Brine Shrimp Direct, Odgen, UT) given 1 hour after

Aquatox was offered. Embryos were collected from fish 1-2 hours after introduction of brine shrimp and were cultured at 25°C in embryo rearing medium (ERM - 17 mM NaCl, 0.40 µM KCl, 0.36 mM CaCl$_2$, 0.66 mM MgSO$_4$ 7H$_2$0) amended with methylene blue (0.0002%) for the first 24 hours to reduce fungal growth. Medaka brook stock maintenance was carried out under Stony Brook University's IACUC approved protocol 1470 to A. McElroy (IRB 206848).

2.2 Experimental design

All exposures were done using a *Microcystis* clone (LE-3) collected from Lake Erie assemblages, isolated, and maintained at 21°C in BG-11 medium. Preliminary experiments determined that BG-11 was toxic to developing fish (data not shown) so cultures were centrifuged at 3300 rpm for 15 minutes, the BG-11 media was removed, and pelleted algal cells were resuspended in ERM (Harke and Gobler 2013) prior to use in experiments. Under these conditions, percent survival to the end of the experiment (16 dpf for experiments 1 and 2, 15 dpf for experiments 3 and 4) averaged 90 ± 10 sd (n=4). Resuspended cultures were used for experiments not only allowing removal of the BG-11 media, but also allowing algal density to be manipulated. Algal cultures were resuspended to a standard color quantity for experiments, and then actual concentrations of algae and MC measured later as described below.

Embryos developing normally at 1 dpf were exposed to 2 mL of various treatment solution in individual 4 mL glass vials and incubated at 25°C with 12:12 hr light dark cycles. Survival was assessed throughout each experiment until 16 or 15 dpf, depending on the experiment, while other measures of effects were assessed only at earlier time points. Heart rate was measured at 6 dpf in each experiment, since this is when the heart is fully developed, and at day 10 and 16 in experiment 4 that assessed recovery potential of embryos. Heart rate measurements were done to verify the presence of measurable cardiac impacts. Four separate

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experiments were performed, each with different primary objectives, but all examining effects on heart rate and survival. Experiment 1 addressed the effects of *Microcystis* exposure on heart length at 6 dpf. Images were taken through the chorions of embryos, allowing them to normally develop until 16 dpf. Experiment 2 examined the effects of *Microcystis* exposures on apoptosis in the heart, body length, and respiration at 16 dpf. Experiments 3 and 4 assessed the potential of fish to recover from *Microcystis* exposures by exposing embryos for a period of time and then transferring them to clean ERM until the end of the experiment. Experiment 3 assessed the effects of early exposure (1-6 dpf) and long recovery (10 days in ERM alone) on body length, apoptosis, and oxygen consumption, which were measured at 6 and 16 dpf. Experiment 4 looked at the effects of both early (1-6 dpf) and late (6-10 dpf) exposure times and shorter recovery periods where one group of fish was exposed from 1 – 6 dpf followed by a recovery period from 6 – 10 dpf and another group was exposed from 6 – 10 dpf and recovered from 10 – 15 dpf. Early exposure embryos were studied until 10 dpf while late exposure embryos were studied until 15 dpf. Apoptosis, body length, and oxygen consumption rates were examined at 6 and 10 dpf for early exposure embryos and 6, 10, and 15 days for late exposure embryos. A subsample of embryos were removed from treatment groups at 6, 10, and 15 dpf for TUNEL analysis. A schematic describing these experiments and the analyses conducted is provide in Figure 1.

Heart rate was assessed by first equilibrating embryos to room temperature for 15 minutes prior to recording heart rate. Then, embryos were chosen at random and heart rate was counted twice for 15 seconds each using light microscopy. Embryos were not aestheticized for measurements. Heart rate was only done using embryos still in their chorions. Due to their ability to swim, heart rates of hatched larvae could not easily be assessed in unanesthetized fish.

Heart length was determined by measuring the distance from the SV to the BA in the heart at 6 dpf. Blood enters the heart through the SV (i.e. the pacemaker) where it is then pumped to the atrium, ventricle, and finally out the BA, which dampens the pressure of the blood before entering the gills. Larvae hearts were imaged twice at two magnifications to provide a more robust measure of length; these measurements were within 0.3 mm of one another. All measurements were averaged to provide the best estimate of heart length for each embryo.

Oxygen consumption rates were determined using a Loligo microplate respirometer (Loligo Systems, Viborg, Denmark). Embryos were rinsed in clean ERM and transferred to individual wells on plates that were filled with ERM. Then, wells were sealed off and oxygen concentrations were recorded every 15 seconds for approximately 1 hour. Oxygen consumption rates were determined by plotting oxygen concentrations over time and calculating the slope using the linear portions of the plots. Rates were corrected for size using mean body length of five fish from each treatment group. Mean body length was used since body weight was not measured for individual fish and body lengths were not statistically different within each treatment group.

Apoptosis was detected using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, dechorionated embryos or hatched larvae were fixed with 4% paraformaldehyde overnight at 4°C. Embryos were dechorionated by incubating embryos in 10% pronase at 25°C for 40-60 minutes, then manually removing the chorion with fine tipped tweezers under a dissecting microscope. The next morning, fish were washed twice with 1X PBS and then endogenous peroxidases were blocked with 3% H_2O_2 in methanol for 15 minutes. After, fish were washed with 1X PBS. Then, the TUNEL reaction mixture was added to tubes, where

the labeling solution was diluted 1:10 prior to being added to the enzyme. Fish were incubated at 37°C for 40 minutes and then were rinsed twice with 1X PBS and imaged. Apoptosis in the heart was quantified as a presence/absence measurement.

2.3 Microcystis and MC analysis

Cell densities of cultures were determined after fixation with Lugol's Solution using a Beckman Coulter Multisizer 3 Coulter Counter and a gridded Sedgewick-Rafter. Total MC concentration was quantified using a commercial Microcystins (Adda specific) Enzyme-Linked Immunosorbent Assay (ELISA—Abraxis, Warminster, PE). Concentrations were determined once for each sample, with initial and final readings done on the treatment sample or the highest concentration of *Microcystis* to ensure that concentrations did not drastically change over the course of the experiment. *Microcystis* concentrations, on average, had a coefficient of variation of 20% from the start to end of the experiment while MC concentrations had an average coefficient of variation of 4.4% (data not shown). Despite attempts to normalize culture conditions and preparation of algal suspensions between the experiments, all three of these parameters (*Microcystis* density, MC concentration, and MC per cell) varied between experiments, especially MC per cell. This almost certainly influences our results and also points out the need to quantify both parameters when assessing impacts of *Microcystis* blooms. In our experience, *Microcystis* in culture typically exist as single or double cells. Unusually, experiment 1 cultures appeared to have colonies, with clumps of algae visible (more similar to the morphology observed during natural blooms in the field (REF)).

2.4 Imaging

Images were taken using a Nikon i80 microscope. TUNEL assay images were viewed using a Nikon GFP filter (ex 450-500, em 515-565 nm). Larvae images were measured using Photo Measure app from Windows (Braddock Software Ltd.).

2.5 Statistics

Statistics were done using R statistical software (R Core Team 2015). Survival was examined using Kaplan Meier analysis and a log rank test was used to determine significant differences between treatments. For experiment 1, survival was considered statistically significant at $p < 0.008$ because of the Bonferroni correction to correct for multiple comparisons. Heart rate, heart length, and body length data were tested for normality and equal variances and then were analyzed using ANOVA and Tukey's post hoc analysis. Heart rate was analyzed using a nested ANOVA and heart length and body length data were analyzed using a one-way ANOVA. Respiratory rates were determined using the slope of oxygen consumption over time determined by generalized linear models. Rates were divided by mean body length to account for size dependent differences in respiratory needs. Sample sizes were not always consistent throughout treatments due to use of embryos for previous measurements, death of embryos, or inconsistent hatching among groups in the later time points (6 or 10 dpf) for heart rate data. Sample sizes are indicated in figures. Comparisons were considered statistically significant when $p < 0.05$, except as noted above for survival in Experiment 1.

3. Results

3.1 Properties of Microcystis cultures

The initial measured concentrations of *Microcystis* and MC for each treatment group and experiment are reported in Table 1. *Microcystis* cell densities ranged from 1.0 x10^6 to 26.8x10^6

cells mL^{-1} and MC concentrations varied from 50 to 1270 µg L^{-1} in the algal cultures. For comparison, also shown is the MC per cell that varied between 2.0 x10^{-8} and 78 x10^{-8} µg MC per cell.

3.2 Survival is affected by exposure to Microcystis cultures

Exposure to cultured *Microcystis* tended to reduce survival in all experiments (Figure 2A – D). The range of exposures to both *Microcystis* cells and MC was largest in experiment 1 where a statistically significant, dose dependent treatment effect on survival was observed using a log rank test (with statistical differences observed between control and the medium and high treatments as well as between the low treatment and medium and high treatments). Although survival in exposed groups appeared to be reduced in experiments 2, 3, and 4, these effects were not statistically significant, even though algal densities and MC concentrations were within the range shown to significantly reduce survival in experiment 1.

3.3 Cardiac performance is altered by exposure to cultured algae

Heart rate at 6 dpf was depressed in embryos exposed to *Microcystis* relative to controls in experiments 1 and 2 (Figure 3), despite heart rates in experiment 1 being higher overall than those observed experiment 2. These differences may be due to variation in lab temperature when heart rate was analyzed, with experiment 1 done in the summer months and having temperatures that averaged 24°C and experiment 2 being performed in the fall months with lab temperatures averaging around 20°C. Despite large differences in *Microcystis* densities and MC concentrations (especially in experiment 1), there were no differences in heart rate observed between algal treatments.

Heart length (SV-BA distance) appeared to respond in a dose dependent manner to
Microcystis exposure in experiment 1, although this trend was not statistically significant (Figure
4). It should be noted that data from the highest algal treatment group in this experiment could
not be included due to the low survival of this group and poor imaging of the heart due to the
decreased integrity of chorion structure at 6 dpf when SV-BA distance was measured. Since
there was no significant trend indicating elongation of the heart, interference of and thus
disrupted heart formation, in subsequent experiments, measurements focused on evaluating other
hypotheses to determine the cause of cardiotoxicity.

Occurrence of apoptosis of cardiomyocytes of exposed fish was examined next to see if
cell death could be causing cardiotoxicity in developing fish. Micrographs illustrating incidences
of apoptotic cells in the heart region of fish exposed to *Microcystis* in experiment 2 are shown in
Figure 4. Fish were measured qualitatively at 16 dpf after being exposed from 1 to 16 dpf.
Fluorescence indicative of apoptosis was not seen in the hearts any of the five control fish tested
(Figure 5A, Table 2) but was observed in two of the five fish exposed to low *Microcystis*
concentrations (Figure 5B, Table 2) and all of the five fish exposed to higher *Microcystis*
concentrations (Figure 5C, Table 2). Fluorescence intensity of each individual fish examined are
also reported (Table S1).

3.4 Fish appear to recover from some cardiac effects after removal from Microcystis

Experiments 3 and 4 were conducted to see if embryos could recover from *Microcystis*
exposures or if treatments had lasting effects. The effects of early (1 – 6 dpf – experiments 3 and
4) and late (6 – 10 dpf – experiment 4) exposure timing were tested as well as longer (10 days –
experiment 3) and shorter (4 and 5 days – for early and late experiment, respectively) recovery
periods. Heart rate was measured at 6 dpf for all experiments, but later time points at 10 and 15

dpf were obtained in experiment 4 in embryos that had not yet hatched. Due to the large number of embryos that hatched by 16 dpf in experiment 3, heart rate could not be assessed at the later recovery time point. In both experiments, heart rate was significantly decreased in exposed embryos relative to controls immediately after exposure (Figure 7). Heart rate was able to recover after early exposure and a short (4 day) recovery period in experiment 4 (Figure 7B) while embryos exposed later (from 6-10 dpf) still had significantly decreased heart rates after a slightly longer (5 day) recovery period (Figure 7C).

Apoptosis in the heart region of fish was examined after exposure to *Microcystis* and recovery periods after removal from treatments using the TUNEL assay (Figure 8 and Table 1). Fluorescence intensities of each fish examined are also reported (Table S1). Comparisons of fluorescence intensity cannot be made between experiments 3 and 4 since TUNEL analysis was done on different days. In both experiment 3 and 4, fluorescence intensity of apoptosis in the heart was greatly increased in fish exposed to blooms compared to those that were in ERM. After a recovery period, the fluorescence intensity was relatively the same in control and treatment groups. One control fish in experiment 4 in the late exposure group had increased fluorescence intensity that was comparable to the treated groups.

3.6 Effects of Microcystis on bioenergetics of fish

Neither body length nor oxygen consumption rates were significantly affected by algal treatment in experiment 2 (Figure 6A & B), although there was a slight trend in increasing oxygen consumption rates with treatment. However, differences in both these measurements were evident in the experiments with exposures followed by recovery periods (experiments 3 and 4). Body lengths of exposed fish were generally shorter than body lengths of control fish after exposure and recovery in experiments 3 and 4, however this was not statistically significant

(Figure 9). The only statistically significant decrease in body length was seen after recovery from late exposure in experiment 4, where exposed fish had, on average, 13% smaller body lengths than control fish (Figure 9C). Oxygen consumption rates were highly variable. In experiment 3, oxygen consumption rates corrected for body length were not statistically different from one another in experiment 3(Figure 10A). However, in experiment 4, early exposure caused increased oxygen consumption rates both after exposure and a 4 day recovery period (Figure 10B). Despite being older, there was no difference observed in oxygen consumption rates between control embryos and embryos that had not been previously exposed to *Microcystis* at 6 dpf. Following exposure to *Microcystis* from 6-10 dpf, oxygen consumption rates appeared to increase, but the effects was not statistically significant. However, when these same embryos were removed from the *Microcystis* culture for 5 days, their oxygen consumption rates remained high, now being significantly different from embryos never exposed to *Microcystis* (Figure 10C). Oxygen consumption per body length decreased with the age and size of fish in both experiments 3 and 4.

4. Discussion

Microcystis blooms differed in cell density and MC concentration in the four experiments described here. Several factors likely contributed to these differences, including the differing densities of cultures subsampled for testing, differences in times since last nutrient additions, and the process of centrifugation and resuspension of algae in our own media. MC concentrations have been shown to increase with the addition of nutrients, especially nitrogen (Davis et al. 2009, Harke and Gobler 2013, Horst et al. 2014), so timing of nutrient additions likely plays a role in the MC production and therefore, the toxicity of the blooms. There was dose dependent mortality in experiment 1, although concentrations were in the range of other experiments that did not have

significant decreases in survival. This could be due to the nature of the culture prior to exposures since the algal cells in experiment 1 appeared to be grouped together rather than existing as single celled or groups of two cells which is generally how *Microcystis* cultures behave (Bolch and Blackburn 1996). Previous work in our laboratory observed that field collected blooms produce more severe effects than cell cultures or even pure MC-LC toxin exposures likely due to clumps of algae attaching to and reducing the structural integrity of the chorion of exposed embryos (Saraf et al. in review). Since experiment 1 appeared to have colonies of cells rather than single or double cell groups, this could have caused increased mortality as compared to other experiments at similar algal densities and MC concentrations because of the algal cell – chorion interactions. Enhanced mortality has an obvious significant effect on the recruitment of fish populations for the following year, but sublethal effects on development, cardiac function, respiration, and growth observed here, could lead to reduced survival as fish grow and develop, particularly in the wild where their survival would depend on competing effectively for food and avoiding predation.

Heart development and function are important for proper fish development and survival post hatch. Adequate blood flow is essential for the transport of nutrients, respiratory gases, waste products, antibodies, and salts throughout the body (Eckert and Randall 1983) and toxic effects on the heart will likely inhibit or slow down this process, limiting the delivery of nutrients and gases needed for life functions or impeding excretion of waste products. During development, the heart is especially important since insufficient availability of biomolecules or nutrients for growth will negatively affect further development. This was observed in ELS zebrafish with cardiac dysfunction resulting from exposure to polycyclic aromatic hydrocarbons which caused a suite of morphological and functional abnormalities (Incardona et al. 2004).

The present study found that heart rate was reduced in fish exposed to *Microcystis* relative to their controls in all experiments, thus validating the cardiotoxic effects of *Microcystis*. Similar findings have been previously reported in zebrafish embryos exposed to cyanobacterial extracts (Oberemm et al. 1997) and more recently by our laboratory in Japanese medaka exposed to MC-LR and cultured and field collected *Microcystis* (Saraf et al. in review). Heart rate of control embryos was different in experiment 1 and experiments 2, 3, and 4, likely due to differences in lab temperature. Treated embryos had heart rates consistently lower than those of their respective controls, and reduced heart rate could have implications for development in later stages.

The goal of this study was to determine what causes the cardiotoxicity observed, determine what stages are most sensitive, evaluate potential co-occurring physiologic effects, and whether or not effects observed are reversible.

One of the possible causes of cardiotoxicity could be inhibition of the looping stage during heart development, where the tubular heart folds to form the two chambers. In medaka, looping occurs in the first days of development, with a fully developed and functional atrium and ventricle at 6 dpf (Iwamatsu 2004). Exposure to *Microcystis* during this time could disrupt the looping process and result in incomplete folding and thus, an elongated heart. In this experiment however, there was no significant alteration in heart length (the SV-BA distance) observed, indicating that the gross architecture of the heart was not affected by exposure. This, along with the fact that bradycardia was observed in embryos after completion of the looping stage in experiment 4, suggests that bradycardia is not likely from incomplete formation of the heart chambers.

Another possible cause of bradycardia could be damage to the cardiac cells responsible for initiating contractions necessary for an effective heartbeat. Heartbeats are generated by cells in the pacemaker region of the heart, cells undergo rapid depolarization, creating action potentials that spread to neighboring cells through gap junctions and to cells in other chambers of the heart through small junctional fibers (Eckert and Randall 1983). Heart contractions are myogenic, meaning that they are initiated by muscle cells rather than neurons (Eckert and Randall 1983). Thus, heart rate could be slowed if the cells initiating contractions or the connections between these cells are damaged. Apoptosis is a normal occurrence in the body, especially during early development where cellular architecture is changing and cellular remodeling is occurring (Ulukaya et al. 2011). Evidence of accelerated (or depressed) apoptosis in response to experimental treatment is an indication of significant alteration in normal development. Evidence of increased apoptosis in cardiac cells was observed in response to *Microcystis* exposure in all three of the experiments where it was assessed, whereas significant apoptosis in the cardiac region was not observed in control embryos. Apoptosis coincided with reductions in heart rate of medaka, suggesting that apoptosis is mechanistically associated with bradycardia in treated fish. Apoptosis could be in response to either cellular injury from exposures that initiate an apoptotic response or some component of *Microcystis* may be affecting pro-apoptotic factors that would cause apoptosis to occur. Cellular death in the heart could be more extensive than described here since apoptosis and necrosis generally occur simultaneously in response to toxins and the TUNEL assay only quantifies apoptosis in nonmyocytes (Klassen 2008). Apoptosis of muscle tissues (i.e. myocytes) or necrosis of heart cells could be inducing further damage that was not identified by these experiments but could be considered in future experiments.

Since *Microcystis* exposures were shown to affect the hearts of developing medaka, we also examined if cardiotoxicity was associated with alterations in the bioenergetics of fish measuring both oxygen consumption rates and body length. Fish obtain energy from oxidative metabolism which can then be used for production (i.e. growth and gamete production), lost as heat during metabolism, or lost in excretory products (Wootton 1999). Energy demands of basic life processes (metabolism and excretory processes) are first met and then remaining energy can be used for growth and gamete production. In this study, metabolism was estimated by measuring oxygen consumption rates (Wootton 1999) and body length was used as a proxy for growth. In general, embryo oxygen consumption rates tended to increase after exposure to *Microcystis*, which coincided with trends of decreasing body length of exposed fish. This apparent increased energy demand could be associated with costs of processing the MC toxin or eliminating reactive oxygen species in fish or other compensatory responses. Both increased metabolic costs and subsequent decreased body length could have effects on the success of the fish since studies have shown that decreases in size greatly increase mortality rates in ELS fish (Houde 1997).

It was somewhat surprising that increased oxygen consumption rates were associated with decreased heart rate in embryos exposed to *Microcystis*. Decreased heart rate generally means that less oxygen is transported throughout the body and is available for use. However, ELS fish have been shown to predominately obtain oxygen through diffusion in both embryonic (Rombough 1988) and larval stages (Rombough 2002), with gills used primarily for ion regulation (Rombough 2002). If respiratory demands can be met through diffusion, heart and oxygen consumption rates may be partially, if not fully, decoupled during these early life stages.

The last two sets of experiments reported here examined the potential of fish to recover from effects induced by *Microcystis* exposures and compared effects and potential to recover from exposures during early (during heart development) and later (after heart development) periods of development. Exposures resulted in decreased heart rate, increased respiration, decreased body length, and incidences of apoptosis in treated fish. These effects were generally recovered after *Microcystis* exposure was terminated in the early exposure experiments, but in later exposures, many of the effects persisted or even became more severe after exposures ceased. This suggests that exposure later in development causes more lasting effects than earlier exposures. Similar enhanced sensitivity was reported from a study on loach embryos exposed to MC-LR during different developmental stages (Liu et al. 2001). This suggests that blooms don't necessarily have to coincide with a spawning event to affect development of fish since later stages were still affected by exposure to algae. More lasting and delayed effects that remain after bloom exposure ends are important to the success of fish in the wild, since fish have to compensate for these physiological differences and that could affect their success in foraging or reproduction.

5. Conclusions

Microcystis blooms have been previously shown to cause early cardiotoxic effects in embryos of the Japanese medaka by our lab (Saraf et al. in review) and zebrafish (Oberemm et al. 1997). The present study provides data indicating that apoptosis is involved in *Microcystis*-induced bradycardia. Further studies will be need to be done to differentiate if apoptosis is caused by triggering of some pro-apoptotic factor or if cell damage resulting from exposures is initiating apoptosis and to determine the cellular mechanism triggering the response. This study has also shown that *Microcystis* exposures, in some cases, also caused changes to the

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bioenergetics of exposed fish, including increased oxygen consumption rates and reduced body length in some but not all cases. Experiments varying time of exposure and recovery indicated differences in sensitivity of persistence of response with apoptosis being a transitory response, and respiration, heart rate and even body length more persistent after exposures at slightly later developmental stages. This indicates the potential for chronic effects that might negatively impact survival in later life stages. Future work should focus on further elucidation of the mechanisms of cellular injury and how they influences respiration and growth as well as repair mechanisms and the persistence of effects from ELS *Microcystis* exposure to free living larval and juvenile fishes.

Acknowledgements

Figure Legends

Figure 1: Schematic describing timing of exposure and recovery periods in experiments 3 and 4.

Figure 2: Survival of fish throughout exposures in experiment 1 (A), 2 (C), 3 (B), and 4 (D). Significant differences were determined using log rank test, with $p < 0.05$ for experiments 2-4 and $p < 0.008$ for significance for experiment 1 to correct for multiple comparisons (Bonferroni correction).

Figure 3: Heart rate at 6 dpf in experiments 1 (A) and 2 (B) plotted with the *Microcystis* and MC concentration on the x axis. Experiments were analyzed independent of one another, with letters

representing statistically significant differences in heart rate of treatment groups of each respective experiment. Significance was determined using a nested ANOVA followed by Tukey's post hoc test.

Figure 4: Heart length (SV-BA distance) in exposed embryos in experiment 1. Letters represent statistically significant differences in heart length of each treatment group, determined using nested ANOVA followed by Tukey's post hoc test.

Figure 5: Apoptotic cells in the hearts of fish exposed to control conditions (A), lower (B), and higher *Microcystis* densities (C) from 1-16 dpf in experiment 2. Apoptotic cells were visualized using the TUNEL assay. The scale bar is 250 µm.

Figure 6: Body length (A) and oxygen consumption rates per body length (B) in embryos exposed to *Microcystis* in experiment 2. Embryos were exposed to treatments from 1 – 16 dpf and measurements were taken at the end of exposure at 16 dpf. Statistically significant differences were determined using one-way ANOVA analysis.

Figure 7: Heart rate in exposed and recovered medaka in experiments 3 and 4. Asterisks represent statistically significant differences in heart length of each pair, determined using one-way ANOVA followed by Tukey's post hoc test. Note, heart rate could not be reliably measured on embryos recovering from early exposures in experiment 3 because most of them had hatched by 15 dpf.

Figure 8: Apoptosis in fish in experiments 3 and 4 after exposure. Apoptosis was visualized using the TUNEL assay. The scale bar is 250 µm.

Figure 9: Body length changes exposed (E) and recovered (R) embryos in experiment 3 (A) and 4 (B, C). Statistical significant was determined using one-way ANOVA and Tukey post-hoc analysis. There was one outlier in the NE group at 6 dpf in panel B that was greater than 4 standard deviations outside of the mean that was not included in the figure to show variability of lengths for other embryos, this point was used in the analysis however.

Figure 10: Changes in oxygen consumption per body length (m) in not exposed or control embryos (NE), exposed (E), and recovered (R) embryos in experiments 3 (A) and 4 (B, C). Statistically significant differences between exposed or recovered embryos and their respective controls are denoted by asterisks. Significant differences were determined using nested ANOVA and Tukey's post hoc tests.

Figures and Tables – Chapter 3

Table 1: Summary of experiments with culture densities and MC concentrations.

Experiment	n	*Microcystis* (10^6 cells mL^{-1})	MC (µg L^{-1})	µg MC per cell (10^{-8})
EXP 1	15	0	0	---
	15	2.46	50	2.0
	15	13.8	480	3.5
	15	26.8	1270	4.7
EXP 2	30	0	0	---
	30	19.5	550	2.8
	30	22.0	820	3.7
EXP 3	20	0	0	---
	20	13.6	440	3.2
EXP 4	20	0	0	---
	20	1.0[a]	780[a]	78
	20	7.0[b]	630[b]	9.0

a: Concentrations for early exposures (1-6 dpf) in experiment 4
b: Concentrations for later exposures (6-10 dpf) in experiment 4

Figure 1

Figure 2

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Figure 3

(A) Experiment 1 — **(B) Experiment 2**

Heart rate (beats min^{-1})

Microcystis 10^6 cells mL^{-1} (MC µg L^{-1})

Figure 4

Experiment 1

SV-BA distance (mm)

A A A

n=15 n=15 n=8

0 (0) 2.48 (50) 13.8 (480)

Microcystis 10^6 cells mL^{-1} (MC µg L^{-1})

Figure 5

Figure 6

(A) Experiment 2

Body length (mm)

A   A   A

n=4

0 (0)   19.5 (550)   22.0 (820)

(B) Experiment 2

Oxygen consumption per length

A   A   A

n=15   n=14   n=15

0 (0)   19.5 (550)   22.0 (820)

Microcystis 10^6 cells mL^{-1} (MC µg L^{-1})

Figure 7

Figure 8

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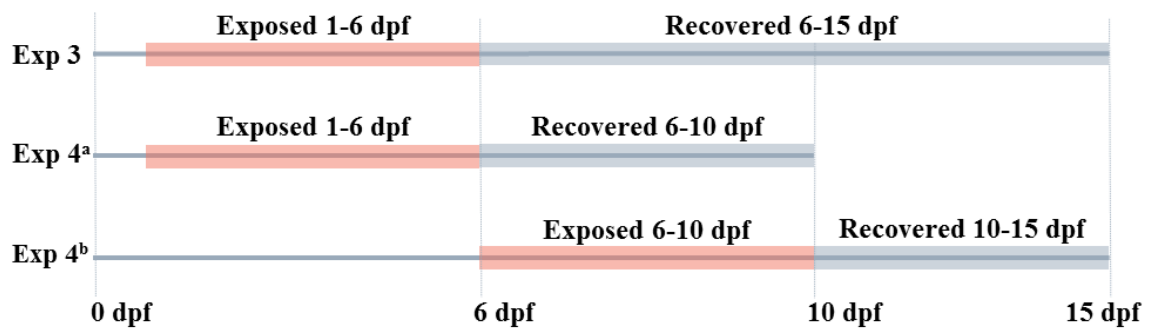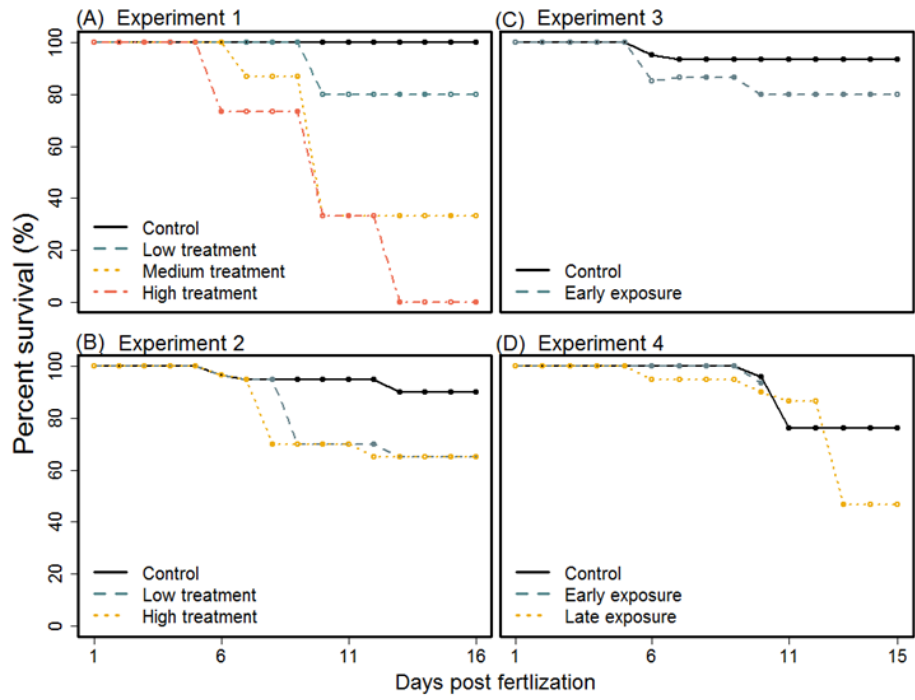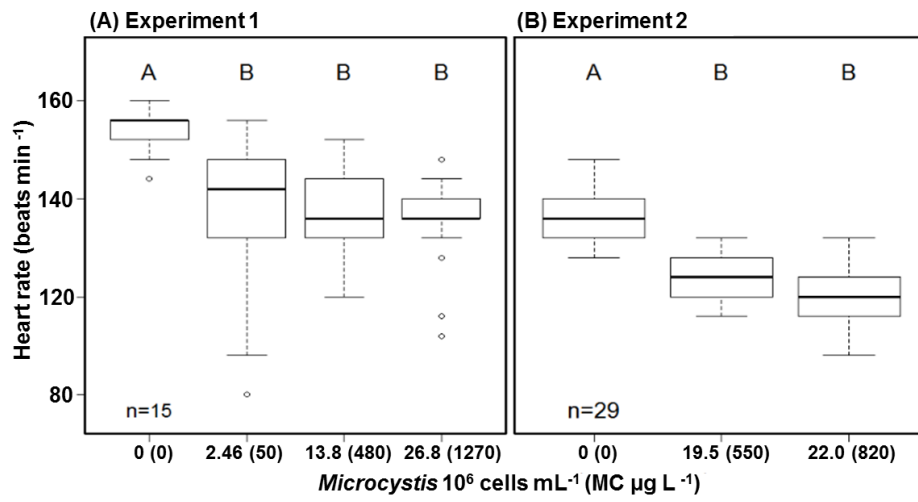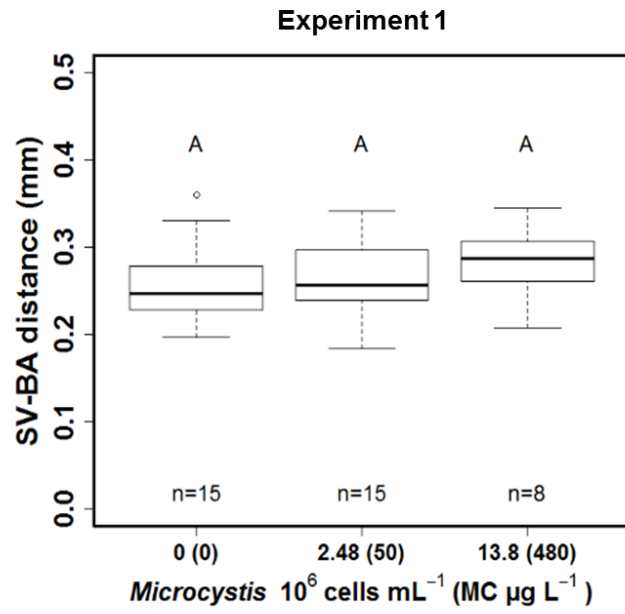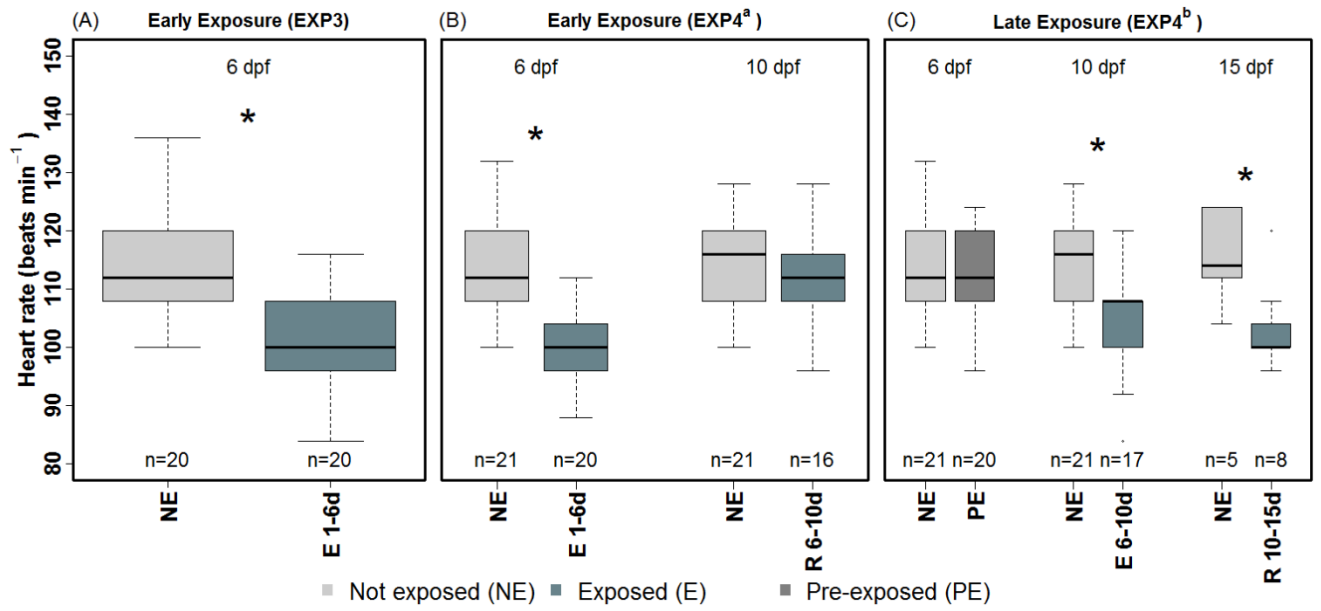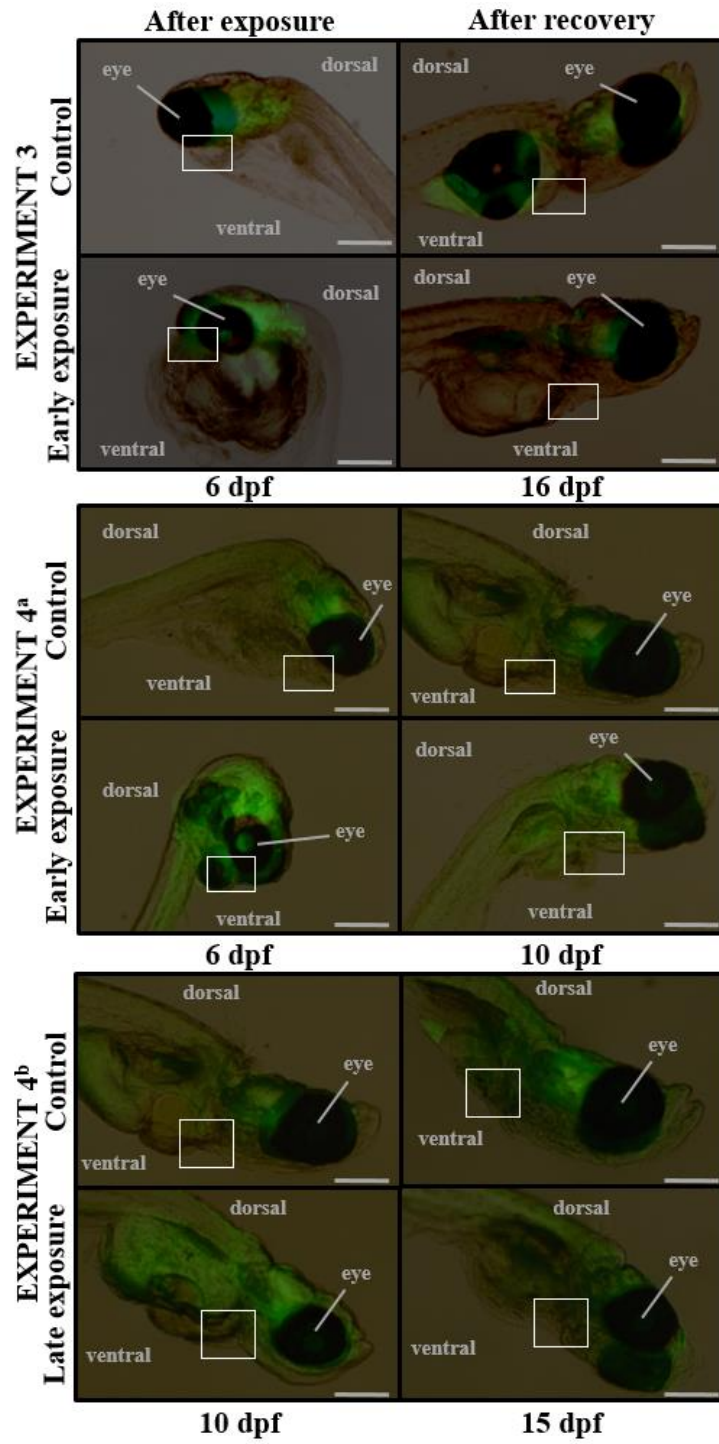
Table 2: Occurrence of apoptotic cells in fish tested using TUNEL assay
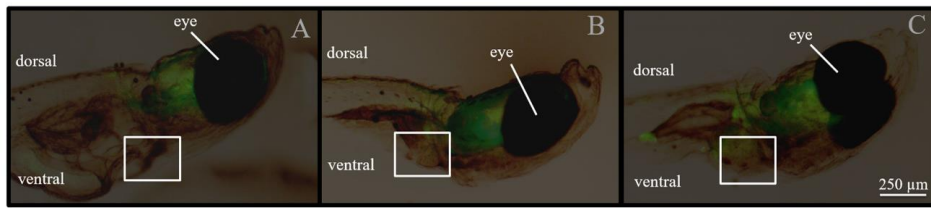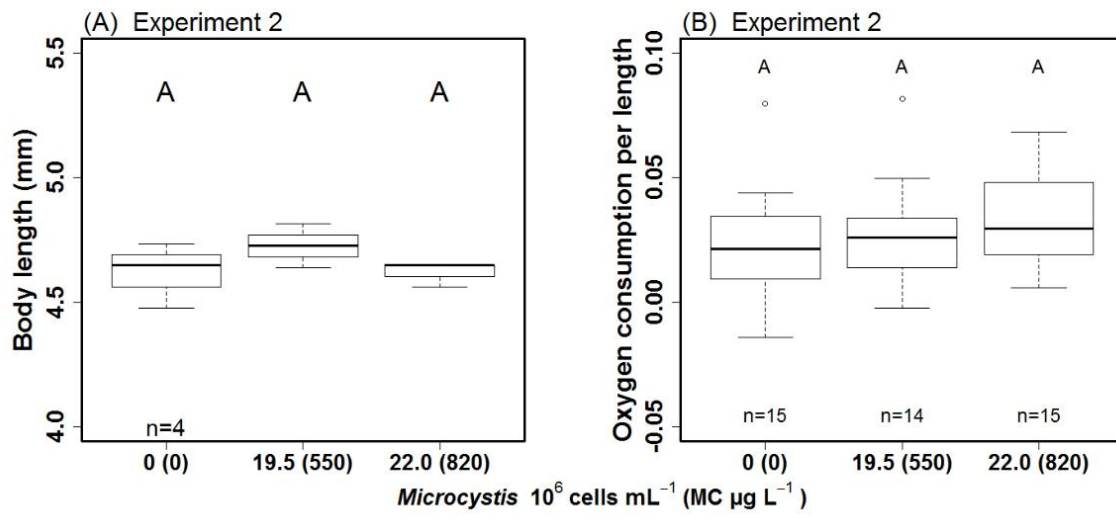
Experiment	Treatment	dpf	Fish with apoptotic cells in hearts (n=5)
Experiment 2 – full exposure	Control	16	0
	Exposed (low conc. 1 – 16 dpf)	16	2
	Exposed (high conc. 1 – 16 dpf)	16	5
Experiment 3 – early exposure	Control	6	0
	Exposed 1 – 6 dpf	6	4
	Control	16	0
	Recovered 6 – 16 dpf	16	1
Experiment 4[a] – early exposure	Control	6	0
	Exposed 1 – 6 dpf	6	5
	Control	10	0
	Recovered 6 – 10 dpf	10	2
Experiment 4[b] – late exposure	Control	10	0
	Exposed 6 – 10 dpf	10	5
	Control	15	1
	Recovered 10 – 15 dpf	15	2

Figure 9

Figure 10

Supplemental Tables – Chapter 3

Table 1: Fluorescence intensity of the heart region of fish from the TUNEL assay in experiments 2, 3, and 4.

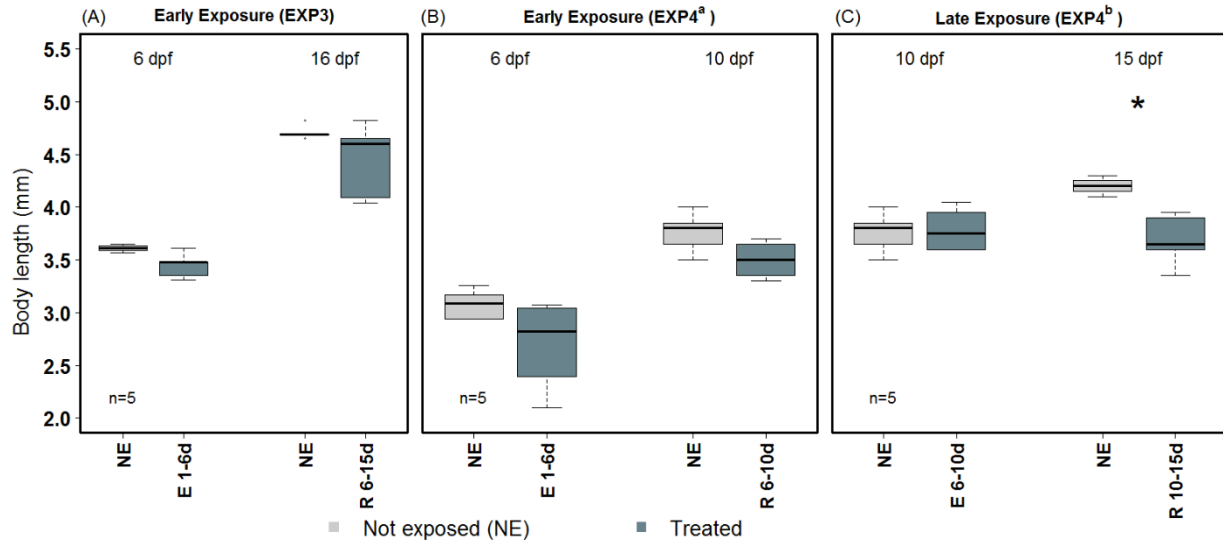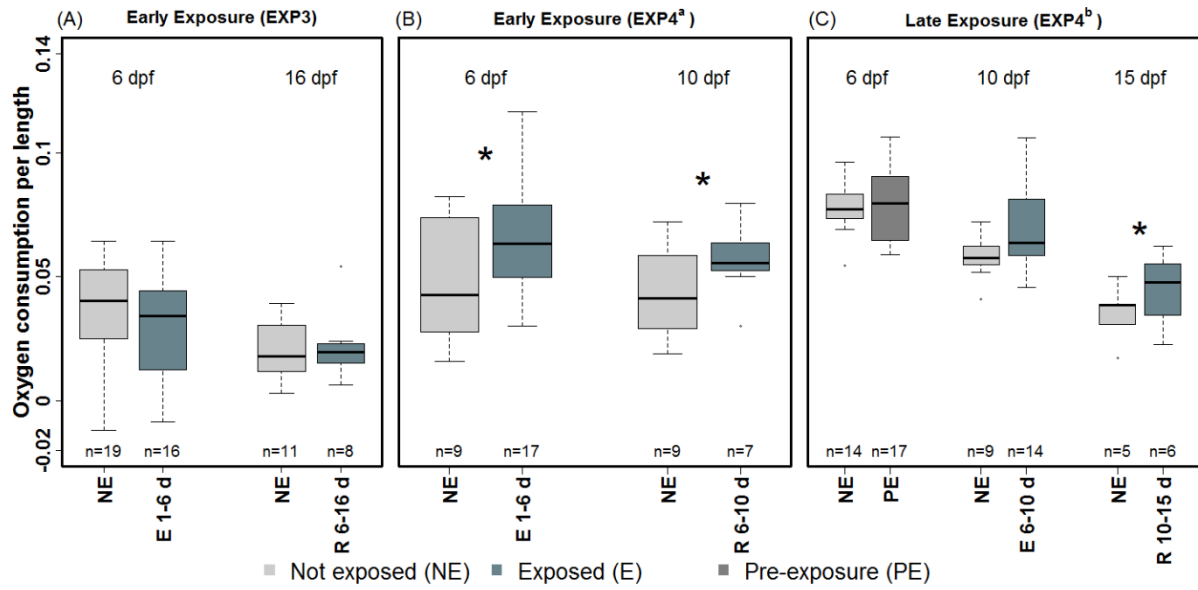|	Treatment	day	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Avg
EXP 2	Control	16	2.80±0.37	2.00±0.73	0.72±0.32	4.14±1.8	2.38±0.08	2.41±1.35
	Low treatment	16	8.34±1.2	3.74±1.3	2.12±0.22	13.0±2.1	2.79±0.15	6.00±4.45
	High treatment	16	8.62±0.96	12.4±0.39	13.1±2.5	6.91±0.07	10.2±0.23	10.2±2.59
EXP 3	Control	6	3.08±2.97	1.54±2.2	1.82±0.03	0.05±0.04	1.11±0.13	1.52±1.61
	Treatment	6	17.1±1.9	5.82±0.17	5.32±0.21	18.8±0.28	18.2±0.42	13.1±6.5
	Control	15	0.77±0.49	0.98±0.55	1.65±0.20	1.83±0.24	2.14±0.52	1.47±0.63
	Treatment	15	0.27±0.18	6.45±1.27	0.02±0.01	0.47±0.56	1.72±0.06	1.78±2.58
EXP 4[a]	Control	6	2.79±0.28	2.42±0.02	2.64±0.65	3.64±0.12	3.82±0.04	3.06±0.64
	E 1-6 dpf	6	12.4±0.27	34.9±0.61	7.80±0.39	9.35±0.81	7.66±0.04	14.4±10.9
	Control	10	4.96±0.24	5.47±0.01	6.03±0.55	6.57±0.45	5.74±0.11	5.75±0.62
	R 6-10 dpf	10	7.24±0.27	8.35±0.62	7.95±1.00	7.71±0.54	8.53±0.47	7.96±0.67
EXP 4[b]	Control	10	4.96±0.24	5.47±0.01	7.03±0.86	6.57±0.45	5.24±0.60	5.85±0.93
	E 6-10 dpf	10	11.4±1.1	12.3±0.36	11.0±0.34	10.4±0.25	12.5±0.68	11.5±0.96
	Control	15	6.53±0.09	5.69±1.19	3.45±0.16	6.77±0.06	5.95±0.17	5.68±1.31
	R 10-15	15	6.38±0.44	7.55±0.84	6.66±0.28	5.69±0.03	9.64±0.56	7.18±1.49

Mean ± standard deviation, E = Exposed, R = Recovered
Fluorescence intensity for each fish is an average of two measured values. Bold numbers indicate the representative fish from each treatment group that were used for images in this paper.

Chapter 4

Synthesis and Future Directions

Microcystis blooms are complex and each is unique, making it difficult to capture their complexity in experimental studies. To date, most toxicological studies examining the effects of *Microcystis* on ELS fishes have employed isolated MC-LR administered through microinjection into embryos through their chorions to examine effects of blooms on developing organisms. My thesis examined more environmentally relevant exposure methods by using bath cultures and whole cell *Microcystis* either from natural blooms or algal cultures for assessment as compared to effects elicited by the pure MC-LR toxin. Further, my work is unique in evaluating the effects produced by natural blooms, algal cultures and the pure toxin at environmentally realistic concentrations in controlled experiments with similar exposure routes allowing me to examine the relative toxicity of each.

In Chapter 2 I demonstrated the greater toxicity of field collected blooms and cultured *Microcystis* compared to MC-LR toxin. Natural blooms induced higher mortality and more severe effects than cultures (at comparable MC concentrations), which was likely associated with the formation of colonies as observed in the field collected samples and their interaction with the chorion as compared to the singlet or doublet cells found in *Microcystis* grown in culture (Bolch and Blackburn 1996). Another contributing factor that cannot be ruled out by my experiments is the possibility that other toxic alga or chemical or biological factors present in waters from the field collected samples may have caused greater toxicity. The inclusion of more detailed water analysis, as well as including environmental samples from similar water bodies not experiencing

Microcystis blooms as reference treatments would be a good first step towards identifying what factors may be enhancing the toxicity observed under natural bloom conditions.

I also attempted to simulate end of bloom conditions and increase release of MC by vigorous vortexing and homogenization of cultures prior to testing appeared to enhance mortality, but these treatments failed to produce enhanced toxicity, further supporting the conclusions that MC alone is not the only factor causing toxicity in *Microcystis* blooms.

Another important finding from my work was that algal cells from both natural blooms and some cultures appear to weaken the chorion. Although I did not have the opportunity to follow up on this finding, it could present a new mode of toxic action that would have important ecological consequences such as early hatching, where underdeveloped larvae would be expected to be at a competitive disadvantage. This also supports the use of bath cultures rather than microinjections since cells can interact with the chorion and alter the amount of toxic components made available to the developing embryo. Future work could employ transcriptomics to evaluate mechanisms associated with the effects seen in experiments as well as chorion effects. Transcriptomic studies have previously shown differential responses of larval zebrafish to MC-LR or *Microcystis* extracts (Rogers et al. 2011), and work with a different model species, like medaka, and with whole cell algae would contribute to this work, identifying changes across species in order to determine a robust analysis of transcriptomic responses associated with *Microcystis* exposure. Structural analysis of the chorion through electron microscopy could also help to elucidate structural damage related to *Microcystis* exposure.

Heart rate effects were found to precede other effects in ELS fish exposed to *Microcystis*. Since MC has generally been categorized as a hepatotoxin in terrestrial and adult vertebrates

(Butler et al. 2009), I hope this thesis will encourage more studies to look into the mechanism behind this cardiotoxicity. In Chapter 3 I showed that apoptosis is involved in the cardiotoxicity in developing fish. However, apoptosis appears to be a transient response which is no longer observed or occurrence is much less severe days after exposure ceases. This suggests that some factor of *Microcystis* is triggering these apoptotic events. However, more work is needed to elucidate the mechanism behind the apoptosis occurring since it could be an indirect effect, as the result of cellular injury, instead. My work also indicated that effects on the heart have the potential to affect the bioenergetics of fish. In some cases, oxygen consumption rates were increased by *Microcystis* exposure and remained elevated after short term recovery, showing the possibility of long term effects on fish as they continue to develop. Studies could be done examining these effects by monitoring early exposed fish into juvenile and adult stages to see if growth or reproduction are affected, both of which would have negative impacts at the population level. One studies examining the effects of MC-LR exposure of adult fish on subsequent generations showed that offspring had reduced body length and weight and that differences in gene expression were evident for four generations (Lui et al. 2014). While reproduction was not shown to be affected in that study, exposure to whole cell blooms rather than the isolated toxin may yield different results. Further work describing these long-term effects would help in describing the impacts of blooms on developing fish populations since ELS stages are important to the recruitment and longevity of a species in an ecosystem.

A better understanding of the ecological effects of *Microcystis* blooms is important as blooms have been increasing in distribution in recent decades, as well as becoming more toxic, and these trends are likely to continue (O'Neil et al. 2012, Paerl and Paul 2011). Temperature increases from climate change as well as increased nutrient input from anthropogenic sources,

runoff from heavy storms, and airborne sediment from drought conditions will all fuel the

growth of blooms and many have the potential to increase MC production (IPCC 2013).

Increased temperature and nutrient additions have been shown to both promote the production of

MC (Conradie and Barnard 2012, Davis et al. 2009, Harke and Gobler 2013) and favor

production of the most toxic congener, MC-LR (Dziallas and Grossart 2011). To date, most

concern about *Microcystis* blooms have focused on potential harm to humans through drinking

water, or catastrophic effects like fish kills. Evaluation of chronic effects on a diversity of lower

trophic level organisms may be more important from an ecological perspective. These studies

will be helpful to gauge the severity to which *Microcystis* blooms could impact the environment

in the future and possibly lead to increased effort in remediating our actions that contribute to

blooms.

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