ECOLOGICAL IMPACTS OF MICROCYSTINS ON BIODIVERSITY STRUCTURE AND ECOSYSTEM FUNCTIONING IN FRESHWATER SYSTEMS

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Conference Abstracts

Adekolurejo, O., Kay, P. and Hassall, C. Survival and functional responses of native (*Gammarus pulex*) and invasive amphipods (*Dikerogammarus villosus*) to environmentally relevant microcystin concentrations. A poster presentation and a lightning talk at the British Ecology Society (BES) Annual Meeting, Birmingham ICC, UK 6 - 19 December 2018.

Adekolurejo, O., Kay, P. and Hassall, C. Acute, chronic and sublethal effects of a cyanobacterial toxin on the water flea, *Daphnia magna*. Faculty of Biological Sciences Postgraduate Symposium, University of Leeds, UK May 2019.

Adekolurejo, O., Floyd, M., Hassall, C. and Kay, P. Interactive effects of temperature and microcystins on the survival and ecosystem functions of key freshwater species. British Ecology Society (BES) Annual Meeting, Belfast ICC, Northern Ireland, 10- 13 December 2019.

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Abstract

Microcystin, a prominent cyanotoxin produced by cyanobacteria during harmful blooms, has been widely reported from freshwaters worldwide, affecting humans and causing high mortalities and reproductive defects among fish, amphibians, and zooplankton. However, the ecological implications of microcystin exposure on structure and functions in freshwater ecosystems remain unclear. In this thesis, to evaluate the ecological impacts of microcystin on survival and ecosystem functions among freshwater species, populations, and communities, I used a scaling up experimental approach to test a set of explicit hypotheses. First, using a literature-based approach, I illustrated that environmentally relevant microcystin concentrations (0.01-10.0 $\mu g \cdot L^{-1}$) used in this study were comparable to the range of safe levels recommended by the WHO for human health in freshwaters. Second, I used a suite of standard ecotoxicological assays to test the sensitivity of five key food web components (namely, *Scenedesmus quadricauda* (phytoplankton), *Daphnia magna* and *D. pulex* (zooplankton grazers), *Gammarus pulex* and *Dikerogammarus villosus* (native and invasive amphipod shredders) to the purified microcystin LR (MC-LR) and crude extract of *Microcystis aeruginosa*. Purified MC-LR significantly inhibited *S. quadricauda* growth by ca. 22% and slightly elevated its photosynthetic pigment contents at low concentrations compared to the control, suggesting hormesis is potentially a physiological response associated with the putative allelopathic role of microcystin during harmful blooms. In contrast, increased crude extract concentrations significantly reduced survival of *D. magna* by ca. 70%, suggesting the effect of low environmental concentrations may vary across taxa, biological endpoints, and treatments. Third, using a combination of sublethal and chronic toxicity tests, I evaluated the effects of two microcystin treatments on survival, feeding, growth and reproduction among three ecologically important freshwater species. Individual survival was unaffected at low exposure concentrations, however, feeding, growth and fecundity were significantly altered, thereby stimulating the population growth rate. Fourth, using a multiple stressor approach, I tested the individual and combined effects of two microcystin treatments and three water temperatures (15, 20 and 25°C) on survival and ecological processes among key freshwater taxa. Purified MC-LR had a higher growth inhibitory effect on *S. quadricauda* compared to the crude extract, while increasing microcystin concentrations in the crude extract reduced the grazing rate of *D. pulex* and survival of *Ischuran elegans*. Reduced prey handling time and higher predation rate induced by warming in *I. elegans* were offset by a 50% reduction in survival caused by the synergistic interaction between temperature and microcystin, suggesting

complex effects on survival and ecological processes of species across multiple trophic levels. Finally, using a set of community microcosms, I report for the first-time complex effects of sublethal microcystin concentrations on structure and functioning in freshwater ecosystems. The results showed indirect effects of sublethal microcystin exposure on community composition and organic matter decomposition may be compounded by changes in abiotic factors such as water pH. Taken together, these studies suggest strongly that microcystin concentrations considered safe for human drinking and recreation may still have significant but subtle effects on the structure and functioning of aquatic communities.

Table of Content

List of Figures

List of Tables

List of Equations

Chapter 1

General Introduction

1.1 Global Change and Freshwater Biodiversity Decline

Freshwaters, including lakes, rivers and reservoirs, are limited in terms of global extent (ca. <0.1%); however, these ecosystems constitute a disproportionately significant component of the Earth's aquatic resources (Grzybowski and Glińska-Lewczuk 2019). Freshwater ecosystems support a vast variety of life forms, accounting for approximately 12% of all known species in the world (Reid *et al.* 2019), comprising of more than 140 000 species (Dudgeon *et al.* 2006). This incredible diversity of life mediates fundamental ecological processes and functions that underpin the provisioning of valuable ecosystem services for human wellbeing (Oliver *et al.* 2015; Sandin and Solimini 2009). However, there are compelling concerns that freshwaters are particularly among the most threatened ecosystems worldwide in the face of global change (Van Rees *et al.* 2021; Carpenter, Stanley and Vander Zanden 2011), being increasingly degraded through intense human activities and widespread environmental degradation (Sandin and Solimini 2009; Dudgeon *et al.* 2006; Dudgeon 2010). A broad variety of anthropogenic impacts have now been associated with ecosystem degradation, leading to biodiversity decline in aquatic systems (Oliver *et al.* 2015; Dudgeon *et al.* 2006). Importantly, freshwater biota have become more increasingly at risk under rising incidence of anthropogenic threats (McRae, Deinet and Freeman 2017; He *et al.* 2019), while research priorities on their sustainable management and conservation have received far less attention compared to marine and terrestrial species (Van Rees *et al.* 2021; Tydecks *et al.* 2018).

Recent reports suggest population abundance among freshwater species has declined by approximately 84% since 1970 (Almond, Grooten and Peterson 2020). These population declines have been mostly observed among amphibians, reptiles and fishes and an annual average decline of 4% has been predicted over the coming decades, under global change (Almond, Grooten and Peterson 2020). This evidence and others in the literature (Tickner *et al.* 2020; Dudgeon *et al.* 2006) highlight the need to urgently prioritise research efforts

and policy interventions towards sustainable management and conservation of freshwater ecosystems across local, regional, and global landscapes (Van Rees *et al.* 2021). For instance, Van Rees *et al.* (2021) recently argued that to safeguard freshwater biodiversity from further decline, there is need for future policy intervention and management efforts to place greater emphasis on understanding how multiple anthropogenic drivers of biodiversity decline may affect the ecology of freshwater biota. This recommendation is in line with the policies and core mandates of regulatory environmental agencies such as, the US Clean Water Act and the European Union Water Framework Directive, which were primarily established to ensure sustainable ecological conditions in freshwaters (Sandin and Solimini 2009; European Commission 2000). Furthermore, increasing evidence suggests that multiple stressors associated with increased anthropogenic impacts (such as, nutrient pollution, biological invasion, and climate change) may act individually (Woodward, Perkins and Brown 2010; Dodds *et al.* 2009) or in concert (Gunderson, Armstrong and Stillman 2016; Segner, Schmitt-Jansen and Sabater 2014). The consequence of such stressor combinations may result in additive and non-additive synergistic or antagonistic interactions which may potentially accelerate deterioriation of ecological conditions and biodiversity decline among freshwater ecosystems (Côté *et al.*, 2016; Brook et al., 2008; Jackson *et al.* 2016; Ormerod *et al.* 2010). Although a growing body of research has examined individual effects of single stressors on freshwater biota and their ecosystem functioning (Woodward, Perkins and Brown 2010; Camargo and Alonso 2006; Vilà *et al.* 2011), aquatic species are rarely exposed to single stressors in isolation in natural water bodies (Segner, Schmitt-Jansen and Sabater 2014). Therefore, understanding the response of freshwater biota and their key ecological processes to the cumulative effects of multiple stressor interactions in freshwater ecosystems remains an expanding area of research priority among aquatic ecologists and conservation biologists (Isaza, Cramp and Franklin 2020; O'neil *et al.* 2012).

1.2 Freshwater Harmful Cyanobacterial Blooms

One of the most important consequences of synergistic interactions between multiple environmental stressors is the ubiquitous proliferation and expansion of cyanobacterial harmful algal blooms in freshwaters (Glibert 2017; Sukenik, Quesada and Salmaso 2015;

Griffith and Gobler 2019). The expansion of freshwater harmful cyanobacterial blooms has emerged a leading environmental problem in aquatic systems (Brooks *et al.* 2016; Briland *et al.* 2020), that have been associated with the repeated incidence of nutrient enrichment (Richardson *et al.* 2019), climate warming (Urrutia-Cordero *et al.* 2020) and thermal stratifications globally (Paerl and Huisman 2008). Furthermore, harmful cyanobacterial blooms have increased in frequency, intensity, and geographical range due to intense anthropogenic environmental change (Glibert 2017; Rigosi *et al.* 2014; Cai *et al.* 2021). Importantly, approximately 75% of all reported freshwater cyanobacterial bloom cases are potentially toxic (Chorus *et al.* 2000; Harke *et al.* 2016), producing a wide range of cyanobacterial toxins (Merel *et al.* 2013). Consequently, freshwater cyanobacterial blooms havs been widely associated with a growing public health, socio-economic and ecological impacts in many of the world's largest freshwater bodies (Rigosi *et al.* 2014; Brooks *et al.* 2016; Dodds *et al.* 2009; Briland *et al.* 2020). For instance, besides impacts on human and animal health (Chorus *et al.* 2000; Svirčev *et al.* 2019), average annual economic losses owing to access restrictions in lakes and reservoirs used for drinking, recreational and fishing purposes have been estimated at US$150 million, US$250 million, and US$4.6 billion in the UK, South Africa and the US, respectively (Park *et al.* 2017; Dodds *et al.* 2009). However, the potential ecological implications of this phenomenon on freshwater ecosystems are still less known compared to other impacts (Šulčius *et al.* 2017; Havens 2008; Briland *et al.* 2020).

Among the most common organisms in algal blooms, cyanobacteria are a group of ecologically successful organisms (Hyenstrand, Blomqvist and Pettersson 1998), dominating approximately 80% of the total phytoplankton composition in eutrophic freshwaters during harmful blooms (Mihaljević and Stević 2011). Phytoplankton communities in a typical freshwater lake undergo a series of seasonal successions, in which diatoms and green algae dominate in winter and spring, green algae dominate in late spring and early summer, and cyanobacteria dominate in late summer and early autumn (Watanabe et al. 1995). Cyanobacteria are a major component of the phytoplankton communities that drive organic matter production, and carbon fluxes to higher trophic levels in aquatic systems (Harke et al. 2016; Jia et al. 2017). These

organisms contribute a significant proportion of the global primary productivity and atmospheric oxygenation (Sánchez-Baracaldo et al. 2021). As one of the earliest life forms on earth (Sánchez-Baracaldo et al. 2021), cyanobacteria are believed to have evolved a wide range of eco-physiological traits which possibly confer certain competitive advantages over eukaryotic green algae in eutrophic waters during freshwater blooms (Shan et al. 2019; Mantzouki et al. 2016). For instance, cyanobacteria possess high anti-grazing resistance, enhanced affinity for nitrogen-fixation, buoyancy regulation and increased tolerance for UV light intensity (Shan et al. 2019; Mantzouki et al. 2016), which have been linked to their successful invasion and colonization in almost every habitat worldwide (Omidi, Esterhuizen-Londt and Pflugmacher 2018). Persistent occurrence of dense cyanobacterial cells, colonies and filament in eutrophic lakes and reservoirs affect abiotic characteristics (such as, reduced light intensity, dissolved oxygen concentration and elevated water pH) and local biotic communities through phytoplankton growth inhibition, zooplankton and fish mortalities., As a result, this may lead to changes in community structure and functioning in impacted ecosystems (Ibelings et al. 2008; Briland et al. 2020; Paerl et al. 2001). Additionally, several studies have shown that changes in the composition of local communities and functioning of the ecosystem may be attributed to cyanobacterial anti-grazing resistance mediated through morphological, nutritional, and chemical defences (Ger et al. 2016b; Dao, Do-Hong and Wiegand 2010).

1.3 Cyanobacterial Toxins

Anti-grazing chemical defence has evolved as a major eco-physiological adaptation in the interactions between cyanobacteria and zooplankton grazers during harmful blooms (Omidi, Esterhuizen-Londt and Pflugmacher 2018; Pohnert, Steinke and Tollrian 2007). Cyanobacteria-zooplankton interaction is believed to be a key ecological process driving the production of cyanobacterial toxins and a wide range of other secondary metabolites in freshwaters (Omidi, Esterhuizen-Londt and Pflugmacher 2018; Kaebernick and Neilan 2001), which may affect higher invertebrate consumers and alter fundamental structure and function in aquatic ecosystems (Hay and Kubanek 2002; Ger *et al.* 2016b). Cyanobacteria produce a variety of cyanotoxins which may be classified into three main

categories based on the site of their toxicity in animals namely; hepatotoxins, neurotoxins and dermatoxins (Bartram and Chorus 1999). Microcystins (MC), a major group of hepatotoxins, currently the most widespread, most reported, and most frequently studied cyanotoxins in freshwater bodies worldwide (Merel *et al.* 2013).

MCs are a group of non-ribosomally produced cyclic heptapeptides, originally known as the Fast-Death Factor (Bishop, Anet and Gorham 1959), which potentially affect liver cells in humans and animals. The general structure of MCs comprises a cyclo (-D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha-), which is made up of two different types of amino acids (in seven and two sequences). D-Ala and D-Glu represent alanine and glutamic acids in their D configurations, while X and Y are the positions of the variable amino acids whose multiple combinations account for the differences between MCs variants (Kaebernick and Neilan 2001; Bownik 2016). There are about 279 variants of MC currently (Bouaïcha *et al.* 2019), among these the most toxic congener is MC-LR (Neumann *et al.* 2016). The toxicity of the MC-LR is associated with the presence of the Adda amino acid chain (Dawson 1998), leucine (L) and arginine (R) as variable amino acids in its structure (see Figure 1.1) (de Figueiredo *et al.* 2004; Merel *et al.* 2013). MC has been shown to mediate its toxicity in organisms primarily through inhibition of protein phosphatase 1 and 2A enzymes in the specific tissues of the vertebrate liver (Schreidah *et al.* 2020). In addition, MC toxicity has also been linked with the induction of cellular oxidative stress in aquatic animals (Amado and Monserrat 2010; Campos and Vasconcelos 2010). Several cyanobacterial genera, including *Microcystis*, *Dolichospermum*, *Planktothrix*, *Anabaenopsis*, *Nostoc* and *Aphanizomenon* have been associated with MC production in freshwater and coastal ecosystems (de Figueiredo *et al.* 2004; Paerl *et al.* 2001), but the notorious cosmopolitan species, *Microcystis aeruginosa*, is widely reported as the predominant producer of MCs during toxic freshwater blooms (Watanabe *et al.* 1995; Harke *et al.* 2016).

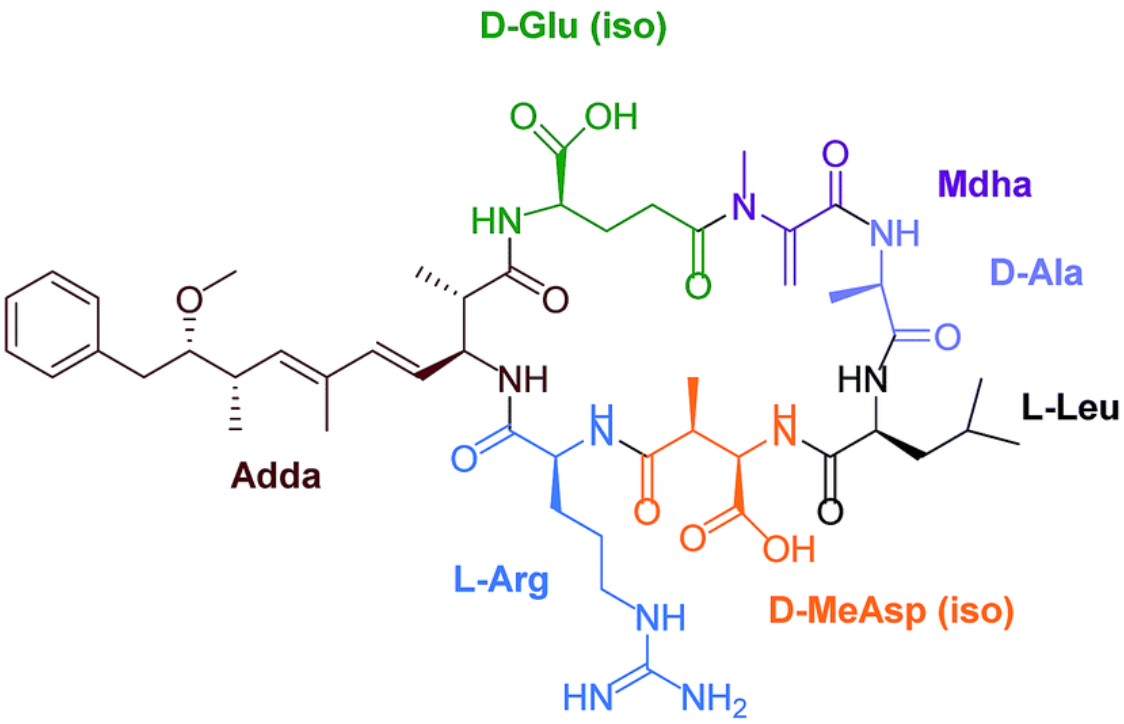

Figure 1.1: Chemical structure of MC-LR

Source: Neumann *et al.* (2016).

1.4 MC Occurrence in Freshwater Bodies

MC occurrence has been widely reported in freshwater bodies around the world (Table 1.1), resulting in severe environmental, socio-economic, and ecological impacts that affect not only water quality and human health, but also aquatic biodiversity and ecosystems (Harke *et al.* 2016; Briland *et al.* 2020; Chen *et al.* 2013). For instance, the repeated high MC concentrations in some of the world's largest lakes, including Lake Erie (USA), Lake Victoria (Tanzania), Lake Taihu (China), Lake Winnipeg (Canada) and Lake Nieuwe Meer (Netherlands), has been linked with increased nutrient pollution and persistent harmful algal blooms dominated by *M. aeruginosa* (Schreidah *et al.* 2020; Michalak *et al.* 2013). The range of MC concentrations reported in these water bodies varied remarkably, depending on the location, time of the bloom event, the dominant cyanobacterial species and the techniques used in MC quantification (Ernst, Hitzfeld and Dietrich 2001; Pham and Utsumi 2018).Therefore, to protect human health, the World

Health Organisation (WHO) recently recommended maximum permissible thresholds of 1 µg/L and 12 µg/L MC-LR equivalents as short term and lifetime exposure levels in drinking waters and 24 µg/L MC-LR concentration in recreational waters (Chorus and Welker 2021). Unfortunately, these regulatory thresholds currently make no provisions for the protection of aquatic species and the ecological integrity of freshwater ecosystems.

MC can occur in aquatic systems, either as intracellularly bound or extracellular toxins. Intracellular MCs are mostly bound in intact cyanobacterial cells (Shahmohamadloo *et al.* 2020) and are usually higher in concentrations than the dissolved extracellular toxins released into the surrounding water bodies (Babica *et al.* 2007; Chen *et al.* 2013). Chen *et al.* (2013) reported that a range of intracellular MC concentrations (0-19.8 µg/mg DW) was found in cyanobacterial biomass collected during bloom event, whereas the typical dissolved MC concentrations in water bodies may rarely exceed 10 µg/L, during persistent incidence of harmful algal blooms (Pham and Utsumi 2018; Babica *et al.* 2007). . One important reason for the occurrence of such low concentrations of dissolved MC is because only approximately 10% of the total MCs produced by cyanobacterial cells are released into freshwaters (Babica *et al.* 2007). Occasionally, high concentrations of dissolved MC may be observed during bloom senescence, following algaecide or hydrogen peroxide treatments (Schmidt 2014; Zheng *et al.* 2004). However, it has been shown that such high MC concentrations rarely persist longer than first few days before they become degraded or metabolized through photolytic and bacterial degradation (Schmidt 2014; Zheng *et al.* 2004). Hence, several studies have shown that persistent long-term environmentally realistic MC concentrations in freshwater bodies worldwide are unlikely to exceed the WHO recommended permissible thresholds (1-24 µg/L) (Chorus and Welker 2021; Zhu *et al.* 2021; Chen *et al.* 2017; Pham and Utsumi 2018; Skafi *et al.* 2021; See Table 1.1).

The highest MC-LR concentrations reported in Lake Champlain (Canada), Lake Taihu and Lake Erie during harmful blooms associated with increased eutrophic conditions and cyanobacterial dominance were 3.8 µg/L (Skafi *et al.* 2021; Table 1.1), 1.82 µg/L (Li *et al.* 2017; Table 1.1) and 3.19 µg/L (Steffen *et al.* 2017; Table 1.1), respectively. Similarly, a nationwide survey of cyanotoxin occurrence in 1161 lakes across the USA detected a

mean MC concentration of 3.0 µg/L in lake water samples (Loftin *et al.* 2016; Table 1.1), while a much lower range of MC concentrations (0.18-0.38 µg/L) was detected in water samples collected from major large rivers across the USA (Graham *et al.* 2020; Table 1.1). These data suggest that while MC levels in the water column in freshwater lakes may usually be < 10 µg/L, much lower concentrations may likely be found in rivers that support only fewer cyanobacterial communities (Graham *et al.* 2020; Reynolds and Descy 1996). Furthermore, this range of environmentally realistic concentrations has been associated with severe public health risks in places where the WHO recommended maximum threshold of 1 µg/L MC-LR equivalent in drinking water sources has been exceeded (Steffen *et al.* 2017) (Schreidah *et al.* 2020). However, the potential ecological impacts of such low environmentally relevant MC concentrations on key components of the freshwater food webs and their ecosystem functions are still poorly known.

1.5 Impacts of MCs on Aquatic Species

MC occurrence in freshwater ecosystems potentially threatens aquatic biodiversity through direct effects on survival and key ecological processes in aquatic species under (Burkholder, Shumway and Glibert 2018). Aquatic species may encounter MC concentrations through several routes, such as direct consumption of toxic cyanobacteria by zooplankton and phytoplanktivorous consumers (Kozlowsky-Suzuki, Wilson and da Silva Ferrao-Filho 2012), or indirectly through feeding on toxin-laced prey, exposure via cell surface contact and uptake of dissolved toxin or cyanobacterial exudate in the water column (Ferrao-Filho, Herrera and Echeverri 2014; Burkholder, Shumway and Glibert 2018). In addition, bioaccumulation and potential trophic transfer may be another important route of MC exposure across different compartments of the aquatic food webs consumers (Kozlowsky-Suzuki, Wilson and da Silva Ferrao-Filho 2012). MC exposure has been shown to reduce survival (DeMott, Zhang and Carmichael 1991; see Table 1.2) and impair behavioural and fitness-related traits in a variety of aquatic species (Dao, Do-Hong and Wiegand 2010; Liang *et al.* 2020; Table 1.2); however, the key questions relating to how MC may affect structure and functions of among species in aquatic food webs remain yet unanswered. MC can mediate several ecological functions in aquatic species, including allelopathic inhibition of growth rate and photosynthetic activities in

algae, as well as anti-grazing chemical cue in cyanobacteria-zooplankton interactions (Omidi, Esterhuizen-Londt and Pflugmacher 2018; Lindholm *et al.* 1992). These effects may cascade through the food web and may alter community structure and ecosystem functioning in freshwaters (Ibelings *et al.* 2008). However, existing empirical evidence on the effects of MCs on aquatic organisms in the laboratory is limited to a few acute and sublethal toxicity studies on single species, such as algae, zooplankton molluscs and fish (Ibelings and Havens 2008; Burkholder, Shumway and Glibert 2018). Current understanding on the ecological effects of MCs on aquatic organisms is based on inferences drawn from acute toxicity on single species in the laboratory (See Table 1.2). Studies investigating the effects of MC and other cyanotoxins at higher levels of biological organisations are still relatively scarce. Several observational field studies have correlated dissolved concentration of toxins in eutrophic lakes with high mortalities among zooplankton, fish, birds, and other terrestrial mammals (White, Duivenvoorden and Fabbro 2005; Kotak *et al.* 1996; Oberholster, Botha and Ashton 2009), but the potential effects of MC exposure on aquatic invertebrates, driving structure and function of food webs in freshwaters are still poorly studied (Bownik, 2016).

1.5.1 Effects on algae

Several studies have demonstrated preliminary evidence of potential inhibitory effects of MC on growth and photosynthetic activities among green algae and diatoms (El-Sheekh, Khairy and El-Shenody 2010; Wang *et al.* 2017; Bittencourt-Oliveira *et al.* 2015; Babica *et al.* 2007; Pereira *et al.* 2018; See in Table 1.2). However, results from many of these studies remain currently inconsistent and inconclusive (Babica, Bláha and Maršalek 2006), using only either crude cyanobacterial extract or the purified toxins at higher concentrations compared to MC levels in freshwater systems. For instance, Pereira *et al.* (2018) observed no significant alteration in growth and photosynthetic pigment when the green algal, *Parachlorella kessleri* was exposed to 55 µg/L and 150 µg/L of MC-LR containing crude extract of *M. aeruginosa*, while Campos *et al.* (2013), demonstrated highest stimulatory effects in the growth of *Chlorella vulgaris* when exposed to 41.5 µg/L MC-LR containing crude extract of *M. aeruginosa* and 179.0 µg/L of the purified MC-LR. These contrasting evidence suggest that the potential allelopathic role of MCs in

interspecific competition during harmful blooms is yet to be fully understood, therefore warrants further studies.

Table 1.1: Dissolved microcystin concentrations recorded in lakes and reservoirs during freshwater harmful cyanobacterial blooms worldwide

Country	Location	Dominant species	Analytical technique	MC variants	Concentrations in (µg/L or µg g/dw)	References
China	Lake Dianchi	*Microcystis spp*	ELISA	MC-LR	0.61-1.00	(Zhang et al., 2012)
	Lake Taihu	*Microcystis spp*	HPLC	Total MCs	1.82	(Li et al., 2017)
	Poyang Lake	*Microcystis spp*	UPLC-MS/MS	MC-RR, MC-YR, MC-LR, MC-LA, MC-LF, MC-LW	1.89	(Zhang et al., 2015)
Mexico	Lake Texcoco	*Planktothrix spp, Anabaenopsis spp Spirulina and Microcystis spp*	ELISA	Total MCs	0.20-2.4	(Barrios et al., 2017)
Lithuanian	James River Esuary/Curonian Lagoon	*Dolichospermium spp, Planktothrix spp*	ELISA	MC-LR	0.30 - 4.34	(Bukaveckas et al., 2017)
USA	1161 Lakes	*Dolichospermium, Microcystis*	ELISA	MC-LR	3.0	(Loftin et al., 2016)

	1038 Lakes	*Planktothrix sp., Dolichospermium sp.*	ELISA	MC-LR	0.06 – 2.05	(Beaver et al., 2018)
	11 Large Lakes	*Dolichospermium spp, Planktothrix spp, Microcystis spp*	ELISA	Total MCs	0.18–0.38	(Graham et al., 2020)
Canada	Pike River, Lake Champlain	Variable	SPE–UHPLC-MS/MS	12 MCs variants	3.8	(Skafi et al., 2021)
	Lakes in Quebec and Ontario	Variable	SPE-UHPLC-HRMS	12 MCs variants	0.03 – 3.5	(Roy-Lachapelle et al., 2019)
Sebia	Lake Ludoš	*Limnothrix redekei, Pseudoanabaena limnetica, Microcystis sp.*	LC-MS	MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-LF	0.03 – 0.30	(Tokodi et al., 2019)
Hungary	Lake Balaton	*Aphanizomenon flos-aquae, Dolichospermum spiroides*	LC-MS	MC-LR, dmMC-LR, MC-RR,	0.01-1.29	(Marinović et al., 2021)

				dmMC-RR, MC-YR, dmMC-YR, MC-LF, MC-LY		
Greece	Lake Pamvotis	*Microcystis aeruginosa*	ELISA	Total MCs	0.01-19.5	(Vareli et al., 2009)
Czech Republic	Vir reservoir	*M. aeruginosa*	HPLC	MC-LR, MC-RR, MC-YR	0-0.12	(Kopp et al., 2013)
	Mostiste Reservoir	*M. aeruginosa*	HPLC	MC-LR, MC-RR, MC-YR	0-0.53	
	Plumlov Reservoir	*Planktothrix agardhii*	HPLC	MC-LR, MC-RR, MC-YR	0.21-0.51	
Ethiopia	Lake Chamo	*M. aeruginosa, D. spiroides*	HLPC-DAD	MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-LF	3.9	(Willén et al., 2011)

Lake Langano	*M. aeruginosa, D.olichospermum*	HLPC-DAD	MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-LF	1.5	(Willén et al., 2011)
Lake Ziway	*M. aeruginosa, D.olichospermum*	HLPC-DAD	MC-LR, dmMC-LR, MC-RR, dmMC-RR, MC-LF	1.3	(Willén et al., 2011)
Lake Kako	*Microcystis, Cylindrospermopsis*	HLPC-DAD, LC-MS	Total MCs	1.73-33.0	(Major et al., 2018)

1.5.2 Effects on zooplankton

Filter-feeding zooplankton grazers are primarily the direct predators of phytoplankton communities, including cyanobacteria, in aquatic systems and are likely to be affected through MC ingestion. While there has been an intense debate regarding the role of MC in cyanobacteria-zooplankton interactions in the literature (Ger *et al.* 2016b; Ger *et al.* 2019; Sarnelle, Gustafsson and Hansson 2010), MC production and toxicity have been linked with the evolution of avoidance mechanism and anti-grazing chemical defence in cyanobacteria (Ger *et al.* 2019; Ger *et al.* 2016a; Chislock *et al.* 2013). Several studies have demonstrated toxic effects of MC on different zooplankton genera, including several species of the generalist large-bodied cladoceran, *Daphnia* (DeMott, Zhang and Carmichael 1991; Bui *et al.* 2020; Ortiz-Rodríguez, Dao and Wiegand 2012; Rohrlack *et al.* 2001; Shahmohamadloo *et al.* 2020; See Table 1.2), rotifers (Cheung, Liang and Lee 2013; Liang *et al.* 2018; Barrios, Nandini and Sarma 2017; Table 1.2) and copepods (Ger *et al.* 2019; Ger *et al.* 2016a; Table 1.2).

One of the major routes of MC exposure in zooplankton is via grazing of toxigenic cyanobacterial cells. Previous studies have tested the toxic effects of MC on zooplankton using either toxigenic cyanobacteria and their mutant strains (Lürling 2003), cyanobacterial bloom samples (Smutná *et al.* 2014), crude cyanobacterial extract (Barrios, Nandini and Sarma 2017) or purified toxins in the laboratory (DeMott, Zhang and Carmichael 1991). These studies demonstrated reduced survival and adverse chronic effects among large *Daphnia* populations at a range of high median lethal concentrations (LC_{50}) or median lethal dose (LD_{50}) of MC exposure (Bui *et al.* 2020; Chen *et al.* 2013), while copepods and smaller cladocerans populations have shown increased resistance to high MC concentrations in the laboratory. This finding is in accordance with the fact that these organisms are highly selective grazers and the observed resistance to high MC concentrations could have resulted from adaptive chemical detection cues (Ger *et al.* 2016b). The range of lethal concentrations reported in these studies is relatively higher than the range of MC concentrations that occur naturally in freshwater bodies (Chen *et al.* 2013), except where there is direct exposure to cell-bound MC concentrations through

ingestion of toxic cyanobacteria (Rohrlack *et al.* 2001). Several studies have shown that the small zooplankton populations, such as smaller cladocerans, copepods and rotifers, but not large-bodied *Daphnia*, are more likely to dominate the freshwater zooplankton communities during bloom peak and senescence when relatively high MC concentrations are released into the water column (Ger and Panosso 2014; Wilson 2006). In contrast, increasing evidence has also shown that *Daphnia* may have evolved adaptive strategies such as, maternal transfer of toxin resistance to offspring (Gustafsson and Hansson 2004; Dao, Do-Hong and Wiegand 2010) and reduced grazing activity which may enable them to avoid ingestion of high MC concentrations through toxic cyanobacteria (Ghadouani *et al.* 2004; Rohrlack *et al.* 2001). While copepods and rotifers may tolerate MC exposure during blooms, the generalist *Daphnia* may rarely encounter relatively high MC concentrations except via ingestion of toxic cyanobacterial cells; however, a more ecologically relevant and ideal scenario will involve a long-term exposure of *Daphnia* to sublethal environmental MC concentrations comparable to the dissolved concentrations in natural waters.

1.5.3 Effects on freshwater invertebrates

Macroinvertebrates are among the most dominant populations in freshwater bodies, occurring in different life stages and occupying different compartment of the aquatic food webs (Collier, Probert and Jeffries 2016). These organisms form a key component of the aquatic food webs, potentially driving structure and functioning in freshwater ecosystems (Peckarsky and Lamberti 2017). Their abundance and diversity have been linked with the integrity and resilience of freshwater ecosystems against chemical stressors (Oliver *et al.* 2015; Clements, Hickey and Kidd 2012). However, unlike zooplankton grazers, only a limited information is currently available on the potential toxic effects of MC exposure on aquatic invertebrates during freshwater harmful blooms (Martins and Vasconcelos 2009; Bownik 2016; Table 1.2). Several field studies have correlated observed decline in invertebrate community composition with the occurrence of MC concentrations in freshwater bodies, suggesting MC may reduce survival and abundance among freshwater invertebrate communities (White, Duivenvoorden and Fabbro 2005; Kotak *et al.* 1996;

Oberholster, Botha and Ashton 2009). White, Duivenvoorden and Fabbro (2005) observed low macroinvertebrate abundance and richness in Lake Elpinstone in Australia, which may be attributed to *Microcystis* toxicity during cyanobacterial bloom event. However, it is uncertain if these effects related to MC toxicity, because there is little understanding of how MC may affect aquatic invertebrates under laboratory exposures (Bownik 2016). Different life stage of invertebrate may encounter varying concentrations of MC and may show variations in their sensitivity to toxin stress. Benthic invertebrates, such as *Hexagenia* have been shown to demonstrate higher tolerance to MC concentrations compared to pelagic invertebrates during a laboratory study (Shahmohamadloo *et al.* 2020; see Table 1.2). However, different life stages of *Hexagenia* can accumulate varying concentrations of MCs, suggesting benthic invertebrates may represent a potential route of toxin bioaccumulation (Woller-Skar *et al.* 2020). MC bioaccumulation has been reported in a wide range of invertebrate taxa such as, bivalves (Poste and Ozersky 2013), gastropods (Lance *et al.* 2010), oligochaetes (Xue *et al.* 2016a), crustaceans (Kim *et al.* 2021), and aquatic insects (Xue *et al.* 2016b), suggesting aquatic invertebrates may be a major vector for trophic transfer of MC to higher consumers. However, it is not clear yet how such MC accumulation may affect individual fitness and behaviour that may potentially influence food web interactions and ecosystem functioning at the community level.

1.6 Impacts of MCs on ecosystem processes

Understanding the intrinsic functioning of ecosystems is important to guide towards effective management of freshwater ecosystems and the sustainability of the ecosystem services they provide mankind (von Schiller *et al.* 2017). However, there is increasing evidence suggesting the fundamental ecological processes underpinning the provisioning of key ecosystem services may be under increasing threats given the rising incidence eutrophication and microcystin-producing blooms (Oberholster, Botha and Ashton 2009). Therefore, assessing how key ecological processes in freshwater ecosystems respond to environmentally relevant MC exposure during cyanobacterial blooms may provide useful information to guide in predicting how persistent harmful cyanobacterial blooms may

affect the beneficial provisioning of ecosystem services for human well-being. There has been an increased interest in the assessment of the potential impacts of diverse natural and anthropogenic stressors on the functional component of freshwater ecosystems in recent decades (European Commission 2000). This is due to the growing realization that functional metrics of ecosystem health do not only constitute a major component of the ecological status in aquatic systems, as described by the EU Water Framework Directive (Monroy *et al.* 2017) but can potentially translate to economic benefits through the provision of ecosystem services European Commission 2000). Functional ecological processes such as metabolism, decomposition of organic matter and secondary productivity have been monitored in freshwater systems as indices of ecosystem functioning. Among these ecological processes, the determination of organic matter decomposition by leaf litter decomposition has been widely employed as one of the most sensitive and reliable surrogate measures of ecological processes in freshwater systems (Tiegs *et al.* 2013). However, studies examining the effects of cyanotoxins, either as purified toxins or as cyanobacterial extracts on these key processes that underpin ecosystem function in organisms, are still lacking in the literature till date.

Table 1.2: Recent experimental studies on the effects of MCs on freshwater species in the laboratory

Taxa	Test species	Treatment	MC Conc., (µg/L or µg/g)	Duration	LC_{50} or EC_{50}	Observed effects	References
Phytoplankton	*Oscillatoria angutissima, Anaebena, Scendesmus obliquus, Chlorella vulgaris*	*Microcystis* crude extract	65.6, 1.2 and 0.8	14 days	24-hr (1.96, 2.4, 2.61) µg/mL	*M. aeruginosa* crude toxin extract inhibited algal growth and chlorophyll-a contents in all the four test species	(El-Sheekh et al., 2010)
Phytoplankton	*Monoraphidium convolutum, S. acuminatus,*	One toxic and nontoxic *Microcystis* strains and MC-	5 and 10	10, 13 days	NA	*Microcystis* strains inhibited algal growth, but crude extract had no effects	(Bittencourt -Oliveira et al., 2015)

		containing crude extract					
Phytoplankton	*Chlamydomonas reinhardtii, C. kesslerii, Pediastrum duplex, Pseudokirchneriella subcapitata, S. quadricauda*	MC-LR, MC-RR	1-25,000	4, 7, 11 days	NA	Higher MC concentrations inhibited algal growth while concentration <10 µg/L had no effects	(Babica et al., 2007)
Phytoplankton	*Aphanizomenon flos-aqua*	*M. ichthyoblabe*, *M. aeruginosa*, *M. viridis*, *M. wesenbergii*, pure MC-LR	380, 290, pure MC-LR (250, 500)	21, 28 days	NA	*Microcystis* strains had differential growth inhibition. *M. aeruginosa* caused the strongest inhibitory effects but no significant inhibition was observed with pure MC-LR	(Ma et al., 2015)

Zooplankton	*Diaptomus birgei, Daphnia pulicaria, D. hyalina, D. pulex*	Purified MC-LR Nodularin, crude extract and intact cyanobacteria cells	500, 2000, 10,000 20,000 and 50,000	24-96 hours	48-hr (450 µg/L) for *D. birgei*, *D. pulex* (9600 µg/L), *D. hyalina* (11400 µg/L) and *D. pulicaria* (21,400 µg/L).	Survivorship varied significantly across the test species, could be attributed to differences in their physiological sensitivities and feeding behaviour	(DeMott et al., 1991)
Zooplankton	*D. lumholtzi*	Purified MC-LR, Crude extract of *M. aeruginosa* and *Cylindrospermo*	0, 100, 200, 300, 400, 500, 600	48 hours, 21 days	Extract 48-hr (299 µg/L) MC-LR 48-hr (409 µg/L)	The purified MC-LR exhibited higher toxicity on *D. lumholtzi* the crude extract possibly due to high proportion of the less toxic	(Bui et al., 2020)

		sis curvispora strain				MC-RR, which in the crude extract.	
Zooplankton	*D. magna*	Complex bloom biomass and crude aqueous extract	(0– 405) mg d.w L^{-1}	48 hours, 21 days	~35.6 mg biomass d.w L^{-1}	The complex biomass, crude aqueous extract, and MC-free SPE permeate all elicited similar and significant lethal effects. The biomass samples increased the date to first brood and reduced fecundity by 50%.	(Smutná et al., 2014)
Zooplankton	*D. magna*, *D. lumholtzi* (cladocerans)	Purified MC-LR, MC-containing crude extract, toxic	1, 5, 50,	21, 60 days	NA	Low MC-LR concentrations slightly reduced the growth and reproduction of parent daphnids. Survivorship	(Dao, T.S. et al., 2010; Dao, T.-S. et al., 2018; Nguyen et

		cyanobacterial strains				decreased with increasing MC concentration.	al., 2020; Ortiz-Rodríguez et al., 2012)
	D. similis, D. laevis, Moina micrura (cladocerans)	Crude extract	434-538	48, 21 days	172-195 mg/L for *M. micrura*	48-h LC50 values varied across species with highest sensitivity in *M. micrura*. MC reduced reproduction and disrupted egg production	(Herrera, N. et al., 2014; Herrera, N.A. et al., 2015)
Zooplankton	*Brachionus calyciflorus* (rotifer)	Purified MC-LR	0, 10, 30, 100	21 days	NA	Higher purified MC concentration (100 µg/L) was associated with significant negative effects on rotifer reproductive timing and	(Liang et al., 2018; Liang et al., 2020)

						fecundity. Grazing rate and timing decreased with increasing MC-LR concentrations.	
Zooplankton	*Eudiaptomus gracilis* (copepod)	MC-containing strains of *M. aeruginosa*	2.88-38.27	10 days	NA	Grazing pressure of *E. gracilis* on *Microcystis* was inversely related to its MC-LR content while selection for alternative prey was positively related to the MC-LR content of *Microcystis*.	(Ger, Kemal Ali et al., 2016)
	Eurytemora affinis *Pseudodiaptomus forbesi* (copepods)	Purified MC-LR	(0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75) mg/L	48 hours	48-hr (1.55) mg/L 48-hr (0.52) mg/L	Mortality increased with higher MC-LR concentrations across the two species. *E. affinis* was less sensitive to	(Ger, Kemal A et al., 2009)

						MC during acute exposure, while such concentrations may exert chronic toxic effects.	
Invertebrate	*Chironomus pallidivittatus* (midges, diptera)	MC-producing and non-MC-producing strains of *M. aeruginosa*,		72 hours, 53 days	nil	*M. aeruginosa* diet had more severe inhibitory effect on the cumulative emergence and burrowing activity of larvae with decreased survival, locomotion ability and adult dry weight.	(Cai et al., 2021)
	Chironomus pallidivittatus (midges, diptera)	Purified MC-LR	Acute (0, 25, 50, 100, 250,	72 hours, 38 days	nil	No morality was observed during acute toxicity test, but chronic exposure inhibited	(Cai et al., 2022)

			500, 1000) Chronic (30)			larvae body length by 35.61% and wet weight by 21.92%, increased emergence time, damaged mitochondria in the intestine, promoted oxidative stress, dysregulated lipid metabolism of chironomid larvae, and increased detoxification enzymes.	
Invertebrate	*Hexagenia spp.*	MC-producing strain of *M. aeruginosa*	(0.5-300 µg/L) total MCs	21 days	NA	No effects of MCs were observed on survival and growth of *Hexagenia spp*	(Shahmohamadloo et al., 2020)

Invertebrate	*Dreissena rostriformis bugensis* (Quagga) *D. polymorpha* (zebra mussels)	Toxic cyanobacterial strains and Purified MC-LR	0, 1, 2.5, 5, 7.5, 10, 15, 20 μg/L	6 days	13.03	The LC_{50} values recorded for MC-LR were within those commonly found during cyanobacterial blooms. This suggests that veligers mortality might have been a consequence of both MC toxicity and poor nutritional qualities.	(Boegehold et al., 2019)

1.7 Thesis aims

The increasing incidence and frequency of freshwater harmful cyanobacterial blooms have been linked with the persistent occurrence of MC concentrations in aquatic ecosystems worldwide. MC has been repeatedly reported to occur in freshwaters at dissolved concentrations that are much lower than the permissible thresholds recommended by the regulatory agencies, while the ecological implications of the phenomenon are still poorly understood. However, the existing lack of empirical studies on the potential effect of environmental MC exposure on freshwater species beyond single-species studies currently limits our understanding and ability to accurately predict the ecological effects of microcystins on impacted freshwater ecosystems. Hence, this thesis aims to investigate the potential ecological impacts of MC on freshwater biodiversity and their ecosystem functioning at environmentally relevant concentrations. To achieve this general aim, I conducted a series of scaling up hierarchical experiments in the laboratory (see Table 1.3). Briefly, I highlight below how each of the chapters in this thesis contribute towards achieving those aims.

In Chapter 1, I conducted literature review, contextualised findings and identified gaps on regarding the environmental occurrence, toxicity, and effects of MC on freshwater organisms and the key ecological processes underpinning their ecosystem functions in freshwaters.

Depending on complexity, uptake and detoxification mechanism, species that form key components of the aquatic food web are expected to vary in their sensitivity to environmentally relevant MC exposure. Therefore, **In Chapter 2**, using a suite of standard ecotoxicological assays, I compared the sensitivity of five key food web components to environmentally relevant treatments of microcystin LR (MC-LR) in purified toxin and crude extract of *Microcystis aeruginosa* (See Table 1.3).

Sublethal effects of MC on behavioural and fitness-related traits, such as survival, feeding, growth and reproduction, are expected to be of more ecological relevance than acute effects on survival which are often expressed as LC_{50}. Such effects are more sensitive and may occur at concentrations that are orders of magnitude lower than the

conventional $LC_{50,}$ which are rarely found in natural waters. **In Chapter 3**, I examined the sublethal and chronic effects of environmentally relevant treatment of MC on survival, feeding, growth and life-history among three ecologically important freshwater species (Table 1.3).

Single stressor experiments are often an over-simplification of the ideal environmental conditions during harmful freshwater blooms because aquatic species are rarely exposed to a single stressor in isolation. The combined effects of toxins and other related abiotic natural stressors on freshwater species during harmful blooms have rarely been studied. Therefore, in **Chapter 4**, I tested the individual and cumulative effects of environmental MC concentrations and increased water temperature on survival and ecological processes among key freshwater species (Table 1.3).

Finally, current ecological risk assessments of the potential effects of MC exposure on freshwater biota and ecosystems are limited and primarily drawn from single-species and single stressor studies in the laboratory. The potential effects of MC on freshwater species and ecosystem functioning are unknown at higher levels of biological organisations. Therefore, in **Chapter 5**, using a set of 32 community microcosms in the laboratory, I evaluate the direct and indirect ecological effects of sublethal MC exposure on ecosystem structure and function in freshwater ecosystems (Table 1.3).

Table 1.3: Experimental Design, Timelines and Thesis Data Chapter Layout

Levels of complexity	Experiment	Test species	Duration of Exposure	Endpoints	Methodology	Thesis Chapter	Timelines
Individual/Species	*Acute toxicity tests*	*D. magna*	48 hours	Mortality/Survival	(OECD, 2004)	2	September/October 2018
		G. pulex	96 hours	Mortality/Survival, body length and weight	(Williams et al., 1984)	2	October/ November 2018
		D. villosus	96 hours	Mortality/Survival, body length and weight	(Williams et al., 1984)	2	October/ November 2018
	Algal growth	*S. quadricauda*	72 hours	Spectrophotometric optical density,	(OECD, 2011)	2	September/October 2018

	inhibition test			percentage growth inhibition, Chlorophyll-a, b, total chlorophyll, and carotenoid pigment concentrations			
Population	Feeding inhibition test	*D. magna*	24 hours	Spectrophotometric optical density, algal biomass concentrations, grazing rate	(Allen et al., 1995)	3	March, 2019
	Chronic life-history test	*D. magna*	21 days	Survival, growth, and fecundity	(OECD, 2008)	3	March-April 2019

	Sublethal toxicity test	*G. pulex*	7 days	Mortality/Survival, body length, weight, leaf mass change and leaf shredding rate	(Kunz et al., 2010)	3	May-June 2019
		D. villosus	7 days	Mortality/Survival, body length, weight, leaf mass change and leaf shredding rate	(Kunz et al., 2010)	3	May-June 2019
Multiple stressors	Feeding response	*Ischnura elegans*	24 hours	Larval head width, survival, number of preys consumed, prey handling rate and attack rate	(Thompson, 1978)	4	June-July, 2019

Acute toxicity tests	*D. pulex*	48 hours	Mortality/Survival	(OECD, 2004)	4	September 2019
Feeding inhibition test	*D. pulex*	24 hours	Spectrophotometric optical density, algal biomass concentrations, grazing rate	(Allen et al., 1995)	4	September 2019
Algal growth inhibition test	*S. quadricauda*	72 hours	Spectrophotometric optical density, percentage growth inhibition, Chlorophyll-a, b, total chlorophyll, and carotenoid	(OECD, 2011)	4	August- October, 2019

				pigment concentrations			
Multiple species	Community microcosms	*S. quadricauda, D. magna, Chironomus spp, G. pulex, and I. elegans*	14 days	Water quality parameters, survival, adult emergence, fine and organic matter decomposition rates	(Choung et al., 2013)	5	April/ May 2019.

Chapter 2

Acute sensitivity of key freshwater food web components to environmentally relevant microcystin concentrations

2.1 Abstract

Widespread anthropogenic eutrophication and climate change have both increased the intensity and frequency of freshwater microcystin-producing cyanobacterial blooms worldwide. However, the specific ecological role of MCs, a group of cyanobacterial toxins produced during blooms in freshwaters is not clear. Therefore, in this study I explored the sensitivity of key food web species across different taxa to environmental MC concentrations. Using a suite of standard ecotoxicological bioassays, I tested the effects of two MC treatments; purified microcystin LR (MC-LR) and crude extract of *Microcystis aeruginosa* at 0.01-10 µg/L and 0.01-0.5 µg/L concentrations, respectively, on (a) 72-hour growth inhibition of *Scenedesmus quadricauda*, (b) 48-hour survival of two species of zooplankton grazer, *Daphnia magna* and *D. pulex*, and (c) 96-hour survival of two species of amphipod shredders, the native *Gammarus pulex* and UK invasive *Dikerogammarus villosus*. Purified MC-LR significantly inhibited *S. quadricauda* growth by ca. 22% but elevated its photosynthetic pigment contents at low concentrations when compared to the control. In contrast, both growth and pigment parameters of *S. quadricauda* were unaffected at the range of crude *Microcystis* extract concentrations tested. Although neither treatment had a significant effect on the survival of *D. pulex*, increased crude extract concentrations significantly reduced survival of *D. magna* by ca. 70% at a concentration equivalent to 50% of the WHO recommended guideline for drinking waters (1 µg/L). No statistically significant effects of either treatment were observed on survival of native and invasive amphipods. These results suggest that low environmental microcystin exposure may exert species-specific negative effects on the abundance of sensitive populations, potentially altering ecological interactions in eutrophic systems. However, further studies are warranted to explore the potential toxic effects of other metabolites produced by cyanobacteria during freshwater blooms.

2.2 Introduction

The expansion of microcystin-producing cyanobacterial blooms in freshwaters is a serious public health threat worldwide (Brooks *et al.* 2016), with potential ecological implications for biodiversity and ecosystem functioning (Shahmohamadloo *et al.* 2020). Driven by the complex interactions between anthropogenic eutrophication (Schulhof *et al.* 2019), climate change (Urrutia-Cordero *et al.* 2020), and biological invasion (Sukenik, Quesada and Salmaso 2015), microcystin-producing cyanobacterial blooms have increased in frequency, magnitude, and intensity in freshwater bodies around the world (Harke *et al.* 2016; Porojan *et al.* 2020b). A major compelling concern regarding this phenomenon in freshwaters is associated with its potential release of microcystins (MCs) and other hazardous cyanotoxins in water bodies (Harke *et al.* 2016; Turner *et al.* 2018). MCs are a variety of well-known natural toxins produced by several cyanobacterial genera, such as *Microcystis*, *Dolichospermum*, *Planktothrix*, *Anabaenopsis*, *Nostoc* and *Aphanizomenon* (de Figueiredo *et al.* 2004; Paerl *et al.* 2001). Harke *et al.* (2016) surveyed 257 bloom events in 108 countries, ca. 42% of these blooms were dominated by the cyanobacterium *Microcystis aeruginosa*, whereas ca. 75% of *Microcystis*-dominated blooms were associated with MC release. This coincidence of *M. aeruginosa* with toxic blooms suggests that this cosmopolitan cyanobacterium is possibly a predominant bloom-forming species and a major producer of MCs during harmful freshwater blooms. Hence, the increasing incidence of toxic *Microcystis* blooms and MC release in the world's freshwater lakes and reservoirs may constitute a significant ecological threat to survival and ecosystem functions among key freshwater biota.

MC occurs as highly toxic, stable, and diverse hepatotoxins in freshwater bodies, with more than 279 MC variants already known (Bouaïcha *et al.* 2019; Rastogi, Sinha and Incharoensakdi 2014; Turner *et al.* 2018). Among these variants, MC-LR remains the most toxic and prevalent congener found in freshwater systems (Martins and Vasconcelos 2009; Rastogi, Sinha and Incharoensakdi 2014). MC occurrence and toxicity in aquatic systems have been shown to vary depending on the nature and duration of the bloom, concentration, and type of MC variants present, as well as the structure of microbial communities (Harke *et al.* 2016; Turner *et al.* 2018). Environmental MC concentrations

have been shown to seldom exceed the WHO recommended permissible guidelines of 1µg/L and 24 µg/L for drinking and recreational waters respectively (Chen *et al.* 2017; rus *et al.* 2000), except for occasionally high MC levels (>2500 µg/L) associated with bloom senescence and algicide treatments (Babica *et al.* 2007). A growing body of evidence has shown that MC can exert negative effects on algae (Bittencourt-Oliveira *et al.* 2015), zooplankton (Rohrlack *et al.* 2001), molluscs (Boegehold, Johnson and Kashian 2019), and fish (Jos *et al.* 2005) during acute and chronic exposures. However, many of these studies have only reported negative effects at very high concentrations of either purified MC-LR, toxic cyanobacterial cells, or crude extract in the laboratory (DeMott, Zhang and Carmichael 1991; Smutná *et al.* 2014). Such high MC concentrations are, at best, only representative of very short periods of peak toxin concentration and may not be readily found in natural waters. Therefore, for more comprehensive predictions and effective management of toxic cyanobacterial blooms in freshwater systems there is need to understand how key species across different food web compartments may respond under ecologically realistic MC concentrations (e.g., around "safe" limits for drinking and bathing water).

MC occurrence at environmentally relevant levels may exert a broad range of negative effects on key freshwater populations, thereby disrupting fundamental ecological processes in impacted systems (Christoffersen 1996; Lindholm *et al.* 1992). MC has been hypothesized to mediate several ecological functions within aquatic ecosystems which may improve the understanding of its impacts on sensitive freshwater populations (Babica, Bláha and Maršalek 2006; Omidi, Esterhuizen-Londt and Pflugmacher 2018). For instance, several studies have suggested that MC may be involved in promoting cyanobacterial dominance and community succession through allelopathic inhibition of phytoplankton growth and photosynthetic activities (Babica *et al.* 2007; Bittencourt-Oliveira *et al.* 2015; Wang *et al.* 2017). However, existing evidence supporting this hypothesis is still relatively small in quantity and controversial, thus suggesting the need for more research to explore the allelopathic potential of MCs (Babica, Bláha and Maršalek 2006). Bittencourt-Oliveira *et al.* (2015) argued that findings from existing studies on allelopathic potentials of MC on phytoplankton growth may be unrealistic and

difficult to extrapolate to the field, as those conclusions were based on the use of unusually high MC concentrations not obtainable in the environment. Furthermore, MC has also been postulated to serve as a major deterrent to zooplankton grazers, influencing cyanobacteria-zooplankton interactions and may prevent energy transfer from cyanobacteria to higher trophic levels (Ger *et al.* 2016b). Previous laboratory studies have attributed observed declines in survival, feeding and grazing pressure among large-bodied *Daphnia* and some copepods to MC toxicity (DeMott, Zhang and Carmichael 1991; Ger *et al.* 2016a). Yet, among these studies, only a few have shown reduced survival (DeMott, Zhang and Carmichael 1991; Reinikainen, Ketola and Walls 1994), inhibition of growth and feeding (Perez-Morales, Sarma and Nandini 2014), and impaired reproductive fitness (Ferrao-Filho, Azevedo and DeMott 2000) among *Daphnia* populations when exposed to dissolved, purified MC-LR and/or crude cyanobacterial extract treatments. In contrast, several studies have failed to support the earlier assumption that MC may be involved in anti-grazing chemical defence (Chislock *et al.* 2013; Smutná *et al.* 2014). Altogether, these contrasting results highlight the need for further investigations to explore how key species across different trophic levels may respond under exposure to environmentally relevant MC concentrations.

Therefore, in this study, I evaluated the sensitivity of five key freshwater species to environmentally relevant concentrations of purified MC-LR and crude extract of microcystin-producing *M. aeruginosa.* The key freshwater species tested in this study include *Scenedesmus quadricauda* (green alga), *Daphnia magna* (zooplankton grazer), *D. pulex* (zooplankton grazer), *Gammarus pulex* (native amphipod) and *Dikerogammarus villosus* (invasive amphipod). These test species in this study were selected based on their significant positions in the trophic web and their reliability as sentinel experimental models in many ecotoxicological studies (Kennedy *et al.* 2002; Šulčius *et al.* 2017). *D. villosus* is included as a comparison with *G. pulex as* it is expanding its range rapidly in Europe and North America, poses a risk to native fauna, and has been shown to respond differently to environmental stressors (Kenna *et al.* 2017).

In this study, I tested the hypothesis that environmentally realistic MC concentrations comparable to those recommended by the WHO for freshwater bodies would (a) inhibit

growth and photosynthetic pigment contents of *S. quadricauda*, following the assumptions of Babica *et al.* (2007) on allelopathic interactions, (b) reduce survival of individuals among *D. magna* and *D. pulex*, in accordance with the assumptions of Ger and Panosso (2014) that MC may be involved in anti-grazing chemical defense, (c) reduce survival of individuals among *G. pulex* and *D. villosus* due to increased MC uptake and assimilation in amphipods through benthic detrital feeding (Babcock-Jackson, Carmichael and Culver 2002) and (d) have a smaller effect on the invasive *D. villosus* than the native *G. pulex* based on previous work demonstrating higher heat stress tolerance in the non-native that may also confer resistance to toxins (Kenna *et al.* 2017). Overall, the hypotheses tested in this study were based on the understanding that variations in the mechanisms and rate of uptake, assimilation, metabolism, and detoxification may influence sensitivity among different species exposed to chemical stressors (Kozlowsky-Suzuki, Wilson and da Silva Ferrao-Filho 2012; Nilsen *et al.* 2019). I tested these hypotheses by conducting a battery of 72-hour algal growth inhibition tests, 48-hour *Daphnia* acute toxicity tests, and 96-hour macroinvertebrate acute toxicity tests following standard methods (OECD 2004; OECD 2011; Williams, Green and Pascoe 1984).

2.3 Materials and Methods

2.3.1 Chemicals

The purified MC-LR (CAS No. 101043-37-2 and purity ≥ 95%) used for this study was supplied by Cayman Chemical Company, UK. 1mg of the purified toxin was dissolved in 1mL methanol to make an aqueous stock solution (1mg/mL, MC-LR), which was later calibrated up to a litre with Milli Q water, following Dao *et al.* (2018). Experimental aliquots were made by serially diluting the stock solution in the magnitude orders of 10 to obtain a range of working nominal concentrations. Aliquot solutions were immediately stored at -20°C until the start of experiments. Microcystin (Adda-specific) ELISA kits (CAT No. ALX-850-391-KI01) used for MC assays were purchased from Enzo Life Sciences, while methanol (anhydrous, 99.8% Sigma-Aldrich) and other chemical reagents used for the preparation of algal and zooplankton culturing media were of purity ≥ 95%.

2.3.2 Cyanobacterial and Algal culture

Stock strains of the cyanobacterium, *Microcystis aeruginosa* (CCAP/1450/16) and the green micro-alga, *Scenedesmus quadricauda* (A950) were obtained from the Culture Collection of Algae and Protozoa (CCAP) of the Scottish Marine Institute in Scotland and Sciento Stores in Manchester, UK respectively. Both strains were cultured individually in the laboratory by inoculating 150 mL of sterile BG-11 medium in 250 mL Erlenmeyer flasks with 3 mL of the stock strains as inoculants. The BG-11 culture medium used was prepared following the CCAP's recipe for growth media preparation. Both organisms were maintained in the laboratory as batch axenic cultures kept under controlled conditions of constant light and temperature. *M. aeruginosa* cultures were maintained at a constant temperature of 25±1°C, fluorescent light intensity of 25µmol quanta $m^{-2}s^{-1}$ and 12:12 hour photoperiod cycle in a shaking incubator, while the growth of *S. quadricauda* was monitored at 21±1°C; 54 µmol quanta $m^{-2}s^{-1}$ light intensity and 12:12-hour photoperiod under an illuminated cabinet chamber in a controlled temperature (CT) room. *S. quadricauda* cultures were manually shaken twice daily to ensure optimal growth (Wang *et al.* 2017).

The daily biomass increases of *M. aeruginosa* and *S. quadricauda* were determined from cell counts using a compound microscope and Sedgewick-Rafter Counting Chamber and the spectrophotometric optical density (OD) at 680nm. The data obtained was used to establish a relationship between the optical density values and the microscopic cell counts by fitting linear regression lines for the cyanobacterium ($R^2 = 0.97$) and the green alga ($R^2 = 0.97$) respectively. *M. aeruginosa* cultures at the stationary growth phase (2.0×10^5cells/mL) were harvested, freeze-thawed thrice at -18°C and centrifuged for 10 minutes at 7500 rpm and 4°C. The pellets were resuspended with deionized water, freeze-thawed again and centrifuged for 10 minutes. The supernatants were decanted into new flasks, while the pellets were discarded, and the total MC content was assayed using an MC-coated 96 well plates ELISA kit (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).

2.3.3 Animal Culture

2.3.3.1 Daphnids

The two microcrustacean zooplankton species used in this study - *D. magna* and *D. pulex* were obtained from Sciento stores in Manchester, UK, and Blades Biological Limited in Kent, UK, respectively. Both cultures were reared in a controlled temperature (CT) room kept at 21±1°C and 16:8-hour photoperiod cycle in the laboratory for 2-3 weeks before the commencement of the study. Fifteen adult individuals from each species were maintained in 500 mL beakers containing 350 mL of modified artificial media for zooplankton, "Aachener Daphnien Medium" (ADaM) (Klüttgen *et al.* 1994). Freshly prepared ADaM media were autoclaved, aerated for 1 hour and seeded with 10 mL of *S. quadricauda* (equivalent to biomass concentration of 1.0×10^5 cells per mL in the medium) as food source for daphnids. This procedure was repeated each time the culture media were renewed. The *Daphnia* culture media used in this study were renewed three times per week and on each occasion, the media were furnished with *S. quadricauda* (1.0 x 10^5 cells per mL) as food material. Neonates less than 24 hours old and produced after the second brood were enumerated and used for the experiment.

2.3.3.2 Amphipods

Gammarus pulex were collected from Meanwood Beck in Leeds, West Yorkshire (53°500′N; 1°350′W), and *D. villosus* were collected from Grafham Water Centre, Perry in Cambridgeshire (52°18′N; 0°19′W), between August and October 2018 (Kenna et al. 2017). *G. pulex* individuals were sampled using a hand net, while populations of D. villosus were collected by dislodging them from a woven net used to provide artificial substrate. Both the native and the invasive gammarids were conveyed to the laboratory in separate cool boxes containing stream water. Animals were acclimatized to laboratory conditions for two weeks in plastic 300 mL tanks, made up of 30 gammarids in 250 mL old-dechlorinated tap water furnished with glass pebbles as shelter for the animals. Both species were fed with dried sycamore leaves (*Acer pseudoplatanus*) and maintained in a CT room conditioned at 15±1°C and 14:10hr photoperiod, while the culture medium was renewed every 72 hours with old, dechlorinated tap water. Before the experiment, all

animals were initially sorted into different 300 mL plastic tanks depending on the species. After that, animals were randomly distributed into replicate tanks without size matching because of variations in the sizes of animals used for the experiment

2.3.4 Experimental Design

2.3.4.1 Algal growth inhibition and photosynthetic pigment tests

The sensitivity of the green alga, *S. quadricauda* to both purified MC-LR and crude extract of MC-producing *M. aeruginosa* treatments was evaluated in accordance with the recommended 72-hour algal growth inhibition test (OECD 2011). Thirty sterile 150 mL Erlenmeyer flasks, each containing 100 mL of freshly prepared and autoclaved BG-11 medium were inoculated with 5 mL of 8.0×10^5 cells per mL of *S. quadricauda.* The cultures were maintained for 7 days, under illuminated florescent bulbs kept at 24±1°C, 54 µmol quanta $m^{-2}s^{-1}$ light intensity and 12:12-hour photoperiod before exposure to experimental treatments (OECD 2011). Concentrations, equivalent to 0.01, 0.1 and 0.5µg/L crude *M. aeruginosa* extract and 0.01, 0.1, 0.5, 1, 10µg/L purified MC-LR respectively were used as treatments with three replicates per concentration. The control group, comprising the blank and the solvent controls, was made in three replicates. The blank control contained the same conditions as the treatments except without the extract or purified MC-LR, while the solvent control had in addition to the blank, 1mL of 0.1% *v/v* methanol to control for the possible effects of methanol diluent used. Each replicate was manually shaken twice daily, and the optical density (OD) values were measured at 680nm every 24 hours for 3 days, using spectrophotometry. The cell number in each sample was calculated from the linear regression calibration curve between the spectrophotometric OD_{680} values and the microscopic cell counts. The growth rate µ, and the percentage inhibition of *S. quadricauda* growth rate *X*, were calculated from the solvent control using the equations described by (Fang *et al.* 2018a). Where $N_{1,}$ is the initial number of algal cells and N_n, the number of cells at $t_{1,}$ which is day 0 and $t_{n,}$ days respectively.

$$\mu = \left(\frac{(lnN_n - lnN_1)}{(t_n - t_1)}\right) \qquad \text{Equation 1}$$

$$X = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$ Equation 2

After 72 hours of exposure, 3 mL each of the algal culture sample was taken from the experimental and the control groups and centrifuged at 4000rpm and 4°C for 10 minutes. The supernatants from each of the samples were decanted and discarded, while the pellets were resuspended in 80% methanol in the dark for 24 hours. The extracts were centrifuged again at 4000rpm and 4°C, while the supernatant was decanted into a clean cuvette for the determination of photosynthetic pigment contents. The chlorophyll and the carotenoid contents in the treatment and control samples were determined by taking the spectrophotometric optical density measurement of the supernatants at 440, 645 652, 663nm (Fang *et al.* 2018a).

2.3.4.2 Acute toxicity in *Daphnia*

The sensitivities of *D. magna* and *D. pulex* neonates to MC-containing extract of *M. aeruginosa* and purified MC-LR were investigated using a 48-hour static acute bioassay, according to OECD Test 202 guidelines (OECD 2004). The treatment concentrations used in this experiment were the same as those used for algal and gammarid acute toxicity experiments. In all, three concentrations of cyanobacterial extract, five of purified toxin, and a control were used, with three replicates each. Ten *Daphnia* neonates per concentration per species (aged <24 hours) were kept in 100 mL-plastic cups containing 50 mL of ADaM medium and exposed to crude *M. aeruginosa* extract and purified MC concentrations as treatments. The experimental conditions in the control replicates were the same as those of the treatment except that no extract or purified toxin was added. All experimental conditions were maintained under illuminated white florescent bulbs in a CT room set at 21±1°C and 16:8-hour photoperiod. Daphnids were not fed throughout the experiment and the number of individuals that survived were estimated by enumerating the total number of immobilized neonates per replicate per species at the end of 24 and 48 hours.

2.3.4.3 Acute toxicity in amphipods

Both the native (*G. pulex*) and the invasive amphipod species (*D. villosus*) were individually exposed to a range of concentrations of purified MC-LR and MC-containing

cyanobacterial extracts treatments for 96-hour in a static non-renewal acute toxicity experiment. One animal per species per replicate was exposed in a 150 mL-plastic cup comprising a mixture of 100 mL of aged, dechlorinated tap water to the same treatment concentrations as those used for the algal toxicity tests with 10 replicates per concentration and control. The experiment was maintained in a controlled incubator conditioned at 15±1ºC and 14:10hr photoperiod cycle (Boets *et al.* 2012). To avoid stress and cannibalism through intraguild predation, animals were exposed individually to MC treatments without food material throughout the experiment (Bundschuh *et al.* 2013). Survival of individuals across treatment concentrations and species were monitored and estimated as the difference between the total number of animals exposed per treatment concentration and the number of animals that died at end of every 24 hours. Animals were considered dead when no movement or swimming was observed after touching with forceps.

2.3.5 Statistical analysis

Algal growth inhibition data was expressed as the percentage reduction in growth relative to the control. Normality assumption tests were checked on each model using Shapiro-Wilk test. Hypothesis testing for a significant difference in percentage growth inhibition between the treatments was carried out by fitting a Gaussian generalized linear model (GLM) with square root link functions into the growth inhibition data, while the pigment data was analysed by fitting a Gaussian GLM with log link function individually to the data for each of the photosynthetic pigments measured. The 0.1% v/v methanol diluent used in the solvent control had a negligible effect on growth and pigment concentrations of *S. quadricauda* relative to the blank control (t = 0.53; df =4; p = 0.60; Appendix 1). Hence, all statistical tests of the difference between the treatments and the control in all the experiments were conducted relative to the blank control.

The overall proportion of surviving animals observed among the test organisms used in this study was greater than 50% during the 48-hour *Daphnia* and 96-hour amphipod acute toxicity tests. As a result, the dose-response relationship in the *Daphnia* and amphipod data could not be expressed as median lethal concentration (LC_{50}), which estimates the

concentration that would reduce survival among the exposed test organisms by 50%. Alternatively, using the *survival* (Therneau 2015) and *survminer* (Alboukadel, Marcin and Przemyslaw 2019) packages in R, I fitted Cox proportional hazard (CPH) models to test for an effect of treatment on the time to death in the exposed population. All statistical analyses were performed using R 3.6.0 (R CoreTeam 2019) at significance level of ($p \leq 0.05$).

2.4 Results

2.4.1 Algal toxicity test

2.4.1.1 Effects of MC on growth inhibition

The purified MC-LR treatment tested in this study was associated with a strong overall inhibitory effect on the growth of *S. quadricauda* ($t = 3.94$; $df = 29$; $p < 0.001$; Figure 2.1), while no significant overall effect of crude *Microcystis* extract treatment was observed on growth inhibition of *S. quadricauda* relative to the control ($t = 1.77$; $df = 29$; $p = 0.09$; Figure 2.1). In addition, the range of concentrations tested across the two treatments showed no significant difference ($t = 0.36$; $df = 29$; $p = 0.73$; Figure 2.1). However, there was evidence of non-linear, inverted U-shaped hormetic dose response relationship, such that growth inhibition increased linearly with increasing concentrations of the purified MC-LR at lower concentrations, whereas higher toxin concentrations reduced the percentage growth inhibition. The highest growth inhibitory effect (ca. 22%) was observed at the intermediate treatment concentration of 0.5 ug/L which is equivalent to 50% of the WHO recommended permissible guideline for microcystin occurrence in drinking water sources.

2.4.1.2 Effects of MC on pigments

Effects of both treatments on chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid pigment contents (Figure 2.2A-D) measured in this study followed a similar pattern. Overall, the two treatments demonstrated evidence of linear concentration-dependent pattern of effects relative to the control across all the parameters examined (*p*

< 0.05; Figure 2.2A-D; see Appendix 1). Increased concentration of the crude *Microcystis* extract had no significant effects on the contents across all the pigment parameters (p =0.303; Figure 2.2A-D; Appendix 1). However, lower concentrations of purified MC-LR treatment resulted in a significant increase in pigment contents (p = 0.001; Figure 2.2A-D; see Appendix 1). This result suggests the effect of purified MC-LR treatments on chlorophyll-a and other photosynthetic pigment contents followed an hormetic, non-linear dose-response relationship.

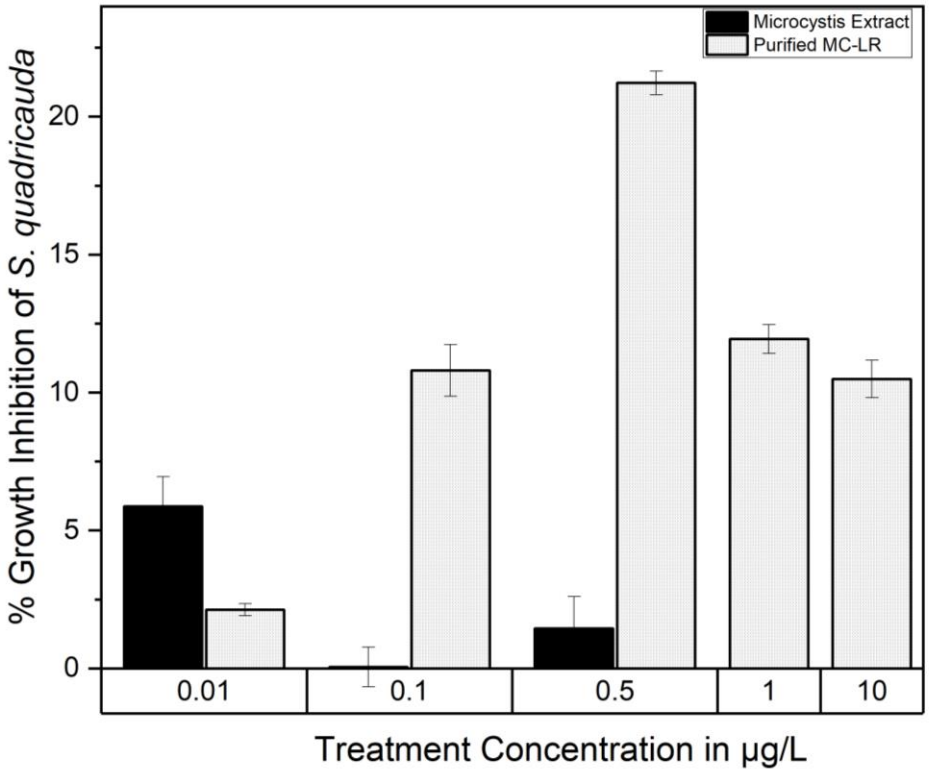

Figure 2.1: Percentage growth inhibition of *S. quadricauda* exposed to purified microcystin (MC-LR) and crude *Microcystis* extract treatments for 72 hours. Note that error bars represent mean ±standard errors of mean.

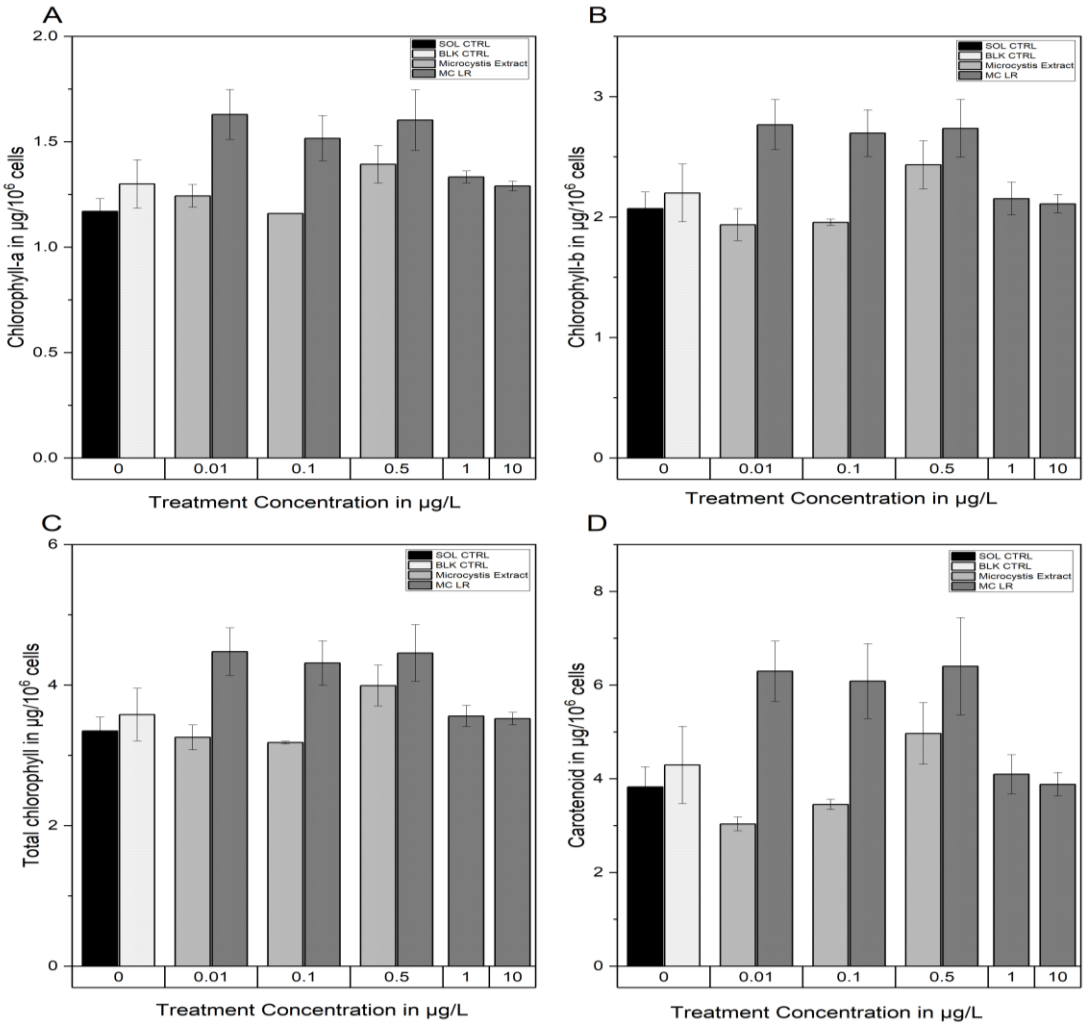

Figure 2.2: Photosynthetic pigment content of *S. quadricauda* exposed to purified MC-LR and crude MC-producing cyanobacterial extract on (A) chlorophyll a, (B) chlorophyll b concentration, (C) total chlorophyll and (D) carotenoid concentrations in µg/10^6 cells. Pigment concentrations are considered significant relative to the blank control (general linear models, $p < 0.05$). Error bars represents mean ±standard errors of mean.

2.4.2 48-hour survival rate of Daphnia

No significant effects of purified MC-LR treatment were observed on the survival of *D. magna* at the range of concentrations tested in this study relative to the control ($p = 0.99$; Table 2.1; Figure 2.3A). However, increased concentration of the crude *M. aeruginosa* extract significantly reduced survival of *D. magna* by ca. 70% compared to the control ($p = 0.03$; Table 2.1; Figure 2.3A). The lowest survival rate (0.2) among *D. magna*

individuals was observed at the highest treatment concentration of the crude extract (0.5µg/L; Figure 2.3A), suggesting that *D. magna* individuals exposed to crude extract were approximately 10x (Hazard ratio (HR) = 9.75; Table 2.1) more likely to die compared to those in the control.

The results of this study showed that purified MC-LR treatment at environmentally relevant concentrations tested in this study had no statistically significant effects on the survival of *D. pulex* relative to the control ($p = 0.26$; Table 2.1; Figure 2.3B). However, crude *Microcystis* extract resulted in a marginally non-significant increase in the survival of *D. pulex* ($p = 0.05$; Table 2.1; Figure 2.3B), such that animals exposed to *Microcystis* extract treatment showed only 39% of the risks of mortality of those in the control group (*HR*: 0.39; Table 2.1).

2.4.3 96-hour survival rate of amphipods

The results of the present study showed purified MC-LR ($p = 0.12$; Table 2.1; Figure 2.4A) and crude extract ($p = 0.49$; Table 2.1; Figure 2.4A), per se, had no statistically significant effects on the survival of *G. pulex* relative to the control during the 96-hour acute toxicity exposure. However, there was a significant effect of the treatment concentration on the survival of *G. pulex* relative to the control ($p = 0.002$; Table 2.1; Figure 2.4A). There was no evidence of statistically significant differences between survival of *D. villosus* exposed to the purified MC-LR ($p = 0.37$; Table 2.1; Figure 2.4B) or crude extract treatments ($p = 0.74$; Table 2.1; Figure 2.4B) relative to the control.

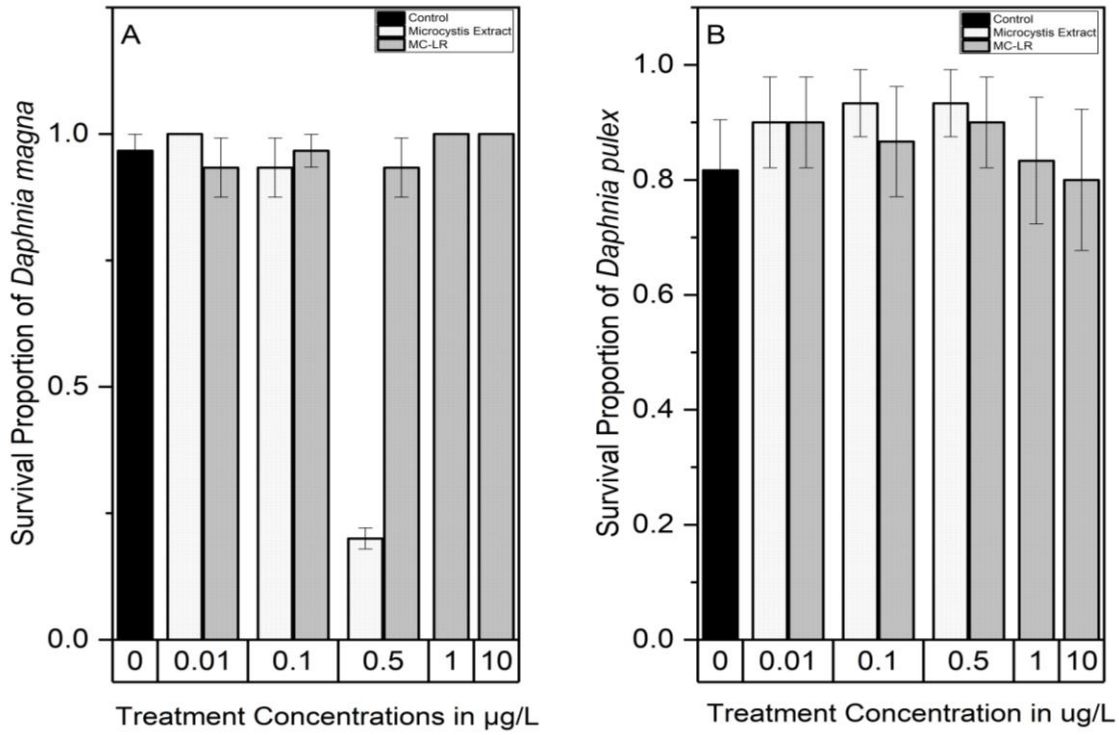

Figure 2.3: Survival Proportion of two cladoceran zooplankton species; *Daphnia magna* (A) and *D. pulex* (B) exposed to environmentally relevant concentrations of purified MC-LR and crude extracts of *M. aeruginosa*. Survival of *Daphnia* was significant if the associated probability for the Cox proportional hazard (CPH) model is <0.05. Error bars represents mean ± 95% confidence interval.

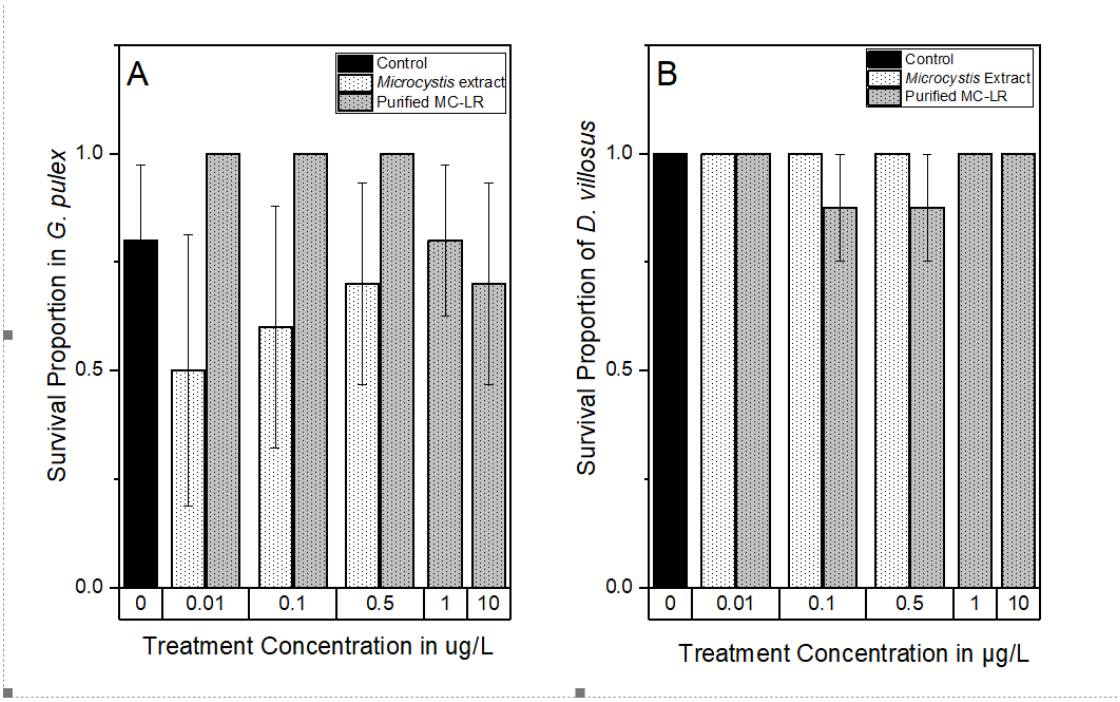

Figure 2.4: Survival Proportion of two amphipod species; (A) *Gammarus pulex* (native sp.) and (B) *Dikerogammarus villosus* (invasive sp.) exposed to environmentally relevant concentrations of purified MC-LR and crude *Microcystis* extracts. Amphipod survival was significant if the associated probability for the Cox proportional hazard (CPH) model is <0.05. Error bars represents mean ± 95% confidence interval.

Table 2.1: Summary of Cox proportional hazard models with their associated parameter estimates, standard errors (SE), Wald statistic, 95% confidence intervals (95% CI) and p-values of the effects of MC treatments on survival of two zooplankton spp. (*D. magna* and *D. pulex)* and two amphipod spp. (*G. pulex* and *D. villosus*).

Species	Variable	Beta	SE	HR (95% CI)	Wald statistic	P-value
D. magna	MC (Extract)	2.28	1.02	9.75 (1.32-71.90)	2.23	0.03
	MC (Purified)	0.01	1.12	1.00 (0.11-9.08)	0.01	0.99
	Concentration	-0.04	0.13	0.96 (0.75-1.25)	-0.27	0.78
D. pulex	MC (Extract)	-0.93	0.48	0.39 (0.15-1.02)	-1.93	0.05
	MC (Purified)	-0.46	0.40	0.63 (0.29-1.40)	-1.13	0.26
	Concentration	0.05	0.05	1.05 (0.95-1.16)	1.02	0.31
G. pulex	MC (Extract)	0.76	0.76	2.14 (0.48-9.56)	0.99	0.32
	MC (Purified)	-1.55	1.03	0.21(0.03-1.59)	-1.51	0.13
	Concentration	-0.20	0.10	1.23 (1.02-1.48)	2.14	0.03
D. villosus	MC (Extract)	-0.40	1.23	0.67 (0.06-7.41)	-0.33	0.74
	MC (Purified)	0.95	1.06	2.58 (0.32-20.71)	0.89	0.37
	Concentration	-0.30	0.28	0.74 (0.43-1.27)	-1.092	0.28

2.5 Discussion

This study demonstrates that sensitivity to environmentally relevant MC exposure among key freshwater species may vary across different taxa and trophic levels. Previous laboratory studies have shown that MCs can affect individual survival, growth, and other physiological parameters among sensitive populations (Babica *et al.* 2007; DeMott, Zhang and Carmichael 1991). However, such evidence is still limited to individual species studied in isolation among algae, zooplankton, and fish exposed to high MC concentrations above what is representative of environmental levels in freshwater bodies (Chen *et al.* 2013; Niu *et al.* 2018). Here, I report the first evidence of how environmentally realistic MC concentrations (1-10 µg/L), comparable to the WHO recommended safe levels, affected the survival of species across key freshwater food web compartments (primary producer, grazer, and shredder). As predicted, the data presented in this study illustrates that low environmental MC concentrations can affect survival and

inhibit growth among key freshwater organisms, with significant variations across different trophic levels and MC treatment type. Purified MC-LR strongly inhibited growth and increased photosynthetic pigment concentrations of *S. quadricauda* but no significant effect of crude *Microcystis* extract treatment was observed compared to the control. Although *D. pulex* survival was unaffected by both treatments, however, *Microcystis* extract reduced survival of *D. magna* relative to the control. Additionally, both purified MC-LR and crude extract treatments had no effects on the survival of native or invasive amphipods. Altogether, these results highlight a major nuance in species' sensitivity to environmental MC exposure, suggesting cyanotoxin effects may be complex and species-specific, with potential effects on community structure and ecosystem functioning. Hence, it is imperative to prioritize regular biomonitoring programmes for effective management of ecological impacts of toxic blooms on freshwater bodies.

2.5.1 Allelopathic effects of MC on algae

The results of this study showed that environmentally relevant MC exposure can exert a broad range of effects on the sensitivity of *S. quadricauda*. As expected, purified MC-LR treatment strongly inhibited growth and increased photosynthetic pigment contents of *S. quadricauda*, but no effects were observed in the crude extract treatment. The effects of purified MC-LR on growth and pigment parameters of *S. quadricauda* observed in this study followed an inverted U-shaped non-linear dose-response relationship and may be explained in accordance with the phenomenon of hormesis (Calabrese 2008). Hormesis is an emerging adaptive stress response in organisms, primarily resulting from overcompensation in response to stress (Calabrese 2008; Liang *et al.* 2017a; Shi *et al.* 2016). This phenomenon is generally characterized by induced stimulatory effect at low dose and inhibitory effect at high dose or, conversely, by low-dose inhibition and high-dose stimulation (Calabrese 2008). In the present study, it was observed that algal growth inhibition was reduced both at lower and higher concentrations, while inhibition was highest at intermediate concentrations, suggesting an inverted U shaped hormetic response. Similarly, I also observed evidence of hormesis when lower MC-LR

concentrations stimulated photosynthetic pigment contents of *S. quadricauda,* although pigment parameters were unaffected at higher toxin concentrations. Therefore, these results suggest hormesis may represent an important ecological phenomenon influencing the toxicity and fate of chemical stressors in freshwater systems. It is also likely that this phenomenon may explain why occurrence of stressors such as MCs at relatively low concentrations potentially poses serious threats to a variety of aquatic organisms (Liang *et al.* 2017a; Liang *et al.* 2020).

A key postulated ecological function of MC in freshwater systems is related to its potential to mediate allelopathic interactions between cyanobacteria and other phytoplankton communities during inter-specific competitions (Babica, Bláha and Maršalek 2006; Bittencourt-Oliveira *et al.* 2015). In accordance with the hypothesis of this study, environmentally realistic concentrations of purified MC-LR strongly inhibited the growth of the green alga, *S. quadricauda*. The observed negative effects of MC on *S. quadricauda* growth in this study may be due to cellular oxidative stress and inhibition of growth enzymes induced by low MC concentrations (Babica, Bláha and Maršalek 2006). This impact on a common alga suggests that environmental MC concentrations below the WHO recommended safe levels for human health may affect freshwater phytoplankton during blooms. This finding agrees with the existing assumptions in literature that MC biosynthesis may be associated with its putative role in seasonal phytoplankton succession and cyanobacterial dominance in eutrophic waters (El-Sheekh, Khairy and El-Shenody 2010; Wang *et al.* 2017). Wang *et al.* (2017) demonstrated strong inhibitory effects of crude extract of *M. aeruginosa* from different growth phases on growth of *S. quadricauda* and other freshwater phytoplankton. Arguably, the observed growth inhibition in *S. quadricauda* might have been due to the presence of MCs as an important allelochemical in the extract treatment, although no reference was made to the role of MC in the study. El-Sheekh, Khairy and El-Shenody (2010), in a 14-day exposure, reported that higher MC concentration from exponential growth phase of *M. aeruginosa* resulted in strong inhibition of growth parameters in two green algae, *S. obliquus* and *Chlorella vulgaris*. Although these findings suggest MC can mediate allelopathic interspecific interactions between MC-producing *M. aeruginosa* and *S. quadricauda*

during toxic blooms, the mechanisms behind this allelopathy are not yet clear. However, certain contrasting evidence suggests that the postulated allelopathic role of MCs in interspecific competition among freshwater phytoplankton communities may be unlikely (Babica, Bláha and Maršalek 2006).

S. quadricauda has been shown to successfully co-exist with toxigenic *M. aeruginosa* during blooms (Babica *et al.* 2007; Mohamed 2008; Sedmak and Kosi 1998). Mohamed (2008) attributed this co-existence to the evolution of certain adaptive physiological mechanisms, such as detoxification and protective polysaccharides against MC-induced oxidative stress in *S. quadricauda*. Supporting this finding, Babica *et al.* (2007) observed no significant alteration in the growth rate of *S. quadricauda* exposed to environmentally relevant concentrations (1-10 µg/L) of MC-LR and MC-RR variants. On the other hand, Bittencourt-Oliveira *et al.* (2015) reported no significant effects of crude extract of MC-producing *M. aeruginosa* on growth rate of *S. acuminatus* at 5 and 10 µg/L treatment concentrations. These studies showed that laboratory findings on allelopathic effects of environmental MC concentrations on green algae may depend on the type of MC treatment, therefore highlighting the need for future studies to incorporate a broad range of experimental designs and treatments. Moreover, in this study, environmental concentrations of the crude extract treatment had no effects on growth and photosynthetic pigment contents of *S. quadricauda*. These results were possibly due to the composite nature of the crude extract treatment used in this study, suggesting that potential antagonistic interactions among heterogenous secondary metabolites present in the extract could have nullified the allelopathic effects of its MC content (Pereira *et al.* 2018). Besides, the purified toxin-induced increase in the photosynthetic pigment contents of *S. quadricauda* observed in this study may have resulted from hormetic overcompensation response to low environmental concentrations of the purified MC-LR as indicated earlier in this study (Calabrese 2008; Shi *et al.* 2016). It is also possible that such low concentrations might have induced and accelerated metabolic responses such as increased pigmentation in our focal species. Nevertheless, the present results suggested that stimulatory effects decreased with increasing toxin concentrations. Hence, MC at the range of concentrations tested in this study is unlikely to affect photosynthetic activities

and pigment contents in green algae. Therefore, this finding is in accordance with Pereira *et al.* (2018) who demonstrated no significant effects of crude extract treatment containing 55 and 150 µg/L of MC and cylindrospermopsin, respectively on chlorophyll-a and other pigment contents of the green alga, *Parachlorella kessleri*.

2.5.2 Effects of MC on Daphnia survival

I predicted that dissolved environmental MC concentrations (thought to be released as anti-grazing deterrent by cyanobacteria) would reduce survival among generalist zooplankton grazers. In contrast to this expectation, *D. pulex* survival was unaffected by purified MC-LR and crude extract treatments, whereas increased concentration of crude extract but not purified MC-LR strongly reduced *D. magna* survival. Purified MC-LR treatment at the range of concentrations tested in this study had no significant effect on survival of either *D. magna* or *D. pulex*. The observed tolerance to low MC-LR concentrations in our study could be attributed to increased secretion of detoxification enzymes, such as glutathione-S-transferase, to combat MC-induced oxidative stress at low exposure concentrations (Dao *et al.* 2018; Ortiz-Rodríguez, Dao and Wiegand 2012). Our present result is consistent with findings from previous studies (Chen *et al.* 2005a; Lürling and van der Grinten 2003), suggesting that low MC concentrations which typically reflect most frequently reported dissolved MC concentrations in freshwater bodies (Chen *et al.* 2017) may not affect individual survival and species abundance among *Daphnia* populations during blooms.

Survival of the two *Daphnia* species used in this study was unaffected by purified MC-LR at the range of concentrations tested during the 48-hour exposure. However, one cannot rule out more subtle effects of low MC concentrations, such as effects on development, growth and reproduction where animals are chronically exposed to toxic blooms in the field (Chen *et al.* 2017; Sarnelle, Gustafsson and Hansson 2010). For instance, an ecologically relevant concentration as low as 1 µg/L of MC in crude extract and purified toxin was associated with significant transgenerational effects on survival, growth, and reproduction of F_1 and F_2 generation offspring of *D. lumholtzi* (Dao *et al.* 2018). Therefore, I suggest that future studies should prioritize understanding sublethal

and chronic effects of low environmentally realistic MC concentrations on life history characteristics and fitness-related traits. Effects at such levels may have potential ecological implications on population dynamics, food web structure and functioning in blooming waters.

Contrary to the results of this study, some other studies have also argued that the observed reduction in *Daphnia* survival and fitness in several laboratory experiments may be linked to MC toxicity (DeMott, Zhang and Carmichael 1991; Lürling and van der Grinten 2003; Rohrlack *et al.* 1999). Cyanobacteria-zooplankton interactions represent an important ecological link between phytoplankton producers and consumers at higher trophic levels, driving carbon and energy transfer within aquatic systems (Ger *et al.* 2016b; Sarnelle, Gustafsson and Hansson 2010). These ecological interactions in freshwater bodies may become disrupted by MC toxicity during toxic blooms (Christoffersen 1996; Lindholm *et al.* 1992). However, current understanding on MC toxicity on zooplankton has been mainly drawn from empirical studies using either wild-type or mutant intact *Microcystis* cells containing high MC concentrations (Lürling and van der Grinten 2003; Rohrlack *et al.* 1999; Sarnelle, Gustafsson and Hansson 2010) or purified MC-LR at dissolved concentrations usually above what may be typically present in the environment (DeMott, Zhang and Carmichael 1991; Ghadouani *et al.* 2004). To date, studies demonstrating the impacts of MC toxicity on zooplankton populations under environmentally realistic conditions comparable to the dissolved MC concentrations in freshwater bodies are still relatively scarce. Therefore, to the best of my knowledge, the data presented here is among the first evidence reporting on the sensitivity of key *Daphnia* species to MC toxicity under a more environmentally realistic scenario.

As pointed out in previous studies (Jungmann 1992; Rohrlack *et al.* 2003; Smutná *et al.* 2014), the adverse effects of crude extract treatment observed on *D. magna* survival in this study may not be due to the presence of MC in the *Microcystis* extract treatment. Given the heterogeneous composition of the crude extract, the reduction in *D. magna* survival might have resulted from the synergistic effects of several other toxic secondary metabolites whose toxicity and chemical behaviour remain yet unravelled (Ger *et al.* 2016b; Jungmann and Benndorf 1994). Although t no specific information regarding the

chemical and biological characteristics of the suspected secondary metabolites is given in this study, there has been increasing interest in characterizing the potential toxicity and chemical behaviour of cyanobacterial secondary metabolites other than MCs (Beversdorf *et al.* 2018; Rohrlack *et al.* 2003). Janssen (2019) recently provided a detailed overview of the occurrence and toxicity of potential secondary metabolite candidates other than MCs in freshwater systems. Therefore, in this study, this data build on the existing knowledge that MC production in cyanobacteria may have evolved as a key anti-grazing chemical deterrent to zooplankton grazers. Moreover, these results also suggest that sensitivity among large-bodied zooplankton grazers to environmental MC concentrations may vary across closely related species within the same genera, depending not only on the exposure concentrations but on the MC treatment type.

2.5.3 Effects of environmental MC on amphipod survival

Contrary to the hypothesis of this study, environmental MC concentrations in the purified MC-LR and crude *Microcystis* extract treatments had no effects on the survival of native or invasive amphipod species. The observed tolerance to MC in this study could be due to low MC assimilation in amphipods (Consolandi, Ford and Bloor 2019), together with their robust antioxidant and detoxification strategies against MC-induced oxidative stress (Sroda and Cossu-Leguille 2011). This suggests that exposure to dissolved MC concentrations via the benthic food webs may potentially pose no significant threats to freshwater detritivores. However, their prolonged exposure to low environmental MC concentrations may potentially alter the dynamics of key ecosystem functions mediated by these organisms. These results are consistent with previous findings on the sensitivity of amphipods to cyanobacterial toxins in the existing literature (Korpinen, Karjalainen and Viitasalo 2006). For instance, Korpinen, Karjalainen and Viitasalo (2006) observed no effect of nodularin on survival of different life stages of the common littoral amphipod, *G. zaddachi*, but found reduced survival among adult gammarids exposed to intact toxic cyanobacterium, *Nodularia sphaerocarpa*. Similarly, exposure of benthic mayflies (*Hexagenia* spp.) to a range of MC concentrations (0.5-300 µg/L) did not affect the survival and growth of *Hexagenia* (Shahmohamadloo *et al.* 2020). The results of this

study suggest that both native and invasive amphipod populations are unlikely to be negatively affected by environmental MC concentrations during *M. aeruginosa* blooms in freshwater bodies.

Amphipod shredders, particularly gammarids, are a keystone component of the freshwater benthic food webs (Kunz, Kienle and Gerhardt 2010), driving the processing of allochthonous organic resources as a major source of carbon and energy for higher consumers in forested streams (Tank *et al.* 2010). Consequently, these organisms are among the most dominant macroinvertebrate communities in temperate freshwater bodies (Kunz et al., 2010). Several studies have evaluated differences in sensitivity to thermal and other environmental stressors among native and invasive amphipods (Boets *et al.* 2012; Bundschuh *et al.* 2013; Kenna *et al.* 2017; Sroda and Cossu-Leguille 2011). This evidence suggests that the invasive amphipod, *D. villosus*, has consistently shown a higher preference towards warmer temperatures (Kenna *et al.* 2017; Truhlar, Dodd and Aldridge 2014) and increased tolerance towards copper and pesticide pollution (Bundschuh *et al.* 2013; Sroda and Cossu-Leguille 2011). However, the sensitivity of gammarids to environmental MC exposure under the increasing incidence of cyanobacterial blooms in freshwater has remained largely unknown. Therefore, to the best of my knowledge, this current study, is one of the first evidence reporting on the sensitivity of native and invasive freshwater gammarids to environmentally realistic MC concentrations.

Moreover, the data presented in this study showed that environmental MC concentrations had no significant effects on survival among native and invasive amphipods. However, as Kratina et al. (2019) predicted, future climate change scenarios are expected to exacerbate vulnerabilities among key freshwater species to climate-driven multiple stressors. Therefore, it is probable that the abundance and key ecosystem function mediated by native amphipod species may become impaired under combined scenarios of climate warming and toxic *M. aeruginosa* blooms.

2.6 Conclusion

In this study, the results illustrate that environmentally realistic MC concentrations can affect sensitivity (including survival and allelopathic growth rate inhibition) among key freshwater food web components. However, while such effects may vary not only across closely related species but also across different trophic levels and MC treatments, we demonstrate how environmental MC concentrations that have been adjudged safe for human health can influence survival and abundance among key freshwater species. The findings here provide important insights into why environmental stressors at relatively low concentrations may elicit significant adverse effects on food web components. Furthermore, these results suggest that current regulatory guidelines and environmental risk assessment may not protect key food web components against cyanotoxin impacts during toxic freshwater blooms. Hence, I highlight the need to prioritize regular biomonitoring efforts to effectively manage the ecological impacts of toxic blooms on freshwater bodies.

Chapter 3

Low environmentally relevant microcystin concentrations altered sublethal endpoints but not survival among freshwater keystone species

3.1 Abstract

MC occurrence in freshwaters remains an emerging global environmental threat in the face of increasing incidence of cyanobacterial harmful algal blooms. MC has been shown to exert severe adverse effects on human health and increase the incidence of animal mortalities among fish, birds and zooplankton. However, the sublethal and chronic ecological effects of MC exposure on survival and fitness-related traits underpinning ecosystem functions among freshwater keystone populations are not fully known. Therefore, using a suite of sublethal and chronic toxicity tests, the present study evaluates the, population-level effects of two environmentally relevant MC treatments (purified MC-LR and crude extract of microcystin-producing *Microcystis* aeruginosa) on individual fitness-related endpoints among three ecologically important freshwater species, *Daphnia magna*, *Gammarus pulex* and *Dikerogammarus villosus*. Low environmental MC concentrations tested in this study had no effects on survival but altered a range of ecologically relevant sublethal responses across the three test species studied. Purified MC-LR increased the feeding rate and stimulated parameters of reproduction in *D. magna*. Non-monotonic responses were observed on the mean number of broods produced per female, mean number of neonates produced per female and the intrinsic rate of natural increase (r), resulting in the stimulation of the population growth rate. However, the somatic growth rate of daphnids was unaffected at the range of purified MC-LR concentrations tested in this study. Both treatments reduced the feeding rate but only the purified MC-LR reduced the growth rate of *D. villosus*. In contrast, neither treatment affected the feeding rate of *G. pulex*, but the crude extract treatment was associated with reduced growth rate among the native amphipods. Altogether, these results suggest sublethal and chronic exposure to low environmental MC concentrations

may induce subtle shifts on key ecological processes, including energy acquisition and biological fitness, thereby influencing population size, community structure and ecosystem functioning.

3.2 Introduction

Harmful cyanobacterial blooms are serious environmental and ecological threats worldwide, potentially releasing hazardous MCs and other cyanotoxins in freshwaters (Briland *et al.* 2020; Brooks *et al.* 2016; Zhu *et al.* 2021). Microcystin occurrence has become ubiquitous in freshwater bodies around the world (Pham and Utsumi 2018), with over 75% of cyanobacterial bloom incidences reported in 108 countries associated with the release of dissolved MC concentrations in freshwater systems (Harke *et al.* 2016). While this phenomenon has been primarily driven by the synergism between anthropogenic nutrient enrichment and climate change (Hall and Calandrino 2011; Glibert 2017), the persistence and toxicity of MC in water bodies (Zhu *et al.* 2021; Harke *et al.* 2016) have been widely correlated with the increasing expansion and incidence of microcystin-producing cyanobacterial blooms in freshwaters (de Figueiredo *et al.* 2004; Glibert 2017). Moreover, a growing body of evidence has linked the occurrence of dissolved MC concentrations in freshwaters with serious water quality issues (Brooks *et al.* 2016), severe adverse effects on human and animal health (Chen *et al.* 2009; Svirčev *et al.* 2019; Wood 2016), and potential threats for freshwater biodiversity and ecosystem integrity (Bukaveckas *et al.* 2017; Shahmohamadloo *et al.* 2020). However, little is currently known about the population-level ecological effects of MC exposure on freshwater biota and ecosystem functioning under environmentally realistic conditions (Ibelings *et al.* 2008; Ibelings and Havens 2008). One major reason associated with this limitation is the existing lack of accurate understanding of the typical exposure regime and what may be considered as environmentally relevant MC exposure concentrations in surface freshwaters (Ibelings *et al.* 2008; Skafi *et al.* 2021; Zhu *et al.* 2021).

MC exposures via intact toxic cyanobacterial cells (Ger and Panosso 2014), crude extract (Herrera, Echeverri and Ferrao-Filho 2015) or purified toxins (DeMott, Zhang and Carmichael 1991) have been shown to increase lethality and reduce survival among

freshwater species during acute toxicity experiments in the laboratory. Several studies have reported a wide range of median lethal concentrations (48hr-LC_{50}) for different zooplankton species exposed to purified MC-LR in the laboratory, such as 247 µg/L (*Daphnia lumholtzi*), 450 µg/L (*Diaptomus birgei*), 9,600 µg/L (*D. pulex*), 11,600 µg/L (*D. hyalina*) and 21400 µg/L (*D. pulicaria*) (Bui *et al.* 2020; DeMott, Zhang and Carmichael 1991). Moreover, 48-hr LD_{50} or 48-hr effective concentration (EC_{50}) values reported for *Daphnia* species exposed to crude extract of microcystin-producing *Microcystis* in the literature ranged from 36,000 to 162,450 µg/L DW for *D. pulicaria* (Jungmann and Benndorf 1994; Sotero-Santos *et al.* 2006; Okumura *et al.* 2007), 34 to 1906 µg/g DW and 84 to 2,550 µg/L for *D. similis* and *D. magna* respectively (Smutná *et al.* 2014). However, the range of MC concentrations reported in these studies are rarely found in natural waters, as dissolved concentrations detected in freshwaters typically range from tens of ng/L to a few µg/L levels (Chen *et al.* 2013; Zhu *et al.* 2021). Therefore, short-term laboratory exposures to high MC concentrations are unlikely to represent environmentally realistic exposure conditions that freshwater organisms may chronically and repeatedly encounter for a greater part of their life cycles during harmful blooms (Chen *et al.* 2017; Wei *et al.* 2020).

Increasing evidence from laboratory studies has shown that assessing chronic effects of MC exposure on sublethal endpoints, such as survival, growth and reproduction may be of more ecological relevance than acute lethal effects (Ibelings and Havens 2008; Chen *et al.* 2005b). Chronic tests are a superior ecotoxicological assays that can integrate lethal and sublethal effects into a single, reliable measure of reproductive success, known as the intrinsic rate of natural increase (r) (Villarroel *et al.* 2003). Therefore, chronic toxicity studies may reflect long-termer ecologically relevant effects of sublethal MC concentrations on fitness components, such as individual survival, feeding, growth and reproduction (Ibelings *et al.* 2008; Billoir, Péry and Charles 2007). These effects may become manifest at relatively low exposure levels, which are usually several orders of magnitude lower than those reported as lethal concentrations (LC_{50}) (Billoir, Péry and Charles 2007). Hence, the ecological consequence of sublethal MC exposure on individuals may likely affect population size among sensitive species, thereby resulting

in long-term alterations of community structure and ecosystem functioning (Nilsen *et al.* 2019; Burkholder, Shumway and Glibert 2018). A considerable number of studies have shown evidence of chronic toxicity on life-history traits among zooplankton exposed to varying MC concentrations (Lürling and van der Grinten 2003; Dao, Do-Hong and Wiegand 2010; Smutná *et al.* 2014; Liang *et al.* 2020). However, only a few studies have demonstrated the effects of chronic MC exposure on important keystone species across different compartments of the freshwater food webs at low environmentally relevant concentrations (Duc *et al.* 2020).

Aquatic keystone species, especially freshwater microcrustacean zooplankton, *Daphnia magna*, the UK native amphipod, *Gammarus pulex* and the invasive killer shrimp, *Dikerogammarus*, represent important components of the food webs, capable of influencing community structure and ecosystem functions (De Castro-Català *et al.* 2017; Sperfeld and Wacker 2009; Rewicz *et al.* 2014). *D. magna* is a generalist pelagic filter-feeder, regulating phytoplankton biomass and providing carbon and energy for consumers at higher trophic levels (Sarnelle, Gustafsson and Hansson 2010). The native amphipod shredder, *G. pulex* is an ecologically important benthic detritivore in forested freshwater streams, driving allochthonous organic-matter processing, a major ecological process and responsible for carbon and energy transfer among different food web components (Kenna *et al.* 2017; von Schiller *et al.* 2017). *D. villosus*, a Ponto-Caspian amphipod crustacean has recently expanded its geographical range and invaded many freshwater bodies across Europe (Rewicz *et al.* 2014). The successful colonization of this generalist amphipod predator in temperate freshwaters has been associated with the ability to maintain normal metabolic activities under intense climatic conditions and switch feeding modes (Kenna *et al.* 2017). These species have been widely employed in many ecotoxicological studies on different environmental stressors (De Castro-Català *et al.* 2017; Kratina *et al.* 2019) and may serve as reliable experimental models to evaluate chronic effects associated with environmental MC exposure (De Castro-Català *et al.* 2017; Kenna *et al.* 2017; Rivetti, Campos and Barata 2016). Hence, to predict the ecological impacts of microcystin on freshwater food webs, it is important to understand how keystone species capable of

structuring freshwater communities may respond to chronic exposure to low environmentally relevant microcystin concentrations.

In this study, I tested sublethal and chronic effects of low range of environmentally relevant MC concentrations (1-10 µg/L), comparable to the WHO recommended guidelines on population-level fitness-related traits across three ecologically important freshwater keystone species, occupying different compartments of the freshwater food web (*D. magna*, *G. pulex* and *D. villosus*). Using a combination of purified MC-LR and crude *Microcystis* extract treatments, I performed a series of sublethal and chronic toxicity assays to test the following two hypotheses. Firstly, in accordance with increasing evidence linking low dose exposure to chemical stressors (such as pesticides and pharmaceuticals) below permissible safe thresholds with adverse effects on chronic and sublethal endpoints in zooplankton and amphipods (Beausoleil *et al.* 2013; Rivetti, Campos and Barata 2016; De Castro-Català *et al.* 2017), I predicted that environmentally relevant concentrations of the purified MC-LR will have negative effects on survival, growth and reproduction of *D. magna*. Secondly, I expected that the subtle stress associated with environmental concentrations of both purified MC-LR, and crude *Microcystis* extract treatments would impose higher metabolic costs and energy demands on *D. magna*, *G. pulex* and *D.* villosus, thereby leading to increased feeding rates as a compensatory response.

3.3 Materials and Methods

3.3.1 Test chemical

The commercial microcystin toxin, subsequently referred to in this study as purified MC-LR (CAS No. 101043-372-2 and purity ≥ 95%), was purchased from the Cayman Chemical Company in the UK. Experimental aliquots were made by serial dilutions from the original stock of 1mg/L prepared in line with the manufacturer's guidelines. Briefly, 1 mg of the purified toxin from the manufacturer was dissolved into 1mL of methanol (anhydrous, 99.8% Sigma-Aldrich) and graduated up to 1 litre with Milli Q water. Working concentrations of the purified MC-LR for this study were prepared through

serial dilutions in the magnitude orders of 10 to obtain the nominal concentrations of 100, 10, 1 and 0.1 µg/L MC-LR used in this study. All aliquot solutions of the purified MC-LR were immediately stored at -20°C until the start of the experiments. Methanol and other chemical reagents used to prepare phytoplankton and zooplankton culturing media were of the highest commercial grade (purity ≥ 95%). Enzo Life Sciences manufactured and supplied MC (Adda-specific) ELISA kits (CAT No. ALX-850-391-KI01) used for MC assays in this study.

3.3.2 Test organisms

3.3.2.1 Algae and cyanobacteria

A toxin-producing strain of the cyanobacterium *Microcystis aeruginosa* (CCAP/1450/16) was obtained from the Culture Collection of Algae and Protozoa (CCAP) at the Scottish Marine Institute, Scotland. While a stock culture of the green alga, *Scenedesmus quadricauda* (A950) was purchased from Sciento Stores Manchester, UK. These organisms were individually cultivated as continuously growing cultures in the laboratory in 250 mL Erlenmeyer flasks comprising of 200 mL sterile BG 11 culture medium. The BG-11 culture medium used in this study was prepared in accordance with the recipes provided by CCAP. *Microcystis* cultures were maintained at constant temperature 25±1°C, light intensity 25µmol quanta $m^{-2}s^{-1}$ and 12 h: 12 h light/dark cycle in a shaking incubator. *Scenedesmus* cultures were cultivated in a controlled temperature (CT) room conditioned at 21±1°C and 25µmol quanta $m^{-2}s^{-1}$ with manual shaking twice daily. To estimate the cell growth rates in both cultures, the daily cell biomass increase was monitored microscopically under a compound light microscope with the aid of a Sedgewick-Rafter counting chamber and spectrophotometrically by taking the daily measurement of the optical density at 680 nm. A simple linear regression line each, was fitted to model the relationship between daily microscopic counts and spectrophotometric readings for *S. quadricauda* (R = 0.97) and *M. aeruginosa* (R = 0.97). *Microcystis* cultures were harvested at the stationary growth phase (biomass concentration, 5.0×10^5 cells/mL), freeze-thawed thrice to lyse the cells and release their intracellularly bound MC contents. The total MC concentration in the laboratory-grown cyanobacterial culture

was quantified using a commercial MC (Adda-specific) ELISA kit and expressed as MC-LR in µg/L. Moreover, water bloom samples collected during summer (July/August 2019) from Grafham waters in Cambridgeshire, UK (52°180′N; 0°190′W) were centrifuged and analysed for congener specific MC content using LC-MS techniques. However, experiments done with the bloom samples could not be completed; hence, they were not reported here due to COVID-19 pandemic disruptions.

3.3.2.2 *Daphnia magna*

Start cultures of the cladoceran zooplankton, *Daphnia magna* were obtained from Sciento Stores in Manchester, UK and maintained for three weeks under constant laboratory conditions at 21±1ºC and 16 h:8 h light/dark cycle. Adult *D. magna,* (15) individuals per container were cultured in 500 mL beakers containing 300 mL of a modified artificial media for zooplankton "*Aachener Daphnien Medium*" (ADaM) (Klüttgen *et al.* 1994). In addition, 10 mL of *S. quadricauda* culture (5.0x 10^6 cells per mL) were added daily to serve as a food source, while the culture medium was renewed every three days. The number of neonates and broods produced were enumerated daily, neonates produced at the first brood were discarded while only neonates at the second brood were used in this study.

3.3.2.3 Amphipods

Two amphipod species, the UK native, *Gammarus pulex* and the invasive killer shrimp, *Dikerogammarus villosus* were collected from the Meanwood Beck, Meanwood Road, Leeds, West Yorkshire (53°500′N; 1°350′W) and Grafham Water, Cambridgeshire, UK (52°180′N; 0°190′W) respectively in March 2019 (Kenna *et al.* 2017). *G. pulex was* sampled with the aid of a pond net, while *D. villosus* individuals were dislodged from an artificial attachment that served as a refuge. Each species was collected in separate cool boxes containing stream water and acclimatized separately for seven days in aerated 2 L-plastic tanks (22 cm×16 cm×9 cm) filled with aged, dechlorinated tap water and glass pebbles. Animals were fed *ad libitum* with air-dried alder leaves (*Alnus glutinosa*) and conditioned at 15±1ºC and 14:10 h light/dark cycle in a CT room, while the culture medium was renewed every 72 hours. Before the experiment, body length (mm) and wet

weight (mg) for each animal were measured using a stereomicroscope and weighing balance. Animals were later sorted into new tanks based on body length size and starved for 24 hrs before the experiment. *G. pulex* (mean body length = 13.70±1.33 mm (SE); mean wet weight; 29.60±9.08 mg) and *D. villosus* (mean body length = 15.07±1.27 mm; mean wet weight; 41.91±8.84 mg) individuals within relatively close size range were used for the amphipod feeding experiment. Alder leaves used for this experiment were collected during leaf fall in autumn, air-dried and stored in cardboard boxes in the laboratory. In preparation for this experiment, dried alder leaves were conditioned at 15ºC in a CT room for 14 days in stream water to facilitate microbial activities and leaching of soluble secondary metabolites (Bloor 2011). Afterwards, conditioned leaves were cut into small discs using a 6mm-diameter cork-borer, air-dried, weighted and stored in small envelopes. To increase leaf palatability, discs were reconditioned in aged, dechlorinated tap water for 24 hours prior to the feeding experiment (Kenna *et al.* 2017).

3.3.3 Experimental Design

3.3.3.1 Rationale

The sub-lethal effects of environmentally relevant concentrations of the purified MC-LR (0.01, 0.1, 0.5, 1 and 10 µg/L) with the control were tested on survival, feeding, growth and reproductive parameters of *D. magna* in a 21-day chronic exposure in line with OECD recommended guidelines (OECD 2008). These sets of experiments were conducted consecutively between March and May 2019. Five purified MC-LR concentrations and three concentrations (0.01, 0.1, 0.5 µg/L) of the crude extract treatment, together with the control, were also tested on survival, feeding and growth rates of *G. pulex* and *D. villosus* during a 96-hr feeding exposure. These treatment concentrations were selected to test the sublethal and chronic effects of low environmental MC levels, comparable to the WHO recommended permissible guidelines of no more than 1 µg/L and 10 µg/L MC levels in drinking and recreational freshwater bodies, respectively on freshwater species (Chorus *et al.* 2000). In my previous experiments, this range of concentrations and treatments was associated with low lethal toxicity on 48-hour survival of *D. magna* and 96-hour survival of amphipods in the laboratory. However, mortalities among organisms exposed to the

treatment concentrations were less than 50%; as a result, MC toxicity on test organisms could not be estimated as median lethal concentration (LC_{50}) used in toxicity studies. Hence, only the sub-lethal effects of MC treatment concentrations on the test organisms are reported in this study.

3.3.3.2 Chronic toxicity test in *Daphnia magna*

Fifteen (15) *D. magna* neonates from the second brood (<24 hours old) were randomly selected from the laboratory raised culture and individually assigned to each treatment concentration of purified MC-LR in 100 mL cups containing 50 mL of ADaM medium. The control was made up of the same experimental conditions, but without MC treatment and this was replicated fifteen times. In all, seventy-five (75) replicates, comprising of the control and purified MC-LR treatments, were assigned one neonate per cup and fed with *Scenedesmus* at a concentration of 5.0×10^6 cell/mL daily during the experiment. The water quality in each replicate was maintained by transferring *Daphnia* neonate individually into a new cup containing fresh ADaM medium every 72 hours. This experiment was set up for three weeks in a controlled temperature room, conditioned at 21±1ºC constant temperature and illuminated with white fluorescent bulbs at 16:8h light/dark cycle. The effects of MC treatment on the survival, growth, and reproduction of daphnids were monitored and recorded daily. In contrast, effects on survival were estimated from the daily mortality of daphnids across all the replicates during the experiment. Parameters such as time to first reproduction, the total number of neonates per female and number of broods per female, and the mean brood size per female were estimated to evaluate the effect of treatment on reproductive success (Sancho *et al.* 2018). The number of neonates produced per female was monitored, counted and new neonates were discarded daily. Daphnid growth was estimated by measuring the initial body length (l_i) and the final body length (l_f) of each daphnid from the apex of the helmet to the base of the tail spine, using a stereoscopic microscope with ocular rule (Nikon SMZ1500) at the beginning (day 0) and at the end of the experiment (day 21) respectively. The growth rate (GR) for day *n* was determined using the equation below.

$$\text{Growth rate } (GR_n) = \frac{ln(l_f) - ln(l_i)}{t_f - t_i} \quad \text{Equation 3}$$

Where l_f and l_i are the final and the initial body length of daphnids (in mm) at day 0, and day 21 respectively while t_f and t_i represent the final and the initial times in days.

The intrinsic rate of natural increase (r) was calculated and used to estimate the population-level effects on daphnids using the Euler-Lotka equation (Lotka 1913)

$$\sum_{x=0}^{n} l_x m_x e^{-rx} = 1 \quad \text{Equation 4}$$

Where l_x represents the proportion of individuals surviving to age x, m_x is the age-specific fecundity (number of neonates produced per surviving female at age x) and x is days.

3.3.3.3 Feeding rates in *Daphnia*

A 24-hour feeding inhibition assay was conducted in line with the procedures described in Agra *et al.* (2010), to test the effects of low environmentally relevant MC concentrations on the feeding rates of *D. magna*. Five (5) 4-day old juvenile daphnids per replicate were exposed to the same experimental conditions described above for the chronic toxicity experiment. This assay was carried out in 50 mL plastic cups containing 20 mL of ADaM medium, treatment concentrations and a food source. Each treatment concentration had five replicates (with both algae and daphnids) and five blank replicates (with algae but without daphnids) as control. Both treatment and control replicates were seeded with 5 mL of the green alga, *Scenedesmus quadricauda* (2.0×10^6 cell/mL) as food before the start of the experiment. To avoid increase in the algal biomass, the experiment was conducted in the dark at 21+1°C and daphnids were allowed to feed for 24 hours after which the experiment was terminated. At the end of tests, daphnids were carefully removed and cups were vigorously shaken before taking the spectrophotometric optical density (OD) at 680 nm. The concentration of algal biomass (number of cell/mL) was estimated from the standard calibration curve of linear regression between the OD_{680} values of *Scenedesmus* and the cell number. (R^2 = 0.97). The change in algal biomass concentration at the end of the experiment was used to estimate the individual feeding rate of daphnids (F), as described in Jesus, Martins and Nogueira (2014) using the equation below.

$$F = \frac{V(C_o - C_t)}{tN}$$ Equation 5

Where F is the feeding rates of single individual; V is the volume of medium (mL); Co is the algal biomass concentration (in number of cells/mL) of the blank (without daphnids); Ct is the final algal biomass concentration in the treatment (in number of cells/mL); t is time (hours) or the duration of the experiments and N, is the number of daphnids per replicate.

3.3.3.4 Amphipod feeding experiment.

One gammarid per species per replicate was individually assigned to the same range of crude extract and purified MC-LR treatment concentrations tested in the *Daphnia* experiments. The 96-hour amphipod feeding experiment was set up in 150 mL-plastic cups filled with 100 mL solution of the treatment and aged dechlorinated tap water in accordance with standard methods (Consolandi, Ford and Bloor 2019). The experimental conditions in each treatment concentration and control were made in ten replicates. Each replicate was furnished with four glass pebbles and four pre-weighed leaf discs to provide refuge and food source, respectively for the animal. The control replicates were made up of the same experimental conditions as the treatment but without MC. This experiment was incubated at 15°C and 14:10 h light/dark cycle for 96 hours in an incubator and every 24 hours, the number of survivors per species per treatment concentrations were monitored in each replicate till the end of the experiment. Animals that died before and at the end of the experiment were immediately removed and kept in 70% ethanol, while the remaining leaf discs were oven-dried at 60°C for 2hours and weighed to estimate the amount of leaf consumed by each animal. The final wet weight, W_f (mg) of each surviving animal, was measured and the growth rate for each animal was estimated as the difference between the final and initial wet weight per animal, per day at the end of the experiment. The amount of leaf consumed was estimated as the difference between the initial air-dried and the final oven-dried leaf weight (mg), while the shredding (feeding) rates, *FR*, of individual animals per day was calculated as expressed in equation (7). Where W_1, is the initial air-dried weight leaf disc (mg), W_2, is the final oven-dried weight of the leaf disc

remaining after 4 days (mg) and *W* is the wet weight of gammarids at the beginning of the assay (mg) (Consolandi, Ford and Bloor 2019; Maltby *et al.* 2002).

$$\text{Growth rate } (GR_n) = \frac{ln(W_f)/\ln(W)}{t_f - t_i} \quad \text{Equation 6}$$

$$Feeding\ Rate\ (FR) = \frac{W_1 - W_2}{W \times 4} \quad \text{Equation 7}$$

3.3.4 Data Analysis

Survival of *D. magna, G. pulex* and *D. villosus* exposed in the treatment and control groups were analysed for significant difference by fitting Cox proportional hazard (CPH) models in *survival* (Therneau 2015; Therneau and Grambsch 2000) and s*urvminer* (Alboukadel, Marcin and Przemyslaw 2019) packages in *R*. Survival proportions of animals in each treatment concentration and control were estimated and their associated 95 % confidence intervals (CI) were obtained using the *PropCIs* package (Scherer 2018) in R. All reproductive parameters measured during the chronic test in *D. magna* (date to first brood, brood size, number of broods produced per female, number of neonates per female and longevity) were expressed as integers. However, because of zero-inflation observed in the dataset, hypothesis testing for significant differences between the treatment and the control groups were conducted by fitting a generalized linear model (GLM) with negative binomial family error distribution in *MASS* package in R (Venables and Ripley 2002). All feeding and growth rates data were checked for normality assumptions using Shapiro-Wilk test and outliers were carefully removed where necessary before GLM analyses were conducted. All statistical analyses were performed at significance level of ($p \leq 0.05$) using R 3.6.0 (R CoreTeam 2019).

3.4 Results

3.4.1 Feeding, growth and reproduction in *D. magna*

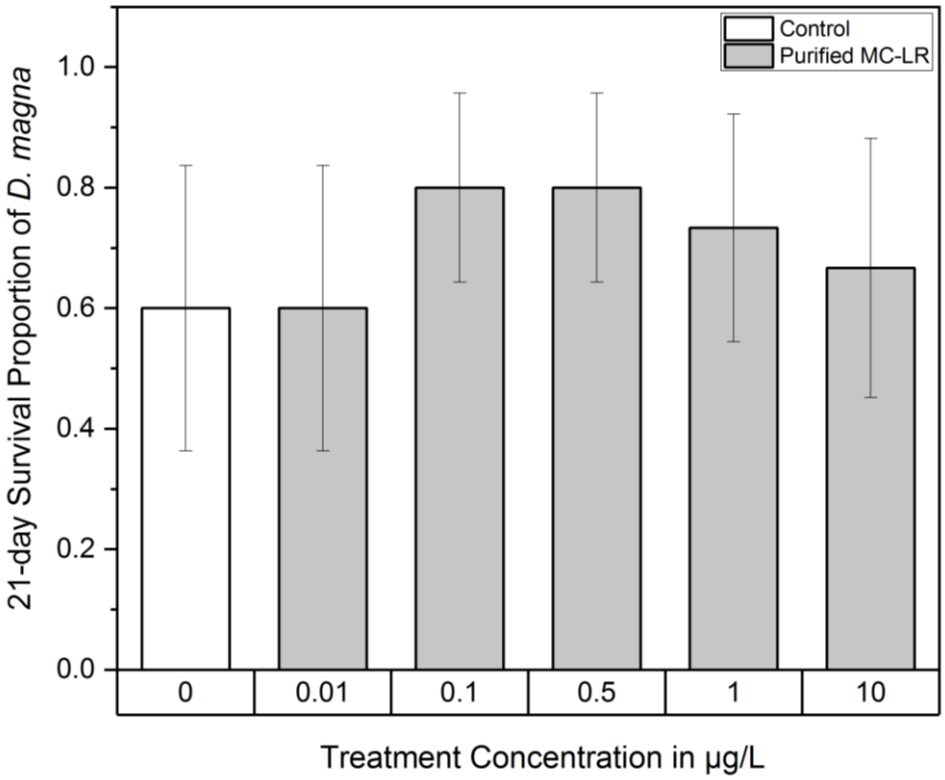

Figure 3.1: Survival Proportion of *D. magna* after 21-day chronic exposure to environmentally relevant purified MC-LR concentrations (error bars represent mean ± 95% confidence intervals and N= 15).

Mortalities of *D. magna* individuals were observed in the control and across all the treatment concentrations (Figure 3.1). Survival of *D. magna* in the control decreased from 100% at the beginning to 60% at the end of the 21-day exposure (Figure 3.1). This decline observed in survival of animals in the control was possibly due to the stress from the long duration of exposure. The purified MC-LR treatment (p = 0.21) and toxin concentration (p = 0.62) showed no statistically significant effect on the survival of *D. magna* when compared to the control at the end of the 21-day chronic exposure (Table 3.1; Figure 3.1).

Similarly, purified MC-LR (p = 0.54) and toxin concentrations (p = 0.15) did not affect the growth rate of *D. magna* relative to the control at the end of the experiment (Table 3.3; Figure 3.2).

Purified MC-LR concentrations demonstrated evidence of non-monotonic effects on different parameters of reproduction measured in *D. magna* (Table 3.2). Although onset of reproduction was slightly prolonged at the lowest concentration (0.01 µg/L), there was a significant decrease and increase on the date to first reproduction at the intermediate and highest concentrations, respectively (p< 0.05; Table 3.2). Such that, the mean date to first brood increased from 15.17 days in the control to 16.25 (18%), 18.00 (19%) and 17:00 (12%) days at 0.01,1 and 10 µg/L MC-LR, respectively. However, onset of reproduction was significantly reduced to 13.55 days (11%) at the intermediate concentration 0.5 µg/L (p< 0.05; Table 3.2).

The mean brood size of *D. magna* across the purified MC-LR treatment concentrations tested in this study did not differ significantly from the control (p< 0.05; Table 3.2). Whereas intermediate concentrations of the purified MC-LR (0.1 and 0.5 µg/L) were associated with increased mean number of broods produced per female, no significant effects were found at lower (0.01µg/L) and higher concentrations (1 and 10 µg/L) relative to the control. There was a non-linear increase in the effect of MC-LR concentrations on the total number of neonates produced per female compared to the control, such that only medium (0.5 µg/L) and (high 10 µg/L) purified MC-LR concentrations significantly increased the fecundity of *D. magna* (p < 0.05; Table 3.2).

The intrinsic rate of natural increase (r) values for *D. magna* exposed to purified MC-LR treatment concentrations in this study showed a significant non-monotonic increase compared to the values in control (p < 0.05; Table 3.2). In addition, the mean calculated r values slightly reduced at the lowest concentration of the purified MC-LR (0.01 µg/L), but significantly increased non-linearly with increasing MC-LR concentrations relative to the control (p< 0.05; Table 3.2), suggesting a toxin-induced increase in the population growth.

Purified MC-LR (p = 0.002) and crude *Microcystis* extract treatments (p = 0.007) significantly increased the feeding rate of *D. magna* relative to the control (Table 3.3; Figure 3.3). However, effects of treatment concentrations on the feeding rate of *D. magna* were not significantly different from the control (p = 0.312; Table 3.3; Figure 3.3).

3.4.2 Survival, feeding and growth in Amphipods

No significant effects of the purified MC-LR and crude *Microcystis* extract treatments were observed on the survival of *G. pulex* and *D. villosus* (Figure 3.4A-B; Table 3.1). Besides, the range of concentrations tested had no significant effects on the survival of *G. pulex* and *D. villosus* relative to the control (Figure 3.4A-B; Table 3.1). However, crude *Microcystis* extract (p = 0.046; Figure 3.4A) and the range of concentrations (p = 0.011; Figure 3.6A) tested in this study significantly reduced the mean growth rate of *G. pulex* compared to the control (Table 3.3). Whereas purified MC-LR (p = 0.012; Figure 3.6B) significantly reduced mean growth rates of *D. villosus*, however, no significant effect of the treatment concentration (p = 0.939; Figure 3.6B) was observed relative to the control (Table 3.3). Interestingly, increased purified MC-LR concentrations consistently increased mass loss in *G. pulex*, but were associated with a steady increase in the growth rate of *D. villosus* until there was a decline at higher concentrations.

Purified MC-LR (p = 0.044) and crude *Microcystis* extract (p = 0.002) significantly reduced the feeding rate of *D. villosus* relative to the control (Figure 3.5; Table 3.3). Neither treatment significantly affected the feeding rate of *G. pulex*, however there was a significant effect of treatment concentration on the feeding rates of *G. pulex* relative to the control (t = 2.58; df = 88; p = 0.01; Figure 3.5; Table 3.3).

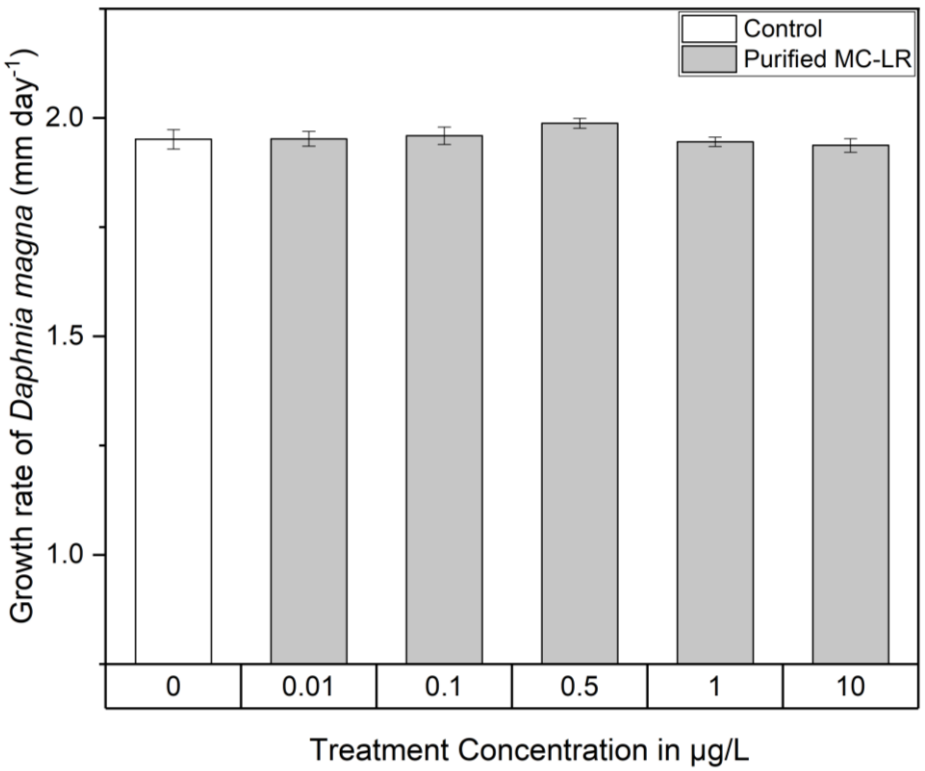

Figure 3.2: Mean growth rate of *D. magna* (per day) exposed to environmentally relevant concentrations of purified MC-LR (error bars represent mean ± standard error of means (SE) and N= 15). Significant levels at ($p < 0.05$) when compared with the control.

Table 3.1: Summary of Cox proportional hazard models with their associated parameter estimates, standard errors (SE), Wald statistic, 95% confidence intervals (95% CI) and p-values of the effects of purified MC-LR on survival of a zooplankton (*D. magna*) and two amphipod species. (*G. pulex* and *D. villosus*).

Species	Variable	Beta	SE	HR (95% CI)	Wald statistic	P-value
D. magna	MC-LR	-0.60	0.48	0.55 (0.21-1.41)	0.55	0.21
	Concentration	0.03	0.05	1.03 (0.92-1.14)	1.03	0.62
G. pulex	Extract	0.27	0.80	1.3 (0.27-6.30)	0.34	0.74
	MC-LR	1.36	0.75	3.89(0.90-16.82)	1.82	0.70
	Concentration	-0.15	0.08	0.86(0.73-1.00)	-1.87	0.06
D. villosus	Extract	-0.005	1.15	1.00 (0.10-9.57)	-0.004	0.99
	MC-LR	0.58	1.10	1.80 (0.22-14.79)	0.55	0.85
	Concentration	-0.07	0.11	0.93 (0.75-1.16)	-0.64	0.80

Table 3.2: Reproductive parameters of *D. magna* after 21-day exposure to environmentally relevant concentrations of purified MC-LR (values are mean ± standard error of means and significant level at $p < 0.05$).

MC-LR (µg/L)	Days to first brood	Brood size	No. of brood/female	No. of neonate/female	Longevity (days)	Intrinsic rate (r)
Control	15.17±0.55	2.67±0.51	1.17±0.11	3.67±1.09	14.20±2.19	0.03±0.00
0.01	16.25±0.61*	2.00±0.30	1.25±0.13	2.25±0.25	12.87±2.11	0.01±0.00*
0.1	14.89±0.68	3.17±0.52	1.67±0.12*	5.56±1.04	18.87±1.39	0.12±0.00*
0.5	13.55±0.47*	5.36±0.85	1.64±0.13*	8.45±1.36*	19.93±0.80*	0.15±0.00*
1	18.00±0.66*	5.60±2.25	1.20±0.12	5.80±2.22	17.53±1.80	0.08±0.00*
10	17.00±0.56*	4.89±0.75	1.44±0.14	7.56±1.40*	17.47±1.85	0.12±0.00*

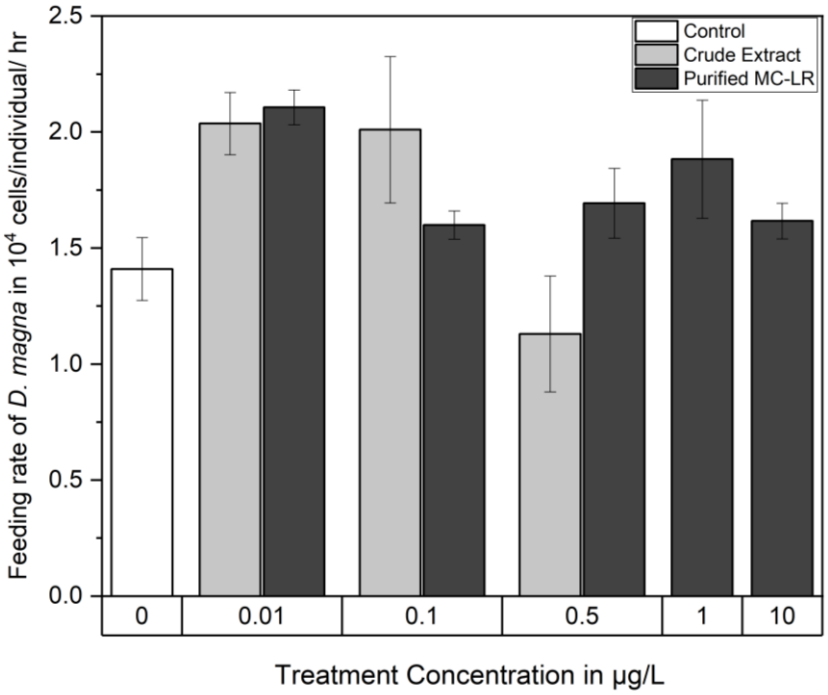

Figure 3.3: Feeding rates of *D. magna* exposed to two treatments of environmental MC concentrations (error bars represent mean ± standard error of means (SE) and N= 5). Significant levels at ($p < 0.05$) when compared with the control.

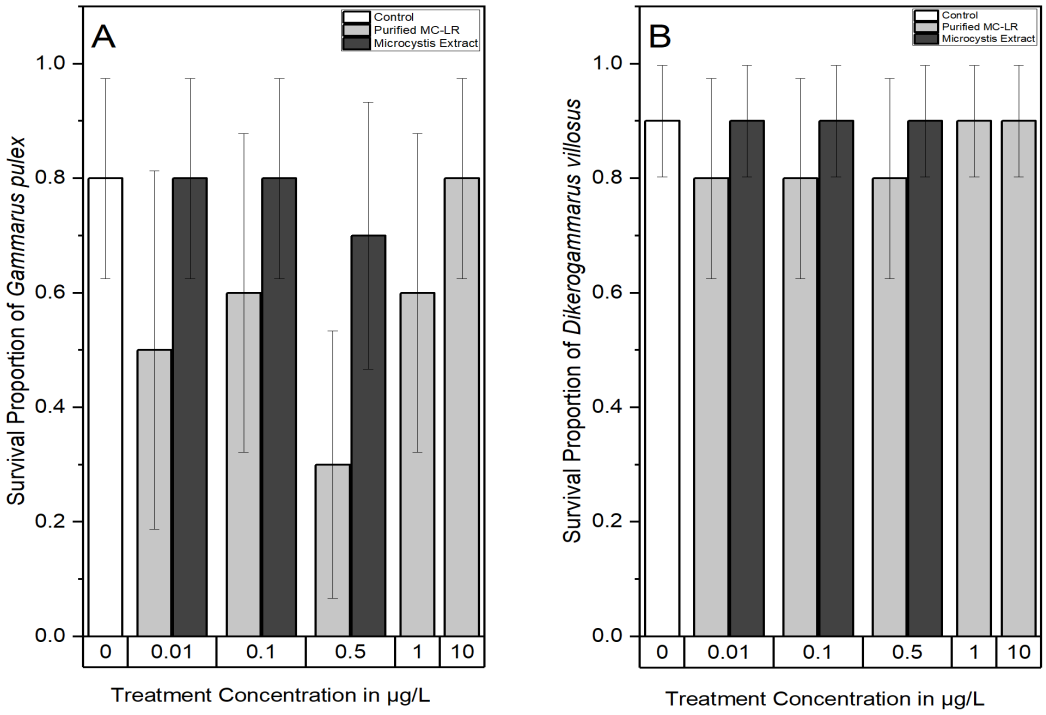

Figure 3.4: Survival Proportion of two amphipod species (A) *G. pulex* and (B) *D. villosus* exposed to sublethal concentrations of the purified MC-LR and crude *Microcystis* extract treatments for 7 days (error bars represent mean ± 95% confidence intervals and N= 10).

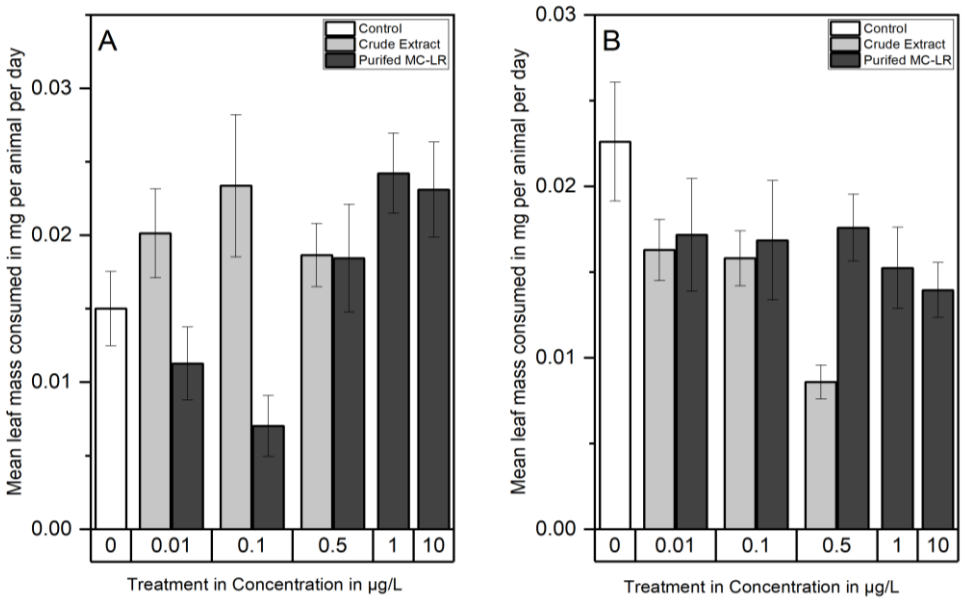

Figure 3.5: Mean leaf mass consumed (in mg per animal per day) by; (A) the UK native amphipod species (*G. pulex*) and (B) the invasive species (*D. villosus*) after a 7-day exposure to environmentally relevant concentrations of purified MC-LR and crude *Microcystis* extract (error bars indicate mean ± standard error of means and N= 10).

Table 3.3: Summary GLM parameter estimates with associated standard errors (SE), t-values, and p-values of purified MC-LR and crude Microcystis extract treatments and concentration on the feeding and growth rates of *D. magna*, *G. pulex* and *D. villosus* relative to the controls (Note that the p-values in bold are significant if $p < 0.05$).

Species	Response	Parameter	Estimate	SE	t-value	p-value
D. magna	Feeding rate	Intercept	1.038	0.210	4.95	<0.001
		MC (Extract)	0.692	0.242	2.86	0.007
		MC (Purified)	0.800	0.237	3.38	0.002
		Concentration	-0.025	0.024	-1.03	0.311
	Growth rate	Intercept	1.952	0.018	107.87	<0.001
		MC (Purified)	0.012	0.020	0.62	0.539
		Concentration	-0.003	0.002	-1.47	0.146
G. pulex	Feeding rate	Intercept	0.119	0.013	9.49	<0.001
		MC (Extract)	0.019	0.015	1.34	0.183
		MC (Purified)	-0.004	0.014	-0.31	0.757
		Concentration	0.004	0.002	2.58	0.012
	Growth rate	Intercept	0.532	0.370	1.44	0.155
		MC (Extract)	-0.885	0.436	-2.03	0.046
		MC (Purified)	0.455	0.419	1.09	0.281
		Concentration	-0.112	0.043	-2.60	0.011
D. villosus	Feeding rate	Intercept	0.023	0.002	9.18	<0.001
		MC (Extract)	-0.009	0.003	-3.16	0.002
		MC (Purified)	-0.006	0.003	-2.05	0.044
		Concentration	-0.0003	0.0003	-1.15	0.253

Growth rate	Intercept	0.311	0.245	1.27	0.208
	MC (Extract)	-0.030	0.287	-1.04	0.300
	MC(Purified)	-0.720	0.280	-2.57	0.012
	Concentration	-0.002	0.029	-0.08	0.939

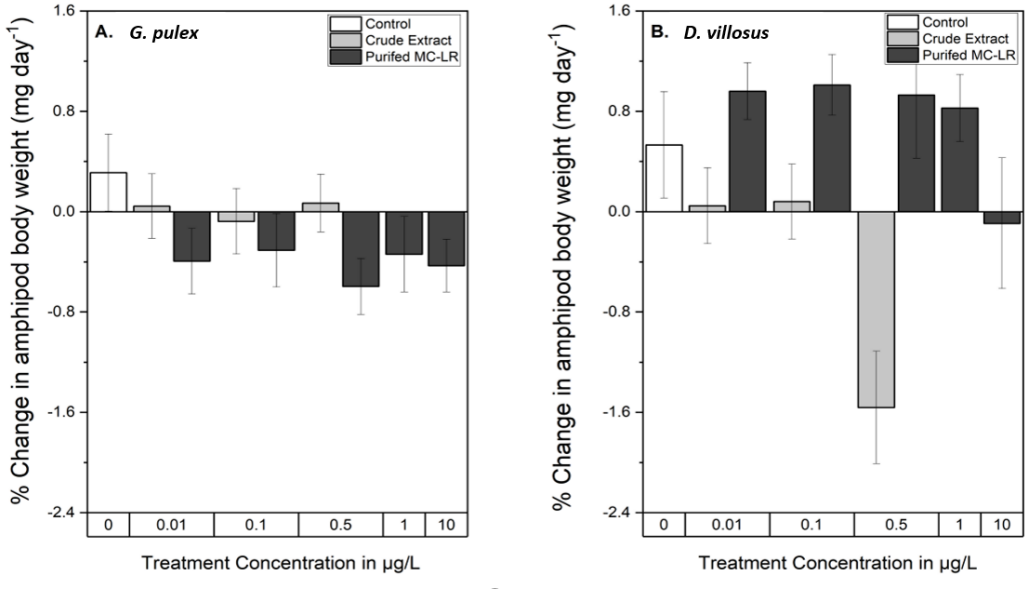

Figure 3.6: % change in amphipod body weight (in mg per day) by; (A) the UK native amphipod species (*G. pulex*) and (B) the invasive species (*D. villosus*) after a 7-day exposure environmentally relevant concentrations of purified MC-LR and crude *Microcystis* extract (error bars indicate mean ± standard error of means and N= 10).

3.5 Discussion

Previous studies have shown that laboratory exposures to high MC concentrations can reduce individual survival and impair physiological fitness among freshwater zooplankton during acute and sublethal toxicity tests (DeMott, Zhang and Carmichael 1991; Bui *et al.* 2020; Chen *et al.* 2005b). However, such short-term exposures to high MC concentrations are unlikely to represent typical environmentally realistic scenarios that freshwater organisms chronically and repeatedly encounter during harmful blooms for a greater part of their life cycles (Chen *et al.* 2017; Wei *et al.* 2020). In this study, I tested for the first time, the hypothesis that sublethal and chronic exposures to a range of low environmentally relevant MC concentrations will affect survival and sublethal population-level responses in three important freshwater keystone species (*D. magna, G. pulex* and *D. villosus*). I demonstrate that, low environmental MC concentrations had no effects on survival but altered a range of ecologically relevant sublethal physiological responses across the three test species. I also illustrate that, environmentally relevant concentrations of the purified MC-LR increased the feeding rate of *D. magna* and induced non-monotonic increase on the intrinsic rate of natural increase and other indices of reproduction, suggesting a population growth stimulation, but the individual growth rate of daphnids was unaffected by MC-LR concentrations. Although both treatments reduced the feeding rate of *D. villosus*, increased purified MC-LR concentrations resulted in increased mass loss in *G. pulex*, while *D. villosus* maintained a steady growth rate under increased purified toxin exposure until there was a decline at higher concentrations. Crude extract significantly reduced the growth rate of *G. pulex*, feeding rate in this amphipod was unaffected by neither treatment. Altogether, these results suggest long-term exposure to low environmentally relevant MC concentrations may induce subtle shifts on key ecological processes, such as energy acquisition and biological fitness, thereby influencing population size, community structure and ecosystem functioning.

3.5.1 Effects on survival, feeding and chronic life history in *D. magna*

The key assessment criteria for testing the chronic effects of environmental stressors on aquatic organisms are survival, growth, and reproduction (Villarroel *et al.* 2003). Among these criteria, survival has been shown to provide a reliable index of chronic toxicity compared to growth and reproductive parameters because of its low variability among organisms with shorter generation times such as zooplankton (Sancho, Villarroel and Ferrando 2016; Ferrando, Sancho and Andreu-Moliner 1995). However, contrary to this expectation, purified MC-LR at the range of environmentally relevant concentrations tested in this study did not affect the survival of *D. magna* during the 21- day chronic exposure. This is in accordance with earlier studies (Chen *et al.* 2005b; Hulot *et al.* 2012; Duc *et al.* 2020), suggesting chronic exposures to low environmental MC concentrations are unlikely to have adverse effects on survival among *D. magna* populations during toxic blooms.

Chen *et al.* (2005b) found no significant effect of chronic exposure to low concentration of purified MC-LR (10 and 113 µg/L) on the survival rate of *D. magna*. Similarly, the mean survival rate of *D. magna* was unaffected during an exposure to 3 µg/L concentration of the pure MC-RR (Hulot *et al.* 2012). However, adverse effects of low environmental MC concentrations may become visible on survival under a more prolonged scenario than the 21-day exposure period used in the present study. Such effects are likely to manifest in the long term because of altered sublethal fitness-related traits in organisms. Therefore, the observed tolerance of *D. magna* to MC in this study could be attributed to the fact that purified MC-LR is possibly less toxic to *Daphnia* compared to intact toxic cyanobacterial cells or crude extracts (Jungmann 1992; Jungmann and Benndorf 1994). However, only purified MC-LR treatment was tested in the *Daphnia* chronic life history experiment in this study. Therefore, the result of the present study supports the ongoing debate that cyanobacterial metabolites other than MC may be responsible for the adverse effects observed in *Daphnia* fed with whole toxic cell diet or exposed to aqueous crude extracts treatments during laboratory experiments

(Jungmann and Benndorf 1994; Smutná *et al.* 2014). This finding further suggests that, to eliminate the confounding effects of other potential metabolites, there is need for more robust experimental designs that may incorporate a broad range of treatment conditions that can reflect the ideal environmentally realistic exposure conditions during toxic blooms (Dao *et al.* 2018; Bui *et al.* 2020).

In addition to the effect on survival, somatic growth rate of *D. magna* was not affected by low environmental concentrations of the purified MC-LR at the end of the chronic experiment. This result agrees with Lürling and van der Grinten (2003) who found no effect of microcystin on growth parameters in *Daphnia* when exposed to low concentrations (3.5µg/L and 1.75 µg/L). However, Dao, Do-Hong and Wiegand (2010) observed reduced growth performance in *D. magna* neonates exposed to two microcystin treatments. These contrast observations may be due to clonal differences in the sensitivity of *D. magna* to MC concentrations, suggesting that it is possible that some clones may have evolved effective detoxification mechanisms that enhance tolerance to chronic microcystin toxicity. Besides, the range of environmentally MC concentrations tested in the present study are relatively much lower than the lethal concentrations reported in the literature (DeMott, Zhang and Carmichael 1991; Bui *et al.* 2020). Hence, these results suggest that one of the possible adaptive strategies to low environmental MC exposure in *D. magna* populations is to invest greater metabolic energy towards maintenance of survival and growth through increased biotransformation and detoxification (Dao *et al.* 2018; Ortiz-Rodríguez, Dao and Wiegand 2012).

Low environmental MC concentrations exerted a range of non-monotonic dose-response effects on reproductive parameters of *D. magna* in this study. These results are in contrast with the general assumption that biological response in organisms would increase linearly with increasing cyanotoxin concentrations (Lagarde *et al.* 2015). Here, date to first reproduction of *D. magna* was increased at lower and higher MC-LR concentrations but reduced at intermediate concentrations, suggesting a U-shaped non-monotonic dose-response relationship. Similarly, evidence of inverted U-shaped hormesis was observed

as mean number of broods produced per female and fecundity in *D. magna* increased only intermediate MC-LR concentrations but no significant effects were observed at lower and higher concentrations. These results corroborate findings from previous studies (Rivetti, Campos and Barata 2016; de Souza Machado *et al.* 2017; Lagarde *et al.* 2015), suggesting that several environmental stressors originating from natural and anthropogenic processes may induce non-linear non-monotonic dose-response effects on reproductive traits in freshwater zooplankton, especially at exposure concentrations much lower than the permissible environmentally safe thresholds (Beausoleil *et al.* 2013; Liang *et al.* 2020).

The implication of such low-dose effects presents a severe challenge for existing regulatory guidelines and may pose significant ecological threats to ecosystem structure and functions in freshwaters. Although the WHO recommends a maximum of 1 µg/L and 10 µg/L MC-LR equivalent as permissible safe thresholds for drinking and recreational freshwaters respectively (Chorus *et al.* 2000; Bartram and Chorus 1999), several authors have associated exposure to high MC concentrations with impaired reproduction and life-history traits among zooplankton in the laboratory (Dao, Do-Hong and Wiegand 2010; Shahmohamadloo *et al.* 2020; Liang *et al.* 2018; Liang *et al.* 2020). Dao, Do-Hong and Wiegand (2010) observed a 60% decline in fecundity of *D. magna* exposed to 50 µg/L MC-LR concentration for 60 days, while onset of reproduction in *D. magna* was delayed at MC concentrations ranging from 50 µg/L to 100 µg/L during a 21-day chronic experiment (Shahmohamadloo *et al.* 2020). However, unlike the treatment concentrations tested in this study, MC levels reported in those studies exceed the WHO permissible thresholds and may rarely occur in freshwaters, except occasionally during bloom senescence (Zhu *et al.* 2021; Pham and Utsumi 2018; Graham *et al.* 2020).

Another important key finding in this study demonstrates that exposure to low environmental MC concentrations can stimulate both reproduction and the population-level parameter, intrinsic rate of natural increase (r) in *D. magna*. Here, increased MC-LR concentrations had a non-linear stimulatory effect on the values of r parameter, suggesting hormesis is likely an important adaptive mechanism through which freshwater

zooplankton respond to toxic stress resulting from chronic exposure to low environmental MC concentrations (Forbes 2000; Rivetti, Campos and Barata 2016). The observed increase in r parameter values was directly a consequence of MC-induced stimulation of reproduction in *D. magna* at the low range of concentrations tested in this study. Increased r parameter was only possible because MC concentrations resulted in increased number and size of broods at first reproduction (Sancho, Villarroel and Ferrando 2016; Hulot *et al.* 2012). This result is consistent with the findings of Duc *et al.* (2020) and Dao, Do-Hong and Wiegand (2010) who also observed increased population growth, resulting from increased values of r parameter and reproduction among *D. magna* exposed chronically to different microcystin treatments. Several authors have argued that survival may serve as a reliable measure of chronic toxicity among freshwater species (Villarroel *et al.* 2003; Sancho, Villarroel and Ferrando 2016). However, in contrast to this argument, the present study demonstrates and corroborates existing studies that suggest the intrinsic rate of natural increase is a more sensitive and superior measure of chronic toxicity in organisms than survival (Sancho *et al.* 2018; Duc *et al.* 2020). This is because the intrinsic rate of increase is a robust integrative parameter that can combine both lethal and sublethal effects of exposure to stressors into a single estimate (Sancho *et al.* 2018). Therefore, the present study suggests that one probable adaptive response in *D. magna* is to compensate the toxicity of low environmentally relevant MC concentration by allocating more energy to reproduction at the cost of individual growth and survival (Forbes 2000; Sokolova *et al.* 2012).

Feeding rate is an important physiological response in aquatic organisms (Brown *et al.* 2004) and may provide a reliable sublethal endpoint to assess chronic effects of low environmental MC exposure in freshwater zooplankton (Barata and Baird 2000). Individual organisms require an adequate supply of energy through feeding to ensure survival, growth, reproduction, and metabolic maintenance (Sancho, Villarroel and Ferrando 2016; Villarroel *et al.* 2003). In this study, environmental MC concentrations were associated with the stimulation of feeding and energy acquisition rates in *D. magna*. This stimulatory effect was found in both treatments, suggesting the observed feeding

rate stimulation in *D. magna* exposed to crude *Microcystis* extract treatment was possibly due to MC toxicity. This result agrees with Duc *et al.* (2020), who reported similar stimulatory effects on the feeding rate of *D. magna* exposed to 1-50 µg/L of MC-containing crude extract (MCCE). The observed feeding rate stimulation in *D. magna* may be due to increased energy requirement for MC detoxification and maintenance of reproduction (Sokolova *et al.* 2012; Ortiz-Rodríguez, Dao and Wiegand 2012).

3.5.2 Effects on survival, feeding and growth in amphipods

Survival of native and invasive amphipod species was not affected by low environmental microcystin concentrations tested in this study. The observed tolerance to MC treatments among these benthic detritivores corroborates existing findings in the literature (Shahmohamadloo *et al.* 2020; Karlson and Mozūraitis 2011). This finding suggests that long-term exposures to sublethal MC concentrations are unlikely to pose significant threats to the survival of amphipods that constitute the most dominant and abundant benthic invertebrate communities in freshwaters (Consolandi, Ford and Bloor 2019). This observation could be attributed to the low uptake of dissolved MC from the water column compared to uptake from detrital substrates (Kim *et al.* 2021) and may possibly be due to the presence of protective mechanisms of detoxification in amphipods (Sroda and Cossu-Leguille 2011; Galanti, Amé and Wunderlin 2013). However, besides effects on amphipod survival, sublethal MC concentrations were also associated with a reduced feeding rate of *D. villosus*. Interestingly, one important finding in this study showed increased purified MC-LR concentrations consistently increased body mass loss in *G. pulex* (reduced growth) but *D. villosus* grew steadily at higher concentrations under increasing purified MC-LR concentrations. This finding is consistent with previous studies (Bundschuh *et al.* 2013; Sroda and Cossu-Leguille 2011), suggesting that the UK native amphipod, *G. pulex* are potentially more sensitive to prolonged exposure to environmental stressors, including low MC concentrations, compared to the invasive killer species, *D.villosus*. Although the present study examined the effects of MC on amphipods' behaviour under acute experimental conditions (7 days), however, the

observed effects on growth may lead to reduced individual survival under longer exposure to MC. In contrast, the observed tolerance in *D. villosus* at low concentrations supports the findings of Kenna *et al* (2017) and Truhlar *et al* (2014) who reported higher thermal tolerance in invasive amphipods, suggesting that the MC tolerance in *D.villosus* may be associate with thermal preference.

Moreover, survival of *G. pulex* and *D. villosus* under chronic environmental MC stress may require buffering toxic effects of low MC exposure through detoxification (Sokolova *et al.* 2012), and this may potentially impose higher metabolic costs on amphipods (Karlson and Mozūraitis 2011). As shown in the present study, it is probable that both species reduced individual growth rate as an adaptative compensatory trade-off to invest more metabolic energy towards maintaining individual survival under low MC stress (Knops, Altenburger and Segner 2001; Sokolova *et al.* 2012). Interesting, while a greater reduction in the growth rate of *G. pulex* observed in this study may be due to synergistic interactions between MC and other secondary metabolites in the crude *Microcystis* extract treatment (Jungmann 1992; Smutná *et al.* 2014), the reduced growth rate of *D. villosus* only at higher concentrations reported here might have resulted from the reduced feeding rate and energy allocation for growth induced by the purified MC-LR treatment (Sokolova *et al.* 2012). One possible explanation for this observation may be related to the fact that *D. villosus* is a generalist omnivorous feeder (Pellan *et al.* 2016); therefore, feeding this species with only conditioned leaves in this study might possibly have contributed to reducing both the feeding and growth rates of *D. villosus.*

3.6 Conclusion

The present study demonstrates low environmentally relevant MC concentrations below the WHO levels recommended guidelines for freshwaters can affect population-level sublethal endpoints across freshwater keystone species. The results of this study showed that although individual survival among key species may be unaffected at the range of concentrations regularly encountered in natural waters, other more ecologically relevant sublethal effects on feeding, growth and reproduction may become evident, affecting

population dynamics, community structure and ecosystem functioning. However, these effects are usually non-monotonic across different traits and taxa. Moreover, this study highlights the fact that hormesis is possibly a pervasive eco-physiological response that may explain the effects of MCs and other chemical stressors at low exposure concentrations.

Chapter 4

Combined effects of increased water temperature and microcystins exert heterogeneous effects on survival and ecological processes in key freshwater species

4.1 Abstract

Climate change is increasing water temperature and intensifying the incidence of freshwater microcystin-producing cyanobacterial blooms worldwide. However, it is unclear how increased temperature and microcystins as co-occurring stressors may jointly affect survival and ecological processes underpinning ecosystem functions in key freshwater species during toxic blooms. Here, using purified MC-LR and crude extract of toxic *Microcystis* treatments, we tested the individual and combined effects of three water temperatures (15, 20 and 25°C) and a range of environmentally relevant microcystin concentrations (0.01-10µg·L^{-1}) on survival and ecological processes in key freshwater taxa: phytoplankton (*Scenedesmus quadricauda*), zooplankton (*Daphnia pulex*), and invertebrate predators (*Ischnura elegans*). Purified MC-LR exerted a significantly higher inhibitory effect on the growth of *S. quadricauda* compared to crude extract, while neither treatment affected its chlorophyll-a content or survival of *D. pulex*. Crude extract alone reduced the grazing rate of *D. pulex* and survival of *I. elegans*. A synergistic interaction between warmer temperature at 25°C and microcystin concentration in the extract led to a reduction of up to 50% in the survival of *I. elegans*. Increased temperature reduced prey handling time of *I. elegans* by 49%, suggesting a higher predation rate. However, evidence of significant antagonistic interactive effects was observed on grazing rate of *D. pulex* and predation rate of *I. elegans* when warmer temperature at 25°C coincided with increased microcystin concentration in the crude extract treatment, resulting in higher zooplankton grazing and reduced damselfly predation. Taken together, these results indicate microcystin can have a broad range of effects on survival and processes in key freshwater species, suggesting these effects may

be unevenly distributed across trophic levels. Hence, our results highlight the importance of neglected and complex ecological mechanisms by which climate warming can exacerbate the effects of low, environmentally realistic microcystin concentrations on key species and their ecosystem functions in eutrophic freshwater habitats.

4.2 Introduction

Harmful cyanobacterial blooms are among the biggest threats impacting freshwater quality, biodiversity and ecosystem functioning globally (Briland *et al.* 2020; Brooks *et al.* 2016; Shahmohamadloo *et al.* 2020). Owing to the cumulative impacts of complex synergies between multiple environmental stressors associated with anthropogenic climate change, eutrophication, and biological invasions (Schulhof *et al.* 2019; Sukenik, Quesada and Salmaso 2015; Urrutia-Cordero *et al.* 2020), harmful freshwater cyanobacterial blooms have increased in frequency and intensity worldwide (Svirčev *et al.* 2019). With synergistic interactions between anthropogenic nutrient enrichment, increased water temperature and thermal stratification intensifying microcystin-producing cyanobacterial blooms in freshwaters (Bui *et al.* 2018; Harke *et al.* 2016), such blooms have emerged a serious environmental concern worldwide (Briland *et al.* 2020; Brooks *et al.* 2016), causing an increase in the occurrence of hazardous microcystins and other secondary metabolites in water bodies (de Figueiredo *et al.* 2004; Porojan *et al.* 2020a). While the public health and socio-economic implications of algal blooms have received considerable attention (Brooks *et al.* 2016; de Figueiredo *et al.* 2004; Dodds *et al.* 2009), little is known about the ecological consequences of environmental microcystin exposure on biodiversity and ecosystem functioning in freshwaters (Briland *et al.* 2020; Shahmohamadloo *et al.* 2020; Šulčius *et al.* 2017). Therefore, as harmful blooms become intensified, environmental microcystin exposure may potentially threaten survival and ecological processes that underpin ecosystem functions among key freshwater species.

Microcystins (MCs) are a variety of hazardous cyanobacterial toxins produced as bioactive secondary metabolites by several bloom-forming cyanobacteria (Bartram and Chorus 1999; de Figueiredo *et al.* 2004; Janssen 2019). MC occurrence in freshwaters

have been linked with severe adverse effects in humans and a wide range of animals, such as birds, amphibians, fish, and zooplankton (Chen *et al.* 2009; Jos *et al.* 2005). The primary mechanisms behind these adverse effects in humans and animals have been causally related to the role of MCs in the inhibition of protein phosphatase enzymes of the liver cells and induction of cellular oxidative stress (Amado and Monserrat 2010; Campos and Vasconcelos 2010). Therefore, in recognition of its potential threats, the World Health Organisation (WHO) recently recommended provisional guidelines of 1 µg/L and 12 µg/L as short term and lifetime permissible MC-LR concentrations in drinking waters, while the permissible threshold in recreational waters is 24 µg/L MC-LR concentration (Chorus and Welker 2021). Although there have been a few reports of occasional occurrence of MC concentrations as high as 6300 µg/L (Babica *et al.* 2007) during cell lysis at senescence, or immediately after algicide treatment (Kotak and Zurawell 2007; Sivonen and Jones 1999), more than 50% of the released MC concentrations in surface waters are usually degraded within five days (Zheng *et al.* 2004). This rapid degradation of toxin suggests that such high MC concentrations are unlikely to persist for longer periods. Typical environmental MC concentrations in freshwater bodies are <10 µg/L (Zhu *et al.* 2021), and rarely exceed the WHO recommended guidelines (1−24 µg/L) (Chorus and Welker 2021; Chen *et al.* 2017). Hence, there is need to understand how exposure to sublethal environmental MC concentrations may affect survival and ecological processes driving ecosystem functions among key freshwater food web components.

As key components of the aquatic food webs, freshwater species mediate important ecological processes, such as primary production, herbivory, and predation within ecosystems (Galic, Grimm and Forbes 2017). The dynamics of these ecological processes in individual organisms has been associated with their vital ecosystem functions in freshwater systems (McKie *et al.* 2009; Oliver *et al.* 2015). However, key ecological processes in freshwater organisms may become impaired either directly or indirectly through reduced survival when exposed to sublethal environmental MC concentrations (Lindholm *et al.* 1992). Previous studies have linked MC exposure with a variety of

adverse effects on a few freshwater taxa, including allelopathic inhibition of growth and photosynthetic pigments in algae (El-Sheekh, Khairy and El-Shenody 2010), altered feeding and life history in zooplankton (Ghadouani *et al.* 2004; Smutná *et al.* 2014) and bioaccumulation and oxidative stress in molluscs (Zhang *et al.* 2016). Nevertheless, these studies only demonstrated effects of either intact cells, crude extracts, or purified toxin on single species at concentrations higher than what may be found in freshwaters (Burkholder, Shumway and Glibert 2018). MC content in intact cyanobacterial cells is usually several orders of magnitude higher than dissolved levels in water bodies (Chen *et al.* 2017). Therefore, findings from these studies may be difficult to extrapolate to the field because MC levels reported were not representative of the dissolved concentrations in freshwaters (Nilsen *et al.* 2019). Moreover, inferences drawn from single-species or single-stressor studies may be inaccurate to make real-world generalisations due to over-simplification as experiment designs may not replicate complex environmental conditions found in natural waters (Edwards and Pascoe 2018). Hence, empirical studies on the effects of sublethal MC concentrations on multiple species across multiple trophic levels under environmentally realistic scenarios are needed.

Water temperature and thermal stratification in freshwaters are expected to rise in view of the ongoing global climate change (Mullin et al., 2020). The earth's mean annual atmospheric temperature has been predicted to rise by 1.5°C over the coming decade (Pachauri *et al.* 2014). Given this prediction, increased water temperature may act independently (Woodward, Perkins and Brown 2010), or in conjunction with other environmental stressors, such as MC concentrations to influence survival and ecological processes among freshwater species during toxic blooms (Walls *et al.* 2018). While increased water temperature accelerates rates of metabolic and cellular activities in freshwater organisms (Galic, Grimm and Forbes 2017), these effects may likely vary across species depending on species-specific thermal thresholds (Brown *et al.* 2004). Moreover, under environmentally realistic scenario during toxic blooms, low MC concentrations may interact with increased water temperature to affect survival and ecological processes in freshwater species (Burkholder, Shumway and Glibert 2018). The

consequence of such complex interactions may involve additive, non-linear synergistic or antagonistic effects which can lead into unexpected ecological outcomes (Vinebrooke *et al.* 2004; Birk *et al.* 2020), and subsequently influence trophic interactions (Woodward, Perkins and Brown 2010). However, the effects of temperature and MC interactions on freshwater species are rarely known beyond individual species or populations (Kim *et al.* 2014; Lamb, Kimmel and Field 2019; Xiang *et al.* 2017) and this limits our understanding of the ecological impacts of toxic freshwater blooms in the face of environmental change (Burkholder, Shumway and Glibert 2018; Šulčius *et al.* 2017; Chen *et al.* 2013).

To address this gap, we tested the combined effects of temperature and MCs on survival and key ecological processes relating to resource-consumer interactions in three key species across freshwater food web taxa: *Scenedesmus quadricauda* (freshwater green alga), *Daphnia pulex* (zooplankton grazer) and *Ischnura elegans* (predatory damselfly larva). These study species were selected based on their significant positions in the freshwater food webs, and their use as sentinel experimental models in many ecotoxicological studies (Šulčius *et al.* 2017). We conducted a suite of factorial laboratory experiments comprising of three temperatures (15°C, 20°C, 25°C) and two MC treatments (purified MC-LR and crude extract) at environmentally realistic concentrations (0.01-10 µg/L). The temperature range tested is in accordance with those reported in previous studies, suggesting characteristic average (15°C), slightly elevated (20°C) and elevated (25°C) thermal conditions in European freshwaters (Henry *et al.* 2017). MC concentrations tested were within the WHO permissible guidelines for freshwaters and were in accordance with existing evidence showing that MCs typically occur at relatively low concentrations ranging from tens of ng/L to a few µg/L in water bodies (Chen *et al.* 2013; Wei *et al.* 2020). Here, we measured algal growth inhibition as a proxy for survival and photosynthetic chlorophyll-a content as a measure of ecological process in algae. We measure survival along with herbivorous grazing and predation rates as surrogates of ecological processes in zooplankton and damselfly larva respectively in freshwater food webs.

The following hypotheses were tested in this study; firstly, we expected negative effects of sublethal environmental MC concentrations on survival and ecological processes in our study species based on previous studies (Burkholder, Shumway and Glibert 2018). Secondly, we predicted negative effects of increased temperature on survival and negative effects on feeding-related processes beyond certain thermal thresholds (Heugens *et al.* 2003; Kenna *et al.* 2017; Verheyen, Delnat and Stoks 2019). Finally, we predicted synergistic interactive effects of temperature and MCs on survival and ecological processes in these organisms, such that temperature exacerbated toxic effects. This third prediction is based on greater metabolic rates at higher temperatures resulting in greater active uptake and metabolism of the toxin.

4.3 Materials and Methods

4.3.1 Cyanobacterial culture

A toxigenic cyanobacterial strain, *Microcystis aeruginosa*, (CCAP/14/50/16) was obtained from the Culture Collection for Algae and Protozoa (CCAP), Scottish Marine Institute, Scotland. *M. aeruginosa* was cultivated in the laboratory as batch cultures under axenic conditions in 250 mL Erlenmeyer flasks containing 150 mL autoclaved BG-11 culture media prepared in line with the CCAP's recipe. These cultures were maintained under constant temperature 25±1ºC, light intensity 25µmol quanta $m^{-2}s^{-1}$ and photoperiod cycle 12 h: 12 h light/dark in a shaking incubator until cells were harvested. Before harvest, daily cell density was monitored by spectrophotometric measurement of the optical density (OD) at 680 nm and light microscopy using a compound microscope and Sedgewick-Rafter Counting Chamber. The relationship between the daily spectrophotometric OD measurement and the cell density was established by fitted linear regression (R^2=0.97). Cells were harvested at the stationary phase and cell density estimated by spectrophotometry, while cells were lysed through a freeze-thaw cycle to release the intracellularly bound MC content. The total MC content in the crude *Microcystis* extract was quantified and expressed as MC-LR using a semi-quantitative

Adda-specific MC ELISA kit (CAT No. ALX-850-391-KI01, Enzo Life Sciences) according to Sarnelle, Gustafsson and Hansson (2010). The purified MC-LR (CAS No. 101043-37-2, purity ≥ 95%) was supplied by Cayman Chemical Company, UK.

4.3.2 Algal biomass accrual and photosynthetic chlorophyll-a

Scenedesmus quadricauda (A950) was purchased from Sciento Scientific Ltd., Manchester, UK and maintained in BG-11 media on a cool white fluorescent lamp-illuminated shelf in a controlled temperature room kept at 21±1ºC; 54µmol quanta $m^{-2}s^{-1}$ and 12:12 h light/dark photoperiod. Daily cell density increases were monitored by spectrophotometric measurement of the optical density (OD) at 680 nm and light microscopy using a compound microscope and Sedgewick-Rafter Counting Chamber.

We set up 40 experimental replicates of 100 mL cultures of *S. quadricauda* in 150 mL Erlenmeyer flasks, in accordance with the OECD guidelines (OECD 2011). Each replicate culture was made up of sterile BG-11 medium, a known concentration of MC-LR treatment (either in the crude *Microcystis* extract or the purified toxin) and 10 mL of the *S. quadricauda* inoculum (2.64 $\times 10^4$ cells/mL) which had been incubated and acclimated as an exponentially growing culture for 4 days before the experiment. We tested the effect of two MC treatments at environmentally realistic concentrations on the growth inhibition and photosynthetic pigments content of *S. quadricauda* at three different temperatures: 15, 20 and 25 ºC. Five concentrations of the purified MC-LR treatment (0.01, 0.1, 0.5, 1.0 and 10.0µg/L) and three concentrations (0.01, 0.1 and 0.5µg/L) of crude *Microcystis* extracts were tested. The MC concentrations in the crude *Microcystis* extract MC were determined by the toxigenicity of the strain which potentially represents an ecologically relevant mixture of secondary metabolites released during cell lysis. Each treatment concentration and control had four replicates. The control group consisted of a blank control and a solvent control. The blank control comprised all the experimental conditions without MC treatment, while the solvent control had in addition to the blank control 0.1mL of methanol (0.1% (*v*/*v*) to isolate the effects of the organic solvent used as diluent from the effects of the purified toxin. The

experiment was incubated at three temperatures 15, 20 and 25 ºC, and constant light intensity (54 µmol quanta $m^{-2}s^{-1}$) over a fixed photoperiod (14 h:8 h hour light/dark cycle) for 72 hours. On each day of the experiment 5 mL samples were taken and replaced with equal volume of the growth medium. The daily biomass increase for each replicate was estimated from the linear regression between the spectrophotometric OD_{680} data and the microscopic cell counts (Ma *et al.* 2015). The percentage growth inhibition of *S. quadricauda* was calculated from the specific growth rate of the treatment relative to the solvent control as described by (Wang *et al.* 2017). The effects of temperature and MC treatments on chlorophyll a content as a surrogate measure of photosynthetic processes were determined by taking the spectrophotometric optical density reading of the methanol extracted samples at OD_{440}, OD_{645}, OD_{652} and OD_{663}. The pigment content was calculated as described by Fang et al. (2018).

4.3.3 Zooplankton survival and grazing

Adults of the cladoceran zooplankton, *Daphnia pulex* were obtained from Blades Biological Ltd., Kent, UK. Daphnids were cultured in a controlled temperature (CT) room at 21±1ºC and 16 h:8 h light: dark photoperiod for three weeks, until third broods of neonates were produced by each adult female. Fifteen adult animals were maintained in 500 mL glass beakers filled with 300 mL of “Aachener Daphnien Medium" (ADaM), a modified artificial media for zooplankton (Klüttgen *et al.* 1994). Daphnids were fed with 10 mL of *S. quadricauda* ($5.0x\ 10^6$ cells per mL) three times weekly. The culture medium and algal food material were renewed every three days and the neonates produced were enumerated and removed daily (Rohrlack *et al.* 2001). Only neonates produced after the second brood were used for experiments.

A 48-hour static acute toxicity test was conducted to test the combined effects of temperature and MC on the survival of *D. pulex* following the OECD guidelines (OECD 2004). Daphnid neonates less than 24 h old and in their third brood were exposed to five treatment concentrations (0.01, 0.1, 0.5, 1.0, and 10.0 µg/L) of purified MC-LR and three concentrations of crude *Microcystis* extract (0.01, 0.1, and 0.5 µg/L). Tests were

conducted in 100 mL plastic beakers, containing 50 mL of the experimental medium (ADaM), MC treatments and 10 daphnid neonates per cup. Six replicates of the control were used, comprising the same experimental conditions without MC, while three replicates per treatment concentration were tested. Replicates were incubated under controlled conditions at three different temperatures 15, 20, and 25 ºC, 54 µmol quanta $m^{-2}s^{-1}$ and 14 h:10h hour light/dark cycle. The proportion of immobilised neonates per replicate was counted as a surrogate measure of mortality (endpoint) at 24 and 48 hours (Luo *et al.* 2018).

The grazing inhibition tests in *D. pulex* were conducted in accordance with the method described by Jesus, Martins and Nogueira (2014). Five 4-day old daphnids were exposed to environmentally relevant concentrations of the two MC treatments at three temperatures 15, 20, and 25 ºC in 50 mL plastic beakers. Treatment concentrations used in this test were the same as those used for the survival experiments. Each beaker contained a 20 mL solution made up of a combination of the test medium (ADaM), MC treatments and 5mL of *Scenedesmus* culture (1.45×10^6 cells/mL) as a food source for daphnids. The control group was made up of a blank with the same experimental conditions as the treatment but without daphnids and MC treatments while the control had in addition to the blank five daphnids. Four replicates of both the control and treatments were tested. To reduce the influence of light on algal growth during the experiment, the tests were incubated in the dark while animals were only allowed to graze for 24 hours. At the end of the test, animals were gently removed with the aid of plastic pipette, and each beaker was shaken vigorously to ensure uniform concentration of algae before taking the spectrophotometric optical density (OD_{680}) at 680nm. The individual grazing rate for each animal was estimated relative to the blank control and expressed as the change in the cell density during the 24-hour grazing test (Allen, Calow and Baird 1995).

4.3.4 Damselfly survival and predation

Blue-tailed damselfly larvae, *Ischnura elegans*, were collected between June and July 2019 from ponds located at None-Go-By Farm (53° 52' 33.75" N, 1° 38' 37.57" W) in Horsforth, West Yorkshire, UK. Larvae were sampled using a pond net, sorted in a plastic tray, and transported to the laboratory in a small cool box containing pond water for further identification using taxonomic keys (Cham 2012). *I. elegans* were acclimated to laboratory conditions in plastic tanks containing 100 mL of aged, dechlorinated tap water, maintained at constant temperature of 20ºC and 14 h:10 h light: dark photoperiod cycle. Larvae were fed *ad libitum* with *Daphnia magna*, while a wooden dowel rod was used to provide a perch for the larvae in each tank (Villalobos-Jiménez, Dunn and Hassall 2017).

A 24-hour functional response experiment was conducted to test the effects of MC and temperature on survival and prey consumption using methods in Villalobos-Jiménez, Dunn and Hassall (2017). Larvae with a size range of 1.5 mm - 3.1 mm at the 9^{th} -11^{th} instars were acclimated to the experimental temperature and starved for a minimum of 24 hours before the start of the experiment. Two sets of larvae were used, the larger larvae at the 11^{th} instar with a size range between 2.5 mm and 3.1mm were used for the 20 °C treatments while the smaller larvae at the 9^{th} and 10^{th} instar (size range between 1.5 mm and 2.5 mm) were used for the 15 °C and 25 °C treatments. Animals were housed individually in 200ml plastic containers in aged, dechlorinated tap water, incubated at 14 h:10 h light: dark photoperiod cycle, and exposed to two concentrations of the MC-containing crude extract treatments (0.05 and 0.2 µg/L) or a control containing no extract at three different temperatures 15, 20 and 25 °C. The abundance of damselfly larvae is seasonal and the capture rate during pond sampling may vary across different seasons of the year. Therefore, these two concentrations were considered as the most typical and ecologically relevant MC concentrations during chronic exposure in freshwater bodies. Purified toxin was not available for this experiment. Larvae were fed with one of five different prey densities, comprising 5, 10, 15, 30, and 50 *D. magna* individuals (body size 1.0-1.4 mm) as prey. Seven replicates of each prey density were used for the experiment

at 15 °C and 25 °C, while five replicates were used for the experiment at 20 °C due to the limited number of size-matched damselfly larvae captured. No feeding experiment was performed at the 20 °C temperature treatment due to low capture rate of *I. elegans*, therefore, only the data on survival at this temperature treatment is reported here. Each damselfly was only used in one replicate. At the end of the 24-hour exposure, the numbers of *Daphnia* consumed were enumerated and functional response curves were generated as discussed below.

4.3.5 Data Analysis

Algal response data (growth inhibition and chlorophyll-a pigment) were checked for the model assumptions of normally distributed residuals using the Shapiro-Wilk test. The 72-hour percentage algal growth inhibition data were log-transformed to avoid non-normal distribution of the residuals and Gaussian generalised linear models (GLMs) with interaction terms were fitted to test the main and the interactive effects of MC and temperature treatments on the response variables.

Survival of *D. p*ulex individuals exposed to MC treatments were found to be lower than 50%, therefore, survival of daphnids could not be expressed as the median lethal concentrations (LC_{50}); a concentration that would kill 50% of the exposed population. Instead, Cox proportional hazard (CPH) models in the *survival* (Therneau 2015; Therneau and Grambsch 2000) and *survminer* (Alboukadel, Marcin and Przemyslaw 2019) packages in R were fitted to test for variation in time to death across toxin and temperature treatments. The 24-hour grazing in zooplankton was checked for normality assumptions and analysed by fitting generalized linear models. Significant differences among treatment and temperature levels were determined using Tukey's multiple comparison test in the *multcomp* package in R (Torsten, Frank and Peter 2008).

The individual and combined effects of temperature and crude *Microcystis* extract treatments on the survival of *I. elegans* during the 24-hour experiment were tested by fitting a binary logistic regression. We explored the differences in the effects of temperature and crude extract treatments on the attack rate and handling time of *I. elegans*

using the *frair* package (Pritchard 2017; Pritchard *et al.* 2017) in R with a Benjamini-Hochberg correction. Prey consumption rates followed a type II functional response. Since there was no prey replacement during the experiment, we estimated handling time (*h*) and attack rate (*a*) following Rogers' type II formula (Rogers 1972) with Lambert's W function (Bolker 2008). We tested the difference in the handling times and attack rates between treatments using bootstrapping.

4.4 Results

4.4.1 Algal biomass accrual and photosynthetic chlorophyll-a

Purified MC-LR (p = 0.001) and the treatment concentrations (p = 0.003) tested in this study had a significantly higher inhibitory effect on the growth of *S. quadricauda* compared to the crude *Microcystis* extract treatment (Figure 4.1A; Table 4.1). Also, there was a significant growth inhibition on *S. quadricauda* when water temperature increased from 15°C to 25°C in this study (p <0.001; Figure 4.1A).

Our results showed no evidence of statistically significant effects of purified MC-LR (p = 0.180), crude *Microcystis* extract (p = 0.109) or toxin concentration (p = 0.131) on the chlorophyll-a pigment content of *S. quadricauda* (Figure 4.1B; Table 4.1). However, increased water temperature from 15°C to 25°C strongly reduced chlorophyll-a pigment content of *S. quadricauda* (p < 0.001; Figure 4.1B). The highest chlorophyll-a content in this study was observed at the 15°C temperature, whereas there was a significant reduction in the chlorophyll-a pigment content when water temperature increased from 20°C and 25°C (p < 0.001; Figure 4.1B).

4.4.2 Zooplankton survival and grazing

Neither purified MC-LR nor crude *Microcystis* extract treatments had significant effects on the survival of *D. pulex* at the range of concentrations tested in this study when compared with the control (Table 4.1, Figure 4.1C). However, increased water

temperature from 15°C to 25°C significantly reduced survival of *D. pulex* individuals ($p < 0.001$; Table 4.1, Figure 4.1C).

Crude *Microcystis* extract only ($p = 0.045$), but not the purified MC-LR ($p = 0.280$) significantly reduced the grazing rate of *D. pulex* relative to the control (Table 4.1, Figure 4.1D). However, these effects did not differ significantly across the range of toxin concentrations tested in this study ($p = 0.562$; Table 4.1, Figure 4.1D). Although increased temperature from 15°C to 25°C significantly reduced the grazing rate of *D. pulex* compared to the baseline temperature ($p = 0.005$; Table 4.1, Figure 4.1D), a significant antagonistic interaction between increased temperature and crude *Microcystis* extract treatment increased the grazing rate of *D. pulex* relative to the control ($p = 0.025$, Figure 4.1D).

4.4.3 Damselfly survival and predation

Increased crude *Microcystis* extract concentrations (from 0.05 µg/L to 0.2 µg/L) significantly reduced survival of *I. elegans* relative to the control ($p = 0.036$; Figure 4.2A, Table 4.2). Overall, the survival odds ratio of *I. elegans* in the control was approximately 6 times higher than in the extract treatment (Table 4.2). Although increased temperature alone had no significant overall effect on the survival of *I. elegans* ($p = 0.512$; Figure 4.2A, Table 4.2); there was a significant synergistic interaction between increased temperature and crude *Microcystis* extract treatment, such that the highest crude extract concentration (0.2 µg/L) and increased temperature at 25°C jointly reduced survival of *I. elegans* by almost 50% compared to the control ($p = 0.007$; Figure 4.2A, Table 4.2).

Increased crude extract concentration showed no significant effects on the attack rate ($p = 0.45$) and prey handling time of *I. elegans* ($p = 0.92$) compared to the control at 15°C (Table 3). Increased temperature had no statistically significant effect on the attack rate of *I. elegans* ($p = 0.92$; Table 4.3), however, the prey handling time of *I. elegans* was significantly reduced by 49% when temperature increased to 25°C, indicating a higher predation rate ($p < 0.001$; Table 4.3; Figure 4.2A). A significant antagonistic interaction between MC toxicity and warming was observed when increased crude extract

concentration (0.2 µg/L) significantly increased the prey handling time of *I. elegans* at 25°C temperature ($p < 0.001$; Table 4.3; Figure 4.2B). However, no significant effect was observed on the attack rate of *I. elegans* when increased crude extract treatment coincided with increased temperature ($p = 0.38$; Table 4.3).

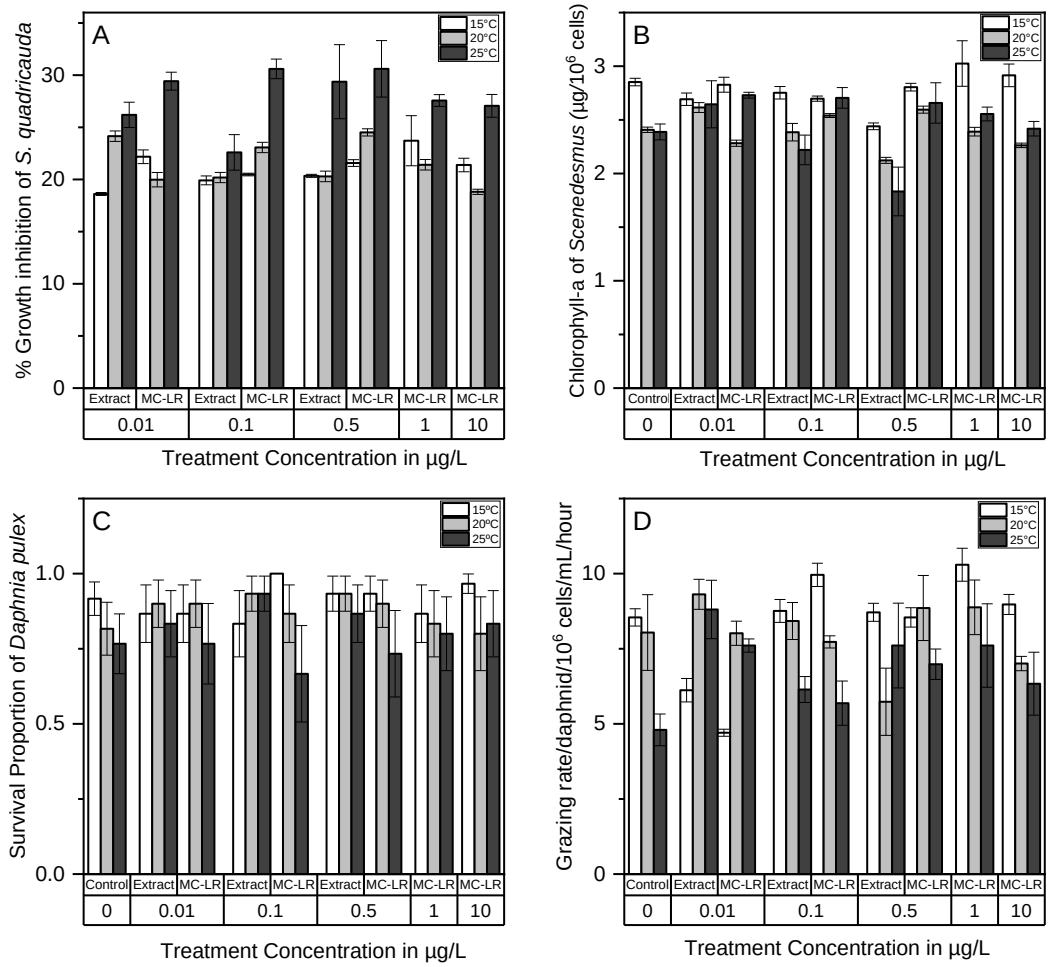

Figure 4.1: Effect of temperature and microcystin treatment concentration on (A) growth inhibition and (B) chlorophyll-a in the freshwater alga *Scenedesmus qudricuada* and survival (C) and grazing rate (D) in the zooplankton *Daphnia pulex*, Note that each point represents mean ± SEM error bars in (A,B & D) but mean± 95% confidence levels error bars in (C) .

Table 4.1: GLM output showing the parameter estimates with associated standard errors (SE), t-values, and p-values of the main and interactive effects of MC treatments and temperature on the percentage growth inhibition and chlorophyll-a content of *S. quadricauda* and grazing rate in *D. pulex*, respectively, relative to the controls (Note that the p-values in bold are significant, if $p < 0.05$).

Species	Response	Parameter	Estimates	SE	t-value	p-value
S. quadricauda	% Growth inhibition	Intercept	2.541	0.0657	38.67	**<0.001**
		MC (Purified)	0.094	0.0277	3.39	**0.001**
		Concentration	-0.009	0.0042	-2.18	**0.03**
		Temperature	0.028	0.0031	8.92	**<0.001**
S. quadricauda	Chlorophyll-a	Intercept	2.781	0.0798	38.84	**<0.001**
		MC (Extract)	-0.135	0.0833	-1.62	0.109
		MC (Purified)	0.110	0.0814	1.35	0.180
		Concentration	-0.014	0.0091	-1.52	0.131
		Temperature	-0.032	0.0064	-4.98	**<0.001**
D. pulex	Grazing rate	Intercept	14.616	2.639	3.42	**<0.001**
		MC (Extract)	-6.185	3.048	-2.03	**0.045**
		MC (Purified)	-3.418	2.894	-1.18	0.240
		Concentration	-0.036	0.061	-0.58	0.562
		Temperature	-0.374	0.129	-2.90	**0.005**
		Extract × Temp	0.340	0.149	2.28	**0.025**
		Purified× Temp	0.209	0.142	1.48	0.143

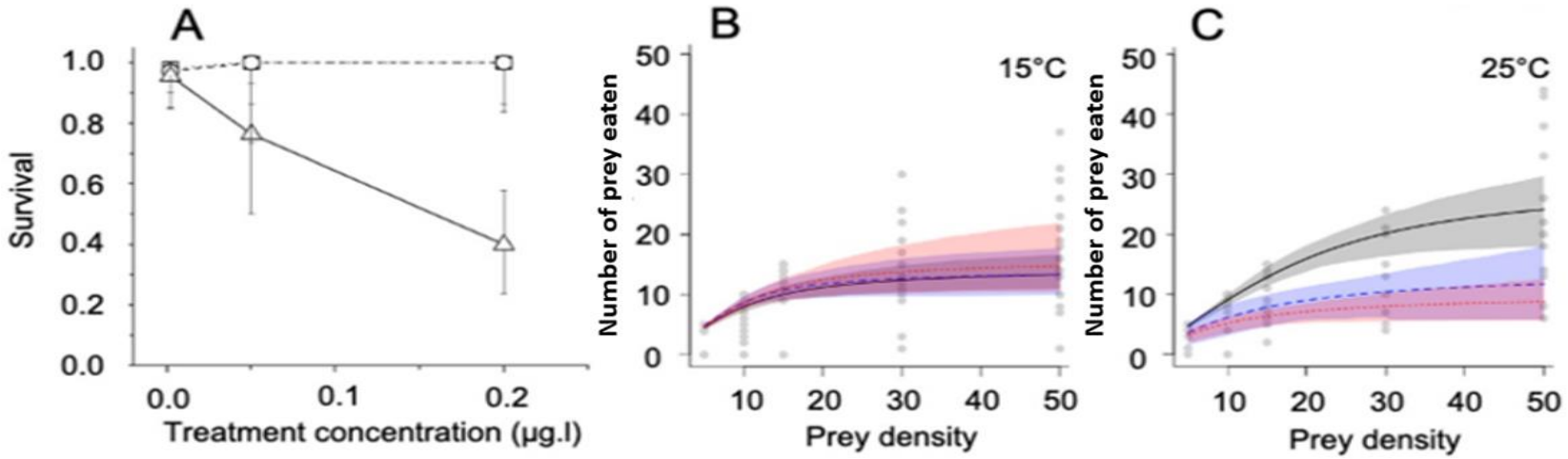

Figure 4.2: Combined effects of temperature and MC on survival and predatory functional response of damselfly larvae, *I. elegans*. (A) on survival (circle and triangle shapes are 15°C and 25°C temperatures respectively and error bars represent 95% confidence levels (B) on predation at different temperatures (15°C and 25°C, note that colours red and blue represent control at 25°C, and control at 15°C respectively) and (C) on predation at different MC concentrations (control, 0.05 and 0.2 µg/L, note that colour ash shows control at 25°C, blue, 0.05 µg/L at 25°C and red is 0.2 c at 25°C. Area shaded in colours represent 95% confidence levels.

Table 4.2: Logistic regression models and their associated parameter estimates (odds ratios), Z-value 95% confidence intervals (95% CI) and p-values summarising the combined effects of two MC treatments (purified MC-LR and crude *Microcystis* extract) and temperature on the survival of the microcrustacean zooplankton, *D. pulex* and damselfly larva, *Ischnura elegans*.

Species	Predictors	Odds Ratios	Z-value	95% CI	p-value
Daphnia pulex	Intercept	0.03	-6.57	0.01- 0.08	<0.001
	MC (Extract)	0.60	-1.81	0.34 -1.04	0.070
	MC (Purified)	0.95	-0.21	0.58 – 1.57	0.835
	Concentration	0.98	-0.61	0.91 – 1.05	0.539
	Temperature	1.10	4.04	1.05 – 1.16	<0.001
Ischnura elegans	Intercept	61.53	2.14	1.82 - 4450	0.032
	MC (Extract)	360.16	2.10	1.42 – 113277	0.036
	Temperature	0.94	-0.66	0.78 - 1.13	0.512
	Extract × Temp	0.71	-2.71	0.55 – 0.91	0.007

Table 4.3: Pairwise comparison of attack rate and handling time coefficients obtained from the functional response of *I. elegans*, showing estimate, z values with the associated standard error and Benjamini-Hochberg correction adjusted p-vales at 0.05 µg/L, 0.2 µg/L with control and two temperatures 15 and 25°C.

Pairwise comparison	Attack rate				Handling time			
	Estimate	SE	Z value	p-value	Estimate	SE	Z value	p-value
15°C (control): 15°C (0.05)	-0.470	0.701	-0.671	0.656	0.010	0.014	0.729	0.635
15°C (control): 15°C (0.2)	-0.104	0.944	-1.105	0.448	-0.004	0.015	-0.236	0.917
15°C (control): 25°C (control)	0.085	0.598	0.143	0.916	0.058	0.013	4.526	<0.001*
15°C (control): 25°C (0.05)	1.116	0.633	1.761	0.180	-0.006	0.030	-0.188	0.917
15°C (control): 25°C (0.2)	1.228	0.658	1.857	0.158	-0.052	0.039	-1.326	0.370
15°C (0.05): 15°C (0.2)	-0.574	0.960	-0.598	0.687	-0.014	0.013	-1.034	0.475
15°C (0.05): 25°C (control)	0.550	0.621	0.886	0.537	0.048	0.010	4.597	<0.001*
15°C (0.05): 25°C (0.05)	1.580	0.661	2.392	0.067	-0.016	0.030	-0.540	0.706

15°C (0.05): 25°C (0.2)	1.693	0.682	2.471	0.067	-0.062	0.038	-1.617	0.226
15°C (0.2): 25°C (control)	1.129	0.887	1.272	0.375	0.062	0.012	5.084	<0.001*
15°C (0.2): 25°C (0.05)	2.159	0.116	2.368	0.067	-0.002	0.030	-0.071	0.943
15°C (0.2): 25°C (0.2)	2.267	0.928	2.441	0.067	-0.048	0.039	-1.239	0.375
25°C (control): 25°C (0.05)	1.031	0.544	1.891	0.158	-0.064	0.029	-2.191	0.097
25°C (control): 25°C (0.2)	1.139	0.573	1.986	0.141	-0.109	0.038	-2.904	<0.05*
25°C (0.05): 25°C (0.2)	0.099	0.610	0.164	0.917	-0.046	0.047	-0.985	0.487

4.5 Discussion

Climate change is increasing water temperature and intensifying the incidence of microcystin-producing cyanobacterial blooms in freshwater bodies around the world (Harke *et al.* 2016; Richardson *et al.* 2019). This suggests that a likely scenario during such blooms in eutrophic freshwaters is a joint exposure to sublethal environmental MC concentrations and increasing water temperature (Burkholder, Shumway and Glibert 2018). However, the combined effects of these ecologically relevant, co-occurring stressors on survival and key ecological processes among freshwater species remain unknown. In this study, we demonstrate wide-ranging effects of low environmental toxin concentrations on survival and ecological processes in species across multiple trophic levels. MC concentrations below those currently adjudged safe for drinking and recreational waters by the WHO (1-10 µg/L) (Turner *et al.* 2018) were associated with significant effects on survival and key processes. Importantly, these effects vary among key freshwater taxa, influencing individual survival and ecological processes in different ways. Moreover, we provide the first evidence of significant interactive effects between increased temperature and low environmental MC concentrations on survival and feeding in key aquatic invertebrates. Increased MC concentrations in the crude extract together with elevated temperature jointly reduced survival in *I.* elegans, whilst predation rate was increased at higher temperature in this freshwater predator. Increased temperature while in concert with increased crude extract concentration reduced predation rate in *I. elegans* but increased grazing rate in *D. pulex*. Taken together, our findings highlight the need to re-evaluate the ecological impacts of cyanobacterial toxins given the predictions of more toxic blooms in freshwater systems under global change.

4.5.1 Effect of low environmental MC concentrations

As predicted in our first hypothesis, low environmental MC concentrations influenced survival and key ecological processes in our experimental organisms. These effects varied in a species-specific way across our study organisms, influencing individual survival (including growth inhibition in algae) and feeding in aquatic invertebrates. Our data also

illustrates that the effects of MC on survival and key processes in aquatic organisms can vary across key freshwater taxa. We observed a significantly higher inhibitory effect of purified MC-LR on the growth of *S. quadricauda* while the photosynthetic chlorophyll-a content was unaffected by the two treatments. This inhibitory effect of MC on the growth of *S. quadricauda* is in accordance with previous findings in literature (Babica *et al.* 2007; El-Sheekh, Khairy and El-Shenody 2010; Pflugmacher 2002), supporting the argument that MC may potentially be involved in the allelopathic interactions between cyanobacteria and other phytoplankton during interspecific competition (Omidi, Esterhuizen-Londt and Pflugmacher 2018). As an important allelochemical, MC is believed to play a critical role in shaping the overall community structure and phytoplankton succession during harmful freshwater blooms (Figueredo, Giani and Bird 2007). MC's role in allelopathic interactions has been attributed to its potential to inhibit growth, photosynthesis, and induce cellular oxidative stress during interspecific competition (Bittencourt-Oliveira *et al.* 2015; Omidi, Esterhuizen-Londt and Pflugmacher 2018). However, the complex mechanisms behind these adverse effects are yet to be fully understood as existing evidence from previous laboratory studies indicate contrasting effects across MC treatments (Babica, Bláha and Maršalek 2006; Ma *et al.* 2015).

Growth inhibitory effect of crude *Microcystis* extract on *S. quadricauda* in this study was relatively low compared to the effect of purified MC-LR treatments. This may be due to the nature of the crude extract and possibly due to the cumulative interactions among other co-occurring unknown secondary metabolites which may be present in the crude extract (Janssen 2019). Crude cyanobacterial extracts have been shown to comprise of a variety of heterogeneous secondary metabolites produced by cyanobacteria other than MCs (Janssen 2019). The synergistic or antagonistic interactions among these heterogeneous metabolites can potentially mask or compound adverse effects observed during harmful blooms (Ibelings *et al.* 2008; Janssen 2019). However, in contrast to our expectation, neither treatment affected photosynthetic chlorophyll-a content in our study. Chlorophyll-a represents a key antenna pigment responsible for harvesting light and

transfer of excitation energy into photosystems, where solar energy is converted into chemical energy in green algae during photosynthesis (Cheng *et al.* 2015; Krause and Weis 1991). As a reliable biomarker of photosynthetic and respiratory rates in algae, the quantification of chlorophyll-a and other photosynthetic pigments have been widely used to evaluate sensitivity of algae to xenobiotic exposures (Fang *et al.* 2018a; Li *et al.* 2005). As reported in previous studies (Cheng *et al.* 2015; Wei *et al.* 2010), we expected that photosynthetic pigmentation would serve as an early-warning and a more sensitive signal to MC stress compared to growth inhibition. This is because changes in photosynthetic pigments are believed to occur earlier at the molecular level of the cells than growth inhibition during exposure to chemical stressors (Fang *et al.* 2018a). However, in contrast to our expectation, our results indicate that low environmental MC concentrations comparable to those tested in this study are unlikely to affect the ability of algae to synthesise chlorophyll-a pigments during toxic blooms. This observation could be due to the short duration of exposure in the present study and adverse effects on photosynthetic pigments may be observed during a longer period of exposure.

Additionally, MC exposure in the purified MC-LR and crude *Microcystis* extract treatments used in this study varied in their effects on survival and processes in *D.* pulex and *I. elegans*. The observed variations in species' sensitivity to low environmentally relevant MC concentrations among these experimental models may be explained by differences in factors related to assimilation, metabolism, and detoxification mechanisms in these organisms (Kozlowsky-Suzuki, Wilson and da Silva Ferrao-Filho 2012). This finding is consistent with earlier studies that reported no significant effects on survival when *Daphnia* was exposed MC concentrations as low as 3.5 µg/L (Lürling and van der Grinten 2003). However, in this study, our results on survival of *D. pulex* are in contrast with Ferrao-Filho, Azevedo and DeMott (2000) who showed MC concentration as low as 1 µg/L reduced survival. In related studies, Shahmohamadloo *et al.* (2020) and Gene *et al.* (2019) associated low MC concentrations with reduced individual survival among pelagic freshwater zooplankton, *Ceriodaphnia dubia* (LC_{50} = 5.53 µg/L) and juvenile fatmucket mussel, *Lampsilis siliquoidea* (LC_{50} = 2.1 µg/L) during 7-d and 28-d chronic

experiments respectively. Sublethal MC concentrations as low as 2.5 µg/L resulted in impaired reproductive fitness in *C. dubia* (Shahmohamadloo *et al.* 2020). As observed in this study, although low MC concentrations are unlikely to cause acute toxicity in organisms (Kotak *et al.* 1996), prolonged exposure to such concentrations in the environment can potentially exert subtle chronic effects on individual survival, physiological fitness, and key processes, which eventually may propagate across to higher levels of biological organisation (Nilsen *et al.* 2019). Besides, a more realistic environmental scenario in the face of the ongoing global climate change would be such that the effects of sublethal MC concentrations on freshwater species become compounded by increasing water temperature during harmful blooms (Ferrão-Filho and Kozlowsky-Suzuki 2011;Raven and Geider 1988)

4.5.2 Effects of increased environmental temperatures

Increased environmental temperature was associated with reduced survival and ecological processes in our model organisms. In support of the second hypothesis in this study, increased temperature reduced growth and photosynthetic chlorophyll-a content of *S. quadricauda*. These results are consistent with findings in the existing literature on the effects of environmental warming on algae (Chalifour *et al.* 2014; Chalifour and Juneau 2011; Gomes and Juneau 2017; Larras *et al.* 2013). These results suggest warming beyond optimum thermal thresholds can induce adverse physiological changes at molecular (pigment alterations) and cellular levels (growth inhibition) in algae (Gomes and Juneau 2017). Here, the observed growth inhibition of *S. quadricauda* was probably due to induce oxidative stress, reduced cell division and apoptosis at warmer temperatures (Raven and Geider 1988), suggesting that increased temperature from climate change-induced extreme heatwaves can have adverse ecological consequences on population growth, community structure and key ecological processes among freshwater phytoplankton (Burkholder, Shumway and Glibert 2018).

Increased temperature has been hypothesized to accelerate metabolic rate, energy requirement and food intake in ectotherms (Galic, Grimm and Forbes 2017; Brown *et al.*

2004), potentially causing shifts in key processes that affect species survival, abundance, and ecosystem functions (Morgan 2016). In this study, following our expectation and evidence from existing studies (Thompson 1978; Verheyen, Delnat and Stoks 2019), increased temperature had no effect on survival but decreased handling time in *I. elegans*, thereby resulting in increased predation rates. The observed thermal tolerance in *I. elegans* may be due to the short duration of our experiment in this study as animals were only exposed for just 24 hours. It is also possible that the exposure conditions and duration were within thermal preference in these organisms (Brown *et al.* 2004). However, in contrast to our results, reduced survivorship in different developmental stages among freshwater odonates is associated with higher temperatures in previous studies (McCauley *et al.* 2015; Verheyen, Delnat and Stoks 2019). We also observed reduced survival and grazing rates in *D. pulex* at higher temperatures. These results suggest that not only the abundance of these keystone species may potentially be at risk as climate becomes warmer, but more importantly ecological processes that underpin ecosystem functions among key freshwater species may become impaired (Ger *et al.* 2016b). In line with these findings, Müller, Colomer and Serra (2018) observed reduced survival among *D. magna* populations at temperatures above 29°C, whereas filtration rate in *Daphnia* was reduced when the optimal temperature (20°C) was exceeded.

4.5.3 Combined effects of temperature and environmental MC

More ecologically significant and environmentally realistic than the direct individual effects of temperature and MC earlier reported in this study are key findings of their synergistic and antagonistic effects across freshwater taxa. Here, a strong indication of synergistic interaction between increased MC concentration and elevated temperature was associated with reduced survival of *I. elegans*. Warming increases metabolic activities and accelerates the toxicokinetics of chemical stressors in ectotherms (Brown *et al.* 2004; Noyes *et al.* 2009). Perhaps, warming may have increased the rate of MC uptake, assimilation, and toxicity, even at such low environmental concentrations studied here (Buchwalter, Jenkins and Curtis 2003; Kim *et al.* 2014; Kozlowsky-Suzuki, Wilson

and da Silva Ferrao-Filho 2012). Such temperature-mediated toxicity might have been responsible for the 50% decline observed in the survival of *I. elegans* exposed to high MC concentration at 25°C in the present study. In agreement with our experimental results, Walls *et al.* (2018) predicted that the proportion of extracellular MC released into the water column in freshwater bodies during microcystin-producing blooms is likely to be greatest at 25°C. This is consistent with our third hypothesis and corroborates earlier works showing evidence of synergistic interactive effects of MC toxicity and environmental warming on freshwater biota (Kim *et al.* 2014; Lamb, Kimmel and Field 2019; Xiang *et al.* 2017). Therefore, our finding suggests that the population dynamics and key ecosystem functions in freshwater predators may be at great risks under a combined scenario of increased MC toxicity and elevated water temperature during climate-driven freshwater blooms (Paerl and Huisman 2008; Walls *et al.* 2018).

In addition to the synergistic effect on survival, strong evidence of antagonistic interactive effects between MC concentrations and increased temperature were observed on the grazing rate of *D. pulex* and predation rate of *I. elegans*. While increased temperature reduced prey handling time in *I. elegans*, the combined effects of both MC concentrations in the crude extract treatment and increased temperature jointly increased the prey handling time to a greater extent, thus suggesting a further reduction in predation. Similarly, the combined effects of crude extract and increased temperature further increased grazing rates in *D. pulex*. These results are in contrast with our earlier predictions in this study. Existing literatures have shown evidence of synergistic interaction between MC and other environmental stressors at lower concentrations, while antagonistic interactions were observed at higher MC concentrations in other freshwater species (Liang *et al.* 2018; Liang *et al.* 2017a; Wei *et al.* 2020). MCs are rarely found at high concentrations in natural waters, apart from occasional release during bloom senescence or algicide treatment (Chen *et al.* 2013), indicating that a more realistic scenario for toxic blooms in most freshwater bodies is rather a combination of low MC levels with increased environmental warming. Here, our data illustrated that MC at lower concentration significantly reduced the predation rate of the damselfly larva but increased

grazing rate of *D. pulex* in the presence of increased temperature. This suggests that predation and other general body size relate effects on key ecological processes mediated by zooplankton grazers and their predators in freshwater food webs, may become altered during toxic freshwater blooms. These may have potential indirect ecological consequences on species interactions and trophic cascades, which may likely propagate to other trophic levels within aquatic food webs (Burkholder, Shumway and Glibert 2018; Knight *et al.* 2005). Additionally, the results of the present study suggest that animals with larger body size are more likely to be adversely affected by the cumulative effects of MC concentrations and increased temperature. Therefore, there is need for future studies to evaluate the effects of these stressors on individuals with similar size range and functional groups.

4.5.4 Wider ecological impacts

Our results suggest that environmental exposure to cyanotoxins may affect survival and ecological processes in species-specific ways across different components of the aquatic food web. These species-specific effects may alter relative abundance of key freshwater taxa and have profound ecological implications on the strength of species interactions within freshwater food webs. Furthermore, as climate change intensifies, water temperature and toxin burdens in freshwater bodies are expected to rise, resulting in complex, non-linear interactive effects on individual species as observed in this study (Glibert 2017; Walls *et al.* 2018). The knock-on effects of these interactions on individual species may propagate through populations and communities via the food web, resulting in complex shifts in community structure and species interactions (Burkholder, Shumway and Glibert 2018). For instance, as observed in this study, changes such as, algal growth inhibition and increased zooplankton grazing may potentially exert both negative bottom-up and top-down effects on trophic cascades. Higher algal growth inhibition would imply a decrease in phytoplankton biomass for primary production which may become further complicated by increased grazing pressure by zooplankton. Although the observed increase in the predation rate in *I. elegans* at higher temperature may be associated with

negative top-down effects on the zooplankton population, however, these effects may be offset as survival and abundance may become reduced in a joint scenario of thermal and toxin stress.

4.6 Conclusion

Here, we demonstrate using a suite of laboratory microcosms that increased temperature and environmentally relevant toxin exposure can affect survival and ecological processes in key freshwater species. We showed that low environmental MC concentrations adjudged to be safe for human health by the WHO can have significant species-specific effects on survival and ecosystem functions across key freshwater taxa. Importantly, a more ecologically relevant effect on freshwater communities may become apparent when low environmental MC exposure coincides with increased water temperature in eutrophic waters. Our results provide the first evidence of significant synergistic and antagonistic interactive effects of temperature and low environmental MC exposure on survival and feeding in key freshwater invertebrates. Moreover, an important key finding in our study suggests that synergy between increased temperature and low environmental MC concentrations would have potential indirect ecological implications on trophic networks, which may cascade to other trophic levels. Overall, given the increasing pace of global change, our findings highlight the need to prioritize further research towards understanding and predicting the ecological impacts of cyanobacterial toxins as an important stressor in freshwater ecosystems.

Chapter 5

Direct and indirect ecological effects of microcystin exposure on structure and function of experimental freshwater ecosystems

5.1 Abstract

Freshwater cyanobacterial blooms and their associated microcystin production are a major environmental, socio-economic, and ecological stressor in freshwater bodies worldwide. However, besides adverse effects on human and animal health, the potential ecological consequences of microcystin exposure on aquatic biota and ecosystem functioning are rarely known at higher levels of biological organisations, such as communities and ecosystems. Here, I present a first report on the ecological effects of long-term sublethal microcystin exposure on the structure and functioning of freshwater ecosystems. Thirty-two experimental microcosms were established in the laboratory for two weeks and used as model freshwater ecosystems to monitor the direct and indirect effects of three microcystin treatments (*Microcystis aeruginosa* culture, crude *Microcystis* extract and purified MC-LR) at environmentally relevant concentrations comparable to the WHO guidelines on indices of community structure (phytoplankton biomass, zooplankton and invertebrate abundance) and ecosystem functioning (primary productivity, predation and organic matter decomposition). Except for dissolved oxygen (DO) concentration, water quality variables varied significantly across the treatment and the control microcosms. While both *Microcystis* culture and increased concentration of MC-LR equivalent in the crude extract significantly reduced chlorophyll-a concentration (phytoplankton biomass), no effect of the purified MC-LR was observed. *Daphnia magna* and *Chironomus* spp, were lost from all the treatment and control microcosms, suggesting that this effect was unrelated to microcystin treatments, thereby limiting the strength of the potential inference on indirect community-level effects in this study. However, increased concentrations of crude extract and purified MC-LR significantly reduced the rate of invertebrate-mediated organic matter decomposition whereas *Microcystis* culture

treatment significantly increased the rate of microbial organic matter decomposition relative to the control. This study suggests the consequence of these effects could be complex because direct effects of MC exposure may become compounded by water physico-chemical parameters, such as elevated water pH during harmful freshwater blooms.

5.2 Introduction

Freshwater harmful cyanobacterial blooms are a serious ubiquitous environmental, socio-economic, and ecological stressor in many aquatic systems worldwide (Harke *et al.* 2016; Ibelings *et al.* 2008; Cai *et al.* 2021). *Microcystis aeruginosa,* the predominant toxigenic cyanobacterial species in freshwaters (Rastogi, Sinha and Incharoensakdi 2014), has increased in its frequency of occurrence, magnitude of bloom size and geographic range under a combination of human-induced climate change and widespread eutrophication (Cai *et al.* 2021; Harke *et al.* 2016). Moreover, *Microcystis* blooms have been widely associated with the occurrence of cyanobacterial toxins, such as MC and other secondary metabolites in freshwater bodies globally (Walls *et al.* 2018; Porojan *et al.* 2020a). The expansion of MC-producing blooms in freshwater lakes, rivers and reservoirs used for drinking and recreational purposes have caused considerable public health and socio-economic concerns (Brooks *et al.* 2016). More than 75% of such blooms have been linked with severe adverse effects on human and animal health (Harke *et al.* 2016; Wood 2016) with an average annual economic loss valued at US$4.6 billion in the US alone (Dodds *et al.* 2009). However, an increasing fundamental concern among aquatic ecologists is that the consequence of MC exposure on aquatic biota and ecosystem functioning is yet to be fully understood (Briland *et al.* 2020; Ibelings *et al.* 2008).

MC has demonstrated a wide range of acute, sublethal and chronic adverse effects on aquatic species, such as fish (Isibor 2017; Ibrahem *et al.* 2012), zooplankton (DeMott, Zhang and Carmichael 1991; Smutná *et al.* 2014) and other benthic invertebrates (Cai *et al.* 2021; Shahmohamadloo *et al.* 2020) as well as algae (Babica *et al.* 2007) in the laboratory. While these studies suggest individual-level effects may vary from reduced

survival, feeding and life-history traits in Daphnia and other zooplankton (DeMott, Zhang and Carmichael 1991; Liang *et al.* 2020; Ghadouani *et al.* 2004), growth inhibition in freshwater algae (El-Sheekh, Khairy and El-Shenody 2010), to bioaccumulation and impaired physiological fitness in fish and benthic invertebrates (Toporowska, Pawlik-Skowronska and Kalinowska 2014; Isibor 2017), such effects have been mostly observed at higher purified toxin concentrations during laboratory exposures, above what may naturally persist for a long time in freshwaters (Chen *et al.* 2017). However, beyond the evidence of direct effects from single-species laboratory studies (Bownik 2016; Martins and Vasconcelos 2009), toxin bioaccumulation (but not biomagnification see (Ibelings *et al.* 2005) and potential trophic transfer of MC across different freshwater food web taxa have also been reported (Poste and Ozersky 2013; Kozlowsky-Suzuki, Wilson and da Silva Ferrao-Filho 2012), suggesting there may be potential indirect effects of MC at higher levels of biological organisation, such as communities and ecosystems.

Communities are important intermediates between populations and ecosystems in the hierarchy of biological organisation (Clements and Rohr 2009) and may represent a suitable level for investigating the ecological effects and fate of microcystin and other chemical stressors on freshwater ecosystems (Clements and Rohr 2009; Ibelings *et al.* 2008). Although single-species laboratory experiments may provide early-warning signals of toxin exposure (Brooks, Gaskell and Maltby 2009), they are usually limited because such studies can only predict direct effects of MC on sensitive taxa, including reduced abundance or elimination of sensitive populations, which are usually easy to interpret (Clements and Newman 2003; Burkholder, Shumway and Glibert 2018). Community studies are rather more difficult to interpret and may integrate long-term direct effects of MC exposure with more subtle indirect effects on species-interactions, such as resource-consumer interactions that drive energy and toxin transfer across different trophic levels, thus influencing ecosystem functioning (Clements and Newman 2003; Gardham, Chariton and Hose 2015). For instance, long-term sublethal MC exposure may lead directly to increased mortality or total elimination among top predators, which may potentially increase zooplankton grazing pressure on phytoplankton

biomass, thereby reducing primary productivity (Coll and Hargadon 2012). The consequences of such indirect effects may become profound, potentially cascading to other trophic levels under long-term MC exposure during freshwater blooms (Burkholder, Shumway and Glibert 2018). However, there is currently little or no empirical information regarding the potential effects of long-term MC exposure on the fundamental structure and function of freshwater ecosystems at the community level (Ibelings *et al.* 2008; Burkholder, Shumway and Glibert 2018).

The use of manipulated model aquatic ecosystems, such as experimental microcosms and mesocosms may offer a reliable empirical approach to understand community-level ecological response of freshwater ecosystem structure and functions to microcystin and other chemical stressor exposure (Ibelings *et al.* 2008; Clements and Newman 2003). Although single-species laboratory studies have been shown to rarely reflect potential ecological impacts of stressors on communities and ecosystems (Brooks, Gaskell and Maltby 2009), data obtained from field observations may be difficult to interpret because of the problem of complexity, poor replication and highly variable environmental conditions involved (Daam, Van den Brink and Nogueira 2008). In contrast, experimental microcosms have been shown to provide a simplified approximation of the ideal natural conditions in freshwater ecosystems (Pestana *et al*. 2009), where manageable hypotheses may be tested with higher power of statistical replication and control (Barry and Logan 1998). Increasing evidence has demonstrated the potential of laboratory microcosms as reliable empirical tools in disentangling direct effects of long-term exposure to a variety of environmental stressors from their subtle ecologically relevant indirect effects at the community level (Gardham, Chariton and Hose 2015; Daam *et al.* 2010; Rico *et al.* 2014; Lamonica *et al.* 2016; Choung *et al.* 2013). However, to date, little is known about the indirect long-term ecological effects of sublethal MC exposure on community structure and ecosystem functioning in freshwaters (Ibelings *et al.* 2008; Burkholder, Shumway and Glibert 2018).

Therefore, to fill this gap in knowledge, the present study was designed to evaluate the long-term ecological effects of sublethal MC exposure on measures of community structure (such as, phytoplankton biomass, invertebrate abundance, and community composition) and ecosystem functioning (primary productivity, predation, and organic matter decomposition). To achieve this objective, I established 32 artificial communities in experimental microcosms used as model freshwater ecosystems in the laboratory for two weeks. Each community microcosm consisted of representatives of six freshwater food web taxa: *Scenedesmus quadricauda* (phytoplankton), *Daphnia magna* (zooplankton grazers), dipteran larvae *Chironomus spp* (benthic detritivore), *Gammarus pulex* (amphipod detritivore) and damselfly larvae, *Ischnura elegans* (predators). The test organisms in this study were selected because they are naturally found to occupy significant positions in the aquatic food webs in many European freshwater bodies (Šulčius *et al.* 2017; Kennedy *et al.* 2002; Kenna *et al.* 2017). In addition, these species have been widely used in many ecotoxicological tests as sensitive and reliable experimental organisms to a wide range of chemical stressors (Heugens *et al.* 2003; Boets *et al.* 2012). Using three different types of MC treatments and environmentally relevant concentrations; *Microcystis aeruginosa* culture, crude *Microcystis* extract (0.01 and 0.1 µg/L) and purified MC-LR (0.01, 0.1, 1, and 10 µg/L), the following hypotheses were tested (a) based on the results from short-term single-species studies (DeMott, Zhang and Carmichael 1991; Pflugmacher 2002), increased MC concentrations were expected to inhibit phytoplankton biomass and primary productivity as well as reduce the abundance of invertebrate populations, (b) direct lethal and sublethal effects of MC on invertebrate population abundance were expected to reduce the rate of invertebrate-mediated coarse-particulate organic matter decomposition (CPOM) with increasing treatment concentrations and increase the rate of microbial activity on fine-particulate organic matter decomposition (FPOM) and (c) direct effects on population abundance in a particular taxa is expected to affect community structure and ecological processes mediated by the taxa.

5.3 Material and Methods

5.3.1 Collection and Maintenance of Study Organisms

Representatives of five freshwater species across different functional groups were collected and used as study organisms to establish artificial communities in the laboratory using model freshwater ecosystems (microcosms). These include the green alga, *Scenedesmus quadricauda* (phytoplankton), the micro-crustacean, *Daphnia magna* (zooplankton grazers), dipteran larvae *Chironomus spp* (benthic detritivore), *Gammarus pulex* (amphipod detritivore) and damselfly larvae, *Ischnura elegans* (predators).

Stock cultures of *Microcystis aeruginosa* (CCAP/1450/16) and *S. quadricauda* (A950) were obtained from the Culture Collection for Algae and Protozoa (CCAP), Scottish Marine Institute and Sciento Stores in Manchester, UK, respectively. *D. magna* and *Chironomus spp* were purchased from Sciento Stores and Northampton Reptile Centre, Northampton, UK respectively. *G. pulex* and *I. elegans* were sampled from a slowly flowing stream at the Meanwood Park (53°50′N; 1°35′W) and "None-Go-Bye- Farm" (53°52′N; 1°36′W) in Leeds respectively.

G. pulex was acclimated in aged, dechlorinated tap water and maintained for 7 days in a CT room conditioned at 15±1ºC and 14 h:10 h light: dark photoperiod and dried alder leaves (*Alnus glutinosa*) were supplied as food material before the experiment. *I. elegans* individuals were maintained in 250 mL plastic tanks containing 100 mL of aged, dechlorinated tap water and kept at 20±1ºC and 14 h: 10 h light: dark photoperiod cycle in a CT room, while animals were fed *ad libitum* with *Daphnia magna*, and wooden dowel rods were supplied to provide perch for the larvae. *Microcystis aeruginosa* and *S. quadricauda* were maintained aseptically in the laboratory in 250 mL Erlenmeyer flasks, filled with 150 mL of sterile BG 11 medium and conditioned at 25±1ºC, 25µmol quanta $m^{-2}s^{-1}$ and 12 h: 12 h light/dark photoperiod cycle. *M. aeruginosa* cultures were harvested at the stationary growth phase, freeze-thawed thrice and centrifuged for 10 minutes at 7500 rpm and 4°C. The pellets were resuspended with deionized water, freeze-thawed

again and centrifuged for 10 minutes. The supernatants were decanted and assayed for total MC content with an MC-coated ELISA kit, while the pellets were discarded. Absorbance values were read at 450nm, analysed using a 4-parameter logistic regression and the total MC content in the samples were presented as MC LR concentration (µg/L).

5.3.2 Chemicals

Purified microcystin, MC-LR (CAS No. 101043-37-2 and ≥ 95% purity) was obtained from Cayman Chemical Company, UK. Stock solution of the purified MC-LR was prepared by dissolving 1 mg of the toxin into 1 mL of methanol and made up to 1 Litre with milli-Q® water, following the procedure described by Dao *et al.* (2018). Aliquot solutions were made by serially diluting the stock solution in the magnitude orders of 10 to obtain a range of nominal treatment concentrations for this experiment. The stock and aliquot solutions were stored in a -20 ºC freezer until the time of application. The commercial microcystin ELISA kits (CAT No. ALX-850-391-K101) for microcystin quantification were supplied by Enzo Life Sciences, UK, while methanol and other chemical reagents used in this study were of purity ≥ 95%.

5.3.3 Leaf-Litter Bag Techniques and Organic Matter Decomposition

Leaves of the UK native riparian Alder plant (*Alnus glutinosa*) were collected in October 2018, during autumn leaf fall around the Chancellor's Court of the University of Leeds, Leeds (53.8046° N, 1.5566° W). The leaf materials were air-dried in the laboratory for four weeks and stored in cardboard boxes until the commencement of this experiment. Sixty-four replicates of leaf litter bags, comprising of 32 coarse-mesh (10 × 10 cm^2, mesh size: 10 mm) and 32 fine-mesh bags (10 × 10 cm^2, mesh size: 0.5 mm) were constructed, while each litter bag was filled with 1.50g of dried and pre-weighed *A. glutinosa* leaves. All leaf packs were pre-conditioned for 48 hours in stream water obtained from Meanwood Beck to enhance microbial activity and leaching of toxic metabolites before the leaf packs were introduced into each microcosm. For the purpose of this study, the effects of MC treatments on organic matter decomposition were evaluated by gently

introducing one coarse-mesh and one fine-mesh leaf bags into each microcosm. The coarse-mesh leaf bags in this study measured the effects of treatment on invertebrate mediated coarse particulate organic matter (CPOM) decomposition while the fine-mesh leaf bags were used to assess the effects on microbe-mediated fine particulate organic matter (FPOM) decomposition. The leaf bags were left in each microcosm and animals were allowed to feed until the experiment was terminated after two weeks.

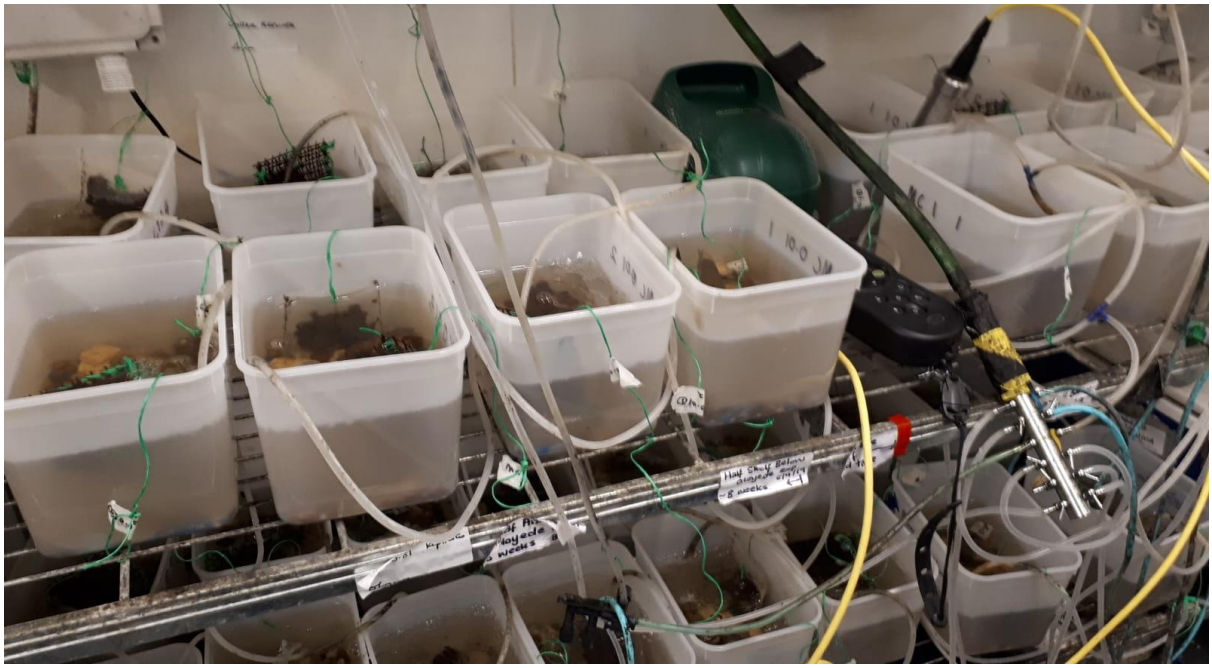

Figure 5.1: Experimental microcosm setup

5.3.4 Microcosm Establishment and Experimental Design

Thirty-two (32) artificial microcosms were set up and established in a controlled temperature (CT) room in the laboratory for five weeks (See Figure 5.1). Each microcosm consisted of a 4-L plastic ice cream container (22.1 cm height ×15.8 cm width ×15.0 cm length) filled with 3 L of aged, dechlorinated tap water and 0.5 L of stream water to provide nutrient and microbial inoculation. Each container was furnished with 400g of aquarium gravels and pebbles to serve as substrates, while continuous aeration was

supplied for 14 days to allow de-chlorination of the tap water used as experimental medium. The microcosms were individually cross seeded with one another to allow homogeneity of the experimental medium before introducing the study organisms. Each microcosm was stocked with representatives of the study organisms by gently seeding each container consecutively with the following organisms: 3 mL of *S. quadricauda* culture (ca. 5.03×10^6 cells per mL) to stimulate algal growth, 20 adult individuals of *D. magna*, 15 individual of *Chironomus spp.*, 10 individuals of *G. pulex* and 3 individuals of *I. elegans* larvae. The communities were allowed to acclimate for four days in each container before introducing the leaf packs and the microcystin treatments. Two leaf packs, comprising of one coarse-mesh and one fine-mesh litter bags were administered to each microcosm alongside with the MC treatments. Three different MC treatments namely, purified MC LR (MC-LR), MC-containing crude *Microcystis* extract, and laboratory simulated blooms of the cyanobacterium, *M. aeruginosa* were tested at different concentrations with control. Four treatment concentrations (0.01, 0.1, 1.0, and 10.0 µg/L) of the purified MC LR, two concentrations (0.01and 0.1µg/L) of the MC-containing crude extract and one simulated bloom treatment with biomass concentration of 6.38×10^4 cells per mL with control were all tested with four replicates per treatment concentration and control. The experiment lasted for additional two weeks after the application of the MC treatments while all the microcosms were maintained in a CT room conditioned at 15±1ºC and 14 h:10 h light: dark photoperiod cycle. All microcosms were covered with net to prevent loss of animals, and aerated throughout the experiment, water physico-chemical parameters such as water temperature (in °C), dissolved oxygen (DO in mg/L), pH and conductivity (in S/cm) were measured weekly and at the end of the experiment before collection of organisms using Hach portable Multi-Parameter Meter (HQ40D53000000 HQ40d). The experiment was terminated two weeks after the application of the treatment, all leaf packs were retrieved, gently rinsed with tap water and the remaining leaf material were transferred into foil cups and dried at 60 ºC for 48 hours in a drying oven. Oven-dried leaf materials were weighed to the nearest mg and the leaf mass losses were calculated for the invertebrate and microbe-mediated leaf-litter

decompositions. Representatives of each taxon were gently removed with the aid of a mesh sieve and the number of survivals per taxa were estimated before storage in 70% ethanol. The number of adult emergences, moulting and exuviae among *I. elegans* and *G. pulex* were enumerated and recorded (See Table 5.1). Water samples were collected in 50 mL falcon tubes from each experimental microcosm, sieved through a 45mm diameter glass fibre (GF/D) filters with 1.6 µm pore size and the phytoplankton chlorophyll-a concentrations as surrogate estimate of the phytoplankton abundance were determined by spectrophotometry using methanol extraction method described by Fang *et al.* (2018b) and Li *et al.* (2005).

5.3.5 Statistical Analyses

Water quality parameters and phytoplankton chlorophyll-a data were individually subjected to a series of Gaussian general linear model (GLM) and tested for the assumptions of normally distributed residuals with Shapiro Wilk test for normality. Non-normally distributed residuals were transformed by adding square-root or natural log-transformation terms to the fitted Gaussian general linear models (GLM) which were used to test for significant differences across treatments. The invertebrate-mediated organic matter decomposition (in mg of leaf mass loss per mg of animal per day) and the microbial organic matter decomposition (in mg of leaf mass loss per day) were estimated from the leaf mass loss in coarse-mesh and fine-mesh leaf litter bags respectively. The two datasets were checked for normality and homogeneity of variance assumptions before fitting a GLM model for hypothesis testing for significant differences across the treatments and control. All analysed datasets (including the water quality parameters, chlorophyll-a content of the phytoplankton and the rate of organic matter decomposition) were summarized and visualised as descriptive statistics (mean ± standard error of means). The community response data were expressed as the proportion of individuals surviving, which was used as a surrogate measure of abundance across the different populations in each community microcosm. A GLM binomial regression model was fitted to test for the significant difference in the survival of individuals among populations in the control and

treatments. The survival data were summarised and expressed as mean ± 95 % confidence interval (CI). The 95% CIs were calculated from the proportion of surviving individuals in all experimental microcosms using the *PropCIs* package (Scherer 2018) in R. All statistical analyses were performed at significance level of ($p \leq 0.05$) using R 3.6.0 (R CoreTeam 2019), while all data visualizations and figures were made in Origin Pro version 2020 (OriginPro 2020).

5.4 Results

5.4.1 Water quality parameters

The mean water temperatures in the control and treatment microcosms recorded in this study ranged from 14.53 ±0.025 ºC to 15.18 ±0.207 ºC (Figure 5.2A). Mean water temperature reduced significantly in microcosms treated with *Microcystis* culture (t = -3.51; df = 31; p = 0.002; Figure 5.2A), and crude extract (t = -3.58; df = 31; p = 0.001; Figure 5.2A) with higher purified MC-LR concentrations resulting in significant decreases in the mean water temperature compared to the control (t = -2.82; df = 31; p = 0.031; Figure 5.2A). The mean dissolved oxygen (DO) concentrations measured across all the experimental microcosms ranged from 10.31±0.011 mg/L to 9.77±0.280 mg/L (Figure 5.2B). Although the DO concentrations across all the experimental microcosm followed a relatively stable pattern (Figure 5.2B), there was a significant reduction in DO at the lowest crude extract (0.01 µg/L; t = -7.14; df = 31; p < 0.001; Figure 5.2B) and the highest purified MC-LR concentrations (10 µg/L) relative to the control (t = -14.25; df = 31; p < 0.001; Figure 5.2B). The mean water pH values measured in experimental microcosms fluctuated between 7.87±0.03 and 8.07±0.03 across all the treatments and control (Figure 5.2C). There was an evidence of significant declines in the mean water pH values at the lowest concentration of crude extract (0.01 µg/L; t = -7.14; df = 31; p = 0.0034) and higher concentrations of the purified MC-LR (1 µg/L; t = -2.15; df = 31; p = 0.042 and 10 µg/L; t = -5.57; df = 31; p < 0.001) compared to the control (Figure5.2C).The mean electrical conductivity values varied significantly across all the

treatments and the control, ranging from the lowest value of 456 ±5.19 µS/cm in microcosms exposed to 10 µg/L concentration of the purified toxin to 854±22.78 µS/cm in the *Microcystis* culture. Except in microcosms exposed to higher concentrations of the crude extract (z = -0.37; df = 31; p = 0.712; Figure 5.2D) and *Microcystis* culture, where conductivity was significantly increased relative to the control (z = 14.52 df = 31; p < 0.001; Figure 5.2D), conductivity values significantly reduced across all crude extract (z = -3.01; df = 31; p = 0.003; Figure 1D) and purified MC-LR treatments (z = -6.87; df = 31; p < 0.001; Figure 5.2D) compared to the control.

5.4.2 Chlorophyll-a

Phytoplankton chlorophyll-a concentrations varied significantly in the microcosms exposed to *Microcystis* culture (t = -2.86; df = 31; p = 0.009) and crude extract (t = -3.81; df = 31; p = 0.001) compared to the concentrations measured in the control (Figure 5.3). Increased MC concentration in the crude extract treatment was associated with significant reduction in chlorophyll-a concentrations (p < 0.01; Figure 5.3) while increased concentrations of the purified MC-LR treatment showed no evidence of significant effects on chlorophyll-a concentration relative to the control (t = 0.171; df = 31; p = 0.865; Figure 5.3).

5.4.3 Organic matter decomposition

The relative leaf mass losses measured in the coarse and fine-mesh leaf bags ranged from 12.9 to 51.9% and 8.1 to 37.6% respectively in all the treatments and control microcosms throughout the duration of exposure. A significant, linear decrease was observed in the mean amphipod-associated organic matter decomposition rates across the crude extract and purified MC-LR treatments relative to the control ($F_{3, 28}$ = 8.93; p < 0.001; Figure 5.4A). Increased concentrations of the crude extract (t = -3.66; df = 31; p = 0.001; Figure 5.4A) and purified MC-LR treatments (t = -3.43; df = 31; p = 0.002; Figure 5.4A) significantly reduced amphipod shredding rates (expressed as mg leaf mass loss/amphipod/day) compared to the control. However, no significant difference was

observed in the mean leaf shredding rates of amphipods in microcosms treated with *Microcystis* culture relative to the control (t = -0.009; df = 31; p = 0.993; Figure 5.4A), suggesting *Microcystis* culture had no effect on the rate of invertebrate-mediated organic matter decomposition. The mean values of microbe-mediated organic matter decomposition rates (in mg leaf mass loss per day) varied significantly across experimental treatments ($F_{3, 28}$ = 11.15; $p < 0.001$) and concentrations ($F_{1, 27}$ = 11.20; p = 0.002) when compared with the control (Figure 5.4B). Experimental microcosms exposed to *Microcystis* culture (t = 3.72; df = 31; p = 0.001) and purified MC-LR concentration (1.0 µg/L; t = 3.23; df = 31; p = 0.004) were associated with a significant increase in the mean rates of microbial organic matter decomposition relative to the control (Figure 5.4B). However, the mean values of decomposition rate reduced significantly in microcosms exposed to crude extract at 0.01 µg/L (t = -4.93; df = 31; $p < 0.001$; Figure 5.4B) and purified MC-LR 10.0 µg/L concentrations (t = -2.58; df = 31; p = 0.017; Figure 5.4B) when compared to the control.

5.4.4 Community level effects

Four invertebrate taxa, comprising *Chironomus spp*, *D. magna*, *G. pulex* and *I. elegans* were introduced into each microcosm prior to the treatment application. The data survival and adult emergence of *I. elegans* across different treatments and replicate tanks are presented in Table 5.1. There was an overall decline in population abundance among each taxon in the control and treatment microcosms. Only 35.9% of *G. pulex* and 32.3% *I. elegans* populations survived till the end of the experiment. Survival proportion among *Chironomus* population was as low as 0.41% while no evidence of survival was found among *D. magna* population when the experiment was terminated, suggesting *D. magna* and *Chironomus* were the most affected populations among the exposed communities. However, given the observed elimination and complete disappearance of *D. magna* and *Chironomus* populations, as well as the marked decline in survival of *G. pulex* (Figure 5.5A) and *I. elegans* (Figure 5.5B) in the control relative to the treatment, it is unlikely that the observed response in this study was related to MC treatment. Contrary to

expectations, higher concentrations of the crude extract (0.1 µg/L; z = -3.92; df = 31; p = 0.001) and purified M-CLR treatments (z = -3.09; df = 31; p = 0.002) were associated with significant increase in survival proportion of *G. pulex* relative to the control (Figure 5.5A). However, survival proportions of *G. pulex* in *Microcystis* culture treatment and in lower concentrations of the extract and purified toxin treatments did not differ significantly from that of the control ($p > 0.05$; Figure 5.5A). The survival proportion of *I. elegans* in all the treatment microcosms showed no evidence of significant difference when compared to the control ($p > 0.05$; Figure 5.5B).

Table 5.1: Survival and adult emergence of I. elegans larvae across treatments and replicates

Treatment	**Concentrations**	**Number of replicates**	**Initial number of larvae**	**Adult emergence**	**Mortality**	**Survival**
Control	0	4	12	1	9	2
Microcystis culture	0	4	12	4	7	1
Crude Extract	0.01	4	12	0	5	7
Crude Extract	0.1	4	12	2	7	3
Purified MC-LR	0.01	4	12	3	6	3
Purified MC-LR	0.1	4	12	0	8	4
Purified MC-LR	1	4	12	1	6	5
Purified MC-LR	10	4	12	0	6	6

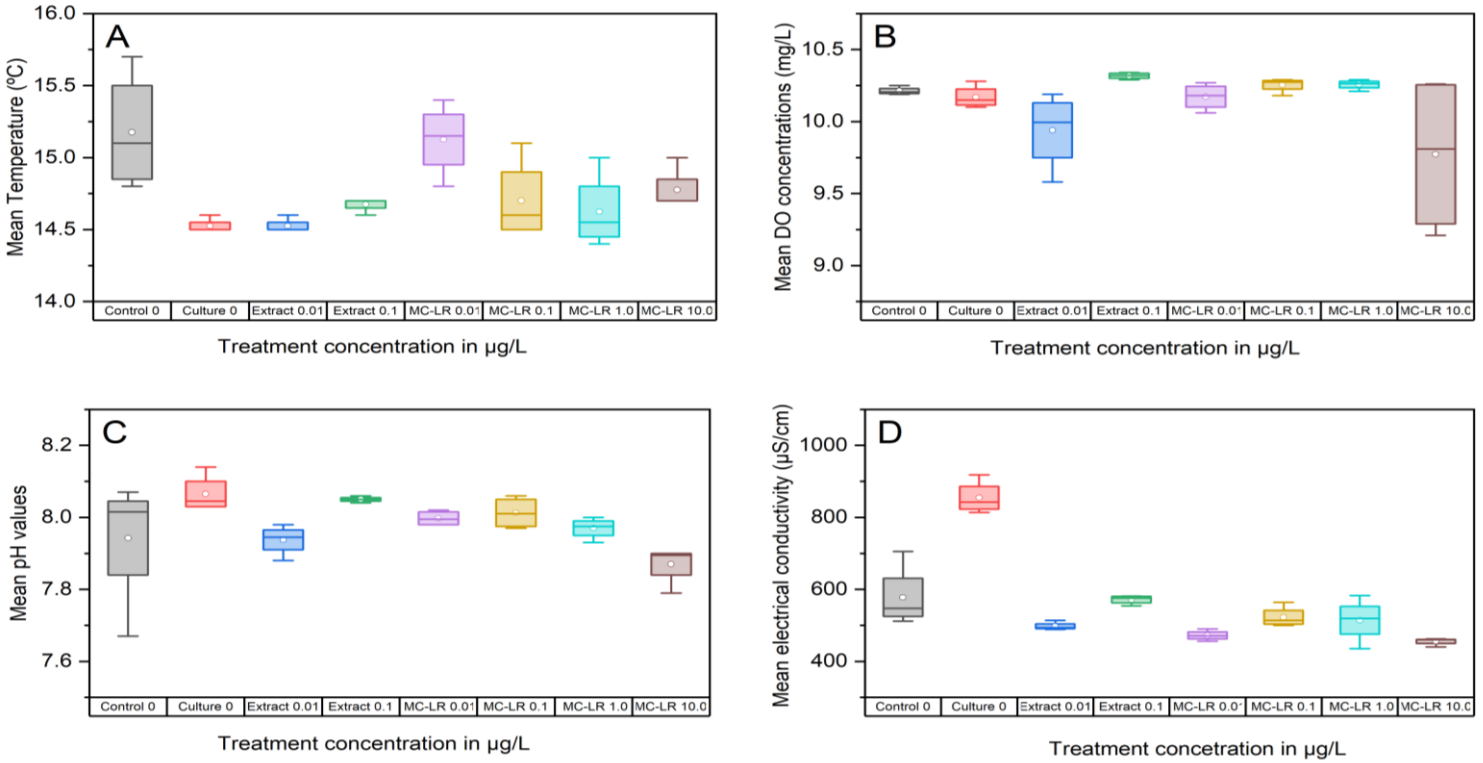

Figure 5.2:Water quality parameters measured across all treatments and control during the experiment. Note that mean (±SE) (A) water temperature; (B) dissolved oxygen concentrations (DO, mg/L), (C) pH and (D) electrical conductivity (µS/cm) values are significant if $p < 0.05$.

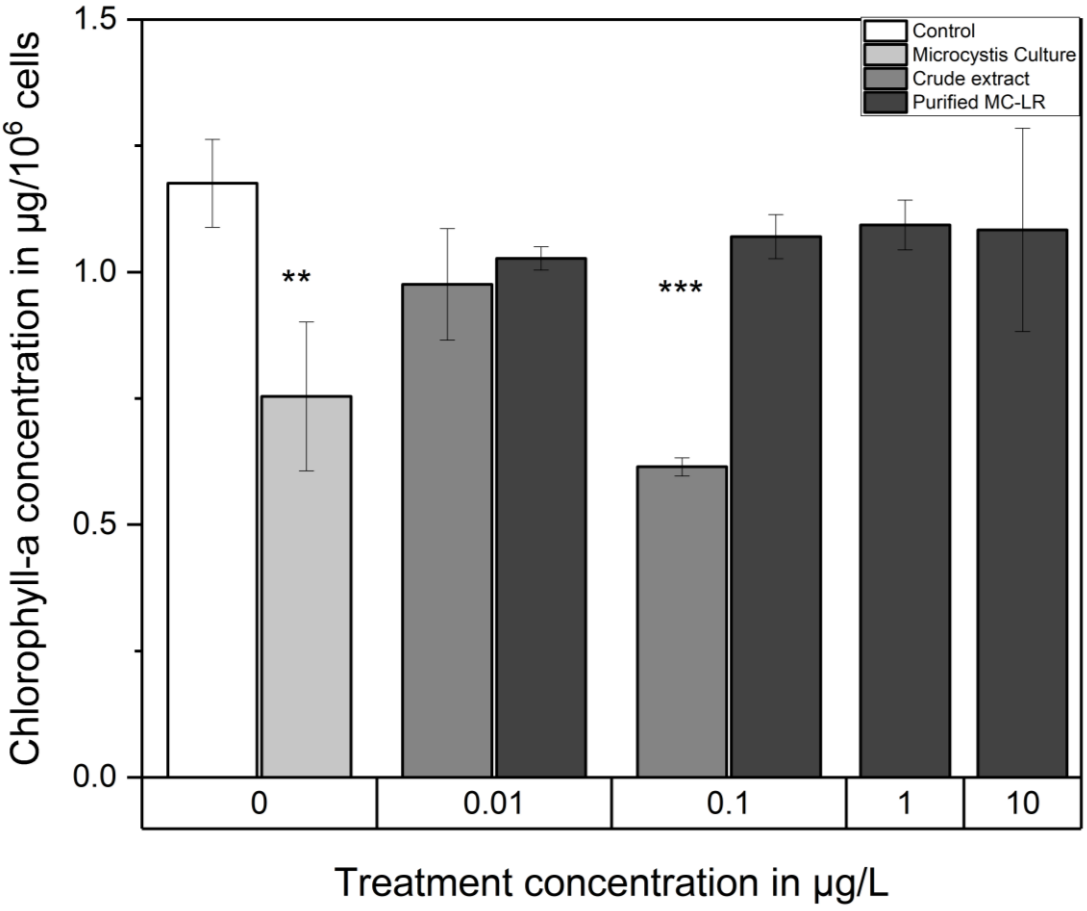

Figure 5.3: The mean (±SE) concentration of chlorophyll a measured in water samples obtained from the control, experimental treatments, and concentrations across all microcosms. Note that mean ±SE are significant if $p < 0.05$.

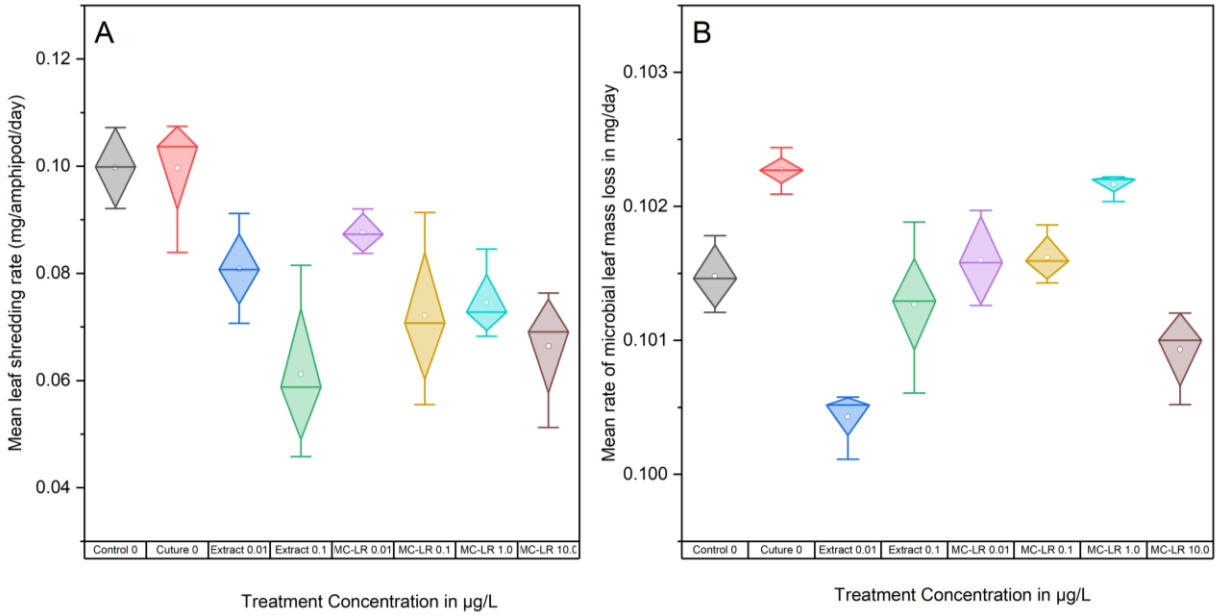

Figure 5.4: (A) The mean (±SE) leaf shredding rate of *G. pulex* per day measured in the coarse-mesh size leaf bags in the control and treatment microcosms. (B) The mean (±SE) rate of microbe-mediated leaf mass loss measured in the fine-mesh size leaf bags in the control and treatment microcosms.

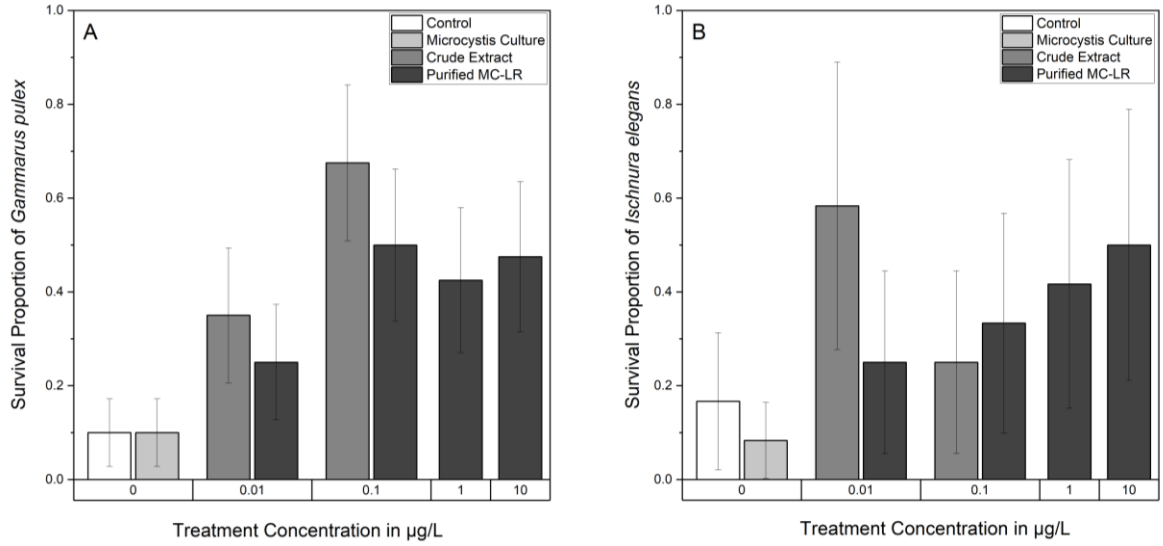

Figure 5.5: The proportion of survival and ± 95% CI among (A) native amphipod population, *G. pulex* and (B) damselfly larval predator, *I. elegans* in the control and experimental microcosms at the end of 14-day exposure.

Discussion

Human-induced climate change and widespread eutrophication associated with environmental changes have intensified the incidence and frequency of microcystin-producing cyanobacterial blooms in freshwaters worldwide (Harke *et al.* 2016). However, the ecological consequence of this phenomenon on aquatic biota and ecosystem functioning is rarely known at higher levels of biological organisations, such as community and ecosystems (Briland *et al.* 2020; Ibelings *et al.* 2008). Beyond evidence of direct effects of MC from single-species laboratory studies (Bownik 2016; Martins and Vasconcelos 2009), the potential long-term effects of sublethal MC exposure on community structure and functions in freshwater ecosystems are still poorly understood (Ibelings *et al.* 2008; Burkholder, Shumway and Glibert 2018). Therefore, I present here one of the first laboratory reports of the community-level effects of sublethal MC exposure on ecosystem structure and functioning using model freshwater ecosystems. The results showed that although some of the effects observed on community structure and ecosystem functioning did not fully support the initial hypotheses in this study, there were a few significant effects of the treatment on water quality parameters, phytoplankton biomass and microbial organic matter decomposition. Moreover, the results on invertebrate community response and organic matter decomposition showed weak, albeit statistically significant effects, which are unlikely to be related to MC treatments. Therefore, these results suggest that indirect community responses to cyanotoxin exposure may be complex and there is a need for caution as the strength of inferences from the present study may be limited. This problem notwithstanding, the potential implications of these results have been discussed in the light of other findings from relevant literature below.

In contrast to the initial prediction that MC may inhibit phytoplankton biomass and primary productivity by reducing chlorophyll-a concentration, only *Microcystis* culture and increased concentrations of the crude extract reduced chlorophyll-a concentrations,

while increased purified MC-LR concentrations had no effect. The present results showed MC may not directly be involved in the observed inhibitory effects on phytoplankton chlorophyll-a concentrations in the experimental microcosms exposed to *Microcystis* culture and crude extract treatments. This evidence is in contrast with the putative explanation that allelopathic growth inhibition among phytoplankton communities during interspecific competition may be among the key ecological functions MC play in freshwaters during harmful blooms (Pflugmacher 2002; Omidi, Esterhuizen-Londt and Pflugmacher 2018; Babica, Bláha and Maršalek 2006). However, while there has been contrasting evidence regarding whether MC may be implicated as the sole allelochemical driving interspecific competition and community dynamics during harmful freshwater blooms (Babica, Bláha and Maršalek 2006; Babica *et al.* 2007), several studies have argued that these effects may be due to toxic cyanobacterial secondary metabolites other than MCs (Janssen 2019; Ma *et al.* 2015). In the present study, increased crude extract concentrations reduced chlorophyll-a content, suggesting phytoplankton growth inhibition. In accordance with previous studies (El-Sheekh, Khairy and El-Shenody 2012; Jungmann 1992; Jungmann and Benndorf 1994), the observed negative effects in this study were possibly due to the potential individual or interactive effects of other secondary metabolites present in the culture and extract treatments. This evidence is potentially representative of the realistic cyanotoxin exposure scenarios during freshwater harmful blooms, and it is consistent with earlier studies (Pietsch *et al.* 2001; El-Sheekh, Khairy and El-Shenody 2010; Babica *et al.* 2007), suggesting aquatic species may differ in their sensitivity depending on the nature of the allelochemical involved that demonstrated higher inhibitory effects (El-Sheekh, Khairy and El-Shenody 2010).

In this study, a marked decline was observed in the proportion of survival across all the four invertebrate taxa, suggesting a reduced population abundance and a potential shift in the community structure. Among the four taxa studied, *Daphnia* and *Chironomus* were mostly affected, with a complete elimination of the former, and only a negligible survival (0.41%) among the latter. Previous studies have associated MC accumulation in *Daphnia* grazers (Thostrup and Christoffersen 1999) and benthic *Chironomus* larvae (Toporowska,

Pawlik-Skowronska and Kalinowska 2014) with reduced survival among these organisms under acute and sublethal laboratory exposures (DeMott, Zhang and Carmichael 1991; Cai *et al.* 2021). In contrast, other studies have suggested that *Chironomus* may be less sensitive to MC toxicity compared to *Daphnia* (Blom *et al.* 2006), as benthic *Chironomus* are more likely to tolerate adverse conditions associated with toxic freshwater blooms (Strandberg *et al.* 2020). The reduced survival reported among *Daphnia* and *Chironomus* populations in previous studies (DeMott, Zhang and Carmichael 1991; Cai *et al.* 2021), were found at MC concentrations higher than the range used in this study, suggesting the observed elimination of *Daphnia* and *Chironomus* populations in the present study were possibly unrelated to microcystin treatments. Similar dramatic population declines have been observed among *Daphnia spp* (Choung *et al.* 2013; van den Brink *et al.* 1995; Detenbeck *et al.* 1996) and chironomids (Choung *et al.* 2013; Rohr and Crumrine 2005) exposed to low pesticide concentrations in previous micro/mesocosms studies. However, the observation in the present study could possibly have resulted from increased oxygen saturation, due to the prolonged aeration during this experiment (Wright and Shapiro 1990). Aeration allowed for the maintenance of relatively stable DO concentrations observed across the treatments and control in this study. The slight variations observed in the DO concentrations across the treatments could possibly be explained by the non-randomized arrangement of the replicates on the shelves during the experimental set up. This suggests that some of the replicates arranged in the outer shelves might have received greater oxygen saturation than the replicates in the inner shelves because of the distance from the aerator pump. Besides, possible effects of biotic factors, such as increased predation by *I. elegans* might also have contributed to the disappearance of *Daphnia* population in this study.

Surprisingly, higher survival among *Gammarus* and *Ischnura* populations was observed in the treatment compared to the control. Contrary to the expectation that increased MC treatments would be associated with reduced survival proportion and population abundance among invertebrates, these results suggest more animals would have died in the control than in the treatments. Perhaps the observed mortality among amphipods in

this study occurred earlier during the experiment, which may not be related to the MC treatments but possibly due to the influence of other environmental factors, such as the health of the animal or the condition of the experimental medium. On the other hand, the monotonic increase observed in the survival of *I. elegans* in this study may be associated with MC treatments. In this study, more of the *I. elegans* larvae in the control and culture replicates moulted and emerged into adults than in the treatment, which may partially account for the apparent reduced survival and suggesting that increased MC concentrations in the treated may be responsible for the delayed emergence of larvae into adults. This finding is consistent with previous studies (Liu *et al.* 2002; Jacquet *et al.* 2004; Wang *et al.* 2005) that reported also delayed adult emergence or reduced embryonic development in aquatic vertebrates, such as, fish exposed to MC concentrations. However, while existing evidence suggests such inhibitory role of MC on embryonic development in fish and other aquatic vertebrates are still controversial (Zhang *et al.* 2020; Wang *et al.* 2005), it is not known how MC may potentially affect embryo/larval development in aquatic invertebrates. Hence, this study partially demonstrates first evidence of how MC exposure may influence the ontogeny of freshwater invertebrates.

In accordance with one of the initial hypotheses in this study, increased MC concentrations were associated with reduced rates of invertebrate-mediated organic matter decomposition. Higher decomposition rates were observed in the control and *Microcystis* culture treatment, whereas increasing MC concentrations significantly reduced the rate of organic matter decomposition in the crude extract and purified MC-LR treated-microcosms. However, the higher rate of leaf decomposition observed in the culture treatment and control, despite a low proportion of amphipod survival at the end of this experiment suggests higher leaf shredding rates might possibly have occurred in the control and culture treatments very early in the experiment before most of the animals died. Hence, the increased rate of invertebrate-mediated organic matter decomposition observed in the control and *Microcystis* culture treatments in this study is unlikely to be a treatment-related effect. Rather, this observation was presumably due to the low amphipod survival in the control and *Microcystis* culture treatments, which might have

resulted from biological explanations other than MC. Besides, increased toxin concentrations significantly reduced the rate of invertebrate-mediated organic matter decomposition. This observation is contrary to the expectation that a higher population abundance of amphipods would result in a higher rate of organic matter decomposition, suggesting the reduced rate of decomposition observed in this study may be related to sublethal effects of MC concentrations on physiological responses, such as reduced feeding/shredding rates in amphipods (Burkholder, Shumway and Glibert 2018; Ibelings *et al.* 2008; Chen *et al.* 2005a). While laboratory exposures to high MC concentrations have demonstrated acute toxic effects leading to reduced survival among aquatic species, including fish (Isibor 2017; Ibrahem *et al.* 2012) and zooplankton (DeMott, Zhang and Carmichael 1991; Smutná *et al.* 2014; Bui *et al.* 2020), a few empirical studies have associated sublethal MC concentrations with subtle effects on physiological and behavioural responses, such as feeding and growth inhibition (Lürling 2003; Ghadouani *et al.* 2004) as well as reproductive impairment (Shahmohamadloo *et al.* 2020; Liang *et al.* 2017a; Liang *et al.* 2020) in *Daphnia* and other zooplankton. However, sublethal effects of MC exposure on behavioural and fitness-related traits among native gammarid populations that mediate key ecosystem processes, such as leaf litter processing in forested freshwaters, are poorly known.

On the other hand, the rate of leaf litter decomposition associated with microbial activities increased significantly in experimental microcosms treated with *Microcystis* culture compared to the control. This observation was possibly related to the high mean pH values recorded in the *Microcystis* culture replicates (Figure 1C), presumably suggesting a prevailing high alkaline pH range, which might have favoured increased microbial colonization (Okano *et al.* 2009) and elevated the rate of microbe-mediated organic matter decomposition in these replicates (Chamier 1987; Thompson and Bärlocher 1989). However, the values reported here are not high enough and may not fully explain the observed effects. In this study, although the effect of a typical freshwater microcystin-producing bloom was tested on organic matter decomposition using a simulated *Microcystis* bloom in the laboratory, the present experimental approach is in line with the

existing recommendations on the need for more environmentally relevant experimental designs during freshwater blooms (Ibelings *et al.* 2008; Burkholder, Shumway and Glibert 2018; Zepernick *et al.* 2021). Moreover, in accordance with the present results, several studies have highlighted elevated pH and increased alkalinity to be among the key environmental variables promoting the growth and dominance of toxic *M. aeruginosa* during freshwater blooms (Ibelings *et al.* 2008; Yang *et al.* 2018; Zepernick *et al.* 2021). Besides, the reduced rates of microbial decomposition observed in the microcosms treated with crude extract and the purified toxin treatment could also be attributed to MC degradation due to the dominance of MC-degrading bacteria in the microbial flora at the range of alkaline pH recorded in this experiment. While MCs are relatively stable (Omidi, Esterhuizen-Londt and Pflugmacher 2018) and may exhibit high resistance under different stress conditions, including pH, temperature, sunlight, and certain enzymes (Okano *et al.* 2009; Tsuji *et al.* 1994), several studies have attributed MC degradation in natural waters with the proliferation of MC-degrading bacteria in freshwater bodies due to increased alkaline pH associated with cyanobacterial blooms (Okano *et al.* 2009; Lopez-Archilla *et al.* 2004). Hence, this study suggests that accurately predicting the effects of cyanotoxins on ecological processes during freshwater blooms may be complex, as the direct effects of toxins may become compounded by other associated stressors, such as changes in water physico-chemical parameters.

5.6 Conclusion

Existing knowledge on the potential impacts of MC on freshwater biota and ecosystems are currently based on evidence from single-species and single stressor studies in the laboratory (Brooks, Gaskell and Maltby 2009; Ibelings *et al.* 2008; Kotak *et al.* 1996). However, the ecological consequences of MC exposure on community structure and ecosystem functions are rarely unknown at higher levels of biological organisations (Ibelings *et al.* 2008; Burkholder, Shumway and Glibert 2018). In this study, I report one of the first laboratory evidence of the community-level effects of sublethal MC exposure on ecosystem structure and functioning using indoor community microcosms as model

freshwater ecosystems. This study suggests sublethal MC exposure can have significant direct and indirect effects on community response and ecosystem functions, such as reduced phytoplankton biomass, and altered organic matter decomposition. The consequence of these effects are complex, as direct effects of MC exposure may become compounded by other co-occurring stressors, such as changes in water physico-chemical parameters during harmful freshwater blooms. This complex scenario may lead to elimination of important components of the food web, such as cladocerans and chironomids, which potentially may have profound ecological implications on community structure and ecosystem functions in impacted freshwater systems through trophic cascades (Choung *et al.* 2013; Burkholder, Shumway and Glibert 2018).

Chapter 6

General Discussion

6.1 Synopsis of the Thesis and Overview of Chapters

Overall, the aim of this thesis was to evaluate the ecological effects of environmentally relevant MC exposure on biodiversity structure and ecosystem functioning in freshwater systems. To achieve this aim, I designed and conducted a range of scaling-up experiments to test a set of hypotheses relating to the ecological effects of MC on survival and ecosystem functions of freshwater species across different levels of biological complexity. The results suggest that environmentally relevant MC concentrations can influence survival and ecological processes among key freshwater species. However, these effects may depend on exposure regime, study taxa and the scale of experiment. Hence, the findings reported in this thesis offer new insights into the existing understanding of the ecological impacts of cyanobacterial harmful blooms on freshwater ecosystems.

6.1.1 Overview of Key Findings

Chapter 1 (Literature Review), introduces freshwater cyanobacterial harmful algal blooms and the associated cyanotoxin production as one of the emerging environmental issues, threatening aquatic biodiversity and ecological conditions in freshwater systems around the world. Focusing on MC as the prominent and most studied group of cyanotoxins in freshwaters, I conducted an extensive literature review on the environmental occurrence and ecological effects of MC on freshwater species and their ecosystem functions in aquatic systems. Furthermore, based on evidence from existing literature, I argued that environmentally relevant MC concentrations may rarely exceed the range recommended as permissible guidelines for MC-LR equivalent in freshwaters used for drinking and recreational purposes, except for some occasional episodes of high

toxin concentrations during bloom senescence. Finally, I made a case for the need to test the effects of low MC concentrations comparable to those recommended by regulatory agencies on freshwater species and ecosystem functioning across hierarchies of biological complexity.

In Chapter 2, I tested the sensitivity of five key food web species across different taxa to environmental MC concentrations. The results suggest low environmental MC concentrations can have species-specific effects on individual survival and growth among key freshwater taxa, depending on the treatment type. The data also illustrate that low toxin concentrations can exert a range of effects on freshwater species and these effects may follow a non-linear, hormetic dose-response pattern.

To predict the population-level effects of environmentally relevant microcystin exposure in **Chapter 3**, I performed a series of sublethal and chronic laboratory experiments to test effects of long-term environmental microcystin exposure on population-related endpoints among three ecologically important freshwater species, *D. magna, G. pulex* and *D. villosus*. Here, the results suggest that individual survival among these key species may be unaffected at the range of concentrations regularly encountered in natural waters. However, more ecologically relevant sublethal effects on feeding, growth and reproduction may occur, potentially causing significant changes in population dynamics, community structure and ecosystem functioning.

Chapter 4 investigates the combined effects of increased water temperature and low MC concentrations, as co-occurring stressors associated with harmful freshwater blooms on survival and key ecological processes underpinning ecosystem functions in freshwater species. I found uneven effects of low environmental toxin concentrations on survival and key processes in organisms across different taxa. Additionally, the results also suggest that effects of low toxin concentrations at higher trophic levels were temperature dependent.

Finally, **Chapter 5** provides a first report of the community-level effects of sublethal MC exposure on the structure and functioning of freshwater ecosystems. Using a set of

laboratory-based community microcosms, I tested the direct and indirect ecological effects of sublethal MC exposure on ecosystem structure and function in freshwater ecosystems. The results suggest that indirect effects of sublethal MC exposure on structural and functional traits in freshwater ecosystems are complex and may become compounded by changes in abiotic factors such as water pH and dissolved oxygen concentration.

6.2 Environmental MC Occurrence and Exposure Regimes in Freshwater Ecosystems

MC has been regarded as one of the most widespread environmental threats associated with the increasing incidence of freshwater harmful cyanobacterial blooms in aquatic systems (Preece *et al.* 2021). Harke *et al.* (2016) reported that approximately 75% of the global rise in freshwater harmful cyanobacterial blooms may be associated with the MC release in water bodies owing to increased eutrophication and climate change, following a trajectory described by Glibert (2017) as "*more blooms, more toxins, more often, in more places*". MC occurrence in freshwater lakes, rivers and reservoirs used for drinking and recreational purposes has been implicated in severe cases of human and animal poisoning (Svirčev *et al.* 2019; Preece *et al.* 2017), leading to a variety of adverse effects during freshwater blooms (Soares *et al.* 2006; Falconer 2001). This incidence has led to the recommendations on regulatory permissible guidelines for freshwaters used as drinking and recreational water sources (Bartram and Chorus 1999) and which has just been recently updated (Chorus and Welker 2021). While these guidelines aimed primarily at protecting human health with little or no consideration for the ecological integrity of freshwater systems (Chorus and Welker 2021; Chorus *et al.* 2000), few studies have suggested that many of the attributed effects on human health have only been described and have rarely been measured (Carmichael and Boyer 2016; Soares *et al.* 2006). There have been a few recorded cases of human poisoning which have been linked with MC exposure during blooms (Carmichael and Boyer 2016), however, cases of animal poisoning relating to MC exposure have been more frequently reported (Svirčev *et al.*

2019; Preece *et al.* 2017), suggesting that terrestrial and aquatic animals may be at risk to MC exposure.

In chapter 1, I argued that one primary route of MC exposure among freshwater organisms is likely via uptake of the dissolved toxin concentrations from the surrounding water column (Ferrao-Filho, Herrera and Echeverri 2014). *Daphnia* and other zooplankton are important phytoplankton grazers and may naturally encounter higher concentrations of the intracellular, cell-bound MC concentrations through grazing on toxic cyanobacteria (Rohrlack *et al.* 2001). Several studies have shown that selective feeders, such as copepods and rotifers have a higher feeding preference for large-sized phytoplankton compared to relative smaller ones (Sommer et al., 2001; Sommer and Sommer, 2006). However, increasing evidence suggests that these organisms may have evolved adaptive traits that enable them to selectively avoid feeding on harmful algae such as, toxic cyanobacteria (Ger *et al.* 2016a; Summer and Summer, 2006). Unlike copepods and rotifers, filter-feeders lack the ability to select edible food particles (Sommer and Sommer, 2006). For instance, large-bodied generalist grazers like *Daphnia* have shown to prevent ingestion of toxic cyanobacteria by reducing their feeding rate (Rohrlack *et al.* 2001) or transferring MC tolerance traits to their offspring through maternal exposure to MC concentrations in the previous bloom session (Dao, Do-Hong and Wiegand 2010). In this thesis, the range of MC treatment concentration tested did not affect survival of *D. magna and D. pulex* across a different exposure duration and conditions (Chapters 2, 3, 4), except in the crude extract treatment as observed in Chapter 2, where such effects could possibly be due to other unknown secondary metabolites that may be present in the composite crude extract treatment (Smutná *et al.* 2014; Jungmann and Benndorf 1994; Jungmann 1992). Therefore, the results of this thesis suggest that while exposure to dissolved MC concentrations comparable to the WHO permissible thresholds may not directly affect individual survival among zooplankton, potentially, they may have subtle ecologically relevant effects on population-level traits, such as, feeding, growth and reproduction under prolonged MC exposure during toxic blooms.

Moreover, I reported in this thesis (Chapter 1) that the MC concentrations detected in the water column in many of the world's largest lakes, where there have been repeated incidence of impacts of eutrophication and harmful algal blooms were mostly within the concentrations recommended by the WHO (Skafi *et al.* 2021; Steffen *et al.* 2017; Li *et al.* 2017). This evidence suggests that the range of environmentally relevant concentrations that freshwater organisms will ideally be exposed to may be within the range recommended by the WHO as safe levels (1-24 µg/L) in drinking and recreational waters (Chorus and Welker 2021). In this thesis, I tested a range of hypotheses relating to the ecological effects of low MC concentrations comparable to the regulatory safe levels and found variable significant effects on survival and key processes, depending on the study taxa, scale of the experiment, duration of exposure and the treatment type. Importantly, these observed effects at relatively lower concentrations in this thesis contrast earlier studies that demonstrated adverse effects at higher concentrations which may rarely be found in natural waters (DeMott, Zhang and Carmichael 1991; Smutná *et al.* 2014; Campos *et al.* 2013; Babica *et al.* 2007). Hence, the results presented in this thesis reflect environmentally realistic and ecologically relevant effects which may provide useful guidance in predicting the ecological effects of MC exposure on freshwater organisms and their ecosystem functions.

6.3Effects of low environmental microcystin concentration on freshwater species and their ecosystem functions

Until recently, existing literature in the field of ecotoxicology and environmental risk assessment have suggested that *dose makes the poison*, following the proposition of the 16th-century toxicologist Paracelsus (Shi *et al.* 2016; Bus 2017). This has been based on the expectation that chemical stressors may be harmless at lower dose or concentrations but may become toxic at higher concentrations (Lagarde *et al.* 2015) and have led to the development of several models to explain linear dose-response models in ecotoxicology (Shi *et al.* 2016). Hence, most of the existing toxicity studies and risk assessments have been done at higher concentrations than what is environmentally realistic (Oberdörster

2010; Chapman 2002). In contrast, there is increasing evidence suggesting low-dose exposure to chemical stressors, especially below the adjudged safe thresholds may be associated with a wide range of non-linear dose-responses, such as U-shaped or inverted U-shaped hormesis (Forbes 2000; Beausoleil *et al.* 2013).

Previous laboratory studies demonstrated a range of adverse effects on freshwater species, including algal growth inhibition (Babica *et al.* 2007; Campos *et al.* 2013), reduced survival (DeMott, Zhang and Carmichael 1991; Bui *et al.* 2020) and impaired life-history traits among zooplankton (Smutná *et al.* 2014; Lürling and van der Grinten 2003), at relatively high MC concentrations. However, in thesis, lower range of MC concentrations (1-10µg/L), comparable to those recommended by the WHO as safe levels were associated with significant adverse effects on variety of freshwater species across different experimental scales and levels of biological organisation. For instance, low MC concentrations was associated with algal growth inhibition (Chapter 2, 4), reduced survival among predators (Chapter 4) and stimulation of fecundity and population growth among zooplankton (Chapter3). Interestingly, the effects observed in this thesis did not increase linearly with increasing MC concentrations as expected but followed a non-monotonic pattern which suggests the occurrence of hormetic dose-response. Hormetic behaviour in the biological response to low dose exposure among laboratory animals to a wide range of environmental stressors, including MC-LR has been reported in many toxicity studies (Liang *et al.* 2020; Chen, Giesy and Xie 2018; Liang *et al.* 2017b). Hence, the results of this thesis suggest that environmental MC exposure within the range considered safe by the WHO may have deleterious effects on sensitive components of the aquatic food web, thereby influencing species interaction, community structure and the overall functioning of the ecosystems. Furthermore, this finding highlights the need to incorporate key components of freshwater food webs and their important biological processes into current ecological risk assessment protocols.

6.4 Complex interactions between environmental microcystin concentrations and climate warming

In Chapter 4, I reported the first evidence of complex stressor interactions on survival and processes in key freshwater invertebrates, suggesting indirect ecological impacts at higher levels of biological organisation through trophic cascades. Freshwater harmful cyanobacterial blooms are complex phenomena involving a wide range of multiple co-occurring stressors (Ibelings *et al.* 2008; Paerl *et al.* 2001). Ideally, under environmentally realistic conditions, aquatic organisms are rarely exposed to MC in isolation (Wei *et al.* 2020), but also with other important abiotic stressors associated with harmful cyanobacterial blooms (Ibelings *et al.* 2008). Given the current pace of global climate change, increased water temperature and thermal stratification in lakes are expected, not only to potentially drive the incidence of more toxic cyanobacterial blooms in freshwater systems (Urrutia-Cordero *et al.* 2020) but also to coincide with MC toxicity as multiple co-occurring stressors (Hayes *et al.* 2020; Walls *et al.* 2018). The results presented in this thesis suggest that multiple stressor exposure during harmful freshwater blooms results in a rather a complex scenario that can result either linearly in additive or non-linearly in synergistic and antagonistic interactions between individual stressors. The consequences of this scenario may become compounded through feeding mechanisms at different trophic levels, influencing food web interactions (Woodward, Perkins and Brown 2010) and leading to profound ecological surprises at higher levels of biological organizations (Segner, Schmitt-Jansen and Sabater 2014). Hence, this finding highlights the need to prioritize and integrate multiple stressor approach into the management of ecological impacts of harmful cyanobacterial blooms in freshwaters in the face of increasing global change.

6.5 Crossing-cutting ideas emerging from the thesis

6.5.1 Ecological realism of single-species studies versus multi-species studies

In this thesis, a synthesis of findings from four different but interrelated laboratory-based data chapters, is used to explain the potential ecological effects of environmental MC exposure on survival and ecosystem functions in freshwater species. Three of these chapters reported the effects of MC on survival and functions in freshwater species using single species experimental approach while only one chapter reported effects at the community using multi-species microcosms. The range of single-species experiments used in this thesis includes acute, sublethal and chronic toxicity tests which usually vary in the duration of exposure and the endpoints measured. For instance, the acute toxicity tests reported in Chapters 2 and 4 were short term studies. The duration of MC exposure ranged from 24 hr to 7 days where survival, growth inhibitions, pigment contents, growth and feeding rates among individuals were measured as endpoints. In Chapter 3, a set of chronic toxicity tests was used to measure population-level survival, growth, and reproduction of *D. magna* at a longer term. Standard single-species tests have become internationally recognised ecotoxicological techniques and guidelines, which are now widely being used in assessing the ecological effects of chemicals on aquatic biota and ecosystems (Brooks et al., 2009; Nilsen et al., 2019). Although these tests can produce reproduceable results with high levels of reliability and precision in the laboratory. They are usually too simplified, and the durations of exposure are too short compared to multi-species studies in the field (Noyes et al., 2009; Edwards and Pascoe, 2018). This suggests single-species studies in the laboratory can only offer low degree of ecological realism. For this reason, recent studies have increased emphasis on the need for risk managers to incorporate community-level multiple species studies with higher degree of ecological realism in their assessment programmes (De Laender et al., 2009; Ibelings et al., 2008). Multi-species studies using model-stream ecosystems offer a more reliable technique that can simulate natural conditions in the field and integrate biotic interaction, community dynamics and ecosystem-level effects (Clements and Newman, 2003). Model systems such as, micro and mesocosm are increasing now being used to assess direct and indirect ecological effects of chemicals (Daam et al., 2008; Rico et al., 2014). These model systems are easy to control, manipulate and replicate, therefore may provide reliable

direct and indirect effects of chemical at higher level of biological organisation (Barry and Logan, 1998; Pestana et al., 2009). However, multi-species community studies are usually very expensive to execute and often difficult to reproduce due to environmental variability (Daam et al., 2008). The data present in Chapter 5 of this thesis is among the first evidence reporting on how different MC treatments and concentrations can affect survival and functions among freshwater species. Therefore, there is need for future studies to prioritize experimental studies assessing the effects of chemical stressors at the community level.

6.5.2 Laboratory experimental studies versus field studies

The research reported in this thesis was originally proposed to employ a combination of three different methods of data collection, namely, laboratory experiments, semi-field mesocosms and field studies. While the field study component of the PhD research was unfortunately disrupted by restrictions related to the COVID-19 global pandemic, the proposed outdoor mesocosm study had to be shelved for time and other logistic constraints. For these reasons, the data reported in this thesis (Chapters 2, 3, 4 and 5), were derived from experimental studies conducted in the laboratory. Aquatic ecosystems are generally highly complex biogeochemical systems, characterised with constant interactions among several abiotic and biotic environmental variables (von Schiller et al., 2017; O'Brien et al., 2016). This complexity may potentially limit the assessment and prediction of the direct effects of individual stressor such as, dissolved MC concentrations on aquatic species during observational field studies in freshwater lakes and reservoirs. This shows that current biomonitoring programmes may be limited because most field studies rely on the correlation between measurement of MC concentrations in the water column and indices of ecosystem structure (survival, abundance, and diversity) in blooming freshwaters (Kim et al., 2021; Bukaveckas et al., 2017). Field data obtained from observational studies provide evidence of a longer-term impact of chronic exposure to stressors in natural waters and such impact can integrate responses across different stages of the life cycle in aquatic species (Nilsen et al., 2019). However, there are yet

uncertainties regarding whether observed effects during field studies are entirely due to dissolved MC exposure or other stressors associated with freshwater blooms (Ibelings et al., 2008).

In recent decades, ecologists have increasingly relied on the use of manipulative laboratory experiments to answer important questions relating to how changes in environmental variables may affect ecological patterns among species (Clements and Rohr, 2009). Manipulative experimental studies can potentially isolate and simplify individual stressors from complex environmental variables found in natural waters. Such studies can generate reliable data that may be required to test explicit hypothesis and establish a cause-effect relationships in aquatic species (Clements and Newman, 2003). In this thesis, a series of scaling up manipulative laboratory experiments was used to assess the impact of MC concentrations on survival and functions across multiple levels of biological organisation. The data presented in Chapters 2 of this thesis showed short-term (48-96hr duration) individual-level effects of MC concentrations on endpoints such as, survival and growth inhibition among single-species in the laboratory. Moreover, Chapter 3 reported on effects of MC concentrations on survival and population-level behaviours (feeding, growth, and reproduction) during a longer exposure (21-day). In Chapter 4, the combined effects of MC concentrations and increased water temperature on single-species individual across four species were reported, suggesting a possible effects of multiple stressors. Finally, Chapter 5 reported the community level impacts of MC concentrations on survival and ecosystem functioning in model laboratory ecosystems.

The use of laboratory experiments in hypothesis testing in ecology offers a range of benefits such as, high levels of reliability, replication, control, and reproducibility compared to field work (Preston, 2002). However, certain limitations have been associated with the use of laboratory experimental studies in ecology. Studies have shown that inferences drawn from laboratory experiments may be difficult to extrapolate to real-world situations in the field due to over-simplication of exposure regimes and complex

environmental variables (Preston, 2002; Clements and Rohr, 2009; Nilsen et al., 2019). On the other hand, while these limitations may be overcome during field observation studies, the complex nature and lack of homogeneity among different sampling locations may potentially limit the statistical power, control, and replication during such studies. In view of this, recent studies have highlighted the need to adopt a robust multi-tiered ecological risk assessment approach in assessing the impact of chemical stressor on aquatic biota and ecosystems. Therefore, it is imperative that future research prioritise efforts toward studies that will integrate data obtained from simplified laboratory experiments, semi-field mesocosms and field observational studies for effective freshwater conservation and ecosystem management.

6.5.3 Variable exposure durations between standard ecotoxicological guidelines and ecologically relevant chronic exposure

The thesis of this PhD research is to answer an important ecological question that relating to how chronic exposure to environmental MC concentrations may influence survival and functions in freshwater systems. To answer this overarching question, a suite of scaling up laboratory experiments were conducted to test a set of explicit hypotheses following standard international ecotoxicological guidelines. The experimental protocols adopted in the thesis have been standardized and documented as internationally recognised guidelines by regulatory agencies. These include the American Society for Testing and Materials (ASTM), the Organization for Economic Co-operation and Development (OECD), and the National Toxicology Program (NTP). In line with the guidelines adopted in this thesis, exposure durations during laboratory experiments varied across different chapters depending on the scale of experiment and the hypotheses tested. In Chapters 2 and 4, OECD acute toxicity tests (OECD 2004; OECD 2011; Williams, Green and Pascoe 1984) were conducted to assess the short-term sensitivity of key freshwater species to dissolved environmentally relevant MC concentrations under certain variable exposure conditions. Chapters 3 and 5 reported chronic and sublethal effects under longer

MC exposure regimes in accordance with standard guidelines (OECD 2008; Kennedy *et al.* 2002).

Laboratory experiments like these are very useful and may provide relevant data to establish cause-effect relationships between MC exposure concentrations and observed endpoints. However, laboratory experiments conducted in strict adherence to standard toxicity protocols in the recommended guidelines are less reliable and ecologically relevant. This is because exposure duration recommend in those guidelines rarely reflect the ideal exposure time and conditions in natural waters. For instance, freshwater species are likely to be exposed to MC concentrations longer than the test durations reported in these studies. This suggests that inferences drawn from such laboratory experiments are unrealistic and may not reflect ecologically relevant exposure scenarios in freshwater bodies. Therefore, flexible but well-designed laboratory experiments that can potentially simulate exposure durations comparable to real-world situations are needed to enhance effective freshwater management and policy decisions. Besides, a growing number of experimental studies are now reporting effects of environmental stressors on a wide range of aquatic species under approximate ecologically relevant conditions in the laboratory. However, most of these studies are still currently considered as preliminary investigations because such studies have not followed strictly a set of conventional guidelines. These studies may not be accepted until they have been replicated severally by other laboratories, in order to validate reproducibility of the observed results. Hence, more ecologically relevant and well-designed laboratory experiments are required to answer practical ecological questions relating to biodiversity, ecosystem functioning and conservation.

6.5.4 Summary and comparison of key findings across different chapters

In this thesis, I assessed the impacts of environmental MC concentrations on survival and key ecological processes underpinning ecosystem functions in key freshwater species by measuring a range of endpoints in organisms. These endpoints were measured across different experimental scales and the results are present across different chapters of the

thesis. These endpoints include individual survival among zooplankton, amphipods, damselfly predators and chlorophyll-a pigment concentrations. A comparative summary of the key findings across all the data chapters in the thesis is presented in Table 6.1.

In Chapters 2, *D. magna* showed higher sensitivity to MC concentrations in the crude extract but no significant effects of the purified MC- LR was observed on the survival of *D. magna*. On the other hand, In Chapters 2 and 4 neither treatment affected survival of *D. pulex*. These results suggest that, although MC may affect survival among *Daphnia* populations, however, such negative effects may vary depending on MC treatments and the species of *Daphnia* tested. The observed variations in sensitivity to low environmental MC concentrations between the two species, may be due to differences in the mechanisms of response to cyanotoxin toxicity. While *D. pulex* has been reported to exhibit a more general mechanism of response to MC toxicity, *D. magna* has been associated with more specific response mechanisms to MC stress. In agreement with the findings reported in Chapters 2 and 4 in this thesis, purified MC-LR concentrations had no significant on the survival of *D. magna*, howbeit, sublethal effects on were found on feeding, growth, and reproduction. Again, these results suggest that the observed effects in *D. magna* exposed to MC-containing crude extract of *M. aeruginosa* in Chapter 2 may presumably not due to MC concentrations in the extract. Perhaps, those effects could be attributed to other potentially toxic secondary metabolites present in the cyanobacterial crude extract (Jungmann 1992; Rohrlack *et al.* 2003; Smutná *et al.* 2014).

Moreover, whereas low range of environmental MC concentrations tested in thesis showed no significant adverse effects on survival among *Daphnia* and amphipod populations under acute laboratory exposures (Chapters 2, 3 and 4), however, such concentrations may lead to subtle deleterious effects on fitness-related traits during chronic MC exposure in lakes and reservoir.

Here in this thesis, phytoplankton chlorophyll-a concentration was quantified as surrogate measure of how MC concentrations may influence photosynthetic activities in algae. In Chapter 2, purified MC-LR at lower concentrations was associated with a slight increase

in chlorophyll-a concentrations whereas no significant effect was found at higher purified MC-LR concentrations and in the crude extract treatment. However, in Chapter 4, neither treatment affected chlorophyll-a concentration under a multiple stressor condition but with similar experimental design. The observed effects in Chapter 2 could be attributed to hormesis, which possibly might have resulted from overstimulation of chlorophyll-a synthesis as a response to low MC concentrations. However, it is quite possible that this effect was masked by increased temperature as a co-stressor during the multiple stressor experiment. On the other hand, in Chapter 5, a reduction in chlorophyll-a concentration was associated with crude extract and *Microcystis* culture treatments during a longer term exposure in community microcosms. The purified MC-LR concentrations had no effects on chlorophyll-a, suggesting the observed effects were possible not due to MCs but other stressors in the crude extract and *Microcystis* culture. These results showed that longer term exposure to cyanotoxin under environmentally realistic conditions may lead to inhibition of growth and photosynthetic activities in green algae.

Another important consideration that may potentially confound the interpretations of some the experimental results presented in this thesis are related to temperature and body size scaling. For instance, organisms used for the multiple stressor experiments reported in Chapter 4 were pre-acclimatized at temperatures different from the experimental conditions before the commencement of the experiment. Hence, the thermal shock resulting from the sudden fluctuations in temperature might possibly have exerted some physiological stress on these organisms, thereby contributing to the observed on survival and feeding rates. Furthermore, the non-randomization of replicates during experimental set up and arrangement in the incubators presumably might have also created differential light and thermal conditions which could have influenced the observations reported in this thesis.

In Chapters 2, 3 and 4 where aquatic invertebrates were exposed to MC treatments, the range of body size among animals exposed varied across different species. This variation in body size of animals may serve as potential confounding factor. Since physiological

function and metabolic activities in animals change with age and body size, it is possible that the effects reported in this thesis may be age-specific. Hence, the use of non-uniform body-size range among species may affect the reliability of the data presented in this thesis. For these reason, future experimental designs should focus on testing effects of cyanotoxins on freshwater species using individuals within a range of closely related body sizes and thermal conditions.

Table 6.1: A comparative summary of key findings from experiments across different chapters

Species	Response	Experiment and duration	Chapter	Findings
D. magna	Survival	48-hour acute toxicity test	2	Increased MC concentrations in the crude extract reduced survival but purified MC-LR had no effect.
D. pulex	Survival	48-hour acute toxicity test	2	Purified MC-LR and crude extract had no effect on the survival of *D. pulex*.
D. pulex	Survival	Multiple stressors with 48-hour acute toxicity test	4	Neither treatment affected survival of *D. pulex*.
D. magna	Survival	21-day chronic toxicity test	3	Purified MC-LR concentrations had no effects on survival of *D. magna.*

G. pulex	Survival	96-hour acute toxicity test	2	Neither treatment had effects on the survival of *G. pulex*
D. villosus	Survival	96-hour acute toxicity test	2	Neither treatment had effects on the survival of *G. pulex*
G. pulex	Survival	7 days	3	Neither treatment had effects on the survival of *G. pulex*
G. pulex	Survival	14-day community microcosm	5	A total decline of 64.1% in survival of *G. pulex* was observed across the tanks. This is not likely to be due either of the treatment.
D. villosus	Survival	7 days	3	Neither treatment had effects on the survival of *D. villosus*
S. quadricauda	Chlorophyll-a	72-hour algal growth inhibition	2	Purified MC-LR slightly elevated chlorophyll-a pigment levels at lower concentrations but had no effect at higher concentrations. But crude extract had no effect.

S. quadricauda	Chlorophyll-a	72-hour algal growth inhibition	4	Neither treatment affected chlorophyll-a pigment content.
S. quadricauda	Chlorophyll-a	14-day community microcosm	5	Increased MC concentrations in the crude extract and *Microcystis* culture significantly reduced chlorophyll-a content while purified MC-LR had no effects.
I. elegans	Survival	24-hour feeding response	4	Increased MC concentrations in the crude extract significantly reduced survival of *I. elegans*
I. elegans	Survival	14-day community microcosm	5	A total decline of 77.7% in survival of *G. pulex* was observed across the tanks, which is unlikely to be related to MC treatment.

6.6 Wider impacts of research findings

Harmful cyanobacterial blooms have become intensified in many freshwater ecosystems around the world, because of human-induced climate change and eutrophication (Preece *et al.* 2017). Approximately, 80-95% of the reported bloom cases has been dominated by *Microcystis aeruginosa* (Harke et al. 2016), the chief producer of MCs in freshwater systems which has been widely implicated in a wide range of environmental, socio-economic, and ecological impacts (Briland *et al.* 2020). Research suggests MC is currently one of the leading environmental stressors in freshwater ecosystems. However, the extent of the associated environmental and ecological impacts of MC exposure may have been greatly underestimated (Ibelings *et al.* 2008). Although there has been increased emphasis on the public health and socio-economic implications (Brooks *et al.* 2016), the ecological impacts of MC exposure on the ecological integrity of aquatic ecosystems have been mostly discussed but not quantified.

Current approaches for managing freshwater *Microcystis* blooms have focused mainly on the use of five methods of quantification, including measurement of MC concentrations, measurement of chlorophyll-a concentration, microscopic examination for cell counts, quantitative real-time PCR (qPCR) to amplify potentially toxigenic strains and the use of remote sensing and satellite images to identify broad areas of *Microcystis* blooms (Bridgeman, Chaffin and Filbrun 2013). While none of these approaches offers a reliable surrogate quantification for the impact of toxic freshwater blooms on aquatic systems (Bridgeman, Chaffin and Filbrun 2013), stakeholders have relied on a combination of routine measurement of the toxin concentration in lake water (Boyer 2008) and data obtained from single species toxicity tests at high concentrations to predict the ecological impacts of *Microcystis* blooms on freshwater ecosystems (Šulčius *et al.* 2017). These limitations have led to widespread under or over-estimation of the impacts of harmful cyanobacterial blooms on freshwater ecosystems.

Therefore, the data presented in this thesis suggests that, for effective management of impacts on aquatic systems, there is a need to urgently review the existing regulatory

guidelines and the procedures for assessing the potential risks of harmful cyanobacterial blooms on freshwater systems. The findings in this thesis offer important insights into the need to integrate the assessment of the ecological impacts of cyanotoxins on fundamental structure and functions in freshwater ecosystems across different levels of biological organisations. Besides, the findings presented in this thesis may go on to have wider implications outside academia, such as influencing policy decisions, advisory monitoring, and management of impacted freshwaters for ecosystem restoration.

6.7 Conclusions and recommendations

Freshwater lakes, rivers and reservoirs are an important hotspot for biodiversity and provide a range of ecosystem services for human well-being. However, these important systems are among the most impacted ecosystems in the world under increasing environmental change. Harmful cyanobacterial blooms have emerged one of the leading environmental threats in freshwater ecosystems. This phenomenon has been widely linked with the release of hazardous MC concentrations in freshwater bodies, impacting on survival, food web interactions and ecosystem functioning in aquatic communities. Unfortunately, the ecological implications of this phenomenon have been widely underestimated and, in many places, neglected. In this thesis, I evaluated the ecological impacts of MC on biodiversity and ecosystem functioning in freshwater systems. This thesis demonstrates for the first time that low environmental MC concentrations comparable to the adjudged safe levels may affect survival and ecological processes among key freshwater species across different levels of biological organisations in freshwaters. The findings presented in this thesis offer some interesting insights that may enhance our current understanding of the ecological impacts of cyanotoxins on freshwater species and ecosystem functioning in aquatic systems.

In this thesis, given the constraints imposed by limitations in time, resources and the impacts of the COVID-19 pandemic, only experimental aspects of the PhD studies are reported here. Unfortunately, the proposed outdoor mesocosms experiment was not executed and proposed the ecosystem-scale field study that was disrupted by the COVID-

19 pandemic. However, topmost on my plan for future research is the desire to focus on exploring potential ecological impacts of cyanotoxins at large scales of biological organisations, such as outdoor community mesocosms and whole ecosystem-scale field studies, where indices of ecosystem structure and functions may be assessed for a much longer period. Besides, one of the current challenges in ecological research is related to the lack of reliable links between inferences drawn from laboratory experiments and their extrapolations to real-world situations during field studies. A major reason associated with this limitation is concerned with the difficulty in replicating the complex natural environmental conditions during laboratory experimental studies. Ideally, in natural waters, aquatic species are rarely exposed to a single stressor alone at a particular time. Therefore, another important area that my future research will focus on is to explore the potential effects of other candidate metabolites in isolation as well as in combination with other abiotic stressors.

References

AGRA, A. R., L. GUILHERMINO, A. M. SOARES and C. BARATA. 2010. Genetic costs of tolerance to metals in *Daphnia longispina* populations historically exposed to a copper mine drainage. *Environmental Toxicology and Chemistry*, **29**(4), pp.939-946.

survminer: Drawing Survival Curves using 'ggplot2'. R package. 2019.

ALLEN, Y., P. CALOW and D. J. BAIRD. 1995. A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. *Environmental Toxicology and Chemistry*, **14**(9), pp.1625-1630.

ALMOND, R., M. GROOTEN and T. PETERSON. 2020. *Living Planet Report 2020-Bending the curve of biodiversity loss*. World Wildlife Fund.

AMADO, L. L. and J. M. MONSERRAT. 2010. Oxidative stress generation by microcystins in aquatic animals: why and how. *Environment International* **36**(2), pp.226-235.

BABCOCK-JACKSON, L., W. W. CARMICHAEL and D. A. CULVER. 2002. Dreissenid mussels increase exposure of benthic and pelagic organisms to toxic microcystins. *Internationale Vereinigung für theoretische und angewandte Limnologie: Verhandlungen*, **28**(2), pp.1082-1085.

BABICA, K. HILSCHEROVÁ, K. BÁRTOVÁ, L. BLÁHA and B. MARŠÁLEK. 2007. Effects of dissolved microcystins on growth of planktonic photoautotrophs. *Phycologia*, **46**(2), pp.137-142.

BABICA, P., L. BLÁHA and B. MARŠALEK. 2006. Exploring the natural role of microcystins—a review of effects on photoautotrophic organisms. *Journal of Phycology*, **42**(1), pp.9-20.

BARATA, C. and D. J. BAIRD. 2000. Determining the ecotoxicological mode of action of chemicals from measurements made on individuals: results from instar-based tests with *Daphnia magna* Straus. *Aquatic Toxicology*, **48**(2-3), pp.195-209.

BARRIOS, C. A. Z., S. NANDINI and S. SARMA. 2017. Effect of crude extracts from cyanobacterial blooms in Lake Texcoco (Mexico) on the population growth of *Brachionus calyciflorus* (Rotifera). *Toxicon,* **139**, pp.45-53.

BARRY, M. J. and D. C. J. A. T. LOGAN. 1998. The use of temporary pond microcosms for aquatic toxicity testing: direct and indirect effects of endosulfan on community structure. *Aquatic Toxicology,* **41**(1-2), pp.101-124.

BARTRAM, J. and I. CHORUS. 1999. *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management.* CRC Press.

BEAUSOLEIL, C., J.-N. ORMSBY, A. GIES, U. HASS, J. J. HEINDEL, M. L. HOLMER, P. J. NIELSEN, S. MUNN and G. SCHOENFELDER. 2013. Low dose effects and non-monotonic dose responses for endocrine active chemicals: science to practice workshop: workshop summary. *Chemosphere,* **93**(6), pp.847-856.

BEAVER, J. R., C. E. TAUSZ, K. C. SCOTESE, A. I. POLLARD and R. M. MITCHELL. 2018. Environmental factors influencing the quantitative distribution of microcystin and common potentially toxigenic cyanobacteria in US lakes and reservoirs. *Harmful Algae,* **78**, pp.118-128.

BEVERSDORF, K. RUDE, C. A. WEIRICH, S. L. BARTLETT, M. SEAMAN, C. KOZIK, P. BIESE, T. GOSZ, M. SUHA and C. STEMPA. 2018. Analysis of cyanobacterial metabolites in surface and raw drinking waters reveals more than microcystin. *Water Research,* **140**, pp.280-290.

BILLOIR, E., A. R. PÉRY and S. CHARLES. 2007. Integrating the lethal and sublethal effects of toxic compounds into the population dynamics of *Daphnia magna*: A combination of the DEBtox and matrix population models. *Ecological Modelling,* **203**(3-4), pp.204-214.

BIRK, S., CHAPMAN, D., CARVALHO, L., SPEARS, B.M., ANDERSEN, H.E., ARGILLIER, C., AUER, S., BAATTRUP-PEDERSEN, A., BANIN, L., BEKLIOĞLU, M., BONDAR-KUNZE, E., BORJA, A., BRANCO, P., BUCAK, T., BUIJSE, A. D.,

CARDOSO, A. C., COUTURE, R., CREMONA, F., de ZWART, D., FELD, C. K; FERREIRA, M. T., FEUCHTMAYR, H., GESSNER, M. O., GIESWEIN, A., GLOBEVNIK, L., GRAEBER, D., GRAF, W., GUTIÉRREZ-CÁNOVAS, C., HANGANU, J., IŞKIN, U., JÄRVINEN, M., JEPPESEN, E., KOTAMÄKI, N., KUIJPER, M., LEMM, J. U., LU. S., SOLHEIM, A. L., MISCHKE, U., MOE, S. J., NÕGES, P., NÕGES, T., ORMEROD, S. J., PANAGOPOULOS, Y., PHILLIPS, G., POSTHUMA, L., POUSO, S., PRUDHOMME, S., RANKINEN, K., RASMUSSEN, J. J., RICHARDSON, J., SAGOUIS, A., SANTOS, J. M., SCHÄFER, R. B., SCHINEGGER, R., SCHMUTZ, S., SCHNEIDER, S. C., SCHÜLTING, L., SEGURADO, P., STEFANIDIS, K., SURES, B., THACKERAY, S. J., TURUNEN, J., UYARRA, M. C., VENOHR, M., VON DER OHE, P. C., WILLBY, N., and D. HERING 2020. Impacts of multiple stressors on freshwater biota across spatial scales and ecosystems. *Nature Ecology & Evolution.* **4**(8), pp.1060-1068.

BISHOP, C., E. ANET and P. GORHAM. 1959. Isolation and identification of the fast-death factor in *Microcystis aeruginosa* NRC-1. *Canadian Journal of Biochemistry and Physiology*, **37**(3), pp.453-471.

BITTENCOURT-OLIVEIRA, M. D. C., M. A. CHIA, H. S. B. DE OLIVEIRA, M. K. CORDEIRO ARAÚJO, R. J. R. MOLICA and C. T. S. DIAS. 2015. Allelopathic interactions between microcystin-producing and non-microcystin-producing cyanobacteria and green microalgae: implications for microcystins production. *Journal of Applied Phycology*, **27**(1), pp.275-284.

BLOM, J. F., H. I. BAUMANN, G. A. CODD and F. JÜTTNER. 2006. Sensitivity and adaptation of aquatic organisms to oscillapeptin J and microcystin-RR. *Archiv fuer Hydrobiologie*, pp.547-559.

BLOOR, M. 2011. Dietary preference of *Gammarus pulex* and *Asellus aquaticus* during a laboratory breeding programme for ecotoxicological studies. *International Journal of Zoology* **2011**.

BOEGEHOLD, A. G., N. S. JOHNSON and D. R. KASHIAN. 2019. Dreissenid (quagga and zebra mussel) veligers are adversely affected by bloom forming cyanobacteria. *Ecotoxicology and Environmental Safety*, **182**, p109426.

BOETS, P., K. LOCK, P. L. GOETHALS, C. R. JANSSEN and K. A. DE SCHAMPHELAERE. 2012. A comparison of the short-term toxicity of cadmium to indigenous and alien gammarid species. *Ecotoxicology*, **21**(4), pp.1135-1144.

BOLKER, B. M. 2008. *Ecological models and data in R.* Princeton University Press.

BOUAÏCHA, N., C. O. MILES, D. G. BEACH, Z. LABIDI, A. DJABRI, N. Y. BENAYACHE and T. NGUYEN-QUANG. 2019. Structural diversity, characterization and toxicology of microcystins. *Toxins*, **11**(12), p714.

BOWNIK, A. 2016. Harmful algae: Effects of cyanobacterial cyclic peptides on aquatic invertebrates-a short review. *Toxicon*, **124**(Supplement C), pp.26-35.

BOYER, G. L. 2008. Cyanobacterial toxins in New York and the lower Great Lakes ecosystems. *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs.* Springer, pp.153-165.

BRIDGEMAN, T. B., J. D. CHAFFIN and J. E. FILBRUN. 2013. A novel method for tracking western Lake Erie *Microcystis* blooms, 2002–2011. *Journal of Great Lakes Research*, **39**(1), pp.83-89.

BRILAND, R. D., J. P. STONE, M. MANUBOLU, J. LEE and S. LUDSIN. 2020. Cyanobacterial blooms modify food web structure and interactions in western Lake Erie. *Harmful Algae*, **92**, p101586.

BROOK, B.W. N.S. SODHI, and C.J. BRADSHAW. 2008. Synergies among extinction drivers under global change. *Trends in Ecology & Evolution.* **23**(8), pp.453-460.

BROOKS, P. N. GASKELL and L. L. MALTBY. 2009. Sublethal effects and predator-prey interactions: Implications for ecological risk assessment. *Environmental Toxicology and Chemistry*, **28**(11), pp.2449-2457.

BROOKS, J. M. LAZORCHAK, M. D. HOWARD, M. V. V. JOHNSON, S. L. MORTON, D. A. PERKINS, E. D. REAVIE, G. I. SCOTT, S. A. SMITH and J. A. STEEVENS. 2016. Are harmful algal blooms becoming the greatest inland water quality threat to public health and aquatic ecosystems? *Environmental Toxicology and Chemistry*, **35**(1), pp.6-13.

BROWN, J. H., J. F. GILLOOLY, A. P. ALLEN, V. M. SAVAGE and G. B.. WEST. 2004. Toward a metabolic theory of ecology. *Ecology*, **85**(7), pp.1771-1789.

BUCHWALTER, D. B., J. J. JENKINS and L. R. CURTIS. 2003. Temperature influences on water permeability and chlorpyrifos uptake in aquatic insects with differing respiratory strategies. *Environmental Toxicology and Chemistry*, **22**(11), pp.2806-2812.

BUI, B.-T., C. WIEGAND, K. V. DINH and T.-S. DAO. 2020. Responses of a tropical micro-crustacean, Daphnia lumholtzi, upon exposures to dissolved toxins and living cells of cyanobacteria. *Environmental Technology and Innovation,* **19**, p100973.

BUI, T., T.-S. DAO, T.-G. VO and M. J. T. LÜRLING. 2018. Warming affects growth rates and microcystin production in tropical bloom-forming *Microcystis* strains. *Toxins,* **10**(3), p123.

BUKAVECKAS, P. A., J. LESUTIENĖ, Z. R. GASIŪNAITĖ, L. LOŽYS, I. OLENINA, R. PILKAITYTĖ, Ž. PŪTYS, S. TASSONE and J. WOOD. 2017. Microcystin in aquatic food webs of the Baltic and Chesapeake Bay regions. *Estuarine, Coastal and Shelf Science,* **191**, pp.50-59.

BUNDSCHUH, M., R. GERGS, S. SCHADT and R. SCHULZ. 2013. Do differences in sensitivity between native and invasive amphipods explain their coexistence in Lake Constance? A case study with lambda-cyhalothrin. **92**(5), *Chemosphere* pp.483-489.

BURKHOLDER, J. M., S. E. SHUMWAY and P. M. GLIBERT. 2018. Food web and ecosystem impacts of harmful algae. *Harmful Algal Blooms* pp.243-336.

BUS, J. S. 2017. “The dose makes the poison”: key implications for mode of action (mechanistic) research in a 21st century toxicology paradigm. *Current Opinion in Toxicology*, **3**, pp.87-91.

CAI, S., Y. JIA, O. O. DONDE, Z. WANG, J. ZHANG, T. FANG, B. XIAO and X. WU. 2021. Effects of microcystin-producing and non-microcystin-producing *Microcystis* on the behavior and life history traits of *Chironomus pallidivittatus*. *Environmental Pollution*, **287**, p117613.

CAI, S., SHU, Y., TIAN, C., WANG, C., FANG, T., XIAO, B. and WU, X. 2022. Effects of chronic exposure to microcystin-LR on life-history traits, intestinal microbiota and transcriptomic responses in *Chironomus pallidivittatus*. *Science of the Total Environment.* **823**, p153624.

CALABRESE, E. 2008. Hormesis: why it is important to toxicology and toxicologists. *Environmental Toxicology Chemistry*, **27**(7), pp.1451-1474.

CAMARGO, J. A. and Á. ALONSO. 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. *Environment International*, **32**(6), pp.831-849.

CAMPOS, A., P. ARAÚJO, C. PINHEIRO, J. AZEVEDO, H. OSÓRIO and V. VASCONCELOS. 2013. Effects on growth, antioxidant enzyme activity and levels of extracellular proteins in the green alga *Chlorella vulgaris* exposed to crude cyanobacterial extracts and pure microcystin and cylindrospermopsin. *Ecotoxicology and environmental safety*, **94**, pp.45-53.

CAMPOS, A. and V. VASCONCELOS. 2010. Molecular mechanisms of microcystin toxicity in animal cells. *International Journal of Molecular Sciences*, **11**(1), pp.268-287.

CARMICHAEL, W. W. and G. L. BOYER. 2016. Health impacts from cyanobacteria harmful algae blooms: Implications for the North American Great Lakes. *Harmful algae*, **54**, pp.194-212.

CARPENTER, S. R., E. H. STANLEY and M. J. VANDER ZANDEN. 2011. State of the world's freshwater ecosystems: physical, chemical, and biological changes. *Annual Review of Environment and Resources*, **36**, pp.75-99.

CHALIFOUR, A., M. T. ARTS, M. J. KAINZ and P. JUNEAU. 2014. Combined effect of temperature and bleaching herbicides on photosynthesis, pigment and fatty acid composition of *Chlamydomonas reinhardtii*. *European Journal of Phycology*, **49**(4), pp.508-515.

CHALIFOUR, A. and P. JUNEAU. 2011. Temperature-dependent sensitivity of growth and photosynthesis of *Scenedesmus obliquus*, *Navicula pelliculosa* and two strains of *Microcystis aeruginosa* to the herbicide atrazine. *Aquatic Toxicology*, **103**(1-2), pp.9-17.

CHAM, S. 2012. *Field guide to the larvae and exuviae of British dragonflies: dragonflies (Anisoptera) and damselflies (Zygoptera).* British Dragonfly Society.

CHAMIER, A.-C. J. O. 1987. Effect of pH on microbial degradation of leaf litter in seven streams of the English Lake District. **71**(4), pp.491-500.

CHAPMAN, P. M. 2002. Integrating toxicology and ecology: putting the “eco” into ecotoxicology. *Marine Pollution Bulletin*, **44**(1), pp.7-15.

CHEN, X. DAI, T. SHU, R. D. GULATI and Z. LIU. 2013. Microcystins derived from lysing *Microcystis* cells do not cause negative effects on crustacean zooplankton in Lake Taihu, China. *Aquatic ecology*, **47**(4), pp.379-387.

CHEN, Y. HU, J. HE, J. CHEN, J. P. GIESY and P. XIE. 2017. Responses of the proteome and metabolome in livers of zebrafish exposed chronically to environmentally relevant concentrations of microcystin-LR. *Journal of Environmental Science Technology*, **51**(1), pp.596-607.

CHEN, L. SONG, D. OU and N. GAN. 2005a. Chronic toxicity and responses of several important enzymes in *Daphnia magna* on exposure to sublethal microcystin-LR. *Environmental Toxicology*, **20**(3), pp.323-330.

CHEN, P. XIE, L. LI and J. XU. 2009. First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. *Journal of Toxicological Sciences,* **108**(1), pp.81-89.

CHEN, L., J. P. GIESY and P. XIE. 2018. The dose makes the poison. *Science of The Total Environment*, **621**, pp.649-653.

CHEN, W., L. SONG, D. OU and N. GAN. 2005b. Chronic toxicity and responses of several important enzymes in *Daphnia magna* on exposure to sublethal microcystin-LR. *Environmental Toxicology,* **20**(3), pp.323-330.

CHENG, C., L. HUANG, R. MA, Z. ZHOU and J. DIAO. 2015. Enantioselective toxicity of lactofen and its metabolites in *Scenedesmus obliquus*. *Algal Research,* **10**, pp.72-79.

CHEUNG, M. Y., S. LIANG and J. LEE. 2013. Toxin-producing cyanobacteria in freshwater: A review of the problems, impact on drinking water safety, and efforts for protecting public health. *Journal of Microbiology,* **51**(1), pp.1-10.

CHISLOCK, M. F., O. SARNELLE, L. M. JERNIGAN and A. E. WILSON. 2013. Do high concentrations of microcystin prevent *Daphnia* control of phytoplankton? *Water Research,* **47**(6), pp.1961-1970.

CHORUS, I., I. R. FALCONER, H. J. SALAS and J. BARTRAM. 2000. Health risks caused by freshwater cyanobacteria in recreational waters. *Journal of Toxicology and Environmental Health Part B: Critical Reviews,* **3**(4), pp.323-347.

CHORUS, I. and M. WELKER. 2021. *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management.* Taylor & Francis.

CHOUNG, C. B., R. V. HYNE, M. M. STEVENS and G. C. HOSE. 2013. The ecological effects of a herbicide–insecticide mixture on an experimental freshwater ecosystem. *Environmental Pollution,* **172**, pp.264-274.

CHRISTOFFERSEN. 1996. Ecological implications of cyanobacterial toxins in aquatic food webs. *Phycologia,* **35**(6S), pp.42-50.

CLEMENTS, W. H., C. W. HICKEY and K. A. KIDD. 2012. How do aquatic communities respond to contaminants? It depends on the ecological context. *Environmental Toxicology and Chemistry*, **31**(9), pp.1932-1940.

CLEMENTS, W. H. and M. C. NEWMAN. 2003. *Community Ecotoxicology.* John Wiley & Sons.

CLEMENTS, W. H. and J. R. ROHR. 2009. Community responses to contaminants: using basic ecological principles to predict ecotoxicological effects. *Environmental Toxicology and Chemistry*, **28**(9), pp.1789-1800.

COLL, M. and K. HARGADON. 2012. Trophic and functional cascades in tropical versus temperate aquatic microcosms. *Aquatic Ecology,* **46**(1), pp.55-71.

COLLIER, K. J., P. K. PROBERT and M. JEFFRIES. 2016. Conservation of aquatic invertebrates: concerns, challenges and conundrums. *Aquatic Conservation: Marine*

Freshwater Ecosystems, **26**(5), pp.817-837.

CONSOLANDI, G., A. T. FORD and M. C. BLOOR. eds. 2019. *Feeding Behavioural Studies with Freshwater Gammarus spp.: The Importance of a Standardised Methodology.* Springer.

CÔTÉ, I.M., E.S. DARLING and C. J. BROWN. 2016. Interactions among ecosystem stressors and their importance in conservation. *Proceedings of the Royal Society B.* **283**(1824), p20152592.

DAAM, M. A., K. SATAPORNVANIT, P. J. VAN DEN BRINK, A. J. NOGUEIRA. 2010. Direct and indirect effects of the fungicide carbendazim in tropical freshwater microcosms. *Archives of Environmental Contamination and Toxicology,* **58**(2), pp.315-324.

DAAM, M. A., P. J. VAN DEN BRINK and A. J. NOGUEIRA. 2008. Impact of single and repeated applications of the insecticide chlorpyrifos on tropical freshwater plankton communities. *Ecotoxicology*, **17**(8), pp.756-71.

DAO, T.-S., C. WIEGAND, B.-T. BUI and K. V. DINH. 2018. Transgenerational effects of cyanobacterial toxins on a tropical micro-crustacean *Daphnia lumholtzi* across three generations. *Environmental Pollution*, **243**, pp.791-799.

DAO, T. S., L.-C. DO-HONG and C. WIEGAND. 2010. Chronic effects of cyanobacterial toxins on *Daphnia magna* and their offspring. *Toxicon*, **55**(7), pp.1244-1254.

DAWSON, R. 1998. The toxicology of microcystins. *Toxicon,* **36**(7), pp.953-962.

DE CASTRO-CATALÀ, N., I. MUÑOZ, J. RIERA and A. J. E. P. FORD. 2017. Evidence of low dose effects of the antidepressant fluoxetine and the fungicide prochloraz on the behavior of the keystone freshwater invertebrate *Gammarus pulex*. *Environmental Pollution*, **231**, pp.406-414.

DE FIGUEIREDO, D. R., U. M. AZEITEIRO, S. M. ESTEVES, F. J. GONÇALVES and M. J. PEREIRA. 2004. Microcystin-producing blooms—a serious global public health issue. *Ecotoxicology and Environmental Safety*, **59**(2), pp.151-163.

DE SOUZA MACHADO, A. A., C. ZARFL, S. REHSE and W. KLOAS. 2017. Low-dose effects: nonmonotonic responses for the toxicity of a *Bacillus thuringiensis* biocide to *Daphnia magna*. *Environmental Science Technology*, **51**(3), pp.1679-1686.

DEMOTT, W. R., Q. X. ZHANG and W. W. CARMICHAEL. 1991. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. *Limnology and Oceanography*, **36**(7), pp.1346-1357.

DETENBECK, N. E., R. HERMANUTZ, K. ALLEN and M. C. SWIFT. 1996. Fate and effects of the herbicide atrazine in flow-through wetland mesocosms. *Environmental Toxicology and Chemistry*, **15**(6), pp.937-946.

DODDS, W. K., W. W. BOUSKA, J. L. EITZMANN, T. J. PILGER, K. L. PITTS, A. J. RILEY, J. T. SCHLOESSER and D. J. THORNBRUGH. 2009. *Eutrophication of US freshwaters: analysis of potential economic damages*. ACS Publications.

DUC, N. T., X. Q. NGO, T. L. PHAM and T. S. DAO. 2020. Ecotoxicological investigation of cyanobacterial crude extracts to *Daphnia magna* under subchronic test conditions. *Turkish Journal of Zoology*, **44**(6), pp.498-507.

DUDGEON, D. 2010. Prospects for sustaining freshwater biodiversity in the 21st century: linking ecosystem structure and function. *Current Opinion in Environmental Sustainability*, **2**(5), pp.422-430.

DUDGEON, D., A. H. ARTHINGTON, M. O. GESSNER, Z.-I. KAWABATA, D. J. KNOWLER, C. LÉVÊQUE, R. J. NAIMAN, A.-H. PRIEUR-RICHARD, D. SOTO and M. L. J. B. R. STIASSNY. 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol. Rev.* , **81**(2), pp.163-182.

EC, 2000. European Union Water Framework Directive 2000/60/EC of the Council establishing a framework for community action in the field of water policy. Official *Journal of the European Communities*, **327**, pp. 1–72.

EDWARDS, R. and D. PASCOE. 2018. Single Species Toxicity Tests. *Aquatic Ecotoxicology*. CRC Press, pp.93-126.

EL-SHEEKH, H. M. KHAIRY and R. EL-SHENODY. 2012. Algal production of extra and intra-cellular polysaccharides as an adaptive response to the toxin crude extract of Microcystis aeruginosa. *Iranian Journal of Environmental Health Science Engineering*, **9**(1), pp.1-7.

EL-SHEEKH, M., H. KHAIRY and R. EL-SHENODY. 2010. Allelopathic effects of cyanobacterium *Microcystis aeruginosa* Kützing on the growth and photosynthetic pigments of some algal species. *Allelopathy Journal* **26**(2), pp.275-290.

ERNST, B., B. HITZFELD and D. DIETRICH. 2001. Presence of *Planktothrix* sp. and cyanobacterial toxins in Lake Ammersee, Germany and their impact on whitefish (*Coregonus lavaretus* L.). *Environmental toxicology*, **16**(6), pp.483-488.

FALCONER, I. R. 2001. Toxic cyanobacterial bloom problems in Australian waters: risks and impacts on human health. *Phycologia*, **40**(3), p228.

FANG, B., J. SHI, L. QIN, M. FENG, D. CHENG, T. WANG and X. ZHANG. 2018a. Toxicity evaluation of 4, 4′-di-CDPS and 4, 4′-di-CDE on green algae *Scenedesmus obliquus*: growth inhibition, change in pigment content, and oxidative stress. *Environmental Science and Pollution Research*, pp.1-11.

FANG, B., J. SHI, L. QIN, M. FENG, D. CHENG, T. WANG and X. ZHANG. 2018b. Toxicity evaluation of 4, 4′-di-CDPS and 4, 4′-di-CDE on green algae *Scenedesmus obliquus*: growth inhibition, change in pigment content, and oxidative stress. *Environmental Science and Pollution Research*, **25**, pp.15630-15640.

FERRANDO, M., E. SANCHO and E. ANDREU-MOLINER. 1995. Effects of lindane on *Daphnia magna* during chronic exposure. *Journal of Environmental Science Health Part B,* **30**(6), pp.815-825.

FERRÃO-FILHO, A. D. S. and B. KOZLOWSKY-SUZUKI. 2011. Cyanotoxins: bioaccumulation and effects on aquatic animals. *Marine Drugs,* **9**(12), pp.2729-2772.

FERRAO-FILHO, A. S., N. A. HERRERA and L. F. ECHEVERRI. 2014. Microcystin accumulation in cladocerans: first evidence of MC uptake from aqueous extracts of a natural bloom sample. *Toxicon*, **87**, pp.26-31.

FERRAO-FILHO, A. S., S. M. AZEVEDO and W. R. DEMOTT. 2000. Effects of toxic and non-toxic cyanobacteria on the life history of tropical and temperate cladocerans. *Journal of Freshwater Biology*

45(1), pp.1-19.

FIGUEREDO, C. C., A. GIANI and D. F. BIRD. 2007. Does allelopathy contribute to *cylindrospermopsis raciborskii* (cyanobacteria) bloom occurrence and geographic expansion? 1. *Journal of Phycology,* **43**(2), pp.256-265.

FORBES, V. E. 2000. Is hormesis an evolutionary expectation? *Functional Ecology* **14**(1), pp.12-24.

GALANTI, L. N., M. V. AMÉ and D. A. WUNDERLIN. 2013. Accumulation and detoxification dynamic of cyanotoxins in the freshwater shrimp *Palaemonetes argentinus*. *Harmful Algae,* **27**, pp.88-97.

GALIC, N., V. GRIMM and V. E. FORBES. 2017. Impaired ecosystem process despite little effects on populations: modeling combined effects of warming and toxicants. *Global Change Biology,* **23**(8), pp.2973-2989.

GARDHAM, S., A. A. CHARITON and G. C. J. E. HOSE. 2015. Direct and indirect effects of copper-contaminated sediments on the functions of model freshwater ecosystems. *Ecotoxicology* **24**(1), pp.61-70.

GENE, S. M., R. S. SHAHMOHAMADLOO, X. ORTIZ, R. S. J. E. T. PROSSER and CHEMISTRY. 2019. Effect of *Microcystis aeruginosa*–Associated Microcystin-LR on the Survival of 2 Life Stages of Freshwater Mussel (*Lampsilis siliquoidea*). *Environmental Toxicology and Chemistry* **38**(10), pp.2137-2144.

GER, K.A., TEH, S.J. and GOLDMAN, C.R. 2009. Microcystin-LR toxicity on dominant copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* of the upper San Francisco Estuary. *Science of the Total Environment.* **407**(17), pp.4852-4857.

GER, K. A., E. J. FAASSEN, M. G. PENNINO and M. LÜRLING. 2016a. Effect of the toxin (microcystin) content of *Microcystis* on copepod grazing. *Harmful algae,* **52**, pp.34-45.

GER, K. A., S. NAUS-WIEZER, L. DE MEESTER and M. LÜRLING. 2019. Zooplankton grazing selectivity regulates herbivory and dominance of toxic phytoplankton over multiple prey generations. *Limnology and Oceanography,* **64**(3), pp.1214-1227.

GER, K. A. and R. PANOSSO. 2014. The effects of a microcystin-producing and lacking strain of *Microcystis* on the survival of a widespread tropical copepod (*Notodiaptomus iheringi*). *Hydrobiologia,* **738**(1), pp.61-73.

GER, K. A., P. URRUTIA-CORDERO, P. C. FROST, L.-A. HANSSON, O. SARNELLE, A. E. WILSON and M. LÜRLING. 2016b. The interaction between cyanobacteria and zooplankton in a more eutrophic world. *Harmful Algae*, **54**(Supplement C), pp.128-144.

GHADOUANI, A., B. PINEL-ALLOUL, K. PLATH, G. A. CODD and W. LAMPERT. 2004. Effects of *Microcystis aeruginosa* and purified microcystin-LR on the feeding behavior of *Daphnia pulicaria*. *Limnology and Oceanography* **49**(3), pp.666-679.

GLIBERT, P. M. 2017. Eutrophication, harmful algae and biodiversity—Challenging paradigms in a world of complex nutrient changes. *Marine Pollution Bulletin*.

GOMES, M. P. and P. JUNEAU. 2017. Temperature and light modulation of herbicide toxicity on algal and cyanobacterial physiology. *Frontiers in Environmental Science* **5**, p50.

GRAHAM, J. L., N. M. DUBROVSKY, G. M. FOSTER, L. R. KING, K. A. LOFTIN, B. H. ROSEN and E. A. STELZER. 2020. Cyanotoxin occurrence in large rivers of the United States. *Inland Waters*, **10**(1), pp.109-117.

GRIFFITH, A. W. and C. J. GOBLER. 2019. Harmful algal blooms: A climate change co-stressor in marine and freshwater ecosystems. *Harmful Algae*.

GRZYBOWSKI, M. and K. GLIŃSKA-LEWCZUK. 2019. Principal threats to the conservation of freshwater habitats in the continental biogeographical region of Central Europe. *Biodiversity and Conservation*, **28**(14), pp.4065-4097.

GUNDERSON, A. R., E. J. ARMSTRONG and J. H. STILLMAN. 2016. Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. *Annual Review of Marine Science* **8**, pp.357-378.

GUSTAFSSON, S. and L.-A. HANSSON. 2004. Development of tolerance against toxic cyanobacteria in *Daphnia*. *Aquatic Ecology*, **38**(1), pp.37-44.

HARKE, M. J., M. M. STEFFEN, C. J. GOBLER, T. G. OTTEN, S. W. WILHELM, S. A. WOOD and H. W. PAERL. 2016. A review of the global ecology, genomics, and biogeography of the toxic cyanobacterium, *Microcystis spp*. *Harmful Algae,* **54**, pp.4-20.

HAVENS, K. E. 2008. Cyanobacteria blooms: effects on aquatic ecosystems. *Cyanobacterial harmful algal blooms: state of the science and research needs*, pp.733-747.

HAY, M. E. and J. KUBANEK. 2002. Community and ecosystem level consequences of chemical cues in the plankton. *Journal of Chemical Ecology*, **28**(10), pp.2001-2016.

HAYES, N. M., H. A. HAIG, G. L. SIMPSON and P. R. LEAVITT. 2020. Effects of lake warming on the seasonal risk of toxic cyanobacteria exposure. *Limnology and Oceanography,* **Letters 5,** 393-402

HE, F., C. ZARFL, V. BREMERICH, J. N. DAVID, Z. HOGAN, G. KALINKAT, K. TOCKNER and S. C. JÄHNIG. 2019. The global decline of freshwater megafauna. *Global Change Biology,* **25**(11), pp.3883-3892.

HENRY, Y., C. PISCART, S. CHARLES and H. COLINET. 2017. Combined effect of temperature and ammonia on molecular response and survival of the freshwater crustacean *Gammarus pulex*. *Ecotoxicology and Environmental Safety*, **137**, pp.42-48.

HERRERA, N., PALACIO, J., ECHEVERRI, F. and FERRÃO-FILHO, A. 2014. Effects of a cyanobacterial bloom sample containing microcystin-LR on the ecophysiology of *Daphnia similis*. *Toxicology reports.* **1**, pp.909-914.

HERRERA, N. A., L. F. ECHEVERRI and A. S. FERRAO-FILHO. 2015. Effects of phytoplankton extracts containing the toxin microcystin-LR on the survival and reproduction of cladocerans. *Toxicon,* **95**, pp.38-45.

HEUGENS, E. H., T. JAGER, R. CREYGHTON, M. H. KRAAK, A. J. HENDRIKS, N. M. VAN STRAALEN and W. ADMIRAAL. 2003. Temperature-dependent effects of cadmium on *Daphnia magna*: accumulation versus sensitivity. *Environmental Science and Technology* **37**(10), pp.2145-2151.

HULOT, F. D., D. CARMIGNAC, S. LEGENDRE, C. YEPREMIAN and C. BERNARD. 2012. Effects of microcystin-producing and microcystin-free strains of *Planktothrix agardhii* on long-term population dynamics of Daphnia magna. *In: Annales de Limnologie-International Journal of Limnology*: EDP Sciences, pp.337-347.

HYENSTRAND, P., P. BLOMQVIST and A. PETTERSSON. 1998. Factors determining cyanobacterial success in aquatic systems: a literature review. *Archiv für Hydrobiologie Spec. Iss. Advances in Limnology,* **51**, pp.41-62.

IBELINGS, B. W., K. BRUNING, J. DE JONGE, K. WOLFSTEIN, L. M. D. PIRES, J. POSTMA and T. BURGER. 2005. Distribution of Microcystins in a Lake Foodweb: No Evidence for Biomagnification. *Microbial Ecology,* **49**(4), pp.487-500.

IBELINGS, B. W., J. W. FOURNIE, E. D. HILBORN, G. A. CODD, M. COVENEY, J. DYBLE, K. HAVENS, J. LANDSBERG and W. LITAKER. 2008. Ecosystem effects workgroup report. *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs.* Springer, pp.655-674.

IBELINGS, B. W. and K. E. HAVENS. 2008. Cyanobacterial toxins: a qualitative meta–analysis of concentrations, dosage and effects in freshwater, estuarine and marine biota. *Cyanobacterial Harmful Algal blooms: State of the Science and Research Needs.* Springer, pp.675-732.

IBRAHEM, M. D., H. M. KHAIRY, M. P. IBRAHIM and BIOCHEMISTRY. 2012. Laboratory exposure of *Oreochromis niloticus* to crude microcystins (containing microcystin-LR) extracted from Egyptian locally isolated strain (*Microcystis aeruginosa* Kützing): biological and biochemical studies. *Fish Physiology and Biochemistry* **38**(3), pp.899-908.

ISAZA, D. F. G., R. L. CRAMP and C. E. FRANKLIN. 2020. Living in polluted waters: A meta-analysis of the effects of nitrate and interactions with other environmental stressors on freshwater taxa. *Environmental Pollution,* **261**, p114091.

ISIBOR, P. O. 2017. Oxidative stress biomarkers in *Clarias gariepinus* (Burchel, 1822) exposed to Microcystin-LR. *Beni-Suef University Journal of Basic Applied Sciences,* **6**(1), pp.69-75.

JACKSON, M. C., C. J. LOEWEN, R. D. VINEBROOKE and C. T. CHIMIMBA. 2016. Net effects of multiple stressors in freshwater ecosystems: a meta-analysis. *Global Change Biology,* **22**(1), pp.180-189.

JACQUET, C., V. THERMES, A. DE LUZE, S. PUISEUX-DAO, C. BERNARD, J.-S. JOLY, F. BOURRAT and M. EDERY. 2004. Effects of microcystin-LR on development of medaka fish embryos (*Oryzias latipes*). *Toxicon,* **43**(2), pp.141-147.

JANSSEN. 2019. Cyanobacterial peptides beyond microcystins–A review on co-occurrence, toxicity, and challenges for risk assessment. *Water Research,* **151**(15), pp.488-499.

JESUS, F. T., C. MARTINS and A. J. NOGUEIRA. 2014. Changes in life-history parameters of *Daphnia longispina* (Cladocera, Crustacea) as a function of water chemistry. *Journal of limnology,* **73**(2).

JIA, J., W. SHI, Q. CHEN and T. L. LAURIDSEN. 2017. Spatial and temporal variations reveal the response of zooplankton to cyanobacteria. *Harmful Algae,* **64**, pp.63-73.

JOS, A., S. PICHARDO, A. I. PRIETO, G. REPETTO, C. M. VÁZQUEZ, I. MORENO and A. M. CAMEÁN. 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis sp*.) under laboratory conditions. *Aquatic Toxicology,* **72**(3), pp.261-271.

JUNGMANN. 1992. Toxic compounds isolated from *Microcystis* PCC786 that are more active against *Daphnia* than two microcystins. *Limnology and Oceanography,* **37**(8), pp.1777-1783.

JUNGMANN, D. and J. J. F. B. BENNDORF. 1994. Toxicity to *Daphnia* of a compound extracted from laboratory and natural *Microcystis spp*., and the role of microcystins. *Freshwater Biology*, **32**(1), pp.13-20.

KAEBERNICK, M. and B. A. NEILAN. 2001. Ecological and molecular investigations of cyanotoxin production. *FEMS Microbiology Ecology*, **35**(1), pp.1-9.

KARLSON, A. M. and R. MOZŪRAITIS. 2011. Deposit-feeders accumulate the cyanobacterial toxin nodularin. *Harmful Algae,* **12**, pp.77-81.

KENNA, D., W. N. FINCHAM, A. M. DUNN, L. E. BROWN and C. HASSALL. 2017. Antagonistic effects of biological invasion and environmental warming on detritus processing in freshwater ecosystems. *Oecologia,* **183**(3), pp.875-886.

KENNEDY, J. H., T. W. LA POINT, P. BALCI, J. STANLEY and Z. B. JOHNSON. 2002. Model aquatic ecosystems in ecotoxicological research: considerations of design, implementation, and analysis. *Handbook of Ecotoxicology,* **2**, pp.45-74.

KIM, J., J. K. SEO, H. YOON, P. J. KIM and K. CHOI. 2014. Combined effects of the cyanobacterial toxin microcystin-LR and environmental factors on life-history traits of indigenous cladoceran *Moina macrocopa*. *Environmental toxicology and Chemistry*

33(11), pp.2560-2565.

KIM, M., D. KIM, J. KIM, S. HONG and K.-H. SHIN. 2021. Distribution of microcystins in environmental multimedia and their bioaccumulation characteristics in marine benthic organisms in the Geum River Estuary, South Korea. *Science of the Total Environment,* **757**, p143815.

KLÜTTGEN, B., U. DÜLMER, M. ENGELS and H. RATTE. 1994. ADaM, an artificial freshwater for the culture of zooplankton. *Water Research* **28**(3), pp.743-746.

KNIGHT, T. M., M. W. MCCOY, J. M. CHASE, K. A. MCCOY and R. D. HOLT. 2005. Trophic cascades across ecosystems. *Nature* **437**(7060), pp.880-883.

KNOPS, M., R. ALTENBURGER and H. J. A. T. SEGNER. 2001. Alterations of physiological energetics, growth and reproduction of *Daphnia magna* under toxicant stress. *Aquatic Toxicology* **53**(2), pp.79-90.

KOPP, R., PALÍKOVÁ, M., ADAMOVSKÝ, O., ZIKOVÁ, A., NAVRÁTIL, S., KOHOUTEK, J., MAREŠ, J. and BLÁHA, L. 2013. Concentrations of microcystins in tissues of several fish species from freshwater reservoirs and ponds. *Environmental Monitoring and Assessment.* **185**(12), pp.9717-9727.

KORPINEN, S., M. KARJALAINEN and M. J. H. VIITASALO. 2006. Effects of cyanobacteria on survival and reproduction of the littoral crustacean *Gammarus zaddachi* (Amphipoda). *Hydrobiologia* **559**(1), pp.285-295.

KOTAK, B. and R. ZURAWELL. 2007. Cyanobacterial toxins in Canadian freshwaters: A review. *Lake Reservoir Management,* **23**(2), pp.109-122.

KOTAK, B. G., R. W. ZURAWELL, E. E. PREPAS and C. F. HOLMES. 1996. Microcystin-LR concentration in aquatic food web compartments from lakes of varying trophic status. *Canadian Journal of Fisheries and Aquatic Sciences,* **53**(9), pp.1974-1985.

KOZLOWSKY-SUZUKI, B., A. E. WILSON and A. DA SILVA FERRAO-FILHO. 2012. Biomagnification or biodilution of microcystins in aquatic foodwebs? Meta-analyses of laboratory and field studies. *Harmful Algae,* **18**, pp.47-55.

KRATINA, P., T. J. WATTS, D. S. GREEN, R. L. KORDAS and E. J. O'GORMAN. 2019. Interactive effects of warming and microplastics on metabolism but not feeding rates of a key freshwater detritivore. *Environmental Pollution*, **255**, p113259.

KRAUSE, G. and E. WEIS. 1991. Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Biology*, **42**(1), pp.313-349.

KUNZ, P. Y., C. KIENLE and A. GERHARDT. 2010. *Gammarus spp*. in aquatic ecotoxicology and water quality assessment: toward integrated multilevel tests. *Reviews of Environmental Contamination and Toxicology Volume 205.* Springer, pp.1-76.

LAGARDE, F., C. BEAUSOLEIL, S. M. BELCHER, L. P. BELZUNCES, C. EMOND, M. GUERBET and C. J. E. H. ROUSSELLE. 2015. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. *Environmental Health,* **14**(1), pp.1-15.

LAMB, M. C., D. G. KIMMEL and E. K. FIELD. 2019. The effects of temperature on *Bosmina longirostris* susceptibility to microcystin-LR acute toxicity. *PlosOne,* **14**(7), pe0219342.

LAMONICA, D., B. CLÉMENT, S. CHARLES, C. J. E. LOPES and E. SAFETY. 2016. Modelling algae–duckweed interaction under chemical pressure within a laboratory microcosm. *Ecotoxicology and Environmental Safety,* **128**, pp.252-265.

LANCE, E., M.-R. NEFFLING, C. GERARD, J. MERILUOTO and M. BORMANS. 2010. Accumulation of free and covalently bound microcystins in tissues of *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacteria or dissolved microcystin-LR exposure. *Environmental Pollution,* **158**(3), pp.674-680.

LARRAS, F., A.-S. LAMBERT, S. PESCE, F. RIMET, A. BOUCHEZ, B. J. E. MONTUELLE and E. SAFETY. 2013. The effect of temperature and a herbicide mixture on freshwater periphytic algae. *Ecotoxicology and Environmental Safety,* **98**, pp.162-170.

LI, D.-M., H.-Y. ZHENG, J.-L. PAN, T.-Q. ZHANG, S.-K. TANG, J.-M. LU, L.-Q. ZHONG, Y.-S. LIU and X.-W. LIU. 2017. Seasonal dynamics of photosynthetic activity, *Microcystis* genotypes and microcystin production in Lake Taihu, China. *Journal of Great Lakes Research,* **43**(4), pp.710-716.

LI, X., X. PING, S. XIUMEI, W. ZHENBIN and X. LIQIANG. 2005. Toxicity of cypermethrin on growth, pigments, and superoxide dismutase of *Scenedesmus obliquus*. *Ecotoxicology and Environmental Safety,* **60**(2), pp.188-192.

LIANG, Y., X. CHEN, X. LU, S. JIN, Y. MIN and J. YANG. 2017a. Combined effects of microcystin and nitrite on the growth, lipid peroxidation, and antioxidant responses of the freshwater rotifer *Brachionus calyciflorus*. *Aquatic Toxicology* **192**, pp.78-88.

LIANG, Y., T. GAO, L. SHAO, Y. MIN and J. YANG. 2020. Effects of microcystin-LR and nitrite on the lifespan, reproduction, and heat shock responses of rotifer *Brachionus calyciflorus* at different temperatures. *Aquatic Sciences,* **82**(4), pp.1-14.

LIANG, Y., X. LU, Y. MIN, L. LIU and J. YANG. 2018. Interactive effects of microcystin and ammonia on the reproductive performance and phenotypic traits of the rotifer *Brachionus calyciflorus*. *Ecotoxicology and Environmental Safety* **147**, pp.413-422.

LIANG, Y., Y. SU, K. OUYANG, X. CHEN and J. YANG. 2017b. Effects of microcystin-producing and microcystin-free *Microcystis aeruginosa* on enzyme activity and nutrient content in the rotifer *Brachionus calyciflorus*. *Environmental Science and Pollution Research,* **24**(11), pp.10430-10442.

LINDHOLM, T., J. ERIKSSON, M. REINIKAINEN and J. MERILUOTO. 1992. Ecological effects of hepatotoxic cyanobacteria. *Environmental Toxicology and Water Quality* **7**(1), pp.87-93.

LIU, Y., L. SONG, X. LI and T. LIU. 2002. The toxic effects of microcystin-LR on embryo-larval and juvenile development of loach, *Misguruns mizolepis* Gunthe. *Toxicon,* **40**(4), pp.395-399.

LOFTIN, K. A., J. L. GRAHAM, E. D. HILBORN, S. C. LEHMANN, M. T. MEYER, J. E. DIETZE and C. B. GRIFFITH. 2016. Cyanotoxins in inland lakes of the United States: Occurrence and potential recreational health risks in the EPA National Lakes Assessment 2007. *Harmful Algae,* **56**, pp.77-90.

LOPEZ-ARCHILLA, A. I., D. MOREIRA, P. LÓPEZ-GARCÍA and C. GUERRERO. 2004. Phytoplankton diversity and cyanobacterial dominance in a hypereutrophic shallow lake with biologically produced alkaline pH. *Extremophiles,* **8**(2), pp.109-115.

LOTKA, A. J. 1913. A natural population norm. I. *Journal of the Washington Academy of Sciences* **3**(9), pp.241-248.

LUO, T., J. CHEN, X. LI, S. ZHANG, H. YAO, and W. J. PEIJNENBURG. 2018. Effects of lomefloxacin on survival, growth and reproduction of *Daphnia magna* under simulated sunlight radiation. *Ecotoxicology and Environmental Safety,* **166**, pp.63-70.

LÜRLING. 2003. *Daphnia* growth on microcystin-producing and microcystin-free *Microcystis aeruginosa* in different mixtures with the green alga *Scenedesmus obliquus. Journal of Limnology and Oceanography* **48**(6), pp.2214-2220.

LÜRLING and E. VAN DER GRINTEN. 2003. Life-history characteristics of *Daphnia* exposed to dissolved microcystin-LR and to the cyanobacterium *Microcystis aeruginosa* with and without microcystins. *Environmental Toxicology Chemistry*, **22**(6), pp.1281-1287.

MA, H., Y. WU, N. GAN, L. ZHENG, T. LI and L. SONG. 2015. Growth inhibitory effect of *Microcystis* on *Aphanizomenon flos-aquae* isolated from cyanobacteria bloom in Lake Dianchi, China. *Harmful Algae*, **42**, pp.43-51.

MAJOR, Y., KIFLE, D., SPOOF, L. and MERILUOTO, J. 2018. Cyanobacteria and microcystins in Koka reservoir (Ethiopia). *Environmental Science and Pollution Research.* **25**(27), pp.26861-26873.

MALTBY, L., S. A. CLAYTON, R. M. WOOD and N. MCLOUGHLIN. 2002. Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: Robustness, responsiveness, and relevance. *Environmental Toxicology Chemistry* **21**(2), pp.361-368.

MANTZOUKI, E., P. M. VISSER, M. BORMANS and B. W. IBELINGS. 2016. Understanding the key ecological traits of cyanobacteria as a basis for their management and control in changing lakes. *Aquatic Ecology*, **50**(3), pp.333-350.

MARINOVIĆ, Z., TOKODI, N., BACKOVIĆ, D.D., ŠĆEKIĆ, I., KITANOVIĆ, N., SIMIĆ, S.B., ĐORĐEVIĆ, N.B., FERINCZ, Á., STASZNY, Á. and DULIĆ, T. 2021. Does the Kis-Balaton Water Protection System (KBWPS) Effectively Safeguard Lake Balaton from Toxic Cyanobacterial Blooms? *Microorganisms.* **9**(5), p960.

MARTINS, J. C. and V. M. VASCONCELOS. 2009. Microcystin dynamics in aquatic organisms. *Journal of Toxicology and Environmental Health, Part B,* **12**(1), pp.65-82.

MCCAULEY, S. J., J. I. HAMMOND, D. N. FRANCES and K. E. MABRY. 2015. Effects of experimental warming on survival, phenology, and morphology of an aquatic insect (O donata). *Ecological Entomology*, **40**(3), pp.211-220.

MCKIE, B. G., M. SCHINDLER, M. O. GESSNER and B. MALMQVIST. 2009. Placing biodiversity and ecosystem functioning in context: environmental perturbations and the effects of species richness in a stream field experiment. *Oecologia,* **160**(4), pp.757-770.

MCRAE, L., S. DEINET and R. FREEMAN. 2017. The diversity-weighted living planet index: controlling for taxonomic bias in a global biodiversity indicator. *Plos One,* **12**(1), pe0169156.

MEREL, S., D. WALKER, R. CHICANA, S. SNYDER, E. BAURÈS and O. THOMAS. 2013. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environment international,* **59**, pp.303-327.

MICHALAK, A. M., E. J. ANDERSON, D. BELETSKY, S. BOLAND, N. S. BOSCH, T. B. BRIDGEMAN, J. D. CHAFFIN, K. CHO, R. CONFESOR and I. DALOĞLU. 2013. Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. *Proceedings of the National Academy of Sciences,* **110**(16), pp.6448-6452.

MIHALJEVIĆ, M. and F. J. A. E. STEVIĆ. 2011. Cyanobacterial blooms in a temperate river-floodplain ecosystem: the importance of hydrological extremes. **45**(3), pp.335-349.

MOHAMED, Z. A. 2008. Polysaccharides as a protective response against microcystin-induced oxidative stress in *Chlorella vulgaris* and *Scenedesmus quadricauda* and their possible significance in the aquatic ecosystem. *Ecotoxicology,* **17**(6), p504.

MONROY, S., A. MARTÍNEZ, N. LÓPEZ-ROJO, A. PÉREZ-CALPE, A. BASAGUREN and J. POZO. 2017. Structural and functional recovery of macroinvertebrate communities and leaf litter decomposition after a marked drought: Does vegetation type matter? *The Science of the total environment,* **599**, p1241.

MORGAN, E., BROWN, ALASTAIR, CIOTTI, BENJAMIN AND PANTON, ANOUSKA. ed. 2016. *Effects of temperature stress on ecological processes.* Oxford, GB. : Oxford University Press,.

MÜLLER, M. F., J. COLOMER and T. SERRA. 2018. Temperature-driven response reversibility and short-term quasi-acclimation of *Daphnia magna*. *Plos One,* **13**(12), pe0209705.

MULLIN, C.A., KIRCHHOFF, C.J., WANG, G. and VLAHOS, P. 2020. Future projections of water temperature and thermal stratification in Connecticut reservoirs and possible implications for cyanobacteria. *Journal of Water Resources Research* **56**(11), pe2020WR027185.

NEUMANN, A.-C., X. WANG, R. NIESSNER and D. KNOPP. 2016. Determination of microcystin-LR in surface water by a magnetic bead-based colorimetric immunoassay using antibody-conjugated gold nanoparticles. *Analytical Methods,* **8**(1), pp.57-63.

NILSEN, E., K. L. SMALLING, L. AHRENS, M. GROS, K. S. MIGLIORANZA, Y. PICÓ and H. L. SCHOENFUSS. 2019. Critical review: grand challenges in assessing the adverse effects of contaminants of emerging concern on aquatic food webs. *Environment Toxicology and Chemistry,* **38**(1), pp.46-60.

NIU, Z., L. DU, J. LI, Y. ZHANG and Z. LV. 2018. Ecological risk assessment of microcystin-LR in the upstream section of the Haihe River based on a species sensitivity distribution model. *Chemosphere* **193**, pp.403-411.

NOYES, P. D., M. K. MCELWEE, H. D. MILLER, B. W. CLARK, L. A. VAN TIEM, K. C. WALCOTT, K. N. ERWIN and E. D. LEVIN. 2009. The toxicology of climate change: environmental contaminants in a warming world. *Environment International,* **35**(6), pp.971-986.

O'BRIEN, A., TOWNSEND, K., HALE, R., SHARLEY, D. and PETTIGROVE, V. 2016. How is ecosystem health defined and measured? A critical review of freshwater and estuarine studies. *Ecological Indicators.* **69**, pp.722-729.

O'NEIL, J., T. DAVIS, M. BURFORD and C. GOBLER. 2012. The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. *Harmful Algae*, **14**, pp.313-334.

OBERDÖRSTER, G. 2010. Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. *Journal of Internal Medicine*, **267**(1), pp.89-105.

OBERHOLSTER, P. J., A.-M. BOTHA and P. J. ASHTON. 2009. The influence of a toxic cyanobacterial bloom and water hydrology on algal populations and macroinvertebrate abundance in the upper littoral zone of Lake Krugersdrift, South Africa. *Ecotoxicology*, **18**(1), pp.34-46.

OECD. 2004. *Test No. 202: Daphnia Sp. Acute Immobilisation Test.* OECD Publishing.

OECD. 2008. *OECD Guidelines for Testing of Chemicals, No. 211, Daphnia magna Reproduction Test*. 4th ed.: OECD Paris.

OECD. 2011. 201: Freshwater alga and cyanobacteria, growth inhibition test. *OECD guidelines for the testing of chemicals, section*, **2**.

OKANO, K., K. SHIMIZU, Y. KAWAUCHI, H. MASEDA, M. UTSUMI, Z. ZHANG, B. A. NEILAN and N. SUGIURA. 2009. Characteristics of a microcystin-degrading bacterium under alkaline environmental conditions. *Journal of Toxicology*, **2009**.

OKUMURA, D. T., R. B. SOTERO-SANTOS, R. A. TAKENAKA and O. ROCHA. 2007. Evaluation of cyanobacteria toxicity in tropical reservoirs using crude extracts bioassay with cladocerans. *Ecotoxicology*, **16**(2), pp.263-270.

OLIVER, T. H., M. S. HEARD, N. J. ISAAC, D. B. ROY, D. PROCTER, F. EIGENBROD, R. FRECKLETON, A. HECTOR, C. D. L. ORME and O. L. PETCHEY. 2015. Biodiversity and resilience of ecosystem functions. *Trends in Ecology & Evolution*, **30**(11), pp.673-684.

OMIDI, A., M. ESTERHUIZEN-LONDT and S. PFLUGMACHER. 2018. Still challenging: the ecological function of the cyanobacterial toxin microcystin–What we know so far. *Toxin Reviews*, **37**(2), pp.87-105.

OriginPro. 2020. Northampton, MA, USA: OriginLab Corporation.

ORMEROD, S., M. DOBSON, A. HILDREW and C. TOWNSEND. 2010. Multiple stressors in freshwater ecosystems. *Freshwater Biology,* **55**(s1), pp.1-4.

ORTIZ-RODRÍGUEZ, R., T. S. DAO and C. WIEGAND. 2012. Transgenerational effects of microcystin-LR on *Daphnia magna*. *Journal of Experimental Biology*, **215**(16), pp.2795-2805.

PACHAURI, R. K., M. R. ALLEN, V. R. BARROS, J. BROOME, W. CRAMER, R. CHRIST, J. A. CHURCH, L. CLARKE, Q. DAHE and P. DASGUPTA. 2014. *Climate change 2014: synthesis report. Contribution of Working Groups I, II and III to the fifth assessment report of the Intergovernmental Panel on Climate Change.* Ipcc.

PAERL, H. W., R. S. FULTON, P. H. MOISANDER and J. DYBLE. 2001. Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *The Scientific World Journal*, **1**, pp.76-113.

PAERL, H. W., N. S. HALL and E. S. CALANDRINO. 2011. Controlling harmful cyanobacterial blooms in a world experiencing anthropogenic and climatic-induced change. *Science of the Total Environment,* **409**(10), pp.1739-1745.

PAERL, H. W. and HUISMAN. 2008. Blooms like it hot. *Science,* **320**(5872), pp.57-58.

PARK, Y., J. PYO, Y. S. KWON, Y. CHA, H. LEE, T. KANG and K. H. CHO. 2017. Evaluating physico-chemical influences on cyanobacterial blooms using hyperspectral images in inland water, Korea. *Water Research,* **126**, pp.319-328.

PECKARSKY, B. L. and G. A. LAMBERTI. 2017. Chapter 18 - Invertebrate Consumer–Resource Interactions. *In:* F. R. HAUER and G. A. LAMBERTI, eds. *Methods in Stream Ecology, Volume 1 (Third Edition).* Boston: Academic Press, pp.379-398.

PELLAN, L., V. MÉDOC, D. RENAULT, T. SPATARO and C. PISCART. 2016. Feeding choice and predation pressure of two invasive gammarids, *Gammarus tigrinus* and *Dikerogammarus villosus*, under increasing temperature. *Hydrobiologia* **781**(1), pp.43-54.

PEREIRA, A. L., C. SANTOS, J. AZEVEDO, T. P. MARTINS, R. CASTELO-BRANCO, V. RAMOS, V. VASCONCELOS and A. CAMPOS. 2018. Effects of two toxic cyanobacterial crude extracts containing microcystin-LR and cylindrospermopsin on the growth and photosynthetic capacity of the microalga *Parachlorella kessleri*. *Algal Research,* **34**, pp.198-208.

PEREZ-MORALES, A., S. S. SARMA and S. NANDINI. 2014. Feeding and filtration rates of zooplankton (rotifers and cladocerans) fed toxic cyanobacterium (*Microcystis aeruginosa*). *Journal of Environmental Biology,* **35**(6), pp.1013-20.

PESTANA, J., A. ALEXANDER, J. CULP, D. BAIRD, A. CESSNA and A. SOARES. 2009. Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms. *Environmental Pollution,* **157**(8), pp.2328-2334.

PFLUGMACHER, S.. 2002 Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. *Environmental Toxicology,* **17**(4), pp.407-413.

PHAM, T.-L. and M. UTSUMI. 2018. An overview of the accumulation of microcystins in aquatic ecosystems. *Journal of Environmental Management,* **213**, pp.520-529.

PIETSCH, C., C. WIEGAND, M. V. AME, A. NICKLISCH, D. WUNDERLIN and S. PFLUGMACHER. 2001. The effects of a cyanobacterial crude extract on different aquatic organisms: Evidence for cyanobacterial toxin modulating factors. *Environmental Toxicology,* **16**(6), pp.535-542.

POHNERT, G., M. STEINKE and R. TOLLRIAN. 2007. Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. *Trends in Ecology & Evolution,* **22**(4), pp.198-204.

POROJAN, C., F. ABBAS, M. A. MOWE, M. LEHANE, S. M. MITROVIC, R. P. LIM, D. C. YEO, A. J. M. FUREY and F. RESEARCH. 2020a. Survey of microcystins in Singapore's reservoirs using liquid chromatography–tandem mass spectrometry (LC-MS/MS). *Marine and Freshwater Research*

POROJAN, C., F. ABBAS, M. A. D. MOWE, M. LEHANE, S. M. MITROVIC, R. P. LIM, D. C. J. YEO and A. FUREY. 2020b. Survey of microcystins in Singapore's reservoirs using liquid chromatography–tandem mass spectrometry (LC-MS/MS) %J Marine and Freshwater Research. pp.-.

POSTE, A. E. and T. OZERSKY. 2013. Invasive dreissenid mussels and round gobies: a benthic pathway for the trophic transfer of microcystin. *Environmental Toxicology and Chemistry,* **32**(9), pp.2159-2164.

PREECE, E. P., F. J. HARDY, B. C. MOORE and M. BRYAN. 2017. A review of microcystin detections in Estuarine and Marine waters: Environmental implications and human health risk. *Harmful Algae,* **61**, pp.31-45.

PREECE, E. P., W. HOBBS, F. J. HARDY, L. O'GARRO, E. FRAME and F. SWEENEY. 2021. Prevalence and persistence of microcystin in shoreline lake sediments and porewater, and associated potential for human health risk. *Chemosphere,* **272**, p129581.

frair: Tools for Functional Response Analysis. R package. 2017.

PRESTON, B.L. 2002. Indirect effects in aquatic ecotoxicology: implications for ecological risk assessment. *Environmental Management.* **29**(3), pp.311-323.

PRITCHARD, D. W., R. PATERSON, H. C. BOVY, D. J. M. I. E. BARRIOS-O'NEILL and EVOLUTION. 2017. Frair: an R package for fitting and comparing consumer functional responses. *Methods in Ecology and Evolution,* **8**(11), pp.1528-1534.

R CORETEAM. 2019. *R: a language and environment for statistical computing (version 3.6.0)[software]*.

RASTOGI, R. P., R. P. SINHA and A. INCHAROENSAKDI. 2014. The cyanotoxin-microcystins: current overview. *Reviews in Environmental Science and Biotechnology*, **13**(2), pp.215-249.

RAVEN, J. A. and R. J. GEIDER. 1988. Temperature and algal growth. *New Phytologist*, **110**(4), pp.441-461.

REID, A. J., A. K. CARLSON, I. F. CREED, E. J. ELIASON, P. A. GELL, P. T. JOHNSON, K. A. KIDD, T. J. MACCORMACK, J. D. OLDEN and S. ORMEROD. 2019. Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biological Reviews* **94**(3), pp.849-873.

REINIKAINEN, M., M. KETOLA and M. WALLS. 1994. Effects of the concentrations of toxic *Microcystis aeruginosa* and an alternative food on the survival of *Daphnia pulex*. *Limnology and Oceanography*, **39**(2), pp.424-432.

REWICZ, T., M. GRABOWSKI, C. MACNEIL and K. BACELA-SPYCHALSKA. 2014. The profile of a 'perfect'invader–the case of killer shrimp, *Dikerogammarus villosus*. *Aquatic Invasions*, **9**(3), pp.267-288.

REYNOLDS, C. and J.-P. DESCY. 1996. The production, biomass and structure of phytoplankton in large rivers. *Large Rivers*, pp.161-187.

RICHARDSON, J., H. FEUCHTMAYR, C. MILLER, P. HUNTER, S. C. MABERLY and L. CARVALHO. 2019. The response of cyanobacteria and phytoplankton abundance to warming, extreme rainfall events and nutrient enrichment. *Global change biology*, **25**, pp.3365–3380.

RICO, A., M. R. DIMITROV, R. P. VAN WIJNGAARDEN, K. SATAPORNVANIT, H. SMIDT and P. J. VAN DEN BRINK. 2014. Effects of the antibiotic enrofloxacin on the ecology of tropical eutrophic freshwater microcosms. *Aquatic Toxicology*, **147**, pp.92-104.

RIGOSI, A., C. C. CAREY, B. W. IBELINGS and J. D. BROOKES. 2014. The interaction between climate warming and eutrophication to promote cyanobacteria is

dependent on trophic state and varies among taxa. *Limnology and Oceanography*, **59**(1), pp.99-114.

RIVETTI, C., B. CAMPOS and C. BARATA. 2016. Low environmental levels of neuro-active pharmaceuticals alter phototactic behaviour and reproduction in *Daphnia magna*. *Aquatic Toxicology*, **170**, pp.289-296.

ROGERS, D. 1972. Random search and insect population models. *Journal of Animal Ecology*, **41**, pp.369–383.

ROHR, J. R. and P. W. CRUMRINE. 2005. Effects of an herbicide and an insecticide on pond community structure and processes. *Ecological Applications*, **15**(4), pp.1135-1147.

ROHRLACK, T., K. CHRISTOFFERSEN, P. E. HANSEN, W. ZHANG, O. CZARNECKI, M. HENNING, J. FASTNER, M. ERHARD, B. A. NEILAN and M. KAEBERNICK. 2003. Isolation, characterization, and quantitative analysis of microviridin J, a new Microcystis metabolite toxic to *Daphnia*. *Journal of Chemical Ecology*, **29**(8), pp.1757-1770.

ROHRLACK, T., E. DITTMANN, T. BÖRNER and K. CHRISTOFFERSEN. 2001. Effects of cell-bound microcystins on survival and feeding of *Daphnia spp*. *Applied and Environmental Microbiology*, **67**(8), pp.3523-3529.

ROHRLACK, T., E. DITTMANN, M. HENNING, T. BÖRNER and J.-G. KOHL. 1999. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. *Applied and Environmental Microbiology*, **65**(2), pp.737-739.

ROY-LACHAPELLE, A., DUY, S.V., MUNOZ, G., DINH, Q.T., BAHL, E., SIMON, D.F. and SAUVÉ, S. 2019. Analysis of multiclass cyanotoxins (microcystins, anabaenopeptins, cylindrospermopsin and anatoxins) in lake waters using on-line SPE liquid chromatography high-resolution Orbitrap mass spectrometry. *Analytical Methods*. **11**(41), pp.5289-5300.

SÁNCHEZ-BARACALDO, P., G. BIANCHINI, J. D. WILSON and A. H. KNOLL. 2021. Cyanobacteria and biogeochemical cycles through Earth history. *Trends in Microbiology*, **20**(20), pp.1-15

SANCHO, E., S. BANEGAS, M. J. VILLARROEL and D. FERRANDO. 2018. Impaired reproduction and individual growth of the water flea *Daphnia magna* as consequence of exposure to the non-ester pyrethroid etofenprox. *Environmental Science and Pollution Research*, **25**(7), pp.6209-6217.

SANCHO, E., M. VILLARROEL and M. FERRANDO. 2016. Assessment of chronic effects of tebuconazole on survival, reproduction and growth of *Daphnia magna* after different exposure times. *Ecotoxicology and Environmental Safety*, **124**, pp.10-17.

SANDIN, L. and A. G. SOLIMINI. 2009. Freshwater ecosystem structure–function relationships: from theory to application. *Freshwater Biology*, **54**(10), pp.2017-2024.

SARNELLE, O., S. GUSTAFSSON and L.-A. HANSSON. 2010. Effects of cyanobacteria on fitness components of the herbivore *Daphnia*. *Journal of Plankton Research* **32**(4), pp.471-477.

SAUVÉ. 2021. Occurrence of microcystins, anabaenopeptins and other cyanotoxins in fish from a freshwater wildlife reserve impacted by harmful cyanobacterial blooms. *Toxicon*, **194**, pp.44-52.

SCHERER. 2018. *PropCIs: Various Confidence Interval Methods for Proportions.*

SCHMIDT, J. R. 2014. *Fate and detection of microcystin-LR and its metabolites in the environment*. 3687350 thesis, State University of New York College of Environmental Science and Forestry.

SCHREIDAH, C. M., K. RATNAYAKE, K. SENARATH and A. KARUNARATHNE. 2020. Microcystins: Biogenesis, Toxicity, Analysis, and Control. *Chemical Research in Toxicology*, **33**(9), pp.2225-2246.

SCHULHOF, M. A., J. B. SHURIN, S. A. DECLERCK and D. B. VAN DE WAAL. 2019. Phytoplankton growth and stoichiometric responses to warming, nutrient addition and grazing depend on lake productivity and cell size. *Global Change Biology*, **25**(8), pp.2751-2762.

SEDMAK, B. and G. KOSI. 1998. The role of microcystins in heavy cyanobacterial bloom formation. *Journal of Plankton Research*, **20**(4), pp.691-708.

SEGNER, H., M. SCHMITT-JANSEN and S. SABATER. 2014. Assessing the impact of multiple stressors on aquatic biota: the receptor's side matters. *Environmental Science and Technology*, **48**, pp.7690-7698.

SHAHMOHAMADLOO, R. S., D. G. POIRIER, X. O. ALMIRALL, S. P. BHAVSAR and P. K. SIBLEY. 2020. Assessing the toxicity of cell-bound microcystins on freshwater pelagic and benthic invertebrates. *Ecotoxicology and Environmental Safety*, **188**, p109945.

SHAN, K., L. SONG, W. CHEN, L. LI, L. LIU, Y. WU, Y. JIA, Q. ZHOU and L. PENG. 2019. Analysis of environmental drivers influencing interspecific variations and associations among bloom-forming cyanobacteria in large, shallow eutrophic lakes. *Harmful algae*, **84**, pp.84-94.

SHI, J., M. HUBER, T. WANG, W. DALI, Z. LIN and Y. J. E. D. CHUN-SHENG. 2016. Progress in the studies on hormesis of low-dose pollutants. *Environmental Disease*, **1**(2), p58.

SIVONEN, K. and G. JONES. 1999. Cyanobacterial toxins. *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*, **1**, pp.43-112.

SKAFI, M., S. V. DUY, G. MUNOZ, Q. T. DINH, D. F. SIMON, P. JUNEAU and S.

SMUTNÁ, M., P. BABICA, S. JARQUE, K. HILSCHEROVÁ, B. MARŠÁLEK, M. HAEBA and L. BLÁHA. 2014. Acute, chronic and reproductive toxicity of complex

cyanobacterial blooms in *Daphnia magna* and the role of microcystins. *Toxicon,* **79**, pp.11-18.

SOARES, R. M., M. YUAN, J. C. SERVAITES, A. DELGADO, V. F. MAGALHAES, E. D. HILBORN, W. W. CARMICHAEL and S. M. AZEVEDO. 2006. Sublethal exposure from microcystins to renal insufficiency patients in Rio de Janeiro, Brazil. *Environ Toxicol,* **21**(2), pp.95-103.

SOKOLOVA, I. M., M. FREDERICH, R. BAGWE, G. LANNIG and A. A. SUKHOTIN. 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research,* **79**, pp.1-15.

SOTERO-SANTOS, R. B., C. R. D. S. E. SILVA, N. F. VERANI, K. O. NONAKA and O. ROCHA. 2006. Toxicity of a cyanobacteria bloom in barra bonita reservoir (middle tiete river, Sao Paulo, Brazil). *Ecotoxicology and Environmental Safety,* **64**(2), pp.163-170.

SPERFELD, E. and A. WACKER. 2009. Effects of temperature and dietary sterol availability on growth and cholesterol allocation of the aquatic keystone species *Daphnia*. *Journal of Experimental Biology* **212**(19), pp.3051-3059.

SRODA, S. and C. COSSU-LEGUILLE. 2011. Effects of sublethal copper exposure on two gammarid species: which is the best competitor? *Ecotoxicology,* **20**(1), pp.264-273.

STEFFEN, M. M., T. W. DAVIS, R. M. L. MCKAY, G. S. BULLERJAHN, L. E. KRAUSFELDT, J. M. A. STOUGH, M. L. NEITZEY, N. E. GILBERT, G. L. BOYER, T. H. JOHENGEN, D. C. GOSSIAUX, A. M. BURTNER, D. PALLADINO, M. D. ROWE, G. J. DICK, K. A. MEYER, S. LEVY, B. E. BOONE, R. P. STUMPF, T. T. WYNNE, P. V. ZIMBA, D. GUTIERREZ and S. W. WILHELM. 2017. Ecophysiological Examination of the Lake Erie Microcystis Bloom in 2014: Linkages between Biology and the Water Supply Shutdown of Toledo, OH. *Environmental Science & Technology,* **51**(12), pp.6745-6755.

STRANDBERG, U., J. VESTERINEN, T. ILO, J. AKKANEN, M. MELANEN and P. KANKAALA. 2020. Fatty acid metabolism and modifications in *Chironomus riparius*. *Philosophical Transactions of the Royal Society B* **375** (1804), p20190643.

SUKENIK, A., A. QUESADA and N. SALMASO. 2015. Global expansion of toxic and non-toxic cyanobacteria: effect on ecosystem functioning. *Biodiversity and Conservation*, **24**(4), pp.889-908.

ŠULČIUS, S., D. MONTVYDIENĖ, H. MAZUR-MARZEC, J. KASPEROVIČIENĖ, R. RULEVIČIUS and Ž. CIBULSKAITĖ. 2017. The profound effect of harmful cyanobacterial blooms: From food-web and management perspectives. *Science of the Total Environment*, **609**, pp.1443-1450.

SVIRČEV, Z., D. LALIĆ, G. B. SAVIĆ, N. TOKODI, D. D. BACKOVIĆ, L. CHEN, J. MERILUOTO and G. A. CODD. 2019. Global geographical and historical overview of cyanotoxin distribution and cyanobacterial poisonings. *Archives of toxicology*, **93**, pp.2429–2481.

TANK, J. L., E. J. ROSI-MARSHALL, N. A. GRIFFITHS, S. A. ENTREKIN and M. L. STEPHEN. 2010. A review of allochthonous organic matter dynamics and metabolism in streams. *Journal of North American Benthological Society*, **29**(1), pp.118-146.

A Package for Survival Analysis in S. 2015.

THERNEAU, T. M. and P. M. GRAMBSCH. 2000. *Modeling Survival Data:Extending the Cox Model.* New York: Springer.

THOMPSON, D. 1978. Towards a realistic predator-prey model: the effect of temperature on the functional response and life history of larvae of the damselfly, *Ischnura elegans*. *Journal of Animal Ecology*, pp.757-767.

THOMPSON, P. L. and F. BÄRLOCHER. 1989. Effect of pH on leaf breakdown in streams and in the laboratory. *Journal of the North American Benthological Society*

8(3), pp.203-210.

THOSTRUP, L. and K. A. F. H. CHRISTOFFERSEN. 1999. Accumulation of microcystin in *Daphnia magna* feeding on toxic *Microcystis*. *Archiv für Hydrobiologie* **145**(4), pp.447-467.

TICKNER, D., J. J. OPPERMAN, R. ABELL, M. ACREMAN, A. H. ARTHINGTON, S. E. BUNN, S. J. COOKE, J. DALTON, W. DARWALL and G. EDWARDS. 2020. Bending the curve of global freshwater biodiversity loss: an emergency recovery plan. *BioScience*, **70**(4), pp.330-342.

TIEGS, S. D., J. E. CLAPCOTT, N. A. GRIFFITHS and A. J. BOULTON. 2013. A standardized cotton-strip assay for measuring organic-matter decomposition in streams. *Ecological indicators*, **32**, pp.131-139.

TOPOROWSKA, M., B. PAWLIK-SKOWRONSKA and R. KALINOWSKA. 2014. Accumulation and effects of cyanobacterial microcystins and anatoxin-a on benthic larvae of *Chironomus spp*.(Diptera: Chironomidae). *European Journal of Entomology*, **111**(1), p83.

TORSTEN, H., B. FRANK and W. PETER. 2008. Simultaneous Inferencein General Parametric Models. *Biometrical Journal*, **50**(3), pp.346-363.

TRUHLAR, A. M., J. A. DODD and D. C. ALDRIDGE. 2014. Differential leaf-litter processing by native (*Gammarus pulex*) and invasive (*Dikerogammarus villosus*) freshwater crustaceans under environmental extremes. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **24**(1), pp.56-65.

TSUJI, K., S. NAITO, F. KONDO, N. ISHIKAWA, M. F. WATANABE, M. SUZUKI and K.-I. HARADA. 1994. Stability of microcystins from cyanobacteria: effect of light on decomposition and isomerization. *Environmental Science Technology*, **28**(1), pp.173-177.

TURNER, A. D., M. DHANJI-RAPKOVA, A. O'NEILL, L. COATES, A. LEWIS and K. LEWIS. 2018. Analysis of Microcystins in Cyanobacterial Blooms from Freshwater Bodies in England. *Toxins*, **10**(1), p39.

TYDECKS, L., J. M. JESCHKE, M. WOLF, G. SINGER and K. TOCKNER. 2018. Spatial and topical imbalances in biodiversity research. *Plos One,* **13**(7), pe0199327.

URRUTIA-CORDERO, P., H. ZHANG, F. CHAGUACEDA, H. GENG and L. A. HANSSON. 2020. Climate warming and heat waves alter harmful cyanobacterial blooms along the benthic-pelagic interface. *Ecology,* **0**(0), pe03025.

VAN DEN BRINK, P. J., E. VAN DONK, R. GYLSTRA, S. J. CRUM and T. C. BROCK. 1995. Effects of chronic low concentrations of the pesticides chlorpyrifos and atrazine in indoor freshwater microcosms. *Chemosphere,* **31**(5), pp.3181-3200.

VAN REES, C. B., K. A. WAYLEN, A. SCHMIDT-KLOIBER, S. J. THACKERAY, G. KALINKAT, K. MARTENS, S. DOMISCH, A. I. LILLEBØ, V. HERMOSO and H. P. GROSSART. 2021. Safeguarding freshwater life beyond 2020: Recommendations for the new global biodiversity framework from the European experience. *Conservation Letters,* **14**(1), pe12771.

VARELI, K., PILIDIS, G., MAVROGIORGOU, M.-C., BRIASOULIS, E. and SAINIS, I. 2009. Molecular characterization of cyanobacterial diversity and yearly fluctuations of Microcystin loads in a suburban Mediterranean Lake (Lake Pamvotis, Greece). *Journal of Environmental Monitoring.* **11**(8), pp.1506-1512.

VENABLES, W. N. and B. D. RIPLEY. 2002. *Modern Applied Statistics with S.* Fourth Edition ed. New York: Springer.

VERHEYEN, J., V. DELNAT and R. STOKS. 2019. Increased daily temperature fluctuations overrule the ability of gradual thermal evolution to offset the increased pesticide toxicity under global warming. *Environmental Science Technology,* **53**(8), pp.4600-4608.

VILÀ, M., J. L. ESPINAR, M. HEJDA, P. E. HULME, V. JAROŠÍK, J. L. MARON, J. PERGL, U. SCHAFFNER, Y. SUN and P. J. E. L. PYŠEK. 2011. Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. *Ecology Letters,* **14**(7), pp.702-708.

VILLALOBOS-JIMÉNEZ, G., A. M. DUNN and C. HASSALL. 2017. Environmental noise reduces predation rate in an aquatic invertebrate. *Journal of Insect Conservation,* **21**(5-6), pp.839-847.

VILLARROEL, M., E. SANCHO, M. FERRANDO and E. ANDREU. 2003. Acute, chronic and sublethal effects of the herbicide propanil on *Daphnia magna*. *Chemosphere,* **53**(8), pp.857-864.

VINEBROOKE, D. R., L. K. COTTINGHAM, N. M. SCHEFFER, DODSON, J. and C. S. MABERLY, ULRICH, S. SOMMER. 2004. Impacts of multiple stressors on biodiversity and ecosystem functioning: The role of species co-tolerance. *Oikos*, **104**(3), pp.451-457.

VON SCHILLER, D., V. ACUÑA, I. ARISTI, M. ARROITA, A. BASAGUREN, A. BELLIN, L. BOYERO, A. BUTTURINI, A. GINEBREDA and E. KALOGIANNI. 2017. River ecosystem processes: A synthesis of approaches, criteria of use and sensitivity to environmental stressors. *Science of the Total Environment*, **596**, pp.465-480.

WALLS, J. T., K. H. WYATT, J. C. DOLL, E. M. RUBENSTEIN and A. R. ROBER. 2018. Hot and toxic: Temperature regulates microcystin release from cyanobacteria. *Science of the Total Environment,* **610**, pp.786-795.

WANG, L., J. ZI, R. XU, S. HILT, X. HOU and X. CHANG. 2017. Allelopathic effects of Microcystis aeruginosa on green algae and a diatom: Evidence from exudates addition and co-culturing. *Harmful Algae,* **61**, pp.56-62.

WANG, P.-J., M.-S. CHIEN, F.-J. WU, H.-N. CHOU and S.-J. LEE. 2005. Inhibition of embryonic development by microcystin-LR in zebrafish, *Danio rerio*. *Toxicon,* **45**(3), pp.303-308.

WATANABE, M. F., K.-I. HARADA, W. W. CARMICHAEL and H. FUJIKI. 1995. *Toxic microcystis.* CRC press.

WEI, C., Y. ZHANG, J. GUO, B. HAN, X. YANG and J. YUAN. 2010. Effects of silica nanoparticles on growth and photosynthetic pigment contents of *Scenedesmus obliquus*. *Journal of Environmental Sciences,* **22**(1), pp.155-160.

WEI, H., S. WANG, E. G. XU, J. LIU, X. LI and Z. WANG. 2020. Synergistic toxicity of microcystin-LR and Cu to zebrafish (Danio rerio). *Science of The Total Environment,* **713**, p136393.

WHITE, S., L. DUIVENVOORDEN and L. FABBRO. 2005. Impacts of a toxic *Microcystis* bloom on the macroinvertebrate fauna of Lake Elphinstone, Central Queensland, Australia. *Hydrobiologia,* **548**(1), pp.117-126.

WILLÉN, E., AHLGREN, G., TILAHUN, G., SPOOF, L., NEFFLING, M.-R. and MERILUOTO, J. 2011. Cyanotoxin production in seven Ethiopian Rift Valley lakes. *Inland Waters.* **1**(2), pp.81-91.

WILLIAMS, K., D. GREEN and D. PASCOE. 1984. Toxicity testing with freshwater macroinvertebrates: Methods and application in environmental management. *Freshwater Biological monitoring.* Elsevier, pp.81-91.

WILSON, A. E. 2006. *Cyanobacteria-grazer interactions: Consequences of toxicity, morphology, and genetic diversity*. 3212320 thesis, Georgia Institute of Technology.

WOLLER-SKAR, M. M., A. L. RUSSELL, J. A. GASKILL and M. R. LUTTENTON. 2020. Microcystin in multiple life stages of *Hexagenia limbata*, with implications for toxin transfer. *Journal of Great Lakes Research*.

WOOD, R. 2016. Acute animal and human poisonings from cyanotoxin exposure—A review of the literature. *Environment International,* **91**, pp.276-282.

WOODWARD, G., D. M. PERKINS and L. E. BROWN. 2010. Climate change and freshwater ecosystems: impacts across multiple levels of organization. *Philosophical Transactions of the Royal Society B: ,* **365**(1549), pp.2093-2106.

WRIGHT, D. and J. SHAPIRO. 1990. Refuge availability: a key to understanding the summer disappearance of *Daphnia*. *Freshwater Biology*, **24**(1), pp.43-62.

XIANG, X.-L., Y.-Y. CHEN, Q.-L. XU, L.-Y. ZHU, X.-L. WEN and Y.-L. XI. 2017. Combined Effects of Temperature and the Microcystin MC-LR on the Feeding Behavior of the Rotifer *Brachionus calyciflorus*. *Bulletin of Environmental Contamination Toxicology*, **99**(4), pp.493-499.

XUE, Q., A. D. STEINMAN, X. SU, Y. ZHAO and L. XIE. 2016a. Temporal dynamics of microcystins in *Limnodrilus hoffmeisteri*, a dominant oligochaete of hypereutrophic Lake Taihu, China. *Environmental Pollution,* **213**, pp.585-593.

XUE, Q., X. SU, A. D. STEINMAN, Y. CAI, Y. ZHAO and L. XIE. 2016b. Accumulation of microcystins in a dominant Chironomid Larvae (*Tanypus chinensis*) of a large, shallow and eutrophic Chinese lake, Lake Taihu. *Scientific Reports,* **6**(1), pp.1-10.

YANG, J., H. TANG, X. ZHANG, X. ZHU, Y. HUANG and Z. YANG. 2018. High temperature and pH favor *Microcystis aeruginosa* to outcompete *Scenedesmus obliquus*. *Environmental Science and Pollution Research*, **25**(5), pp.4794-4802.

ZEPERNICK, B. N., E. R. GANN, R. M. MARTIN, H. L. POUND, L. E. KRAUSFELDT, J. D. CHAFFIN and S. W. WILHELM. 2021. Elevated pH Conditions Associated With *Microcystis spp*. Blooms Decrease Viability of the Cultured Diatom *Fragilaria crotonensis* and Natural Diatoms in Lake Erie. *Frontiers in Microbiology*, **12**, p188.

ZHANG, J., CHENCHEN, WANG, Z., SONG, Z., XIE, Z., LI, L. and SONG, L. 2012. Bioaccumulation of microcystins in two freshwater gastropods from a cyanobacteria-bloom plateau lake, Lake Dianchi. *Environmental Pollution.* **164**, pp.227-234.

ZHANG, LIAO, Q., ZHANG, L., WANG, D., LUO, L., CHEN, Y., ZHONG, J. and LIU, J. 2015. Occurrence and spatial distributions of microcystins in Poyang Lake, the largest freshwater lake in China. *Ecotoxicology.* **24**(1), pp.19-28.

ZHANG, J., Z. XIE, and ZHANG, J., Z. XIE, and Z. WANG 2016. Oxidative stress responses and toxin accumulation in the freshwater snail *Radix swinhoei* (Gastropoda, Pulmonata) exposed to microcystin-LR. *Environmental Science and Pollution Research*, **23**(2), pp.1353-1361.

ZHANG, S., X. DU, H. LIU, M. D. LOSIEWIC, X. CHEN, Y. MA, R. WANG, Z. TIAN, L. SHI and H. GUO. 2020. The latest advances in the reproductive toxicity of microcystin-LR. *Environmental Research*, p110254.

ZHENG, L., P. XIE, Y. LI, H. YANG, S. WANG, N. GUO and TOXICOLOGY. 2004. Variation of intracellular and extracellular microcystins in a shallow, hypereutrophic subtropical Chinese lake with dense cyanobacterial blooms. *Journal of Environmental Contaminantion,* **73**(4), pp.698-706.

ZHU, R., H. WANG, H. SHEN, X. DENG and J. CHEN. 2021. The dynamics and release characteristics of microcystins in the plateau Lake Erhai, Southwest China. *Environmental Science Pollution Research*, pp.1-9.

Appendix

Appendix 1: Summary of statistics and parameter estimates with associated standard errors (SE), *t-statistic* and *p-values* for the linear models describing the effects of purified MC-LR and crude *Microcystis* extract on four photosynthetic pigments of *S. quadricauda* relative to the control. (Note that the *p*-values in bold are significant if $p < 0.05$).

Pigment	Parameter	Estimate	SE	t-statistic	p-value
Chlorophyll-a	Intercept	0.15	0.07	2.35	0.027
	Extract	0.08	0.07	1.05	0.303
	MC-LR	0.27	0.07	3.58	0.001
	Solvent Control	0.10	0.09	1.08	0.290
	Concentration	-0.02	0.01	-2.23	0.035
Chlorophyll-b	Intercept	2.07	0.20	10.41	<0.001
	Extract	0.05	0.23	0.22	0.831
	MC-LR	0.54	0.22	2.42	0.023
	Solvent Control	0.13	0.28	0.46	0.648
	Concentration	-0.05	0.02	-2.26	0.033
Total Chlorophyll	Intercept	1.20	0.08	15.64	<0.001
	Extract	0.04	0.09	0.43	0.675
	MC-LR	0.23	0.09	2.65	0.014
	Solvent Control	0.06	0.11	0.56	0.584

	Concentration	-0.02	0.01	-2.03	0.053
Carotenoid	Intercept	1.33	0.14	9.26	<0.001
	Extract	-0.01	0.17	-0.06	0.952
	MC-LR	0.40	0.16	2.48	0.020
	Solvent Control	0.09	0.20	0.45	0.655
	Concentration	-0.04	0.02	-2.33	0.028

Appendix 2: Length and weight of *Gammarus pulex* and *Dikerogammarus villosus* used for the 7-day amphipod leaf-shredding experiment in Chapter 3.

MC Treatment	Concentration (µg/L)	*Gammarus pulex*		*Dikerogammarus villosus*	
		Length (mm)	Weight (mg)	Length (mm)	Weight (mg)
Control	0	12.68	26.30	12.41	28.8
Control	0	14.47	47.70	12.84	26.1
Control	0	14.19	32.10	12.34	28.1
Control	0	13.64	34.30	12.61	22.3
Control	0	12.98	28.40	11.94	23.6
Control	0	14.49	32.80	13.85	28.4
Control	0	16.51	35.80	13.93	37.1
Control	0	14.68	35.40	14.74	38.3
Control	0	16.13	40.90	15.14	45.2
Control	0	16.23	47.50	13.80	35.1
Extract	0.01	12.93	16.00	15.9	46.7
Extract	0.01	13.62	20.62	16.37	50.6
Extract	0.01	12.15	25.50	14.66	36.8
Extract	0.01	13.40	24.83	17.37	60.8
Extract	0.01	12.49	20.44	16.88	51.5
Extract	0.01	13.65	27.21	15.33	37.2

Extract	0.01	14.67	34.40	15.22	43.4
Extract	0.01	12.16	21.27	15.13	44.1
Extract	0.01	12.59	20.20	17.05	51.4
Extract	0.01	12.46	18.20	16.28	50.6
Extract	0.1	14.12	36.3	15.04	42.1
Extract	0.1	12.55	24.6	16.45	52.7
Extract	0.1	13.80	31.6	16.29	48.5
Extract	0.1	14.60	29.4	16.15	43.4
Extract	0.1	13.95	31.8	16.52	51.6
Extract	0.1	11.68	20.1	16.55	52.2
Extract	0.1	12.91	24.5	14.89	43.9
Extract	0.1	13.77	23.3	15.56	45.6
Extract	0.1	12.89	20.8	16.26	46.7
Extract	0.1	12.68	22.9	16.09	49.7
Extract	0.5	12.56	21.8	16.83	51.3
Extract	0.5	12.16	20	15.21	46.5
Extract	0.5	13.42	18.9	15.43	39.7
Extract	0.5	11.67	20.9	16.21	46.5
Extract	0.5	11.36	22.6	14.56	31.8
Extract	0.5	13.50	21.9	14.46	37.1
Extract	0.5	13.15	18.6	15.31	36.9

Extract	0.5	12.20	20.4	15.26	39.6
Extract	0.5	11.66	18.5	14.02	38.3
Extract	0.5	13.00	24.1	15.22	56.6
MC-LR	0.01	15.54	51.5	13.25	32.5
MC-LR	0.01	15.70	35.6	13.11	33.3
MC-LR	0.01	15.76	41	15.31	51.4
MC-LR	0.01	14.87	47	14.2	48.5
MC-LR	0.01	16.48	55.9	13.28	28.8
MC-LR	0.01	14.26	34.7	14.34	37
MC-LR	0.01	15.00	43.4	14.67	35.8
MC-LR	0.01	16.27	40	15.78	44.7
MC-LR	0.01	12.73	38.1	12.74	30.5
MC-LR	0.01	14.80	38.4	16.3	52.8
MC-LR	0.1	14.39	47.9	16.43	51.2
MC-LR	0.1	13.48	33.9	14.84	37.7
MC-LR	0.1	16.51	40.9	16.17	46.8
MC-LR	0.1	17.41	55.2	17.35	58.9
MC-LR	0.1	15.99	40.7	14.38	40.5
MC-LR	0.1	14.75	31.1	15.73	41.5
MC-LR	0.1	15.08	37.5	16.21	52.1
MC-LR	0.1	13.10	24.7	13.06	27

MC-LR	0.1	15.29	36.2	15.87	49.7
MC-LR	0.1	12.79	30.9	15.6	35.8
MC-LR	0.5	14.27	35.4	14.96	39.6
MC-LR	0.5	12.38	19.7	15.23	39.6
MC-LR	0.5	13.52	24.4	14.75	39.9
MC-LR	0.5	13.49	29.4	14.63	39.3
MC-LR	0.5	14.55	35.7	15.3	39.9
MC-LR	0.5	13.43	27.7	13.67	37.7
MC-LR	0.5	13.97	31.1	14.85	37.1
MC-LR	0.5	12.97	23.4	14.61	30.9
MC-LR	0.5	13.71	32.1	15.28	42
MC-LR	0.5	12.56	24.8	14.35	41
MC-LR	1	12.35	28.4	12.57	30.3
MC-LR	1	11.68	16.1	15.06	38.1
MC-LR	1	14.34	30.5	13.1	27.6
MC-LR	1	13.67	26	14.81	29.2
MC-LR	1	13.96	27.9	15.44	47
MC-LR	1	15.21	37.8	16.62	46.6
MC-LR	1	14.49	33.3	15.18	45.6
MC-LR	1	13.04	26.8	13.53	38.5
MC-LR	1	15.01	24.5	15.47	41.2

MC-LR	1	12.27	21.6	16.51	59
MC-LR	10	12.69	21.4	14.45	39.1
MC-LR	10	12.11	20.9	15.89	40.9
MC-LR	10	13.56	23.8	14.97	37.7
MC-LR	10	12.59	21.1	15.99	50.5
MC-LR	10	12.71	29.4	14.3	38.1
MC-LR	10	13.03	29.7	15.15	43.9
MC-LR	10	12.82	24.4	17.89	62.6
MC-LR	10	12.26	20.4	14.83	36.9
MC-LR	10	13.32	24.4	16.02	55.6
MC-LR	10	13.14	25.2	16.27	53.4

Appendix 3: Head width of *Ischnura elegans* used for the survival and functional response experiments in Chapter 4.

Treatment	Head width (mm)
20_0_big	3.1
20_0_big	3.1
20_0_big	3
20_0_big	3.1
20_0_big	3.1
20_0_big	2.7
20_0_big	3
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3
20_0_big	2.9
20_0_big	3.1
20_0_big	3.1
20_0_big	3
20_0_big	2.7

20_0_big	3.1
20_0_big	3
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3
20_0_big	3.1
20_0_big	2.8
20_0_big	2.9
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	2.5
20_0_big	2.7
20_0_big	2.6
20_0_big	3
20_0_big	2.7
15_0_big	2.6
15_0_big	3.1
15_0_big	2.7

15_0_big	3
15_0_big	2.6
15_0_big	3.1
15_0_big	2.5
15_0_big	3.1
15_0_big	3.1
15_0_big	3
15_0_big	2.7
15_0_big	3.1
15_0_big	2.7
15_0_big	3.1
15_0_big	3.1
15_0_big	3.1
15_0_big	2.8
15_0_big	3.1
15_0_big	3.1
15_0_big	3
15_0_big	2.9
15_0_big	2.7
15_0_big	2.6
15_0_big	2.5
15_0_big	2.6

15_0_big	3.1
15_0_big	3
15_0_big	2.5
15_0_big	2.5
15_0_big	3.1
15_0_big	2.5
15_0_big	2.6
15_0_big	3.1
15_0_big	3.1
20_0_big	2.9
20_0_big	3.1
20_0_big	3.1
20_0_big	3
20_0_big	2.7
20_0_big	3.1
20_0_big	3
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	3

20_0_big	3.1
20_0_big	2.8
20_0_big	2.9
20_0_big	3.1
20_0_big	3.1
20_0_big	3.1
20_0_big	2.5
20_0_big	2.7
20_0_big	2.6
20_0_big	3
20_0_big	2.7
15_0_big	2.6
15_0_big	3.1
15_0_big	2.7
15_0_big	3
15_0_big	2.6
15_0_big	3.1
15_0_big	2.5
25_0_big	3.1
25_0_big	3.1
25_0_big	3.1
25_0_big	3.1

25_0_big	3.1
25_0_big	2.9
25_0_big	3.1
25_0_big	3.1
25_0_big	3.1
25_0_big	3.1
25_0_big	3.1
25_0_big	2.8
25_0_big	3
25_0_big	3.1
25_0_big	2.9
25_0_big	2.9
25_0_big	3
25_0_big	2.5
25_0_big	2.9
25_0_big	3.1
25_0_big	2.6
25_0_big	3
25_0_big	3
25_0_big	2.8
25_0_big	3
20_0.05_big	2.5

20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	2.9
20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	3.1
20_0.05_big	2.8
20_0.05_big	3
20_0.05_big	3.1
20_0.05_big	2.9
20_0.2_big	2.9
20_0.2_big	3
20_0.2_big	2.5
20_0.2_big	2.9
20_0.2_big	3.1
20_0.2_big	2.6
20_0.2_big	3

20_0.2_big	3
20_0.2_big	2.8
20_0.2_big	3
20_0.2_big	2.5
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	2.9
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	3.1
20_0.2_big	3
20_0.2_big	3
20_0.2_big	3.1
15_0.2_small	2.5
15_0.2_small	2.4
15_0.2_small	2.4
15_0.2_small	2.5

15_0.2_small	2.4
15_0.2_small	2.5
15_0.2_small	2.1
15_0.2_small	2.4
15_0.2_small	2.4
15_0.2_small	2.4
15_0.2_small	2.1
15_0.2_small	2
15_0.2_small	2.4
15_0.2_small	2.3
15_0.2_small	2
15_0.2_small	2.5
15_0.2_small	2
15_0.2_small	2.5
15_0.2_small	2.2
15_0.2_small	2.4
15_0.2_small	2
15_0.05_small	1.9
15_0.05_small	2.1
15_0.05_small	2.3
15_0.05_small	2.2
15_0.05_small	2.4

15_0.05_small 2.2

15_0.05_small 2.3

15_0.05_small 2.4

15_0.05_small 2.4

15_0.05_small 2.1

15_0.05_small 2.4

15_0.05_small 2.7

15_0.05_small 2.4

15_0.05_small 2.7

15_0.05_small 2

15_0.05_small 2.5

15_0.05_small 2.3

15_0.05_small 2.3

15_0.05_small 3

15_0.05_small 2.3

15_0.05_small 2.4

15_0.05_small 2.3

15_0.05_small 3

15_0.05_small 2.9

15_0.05_small 2

25_0.2_small 2.3

25_0.2_small 2.5

25_0.2_small	2.3
25_0.2_small	1.9
25_0.2_small	2.1
25_0_small	2.3
25_0_small	2.6
25_0_small	1.8
25_0_small	1.9
25_0_small	2.1
25_0_small	2.1
25_0_small	2
25_0_small	1.9
25_0_small	1.8
25_0_small	2.4
25_0_small	1.6
25_0_small	1.7
25_0_small	2.5
25_0_small	2.1
25_0_small	1.8
25_0_small	2
25_0_small	1.8
25_0_small	2.3
25_0_small	2.2

15_0_small	1.8
15_0_small	1.8
15_0_small	2.1
15_0_small	2
15_0_small	1.5
15_0_small	2
15_0_small	2