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Survival and Accumulation of Microcystins in a Native Amphipod Shredder, Gammarus Pulex under Environmentally Relevant Cyanotoxin Exposure

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Abstract:
Microcystin release in freshwaters is a major public health and ecological threat, affecting survival and trophic interactions across food web components. Although bioaccumulation has been suggested to represent an important pathway for microcystin uptake and trophic transfer across different food web compartments, it is rarely known how microcystin exposure may affect survival and accumulation among lower trophic levels under environmentally realistic scenario. Therefore, in this study, survival and microcystin accumulations in the UK native amphipod species, Gammarus pulex were monitored during a 96-hour static acute toxicity experiment. Animals were exposed to a range of environmentally relevant microcystin concentrations (0.01–10µg/L) across two treatment types, purified MC-LR and crude extract of microcystin-producing Microcystis aeruginosa. Survival of G. pulex individuals across the two treatments showed no evidence of significant difference from the control. Microcystin concentrations observed in G. pulex exposed to the purified MC-LR and crude extract ranged from (12.8 – 27.5) ng/g of MC-LR and (5.6-12.8) ng/g of MC-LR respectively. Microcystin concentrations measured in animals showed strong positive correlations with toxin concentrations in the experimental media across the two treatments, suggesting G. pulex can bioaccumulate microcystins, therefore may represent a potential vector for microcystin exposure to higher food web consumers. These results highlight the fact that exposure of benthic food web components to environmental microcystin concentrations may constitute an important route of toxin transfer within freshwater food webs.

Keywords: Harmful algae, cyanobacteria, microcystin, food web, bioaccumulation, amphipods.

I. INTRODUCTION

Freshwater harmful cyanobacterial blooms are expanding rapidly as a major environmental problem worldwide (Cai et al., 2021; Harke et al., 2016), affecting not only human and animal health, but also reducing survival and ecosystem functions among aquatic organisms (Adekolurejo et al., 2022; Shahmohamadloo et al., 2020). Although primarily driven by increased anthropogenic nutrient enrichment (Beaver et al., 2018; Glibert, 2017), freshwater cyanobacterial blooms have become increased in frequency and intensity in the face of climate change (Paerl et al., 2011), leading to the release of microcystins and other toxic hazardous cyanobacterial secondary metabolites in freshwater bodies (Harke et al., 2016). Microcystins are a prominent group of hepatotoxins produced by a variety of bloom-forming cyanobacteria, including Microcystis aeruginosa, which is the leading producer of microcystins in freshwaters (Šejnohová and Maršálek, 2012). Moreover, increasing evidence has shown that laboratory exposure to microcystin may be associated with several adverse effects in zooplankton and other aquatic species, such as reduced survival (DeMott et al., 1991), feeding and growth rate inhibition (Lürling and van der Grinten, 2003) as well as impaired reproduction (Dao et al., 2010). This evidence suggests that microcystin occurrence in freshwaters may represent an emerging environmental stressor, potentially affecting individual fitness among key freshwater species, thus altering fundamental structure and functions in aquatic food webs (Adekolurejo et al., 2022; Briland et al., 2020; Burkholder et al., 2018).

Microcystin concentrations may find its way into different compartment of the aquatic food web through a variety of exposure routes (Kozlowsky-Suzuki et al., 2012; Pham and Utsumi, 2018). For instance, benthic and pelagic aquatic invertebrates may be exposed to microcystin via direct contact or ingestion of dissolved toxins in water (Chen et al., 2013), whereas freshwater consumers and predators may readily take up microcystin through consumption of toxin-laden prey (Burkholder et al., 2018; Shahmohamadloo et al., 2020). However, bioaccumulation of dissolved microcystin concentrations from surrounding water column has been highlighted to be an ecologically important route of toxin exposure and trophic transfer to higher food web consumers (Pham and Utsumi, 2018). Although a few studies have suggested that biodilution is more likely to occur in organisms as opposed to biomagnification of the toxin across different trophic levels of food webs in natural waters (Ibelings et al., 2005; Kozlowsky-Suzuki et al., 2012). Increasing studies have also shown that microcystin may become bioaccumulated in individual taxa across different compartments of the food webs such as, bivalves (Poste and Ozersky, 2013), gastropods (Lance et al., 2010), chironomids (Xue et al., 2016b), crustaceans (Kim et al., 2021), amphipods (Kim et al., 2021) and oligochaete (Xue et al., 2016a), while the highest potential for bioaccumulation was observed among pelagic primary consumers, especially zooplankton (Ferrão-Filho and Kozlowsky-Suzuki, 2011). However, there is limited information on how microcystin uptake via freshwater amphipods at the base of the benthic food webs may serve as a major route of microcystin exposure and a potential vector for

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trophic transfer across consumers at higher levels of the aquatic food web.

The UK native amphipod, Gammarus pulex is an important keystone species (De Castro-Català et al., 2017) and a major component of the benthic food webs in aquatic systems (Tlili et al., 2012). It is the most abundant and predominant macroinvertebrate species in many freshwater bodies across Europe (Consolandi et al., 2019; Kunz et al., 2010), playing a significant ecological role in allochthonous detrital processing (Kenna et al., 2017), thereby altering structure and functions of food webs (Galic et al., 2017; Maltby et al., 2002). G. pulex represents an important link between detritus at the base of the benthic food web and higher trophic level consumers, such as fish and aquatic birds (Tlili et al., 2012). Several studies have shown that gammarids are excellent sentinel organisms used in aquatic biomonitoring (Maltby, 1994; Zubrod et al., 2010), because of their ability to accumulate a wide range of environmental contaminants from diet and water column (Boets et al., 2012; Kunz et al., 2010; Lebrun et al., 2014), thus suggesting G. pulex may be an important intermediate in the transfer of cyanotoxins and other chemical stressors to higher trophic level consumers in aquatic food webs. However, there is currently little or no evidence regarding microcystin bioaccumulation in this native amphipod species and its potential as an important vector for toxin transfer across trophic levels in freshwater food webs.

Hence the present study evaluated the effects of a 96-hour exposure to a range of low environmentally relevant microcystin concentrations across two treatments on survival and toxin bioaccumulation in G. pulex. We anticipated that microcystin uptake and accumulation in G. pulex will correlate with the toxin concentrations in the experimental media across the two treatments, suggesting G. pulex may be a potential vector for trophic transfer of the toxin to higher levels. We also expected the proportion of survival of G. pulex exposed to microcystin treatment to be related to the toxin concentrations in animals across the two treatments.

II. Materials and Methods

COLLECTION OF ANIMALS

The UK native amphipod, Gammarus pulex individuals were collected with the aid of a pond net from the Meanwood Beck, Meanwood Park, Leeds, West Yorkshire (53°500′N; 1°350′W). Animals were immediately transported to the laboratory in a small cool box containing stream water and fallen leaves to provide food materials for the animals. In the laboratory, amphipods were acclimatized for seven days in aerated 2 L-plastic (22 cm×16 cm×9 cm), filled with 1.8 L of aged, dechlorinated tap water and maintained in a controlled temperature (CT) room conditioned at 15±1ºC and 14 h:10 h light: dark photoperiod cycle. During the acclimation, each plastic tank was furnished with glass pebbles to provide artificial substrate and refuge for the animals. Amphipods were fed ad libitum with air-dried alder leaf material (Alnus glutinosa) collected during leaf fall in October 2019 while the culture medium was renewed after every 72 hours. Body length and weight of each animal were measured, and approximately same-age adult animals were sorted into a new tank and starved for 24 hours before the commencement of the experiments.

CYANOBACTERIAL CULTURE

Microcystis aeruginosa (CCAP/1450/16), a toxigenic cyanobacterial strain was obtained from the Culture Collection for Algae and Protozoa (CCAP), Scottish Marine Institute, Scotland and cultivated as batch cultures under axenic conditions in the laboratory. Batch culture were reared in 250 mL Erlenmeyer flasks, comprising of 150 mL freshly prepared and autoclaved BG-11 culture medium in accordance with the CCAP's recipe. Cultures were incubated at constant temperature of 25±1ºC, light intensity of 25µmol quanta m-2s-1 and 12 h:12 h light/dark photoperiod cycle in a shaking incubator until the stationary growth phase was reached. The daily increase in the cell biomass of M. aeruginosa was monitored by taking the spectrophotometric reading of the optical density (OD) at 680 nm and enumerating the cell number under light microscopy with the aid of a compound microscope and Sedgewick-Rafter Counting Chamber. A linear regression line was fitted to establish the relationship between daily spectrophotometric OD measurement and the microscopic enumeration of the cell density (R2 = 0.97). Microcystis cultures were harvested at the stationary growth phase, the cell density was estimated, and cells were ruptured and lysed through a freeze-thaw cycle three times at -18°C to release the intact cell-bound microcystin content. After the freeze-thaw cycle, the culture was centrifuged for 10 minutes at 7500 rpm and 4°C, whereas the pellets were resuspended in deionized water to repeat the freeze-thaw cycle. The supernatants were decanted into new flasks while the total microcystin content of the crude cyanobacterial extract was quantified using a commercial semi-quantitative microcystin ELISA kit with microcystin coated 96 well plates (ALX-850-319). The absorbance values were read at 450nm, analysed using a 4-parameter logistic regression and the total microcystin content in the samples were presented as MC-LR concentration (µg/L). The purified MC-LR used in this study was purchased from Cayman Company. 1mg of the toxin powder was dissolved in 1 mL of methanol (> 95% purity). The solution was graduated to 1 Litre with milli-Q water to make 1mg/L (1000 µg/L).

EXPERIMENTAL DESIGN

Ten individual animals per container were exposed to two microcystin treatments and the control in 250 mL plastic cups containing of 200 mL solution of microcystin treatment and aged, dechlorinated tap water. The two microcystin treatments used in this study were made up of three concentrations of crude extracts of M. aeruginosa containing 0.01, 0.1 and 1 µg/L MC-LR equivalent and three concentrations of the purified toxin containing 0.1, 1 and 10 µg/L MC-LR equivalent with three replicates per concentration and control used. Animals were exposed for 96 hours with non-renewal static acute toxicity. Animals were not allowed to feed during the period of exposure. Every 24 hour, the mortality across each replicate was determined. Mortalities were recorded while the weight of each animal at the time of death was

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determined. Animals were immediately washed with tap water, dried on paper towel and stored in a - 20°C freezer before microcystin analysis. The microcystin contents in each treatment were monitored in the water samples at the beginning and at the end of the experiment.

MICROCYSTIN ANALYSIS

Animal and water samples were defrosted and six animals per replicates were randomly selected, dried on paper towel and weighed. Animal samples were grounded with mortar and pestle and digested by adding 5 mL of 70% methanol. The digested solutions were homogenized using an ultrasonic sonicator and centrifuged at 7500 rpm for 10 minutes. The supernatant was decanted and carefully transferred into microcystin-coated 96-well plates and analysed in triplicates for the total microcystin content. The initial and final microcystin content of the water sample replicates were quantified. Microcystin accumulation per each animal was estimated by dividing the total accumulated microcystin content by the average weight of the animals in the treatment as expressed as MC-LR equivalent in ng/g.

STATISTICAL ANALYSIS

Amphipod survival data were analysed by fitting a Cox proportional hazard (CPH) model in the survival (Therneau, 2015; Therneau and Grambsch, 2000) and Survminer (Alboukadel et al., 2019) packages in R to test for variation in time to event (death) across different microcystin treatments and concentrations. The survival data was summarized as survival proportion with the associated 95% confidence interval extracted using the PropCIs package (Scherer, 2018) in R, while the length and weight data were summarized and presented as descriptive statistics (means ± standard error of means). The nominal microcystin concentrations in the experimental medium and the concentration accumulated in G. pulex were normalized using natural log transformation while Spearman Rank Correlations analyses were conducted to test the association between natural log-transformed microcystin concentrations in G. pulex and the experimental medium. Similarly, associations between survival proportion and microcystin concentrations in G. pulex were also tested using Spearman Rank Correlations. All data analyses were conducted at the significance level of ($p \leq 0.05$) using R 3.6.0 (R CoreTeam, 2019).

III. RESULTS

The G. pulex individuals used in this experiment had average body length and wet weight of G. pulex of 11.02±0.08 mm (range: 8.95-14.99 mm, N=210) and 27.74±0.52 mg, (range:11-59.80 mg, N=210) respectively.

Purified and extracted microcystin treatments exhibited evidence of inverted U-shaped non-linear response on the survival of G. pulex relative to the control. However, Cox proportional hazard (CPH) models showed no statistically significant effects of purified MC-LR (Z = -0.53; HR = 0.74; 95% CI = 0.24-2.28; p = 0.60; Figure 1), crude Microcystis extract treatments (Z = 1.37; HR = 1.94; 95% CI = 0.75-4.99; p = 0.17; Figure 1) or concentration (Z = 0.08; HR = 1.08; 95% CI = 0.98-1.20; p = 0.12; Figure 1) on the proportion of survival among G. pulex individuals at the end of the 96-hour exposure period. The concentration of microcystin accumulated in G. pulex exposed to the purified MC-LR and crude extract ranged from 12.8 – 27.5 ng MC-LR Eq. g-1 and 5.6-12.8 ng of MC-LR Eq.g-1 respectively. The survival proportion of G. pulex exposed to crude extract treatments was positively associated with microcystin concentrations accumulated in animals (r = 0.72; N = 4; p = 0.28), and no relationship was found in the animals exposed to purified MC-LR. However, the small sample sizes tested in this study limit the extent to which inferences could be made on these results difficult.

The range of nominal microcystin concentrations in the experimental medium (water) was positively correlated with microcystin levels in G. pulex, however, the observed association was not significant due to limited number of data points (r = 0.99; N = 4 ; p = 0.08; Figure 2A). Increased nominal concentrations of the purified MC-LR in the water was generally associated with high concentrations of microcystin (MC-LR Eq. L-1) in G. pulex. However, there was a weak evidence of negative, non-significant correlation between nominal microcystin concentrations in water and in G. pulex exposed to crude Microcystis extract (r = -0.2; N = 4; p = 0.26; Figure 2B)

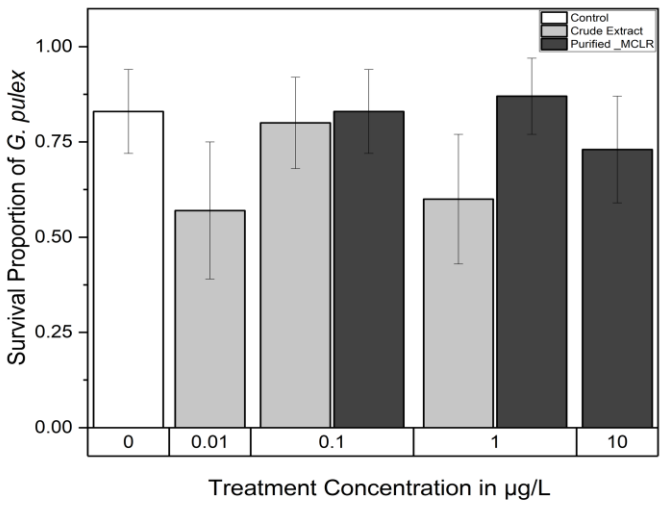

Figure 1: Survival Proportion of *G. pulex* exposed to environmentally relevant concentrations of purified MC-LR and crude *Microcystis* extract treatments during a 96-hour bioaccumulation experiment. Amphipod survival was significant if the associated probability Cox proportional hazard model is <0.05. Error bars represents mean ± 95% confidence interval.

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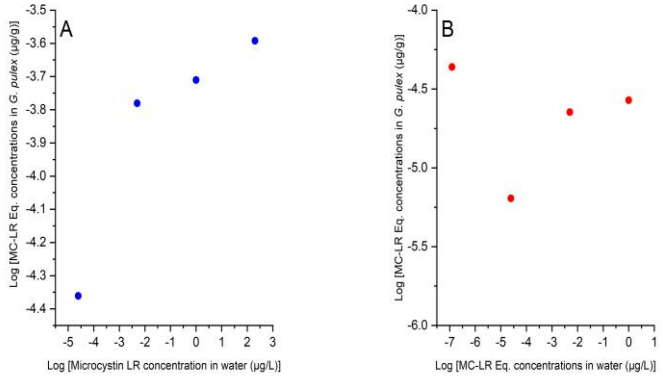

Figure 2: Relationship between natural log-transformed microcystin concentrations (MC-LR equivalent per L) in *G. pulex* and nominal concentrations in water. Note (A) represents microcystin accumulation in animals exposed to purified microcystin treatments and (B) microcystin-containing crude extract of *M.* aeruginosa. Significant (positive or negative) correlations were at *p*-value > 0.05.

IV. Discussion and Conclusion

Microcystin occurrence in dissolved concentrations has become a major public health and ecological risk in freshwaters (Chen et al., 2013; Harke et al., 2016), potentially altering food web structure and function (Bukaveckas et al., 2017; Christoffersen, 1996). Although bioaccumulation has been highlighted to be a major route of microcystin exposure in dissolved concentrations and trophic transfer to higher food web consumers (Ferrão-Filho and Kozlowsky-Suzuki, 2011), it is not known how G. pulex, a UK native amphipod species and an important benthic primary consumer, at the base of most freshwater food webs may serve as a potential vector for toxin transfer to higher trophic consumers under sublethal environmentally relevant microcystin exposure. Here we tested the hypothesis that microcystin uptake and accumulation in G. pulex may be associated with the toxin concentrations in water and secondly, we attempted to show that survival proportion of G. pulex exposed to microcystin treatment may be associated with the concentrations of microcystin accumulated in animals across the treatments. Neither treatment affected survival of G. pulex across the range of low sublethal microcystin concentrations tested in this study. Although inference from the data presented in this study may be limited because of the small sample size used and the range of concentration tested, there was a positive, strong howbeit non-significant association between microcystin concentrations in animals exposed to purified toxin and microcystin levels measured in the water, suggesting possible toxin bioaccumulation in the test animals. Microcystin concentrations in G. pulex was weakly related to the toxin level in crude extract treatment. Hence, these results suggest benthic freshwater amphipods may represent an important route of microcystin exposure and trophic transfer to higher consumers within aquatic food webs.

In line with the prediction that sublethal microcystin exposure are unlikely to reduce survival among sensitive freshwater populations, neither purified MC-LR nor crude extract treatment affected survival of G. pulex in this study. However, survival proportion of G. pulex followed a non-linear, inverted U-shaped pattern with increasing microcystin concentrations across the two treatments, suggesting hormetic dose-response relationship. While similar evidence has been reported in previous studies on the effects of microcystin on aquatic species (Adekolurejo et al., 2022; Liang et al., 2020), this phenomenon of hormesis has been associated with low-dose exposure to chemical stressors (Adekolurejo et al., 2022; Shi et al., 2016). The observed tolerance of G. pulex to microcystin is consistent with the findings of Korpinen et al. (2006) who observed 100% survival rate in G. zaddachi exposed to nodularin at 12-50 µg L−1 and lower concentrations of crude extract of Nodularia spumigena. These results may be attributed to the range of low toxin concentrations tested in this study, compared to higher lethal concentrations which have been reported in many freshwater organisms in the existing literature (Bui et al., 2020; DeMott et al., 1991; Herrera et al., 2015; Smutná et al., 2014). For instance, Bui et al. (2020) recently reported 48hr-LC50 values for purified MC-LR and crude cyanobacterial extract in the tropical micro-crustacean, Daphnia lumholtzi, as 247 µg L−1 and 331 µg L−1 respectively.

Moreover, it could be due to the fact that the rate of microcystin uptake at the range of concentrations tested in the present study was too low to affect individual survival in G. pulex during this short period of exposure. Here, the range of microcystin concentrations observed in G. pulex exposed to purified MC-LR was (12.8 to 27.5) ng MC-LR Eq. g-1 and (5.6 to 12.8) ng of MC-LR Eq.g-1 in the crude extract treatment. These results agree with the findings of de Maagd et al. (1999) and Kozlowsky-Suzuki et al. (2012), suggesting uptake of dissolved microcystin concentrations from water into biota may represent a limited route of toxin accumulation compared to the consumption of toxin-laced prey by freshwater consumers. However, while freshwater organisms are typically more likely to encounter sublethal low microcystin concentrations repeatedly for longer periods in freshwater bodies (Chen et al., 2017; Wei et al., 2020), there is limited information about the subtle ecologically relevant effects of such microcystin exposures on individual behaviour and fitness-related traits in amphipods, which may have adverse ecological outcomes on populations and ecosystem functioning (Burkholder et al., 2018).

Microcystin concentrations observed in the test organism, G. pulex showed strong positive associations with toxin levels in the experimental media across the two treatments. Increased toxin concentration in the experimental media resulted in higher microcystin levels in animals exposed to both purified MC-LR and crude extract treatments, suggesting possible evidence of microcystin uptake and bioaccumulation in G. pulex (Kozlowsky-Suzuki et al., 2012). These observations support the first hypothesis of this study, suggesting microcystin bioaccumulation in benthic native amphipods may possibly represent an important route for microcystin exposure and a pathway its trophic transfer across different food web compartments. Benthic invertebrates are a key ecologically

important components of the aquatic food webs (Burkholder et al., 2018; Xue et al., 2016b), where they influence community structure and mediate important ecosystem functions, such as predation and coarse organic-matter processing in freshwater bodies (von Schiller et al., 2017). Besides, these organisms can accumulate microcystins (Kim et al., 2021) and may serve as important intermediate vectors for microcystin exposure and trophic transfer to organisms at higher levels in the aquatic food webs (Poste and Ozersky, 2013; Woller-Skar et al., 2020). Several studies have reported bioaccumulation of microcystins in a wide range of benthic aquatic organisms, including bivalves (Poste and Ozersky, 2013), gastropods (Lance et al., 2010), chironomids (Xue et al., 2016b), crustaceans (Kim et al., 2021), amphipods (Kim et al., 2021) and oligochaete (Xue et al., 2016a). However, the range of microcystin concentrations observed in G. pulex in this study was much lower than the range reported in other benthic detritivores in previous studies. For instance, a range of (380 to 1200) ng g-1 wet weight of total microcystin was reported in the amphipod species, Mandibulophoxus spp (Kim et al., 2021), while a range of (371 to 636.4) ng g-1 dry weight of total microcystin was found in different life stages of the mayfly, Hexagenia limbata, suggesting that bioaccumulation of microcystin may show species or taxa-specific variations among freshwater benthic detritivores (Kim et al., 2021).

However, the range of microcystin concentrations observed in animals exposed to purified MC-LR treatments was higher than in the crude extract. The reduced concentrations of total microcystin (MC-LR Eq. µg.g-1) observed in the crude extract treatment possibly might have resulted from microbial degradation and complex antagonisms among other metabolites present in the crude extract treatment.

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