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Direct and indirect effects of a common cyanobacterial toxin on amphibian-trematode dynamics

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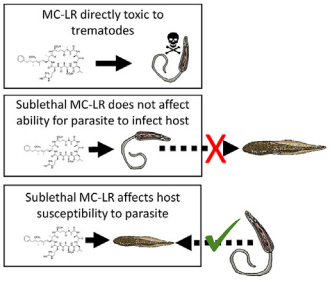
HIGHLIGHTS

- Microcystin-LR is toxic to the cercariae of trematodes.
- Sublethal microcystin-LR exposure does not reduce trematode infectivity.
- Effect of microcystin-LR on tadpole susceptibility to trematodes was non-monotonic.

GRAPHICAL ABSTRACT



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ABSTRACT:

Wildlife diseases are emerging at unprecedented rates. While there are likely several factors at play, human-mediated environmental alterations may play a significant role. Of growing interest is the effect that microcystin-LR (MC-LR), a cyanotoxin, may have on disease outcomes. In this study, using an amphibian-trematode model we examined (1) the lethal effects of MC-LR on cercariae of trematodes; (2) the sublethal effects of MC-LR exposure on the ability for trematodes to infect green frog tadpoles; and (3) the sublethal effects of MC-LR on green frog tadpole susceptibility to trematodes. We found that environmentally-relevant concentrations of MC-LR at 50, 100, and 500 $\mu g\,L^{-1}$ increased cercariae rate of mortality ($LC_{50-14h} = 134.24\,\mu g\,L^{-1}$). However, sublethal exposure of trematodes to 2 and 10 $\mu g\,L^{-1}$ MC-LR did not alter their infectivity. Conversely, sublethal exposure of tadpoles to 2 $\mu g\,L^{-1}$ increased their susceptibility to trematodes by 147%. However, 10 $\mu g\,L^{-1}$ of MC-LR did not affect tadpole susceptibility to trematodes, indicating a non-linear response to sublethal MC-LR exposure. Overall, our findings suggest that high concentrations of MC-LR ($\geq 50\,\mu g\,L^{-1}$) have the potential to limit trematode transmission to amphibian hosts through MC-LR-induced mortality. However, at lower concentrations ($<10\,\mu g\,L^{-1}$) MC-LR's effect on tadpole-cercariae disease outcome is likely driven by its effect on the tadpole host. Collectively, this work highlights the need to consider how toxicants influence both host and parasite at multiple concentrations to better understand the impacts of cyanotoxins on disease dynamics.

1. Introduction

The eutrophication of aquatic ecosystems is a major global issue with consequences that include changing patterns of primary productivity and an increased occurrence, duration, and intensity of

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harmful cyanobacterial algal blooms (cyanoHABs; Paerl and Otten, 2013; Visser et al., 2016; Huisman et al., 2018). CyanoHABs are of particular interest given the diverse direct (i.e. shading of other photosynthetic organisms and increased pH), and indirect (i.e. hypoxia, anoxia; Havens, 2008) effects that they have on aquatic biota and ecosystems. CyanoHABs also commonly produce secondary metabolite toxins (hereafter cyanotoxins), with the most commonly reported cyanotoxin being MC-LR (Humpage, 2008). MC-LR is a cyclicpolypeptide which acts as a potent protein phosphatase inhibitor, causing damage to the hepatic system of vertebrates MacKintosh et al., 1990). Because of its potential to degrade drinking water quality and poison humans, wildlife and livestock (Paerl and Otten, 2013; Backer, 2002), MC-LR is a toxin of emerging concern among ecologists, natural resource managers, and public health officials.

Cyanobacterial blooms have likely occurred for millions of years (Huisman et al., 2018), and the toxic effects of blooms have been implicated in animal mortalities throughout the Pleistocene (Braun and Pfeiffer, 2002; de Boer et al., 2015). However, more recently the frequency, duration, and intensity of toxic cyanoHABs are increasing throughout the world because of the contamination of surfaces waters by anthropogenic inputs such as nutrient enrichment (Taranu et al., 2015). Indeed, concentration of MC-LR within the water column can range from trace amounts up to 80,000 $\mu g\,L^{-1}$ in highly eutrophic systems (Heiskary et al., 2014). Additionally, with expected increases in other facets of global change, such as the rise in atmospheric CO_2 levels or average temperatures, cyanoHABs are also expected to expand to areas traditionally free from blooms (i.e. high latitude and ephemeral systems; Chapra et al., 2017; Przytulska et al., 2017). Because human populations are expected to grow and continue influencing aquatic ecosystem processes, it will become even more critical to develop an understanding of the impacts of cyanoHABs and associated toxins.

Concurrent with the increase of cyanoHABs is the increased rate of disease emergence seen amongst human and wildlife populations worldwide (Daszak et al., 2000; LaDeau et al., 2008; Tompkins et al., 2015). While numerous factors contribute to the rise of disease, there is growing recognition that human-mediated changes to the environment such as climate change (Rohr et al., 2011; Altizer et al., 2013), agro-chemical toxicants (Rohr et al., 2008a), and habitat fragmentation (Jones et al., 2008) play a significant role (Keesing et al., 2010). Despite an increase in recent studies that document the harmful effects of cyanotoxins, their potential impact on disease outcomes are not well understood. Determining the effects of these toxins on disease dynamics will be critical as eutrophication and associated cyanoHABs are predicted to increase over time (Flombaum et al., 2013; Glibert et al., 2014; Chapra et al., 2017).

Evaluating the effects of toxins, such as MC-LR, on disease dynamics can be challenging, as direct and indirect effects of contaminant exposure on both hosts and parasites must be taken into account when determining the net effects of exposure on host-parasite outcomes (Rohr et al., 2008b; Studer and Poulin, 2012). For example, toxicant exposure at lethal concentrations can influence host-parasite interactions directly by reducing the abundance of either host or parasite, thus altering disease outcomes (Rohr et al., 2008a). Alternatively, toxicant exposure at sublethal concentrations can indirectly influence host-parasite outcomes through altering the traits of hosts and their parasites, such as immune response of the host, infectivity of the parasite, or by altering the behavior of either host or parasite (Rohr et al., 2008b; Milotic et al., 2017). Despite this, most studies which have evaluated the effects of toxicants on host-parasite interactions have predominantly focused on the effects of toxicants on either host or parasite alone (but see: Rohr et al., 2008a; Studer and Poulin, 2012; Koprivnikar et al., 2007; Buss and Hua, 2018). Given the potential for toxicants to influence disease outcomes via effects on both hosts and their parasites, it is critical that experiments examine the net direct and indirect effects of toxicants on both hosts and their parasites.

Determining the net effects of toxicants on disease dynamics is especially important for populations of amphibians, which have undergone significant population declines in the previous decades (Blaustein et al., 2011). As both disease and environmental contaminants have been hypothesized to be contributing factors to global amphibian declines (Hayes et al., 2010), such evaluations are critical to the management and conservation of amphibian populations inhabiting contaminated landscapes. For example, environmental contamination has been linked to continental scale patterns in the emergence of amphibian malformations caused by the trematode *Ribeiroia ondatrae*, where pesticide application strengthened the relation of parasite presence and malformations (Haas et al., 2017). Specific to cyanotoxins, Milotic et al. (2018) found that leopard frogs (*Lithobates pipiens*) exposed to an environmentally-relevant concentration of 11 $\mu g\,L^{-1}$ MC-LR were more susceptible to infection by the trematode *Echinostoma* sp. compared to larvae unexposed to MC-LR. Additionally, there is evidence that contaminants, such as heavy metals (Cross et al., 2001) and pesticides (Koprivnikar et al., 2007; Rohr et al., 2008b; Hua et al., 2016), negatively impact free-living life stages of parasites. However, the effects of MC-LR on the longevity or infectivity of free-living stages of parasites are unknown.

To move towards a more holistic framework for how MC-LR contamination may alter host-parasite dynamics, we first investigated the direct, toxic effects of six environmentally-relevant concentrations of MC-LR to the trematode *Echinostoma* sp. Next, we examined the sublethal effects of MC-LR on both trematode parasites (i.e. decreased infectivity) and amphibian hosts (i.e. increased susceptibility to trematodes). We predicted that (1) MC-LR will be directly toxic to cercariae of trematodes; (2) MC-LR exposure will decrease trematode infectivity; and (3) MC-LR exposure will increase the susceptibility of larval amphibians to trematode infection.

2. Materials and methods

2.1. Model system

For our experiments, we chose to work with trematode parasites from the family Echinostomatidae, which are one of the most commonly reported parasites of amphibians. In one survey of amphibians across the US, echinostomes were found in more than one third of amphibians sampled, with some individuals supporting infections of up to 2750 metacercariae within their kidneys (Johnson and McKenzie, 2009). Echinostomes have a complex life cycle utilizing multiple hosts (Huffman and Fried, 2012). First, trematodes in this family reproduce sexually in vertebrates, such as birds or mammals (i.e. definitive hosts). Eggs produced within the definitive host are then passed into the environment, where they hatch into free-swimming miracidia, which seek out and infect gastropod hosts (i.e. first intermediate hosts). When established within the gastropod host, miracidia reproduce asexually to form free-swimming cercariae, which emerge from the snail and enter the aquatic environment. Once cercariae emerge from the snail, they seek out larval amphibians (i.e. second intermediate hosts). Once inside the larval amphibian host, the cercariae encyst within the kidneys, forming metacercariae. If an infected amphibian is then consumed by a definitive host, the trematode life cycle begins again (Huffman and Fried, 2012). In this study, we focus on the cercariae life-stage of trematodes whose abundance has been

shown to increase during the late-summer months (July–August; Wash, 1997) when high water temperatures increase the likelihood of cyanoHABs (Visser et al., 2016). Past work also demonstrates that cercariae of echinostomes are vulnerable to a range of environmental contaminants (Hua et al., 2016; Rohr et al., 2008a) making them a useful model for examining the potential impacts of contamination on free-living stages of parasites. Lastly, as echinostomes display a lifestyle consisting of multiple host species, their presence can be used as bioindicators of ecosystem health. Thus, it is valuable to understand the impacts of MC-LR toxicity on trematodes for the management of systems undergoing eutrophication.

Past studies demonstrate that concentrations of 11 and 82 $\mu g\,L^{-1}$ of MC-LR cause mortality in larvae of northern leopard frogs (*Lithobates pipiens*), with 11 $\mu g\,L^{-1}$ also increasing *L. pipiens* susceptibility to trematode infection (Milotic et al., 2018). Amphibian species commonly differ in susceptibility to contaminants and parasites thus it is important to assess whether the effects of MC-LR on amphibian susceptibility to parasites are generalizable. Therefore, for this study, we chose to use green frogs (*Lithobates clamitans*) as our model amphibian host. *L. clamitans* are relevant hosts because they inhabit and oviposit in permanent water bodies that can host cyanoHABs during periods of nutrient enrichment and high heat (Paerl et al., 2011). Furthermore, *L. clamitans* are in their larval periods during late summer (July–August) when such blooms most commonly occur (Konopka and Brock, 1978) and when trematode infection peaks.

2.2. Animal collection and husbandry

On 12 July 2017, we collected four partial egg masses of *L. clamitans* from Kennedy Pond, a permanent pond located in Rensselaer County, NY (42° 37′ 36.83″ N, 73° 33′ 55.18″ W; Gosner stage 4; Gosner, 1960). The egg masses were transported to the Binghamton University Ecological Research Facility (ERF) and placed in four 120-L plastic pools filled with 100-L of well water. We allowed individuals to develop in common garden conditions until they reached the tadpole stage (Gosner stage 25). Once all individuals reached Gosner stage 25, we mixed individuals from all egg masses and standardized the tadpole densities within each pool. Tadpoles were fed rabbit chow *ad libitum* until the start of the experiment.

To obtain cercariae for our experiments, we collected 30 and 35 *Helisoma trivolvis* on 23 April and 5 October 2017, respectively, from the Binghamton University Nature Preserve (42°05′01.4″N 75°58′22.3″W). We screened all snails for trematode infection by placing individuals into 50 mL falcon tubes filled with 35 mL of UV-filtered well water beneath a heating lamp for 1 h to induce cercarial shedding. For additional details, see Supplemental Materials "Cercariae identification methodology".

2.3. Experiment 1: effect of MC-LR exposure on cercariae survival

To examine the direct effects of MC-LR on cercariae survival, on 28 June 2017, we exposed cercariae to five environmentally-relevant concentrations of MC-LR (0, 5, 10, 50, 100, and 500 $\mu g\,L^{-1}$), measured cercariae time-to-death, and calculated an LC_{50} value for MC-LR exposure (i.e. lowest concentration to kill 50% of a population). To obtain cercariae for this experiment, we placed 10 infected snails that we collected on 23 April 2017 into 50 mL falcon tubes filled with 35-mL of UV-filtered well water and exposed them to a heat lamp for 90 min to induce cercarial shedding. After shedding cercariae from each snail, we mixed cercariae from each tube to maximize genetic variation of cercariae as well as to avoid any bias from any individual snail. Experimental units were five 24-well cell culture plates with 2 mL of 0, 5, 10, 50, 100, or 500 $\mu g\,L^{-1}$ of MC-LR. Each well contained a single cercaria and we replicated the five MC-LR treatments 20 times for a total of 120 wells. All MC-LR treatments were randomized across 4 plates, with a fifth plate reserved for controls to avoid contamination of controls with MC-LR (following Hua et al., 2016). Using a stereo microscope (Olympus SZ240), we pipetted a single cercariae into each well. For each well-plate we used freshly shed cercariae to control for cercariae age and completed each plate before moving to the next (8–12 min per plate). To avoid cross-contamination of wells, we used separate glass pipettes to add cercariae for each MC-LR concentration.

For the first six hours of the experiment, we checked the survival (see Supplemental Materials: Cercariae Survival Criteria) of cercariae every hour. Due to low mortality from hours 0–6 (3%) we shifted checks to every two hours during hours 6–12. Beginning on hour 12, cercariae mortality began to increase across treatments (11%) so we began checking individuals every hour again. We terminated the experiment at hour 16 because we began to detect increased control mortality (40%) and were thus unable to differentiate between natural cercariae mortality and effect of MC-LR treatment on cercariae mortality. The natural life-span for trematode cercariae following snail emergence has been shown to be about 24 h (Rohr et al., 2008a), with mortality occurring as early as 6 h after emergence (Hua et al., 2016).

2.4. Experiment 2: the effect of MC-LR on trematode infectivity

To evaluate the effects of sublethal MC-LR exposure on the ability for trematodes to infect *L. clamitans* tadpoles, we exposed cercariae to three concentrations of MC-LR (0, 2, or 10 $\mu g\,L^{-1}$) and then assessed their ability to successfully encyst in the kidneys of *L. clamitans* tadpoles and form metacercariae. To obtain tadpoles for this experiment, on 8 October 2017, we haphazardly selected 45 *L. clamitans* tadpoles from the common garden pools at ERF and allowed them to acclimate to lab conditions (20 °C and 12:12 light cycle) for a total of five days. For the first 48 h of this acclimation period, we evenly distributed tadpoles into 10-L plastic tubs filled with UV-filtered well water. After this initial 48 h acclimation period, we individually housed tadpoles in 100 mL plastic cups filled with 60 mL of UV-filtered well water containing 0 $\mu g\,L^{-1}$ of MC-LR. Tadpoles were exposed to these two different experimental units to standardize tadpole housing conditions between this experiment and Experiment 3. We conducted water changes every 48 h and fed tadpoles a Tetramin slurry *ad libitum*.

To obtain cercariae for this experiment, on 13 October 2017, we individually placed 10 infected *H. trivolvis* snails that we collected on 5 October 2017, into 50-mL falcon tubes under a 50-W heat lamps to induce cercarial shedding. After allowing snails to shed cercariae for 2 h, we then mixed together cercariae from all ten snails. We then placed 30 cercariae into 10 mL scintillation vials containing 0, 2, or 10 $\mu g\,L^{-1}$ of MC-LR. Each treatment was replicated 15 times for a total of 45 vials. Cercariae were held in these vials for 4 h. At 4 h we poured the contents of each scintillation vial into 100 mL plastic cups filled with 60 mL of microcystin-free UV-filtered well water containing tadpole hosts. We chose an exposure period of 4 h as data from our TTD assay showed that concentrations $\leq$10 $\mu g\,L^{-1}$ of MC-LR were sublethal to cercariae for this exposure period. Further, previous work has shown that cercariae infectivity is age-dependent (Pechenik and Fried, 1995) and is highest for cercariae that are 0–5 h old.

We terminated the experiment 36 h after exposing tadpoles to cercariae, as this is an adequate amount of time for metacercariae to develop within the kidneys of tadpole hosts (Rohr et al., 2008a). At

this time, we removed all tadpoles from experimental units and euthanized individuals using 5 g L^{-1} MS-222 and stored them in buffered 10% formalin solution. To quantify trematode infection, we removed the kidneys from each tadpole and then placed them between microscopes slides, and then scanned them under a stereo microscope. We also measured the mass, stage (Gosner, 1960), and snout-vent-length (SVL) of all tadpoles.

2.5. Experiment 3: the effect of MC-LR on tadpole susceptibility to infection

To evaluate the effects of sublethal MC-LR exposure on *L. clamitans* susceptibility to trematode infection, we exposed *L. clamitans* to three concentrations of MC-LR (0, 2, or 10 μg L^{-1}) then assessed the number of metacercariae (not exposed to MC-LR) that successfully encysted in the kidneys of the tadpoles. Tadpoles were held in identical conditions to those described in Experiment 2, except after 48 h of acclimation to laboratory conditions the 45 tadpoles were haphazardly distributed into individual 100 mL plastic cups filled with 60 mL of UV-filtered well water containing either 0, 2, or 10 μg L^{-1} MC-LR ($N = 15$ tadpoles/treatment). After exposing tadpoles to MC-LR for 72 h, we moved all tadpoles to 100 mL plastic cups filled with 60 mL of microcystin-free UV-filtered well water and then exposed each tadpole to 30 cercariae. We chose these concentrations based on a 72 h pilot study which confirmed that MC-LR at these concentrations are not lethal to green frog tadpoles (100% survival). We used the same methodology described for Experiment 2 to obtain cercariae for Experiment 3 except all cercariae were counted and placed into scintillation vials containing 3-mL of UV-filtered, microcystin-free well water ($N = 45$ vials). After exposing tadpoles to cercariae for 36 h, we terminated the experiment. Tadpole exposed to MC-LR showed 100% survival across the three-day exposure period. For all tadpoles, we measured mass, stage, SVL and quantified trematode infection using the same methodology used in Experiment 2.

For all three experiments, we created experimental MC-LR solutions using dissolvable commercial Microcystin-LR powder (Cyanoginosin-LR; Toxin T 17 *Microcystis aeruginosa* CAS 101043-37-2; ≥95% purity; Cayman Chemical Company, Ann Arbor, Michigan). For details on MC-LR solution preparation and confirmation of final concentrations see Supplemental Materials.

2.6. Statistical analysis

2.6.1. Experiment 1: effect of MC-LR exposure on cercariae survival

We used a Cox proportional hazard model (Cox and Oakes, 1984) to compare survival rates of cercariae in each of our five MC-LR exposure groups relative to the control. To control for spatial effects between our well-plates, we added plate number as a covariate. We also used a probit regression analysis (Finney, 1947) to calculate an LC_{50} value and a 95% confidence interval for hour 14 of our TTD experiment. We chose to calculate our LC_{50} value using data at hour 14 because this time period had the greatest variability in mortality between MC-LR concentrations (Rohr et al., 2008a), and because we began documenting natural cercariae mortality in our control group at hour 16 (30% mortality).

2.6.2. Experiment 2 and 3: effect of MC-LR exposure of cercariae or tadpoles on infection outcome

We used a generalized linear model (GLM; McCullagh and Nelder, 1989) with a Poisson distribution and log-link function to investigate the main effect of exposure to sublethal concentrations of MC-LR on infection outcomes. We chose this statistical analysis because our response variables (i.e. trematode infection) were count data (Warton and Hui, 2011). We included tadpole Gosner stage (Gosner, 1960) as a covariate in both of our GLM analyses as Gosner stage has been shown to be correlated with susceptibility to trematode infection (Rohr et al., 2010). For significant main effects, we conducted Bonferroni-adjusted planned contrast (Quinn and Keough, 2002). We analyzed data in all three experiments using IBM SPSS software (Version 22, IBM INC).

3. Results

3.1. Experiment 1: effect of MC-LR exposure on cercariae survival

We found a significant main effect of MC-LR exposure on the survival rate of cercariae ($\chi^2 = 58.8$; $p < 0.001$; Fig. 1), but no main effect of plate number ($\chi^2 = 0.2$; $p = 0.672$). Relative to cercariae not exposed to MC-LR, cercariae died faster when exposed to 50 ($p < 0.001$), 100 ($p < 0.001$), and 500 ($p < 0.001$) μg L^{-1} of MC-LR. There was no difference in survival rate between cercariae exposed to 5 or 10 μg L^{-1} of MC-LR and cercariae not exposed to MC-LR ($p = 0.282$ and $p = 0.109$, respectively). The LC_{50-14h} value for MC-LR was 134.24 μg L^{-1} (95% confidence interval: 78.8–274.6).

3.2. Experiment 2: effect of MC-LR on trematode infectivity

We did not find a significant main effect of cercariae exposure to sublethal concentrations of MC-LR on trematode infectivity ($\chi^2 = 5.1$; $p = 0.078$; Fig. 2).

3.3. Experiment 3: effect of MC-LR on tadpole susceptibility to trematode infection

We found a significant main effect of tadpole exposure to sublethal concentrations of MC-LR on tadpole susceptibility to trematode infection ($\chi^2 = 29.9$; $p < 0.001$; Fig. 3). Relative to tadpoles not exposed to MC-LR, tadpoles exposed to 2 μg L^{-1} of MC-LR had 147% higher trematode infection ($p < 0.001$). However, exposure to 10 μg L^{-1} of MC-LR did not affect trematode infection relative to tadpoles not exposed to MC-LR ($p = 0.827$). Finally, tadpoles exposed to 2 μg L^{-1} of MC-LR had 135% higher trematode infection compared to tadpoles exposed to 10 μg L^{-1} of MC-LR ($p < 0.001$).

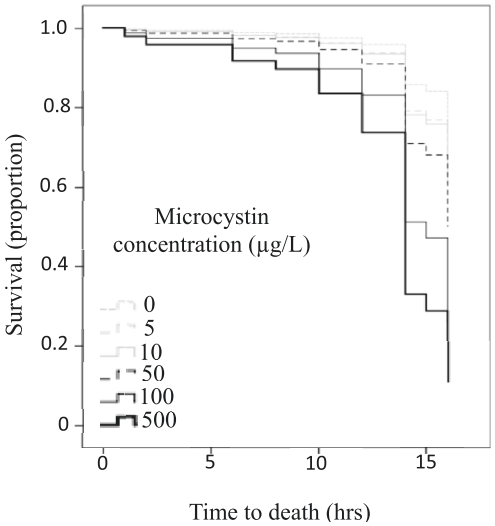

Fig. 1. Survival across time for cercariae that were exposed to 0, 5, 10, 50, 100, and 500 μg L^{-1} of MC-LR.

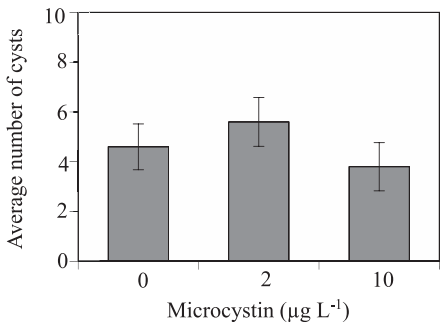

Fig. 2. Number of metacercariae encysted in tadpole hosts (average ± SE) after cercariae had been exposed to 0, 2, or 10 μg L^{-1} of MC-LR for four hours.

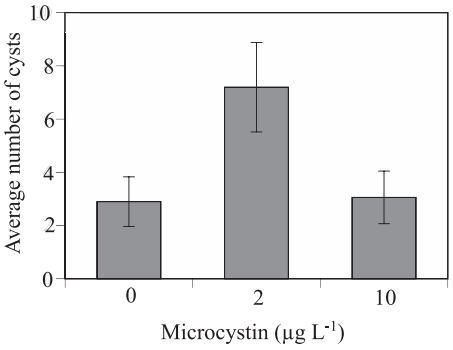

Fig. 3. Number of metacercariae encysted in tadpole hosts (average ± SE) after tadpoles had been exposed to 0, 2, or 10 μg L^{-1} of MC-LR for three days.

4. Discussion

To our knowledge, this is the first study to examine the effects of exposure to MC-LR on trematode survival. Our results were consistent with others which have shown that environmental contaminants are directly toxic to cercariae of trematodes (Rohr et al., 2008a, 2008b; Hua et al., 2016; Koprivnikar et al., 2006). The United States EPA classifies toxicants with an LC_{50} value of 100–1000 μg L^{-1} as being highly toxic to aquatic organisms (EPA, 2010). As microcystins can reach concentrations as high as 80,000 μg L^{-1} in highly eutrophic systems (Heiskary et al., 2014), our calculated LC_{50-14h} value of 134.42 μg L^{-1} suggests that MC-LR may pose a direct toxic threat to populations of trematodes. However, it is important to note that while our calculated LC_{50-14h} value suggest that trematodes may be susceptible to environmentally-relevant concentrations of MC-LR, our TTD assay demonstrates that mortality was minimal in the first eight hours of our experiment. Indeed, in the first eight hours, only 100 and 500 μg L^{-1} showed increased mortality of 10 and 15%, respectively. Infectivity of cercariae is largely age-dependent, with infectivity declining five hours after leaving their intermediate snail hosts (Pechenik and Fried, 1995). Thus, while MC-LR concentrations in nature can be directly toxic to cercariae given enough time, some cercariae may still persist long enough to escape from directly toxic conditions by encysting in their hosts. Such potential outcomes highlight the value of incorporating ecological perspectives with traditional toxicological approaches when evaluating the effects of contaminants on host-parasite interactions.

Although we found a direct, toxic effect of MC-LR on trematode cercariae in Experiment 1, short-term exposure to sublethal concentrations of 2 and 10 μg L^{-1} MC-LR had no effect on infectivity of trematode cercariae to *L. clamitans*. Infectivity of trematode cercariae has been shown to sharply decline five hours after cercariae leave the first intermediate snail host (Pechenik and Fried, 1995). In our study, mortality of cercariae across all MC-LR concentrations was highest at 14 h, thus it is likely that trematodes successfully encysted in the tadpole before MC-LR-induced mortality reached its highest point. Our findings are consistent with others, such as Rohr et al. (2008a), who assessed the indirect effects of sublethal exposure to four common herbicides on cercariae of *Echinostoma trivolvis* and found no effect of herbicide exposure on cercariae infectivity. Similar to Rohr et al. (2008a), our experimental design targeted the effect of MC-LR exposure on cercariae infectivity by using a small volume of water (60 mL) which limits the anti-parasite avoidance behavior of *L. clamitans* tadpoles. However, in nature, *L. clamitans* hosts and trematode cercariae are not likely to be in such a small volume of water, necessitating cercariae to seek out tadpole hosts. Thus, while we found no effect of MC-LR exposure on encystment, 2 and 10 μg L^{-1} MC-LR resulted in a reduction in cercariae movement and motility (NB and MW personal observation, Experiment 1). Thus, concentrations of 2 and 10 μg L^{-1} may still alter infection outcomes in nature where trematodes are forced to seek out amphibian hosts in larger volumes of water. Thus, future consideration of these sublethal effects using experimental designs such as mesocosm experiments, which allow researchers to more closely mimic natural systems than experiments in laboratory settings (Crossland and La Point, 1992) are needed.

In Experiment 3, we found that sublethal exposure to MC-LR (2 μg L^{-1}) for three days increased *L. clamitans* susceptibility to trematodes. Similarly, Milotic et al. (2018), found that tadpoles of northern leopard frogs exposed to 11 μg L-1 MC-LR for a period of five days were more susceptible to infection by trematodes. Despite differences in amphibian species, MC-LR concentration, and duration of exposure, these findings suggests that the effect of MC-LR on increasing trematodes susceptibility in larval amphibians may be somewhat generalizable. While the mechanisms driving the effect of MC-LR on tadpole susceptibility are unknown, other studies examining the effects of contaminant exposure on amphibian-trematode interactions demonstrate that an increase in susceptibility may be mediated by contaminant-induced reductions in anti-parasite avoidance behaviors in tadpoles (Daly and Johnson, 2011). However, in our experiment, any anti-parasite avoidance behavior was likely limited by the size of the experimental units that *L. clamitans* were placed in (60 mL of water). Similarly, Milotic et al. (2018) found no evidence that increased susceptibility to trematodes following exposure to MC-LR was due to a reduction in anti-parasite avoidance behavior. Alternatively, MC-LR exposure has been shown to have immunosuppressive effects through the reduction of lymphocyte proliferation in species of fish (Rymuszka et al., 2007). Thus, the increase in trematode infection may be due to MC-LR reducing the immune response of tadpole hosts. Future examination of lymphocytes and other immune system parameters are needed to better understand potential mechanisms behind increase trematode susceptibility in *L. clamitans* tadpoles following exposure to sublethal concentrations of MC-LR.

Interestingly, while tadpole exposure to 2 μg L^{-1} MC-LR caused an increase in trematode susceptibility, *L. clamitans* tadpoles exposed to 10 μg L^{-1} MC-LR did not show an increase in trematode susceptibility relative to the control. To our knowledge, this is the first example of a non-monotonic response to a parasite challenge following exposure to MC-LR. Traditional toxicological approaches typically assume that organisms respond to toxicant exposure in a dose-dependent manner (i.e. 'the dose makes the poison'; Girbes et al. 2016). However, there is a growing body of literature that suggests that toxicants commonly cause hormetic dose-responses where exposure to low and high concentrations, but not

intermediate, lead to increased rates of a given hazard (e.g. mortality, tumor formation; Fukushima et al. 2005). While testing the mechanisms for hormetic results were beyond the scope of our study, it is possible that cercariae may have been less able to encyst within the kidneys of individuals exposed to 10 μg L^{-1} of MC-LR. MC-LR has been shown to degrade the kidneys cells of rats (Zhang et al., 2002). Thus, the kidneys of tadpoles exposed to 10 μg L^{-1} MC-LR may have been inhospitable for trematode cercariae.

Despite using similar concentrations of MC-LR as Milotic et al. (2018; 10 μg L^{-1} vs. 11 μg L^{-1} MC-LR), MC-LR did not increase green frog tadpole susceptibility to trematodes but did for leopard frog tadpoles. Differences in findings between these studies suggests that there are likely species-specific differences in responses to different concentrations of MC-LR. Overall, these results contribute to the growing body of literature highlighting the challenges of making predictions about the effects of low concentrations of contaminants (Costantini et al., 2010; Calabrese and Baldwin, 2003). Studies examining the effects of toxicants on host-parasite interactions which evaluate only a single concentration of a given toxicant and species may limit conclusions regarding the indirect effects that toxicants may have on infection outcomes in nature. Thus, future studies that expose both multiple hosts and parasites to a broad range of sublethal concentrations may improve our ability to predict how toxicants will indirectly shape disease dynamics in contaminated systems.

To sum, we found that concentrations of MC-LR ≥50 μg L^{-1} had negative effects on trematodes by increasing their rate of mortality. However, the net effects of MC-LR exposure in nature are likely dependent on the timing of MC-LR exposure. Specifically, while MC-LR at concentrations of 50, 100 and 500 μg L^{-1} increased mortality rates of trematodes, the increase in mortality occurred after the parasite's most infective period (0–5 h). As such, depending on the timing of exposure to MC-LR relative to a cercariae's lifespan, it is possible that cercariae may still be able to encyst in their hosts. Future work that considers the effects of these high concentrations of MC-LR on cercariae encystment rates and the sublethal consequences on parasite survival following encystment is needed to evaluate the net effects of MC-LR on disease outcome.

At lower concentrations (<10 μg L^{-1}), our work suggests that MC-LR's effect on tadpole-cercariae disease outcome is likely driven by its effect on the tadpole host. Indeed, in our second experiment, we demonstrate that exposure to 2 and 10 μg L^{-1} of MC-LR early in the lifespan of cercariae had no effect on their ability to encyst in tadpole hosts. In contrast, exposure to 2 μg L^{-1} cause green frog tadpoles to be more susceptible to parasites. Interestingly, we find that the effect of MC-LR on susceptibility to parasites did not increase with increasing concentrations. Collectively, our results highlight the challenges of predicting the effects of contaminants and underscores the need for future studies to consider multiple concentrations when evaluating the effect of contaminants on host-parasite interactions.

4.1. Future directions

Here, we demonstrate that MC-LR can alter tadpole-cercariae dynamics. However, our study focused on evaluating the sublethal effects of MC-LR following only a single period of exposure (72 h). MC-LR in nature has a relatively long half-life (90–120 days; Welker and Steinberg, 2000), and is most commonly released into water bodies across time as cyanobacterial cells age and die (Watanabe et al., 1992). Thus, future consideration of these sublethal effects across different exposure regimes (e.g. pulse exposure) and longer exposure periods will allow us to better evaluate the sublethal effects of MC-LR on trematode transmission within this system. Additionally, we did not examine the effects of MC-LR exposure on the first intermediate planorbid snail host of trematodes. As eutrophication has been shown to indirectly increase parasite infection in amphibian hosts by increasing the abundance of periphyton (Johnson et al., 2007) – a food resource for snails – examining the direct (i.e. toxic), and indirect (i.e. snail fecundity, cercarial shedding-rate) effects of microcystins on snail hosts is needed. Ultimately, it will be through examining the direct and indirect effects of MC-LR across several concentrations and periods of exposure on each player within this, and other host-parasite systems that we will gain a better understanding of the effects that these toxicants may have on disease outcomes within eutrophic environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.12.160.

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