



Research Paper

Effects of *Microcystis* on development of early life stage Japanese medaka (*Oryzias latipes*): 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 effects associated with the rapid increase of biomass 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 the 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, lab cultures of *Microcystis*, 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 indicated 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 solutions 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 isolated *Microcystis* strains influence heart rate of fish exposed to *Microcystis* blooms. Enhanced mortality, depressed heart rate, and abnormal development observed in response to environmentally realistic exposures of *Microcystis* blooms could affect success of fish at both individual or population levels.

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 that are expected to worsen with climate change and eutrophication (O'Neil et al., 2012; Paerl et al., 2011). One of the most common freshwater CyanoHAB species is *Microcystis aeruginosa*, which is found worldwide (Harke et al., 2016). Throughout this paper we will refer to this CyanoHAB only as *Microcystis* since recent genetic analysis has determined that intraspecies variability is greater than the variability measured

across *Microcystis* species, suggesting that all previously named *Microcystis* species be grouped together (Harke et al., 2016). *Microcystis* blooms are problematic due to the rapid increase of algal biomass at the surface of the water which limits sunlight and potentially reduces dissolved oxygen concentration when they decompose. In addition, cyanobacteria are generally not known to be 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 the community.

Toxic strains of *Microcystis* produce a suite of monocyclic heptapeptides called microcystins (MCs), which are known hepatotoxins (Carmichael, 1994). Specifically with *Microcystis*, bloom growth and

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toxin production have been found to be 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), indicating that future climate conditions and continued eutrophication could promote both growth and toxicity of blooms (O'Neil et al., 2012). Furthermore since MCs are endotoxins, during end of bloom conditions when cell lysis or programmed cell death are accelerated (Bidle and Falkowski, 2004), toxin release may be enhanced, potentially making end of bloom conditions more toxic than the bloom itself. Thus, there is a need to evaluate the effects of *Microcystis* under natural conditions.

The toxicity of *Microcystis* blooms has predominately been evaluated by examining the effects of just one of its toxins, MC-LR, which is considered the most toxic and frequently encountered MC congeners (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). 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 and Joo, 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 and Codd, 1995), and reduced growth (Acuña et al., 2012; Bury and Codd, 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 examining the effects of *Microcystis* blooms on development of ELS fish 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 studies were performed by exposing ELS fishes to isolated MC-LR toxin, often with microinjection as the route of exposure. Cyanobacterial extracts have been shown to elicit greater toxicity than MC-LR exposures alone in ELS fish (Ghazali et al., 2009; Oberemm et al., 1997). A recent transcriptomic study using zebrafish larvae revealed differential responses when fish were exposed to *Microcystis* blooms or high or low concentrations of MC-LR toxin alone (Rogers et al., 2011). Therefore, while studies using isolated MC-LR toxin can indicate possible effects of *Microcystis* blooms and mechanisms of toxicity, more realistic exposures to intact algae and natural blooms are needed.

This study examines the 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 above which examined effects on ELS fish resulting from exposure to pure MC toxin and/or dosing through microinjection. Japanese medaka (*Oryzias latipes*) were used in our experiments due to their history as a model organism for vertebrate systems (Shima and Mitani, 2004; Wittbrodt et al., 2002), their transparent chorions that facilitate tracking developmental progress, and

longer development time as compared to zebrafish that allows for flexibility in timing of experiments (9–11 days for medaka vs. 3 days for zebrafish). Examining responses of intact organisms to *Microcystis* lab cultures or field collected blooms allowed us to assess natural interactions of the algae with the chorion of embryos, better mimicking what developing fish would be exposed to in an environmental setting. *Microcystis* lab cultures and field samples of natural *Microcystis* blooms were examined to isolate possible effects due to *Microcystis* alone as well as from whole blooms. Finally, embryos were exposed to isolated MC-LR solutions to determine if effects seen in response to algal exposures could be fully attributed to the presence of the toxin or if other algal components may contribute to the toxicity observed.

2. Material and methods

2.1. Care of brood stock and embryos

Medaka were maintained in tanks at 28 °C and 14:10 h 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 h after the brine feeding, and cultured in embryo rearing medium (ERM – 17 mM NaCl, 0.40 μ M KCl, 0.36 mM CaCl₂, 0.66 mM MgSO₄·7H₂O) at 25 °C. ERM was amended with methylene blue (0.0002%) for the first 24 h 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 h:12 h 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 resorption. Heart rate was measured at 6 dpf, as this is a critical stage of cardiac development in medaka where heart formation is complete. 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 s twice using light microscopy, and the results of both measurements averaged for each individual. 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 min using a Clay Adams Dynac centrifuge

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