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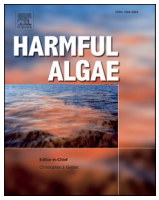

Microcystins bioaccumulate but do not biomagnify in an experimental aquatic food chain

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ABSTRACT

Microcystins—common hepatotoxins produced by cyanobacteria—have been detected in a wide range of organisms, though research examining the trophic transfer of microcystins and whether microcystins bioaccumulate or biomagnify in food webs has generated contradictory results. Here, we explored the trophic transfer of microcystins from the herbivorous water flea, *Daphnia pulex*, to the predatory larvae of a damselfly, *Enallagma* sp. We tested the hypotheses that microcystins transfer from the tissue of herbivorus zooplankton to that of predatory invertebrates and that these toxins biomagnify across trophic levels. We also assessed the relative contribution of toxin transfer from the gut and tissue of *Daphnia pulex* to *Enallagma* sp. We found that microcystins are effectively sequestered in the tissue of *Daphnia pulex*, and that these sequestered toxins are then transferred to the tissue of *Enallagma* sp. The contribution of gut contents to toxin transfer was negligible. Contrary to the pattern predicted by biomagnification, we found that the concentration of microcystins decreased with increasing trophic levels. Our results support the hypothesis that microcystins can be transferred trophically, but do not support the hypothesis that microcystins biomagnify from lower to higher trophic levels. Conversly, we observe biodilution in this system. These results have consequences for the impact of microcystins across trophic levels in a changing world with increasing intensity and duration of harmful algal blooms.

1. Introduction

Several anthropogenic chemicals contaminate aquatic environments. Many of these chemicals accumulate in aquatic organisms that are exposed to them in a process known as bioaccumulation, the biological uptake and short- or longterm storage of a contaminant within an organism's tissue. Some contaminants have the potential to biomagnify—a process in which a contaminant is taken up in a basal trophic organism, stored in specific tissues, and then passed on to consumers who also store the contaminants in specific tissues—with the tissue mass-specific concentration of the contaminant increasing with successive trophic levels within a food web. Generally, water-soluble contaminants bioaccumulate in aquatic organisms, while lipid-soluble compounds—such as mercury (Morel et al., 1998) and DDT (Lushchak et al., 2018)—biomagnify in food webs, reaching deleterious concentrations in apex predators (Ames, 1966; Schaefer et al., 2011). Due to the growing concern regarding toxin-producing cyanobacterial harmful algal blooms, particularly microcystin producers, researchers are interested in whether these toxins bioaccumulate or biomagnify in various aquatic organisms.

Globally, microcystins are among the most reported cyanobacterial toxins in freshwaters. They are produced by members of the genera *Dolichospermum, Fischerella, Gloeotrichia, Nodularia, Nostoc, Oscillatoria, Microcystis,* and *Planktothrix* (Fristachi et al., 2008). These toxins have both lethal and non-lethal effects in invertebrates, including decreases in grazing, heartbeat, growth rates, and reproduction (Bownik, 2016). Microcystins are classified as hepatotoxins and tumor promotors that covalently bind with protein phosphatases in animals (Lambert et al., 1994).

Despite the ability of microcystins to bind to tissue, there is contradictory evidence on whether microcystins biodilute, bioaccumulate, or biomagnify. There are several mechanisms that could lead to biodilution of microcystins across trophic levels. First, microcystins are hydrophilic, unlike many bioaccumulating and biomagnifying toxins that are lipophilic (although this varies between microcystin congeners, see Vesterkvist and Meriluoto (2003). Additionally, removal of microcystins occurs at each trophic level through multiple pathways, including excretion and detoxification through depuration, potentially decreasing

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concentrations (Ibelings and Chorus, 2007; Shingai and Wilkinson, 2022).

Generally, meta-analyses addressing the transfer of microcystins across trophic levels have found evidence for biodilution. A systematic comparison of microcystin concentrations across trophic levels within studies using biomagnification levels found evidence for biodilution (Kozlowsky-Suzuki et al., 2012). In comparing microcystin concentrations among freshwater planktivores, omnivores, and carnivores, Flores et al. (2018) found that omnivores had the highest toxin levels, with concentrations decreasing in carnivores—again supporting the hypothesis of biodilution. However, exceptions to this broad trend exist. For example, zooplankton and some zooplanktivorous fish have displayed biomagnification of microcystins, and substantial variation in biomagnification factor has been observed within these groups (Kozlowsky-Suzuki et al., 2012). Overall, biomagnification of microcystins appears to be uncommon. But even in the absence of biomagnification, phytoplanktivorous zooplankton may act as a vector for the transfer of microcystins to higher trophic levels.

The transfer of microcystins from phytoplanktivorous zooplankton to invertebrate consumers is largely unstudied (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Kozlowsky-Suzuki et al., 2012). Results of experimental studies tracking trophic transfer of microcystins from herbivorous zooplankton to zooplanktivorous invertebrate consumers have been mixed. For example, an experimental study testing microcystin transfer from *Daphnia pulex* to the zooplanktivourus midge larvae *Chaoborus* found no microcystin in *Chaoborus* (Laurén-Määttä et al., 1995). However, a survey of zooplankton in Lake Hallwil (Switzerland) did detect microcystin in *Chaoborus* larvae (Sotton et al., 2014), suggesting potential transfer of microcystins through the intermediary trophic level of zooplankton.

The transfer of microcystins from invertebrate grazers like *Daphnia,* a common invertebrate grazer of cyanobacteria (Nizan et al., 1986), may come from either or both of two sources: tissue-bound toxins or gut contents containing grazed cyanobacteria. Given that *Daphnia* with full guts are more conspicuous to visual predators than *Daphnia* with clear guts (Zaret, 1972), *Daphnia* feeding on cyanobacteria could lead to high algal toxin exposure for visual predators like damselfly larvae.

In this study, we fed *Daphnia pulex* (hereafter *Daphnia*) either microcystin-producing *Microcystis* or the non-toxic algae, *Scenedesmus*. We then fed these individuals to predatory invertebrates, aquatic larvae of *Enallagma* sp. damselflies (hereafter *Enallagma*).

The first hypothesis was that microcystins could bioaccumulate in *Daphnia* tissue. This hypothesis was tested by measuring microcystins (after gut clearance) in *Daphnia* tissue after the *Daphnia* were exposed to *Microcystis*. Next, we tested the hypothesis that microcystins can be transferred to invertebrate aquatic predators through their diet. To do so, we measured the presence of microcystins in *Enallagma* tissue after they consumed *Daphnia* fed *Microcystis*. We also tested the hypothesis that microcystins biomagnify throughout the aquatic food chain. This test was carried out by calculating the biomagnification factor (BMF)—the ratio of microcystin concentration in a consumer to that of its consumed prey—and comparing the BMF to the threshold above which biomagnification is indicated.

Next, we hypothesized that both gut contents and tissue-assimilated toxin—the two possible, non-exclusive routes of toxin transfer from *Daphnia* to *Enallagma*—contribute to the microcystins transferred between trophic levels. The contribution of each route was isolated by comparing the quantity of toxin transferred to *Enallagma* by *Daphnia* with either full or cleared guts. *Daphnia* with cleared guts can only transfer tissue-assimilated microcystins, whereas *Daphnia* with full guts can transfer both tissue-assimilated microcystins and microcystins in their gut contents.

Lastly, we assessed the trophic transfer efficiency of microcystin from *Daphnia* to *Enallagma*. To do so, we calculated the ratio of the quantity of microcystin observed in *Enallagma* tissue to the microcystin dose received by *Enallagma*. This measure broadly assesses the balance of rates of toxin uptake, degradation, and excretion, providing a framework for estimating not only if toxin bioaccumulates, but also how much of the toxin may be expected to transfer.

2. Material and methods

2.1. Experimental organisms

Four organisms were used in this study, including toxic and nontoxic algae (*Microcystis* and *Scenedesmus*, respectively), primary consumers (*Daphnia pulex*), and secondary consumers (*Enallagma* sp.). The first trophic level of our experiment was either *Microcystis* or *Scenedesmus*. *Microcystis aeruginosa* (UTEX LB2385, Kützing, 1846, hereafter *Microcystis*) was used as the microcystin-producing cyanobacteria. This strain has consistently produced microcystins—as previously verified by ELISA tests conducted at the Plankton Ecology and Limnology Laboratory, PEL Lab (Beyer and Hambright 2015)—and is reported in the literature to produce microcystins (Bateman et al., 1995). The green algae *Scenedesmus acutus* (UTEX 72 Meyen 1829, hereafter *Scenedesmus*) was used as a nontoxic food source. Both *Microcystis* and *Scenedesmus* were cultured in 16:1 N:P COMBO (800 μM N, 50 μM P), omitting sodium silicate nonahydrate (Kilham et al., 1998). Cultures were maintained at 25 °C with a 12-hr photoperiod.

Each day on which we conducted an experiment, we used in vivo chlorophyll fluorescence of *Microcystis* and *Scenedesmus* cultures to estimate cell density based on previously derived equations relating cell density to fluorescence (using Trilogy Laboratory Fluorometer, Turner Designs, San Jose, CA, USA). Cultures were diluted with COMBO to achieve similar concentrations for each alga in terms of carbon content, which was standardized to 0.5 mg C L^{-1} based on chlorophyll-carbon relationships previously developed in the PEL Lab. This carbon concentration is just above the incipient limiting concentration for *Daphnia* (Lampert, 1978). A pilot experiment demonstrated that 0.5 mg C L^{-1} of *Microcystis* was sub-lethal to the strain of *Daphnia* used in this experiment as no mortality was observed in *Daphnia* incubated for four hours with *Microcystis* at an even higher carbon concentration (3.6 mg C L^{-1}).

Daphnia pulex (hereafter *Daphnia*, Leydig, 1860) constituted the second trophic level of our experiment. We selected this organism because they are common grazers of cyanobacteria (Fey et al., 2010; Hambright et al., 2007) and because they are commonly consumed by predatory invertebrates (Johnson, 1973). This strain of *Daphnia pulex* was originally collected from the University of Oklahoma's Aquatic Research Facility in 2014 and thereafter maintained in the PEL Lab in COMBO at 20 °C with a 12-hr photoperiod and fed *Chlamydomonas* sp. (CVBS, USA).

The third trophic level was represented by the invertebrate predator, *Enallagma* sp. Damselfly larvae (hereafter *Enallagma*, Charpentier 1840), which were purchased from Carolina Biological Supply (Burlington, NC, USA). The larval *Enallagma* were selected because damselfly larvae readily consume *Daphnia* (Hunt and Swift, 2010). The *Enallagma* larvae were maintained in COMBO at 20 °C with a 12-hr photoperiod cycle for 24-48 hours before the experiment. All *Daphnia* and *Enallagma* were starved for 24 hours before an experiment.

2.2. Experimental treatments

We measured the transfer and accumulation of microcystins across these three trophic levels in a semi-factorial design (Fig. 1). The experimental design was not fully factorial, as not all combinations were necessary to test the outlined hypotheses. In brief, we measured microcystin concentrations in *Microcystis, Daphnia,* and *Enallagma*, combining full and cleared gut treatments to quantify toxins in the tissue and gut. Similar treatments were repeated with the non-toxic alga, *Scenedesmus*, to verify the absence of matrix interference during analysis (i.e., false positives from *Daphnia* or *Enallagma* tissue). Five trials of the feeding experiment described below were conducted across three

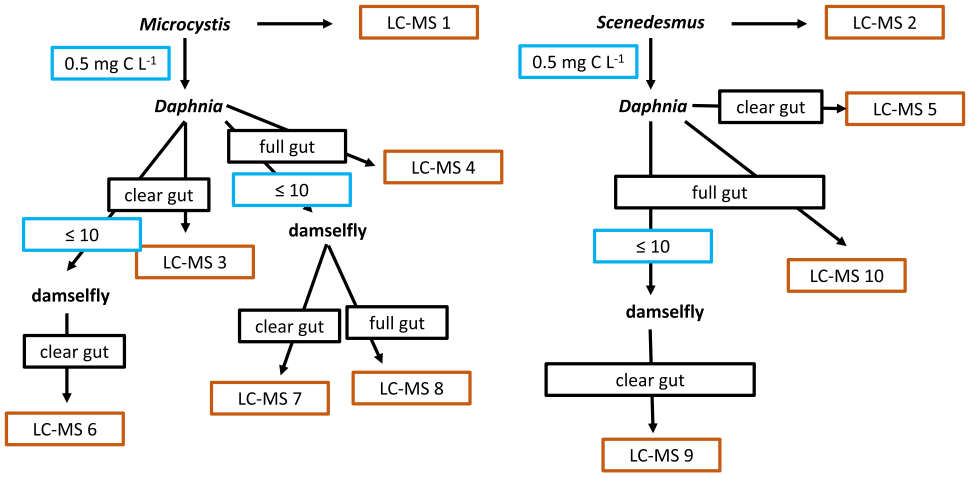

Fig. 1. Schematic of experimental design. Orange boxes indicate samples that were analyzed by LC-MS. The number within the orange box indicates the treatment group. Black boxes indicate whether organisms cleared guts before transfer to next step. Blue boxes represent the quantity of food, either carbon concentration of algae or number of *Daphnia* individuals.

nonconsecutive days, due to the limited availability of comparably sized adult *Daphnia*. Trial one was conducted on August 19, 2021. Trials two and three were conducted on September 12, 2021. Trials four and five were conducted on September 26, 2021.

2.3. Feeding experiment

Experiments were conducted in six-well plates (Millipore, Burlington, MA, USA). Twenty-four hours before the experiment, *Daphnia* and *Enallagma* were transferred to wells containing 10 mL COMBO without algae. Only adult *Daphnia* of similar size, approximately 1.7 mm in body length, were selected for use in experiments. For each treatment combination, ten (Trial 1) or five (Trials 2-5) *Daphnia* were placed in wells. Prior to each trial, samples of 1:10 dilutions of both algal cultures were frozen in Verex vials (Phenomenex, Torrance, CA, USA) at −80 °C for later microcystin analysis. Experiments began by adding 10 mL of 0.5 mg C L^{-1} of either *Microcystis* or *Scenedesmus* to wells containing *Daphnia*. *Enallagma* individuals were placed in their own wells, without algae.

Daphnia were exposed to *Microcystis* or *Scenedesmus* to feed for four hours. After this time, all *Daphnia* were removed from the wells containing food. *Daphnia* from a quarter of the wells were transferred to Verex vials and frozen. *Daphnia* from another quarter of the wells were fed to *Enallagma*. *Daphnia* from the remaining half of the wells were placed in COMBO with *Scenedesmus* for 2 hours to allow their guts to clear of *Microcystis*. Previous results have demonstrated this time period is sufficient for the clearance of gut contents in *Daphnia* (Murtaugh, 1985). Half of the *Daphnia* with cleared guts were then transferred to Verex vials and frozen, while half were fed to *Enallagma*. After consuming *Daphnia, Enallagma* from half of the wells were rinsed in COMBO, transferred to Verex vials, and frozen, while half were transferred to clean COMBO for 12 hr to allow their guts to clear before being transferred to Verex vials and frozen. This time period was sufficient, as six hours has been shown to be the maximum time needed for gut clearance in damselflies that had eaten *Daphnia* (Johnson et al., 1975).

Daphnia were transferred using a plastic pipette with the minimum amount of liquid volume necessary and *Enallagma* were transferred by forceps to minimize potential contamination by *Microcystis* containing microcystins. Animals were rinsed in clean COMBO and placed in Verex vials which were then filled to 1 mL with fresh COMBO and frozen at −80 °C. Animals frozen immediately after feeding were used to assess the accumulation of microcystins in both tissue and guts. Animals frozen after allowing guts to clear were used to assess the accumulation of microcystins in tissue. All samples were stored at −80 °C prior to shipping on dry ice to GreenWater Laboratories (Palatka, Florida, United States) for extraction and analysis of microcystins.

2.4. Sample analysis

Microcystins were quantified following the chemical oxidation and LC-MS/MS method described in Foss et al. (2022). Samples were first homogenized using three freeze-thaw cycles followed by bead-beating. An internal standard (d_3-MMPB) was added to all samples. MC-LR was used to produce a standard curve. Samples were then oxidized, cleaned up using solid phase extraction (SPE), and measured using LC-MS/MS. Total Adda microcystins and nodularins (μg/L) were calculated based on the MC-LR standard curve concentrations (pre-oxidation).

We verified our methods in three ways. First, the microcystin concentration in both algal treatments was compared to verify that microcystins only occurred in *Microcystis* (comparison of Groups 1 and 2 as illustrated in Fig. 1). We then verified that *Daphnia* and *Enallagma* tissue did not cause false positives for the detection of microcystins (i.e., matrix interference) by testing whether *Daphnia* and *Enallagma* that were not exposed to *Microcystis* contained no microcystins (Group 2 = 0, Group 9 = 0).

2.5. Statistical analysis

In both treatments, values less than the limit of detection (0.2 ng/mL) were treated as 0.13 ng microcystins/mL, 65% of the limit of detection (Martın-Fernandez et al., 2003). Toxin concentrations were first converted to ng microcystins per mg dry weight of organism using the following approach. For *Daphnia,* an average weight was used based on length measurements of 10 similarly sized adult *Daphnia*, like those used in the experiment. The length-weight regression used was:

$$W = 0.0116L^{2.67}$$

where W is the dry weight of the *Daphnia* in mg and *L* is the body length in mm (Burns, 1969). For *Enallagma* sp., an average length was determined from three larvae, and the length-weight regression used was:

$$W = 0.0078L^{2.792}$$

where W is the dry weight of the *Enallagma* in mg and *L* is the total length in mm (Benke et al., 1999).

We first tested for bioaccumulation by verifying the presence of microcystins in the tissue of *Daphnia* and *Enallagma*. For both analyses, we considered only individuals with empty guts to isolate tissue-bound

toxins. First, we compared the concentration of microcystins in *Microcystis*-exposed *Daphnia* (Group 3) to zero using a one-sample t-test. We tested whether tissue-bound microcystins in *Daphnia* could be transferred to *Enallagma* and subsequently sequestered in their tissue by comparing the concentration of microcystins in Group 6 to zero using a single-sample t-test. Microcystins were not detected in any *Daphnia* or *Enallagma* on the non-toxic arm of our experiment, so zero is the appropriate baseline comparison.

Next, we compared the relative contribution of two pools of microcystins (gut, tissue) in *Daphnia* to the transfer of microcystins to *Enallagma*. We compared concentrations of microcystins in *Enallagma* tissue between individuals that ate full-gut *Daphnia* (Group 7) versus cleared-gut *Daphnia* (Group 6). If the two values were equal, we would consider the contribution of gut contents to be negligible. Groups were compared using a two-sample t-test.

Finally, we tested for biomagnification by calculating the biomagnification factor (BMF), the ratio of microcystin concentrations (ng microcystins per mg dry weight of organism) in a predator to that of its prey (Gray, 2002). Based on our design, we calculated the BMF for microcystins in *Daphnia* tissue in comparison to microcystins in *Microcystis*. Then we calculated the BMF for microcystins in *Enallagma* tissue versus microcystins in the tissue of *Daphnia* that had consumed *Microcystis*. These ratios were calculated within a given trial, thus controlling for observed variation in the microcystin concentrations between trials. For each trophic level comparison, we tested whether the values were significantly different from unity using a single-sample t-test. A BMF >1 would indicate the toxins have the potential to biomagnify across trophic levels (Gobas et al., 2009). Additionally, we estimated the transfer efficiency of microcystins from prey to predator. We calculated the quantity of microcystins that were ingested by *Enallagma* by multiplying the amount of microcystins per *Daphnia* individual by the number of ingested *Daphnia*. We compared this value to the observed value of microcystins per *Enallagma* individual by dividing the observed microcystins by the microcystin dose. These transfer efficiency values were compared between three treatments: *Daphnia* cleared-gut + *Enallagma* cleared-gut; *Daphnia* full gut + *Enallagma* clear gut; and *Daphnia* full gut + *Enallagma* full gut. These values were compared with an ANOVA test. All statistical analyses were carried out in R (Version 4.2.2).

3. Results

Microcystins were verified to be absent in treatments designed to be non-toxic. Microcystins were not detected in any sample of the *Scenedesmus* treatment. None of the *Daphnia* or *Enallagma* individuals receiving the *Scenedesmus* treatment had detectable microcystin levels. There were no false positives that could indicate either cross contamination or matrix interference in the LC-MS analysis. However, in one of the five trials, microcystins were detected in *Microcystis* but not in the grazing *Daphnia*. This observation may represent a false negative caused by matrix interference from the higher biomass of *Daphnia* included in this trial (n = 10), compared with the other four trials (n = 5). We dropped this trial from subsequent analyses.

Microcystins were confirmed in all samples of the *Microcystis* treatments, but concentrations were variable (mean = 16.3 ng ml^{-1}, sd = 15.0). With the exception of one outlier treatment (see above), microcystins were detected in all *Daphnia* and *Enallagma* in the *Microcystis* treatment arm of the experiment, regardless of feeding status (Fig. 2). Tissue-bound microcystins were transferred across two trophic levels

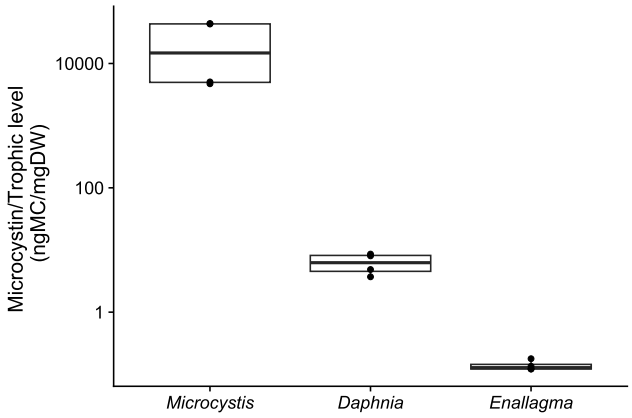

Fig. 3. Boxplots of tissue-bound microcystin concentrations in the second trophic level, *Daphnia*, and the third trophic level, *Enallagma,* compared to that of the first trophic level, *Microcystis*. The measurement of microcystin concentration in both *Daphnia* and *Enallagma* was made on indivduals with empty guts. See Section 2.2 for further description of treatments.

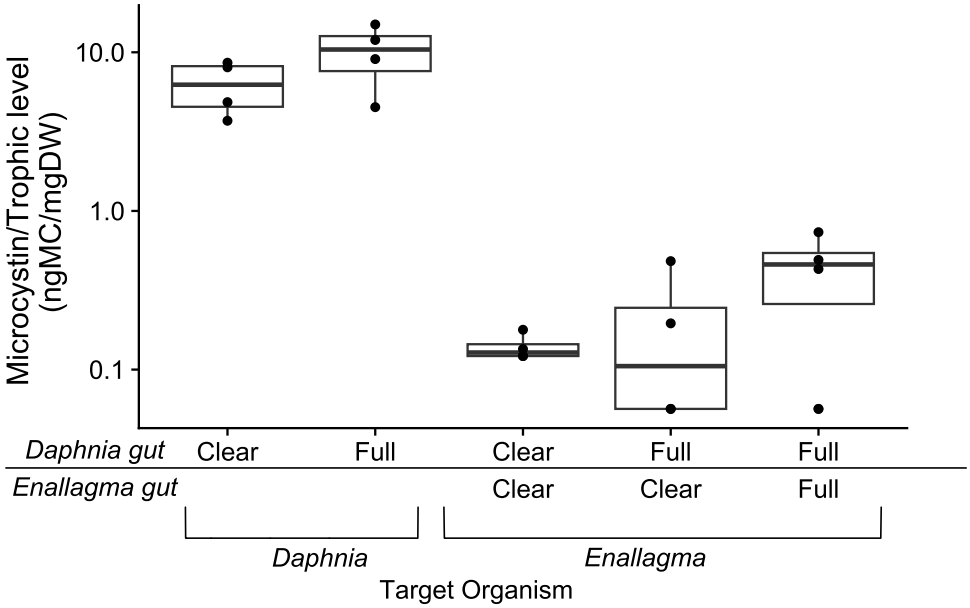

Fig. 2. Microcystin concentration for each treatment. Target organism indicates the species on which the microcystin analysis was performed.

(Fig. 3). The microcystin concentration in the tissue of *Daphnia* exposed to *Microcystis* was greater than zero ($t(3) = 5.27$, $p = 0.013$). The tissue of *Enallagma* that ate cleared-gut *Daphnia* exposed to *Microcystis* also had non-zero levels of microcystins ($t(3) = 10.38$, $p = 0.002$).

There was no significant contribution of *Microcystis* in *Daphnia* gut contents to microcystins in the tissue of *Enallagma* individuals. The relative contribution of tissue-bound microcystins versus that found in gut contents was assessed by comparing the concentration of microcystin in *Enallagma* tissue after consuming *Daphnia* with full versus cleared guts (Fig. 4). No significant effect of *Daphnia* gut contents on tissue-bound microcystins in *Enallagma* was observed ($t(3.11) = -0.58$, $p = 0.602$).

Biomagnification factors were low overall (Fig. 5). The biomagnication factor calculated for *Daphnia* relative to their diet, *Microcystis*, was significantly lower than one ($t(3) = -4807.3$, $p < 0.001$). *Enallagma* also had a low magnification value significantly less than one ($t(3) = -126.09$, $p < 0.001$). Overall, a high fraction of the microcystin dose was transferred to *Enallagma* (Fig. 6). There was no difference in transfer efficiency between the three treatments ($df = 2$, $F = 2.65$, $p = 0.124$).

4. Discussion

Overall, the results of these experiments confirmed the potential for microcystins to be transferred across trophic levels in an experimental aquatic ecosystem. We first confirmed that microcystins bioaccumulated in *Daphnia* tissue by measuring the concentration in *Daphnia* that had been fed *Microcystis* then placed in COMBO with *Scenedesmus* to clear their guts of *Microcystis*. We also confirmed the transfer of these microcystins from *Daphnia* tissue to *Enallagma* tissue by feeding the aforementioned *Daphnia* to *Enallagma*, then starving the *Enallagma* for 12 hr to clear their guts. The presence of microcystins in both grazer and predator tissues supports our hypothesis that microcystins can be transferred across trophic levels. This result is in line with the results of Sotton et al. (2014), where microcystin was found to transfer from *Microcystis* to *Daphnia* and subsequently to the predatory *Chaoborus*.

One key assumption in our study is that the methods of clearing guts for both *Daphnia* and *Enallagma* are effective. Any remaining microcystin-containing tissue in gut contents could artificially inflate the estimate of microcystins sequestered in each animal's tissue. However, *Daphnia* in clear-gut treatments were given ample time to clear their guts (Murtaugh, 1985) and the *Enallagma* in clear-gut treatments left visible fecal material in the wells, suggesting the organisms in clear-gut treatments likely did have empty guts at the time of sampling.

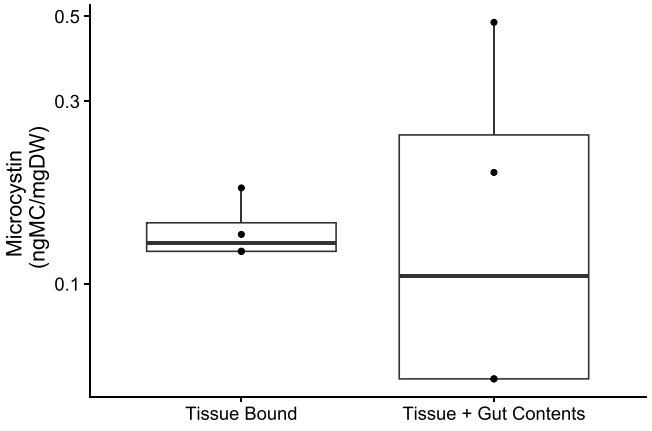

Fig. 4. Comparison of microcystin concentration in the tissue of *Enallagma* individuals that consumed either *Daphnia* with cleared guts (tissue-bound only) or guts containing *Microcystis* (tissue + gut contents). All *Enallagma* were starved for 12 hr before measurement to measure solely the microcystin transferred to their tissue (not gut contents).

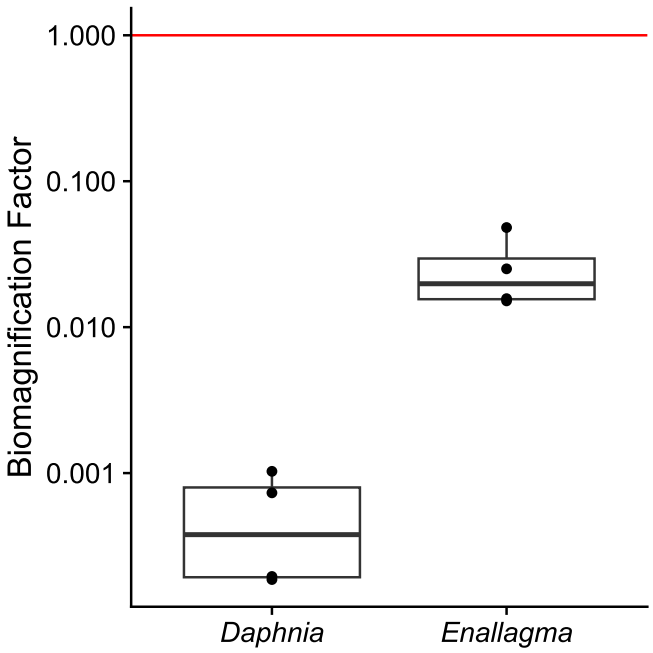

Fig. 5. Biomagnification factor calculated for *Daphnia* relative to *Microcystis* (left) and *Enallagma* compared to *Daphnia* (right). Red line indicates the threshold above which biomagnification is indicated.

Additionally, as mentioned previously, earlier work has demonstrated clearing of gut contents in damselflies after just six hours (Johnson et al., 1975).

In comparing the relative contributions of tissue-bound microcystins versus that in gut contents, the microcystin concentrations in the tissue of *Enallagma* individuals fed on full-gut and clear-gut *Daphnia* were equivalent. This result leads to the rejection of the hypothesis that gut contents contribute to the microcystins transferred across these two trophic levels. This is unsurprising given the lack of substantial difference in microcystin concentration in *Microcystis*-exposed *Daphnia* with either full or clear guts (Fig. 2).

The third hypothesis—that microcystins biomagnify across trophic levels—was rejected. Biomagnification factors for both trophic levels were significantly lower than one (*Daphnia:Microcystis* mean $= 5.36 \times 10^{-4}$; *Enallagma:Daphnia* mean $= 2.60 \times 10^{-2}$), which is the necessary cutoff for evidence of biomagnification. For comparison, a meta-analysis of methyl-mercury in aquatic ecosystems showed an average BMF of 2.4 for seston to zooplankton and an average of 5.6 for zooplankton to preyfish (Wu et al., 2019). A meta-analysis of experimental BMF values from fish also showed that PCBs have an average BMF of 2.5 (Grisoni et al., 2018). The lack of microcystin biomagnification in our study is in line with the meta-analysis of Kozlowsky-Suzuki et al. (2012) who found evidence for biodilution of microcystins within food webs, as opposed to biomagnification. This biodilution agrees with predictions based on the biochemical structure of microcystins. Because microcystins are water soluble (Rivasseau et al., 1998) and organisms such as *Daphnia* do have the ability to depurate them (Castro et al., 2019), they are less likely to biomagnify than lipid-soluble toxins such as mercury or DDT. Generally, lipid-soluble toxins are sequestered into the fat-storage structures of organisms, where they remain due to the organisms' inability to detoxify the compounds (Ibelings and Havens, 2008). This allows the toxins to increase in concentration with trophic level. Detoxification of microcystins through depuration has been shown to operate on the scale of 15-20% per day in bivalves—a much slower rate than the period of our experiment (Yokoyama and Park, 2003). In our experiment, the gut-clearing time may have allowed for some detoxification of ingested microcysins, but we expect this value to be negligible given the aforementioned measurement in mussels.

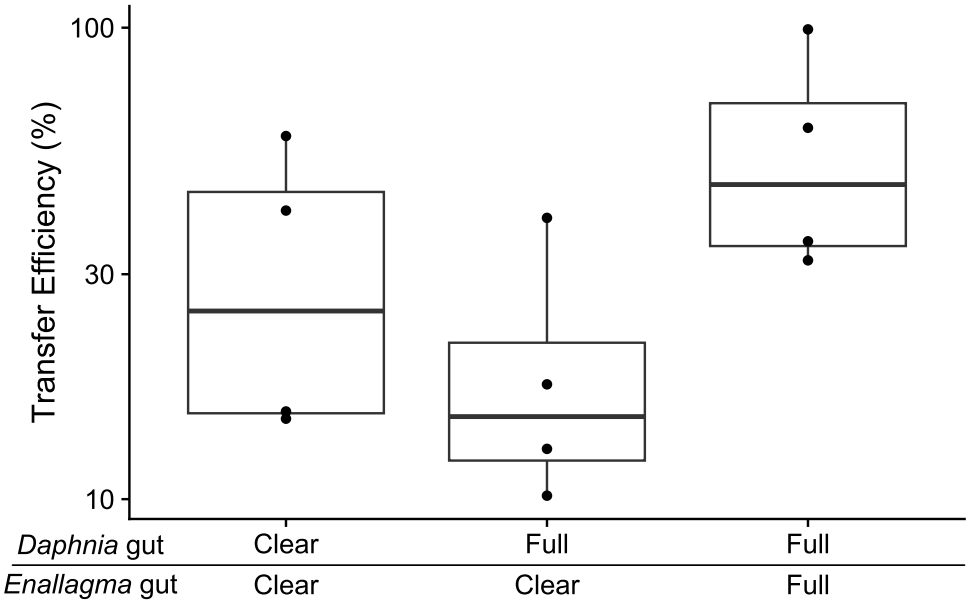

Fig. 6. Comparison of microcystin transfer efficiency from *Daphnia* to *Enallagma* in three different treatments.

The apparent transfer efficiency of microcystins from one trophic level to the next will be the balance of three processes: uptake, detoxification, and excretion. In regards to uptake, microcystins have low membrane permeability, so without active transport they will not accumulate in the tissue of the target organism. In mammals, the uptake of microcystin in the gut is mediated by organic ion transporting polypeptides (OATPs) and organic ion transporters (OATs) (Campos and Vasconcelos, 2010). Generally, the detoxification of microcystin works by the conjugation of microcystin to glutathione, a process mediated by glutathione S-transferase (GST) (Campos and Vasconcelos, 2010). This process has been documented in *Daphnia* (Pflugmacher et al., 1998, Wojtal-Frankiewicz et al., 2013), however, another study demonstrated that GST production is a more generalized response to cyanobacteria as opposed to a specific response specific to microcystins (Sadler and von Elert, 2014). The mechanism underlying the excretion of detoxified microcystin products is less clear. In zebra mussels (*Dreissena polymorpha*), this process has been linked with the expression of P-glycoprotein (P-gp), a generalized xenobiotic transporter (Contardo-Jara et al., 2008). These transporters have also been identified in *Daphnia*, making this a potential excretion pathway (Campos et al., 2014). Any variation in the concentration or rate of activity of these proteins will affect the efficiency of microcystin transfer between trophic levels. Overall variation in these three steps (uptake, detoxification, and excretion) will determine the transfer efficiency between trophic levels. For example, a consumer with a high uptake rate and low detoxification rate would produce a higher transfer efficiency than that of a consumer with a low uptake rate, in which microcystins may pass through the gut without being absorbed at all. This complexity makes it impossible to predict, *a priori*, the microcystin concentrations in prey necessary to produce unsafe concentrations in, for example, predator species used as human food. We do not know enough about uptake, detoxification, and excretion rates to generalize across organisms. With further study on these processes across major taxonomic groups, predictions can be made to support safe fisheries and human health.

Outside of the lab, it is highly unlikely that the diet of a zooplankter would be made up of one hundred percent *Microcystis*. Even in a bloom, grazers are likely to consume a mixed diet, which would dilute the quantity of microcystin ingested—and therefore transferred from the first to second trophic levels. The concentration to which grazers are exposed will be impacted by the relative contributions of autochthonous and allochthonous inputs (Bukaveckas et al., 2017). In systems dominated by allochthonously derived organic matter (i.e. high sediment load), dilution of algal toxins originating from authochthonously derived organic matter will be observed. The degree of omnivory and selectiveness will be critical here, too. For example, Ibelings et al. (2005) found that food webs with *Daphnia* as the primary consumer had bioaccumulation of microcystin, whereas no bioaccumulation was measured when food webs originated with zebra mussels, a pattern that the authors attribute to selective grazing on the part of zebra mussels. The observed differences in bioaccumulation in field studies are likely attributable to mixed diets and feeding selectivity, complications that would not be apparent in our study where each trophic level was made up of only one species.

The bioaccumulation and trophic transfer of microcystins to the *Enallagma* in this experiment demonstrates that damselflies are a potential route of transfer of microcystins to higher trophic levels in aquatic systems. Fish preying on damselflies may be exposed to toxins, and—as demonstrated in earlier work—also have the potential to bioaccumulate microcystins (Kozlowsky-Suzuki et al., 2012). During *Microcystis* blooms, fish can be exposed through two routes, indirectly through prey consumption and directly through water exposure (Ibelings et al., 2008), doubling the potential for transfer of microcystins across the food chain in *Microcystis*-dominated lakes. Given the demonstrated potential for damselflies to accumulate microcystins, they also serve as a potential route for movement of microcystin from aquatic ecosystems to terrestrial ecosystems, perhaps contributing to some of the microcystin contamination seen in terrestrial food webs from mayfly larvae to spiders to warblers (Moy et al., 2016). While damselfly larvae are aquatic, adult damselflies emerge from aquatic habitats after metamorphosis and become terrestrial, where they are preyed upon by several predators such as warblers (Bibby and Green, 1983) and spiders (Rehfeldt, 1992). Methylmercury has also been observed transferring trophically from aquatic to terrestrial ecosystems along the same route, from emergent aquatic insects to spiders to songbirds (Gann et al., 2015), implying this is a common route of trophic transfer for multiple environmental contaminants. This evidence strongly indicates that cyanobacterial toxins in general, and microcystins in particular, are likely to bioaccumulate in aquatic larvae such as damselflies and trophically transfer to terrestrial ecosystems.

5. Conclusion

In summary, microcystins were clearly passed to organisms that had no direct exposure to cyanobacteria, as microcystins sequestered in *Microcystis* grazers were also sequestered in the tissue of their predators. These results support trophic transfer of microcystins between invertebrates and bioaccumulation of microcystins, but not its biomagnification. Instead, we observed decreased concentrations of microcystins per dry mass of organisms moving from primary to secondary consumers. Therefore, we find support for biodilution of these toxins within aquatic invertebrates. Additionally, the demonstrated potential for bioaccumulation of microcystins in invertebrate predators serves as a potential pathway for transfer of microcystins to predatory fish and the bioaccumulation of microcystin in damselflies could present a route of trophic transfer from aquatic to terrestrial ecosystems.

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CRediT authorship contribution statement

Dani Glidewell: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Jessica E. Beyer:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **K. David Hambright:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data deposited in the Dryad digital repository: https://doi.org/10.5061/dryad.280gb5n0n (Glidewell et al., 2024).

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