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Assessing the toxicity of cell-bound microcystins on freshwater pelagic and benthic invertebrates

René S. Shahmohamadloo[a,*], David G. Poirier[b], Xavier Ortiz Almirall[b,c], Satyendra P. Bhavsar[b,d], Paul K. Sibley[a]

[a] School of Environmental Sciences, University of Guelph, Guelph, Ontario, Canada
[b] Ministry of the Environment, Conservation and Parks, Toronto, Ontario, Canada
[c] School of Environmental Studies, Queen's University, Kingston, Ontario, Canada
[d] Department of Physical & Environmental Sciences, University of Toronto, Toronto, Ontario, Canada

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ABSTRACT

Cyanobacterial harmful algal blooms dominated by *Microcystis* frequently produce microcystins, a family of toxins capable of inflicting harm to pelagic and benthic freshwater invertebrates. Research on the effect of microcystins on invertebrates is inconclusive; from one perspective, studies suggest invertebrates can coexist in toxic blooms; however, studies have also measured negative food-associated effects from microcystins. To test the latter perspective, we examined the reproduction, growth, and survival of laboratory-cultured *Ceriodaphnia dubia*, *Daphnia magna*, and *Hexagenia* spp. exposed to cell-bound microcystins through a series of life-cycle bioassays. Test organisms were exposed to a concentration gradient ranging from 0.5 $\mu g\,L^{-1}$ to 300 $\mu g\,L^{-1}$ microcystins, which corresponds to values typically found in freshwaters during bloom season. Lethal concentrations in *C. dubia* (LC50 = 5.53 $\mu g\,L^{-1}$) and *D. magna* (LC50 = 85.72 $\mu g\,L^{-1}$) exposed to microcystins were among the lowest recorded to date, and reproductive effects were observed at concentrations as low as 2.5 $\mu g\,L^{-1}$. Length of *D. magna* was significantly impacted in microcystin treatments great than 2.5 $\mu g\,L^{-1}$. No lethality or growth impairments were observed in *Hexagenia*. This information will improve our understanding of the risks posed by microcystins to food webs in freshwaters.

1. Introduction

Harmful algal blooms dominated by the cyanobacterial species *Microcystis* are a growing, serious problem that threatens the ecological integrity and sustainability of freshwater systems (Paerl and Otten, 2013; Harke et al., 2016; Visser et al., 2016; Huisman et al., 2018). The process by which cyanobacteria becomes "harmful" essentially depends on nutrient enrichment and factors that are affected by climate change, the combination of which can culminate in a critical situation: expansive blooms, increased turbidity, hypoxia and anoxia, and suppressed macrophytes and aquatic (in)vertebrate communities (Scholten et al., 2005; Paerl et al., 2016; Visser et al., 2016; Xiao et al., 2018). In recent decades, eutrophication and climate change have intensified considerably, exacerbating *Microcystis* blooms in many of the world's largest freshwater and brackish water systems, including Lake Victoria (Africa), Lake Erie, (North America), Lake Taihu (East Asia), and the Baltic Sea (Europe) (Paerl et al., 2011). To complicate matters further, *Microcystis* adapt well in temperate systems by overwintering among the benthos and rising to the epilimnion to form blooms during the summer (Reynolds and Rogers, 1976; Ibelings et al., 1991; Harke et al., 2016). Many strains of *Microcystis* can produce microcystins, a class of potent hepatotoxins that have caused death to humans, mammals, fish, birds, mussels, and zooplankton (Yang et al., 2011; Sun et al., 2012; Huisman et al., 2018; Gene et al., 2019). Microcystins are intracellular and produced from viable *Microcystis* cells, typically at high concentrations, and may therefore harm organisms that graze on them (Rohrlack et al., 2001; Sadler and von Elert, 2014; Ger et al., 2016; Harke et al., 2016; Lyu et al., 2016; Xiao et al., 2018; Lyu et al., 2019). In the presence of toxic *Microcystis* blooms, pelagic (*Ceriodaphnia* spp. and *Daphnia* spp.) and benthic (*Hexagenia* spp.) invertebrates that depend on the quality of primary production may be at risk of population decline that could alter food web dynamics (Ger et al., 2016).

Ceriodaphnia and *Daphnia* are pelagic species that constitute an important part of aquatic food chains. They are primary grazers of phytoplankton, as well as the prey of higher trophic level species (Stollewerk, 2010; Davis et al., 2012). *Ceriodaphnia* and *Daphnia* have

* Corresponding author.
E-mail address: rshahmoh@uoguelph.ca (R.S. Shahmohamadloo).

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the potential to be exposed to microcystins in freshwaters experiencing *Microcystis* blooms; however, research on the interaction between microcystins and cladocerans is inconclusive. One perspective suggests that cladocerans are positively impacted in the presence of toxic *Microcystis*. A study by Sarnelle et al. (2010) found that *Daphnia magna* were able to maintain positive population growth (~0.12 d^{-1}) on a diet containing 50% *Scenedesmus* and 50% toxic *Microcystis aeruginosa*. Several other studies have also shown that daphnid populations genetically adapt and phenotypically acclimate to toxic cyanobacterial blooms to ensure survival, growth, and reproduction (Hairston et al., 2001; Gustafsson et al., 2005; Sarnelle and Wilson, 2005). Through adaptation processes, toxic cyanobacteria can become an important food source in the diet of daphnids, and its absence can have consequences on population growth and survival (Sarnelle et al., 2010). Sarnelle et al. (2010) argue that previous exposure to toxic cyanobacteria must be taken into consideration when selecting a suitable organism for study when extrapolating results to the natural environment. A second and opposing perspective suggests that cladocerans are negatively impacted in the presence of toxic *Microcystis*. Several studies have demonstrated that daphnids experience toxic effects from cell-bound (Rohrlack et al., 2001, 2005) and extracellular (Freitas et al., 2014; Herrera et al., 2015) microcystins, including mortality, reproductive impairment, and population decline. Despite opposing views that state *Microcystis* colonies do (Davis et al., 2012) and do not (Sarnelle et al., 2010) inhibit daphnid population growth and development, *Microcystis* species exhibit diverse colonial morphologies (e.g. single cells, sponge-like, spherical, elongated) and may therefore harm animals that are large enough to ingest them (Tillmanns et al., 2008; Xiao et al., 2018). Under normal conditions, microcystins are cell-bound and the toxin content is often high (Rohrlack et al., 2005). Measuring a dose-response relationship for cell-bound microcystins effects is therefore needed in order to estimate the impact of toxic *Microcystis* blooms on daphnid populations (Rohrlack et al., 2001). Since Rohrlack et al. (2001) put forward this thought numerous studies (Maršálek and Bláha, 2004; Chen et al., 2005; Lindsay et al., 2006; Sotero-Santos et al., 2006; Okumura et al., 2007; Smutná et al., 2014; Ferrão-Filho et al., 2014; Freitas et al., 2014; Herrera et al., 2015) have developed dose-response relationships for *Ceriodaphnia* and *Daphnia*. However, while it is generally agreed that the ingestion of cell-bound microcystins by cladocerans is considered an important route of exposure to these toxins (Rohrlack et al., 2001; Ferrão-Filho et al., 2014), none of these studies used microcystins that were cell-bound. Therefore, cell-bound microcystin studies are needed to provide an environmental scenario that depicts the exposure of daphnids to a viable, toxic *Microcystis* bloom in the pelagic zone.

Hexagenia spp. are benthic species native to North America and found in lakes and rivers (Edsall, 2001). They typically hatch from eggs and spend approximately 2 yr as nymphs underwater, burrowed in a substrate of clay and (sandy) silt (Edsall, 2001). Mayfly nymphs are detritivores that constitute a major food source for fish by transferring detrital energy resources directly to them (Price, 1963). They are regarded as important indicators for the ecological integrity of lakes and rivers due to their abundance in mesotrophic habitats and intolerance to pollution (Edsall, 2001). With the reoccurrence of cyanobacterial blooms, especially in the Great Lakes where *Hexagenia* reside, burrowing mayflies may be susceptible to an adverse effect from *Microcystis* (Smith et al., 2008; Schmidt et al., 2014; Woller-Skar et al., 2015; Moy et al., 2016). Laboratory experiments conducted by Smith et al. (2008) found microcystin-LR, the most studied congener in the literature, affected the temporal hatching pattern in eggs after 96 h of exposure in petri dishes to concentrations ranging from 1 to 100 μg L^{-1}. Field samples collected by Woller-Skar et al. (2015) also found microcystins in *Hexagenia limbata* and fecal matter from a maternity colony of little brown bats (*Myotis lucifugus*), suggesting trophic transfer of the hepatotoxin from aquatic insects. In riparian food webs, Moy et al. (2016) detected microcystins in *Hexagenia* and their consumers, including spiders (*Tetragnathidae*) and prothonotary warblers (*Protonotaria citrea*). Moy et al. (2016) further observed higher microcystin concentrations in nestlings that received a higher proportion of mayflies in their diet, suggesting *Hexagenia* have the potential to be a hepatotoxic vector to higher trophic species. No studies have assessed the survival and growth of *Hexagenia* in sediment exposed to cell-bound microcystins. Sediments are frequently an in-situ source of pollutants, and given the potential for *Microcystis* to overwinter in the benthos, further investigation whether viable, toxic cells can harm *Hexagenia* is warranted.

Toxicological research on the effects of *cell-bound* microcystins to pelagic and benthic species is needed in order to estimate the impact from toxic *Microcystis* when blooms are viable in freshwaters. The present study addresses this research gap by measuring dose-response relationships from exposure to *cell-bound* microcystins produced by *M. aeruginosa*, at concentrations environmentally relevant to freshwaters (0.5–300 μg L^{-1}), to laboratory-cultured *Ceriodaphnia dubia*, *Daphnia magna*, and *Hexagenia* spp. through a series of bioassays. Our toxicity tests measure lethal and effective concentrations (LC_x and EC_x) for the survival, growth, and reproduction of pelagic and benthic organisms exposed to *cell-bound* microcystins. The significance of our work is that we simulated a *Microcystis* bloom scenario where cells were viable and microcystins were intracellular. Our interest in pursuing this scenario over the traditional approach —where cells are lysed in order to release microcystins into water— comes in light of the recent work of Janssen (2019) that microcystins never occur alone, and although this class of cyanopeptides are of ecotoxicological concern may operate in concert with other bioactive metabolites within a cyanobacterial cell.

2. Materials and methods

2.1. Culture preparation

M. aeruginosa strain CPCC 300 was provided by the Canadian Phycological Culture Centre (University of Waterloo, Waterloo, Canada). This strain originates from an extract in Pretzlaff Pond, Alberta, Canada, on August 7, 1990 (deposited by E. Prepas/A. Lam as #45-4A in March 1993). Growth of *M. aeruginosa* CPCC 300 was unicellular and produces microcystin-LR and [D-Asp^3]-microcystin-LR, according to Shahmohamadloo et al. (2019). Cultures were kept under continuous light (600 ± 15 lx cool-white fluorescent light) and fixed temperature (24.0 ± 1 °C) for 16:8 h of light:dark cycle in a growth chamber. The diameter of *M. aeruginosa* cells ranged between 1 and 2 μm. *M. aeruginosa* cultures were grown for at least 1 mo before use in the experiments. Prior to running our experiments, we investigated whether BG-11 liquid media recipe could cause lethal and reproductive effects in *C. dubia*. Preliminary trial runs confirmed lethal and reproductive effects in *C. dubia*, and we began to experiment with several dilution series of BG-11 medium. We found that *C. dubia* did not experience lethality or reproductive effects when 10% of the total test vessel volume had BG-11 medium and the remaining 90% was dechlorinated municipal tap water. Next, we explored whether *M. aeruginosa* cells could be successfully centrifuged down into 10% of a vessel containing BG-11 medium without rupturing cells. We found that *M. aeruginosa* cells could be successfully centrifuged for 8 min at 2800 × *g*, with 90% of BG-11 media decanted and the remaining 10% re-suspended in dechlorinated municipal tap water, without compromising the integrity of cells and toxins. Chemical analyses later confirmed > 95% of microcystins were intracellular (cell-bound) and cultures did not crash after re-suspension in dechlorinated municipal tap water. The physicochemical properties of dechlorinated municipal tap water are listed in Table S1. Cell concentration was determined by hemacytometer to be 1.68 ± 0.02 × 10^7 cells mL^{-1} prior to testing. *M. aeruginosa* biomass was further calculated to quantify the contribution of microcystins and take into consideration other compounds may have been present. *M. aeruginosa* biomass was 0.55 mg d.w. mL^{-1}. The

contribution of microcystins was approximately 2.00 μg per mg d.w. of *M. aeruginosa*.

2.2. Test solution preparation

M. aeruginosa CPCC 300 was prepared by centrifuging for 8 min at 2800 × *g* followed by decanting 90% of BG-11 media and re-suspending remaining 10% in dechlorinated municipal tap water. Care was taken to ensure minimal loss of *M. aeruginosa* cells during decanting. The test solution was stockpiled in polyethylene jars and stored in the dark at 6.0 ± 1 °C for 24 h until needed. Storing the test solution at this temperature and darkness suspended growth of *M. aeruginosa* and kept cells viable. Prior to each exposure the viability of cells was confirmed by calculating the cell concentration using a hemacytometer.

In order to provide a realistic exposure scenario reflective of freshwaters systems that experience *Microcystis* blooms, nominal concentrations for microcystins were 2.5, 5, 10, 25, 50, 75, and 100 μg L^{-1} for *Ceriodaphnia* and *Daphnia*, and 6.25, 12.5, 25, 50, and 100 μg L^{-1} for *Hexagenia*. Test solutions were diluted with dechlorinated municipal tap water to achieve the desired concentrations per treatment. Each test included 2 negative controls; 1 control was 10% BG-11 media in dechlorinated water (i.e. 0 μg L^{-1} microcystins), and the other control was dechlorinated water only. The total number of treatments per test was 9 for *Ceriodaphnia* and *Daphnia*, and 5 for *Hexagenia*. For *Ceriodaphnia* and *Daphnia* tests, all treatments received a sufficient amount of green algae to ensure growth and reproduction of daphnids, based, in part, on extrapolation from previous literature (DeMott, 1999; Rohrlack et al., 2005) which showed that the strain of *M. aeruginosa* used for our experiments may have the potential to inhibit feeding in daphnids. Green algae were supplied together with *M. aeruginosa* to eliminate the possibility of lethality from a lack of nutritious food and thus allow us to focus on the possibility of lethality from microcystin toxicity. Temperature, pH, conductivity, ammonia, and dissolved oxygen were measured in all treatments at test initiation, termination, and on days when solution changes were required.

2.3. Toxicity tests

2.3.1. Ceriodaphnia dubia

A 7-d static-renewal life-cycle test developed by Environment Canada (2007) was used to determine the survival and reproduction of *C. dubia*. Neonates of *Ceriodaphnia* used for this test were supplied from a continuous culture at the Ontario Ministry of the Environment, Conservation and Parks, Aquatic Toxicology Unit (OMECP ATU) (Etobicoke, ON, Canada), reared according to Environment Canada (2007). Test organisms had to be less than 24 h old and within 12 h of the same age in order to be used in the tests. At test initiation, each of the 9 treatments received 10 test organisms, and each test organism was placed in a 30-mL glass tube that contained 15 mL of test solution. A total of 90 *Ceriodaphnia* were used for this test. Each *Ceriodaphnia* was fed with 0.6 mL of total food, comprised of 0.5 mL concentrated *Raphidocelis subcapitata* algae (~1.5 × 10^6 cells mL^{-1}) and 0.1 mL yeast-cereal grass-trout chow (YCT; 1.8 g dw L^{-1}). Following test initiation, solution changes were done daily by transferring adult *Ceriodaphnia* from old to new glass tubes, followed by supplying each *Ceriodaphnia* with 0.6 mL of total food. During this time, lethality in adult *Ceriodaphnia* and number of neonates was recorded. Solutions from old glass tubes were pooled per treatment, and water chemistry parameters (temperature, pH, conductivity, dissolved oxygen) were measured. Tubes were incubated under 500–1000 lx cool-white fluorescent light at 25.0 ± 1 °C with a 16:8 light:dark cycle. The test was to be terminated as soon as 60% or more of the control organisms had 3 broods. Within 72 h of the life-cycle test, 90% mortality was observed in 2.5, 5, 10, 25, 50, 75, and 100 μg L^{-1} microcystin treatments, while no mortality was observed in the controls (Table S2). Thus, a second test was initiated, this time with a lower concentration gradient of 0.5, 1, 1.5, 2.5, 5, 10, and 20 μg L^{-1} microcystins, and 2 controls (Table S3).

2.3.2. Daphnia magna

A 21-d semi-static-renewal life-cycle test developed by the Organization for Economic Cooperation and Development (OECD) (2012) was used to determine the survival and reproduction of *D. magna* (Tables S4A–C). Neonate *Daphnia* used for this test were also supplied from a continuous culture and raised according to the OMECP ATU (2014). At test initiation, each of 10 replicates for each of the 9 treatments received *D. magna* that were less than 24 h old (a total of 90 *Daphnia*). Each *Daphnia* was placed in a 50-mL glass tube that contained ~45 mL of test solution and 1.0 mL of total food, subdivided to 0.5 mL of concentrated *R. subcapitata* algae and 0.5 mL of concentrated *Chlorella fusca*, or 1:1 based on cells mL^{-1} mixture of the two. Solutions were changed 3 × wk on Mondays, Wednesdays, and Fridays by transferring adult *Daphnia* from old to new glass tubes, followed by supplying each *Daphnia* with 1.0 mL of total food. During this time, lethality and the number of neonates were recorded. Tubes were incubated under 400–800 lx cool-white fluorescent light at 20.0 ± 1 °C with a 16:8 light:dark cycle. Water chemistry parameters were measured at initiation, solution changes, and termination of the test. Neonates were collected on Day 14 to assess whether the mean total length (mm) decreased between the controls and the microcystin treatments.

2.3.3. Hexagenia spp.

A 21-d semi-static-renewal test developed by the OMECP ATU (2015) was used to determine the survival and growth of *Hexagenia* spp. (i.e. mixed colony of *Hexagenia limbata* and *Hexagenia rigida*) in sediment. Organisms used for this test were supplied from a continuous mixed culture raised at the OMECP ATU (Etobicoke, ON, Canada). At test initiation, each of the 7 treatments received 5 replicates, and each replicate received 10 *Hexagenia* that were 5 mg ± 2 mg and 2–4 mo old. For each replicate, 10 *Hexagenia* were placed in a 1.8 L glass jar that contained 1.3 L of test solution and 325 mL of sediment obtained from Long Point, Ontario, Canada (latitude: 42° 32′ 51.76″ N; longitude: 80° 03′ 33.55″ W). Thus, a total of 35 jars were used for the entire test. 2 controls (i.e. water-only and BG-11 medium with water) were included in this test. The weight of organisms was measured at test initiation and termination. Nominal concentrations for the exposure treatments were 6.25, 12.5, 25, 50, and 100 μg L^{-1} of microcystins. Solutions were changed 3 × wk on Mondays, Wednesdays, and Fridays. During this time, lethality was recorded. Jars were incubated under 400–800 lx cool-white fluorescent light at 23.0 ± 1 °C with a 16:8 light:dark cycle. Water chemistry parameters were measured at initiation, solution changes, and termination of the test.

2.4. Chemical analysis

Water samples from each treatment group were collected at initiation, during solution changes, and at termination of the tests, and submitted to the Ontario Ministry of the Environment, Conservation and Parks, Toxic Organic Section (Etobicoke, ON, Canada) for quantification of microcystins to determine the measured concentrations. Samples were analyzed within 4 wk after test termination using on-line solid phase extraction coupled to liquid chromatography-quadrupole time-of-flight high resolution mass spectrometry (Waters Xevo G2-XS, Milford, MA, USA) (Ortiz et al., 2017). In this targeted and non-targeted high throughput method, samples of *M. aeruginosa* CPCC 300 at each time point were measured and 12 microcystin variants (LR, YR, RR, HtyR, HilR, WR, LW, LA, LF, LY, Dha_7-LR, and Dha_7-RR) and anatoxin-A (all standards purchased from Enzo Life Sciences, Farmingdale, NY, USA) were quantified using nodularin as the internal standard. Detection limits were 0.05 μg L^{-1} with an expanded uncertainty ranging from 4 to 14% for the different variants that accounts for uncertainty coming from the sample preparation, the instrument, and calibration standards. Samples were centrifuged before sample preparation. Cells

containing intracellular toxins were analyzed separately from the supernatant, containing extracellular toxins.

2.5. Statistical analysis

Endpoints measured in the tests were lethality, reproduction, and length for *Daphnia*, and lethality and growth for *Hexagenia*. Lethality was calculated as the percent of organisms per treatment (n = 10 for *Daphnia*; n = 50 for *Hexagenia*) that died at the end of test. Reproduction was calculated as the total number of neonates produced by each organism within the first 3 broods for *C. dubia*, and the total number of neonates produced in each organism at the end of test for *D. magna*. In both tests, if an organism died before producing a brood, the number of neonates recorded was zero; conversely, if an organism died after producing a brood, the number of neonates was recorded (Environment Canada, 2007).

Measured concentrations of total microcystins (i.e. microcystin-LR and [D-Asp^3]-microcystin-LR) were used in all statistical analyses. Normality and equality of variance were tested using the Shapiro-Wilk and Levene's tests, respectively, and the data were transformed prior to statistical analysis, where necessary. If the assumptions of normal and equal variance passed, a one-way analysis of variance (ANOVA; $\alpha = 0.05$) was used to determine whether there was a significant difference in the percentage of lethality, reproduction, and length for *Daphnia*, and the percentage of lethality and growth for *Hexagenia*. If the assumptions of normality and equal variance failed, a Kruskal-Wallis one-way ANOVA ($\alpha = 0.05$) was used. If a significant difference between the treatments was identified by ANOVA, a post hoc Tukey's test ($\alpha = 0.05$) was performed to compare all treatment means. ANOVAs were performed using Sigma Stat (Version 4.0, Systat Software, San Jose, CA, USA).

Dose-response relationships were modeled using log-logistic regression in R version 3.5.0 with the *drc* version 3.0–1 package (Ritz and Strebig, 2005). Regression models were calculated using measured concentrations. Lethal and effective concentrations for 5th, 10th, 25th, 50th, and 75th percentile (LC5/10/25/50/75 and EC5/10/25/50/75) with 95% confidence internals were calculated from the regression models.

3. Results

Toxin concentrations consisted of 40 ± 3% microcystin-LR, 60 ± 3% [D-Asp^3]-microcystin-LR; no other microcystin variants and anatoxin-A were detected (Table 1). Lethality data was fit to a 2-parameter log-logistic model, and the concentration at which *Ceriodaphnia* and *Daphnia* experienced 10%, 25%, and 50% lethality from microcystins was determined (Table 2). Reproduction data was fit to a 3-parameter log-logistic model, and the concentration at which *Ceriodaphnia* and *Daphnia* experienced 10%, 25%, and 50% impairment in reproduction from microcystins was determined (Table 3).

For the *C. dubia* test, survival of controls was ≥80% and water chemistry parameters before and after solution changes were within the test criteria (Table S5). Following the criteria for reproduction of controls outlined in Environment Canada (2007), all test acceptability criteria were met. Mortality was first observed 24 h after exposure to microcystins, however the majority of mortality (i.e. 30/90 or 33% of test organisms) was observed after 72 h (Fig. 1). Immobilization of parent organisms was not observed. Control reproduction was significantly different from the microcystin treatments (Tables S6A–B, one-way ANOVA, F = 67.045, $p = < 0.001$). The controls had a mean total neonate production of 34.0 ± 1.5 in water-only and 37.3 ± 0.8 in BG-11, whereas a gradual decline was observed in the number of neonates as the concentration of microcystins increased (Fig. 1, Table 1).

For the *D. magna* test, survival of controls was ≥80% and water chemistry parameters before and after solution changes were within the test criteria (Tables S7A–C). There was no evidence of hypoxia stress on the behaviour of the organisms. Following the criteria for reproduction of controls outlined in OECD (2012), all test acceptability criteria were met. Mortality was first observed 48 h after exposure to microcystins, however most mortality (i.e. 28/90 or 31% of test organisms) was observed after 72 h (Fig. 1). Immobilization was observed in some parent organisms within 48 h of exposure in the 50, 75, and 100 μg L^{-1} microcystin treatments. Control reproduction was significantly different from the microcystin treatments (Tables S6A–B, one-way ANOVA, F = 37.850, $p = < 0.001$). Where the controls had a mean total of 89.5 ± 5.6 in water-only and 80.3 ± 8.8 in BG-11, a gradual decline was observed in the number of neonates as the concentration of microcystins increased (Fig. 1, Table 1). The controls produced their first brood on Day 7 as expected following the reproduction criteria. However, production of the first broods in the 2.5, 5, 10, and 25 μg L^{-1} microcystin treatments was delayed to Day 8, and in the 50, 75, and 100 μg L^{-1} microcystin treatments production was delayed further to Day 11 (Tables S4A–C). The length of the controls was significantly different in microcystin treatments > 2.5 μg L^{-1} (Tables S6A–B, one-way ANOVA, F = 81.020, $p = < 0.001$).

For the *Hexagenia* test, survival of controls was ≥80% and water chemistry parameters before and after solution changes were within the test criteria (Table S8). Following the criteria for reproduction of controls outlined in the OMECP ATU (2015), all test acceptability criteria were met. No turbidity, signs of stress or abnormal behaviour (e.g. lack of burrowing activity) were observed during the test. Mean control survival and growth was not significantly different from the microcystin treatments (one-way ANOVA, $p = 0.614$).

4. Discussion

We ran a series of life-cycle bioassays on pelagic and benthic invertebrates to measure whether a dose-response exists to microcystins cell-bound within *M. aeruginosa* at a concentration gradient relevant to freshwaters (0.5–300 μg of total microcystins L^{-1}) during harmful algal bloom events. Results from our studies revealed lethal (LC50 = 5.53 μg L^{-1}) and reproductive effects (EC50 = 4.39 μg L^{-1}) in *C. dubia*, lethal (LC50 = 85.72 μg L^{-1}) and reproductive effects (EC50 = 45.85 μg L^{-1}) in *D. magna*, and no lethality in *Hexagenia* spp. Further information from the *D. magna* bioassay revealed a delay by 24–96 h in the production of the first broods, and a significant decrease in length of neonates (Tables S6A–B; one-way ANOVA, F = 81.020, $p = < 0.001$) within microcystin treatments.

4.1. Pelagic species

The size of species, duration of test, feeding regimen, detectable microcystin profile, and lethal concentrations observed in *Ceriodaphnia* and *Daphnia* were compared and contrasted to the literature from 1989 to 2019 (Table S9). We report the lowest lethal concentrations for *C. dubia* and *D. magna* exposed to microcystins, in most cases by an order of magnitude from 10^3 – 10^6 μg L^{-1}. Several distinctions in the experimental design between each study can offer insight into the apparent sensitivities measured in our work.

The first distinction was that 92% of studies we found (DeMott et al., 1991; Maršálek and Bláha, 2004; Chen et al., 2005; Lindsay et al., 2006; Sotero-Santos et al., 2006; Ferrão-Filho et al., 2014; Freitas et al., 2014; Smutná et al., 2014; Herrera et al., 2015) exposed *Ceriodaphnia* and *Daphnia* to extracellular microcystins. However, direct consumption of cell-bound microcystins is considered the main route of exposure to daphnids (Rohrlack et al., 2001, 2005; Kozlowsky-Suzuki et al., 2012; Ferrão-Filho et al., 2014). In a study performed by Rohrlack et al. (2005), *Daphnia galeata* were fed toxic and non-toxic strains of *M. aeruginosa* to assess whether toxicity came from microcystins or cyanobacterial cells. Results confirmed that when toxic cells were ingested, microcystins were rapidly taken up into the blood and induced toxicity

Table 1
Measured concentrations for microcystin-LR and [D-Asp^3]-microcystin-LR (μg L^{-1}) effects on lethality, reproduction, length, and growth for *Ceriodaphnia*, *Daphnia*, and *Hexagenia* tests.

Species	Nominal concentration for each microcystin (μg L^{-1})	Microcystin-LR measured concentration (μg L^{-1})	[D-Asp^3]-microcystin-LR measured concentration (μg L^{-1})	% Mortality	Mean total neonates/female (n = 10) ± SD	Mean length (mm) of neonates/female (n = 10) ± SD	Mean total wet wt (mg) (n = 50) ± SD
Ceriodaphnia dubia	0	–	–	0	42.3 ± 1.8	–	–
	0 (BG-11)	–	–	0	56.2 ± 1.3	–	–
	2.5	–	–	100	0.8 ± 0.5	–	–
	5.0	–	–	70	1.5 ± 0.5	–	–
	10.0	–	–	100	0 ± 0	–	–
	25.0	–	–	100	0 ± 0	–	–
	50.0	–	–	100	0 ± 0	–	–
	75.0	–	–	100	0 ± 0	–	–
	100.0	–	–	100	0 ± 0	–	–
Ceriodaphnia dubia	0	0	0	0	34.0 ± 1.5	–	–
	0 (BG-11)	0	0	0	37.3 ± 0.8	–	–
	0.5	0.5	0.5	0	29.4 ± 1.3	–	–
	1.0	1.0	1.1	0	26.0 ± 0.9 [b]	–	–
	1.5	1.7	1.8	0	24.3 ± 0.7 [a,b]	–	–
	2.5	2.5	2.7	20	12.6 ± 1.5 [a,b]	–	–
	5.0	5.4	5.9	100	0 ± 0 [a,b]	–	–
	10.0	10.3	11.4	100	0 ± 0 [a,b]	–	–
	20.0	21.2	23.4	100	0 ± 0 [a,b]	–	–
Daphnia magna	0	0	0	0	89.5 ± 5.6	1.53 ± 0.07	–
	0 (BG-11)	0	0	0	80.3 ± 8.8	1.28 ± 0.04	–
	2.5	3.2	4.9	0	59.9 ± 9.3 [a]	1.19 ± 0.07 [a]	–
	5.0	7.5	7.7	0	69.8 ± 8.6	1.19 ± 0.05 [a]	–
	10.0	13.9	14.7	20	58.4 ± 6.1 [a]	1.18 ± 0.08 [a]	–
	25.0	31.3	38.4	30	30.5 ± 13.7 [a,b]	1.03 ± 0.12 [a,b]	–
	50.0	49.9	64.5	70	9.8 ± 2.3 [a,b]	0.86 ± 0.06 [a,b]	–
	75.0	75.0	95.5	80	5.8 ± 9.9 [a,b]	0.95 ± 0.12 [a,b]	–
	100.0	130.7	172.2	90	2.0 ± 6.3 [a,b]	0.81 ± 0.07 [a,b]	–
Hexagenia spp.	0	0	0	8	–	–	0.27 ± 0.04
	0 (BG-11)	0	0	16	–	–	0.21 ± 0.03
	6.25	7.0	11.2	4	–	–	0.26 ± 0.03
	12.5	13.7	22.0	2	–	–	0.29 ± 0.03
	25.0	27.0	43.3	8	–	–	0.27 ± 0.03
	50.0	55.9	89.6	6	–	–	0.28 ± 0.05
	100.0	125.7	208.4	4	–	–	0.31 ± 0.02

[a] Statistically significant from the water-only control ($p < 0.05$) as determined by Kruskal-Wallis test with post-hoc Tukey's test.
[b] Statistically significant from the BG-11 control ($p < 0.05$) as determined by Kruskal-Wallis test with post-hoc Tukey's test.

to multiple organs in *D. galeata*; when non-toxic cells were ingested, the appearance and activity of *D. galeata* were normal (Rohrlack et al., 2005). Therefore, lethal concentrations reported in the literature may underestimate exposure scenarios for daphnids in the environment prior to the senescence of a bloom.

The second distinction was that 60% of studies (Maršálek and Bláha, 2004; Chen et al., 2005; Lindsay et al., 2006; Sotero-Santos et al., 2006; Ferrão-Filho et al., 2014; Freitas et al., 2014) did not feed daphnids during testing. To maintain daphnids in a nutritional state that will support their growth, survival, and reproduction, daily feeding is required during testing. Food limitation can exert a strong influence on the health and function of test organisms (DeMott and Kerfoot, 1982). Starvation effects in *Ceriodaphnia* and *Daphnia* may have therefore compromised the health of test organisms. Moreover, given the weight of evidence that shows the main route of exposure to microcystins is when the toxins are cell-bound, the exposure scenario —and associated toxicity— is no longer the same when microcystins are extracellular. Thus, lethal concentrations reported in the literature will vary depending on whether microcystin-LR is intra- or extracellular. In the latter instance, toxic effects may have been diluted because there was no vector (i.e. food in the form of algal cells) for *Ceriodaphnia* and *Daphnia* to feed on.

The third distinction was that 48% of studies (Maršálek and Bláha, 2004; Sotero-Santos et al., 2006; Ferrão-Filho et al., 2014; Smutná et al., 2014; Herrera et al., 2015) exposed daphnids to microcystins that were sourced from a cyanobacterial extract (i.e. from the field, either crude or purified for microcystin-LR). It is important when designing lethality experiments to control for the species/strain or colony type of cyanobacteria as a means to control for microcystin congeners profile. If cyanobacterial extracts from the field are used in such experiments,

Table 2
Summary of total microcystin (microcystin-LR and [D-Asp^3]-microcystin-LR) toxicity values (lethal concentration [LC*x*] values with 95% confidence intervals in parentheses) for lethality of *Ceriodaphnia*, *Daphnia*, and *Hexagenia*.

Species	Duration (d)	Lethality, LC*x* (μg L^{-1})				
		Model	10	25	50	Slope
Ceriodaphnia dubia	7	*No dose-response relationship in first test; LC_{50} < 3.0 μg L^{-1}*				
Ceriodaphnia dubia	7	2-parameter log-logistic	5.02 (2.82–7.21)	5.27 (4.38–6.16)	5.53 (1.42–9.64)	−22.68
Daphnia magna	21	2-parameter log-logistic	27.59 (9.94–45.24)	48.63 (26.98–70.28)	85.72 (55.91–115.52)	−1.94
Hexagenia spp.	21	No dose-response relationship was established				

Table 3
Summary of total microcystin (microcystin-LR and [D-Asp^3]-microcystin-LR) toxicity values (effective concentration [EC*x*] values with 95% confidence intervals in parentheses) for reproduction of *Ceriodaphnia* and *Daphnia*.

Species	Duration (d)	Reproduction, EC*x* (μg L^{-1})[a]				
		Model	10	25	50	Slope
Ceriodaphnia dubia	7	*No dose-response relationship in first test; EC_{50} < 3.0 μg L^{-1}*				
Ceriodaphnia dubia	7	3-parameter log-logistic	1.95 (1.06–2.84)	2.92 (2.01–3.75)	4.39 (3.75–5.02)	2.71
Daphnia magna	21	3-parameter log-logistic	11.83 (1.64–22.03)	23.29 (9.74–36.85)	45.85 (30.16–61.55)	1.62

[a] Reproduction measured as mean total neonates produced per female.

opportunities exist for other compounds to be present and confound the results. In the instance of Maršálek and Bláha (2004) who reported the least toxic lethal concentrations for *Ceriodaphnia* and *Daphnia*, water samples from blooms dominated by *Microcystis* were selected for their studies according to the content of microcystin-LR, namely "toxic" (1.085 mg g^{-1} d.w. microcystin-LR, 98% *M. aeruginosa*), "intermediate" (0.48 mg g^{-1} d.w. microcystin-LR, 20% *M. aeruginosa* and 75% *M. ichtyoblabe*), and "nontoxic" (0.003 mg g^{-1} d.w. microcystin-LR, 98% *M. wesenbergii*). Similarly, in the work of Sotero-Santos et al. (2006), who reported the second highest lethal concentrations in daphnids, water samples dominated by *M. aeruginosa* at time of sampling were collected and lyophilized prior to testing, and results were expressed as microcystin-LR equivalents with no further classification of other algal groups present. Cyanobacteria produce hundreds of cyanopeptides beyond microcystins, and their occurrence is as frequent in surface waters (Janssen, 2019). Extracting toxins and expressing toxicity based solely on microcystins, as was the case in the work of Maršálek and Bláha (2004) and Sotero-Santos et al. (2006), may have diluted the toxicity of microcystin-LR since additional compounds could be present within cyanobacterial cells and contribute to the overall toxicity (Janssen, 2019). Recent advances in analytical techniques have resulted in the identification of hundreds of previously unknown compounds including

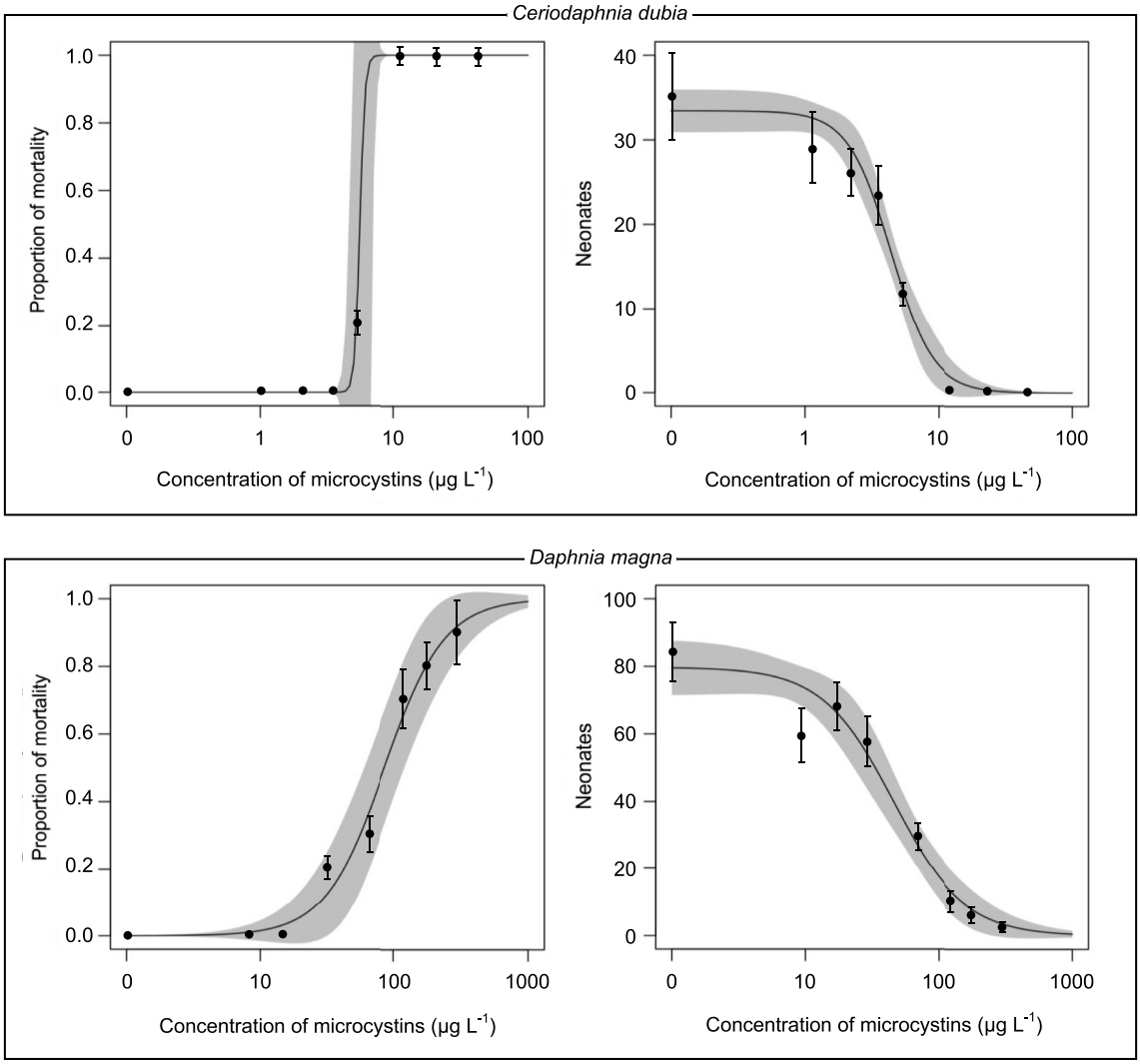

Fig. 1. Effects of total microcystins (microcystin-LR and [D-Asp^3]-microcystin-LR) on lethality and reproduction for *Ceriodaphnia* and *Daphnia*.

new microcystin congeners, as well as other cyanopeptides including cyanopeptolins, aerucyclamides, and aeruginosines (Janssen, 2019). Performing toxicity experiments with microcystin-producing monocultures that are cell-bound and using a high throughput method to quantify several microcystin congeners, as in the present study, enables researchers to predict lethal concentrations with greater accuracy. Furthermore, expressing the relative contribution of microcystins to cyanobacterial biomass offers a holistic perspective on the relative toxicity without ignoring the possibility that other toxic metabolites may have been produced within the monoculture and contributed to lethality and reproductive effects.

The size of the organism in relation to filter feeding particle size preference and period of exposure to microcystins may also be important to consider in explaining the species-specific toxicity differences we observed. In our studies, we observed mortality in 33% and 31% of *C. dubia* and *D. magna*, respectively, after 72 h of exposure. Considering the length of a mature *C. dubia* is from 0.9 to 1.0 mm, and the length of a mature *D. magna* is from 2.0 to 5.0 mm, the possibility exists that the size of the organism matters in relation to the diameter of cyanobacterial cells ingested. This may offer one explanation for the higher toxicity measured in *C. dubia* ($LC50 = 5.53\,\mu g\,L^{-1}$) versus *D. magna* ($LC50 = 85.72\,\mu g\,L^{-1}$) and further support the work of Rohrlack et al. (2005) who observed toxicity in *D. galeata*, an organism that ranges in size from 1.3 to 2.0 mm at maturity and is similarly sized to *C. dubia*. We also observed clear, though qualitative, evidence that *Microcystis* cells were being taken up through the intestinal tract after recording their feeding behaviour under a microscope with no behavioural differences between *C. dubia* and *D. magna* (R.S. Shahmohamadloo, University of Guelph, Guelph, ON, Canada, unpublished data).

4.2. Benthic species

The lack of lethality observed in *Hexagenia* spp. from our studies may also be related to size because the organisms ranged from 20 to 30 mm and were anatomically large enough for *M. aeruginosa* CPCC 300 cells (1–2 μm) to pass through them without rupture and release of microcystins. However, it is also possible that *Hexagenia* in the present study may have avoided exposure to *Microcystis* cells by burrowing in sediments, or that microcystins may have accumulated in the tissues, and lethality or growth impairment may have been observed at a later stage of development had the test been continued. Bearing in mind harmful algal blooms can last anywhere from 1 to 21 d, our laboratory study attempted to incorporate realistic exposure scenarios where *Microcystis* cells are viable, toxic, and occurring in freshwaters.

5. Conclusion

Our data suggest that the uptake, accumulation, and toxicity of food-associated microcystins is possible in pelagic and benthic invertebrates and, in light of the toxicity observed in the present study at realistic concentrations, the potential exists to influence food web dynamics. Having said that, we acknowledge the present study's limitations to emulate the natural environment by providing *Microcystis* colonies of varying sizes, which are more commonly encountered in the environment, and accounting for variations in food sources, temperature, nutrients in water, and so on. To the best of our knowledge, this is the first study to measure survival, growth, and reproduction of pelagic and benthic organisms exposed to cell-bound microcystins. Further studies are needed to characterize the effects, if any, from microcystin dietary exposure and accumulation to *Ceriodaphnia*, *Daphnia*, and *Hexagenia* over multiple generations. Such studies will help us to better understand the ecological risks on pelagic and benthic invertebrate populations who routinely encounter harmful algal blooms such as *Microcystis*. Preliminary evidence from the present study shows a clear reduction in growth of parents and number of neonates produced by *D. magna* exposed to increasing concentrations of cell-bound microcystins within fixed amounts of nutritional algae to support their development. Advances in these areas can strengthen our framework for understanding the direct risks posed by harmful algal blooms to invertebrates and indirect risks to consumers who rely on them as an important component of their diet.

Author contributions

R.S.S., D.G.P., S.P.B. and P.K.S. conceived and designed the experiment. R.S.S. collected the data. R.S.S. and X.O. analyzed the data. R.S.S. wrote the paper. All authors read, amended, and approved the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109945.

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