TOXIC BENTHIC CYANOBACTERIA (*MICROCOLEUS AUTUMNALIS*): GENETIC STRUCTURE AND ECOLOGICAL EFFECTS

BY

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Abstract

Toxic, benthic cyanobacterial proliferations occur in rivers globally and are increasing in both frequency and severity. More than 100 dog deaths have been reported in New Zealand since 2005, and dogs have died due to cyanotoxins from benthic cyanobacteria in California and France in recent years. In New Zealand, toxic, benthic proliferations are typically dominated by the genus *Microcoleus*, which produces the potent neurotoxins called anatoxins. Investigations of the drivers of *Microcoleus* proliferations have been carried out, revealing important relationships between growth, nutrients and flow. Despite increased attention over the past 15 years, significant knowledge gaps remain regarding regulation of anatoxin concentrations in mats and the ecological impacts of toxic *Microcoleus*-dominated proliferations in aquatic ecosystems. In this study, I aimed to address these crucial knowledge gaps.

Concentrations of anatoxins in *Microcoleus*-dominated mats are highly variable, ranging from 0 μg g^{-1} to over 2000 μg g^{-1} dry weight. Like other cyanobacteria, both toxic and non-toxic strains coexist, and it is possible that the relative abundance of these strains is related to the observed variability in anatoxin concentrations. I developed a quantitative PCR assay specific to the *Microcoleus anaC* gene and combined this with a 16S rRNA quantitative PCR specific to cyanobacteria. This assay enabled the relative abundance of toxic and non-toxic strains to be quantified for the first time in environmental samples of *Microcoleus*-dominated mats. The quantitative PCR assay was applied to 122 environmental samples. Toxic cells made up 0% to 30.3% of the samples and significant differences were detectable among rivers, with wide variation evident both within and among rivers. Anatoxin contents in samples were significantly correlated with the proportion of toxic cells, suggesting that variation in anatoxin concentrations are related primarily to the dominance of toxic strains.

A field study was conducted in California to identify cyanotoxin producers, investigate the utility of the primers from the quantitative PCR developed in the previous chapter in an international setting and determine the extent of spatial variability in anatoxin concentrations and quotas. Samples were collected from the Eel and Russian rivers in northern California and gene screening, followed by sequencing, was conducted to identify likely cyanotoxin

producers. Cyanotoxin concentrations were measured using liquid chromatography tandem-mass spectrometry and the anatoxin quotas were determined using droplet digital PCR. Cyanotoxin producers were identified as *Microcoleus* producing anatoxins and *Nodularia* producing nodularin. Anatoxin concentrations were highly variable and differed significantly among sites; however, this variability did not extend to anatoxin quotas, indicating that the abundance of toxic cells also drives anatoxin content in this system. The anatoxin congener dihydroanatoxin-a comprised a significant proportion of the total anatoxins (38%–71%), indicating that this congener should be included in monitoring. Mats dominated by the green alga *Cladophora glomerata* contained both anatoxins and nodularin; thus, they may pose an exposure risk to cyanotoxins.

Microcoleus-dominated mats grow in waters with moderate concentrations of dissolved inorganic nitrogen and low concentrations of dissolved reactive phosphorus. Acquisition of phosphorus from organic sources, in addition to within-mat nutrient cycling, may explain how *Microcoleus* can obtain high biomass in low nutrient environments. In chapter 4, I compare the alkaline phosphatase activity of four toxic and four non-toxic strains under four phosphorus regimes to identify the potential for *Microcoleus* to use organic phosphorus as a nutrient source. Toxic strains exhibited greater alkaline phosphatase activity than non-toxic strains; however, considerable variability was evident among strains with the same toxigenicity. Environmental mat samples were collected from sites on an upstream-downstream gradient and across a diel cycle, and alkaline phosphatase activity measured. There was a significant difference in alkaline phosphatase activity between the most upstream and downstream site. No diel changes in alkaline phosphatase activity were apparent. The presence of alkaline phosphatase activity suggests that organic phosphorus sources may be accessible to *Microcoleus*.

To date, management of, and research on, toxic, benthic cyanobacterial proliferations has been focused largely on identifying drivers of growth and toxin production. As a result, there is a significant lack of information on the effects of *Microcoleus*-dominated proliferations on the wider ecosystem. In chapter 5, an ecotoxicological study was undertaken on *Deleatidium* spp. larvae using purified anatoxins to address this knowledge gap. *Deleatidium* spp. larvae were exposed to a range of doses of anatoxin-a, dihydroanatoxin-a, or a mixture of homoanatoxin-a and dihydrohomoanatoxin-a. No significant mortality was observed for any dose or toxin congener. Larvae exposed to high doses (300 μg L^{-1} to 600 μg L^{-1}) of dihydroanatoxin-a had measurable concentrations of the toxin in their tissues 24-hours post-exposure. The lack of

mortality observed, combined with detectable anatoxins in tissues post-exposure, is indicative of a potential pathway for anatoxin transfer or bioaccumulation, and warrants further investigation.

Overall, this thesis has addressed a number of critical knowledge gaps in our understanding of *Microcoleus*. Continued effort is needed to broaden our understanding of the physiological and ecological role of anatoxins to improve risk assessments and ultimately inform better management of *Microcoleus* proliferations.

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Statement of Authorship

This thesis has been written as a series of manuscripts, either already published or in preparation for submission in the near future, and there is, therefore, inevitable repetition between introductions and discussions of these chapters. This thesis is the intellectual and analytical work of the author. Ken Ryan and Susie Wood provided advice and support throughout. The contributions of co-authors for each chapter are outlined below.

Chapter 2 was published in the journal *Toxins* in October 2018. The structure of this paper reflects the requirements of this journal, with results and discussion ahead of the methods. Author contributions: LTK and SAW conceptualised the research; LTK, SAW and TGM collected the samples; LTK conducted the formal analysis and laboratory work; LTK wrote the manuscript with input from SAW and KGR. The full reference for this paper is as follows:
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CHAPTER 1

General introduction

1.1 Toxic cyanobacteria

Cyanobacteria are an ancient and diverse group of photosynthetic prokaryotes that are characterised by the pigments phycocyanins and phycoerythrins. These pigments give them their characteristic colour and led to their common name – blue-green algae. Cyanobacteria have a global distribution and are capable of surviving in a variety of habitats, including extreme environments such as hot springs, the polar regions and the deep sea (Whitton and Potts 2012). Most cyanobacteria have a high thermal tolerance, enabling their survival at temperatures greater than competing organisms. Some species are adapted to above-average UV exposure, while others are extremely efficient at photosynthesising under low light conditions (Chorus and Bartram 1999, Garcia-Pichel et al. 2003, Kanoshina et al. 2003, Pandey et al. 2004, Paerl and Otten 2013). Many species can fix atmospheric nitrogen, enabling their distribution in areas where nitrogen is limiting for other organisms (Lehtimaki et al. 1997, Berman-Frank et al. 2003). Cyanobacterial morphologies range from solitary single cells to globular colonies held together by an extracellular polysaccharide matrix (e.g. *Microcystis* spp.), filamentous forms and exo- or endo-symbionts (Kaebernick and Neilan 2001). Such variety in life history traits and growth strategies is the key to the ubiquity of cyanobacteria globally.

Harmful algal blooms (HABs) are characterised by the production of toxins, and are receiving increasing attention worldwide as a result of increased scientific awareness, eutrophication and changes in our use of waterbodies (Hallegraeff 1993, Chorus et al. 2000, Spaulding and Elwell 2007, Paerl and Huisman 2009, Paerl and Otten 2013). A number of taxonomic groups are involved in the formation of HABs, including dinoflagellates, diatoms, raphidophytes, haptophytes and cyanobacteria (Smayda 1997, Landsberg 2002, Spaulding and Elwell 2007, Hallegraeff 2010, Huisman et al. 2018). Cyanobacteria are particularly interesting as they produce toxic secondary metabolites (cyanotoxins), which can be categorised as alkaloids, cyclic peptides or lipopolysaccharides. However, they are more commonly grouped by their

mode of action (Kaebernick and Neilan 2001, Pearson et al. 2010). There are four broad classes of cyanotoxins: hepatotoxins, neurotoxins, cytotoxins and dermatotoxins, which affect the liver, nervous system, cells and epidermis, respectively (Kaebernick and Neilan 2001, Pearson and Neilan 2008, Pearson et al. 2010). The presence of cyanotoxins in water bodies used for drinking water and recreation has led to considerable investigation into their chemical structures and toxicity. Research has typically focused primarily on microcystins and saxitoxins (Merel et al. 2013). Growing concern about the proliferation of cyanobacterial blooms and increased pressure on freshwater resources has sparked increasing attention on the factors regulating toxin production in cyanobacteria (Paerl and Huisman 2009, Paerl and Paul 2012, Paerl and Otten 2013).

Research has focused largely on planktonic cyanobacterial blooms, with little research into benthic proliferations (Heath et al. 2011). Research into toxic, benthic cyanobacterial proliferations appears driven by investigation into animal toxicosis events, including dog deaths in Scotland (Edwards et al. 1992), France (Gugger et al. 2005), California (Backer et al. 2013) and New Zealand (Hamill 2001, Wood et al. 2007b, Wood et al. 2017b), and cattle deaths in Switzerland (Mez et al. 1997). While animal toxicosis events from benthic cyanobacteria have been reported since the early 1990s, the frequency and severity of toxic, benthic proliferations appears to have increased (Quiblier et al. 2013, McAllister et al. 2016). In New Zealand the primary taxa in benthic mats are *Microcoleus* spp. (formerly *Phormidium* sp.; Wood et al. 2007b, Heath et al. 2010, Brasell et al. 2015). *Microcoleus* sp. produces the neurotoxins anatoxin-a (ATX), dihydroanatoxin-a (dhATX), homoanatoxin-a (HTX) and dihydrohomoanatoxin-a (dhHTX), collectively referred to as anatoxins (Wood et al. 2010c, Wood et al. 2012b, Heath et al. 2014). Anatoxins are produced by a range of cyanobacteria (Moore 1977, Quiblier et al. 2013) and have been implicated in the deaths of domestic animals around the globe (Devlin et al. 1977, Moore 1977, Hamill 2001, Osswald et al. 2007a, Osswald et al. 2007b, Wood et al. 2007b, Al-Sammak et al. 2014). Despite this, determining the risk benthic cyanobacterial blooms pose to human and animal health remains a significant challenge because of variability in toxin production over time, space and between genotypes of the same species (Devlin et al. 1977, Wood et al. 2010c, Rantala-Ylinen et al. 2011, Wood et al. 2012b).

1.2 Drivers of benthic cyanobacterial proliferations

Benthic and planktonic forms of cyanobacteria exist in both marine and freshwater ecosystems (Golubic et al. 2010, Whitton 2012). When conditions are favourable, rapid growth can lead to large accumulations of cyanobacteria – known as blooms or proliferations (for planktonic and benthic growth, respectively). The growth of cyanobacteria is influenced by a range of biotic and abiotic factors, including nutrient availability, irradiance, grazing pressure, stratification, temperature and flow rates. Nutrient availability is a key driver of cyanobacterial growth and dominance across a range of ecosystems, with many blooms occurring in eutrophic waters (Paerl and Huisman 2009, O'Neil et al. 2012, Paerl and Otten 2013). It must be noted, however, that blooms and proliferations do occur in oligotrophic and mesotrophic waters, and this may be the result of the ability of many cyanobacteria to fix atmospheric nitrogen and to compete effectively for nutrients (Lehtimaki et al. 1997, Berman-Frank et al. 2003, Paerl and Fulton 2006, O'Neil et al. 2012).

Microcoleus-dominated proliferations form thick, cohesive mats, which isolate them from the overlying water column; thus, within-mat conditions can be significantly different from those in the surrounding water (Wood et al. 2015b, McAllister et al. 2016). The relationships between physicochemical drivers of *Microcoleus*-dominated proliferations are complex and vary throughout the accrual cycle. Early in mat development, biofilms are thin and more exposed to the overlying water. Additionally, these thin early-phase biofilms are less likely to have significant within-mat nutrient cycling. At this stage in the accrual cycle nutrient availability is important, particularly the concentrations of dissolved inorganic nitrogen (DIN) and dissolved reactive phosphorus (DRP). Nutrient thresholds for biofilm establishment are DIN concentrations greater than 0.2 mg L^{-1} and DRP concentrations less than 0.01 mg L^{-1}, though these thresholds vary among rivers (Wood et al. 2017a, McAllister et al. 2018a), and while they are reasonable predictors of biofilm presence, they do not predict the extent or severity of *Microcoleus*-dominated proliferations.

Nutrient concentration relationships appear less important later in mat development when mats are thick and conditions are conducive to internal nutrient cycling; for example, large diel shifts in within-mat pH could promote desorption of phosphorus from entrapped sediments (Wood et al. 2015b) and the microbial community includes taxa capable of N-fixation (Brasell et al. 2015). Genes associated with carbon fixation, urea breakdown, nitrite utilisation, phosphatases

and phosphorus transport have been recovered from *Microcoleus*-dominated mats (Bouma-Gregson et al. 2019).

Flow is an important factor in the growth and detachment of *Microcoleus*. Flow velocity influences *Microcoleus* growth, with higher velocities resulting in higher growth rates due to increased flow reducing the thickness of the diffusive boundary layer (McAllister et al. 2018b), allowing greater exchange of nutrients, carbon dioxide and oxygen between the river water and mat. Flushing or scouring flows remove biofilms from the rocks and "reset" the periphyton (Wood et al. 2015a). The more frequently these occur, the less time there is for colonisation and biofilm development. Periods of extended low flow allow considerable cyanobacterial biomass to accumulate (Heath et al. 2015, Wood et al. 2017a). At this point, autogenic detachment can occur due to the accumulation of fine sediment decreasing the strength of attachment to the substrate, and increased buoyancy via photosynthetically generated oxygen bubbles trapped in the mat (Wood et al. 2015a). This autogenic detachment can decrease the cover of *Microcoleus*-dominated mats on riverbeds but poses increased health risks from exposure to detached mats washed up on river and stream margins.

1.3 Drivers of toxin production

Cyanotoxin production can be influenced by a range of environmental conditions, including nutrient availability, salinity, pH, irradiance and temperature (van der Westhuizen and Eloff 1985, Sivonen 1990, Pearson et al. 2008, Harland et al. 2013, Neilan et al. 2013). However, despite considerable research, there is no consensus on the effects of the environment on cyanotoxin production and regulation. Orr and Jones (1998) determined that microcystin (MCY) production by *Microcystis aeruginosa* was positively and linearly correlated with cell division rates under nitrogen limiting conditions. However, while cell division rates are correlated with MCY production, other factors modulate this response, with nitrate availability being the most significant factor (Downing et al. 2005). *Aphanizomenon ovalisporum* had increased cylindrospermopsin (CYN) concentrations under elevated light levels (Shalev-Malul et al. 2008), while Preußel et al. (2014) found that CYN is retained in *Aphanizomenon* cells when nitrogen is limiting and released under elevated nitrogen levels. *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*) produces the most CYN at high light intensities when growth rate is depressed and the least CYN at low light intensities when growth rates are

optimal (Dyble et al. 2006). Many saxitoxin (STX) producers accumulate more toxins under elevated NaCl conditions and high conductivity (Neilan et al. 2013). Temperature and nutrient composition of growth media influence the production of anatoxin-a (ATX) in *Oscillatoria* PCC6506, with suboptimal conditions for growth resulting in the greatest production of ATX and greater HTX when enriched with CO_2 (Neilan et al. 2013).

A significant factor complicating our understanding of anatoxin production in *M. autumnalis* is the coexistence of multiple strains with varying toxicity within the same mat (Heath et al. 2010, Wood et al. 2012b). Some strains do not carry the gene cluster (*anaA* – *anaG*) responsible for toxin synthesis, while others carry the gene cluster but produce different quantities of ATX, HTX, dhATX and dhHTX (Neilan et al. 2008, Heath et al. 2010, Wood et al. 2012b, Neilan et al. 2013, Méjean et al. 2014). The coexistence of these strains indicates that the benefits of toxin production do not always outweigh the costs associated with toxin production. Given the diversity of factors that influence toxin production and that the results differ between species, even when the toxin is the same, it is not currently possible to draw inferences about environmental regulation of toxin production in the absence of species- and strain-specific studies. Improving knowledge of those conditions that favour each *M. autumnalis* strain may provide new insight into potential ecological functions of anatoxin production. Research in this area has thus far been limited by an inability to distinguish toxic and non-toxic genotypes. Morphologically, the strains appear identical, and while it is possible to test for the presence of the *ana*-gene cluster, it is not yet possible to determine the proportion of different genotypes in a mixed sample (Rantala-Ylinen et al. 2011).

The advent of new technologies has enabled more detailed investigation into the genetic and biochemical regulation of cyanotoxin production. The gene clusters responsible for the production of most major cyanotoxins are known for a number of toxin-producing species. Microcystins, for example, are produced by the *mcy* gene cluster, which comprises genes encoding polyketide synthases, tailoring enzymes and non-ribosomal peptide synthetases (Moffitt and Neilan 2004). Transcriptional regulators influence the expression of the *mcy* gene cluster, with key regulators including a nitrogen responsive regulatory protein (NtcA), a ferric uptake regulator (FurA), and a redox-active photoreceptor protein (RcaA) (Boopathi and Ki 2014). These are consistent with some studies that have found that light intensities, nitrogen availability and iron availability influence MCY production in *M. aeruginosa* (Utkilen and Gjølme 1995, Alexova et al. 2011).

The *ana* gene cluster responsible for the production of anatoxins is also known (Méjean et al. 2014); however, less is known about the regulators of this biosynthetic pathway, making it difficult to draw parallels with other cyanotoxins. Factors, like iron deficiency, that upregulate microcystin production appear to have little or no effect on the production of anatoxins in *M. autumnalis* (Harland et al. 2013). Anatoxin concentrations are closely related to the abundance of toxic genotypes (Wood and Puddick 2017), and it is likely that physicochemical factors with differing impacts on toxic and non-toxic strain growth drive changes in the abundance of toxic genotypes and hence anatoxin concentrations. Anatoxin concentrations peak early in the growth phase in *M. autumnalis* growth studies with single toxin-producing strains (Harland et al. 2013, Heath et al. 2014). Iron and copper concentrations had no effect on anatoxin production, despite growth being suppressed at concentrations higher than 40 μg L^{-1} and 250 μg L^{-1} for iron and copper, respectively (Harland et al. 2013). The availability of nutrients influences the composition of the anatoxin congeners, but does not have a significant effect on total anatoxin concentrations (Heath et al. 2014). Concentrations of anatoxins are variable in environmental samples; however, significant differences are evident between rivers (e.g., Wood and Puddick 2017, Kelly et al. 2018, McAllister et al. 2018a), suggesting there are river-specific factors that have not yet been identified.

1.4 Ecological effects of benthic cyanobacterial proliferations

Cyanotoxins can have effects on aquatic organisms, ranging from mortality to liver damage, including reduced growth and fertility (Ibelings and Havens 2008). The majority of ecotoxicology assessments involving cyanotoxins use MCY, NOD or SXT, as these are commonly occurring cyanotoxins (Ibelings and Havens 2008) and anatoxins are considered less stable (Stevens and Krieger 1991), thus less of a concern. Considerable research has focused on the toxicity of cyanotoxins to mammals (Briand et al. 2003, Pearson et al. 2010), particularly livestock, domestic animals and humans. Despite this, until recently, the effects of anatoxins on river organisms has received little attention. There is potential for lethal and sub-lethal effects of cyanotoxin exposure on macroinvertebrates that may feed on cyanobacteria or use blooms as refugia (Hart et al. 2013). Macroinvertebrates are often used as indicator species of river health, and provide a food source for larger invertebrates, fish, birds and people; thus, the risk of lethal effects or bioaccumulation of toxins is of significant concern (Lance et al. 2006). Cyanotoxins can accumulate in the tissues of mussels and fish when exposed to

planktonic sources of STXs, anatoxins and MCYs, but benthic sources of cyanotoxins have not been investigated (Bogialli et al. 2006, Osswald et al. 2008, Osswald et al. 2011, Pawlik-Skowrońska et al. 2012, Al-Sammak et al. 2014). Microcystin-LR reduces growth and photosynthetic efficiency in some aquatic macrophytes, but not in others (Pflugmacher 2002). This raises the concern that cyanobacterial blooms and proliferations could alter the community composition of macrophytes in aquatic ecosystems. In some aquatic plants, exposure to ATX depresses photosynthetic oxygen production and increases the production of glutathione-S-transferase, a detoxification enzyme (Mitrovic et al. 2004, Leflaive and Ten-Hage 2007).

As with other cyanobacterial blooms, it is expected that benthic blooms of *M. autumnalis* will have impacts on the local ecology. Those effects have not yet been well elucidated, as the focus of initial *M. autumnalis* research was, understandably, focused on growth characteristics, toxin production and the biofilm community (Jahnke and Mahlmann 2010, Harland et al. 2013, Heath et al. 2014, Brasell et al. 2015). While the toxic effects of consumption of anatoxins are known for some vertebrates, e.g., dogs, its impact on aquatic ecosystems remains unclear (Quiblier et al. 2013). Planktonic sources of ATX reduce survivorship in rotifers and modify fish behaviour (Snell 1980, Pawlik-Skowrońska et al. 2012, Al-Sammak et al. 2014). Freshwater mussels also accumulate ATX from planktonic cyanobacteria, but sub-lethal effects have not been measured (Osswald et al. 2008). Wood et al. (2014b) found the macroinvertebrate community shifted in response to *Microcoleus*-dominated mats, with increases in species richness, but a decrease in the quantitative macroinvertebrate community index. There is anecdotal evidence that the taste and odour compounds geosmin and 2-methylisoborneol (MIB), which are produced by cyanobacteria, can be found in the tissues of fish in rivers with large benthic cyanobacterial proliferations (Wood et al. 2017a), raising the question of whether anatoxins could be transferred to higher trophic levels alongside these taste and odour compounds. Shifts in the macroinvertebrate community, combined with the presence of taste and odour compounds of cyanobacterial origin in fish, both indicate a pressing need to investigate the effects of anatoxins from benthic cyanobacterial proliferations on aquatic organisms.

1.5 General overview

1.5.1 *Chapter 2: Development and application of a quantitative PCR assay to assess genotype dynamics and anatoxin content in* Microcoleus autumnalis*-dominated mats*

In *M. autumnalis*, relationships between the toxic and non-toxic strains have received little attention due to a lack of tools to distinguish the genotypes from one another. This chapter documents my development of a quantitative PCR (qPCR) assay to resolve this impediment to our understanding of toxic/non-toxic strain dynamics. I then demonstrate the application of this qPCR methodology to environmental samples collected from ten rivers across New Zealand, which allowed the relationships between anatoxin concentration and the relative abundance of toxic cells to be investigated for the first time in *M. autumnalis*.

1.5.2 *Chapter 3: Benthic cyanotoxin producers and spatial variability in anatoxin concentrations in the Eel and Russian Rivers, California*

The coexistence of multiple taxa capable of cyanotoxin production in the field can present challenges to understanding the ecology of benthic cyanobacterial proliferations. This occurs in the Eel and Russian rivers in California, where multiple cyanotoxin producing taxa co-occur. In this chapter I identify the probable cyanotoxin producers in benthic mats from these rivers using molecular techniques. I then apply the qPCR assay from Chapter 2 to investigate anatoxin quota and variability in the abundance of toxic strains in these environmental samples.

I was part of a successful Royal Society Catalyst Seeding Grant (17-CAW-002-CSG) which provided me an opportunity to travel to California to conduct fieldwork as part of an international team. This was an ideal chance to test the assay developed in Chapter 2 in an international context and in a location from which no sequences were available from during the assay development. It also provided me the opportunity to compare the patterns I had observed in samples from New Zealand of highly variable anatoxin concentrations and quota with samples from California. The sites and rivers visited in California were places where dog deaths had previously been reported.

1.5.3 *Chapter 4: Phosphorus source and concentration have differential effects on alkaline phosphatase activity in* Microcoleus autumnalis *strains*

Environmental conditions can have differential effects on toxic and non-toxic strains of the same cyanobacterial taxa, which in turn may impact their relative abundance in the environment. To investigate whether alkaline phosphatase activity (APA) and the subsequent ability to utilise organic sources of phosphorus differs between toxic and non-toxic strains, in Chapter 4, I exposed multiple toxic and non-toxic *M. autumnalis* strains to differing concentrations and sources of phosphorus. In addition, the potential for APA to enable organic phosphorus utilisation in environmental *Microcoleus*-dominated mats was investigated at multiple sites on a river and across a diel cycle.

Chapter 4 focuses on investigating the interactions between *Microcoleus* and the environment it grows in. The previous chapters highlighted that the relative abundance of toxic and non-toxic strains varies within mats and this chapter seeks to enhance knowledge on the differences between growth of toxic and non-toxic strains and how this might be influenced by the form and concentrations of phosphorus.

1.5.4 *Chapter 5: An ecotoxicological assessment of anatoxin congeners on the New Zealand mayfly (*Deleatidium *spp.)*

The preceeding chapters develop our knowledge of *Microcoleus* from molecular biology, to the ecophysiology of strains. Chapter 5 seeks to extend our ecological understanding of *Microcoleus* further by investigating the ecotoxicology of the toxins produced by *Microcoleus.*

A critical knowledge gap exists regarding the broader ecological effects of anatoxins in aquatic ecosystems, which urgently needs addressing. The focus of this chapter was to examine the effects that the toxins *Microcoleus* produces have on a common aquatic macroinvertebrate. Chapter 5 examines the acute toxicity of ATX, dhATX and HTX/dhHTX on a freshwater macroinvertebrate commonly used as an indicator species in New Zealand, the native mayfly (*Deleatidium* spp.). The potential for anatoxin transfer via accumulation in the tissues of the mayfly larvae was also explored.

CHAPTER 2

Development and application of a quantitative PCR assay to assess genotype dynamics and anatoxin content in *Microcoleus autumnalis*-dominated mats

2.1 Abstract

Microcoleus is a filamentous cyanobacteria genus with a global distribution. Some species form thick, cohesive mats over large areas of the benthos in rivers and lakes. In New Zealand *Microcoleus autumnalis* is an anatoxin producer, and benthic proliferations are occurring in an increasing number of rivers nationwide. Anatoxin content in *M. autumnalis*-dominated mats varies spatially and temporally, making understanding and managing proliferations difficult. In this study a *M. autumnalis*-specific TaqMan probe quantitative PCR (qPCR) assay targeting the *anaC* gene was developed. The assay was assessed against 26 non-*M. autumnalis* species. The assay had a detection range over seven orders of magnitude, with a limit of detection of 5.14×10^{-8} ng μL^{-1}. The *anaC* assay and a cyanobacterial specific 16S rRNA qPCR were then used to determine toxic genotype proportions in 122 environmental samples collected from 19 sites on 10 rivers in New Zealand. Anatoxin contents of the samples were determined using LC-MS/MS and anatoxin quota per toxic cell calculated. The percentage of toxic cells ranged from 0% to 30.3%, with significant ($p < 0.05$) differences among rivers. The anatoxin content in mats had a significant relationship with the percentage of toxic cells ($R^2 = 0.38$, $p < 0.001$), indicating that changes in anatoxin content in *M. autumnalis*-dominated mats are primarily related to the dominance of toxic strains. When applied to more extensive sample sets, the assay will enable new insights into how biotic and abiotic parameters influence genotype composition and, if applied to RNA, assist in understanding anatoxin production.

2.2 Introduction

Benthic cyanobacterial proliferations in freshwater ecosystems, in particular *Microcoleus* spp. and closely related taxa, pose a significant risk to ecosystem, animal and human health (Teneva et al. 2005, Wood et al. 2007b, Heath et al. 2010, Wood et al. 2010c). Several *Microcoleus* and closely related species produce the potent neurotoxins anatoxin-a (ATX) and homoanatoxin-a (HTX), and their dihydro-variants dihydroanatoxin-a (dhATX) and dihydrohomoanatoxin-a (dhHTX; Gugger et al. 2005, Heath et al. 2010), hereafter collectively referred to as anatoxins. While the toxic effects of consumption of anatoxins are known for some vertebrates, e.g., dogs, their impact on aquatic ecosystems remains unclear (Quiblier et al. 2013).

A significant factor impeding advancements in knowledge on the ecological function of anatoxins and the ability to predict its concentrations in *Microcoleus*-dominated samples is the coexistence of multiple strains with varying anatoxin quotas (concentration of anatoxin per cell; Heath et al. 2010, Wood et al. 2012b). Some strains do not carry the gene cluster (*anaA* – *anaK*; Méjean et al. 2016) responsible for anatoxin synthesis, while others carry the gene cluster but produce different quantities of each anatoxin variant (Heath et al. 2010, Wood et al. 2012b, Méjean et al. 2014). The coexistence of toxic and non-toxic strains suggests that the benefits of producing anatoxins do not always outweigh the costs associated with anatoxin production. Improving knowledge of the conditions that favour toxic *Microcoleus* strains has the potential to help predict when proliferations are more likely to be toxic. Research in this area has been limited by a lack of tools to quantify toxic and non-toxic *Microcoleus* genotypes in environmental samples. Morphologically, the strains appear identical, and while it is possible to test for the presence of the *ana*-gene cluster, to date the proportion of toxic and non-toxic genotypes in environmental samples has not been assessed (Rantala-Ylinen et al. 2011).

In New Zealand, *Microcoleus autumnalis* (formerly *Phormidium autumnale*) is an anatoxin producer, and proliferations have been reported in an increasing number of rivers nationwide (McAllister et al. 2016). Anatoxin content in *M. autumnalis*-dominated mats exhibit remarkable spatial and temporal variability (Wood et al. 2010c, Heath et al. 2011, Wood and Puddick 2017, Wood et al. 2017a). There are several explanations for this: that the composition of non-*Microcoleus* mat components (e.g., sediment, microbial community) varies over time and between sites (Brasell et al. 2015, Wood et al. 2015b); physicochemical factors within the mats or the overlying water result in up- or down-regulation of anatoxin production (Heath et

al. 2014); toxic and non-toxic genotypes co-occur in the mats and the relative abundance of these genotypes influences the overall anatoxin content in the mats (Wood et al. 2012b, Wood and Puddick 2017). Wood and Puddick (2017) found that anatoxin concentrations closely followed the abundance of copies of anatoxin genes in environmental samples; however, their study did not examine whether the proportion of toxic and non-toxic strains varied. The need for a quantitative method to determine the relative abundance of toxic to non-toxic *M. autumnalis* has been highlighted by numerous studies (Quiblier et al. 2013, McAllister et al. 2016, Wood and Puddick 2017, McAllister et al. 2018a). The present study aimed to develop a *M. autumnalis*-specific quantitative PCR (qPCR) assay to quantify *anaC* gene copies in environmental samples. This assay was then applied, in combination with a cyanobacterial specific 16S rRNA assay (Al-Tebrineh et al. 2012), to environmental samples enabling the proportion of toxic gene copies to total cyanobacterial gene copies to be determined. These data will enhance understanding of how anatoxin quota and the proportion of toxic genotypes varies within and between rivers, with a view to informing future management of proliferation events.

2.3 Results

2.3.1 *Quantitative PCR*

Species-specific primers and a probe targeting a highly conserved 100 bp region of the *M. autumnalis anaC* gene were designed. The sequences for the primers and probe were: Phor-AnaC-F5 5′-ACTAACCGAATCACTTCCACTT-3′, reverse primer: Phor-AnaC-R5 5′-CTCACCCACCTCACCTTTAG-3′, probe: Phor-AnaC-P5 5′-TTCAGTATTAGCGCAGGCTTTGCC-3′. The probe had the fluorescent reporter dye FAM-6-carbyfluoroscein at the 5′ end and was labelled with the non-fluorescent Black Hole Quencher®-1 (IDT, USA). Primer and probe sequences were checked for potential cross-reactivity in GenBank using the BLAST online software (http://blast.ncbi.nlm.gov/Blast.cgi) and no cross-reactivity was detected. *In vitro* tests of specificity did not result in amplification of any non-target species, including a strain of *Cuspidothrix issatchenkoi* (which is the only other known anatoxin producer in New Zealand), three *Aphanizomenon*, two *Aphanocapsa*, two *Raphidiopsis raciborskii*, eight *Dolichospermum*, two *Leptolyngbya*, two *Microcystis*, one *Nostoc*, two *Oscillatoria* (both anatoxin producers), one *Planktothrix* and two *Scytonema*

(Table A1). The assay had a linear range of detection over seven orders of magnitude, with the limit of detection reached at 5.14×10^{-8} ng μL^{-1} DNA.

2.3.2 *Environmental samples*

Samples from 19 sites in 10 rivers (n = 122) were analysed for anatoxin content using LC-MS/MS, and the quantity of *M. autumnalis anaC* and cyanobacterial specific 16S rRNA copies determined using qPCR. Anatoxin quota was determined by dividing weight normalised total anatoxin content by *anaC* copy numbers and the percentage of toxic cells in the total cyanobacterial community was calculated. There were no consistent temporal trends in anatoxin content, anatoxin quota or the percentage of toxic cells among the Ashley, Opihi, Maitai and Temuka rivers (Figure 2.1). The total anatoxin content and percentage of toxic cells were higher in the Ashley and Opihi rivers than in the Maitai and Temuka rivers. The Ashley River had the greatest variability in both total anatoxin content and percentage of toxic cells, ranging from 0.6 mg kg^{-1} to 662.5 mg kg^{-1} and 0.1% to 30.3%, respectively. In this location, there was a trend from higher and more variable anatoxin content and percentage of toxic cells early in the season to lower and less variable anatoxin content and percentage of toxic cells in March – April 2015. In contrast, the Maitai River had a relatively high anatoxin content and a lower percentage of toxic cells in October 2015. Both the Opihi and Temuka rivers exhibited variable anatoxin content and percentage of toxic cells with no apparent temporal trends. Anatoxin quota were variable for all rivers (Figure 2.1); however, with the exception of a single occasion on the Opihi River, the anatoxin quota varied less than twofold.

The total anatoxin content of samples was significantly correlated with the percentage of toxic cells (R^2 = 0.38, $p < 0.001$, Figure 2.2). Samples from the Ashley, Cardrona and Hutt rivers generally had higher anatoxin quota. Samples with a higher than expected anatoxin content relative to the percentage of toxic cells also had a higher anatoxin quota, while those with a lower than expected anatoxin content also had lower anatoxin quota. The proportion of each anatoxin congener (ATX, HTX, dhATX and dhHTX) varied among all samples measured; however, the dihydro-congeners were the most abundant in most of the samples (Figure A1.1, Appendix 1).

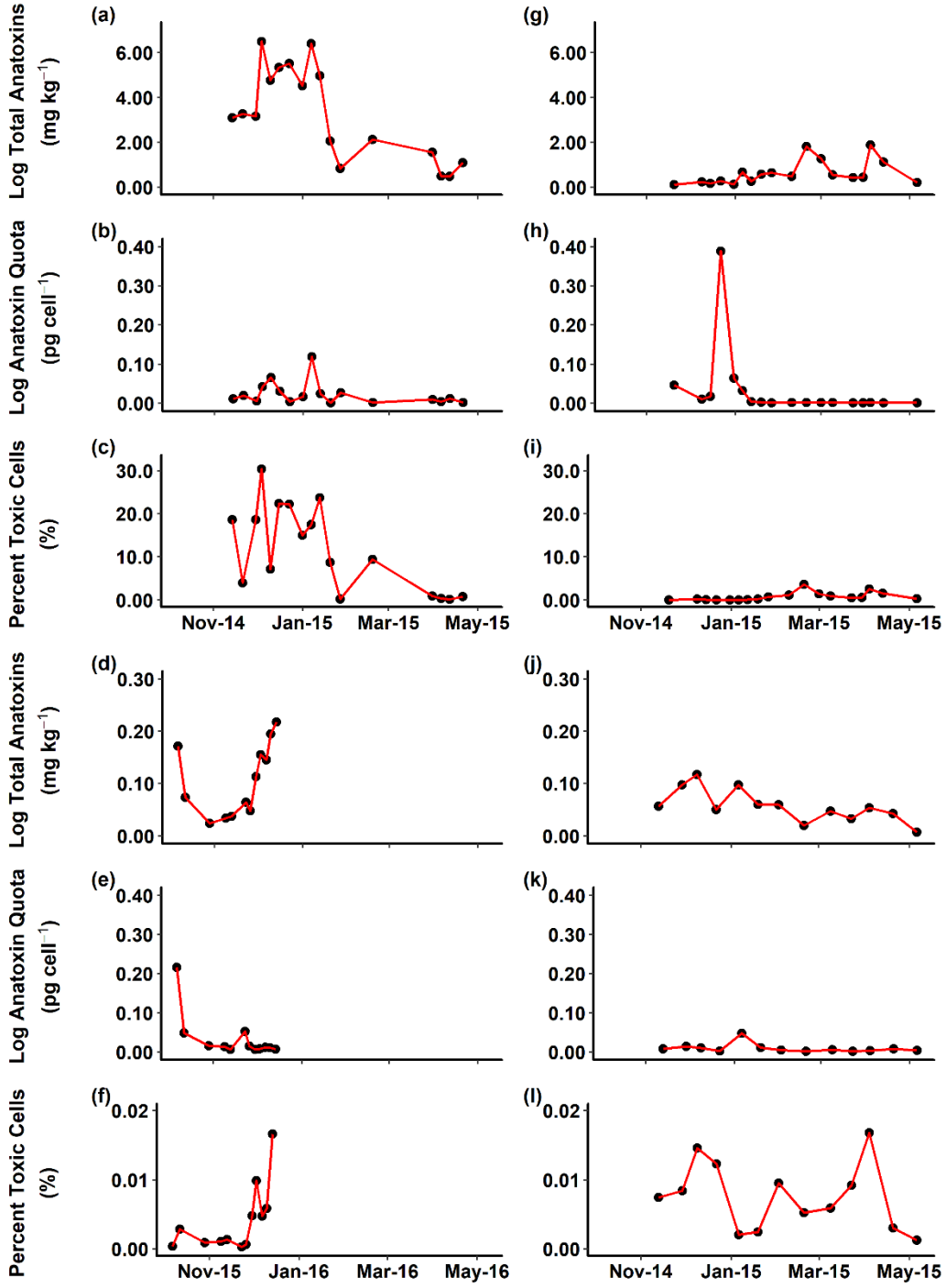

Figure 2.1. Total anatoxins, anatoxin quota and percent toxic cells for: (**a-c**) Ashley; (**d-f**) Maitai; (**g-i**) Opihi; (**j-l**) Temuka rivers between October 2014 and December 2015. All points reflect a pooled 10-mat sample from each river on that sampling date. Note different *y*-axis scales between (a-c)/(g-i) and (d-f)/(j-l).

Anatoxin content of samples from the Ashley, Cardrona and Hutt rivers spanned four orders of magnitude, while those of the other seven rivers generally had lower anatoxin contents and spanned only two orders of magnitude (Figure 2.3a). Statistically significant differences in anatoxin content were identified between rivers (pairwise Wilcoxon rank sum test, $p < 0.05$; Figure 2.3a). Anatoxin quota were highly variable within rivers; however, in contrast to the anatoxin content there was more overlap among rivers (Figure 2.3b). The median percentage of toxic cells was less than 1% in all rivers except the Ashley, where it was 9.3% (Figure 2.3c).

Within rivers, the percentage of toxic cells varied considerably, with the Ashley, Cardrona and Hutt rivers ranging from 0.1%–30.3%, 0.001%–1.8% and 0.009%–3.6% toxic cells, respectively. Within the Hutt and Cardrona rivers, there were significant differences in the proportion of toxic cells among sites (Figure 2.4).

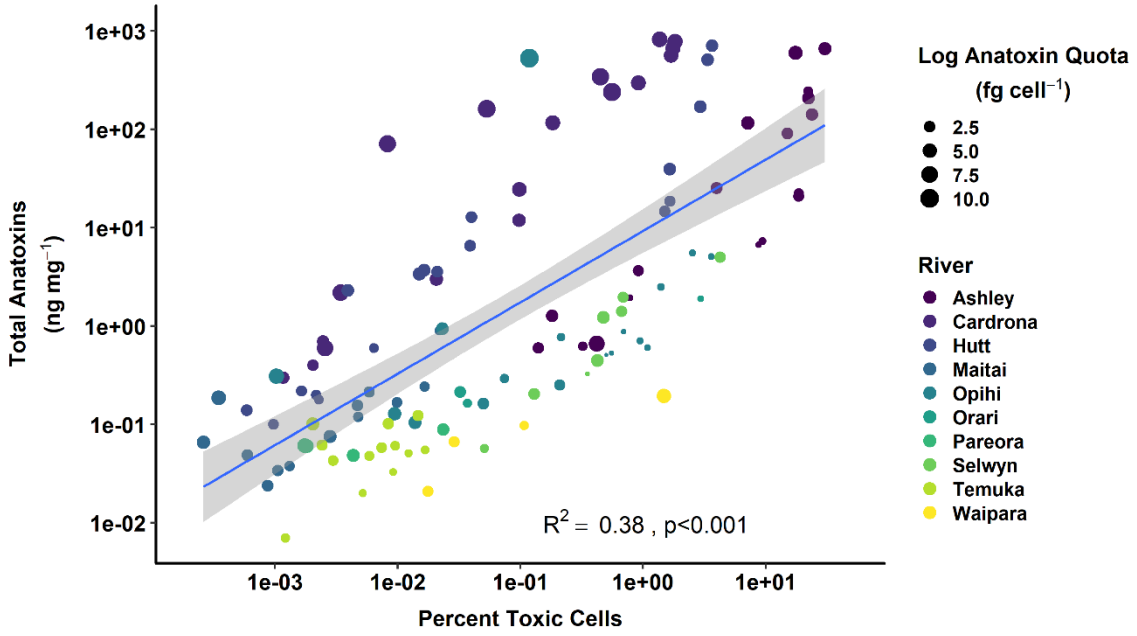

Figure 2.2. Relationship between percentage of toxic cells (determined using quantitative PCR) and total anatoxin content of samples. The blue line is a linear regression (±SE, grey shading). Colours represent rivers and the size of the dots represents anatoxin quota (fg cell^{-1}). The limit of detection/limit of quantitation of total anatoxins were 0.002 ng mg^{-1} and 0.006 ng mg^{-1}, respectively.

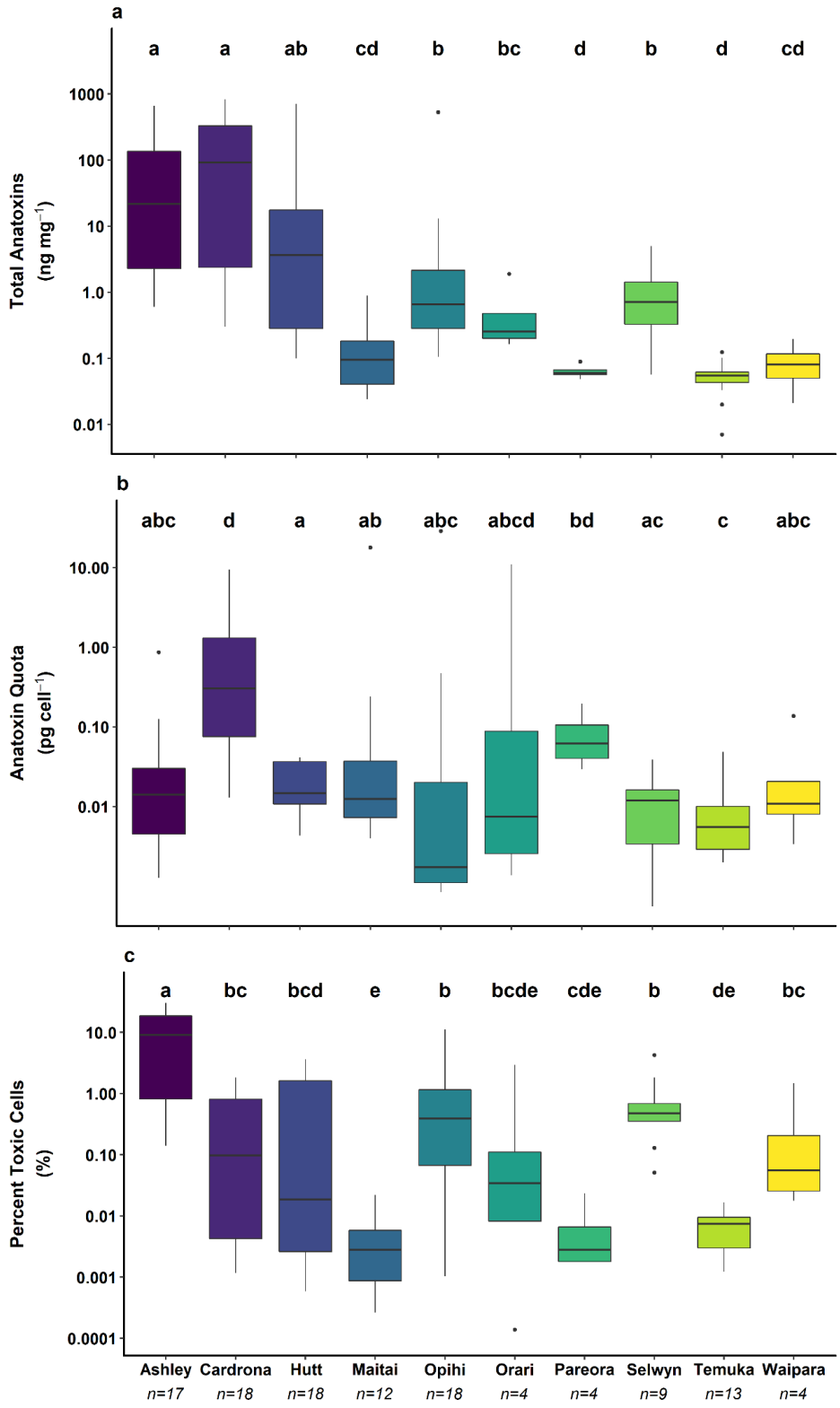

Figure 2.3. Anatoxin concentrations, quota and percent toxic cells among ten New Zealand rivers (a) total anatoxin concentration; (b) anatoxin quota and (c) percent toxic cells. Lines within the boxes are medians, the ends of boxes are quartiles and whiskers extend to the lowest or highest data point ≤ 1.5 × interquartile range. Black dots are outliers. A Kruskal-Wallis test and pairwise Wilcoxon rank sum test with a Benjamini-Hochberg adjustment were used to identify rivers that were significantly different from one another ($p < 0.05$), denoted by the letter above the plot. Where time-series data were available, these were pooled for this analysis. The limit of detection/ limit of quantitation for anatoxins were 0.002 ng mg^{-1} and 0.006 ng mg^{-1}, respectively.

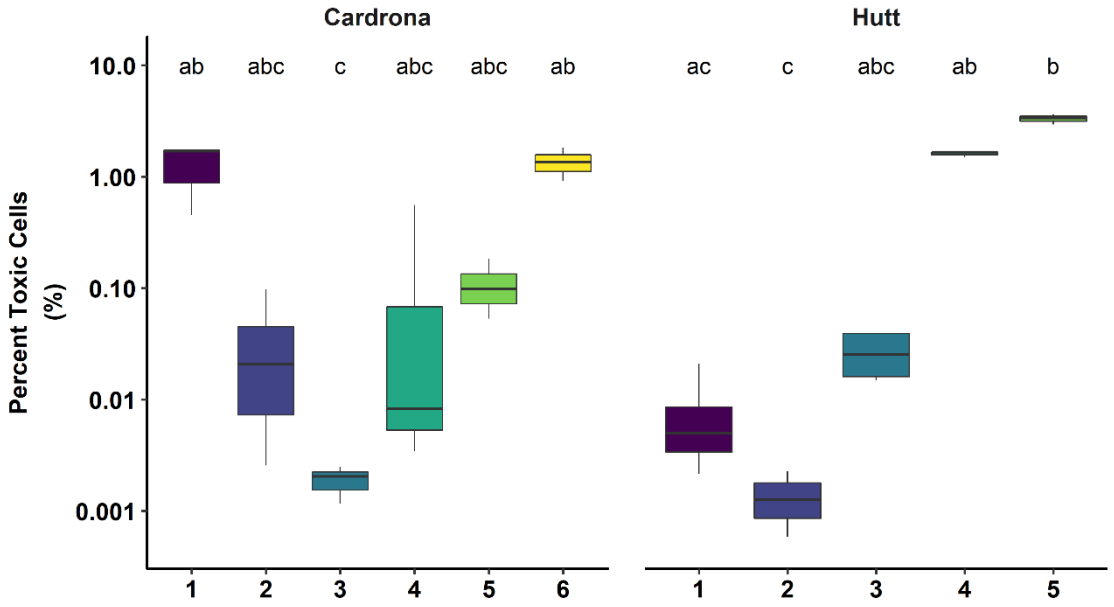

Figure 2.4. Percent toxic cells for sites along the Cardrona and Hutt rivers. See Figure 2.3 for interpretation of boxplots. Sites are in order from upstream to downstream on each river (see Figure 2.5). A Kruskal-Wallis test and Dunn's post-hoc test were used to identify significant differences ($p < 0.05$) among sites, denoted by the letter above the plot.

2.4 Discussion

Microcoleus autumnalis proliferations are increasing in prevalence and there is a growing need to understand more about anatoxin content variability and production (Quiblier et al. 2013, McAllister et al. 2018a). The first aim of this study was to develop a quantitative PCR method to enumerate the copy numbers of *anaC* in *M. autumnalis*-dominated mat samples. When used in concert with LC-MS/MS analysis of anatoxins and a second qPCR enumerating the total 16S rRNA cyanobacterial copy numbers, this enables both anatoxin quota and the percentage of toxic cells to be determined. The *M. autumnalis*-qPCR assay was specific, sensitive and robust. Its specificity was verified using 24 non-*Microcoleus* cultures mostly isolated from New Zealand, including the only other known anatoxin producer in New Zealand (*Cuspidothrix issatchenkoi*), and no amplification was detected. An advantage of such a specific assay is that it may be applied in systems where multiple anatoxin-producing species co-occur, enabling the quantitation of the *anaC* contained within *M. autumnalis*. The qPCR can be easily transferrable to emerging technologies, including droplet digital PCR (ddPCR; Wood and Puddick 2017).

The cyanobacterial 16S rRNA assay used in this study detects other non-*M. autumnalis* cyanobacteria in mat samples; thus, the proportion of toxic cells determined in this study represents the proportion relative to the entire cyanobacterial community within the mat. Previous studies have shown that the abundance of other cyanobacteria in *M. autumnalis*-dominated mats is very low (Brasell et al. 2015, McAllister et al. 2018b). Therefore, detections of other cyanobacteria are unlikely to have a marked impact on the relationships identified. Ideally, a *M. autumnalis*-specific reference qPCR would be used instead of a general cyanobacterial assay. Attempts made to develop such an assay during this study were unsuccessful.

There was no relationship between the anatoxin quota and percentage of toxic cells in the Ashley, Opihi, Maitai or Temuka rivers in multiple samples at different times, suggesting that anatoxin quota is not closely related to the percentage of toxic cells in *M. autumnalis*-dominated mats. This contrasts with *Raphidiopsis raciborskii*, where the cylindrospermopsin quota was highly correlated with the relative proportion of toxic gene copies (Burford et al. 2014). Individual strains of *M. autumnalis* isolated from New Zealand rivers exhibit up to 100-fold differences in their total anatoxin content, in addition to producing different proportions of the four anatoxin variants (Wood et al. 2012b). Environmental samples exhibited variation in the proportion of the different anatoxin congeners produced, and consistent with previous work, the dihydro-congeners were dominant in most of the samples (Wood et al. 2010c, Wood et al. 2012b, Wood and Puddick 2017). It is probable that the decoupling of anatoxin content and the anatoxin quota observed in this study reflects shifts in the dominance of different toxic strains within mats. The anatoxin content of *M. autumnalis*-dominated mats was highly variable within and between rivers, and over time, which is consistent with observations from previous studies (Wood et al. 2010c, Heath et al. 2011, Wood et al. 2017a). Both anatoxin concentrations and percentage of toxic cells varied over time in the Ashley and Maitai rivers. While this is consistent with studies on other rivers in New Zealand (Wood et al. 2017a), datasets spanning multiple years are required to confirm that this trend is consistent over multiple proliferation seasons. Identification of temporal trends in anatoxin content, anatoxin quota and the percentage of toxic cells in *M. autumnalis*-dominated mats is complicated by significant spatial heterogeneity. Often, anatoxin concentrations vary within a very small spatial area (i.e., 5 cm–10 cm), and are significantly different over short time periods (Wood and Puddick 2017). Sampling of *M. autumnalis*-dominated mats is destructive, and the presence of spatial heterogeneity may result in observations reflecting changes in spatial rather

than temporal differences. The use of an experimental system where the inoculum source is controlled and known would help mitigate this issue and identify whether there are temporal or successional trends in the proportion of toxic cells in mats.

The relationship between the total anatoxin content and percentage of toxic genotypes in the present study was significant. A relationship between the total abundance of toxic cells and anatoxin content in *M. autumnalis*-dominated mats has previously been identified (Wood and Puddick 2017); however, our study demonstrates that the proportion of toxic cells is also important. Similar correlations in microcystin concentrations and the proportion of toxic cells to the total cyanobacterial community in environmental samples have been observed in *Microcystis* and *Planktothrix*, though there the relationship was much stronger (Davis et al. 2009). In *M. autumnalis,* anatoxin quota differ among toxic strains (Wood et al. 2012b); thus, some variation in the relationship between anatoxin content and percentage of toxic cells is to be expected. The changes in anatoxin content observed in this study may therefore reflect changes in the dominance of toxic strains with differing anatoxin-producing ability. This is supported by the anatoxin quota data, which show that samples with higher than expected anatoxin content relative to the proportion of toxic cells also had higher anatoxin quota, indicating that the dominant toxic strains in these samples produce more anatoxin than average. The percentage of toxic cells ranged from 0.1% to 30.3% in the Ashley River and from < 0.01% to 3.6% across the other rivers. These results broadly align with similar studies in other genera, including *Microcystis*; toxic *Microcystis* made up 12% to 100% of the total cells in Lake Ronkonkoma (New York) and 0.01% to 6% in other lakes (Davis et al. 2009), while toxic *Microcystis* comprised 0.01% to 27% of the total *Microcystis* population in San Francisco Estuary (Baxa et al. 2010). Similarly, while toxic cells of *Planktothrix* comprised between 75% and 100% of the *Planktothrix* community in the Masurian lakes (Poland), when compared to the total cyanobacterial community, toxic cells made up between 0.01% and 5.89% of the community (Bukowska et al. 2017). The large variability in the proportion of toxic cells in the Hutt and Cardrona rivers is reflected in considerable differences between samples collected from different sites on the same day. It is possible that the variation observed between rivers and sites in this study was the result of physicochemical parameters. Other genera of cyanobacteria including *Microcystis* and *R. raciborksii* exhibit variation in the relative abundance of toxic genotypes or toxin production under different nutrient levels (Davis et al. 2009, Davis et al. 2010, Burford et al. 2014). Previous culture-based studies in *M. autumnalis* suggest that anatoxin quota are lowest under low nitrogen and phosphorus conditions (Heath

et al. 2016) and that toxic strains may be favoured at temperatures greater than 13.4 °C (Heath et al. 2011). The lack of available molecular methods has prevented the relationships between the proportion of toxic cells and physicochemical variables in environmental *M. autumnalis*-dominated mats from being determined until now. Future work should examine physicochemical variables across a range of rivers and over time to allow these relationships to be elucidated.

2.5 Conclusions

The qPCR assay developed in the present study enables the quantification of *M. autumnalis anaC* copy numbers in environmental samples. LC-MS/MS was used to determine anatoxin content and a second qPCR used to determine the total cyanobacterial 16S rRNA copy numbers in environmental samples. These data enabled anatoxin quota and the abundance of toxic/non-toxic strains to be determined in *M. autumnalis*-dominated mats. The results indicate that changes in anatoxin content in these mats are primarily related to the dominance of toxic strains.

The *M. autumnalis anaC* qPCR assay now provides an opportunity to investigate the dynamics of toxic/non-toxic strain dominance in environmental samples and if used on RNA (via complementary DNA) could enable insights into anatoxin regulation in this species to be gained. Future studies should use fine-scale, standardised sampling across a range of physicochemical gradients to identify factors that influence the relative abundance of toxic and non-toxic strains and anatoxin production.

2.6 Materials and Methods

2.6.1 *Samples for quantitative PCR assay development and validation*

Microcoleus autumnalis strains (n = 26) isolated from New Zealand rivers were sourced from the Cawthron Institute Culture Collection of Micro-algae (CICCM, http://cultures.cawthron.org.nz/; Table 2.1). Cultures were non-axenic. An additional 24

Table 2.1. Presence and absence of *anaC* and *anaF* genes and anatoxins in *Microcoleus autumnalis* strains used in this study. Anatoxins were measured using liquid chromatography tandem-mass spectroscopy (LC-MS/MS; Wood et al. 2017b).

Strain	*AnaC*	*AnaF**	Anatoxins** (LC-MS/MS)
CAWBG24	-	nt	-
CAWBG26	-	nt	-
CAWBG32	+	nt	nt
CAWBG36	-	nt	nt
CAWBG37	-	nt	nt
CAWBG38	-	nt	-
CAWBG46	-	nt	-
CAWBG48	-	nt	-
CAWBG50	-	nt	-
CAWBG51	-	nt	-
CAWBG52	-	nt	-
CAWBG53	-	nt	-
CAWBG54	-	nt	-
CAWBG55	-	nt	-
CAWBG56	-	nt	nt
CAWBG57	-	nt	nt
CAWBG58	+	nt	nt
CAWBG71	-	nt	nt
CAWBG503	+	+	+
CAWBG507	-	-	-
CAWBG511	-	-	-
CAWBG512	-	-	-
CAWBG520	+	+	+
CAWBG521	+	+	+
CAWBG556	+	+	+
CAWBG557	+	+	+

* *anaF* detection from Wood et al. (2012b), Wood et al. (2017b); ** Anatoxin detection from Wood et al. (2010c); nt indicates samples were not tested for this attribute.

non-axenic cyanobacterial strains including non-*Microcoleus* anatoxin producers were sourced from CICCM (Table A1.1; Appendix 1) for verification of assay specificity.

Strains tested included: three *Aphanizomenon*, two *Aphanocapsa*, one *Cuspidothrix*, two *R. raciborskii*, eight *Dolichospermum*, two *Leptolyngbya*, two *Microcystis*, one *Nostoc*, one *Planktothrix* and two *Scytonema*. Sub-samples (ca. 200 mg wet weight) of cultures were

harvested into a 1.7 mL microcentrifuge tube and centrifuged (12,000 × g, 1 min). The supernatant was discarded and pellets frozen (–20°C) until extraction. DNA was extracted using a Purelink® Genomic DNA extraction kit (Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions.

2.6.2 *Primer design and optimisation for quantitative PCR analyses*

Potential primer sites for detection of the *M. autumnalis*-specific *anaC* gene were identified using a multiple sequence alignment (ClustalW; Thompson et al. 1994) of a ca. 300 base pair (bp) region within the *anaC* gene from six *M. autumnalis* and five *Oscillatoria* sp. sequences obtained from GenBank (www.ncbi.nlm.nih.gov) and Wood et al. (2017b); Table A1.2, Appendix 1. Species-specific primers and a probe targeting a 100 bp region of the *anaC* gene in *M. autumnalis* were designed. Primers and probe concentrations were optimised for use with the Rotor-Gene-Q real-time rotary analyser (Qiagen, Netherlands) using *M. autumnalis* genomic DNA isolated from CICCM cultures CAWBG556 and CAWBG37. The optimised assay consisted of a 10 µL reaction containing: 5.6 µL KAPA Probe Fast QPCR Kit Master Mix (2×), 0.5 µL of primers (10 µM), 0.3 µL TaqMan probe, 2.1 µL Bovine serum albumin (BSA; Sigma, MO, USA) and 1 µL of template DNA. The optimised cycling profile was 95 °C for 3 min, followed by 40 cycles at 95 °C for 3 s and 60 °C for 20 s. Five-point standard curves ranging from 1.85×10^2 to 10^5 gene copies per µL and no template controls were analysed in triplicate on each qPCR run. The standard curve was constructed using purified (AxyPrep PCR Clean-up Kit, Axygen Biosciences, USA) PCR product using the *M. autumnalis*-specific *anaC* primers described above. Duplicate reactions of PCR product (5.14×10^{-9} ng μL^{-1} to 5.14×10^{-2} ng μL^{-1}) were used to determine the limits of detection (LOD). The number of copies in the PCR product for the standard curves were determined using the equation:

$$anaC \text{ copies } \mu L^{-1} = \frac{(A \times 6.022 \times 10^{23})}{(B \times 1 \times 10^{9} \times 650)}, \quad (1)$$

where A is the concentration of the PCR product and B is the length of the PCR product. The standard curves were linear ($R^2 > 0.99$) and the qPCR efficiency ranged from 0.94 to 1.08.

2.6.3 *Environmental sample collection*

Environmental samples from previous studies (Wood et al. 2017b, McAllister et al. 2018a) were used to explore the variability in anatoxin quota and relative abundance of genotypes among 10 New Zealand rivers (n = 122, Figure 2.5). Samples from the eight rivers in Canterbury and the Maitai River (Nelson) consisted of sub-samples (~2 cm^2) of mats from ten different cobbles at each site pooled into a single collection tube. Samples from the Hutt River and Cardrona River consisted of a mat sample from a single cobble harvested into a collection tube. Samples were stored in the dark and on ice, and frozen (–20 °C) on return to the laboratory. Four rivers (Ashley, Opihi, Maitai and Temuka) were chosen to conduct a time-series analysis to identify changes in the proportion of toxic strains and anatoxin quota over time. A further two of the rivers (Hutt and Cardrona) were sampled at multiple sites along the river, enabling a within-river comparison of the proportion of toxic strains and anatoxin quota.

2.6.4 *DNA extraction and test for inhibition*

Environmental samples were lyophilised and homogenised. A sub-sample of approximately 20 mg was weighed and extracted using a PowerSoil® DNA Isolation Kit (Qiagen, CA, USA) following the manufacturer's directions. All extracted DNA samples were aliquoted into two sub-samples and stored frozen (–20 °C) until use. Samples were screened for the presence of inhibitors using an internal control inhibition assay (Haugland et al. 2005). The assay consisted of an 11 µL reaction containing 6.25 µL KAPA Probe Fast qPCR Kit Master Mix (2×), 0.5 µL of each forward and reverse primer targeting the rRNA internal transcribed spacer region 2 (ITS2) of *Oncorhynchus keta* salmon sperm (10 µM, Sketa F2 and Sketa R3, IDT, USA, Haugland et al. (2005)), 0.2 µL TaqMan probe (10 µM) labelled with FAM-6-carboxyfluorescein at the 5′ end and with the Black Hole Quencher®-1 (IDT, IA, USA) at the 3′ end, 1.55 µL DNA/RNA free water (Thermo Fisher Scientific), 1 µL salmon sperm DNA (Sigma) and 1 µL of template DNA. The cycling profile was: 95 °C for 3 min, followed by 40 cycles at 95 °C for 3 s and 60 °C for 20 s. All samples showed inhibition and were diluted 1:10 with DNA/RNA free water (Thermo Fisher Scientific) and reanalysed for inhibition as above.

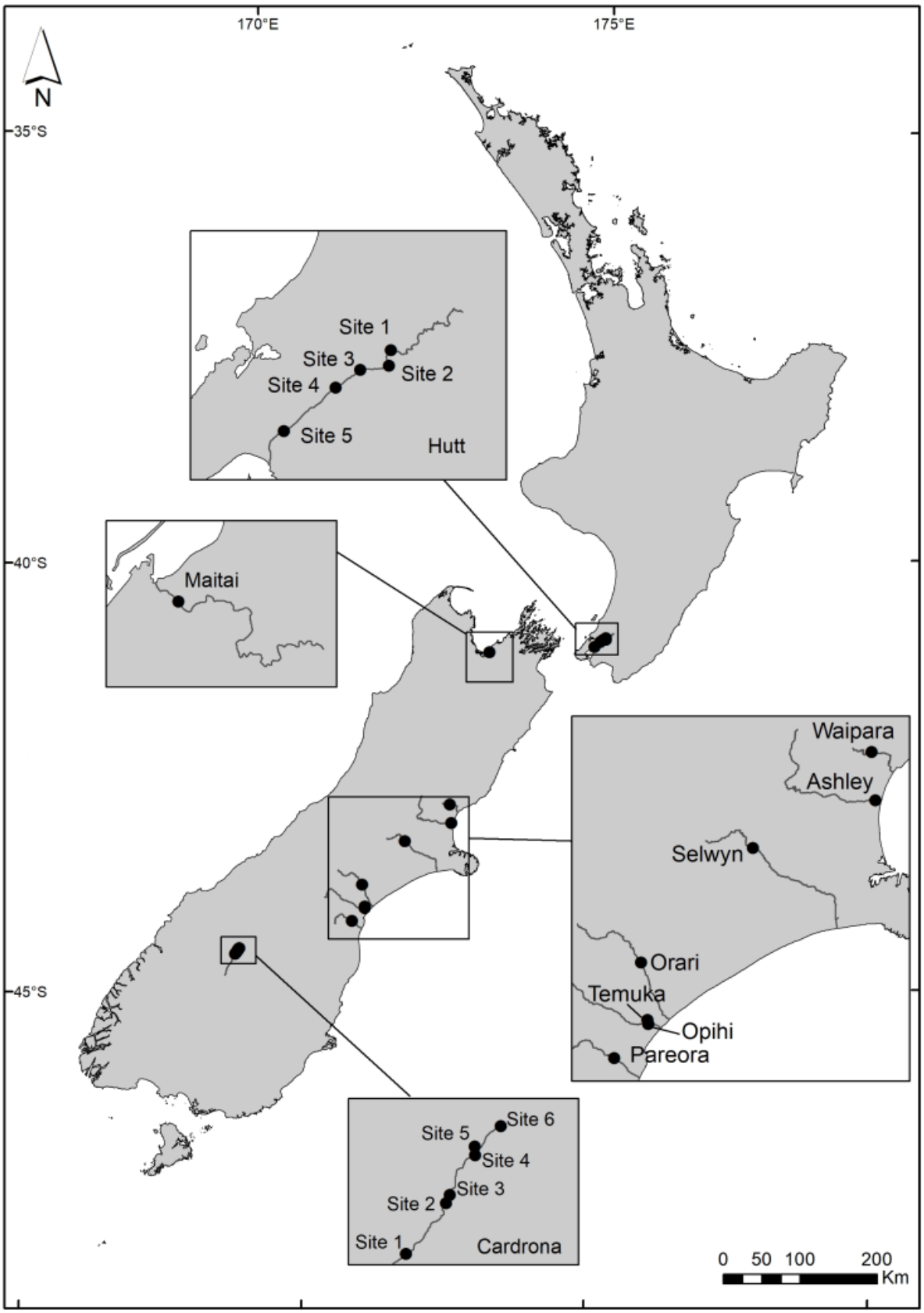

Figure 2.5. Map of sampling locations on 10 rivers in New Zealand. Sites 1–5 and 1–6 on the Hutt and Cardrona rivers, respectively, were collected from upstream to downstream on a single sampling occasion. Samples collected from the other eight rivers were collected over time from the same location.

2.6.5 *Quantitative PCR of 16S rRNA reference gene*

This qPCR assay was used to determine the number of cyanobacterial 16S rRNA gene copies in each sample. Reactions consisted of 5 µL Kapa SYBR Green Super Mix, 0.4 µL of each forward and reverse primer (cyano-real 16sF/R; Al-Tebrineh et al. (2010)), 2 µL BSA (Sigma), 1.2 µL DNA/RNA free water (Thermo Fisher Scientific), and 1 µL template DNA. The qPCR cycling conditions were the same as described for the anaC qPCR assay. Each reaction was run in triplicate using the optimised assay described above. Five-point standard curves ranging from 6.18×10^3 to 10^7 copies per µL and no template controls were analysed in triplicate on each qPCR run. The standard curves were constructed using purified (Nucelospin PCR Cleanup Kit, Machery-Nagel, Germany) PCR product using cyanobacterial 16S rRNA primers: 27F1 5′-AGAGTTTGATCCTGGCTCAG-3′ and 809R 5′-GCTTCGGCACGGCTCGGGTCGATA-3′ (Jungblut et al. 2005). The standard curve was linear ($R^2 > 0.99$) and the qPCR efficiency ranged from 0.82 to 0.93.

2.6.6 *Anatoxin extraction and analysis*

Microcoleus autumnalis-dominated mat samples were lyophilised and homogenised using a sterile metal spatula. Anatoxins were extracted from a known mass according to McAllister et al. (2018a). Each anatoxin extract was analysed for ATX, HTX, dhATX and dhHTX by liquid chromatography tandem-mass spectroscopy (LC-MS/MS), as described in Wood et al., 2017 (Wood et al. 2017b). An external standard curve consisting of dilutions of a certified ATX reference material (National Research Council, Ottawa, ON, Canada; 0.5 ng mL^{-1} – 20 ng mL^{-1} in 0.1% formic acid) was used to determine ATX concentrations. Anatoxin was used as a calibration reference and a relative response factor of 1 was used to quantify HTX, dhATX and dhHTX. The analytical limit of detection (LoD) was 0.02 ng mL^{-1} and the limit of quantitation (LoQ) 0.06 ng mL^{-1}. Detection limits in the extracted samples were 0.002 ng mg^{-1} (LoD) and 0.006 ng mg^{-1} (LoQ). The LC-MS/MS results (ng mL^{-1}) were divided by the weight of the lyophilised sample (in mg) to convert the data to ng mg^{-1}.

2.6.7 *Data and statistical analysis*

The number of copies of the *anaC* and cyanobacterial 16S rRNA genes per microlitre of DNA extract was calculated as described in equation 1. The results were converted to copies mg^{-1} using equation 2:

$$\text{copies mg}^{-1} = \frac{(\text{copies } \mu\text{L}^{-1} \times C \times D \times E)}{\text{weight of starting material (mg)}}, \quad (2)$$

where *C* is the volume of the qPCR reaction, *D* is the dilution factor and *E* is the elution volume. Anatoxin quota per toxic cell were calculated using equation 3:

$$\text{anatoxin quota (pg anaC}^{-1}) = \frac{[\text{ATX} + \text{HTX} + \text{dhATX} + \text{dhHTX}]\text{pg mg}^{-1}}{(\text{anaC copies}) \text{ mg}^{-1}}, \quad (3)$$

where [ATX, HTX, dhATX and dhHTX] are the concentrations of the respective anatoxins congeners (in pg per mg) of lyophilized mat material. To determine the ratio of toxic to non-toxic strains in the samples, their relative percentage was calculated by assuming that there are on average 2.3 16S rRNA copies per cyanobacterial genome, and using equation 4 (Větrovský and Baldrian 2013):

$$\text{percent toxic strains} = \left(\frac{anaC \text{ copies}}{(\text{16S rRNA copies})/2.3} \right) \times 100 . \quad (4)$$

Samples from the 10 study rivers were compared to examine differences in the proportion of toxic strains and anatoxin quota between rivers. All analyses were conducted in R (Version 3.1.3). The relationship between total anatoxins and the percentage of toxic genotypes across all samples was explored using a linear regression model, with the response variables log-transformed. Mean total anatoxins, anatoxin quota and proportion of toxic strains were analysed using a Kruskal-Wallis test and pairwise Wilcoxon rank sum test with a Benjamini-Hochberg adjustment, as the data were non-normally distributed and had heterogeneity of variances. A within-river comparison of the anatoxin quota and proportion of toxic strains was

undertaken for five sites on the Hutt River and six sites on the Cardrona River using a Kruskal-Wallis Test. Pairwise differences between sites were explored with Dunn's post-hoc test.

CHAPTER 3

Benthic cyanotoxin producers and spatial variability in anatoxin concentrations in the Eel and Russian Rivers, California

3.1 Abstract

Benthic cyanobacterial proliferations in rivers are increasing in prevalence worldwide. In the Eel and Russian rivers in California, more than a dozen dog deaths attributable to cyanotoxin toxicosis have been reported since 2000. Benthic proliferations in these rivers comprise multiple cyanobacterial taxa capable of cyanotoxin production and there is uncertainty regarding which taxa are producing cyanotoxins as a result. In this study, samples were screened for potential cyanotoxin producers using PCR and sequencing of genes responsible for anatoxin, microcystin and nodularin production in periphyton samples dominated by *Anabaena* spp., *Microcoleus* sp. and the green alga *Cladophora glomerata*. Cyanotoxin concentrations were measured using liquid chromatography tandem-mass spectrometry and anatoxin quota were determined using droplet digital PCR. Sequencing revealed that *Microcoleus* sp. and *Nodularia* sp. were the most likely producers of anatoxins and nodularins, respectively, regardless of the dominant taxa in the mat. Anatoxin concentrations varied from 0.1 μg to18.6 μg g^{-1} and were significantly different among sites ($p < 0.01$, Wilcoxon test); however, anatoxin quotas were less variable (< 5-fold). Dihydroanatoxin-a comprised 38% – 71% of the total anatoxins measured. Mats dominated by the green alga *Cladophora glomerata* contained both anatoxins and nodularin at concentrations similar to those of cyanobacteria-dominated mats. Our findings highlight that non-dominant taxa can be cyanotoxin producers and that *C. glomerata* mats may represent a significant exposure risk to cyanotoxins in these rivers. This is the first documentation of dihydroanatoxin-a in Californian rivers and its high proportion of total anatoxins indicates that this congener should be included in monitoring programs.

3.2 Introduction

Benthic cyanobacterial proliferations in freshwaters can threaten human health through their production of cyanotoxins (Paerl and Otten 2013). Reports of animal toxicosis resulting from benthic cyanobacterial proliferations have been described over the past 30 years (e.g. Edwards et al. 1992, Mez et al. 1997, Hamill 2001) and are increasing in frequency globally (Quiblier et al. 2013, Hudon et al. 2014, Belykh et al. 2016, McAllister et al. 2016, Cantoral Uriza et al. 2017, Gaget et al. 2017). Despite these reports, investigations into benthic cyanobacterial proliferations have lagged behind those into their planktonic counterparts. Filamentous cyanobacterial genera such as *Anabaena*, *Phormidium*, *Lyngbya*, *Oscillatoria*, *Nostoc*, *Nodularia* and *Microcoleus* can form extensive benthic mats and are capable of producing a range of cyanotoxins (Quiblier et al. 2013).

Cyanotoxins are typically classified by their different toxicological properties into neurotoxins, hepatotoxins, cytotoxins and dermatotoxins (Kaebernick and Neilan 2001, Pearson and Neilan 2008). Some of the more commonly detected cyanotoxins in freshwater environments include the hepatotoxins microcystin (MCY), nodularin (NOD) and cylindrospermopsin (CYN), and the neurotoxins anatoxins and saxitoxin (STX; Merel et al. 2013). Among benthic cyanobacteria, anatoxins are the most commonly reported cyanotoxin. They comprise four congeners: anatoxin-a (ATX), dihydroanatoxin-a (dhATX), homoanatoxin-a (HTX) and dihydrohomoanatoxin-a (dhHTX), and their relative proportions vary in environmental material (Wood and Puddick 2017). In New Zealand, dhATX, HTX and dhHTX are often present in *Microcoleus*-dominated mats at concentrations similar to or higher than ATX (Wood et al. 2007b, Wood et al. 2010a, Wood and Puddick 2017). To date, the presence and proportions of anatoxin congeners other than ATX have not been investigated in Californian rivers, which could mean the risks from anatoxin exposure have been underestimated.

Cyanotoxins occur in many Californian streams and rivers, with detections of MCYs, NOD, CYNs and anatoxins documented in periphyton samples and using solid phase absorption toxin tracking (SPATT) samplers (Fetscher et al. 2015, Bouma-Gregson et al. 2018). The SPATT samplers are semi-time integrated samplers, which can accumulate toxins that can then be extracted and tested. They provide no information on the organisms producing the toxins. Recently, benthic cyanobacterial proliferations and cyanotoxins have been reported in the Eel and Russian rivers in Northern Californian (Bouma-Gregson et al. 2018). These assemblages

comprise a variety of cyanobacterial genera known to produce cyanotoxins and these often co-occur at the same site. As a result, there is uncertainty about which genera in these systems are producing toxins, although *Microcoleus* has been identified as an anatoxin producer in the Eel River using assembled metagenomes (Bouma-Gregson et al. 2019). Culture-based studies could be prohibitively large and costly considering the range of potential toxic taxa present; however, molecular techniques can be used to screen for the presence of genes involved in cyanotoxin biosynthesis. These techniques, coupled with sequencing, can provide a strong indication of the toxin producers in environmental samples and this information can then be used to guide culturing of selected species.

Both toxic and non-toxic genotypes occur in the genus *Microcoleus*, and these genotypes can be present at varying relative abundances in environmental mats (Wood and Puddick 2017, Kelly et al. 2018), resulting in considerable spatial variability in anatoxin concentrations of mats within short distances. Molecular assays to determine the concentration of *anaC* gene copies using quantitative PCR (qPCR) and droplet digital PCR (ddPCR) have recently been developed and allow the toxin quota (amount of toxin per toxic cell) to be determined (Wood and Puddick 2017, Kelly et al. 2018). Comparisons of toxin quota, combined with data on the physicochemical conditions at the time of sampling, may provide insights into the drivers of anatoxin production by *Microcoleus*.

The present study aimed to characterise benthic cyanotoxin producers and to assess spatial variability in anatoxin concentrations and quotas in the Eel and Russian rivers. Ten samples were collected from five sites in the Eel River, and one site in the Russian River. PCR amplification of genes involved in toxin production and sequencing were utilised to identify potential cyanotoxin producers in the benthic cyanobacterial proliferations. Liquid chromatography tandem-mass spectroscopy (LC-MS/MS) was used to quantify anatoxin congeners, nodularin and microcystins. Spatial variability in anatoxin concentrations and anatoxin quotas were investigated by combining toxin concentrations determined by LC-MS/MS with ddPCR quantification of *anaC* gene copies.

3.3 Materials and Methods

3.3.1 *Environmental sample collection*

Sampling sites were chosen in the Eel and Russian rivers in Northern California. The river beds at the sampling sites were comprised of gravel, cobble or boulders. The four sites in the Eel River watershed were sampled on 29 July 2018 and spanned upstream drainage areas of 17 km^2 to 495 km^2. Site 1_ELD was on Elder Creek and sites 2_SFE–4_SFE were on the South Fork Eel River (Figure 3.1). The single Russian River site (5_RUS) was sampled on 31 July 2018 and had an upstream drainage area of 793 km^2 (Figure 3.1). At each site, ten (nine at 5_RUS) samples with visible cyanobacterial mats were selected and attached cyanobacteria/periphyton collected by scraping a small sample (ca. 2 cm diameter) into a 15 mL centrifuge tube. At 4_SFE, fine-scale samples were collected by taking five samples from each of three additional rocks. Additional samples of floating cyanobacterial and *Cladophora glomerata* mat material were collected at 3_SFE on the South Fork Eel River. Samples were stored on ice, frozen (–20 °C) on return to the laboratory (within 8 hours), and subsequently lyophilised prior to further analysis.

All ten samples from each site were analysed for cyanotoxin concentrations. Three samples from each site were screened for cyanotoxin genes, which were sequenced if detected. Anatoxin quotas were determined for the five samples with the highest anatoxin concentrations from sites 3_SFE, 4_SFE and 5_RUS, as well as the fine-scale samples from 4_SFE (Figure A2.1, Appendix 2). These sites were selected as anatoxin concentrations were markedly higher at these locations, and practical constraints limited the number of samples that could be processed.

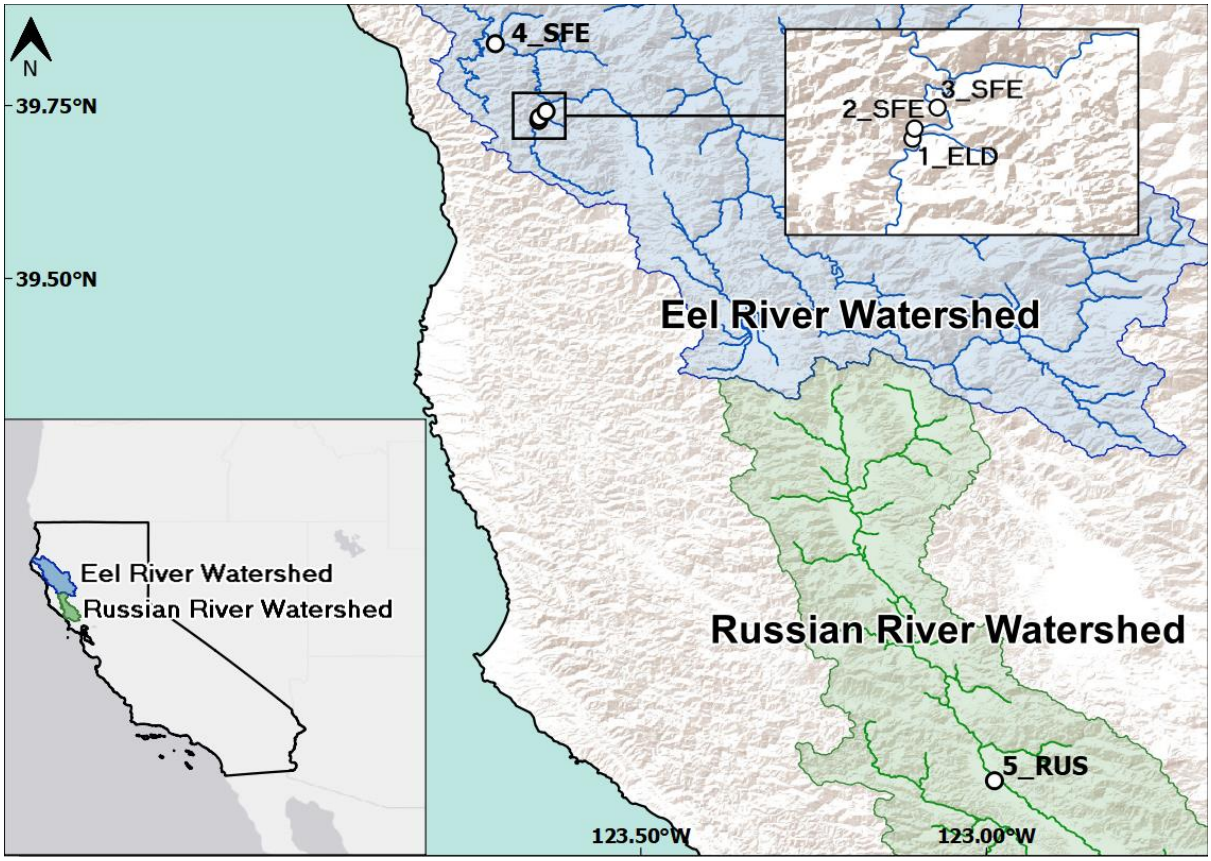

Figure 3.1. Map of sampling sites on the South Fork of the Eel River and on the Russian River in California, USA. Blue areas on the map refer to the Eel River watershed, while green denotes the Russian River and associated watershed. Sites are labelled in order of increasing drainage area from 1 to 5, while the suffix refers to the river: ELD = Elder Creek, SFE = South Fork Eel, RUS = Russian River.

3.3.2 *Toxin analyses*

Lyophilised environmental samples were homogenised with a sterile metal spatula and an extract for cyantotoxin analysis was made from a single sub-sample (ca. 10 mg dry weight) in 1 mL of 0.1% formic acid in water. The samples were frozen at –20 °C and subsequently thawed in a bath sonicator (53 kHz, 30 min; LHC bath sonicator, Kudos, Shanghai, China). Freeze-thaw and sonication steps were repeated two more times for each sample. Cyanotoxin extracts were clarified by centrifugation (14,000 × *g*, 5 mins) and the supernatants transferred to septum-capped glass vials for analysis of anatoxins, CYN, MCYs and nodularin-R (NOD-R) using liquid chromatography tandem-mass spectrometry (LC-MS/MS).

Anatoxins (ATX, HTX, dhATX and dhHTX) and cylindrospermopsins (cylindrospermopsin and deoxycylindrospermopsin; CYN and doCYN) were analysed using the LC-MS/MS methodology described in Wood et al. (2017b). Multiple-reaction monitoring (MRM) channels

for the anatoxins are provided in Wood et al. (2017b) and quantitation MRM channels for CYN and doCYN were 416.3 > 194.15 and 400.3 > 194.15, respectively. The anatoxins and cylindrospermopsins were quantified using a mixed external five-point calibration curve (0.5 ng mL^{-1} – 18 ng mL^{-1} in 0.1% formic acid) made from certified reference materials for ATX and CYN (National Research Council, Canada) and a standard for dhATX calibrated by quantitative nuclear magnetic resonance (NMR) spectroscopy. The concentrations of ATX and HTX were determined using the ATX calibration curve and the concentrations of dhATX and dhHTX were determined using the dhATX calibration curve and the concentration of CYN and doCYN were determined using the CYN standard. The analytical limit of detection (LoD) for ATX, dhATX and CYN was 0.05 ng mL^{-1}, which equates to an approximate LoD of 0.005 mg kg^{-1} in the sample extracts (dependent on the amount of sample weighed out for extraction).

Microcystins and NOD-R were analysed as described in Wood et al. (2012a) using a mixed external four-point calibration curve (2 mL^{-1} – 100 ng mL^{-1} in 50% methanol) made up of standards for NOD-R, MCY-RR, -YR and -LR (DHI Lab Products, Denmark). The analytical LoD for MCY-RR, -YR, -LR and NOD-R was 0.02 ng mL^{-1}, which equates to an approximate LoD of 0.002 mg kg^{-1} in the sample extracts (dependent on the amount of sample weighed out for extraction).

3.3.3 *Molecular analyses*

A sub-sample (ca. 15 mg dry weight) was placed into the first tube of a PowerSoil® DNA Isolation Kit (Qiagen, CA, USA) and DNA extracted according to the manufacturer's protocols. The extracted DNA was stored at –20 °C until utilised in molecular analyses.

Samples were screened by PCR (Table A2.1, Appendix 2) for the presence of *mcyE/ndaF* (primers HEPF/HEPR; Jungblut and Neilan 2006), *sxtA* (primers Sxta/Sxtf; Ballot et al. 2010), *anaC* (primers anaC-gen-F/anaC-gen-R; Rantala-Ylinen et al. 2011) and *cyl* (primers cynsufF/cylnamR; Mihali et al. 2008). The reactions consisted of 12.5 µL MyTaq RedMix (Bioline, London, UK), 1 µL each of the relevant forward and reverse primer (Supplementary Table 1), 3 µL bovine serum albumin (BSA; Sigma, USA), 4.5 µL DNA/RNA free water (Thermo Fisher Scientific) and 3 µL of template DNA. The cycling conditions for all reactions comprised an initial denaturation at 95 °C for 1 min, followed by 30 cycles with denaturation

at 95 °C for 15 sec, annealing at 54 °C for 15 sec,extension at 72 °C for 15 sec and a final extension at 72 °C for 5 min and hold at 4 °C.

For sequencing, positive PCR reactions were purified using a nucleospin PCR cleanup kit (Machery-Nagel, Düren, Germany), according to the manufacturer's directions. Purified PCR product was then quantified (NP80 NanoPhotometer, Implen GmbH, Munich, Germany) and diluted to a concentration of 5 ng μL^{-1} (*mcyE*) or 4 ng μL^{-1} (*anaC*). Amplicons were sequenced bi-directionally with gene-specific primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequences were compared for similarity to reference sequences using Blastn (NCBI). Sequences obtained in this study were deposited in GenBank under accession numbers MK821061 to MK821086.

The absolute number of copies of *anaC* gene were quantified by droplet digital PCR (ddPCR), using the *Phor-AnaC* primers and probes (Table A2.1, Appendix 2) according to the methods of Wood and Puddick (2017). Anatoxin quota were calculated as the summed anatoxins per mg dried weight (dw) divided by the *anaC* copy number per mg dw as described by Kelly et al. (2018).

3.3.4 *Statistical analyses*

All statistical analyses were conducted in the software R Studio (R Version 3.5.1). Mean toxin concentrations, *anaC* gene copy numbers and anatoxin quota were tested for normality using the Shapiro-Wilks test. All variables failed to meet parametric test assumptions, so non-parametric tests were used. Comparisons of mean toxin concentrations and anatoxin quota were undertaken using Kruskal-Wallis tests and pairwise Wilcoxon rank sum tests with Benjamini-Hochberg adjustment for multiple comparisons.

3.4 Results

3.4.1 *Cyanotoxin presence and variability*

All five sites surveyed had visible cyanobacteria biomass present. Sites 1_ELD, 2_SFE and 4_SFE were riffle habitats, and cyanobacterial mats were cohesive, attached to benthic cobbles,

and were a black/brown colour characteristic of *Microcoleus*-dominated mats in these rivers. Site 3_SFE was a slow-moving pool and was dominated by senescing *Cladophora glomerata* and spires of *Anabaena* sp., with *Nostoc* sp. also present upstream of the survey site. Site 5_RUS was a run habitat on the Russian River. It was also dominated by *C. glomerata* and spires of *Anabaena* sp.

Anatoxins were detected by LC-MS/MS at all five sites, though levels were low (< 0.2 μg g^{-1} dw) at sites 1_ELD and 2_SFE (Figure 3.2a). Total anatoxin concentrations at the other three sites were considerably higher, with samples ranging from 0.1 μg g^{-1} dw to 18.6 μg g^{-1} dw. The mean anatoxin concentration at sites 3_SFE, 4_SFE and 5_RUS were significantly different (p < 0.01; Wilcoxon test). Hepatotoxins comprised NOD, MCY-LR, and dmMCY-LR and were detected at all sites, though the concentrations in 4_SFE and 5_RUS were very low and only detected in one (4_SFE) or two (5_RUS) samples (Figure 3.2b). Site 3_SFE had the highest hepatotoxin concentrations (mean 1.4 μg g^{-1} dw), with all other site concentrations below the quantitation limit of 0.1 μg g^{-1} dw. Microcystin/nodularin concentrations were below quantitation limits at most sites and when they were quantifiable, closely followed anatoxin concentrations. The mean concentrations of cyanotoxins in the floating cyanobacterial mats from 3_SFE were (10.2 ± 8.5) μg g^{-1} dw and (0.85 ± 0.6) μg g^{-1} dw for anatoxins and nodularin, respectively. Mean concentrations of anatoxins in the *C. glomerata* mats were (7.4 ± 1.8) μg g^{-1} dw and mean nodularin concentrations were (0.33 ± 0.33) μg g^{-1} dw.

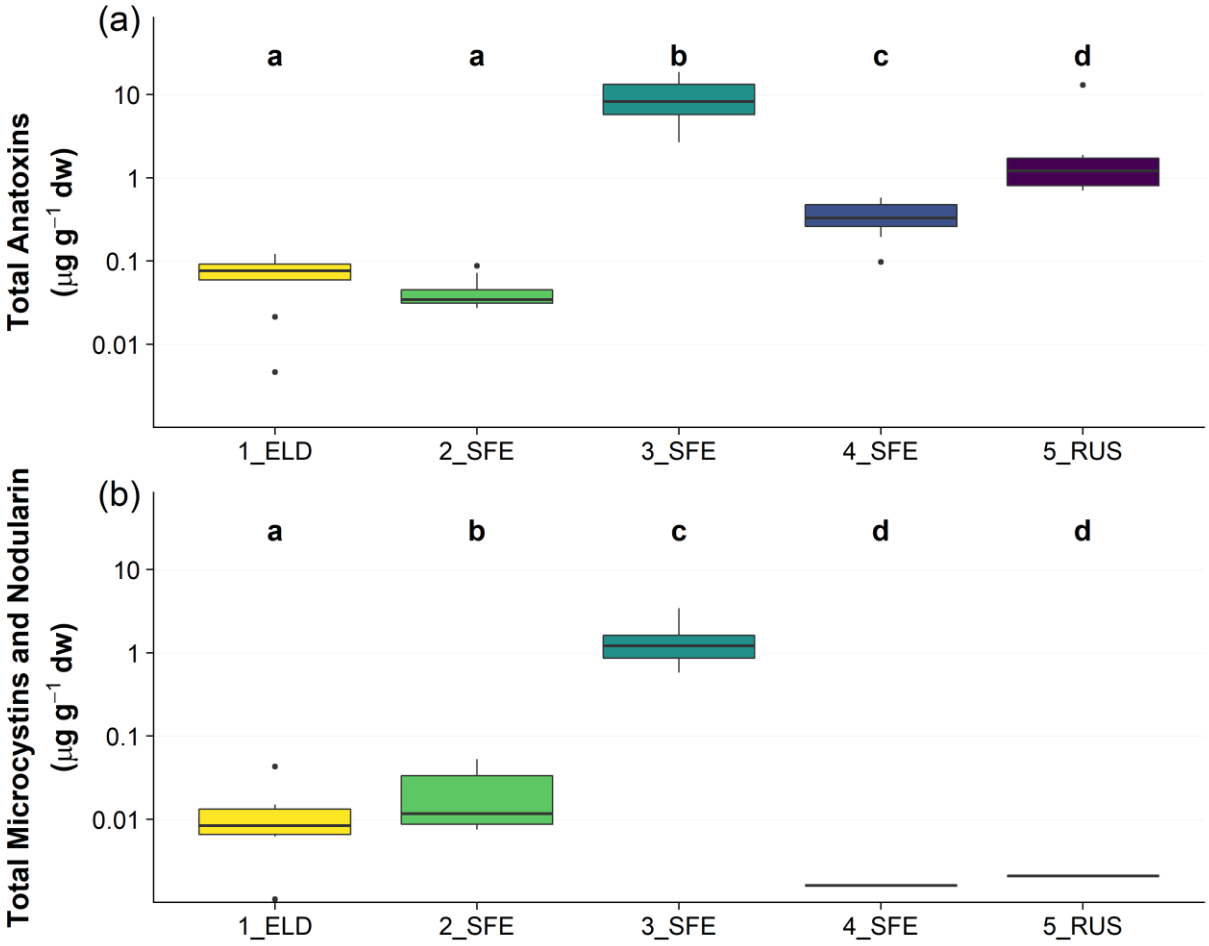

Figure 3.2. Cyanotoxin concentrations in the Eel and Russian rivers, California. (a) Summed anatoxin (the total of the four congeners: anatoxin-a, homoanatoxin-a, dihydroanatoxin-a and dihydrohomoanatoxin-a); and (b) summed microcystins and nodularin from 10 replicate attached periphyton samples collected from each of four sites on the Eel River on 29 July 2018 and 9 attached periphyton samples from one site on the Russian River on 31 July 2018. Dw = dry weight. Note log scale on the *y*-axes. Lines within the boxes are medians, the ends of boxes are quartiles and whiskers extend to the lowest or highest data point ≤ 1.5 × interquartile range. Black dots are outliers. A Kruskal-Wallis test and pairwise Wilcoxon rank sum test with a Benjamini-Hochberg adjustment were used to identify sites that were significantly different from one another ($p < 0.05$), denoted by the letter above the plot.

Anatoxins were almost exclusively comprised of ATX and dhATX, with low levels (< 0.01 μg g^{-1} dw) of HTX and dhHTX only detected at 1_ELD and 2_SFE (Figure 3.3). The composition of congeners was similar within each site (Table 3.1), with the exception of 4_SFE, which had more variable proportions of ATX and dhATX between samples. Hepatotoxins were almost exclusively comprised of NOD, with only low levels (< 0.01 μg g^{-1} dw) of MCY-LR and dmMCY-LR detected. Nodularin was the only hepatotoxin detected at 1_ELD and 5_RUS.

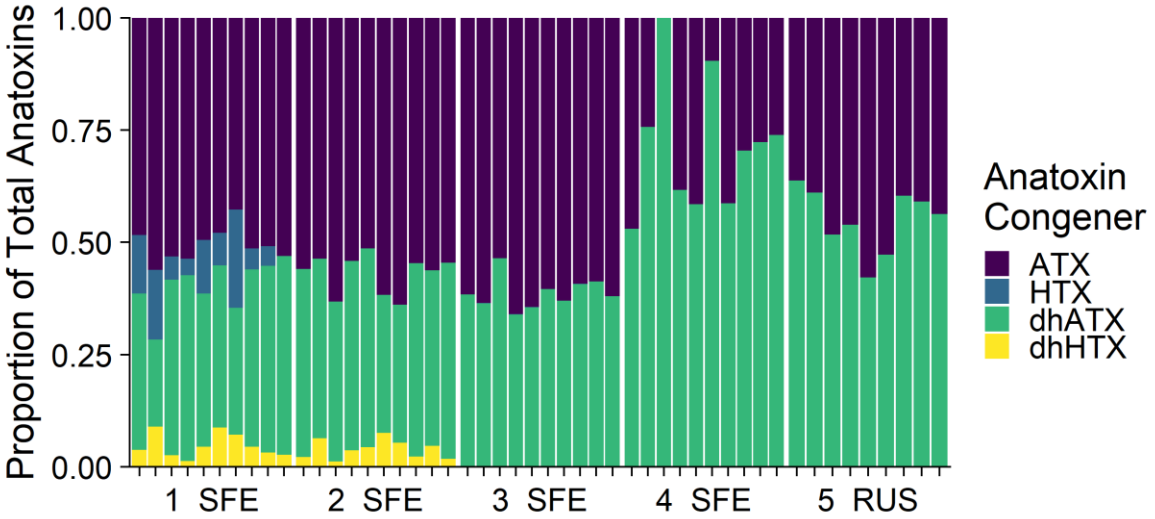

Figure 3.3. The composition of anatoxin congeners from attached periphyton samples collected at the five sites. Each vertical bar represents one periphyton sample. ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydroanatoxin-a, dhHTX = dihydrohomoanatoxin-a.

Table 3.1. The mean proportion of each anatoxin congener in attached periphyton samples collected at the five sites ± standard deviation (n = 10 for all sites except 5_RUS, where n = 9).

Site	ATX	dhATX	HTX	dhHTX
1_ELD	50.7 ± 15.8	38.7 ± 15.4	6.5 ± 7.8	4.1 ± 6.3
2_SFE	56.5 ± 15.7	39.7 ± 15.5	0 ± 0	3.8 ± 6.1
3_SFE	60.5 ± 15.5	39.4 ± 15.5	0.02 ± 0.51	0.02 ± 0.48
4_SFE	28.5 ± 14.3	71.5 ± 14.3	0 ± 0	0.06 ± 0.79
5_SFE	51.4 ± 15.8	48.6 ± 15.8	0 ± 0	0.06 ± 0.79

3.4.2 *Potential cyanotoxin producers*

The PCR reactions were positive for *anaC* at sites 3_SFE, 4_SFE and 5_RUS, but not at sites 1_ELD and 2_SFE. All the *anaC* sequences were identical and most closely matched those of *Oscillatoria* sp. from the Pasteur Culture Collection (*Oscillatoria* sp. PCC 10601, PCC 9240; GenBank accession: JF803652, JF803653; 100% cover and 99.7% identity) and one *Phormidium autumnale* (*Microcoleus autumnalis*; CAWBG618; GenBank accession: KX016036; 93% cover and 99.6% identity) from the Cawthron Institute Culture Collection of Microalgae (http://cultures.cawthron.org.nz/). Comparison of sequences from this study to draft metagenomes from Bouma-Gregson et al. (2019) revealed 100% sequence homology with the *anaC* gene from the draft *M. autumnalis* genomes assembled in their study. Samples from site 3_SFE had PCR detections for the *mcyE/nduF* genes. Sequences from these reactions were

identical and shared the closest sequence similarity to *Nodularia spumigena* (GenBank accession: CP020114; 100% cover and 99.7% identity). Sequences obtained from the attached and floating samples of the filamentous green alga *C. glomerata* were identical to the above sequences from the cyanobacteria-dominated samples. Saxitoxin and cylindrospermopsin genes were not detected in any of the samples.

3.4.3 *Within-site and within-mat variability*

Three sites were assessed for within site variability (3_SFE, 4_SFE and 5_RUS). Low levels of variability in anatoxin concentrations (< 5-fold) were observed within each site (Figure 3.4a). When anatoxin concentrations were normalised to the concentration of *anaC* gene copies (Figure 3.4b), the variability was reduced to < 2-fold in each site (Figure 3.4c). Where samples had high anatoxin concentrations relative to others from the same site (e.g., 5_RUS sample 5; Figure 3.4), normalisation to number of toxic cells reduced the anatoxin quota to levels comparable to the other samples. The mean anatoxin quota of 3_SFE was 0.98 pg cell^{-1}, while 4_SFE and 5_RUS had quotas of 0.23 pg cell^{-1} and 0.12 pg cell^{-1}, respectively. The mean anatoxin quota among all three sites were significantly different ($p < 0.05$; Kruskal-Wallis and Wilcoxon tests).

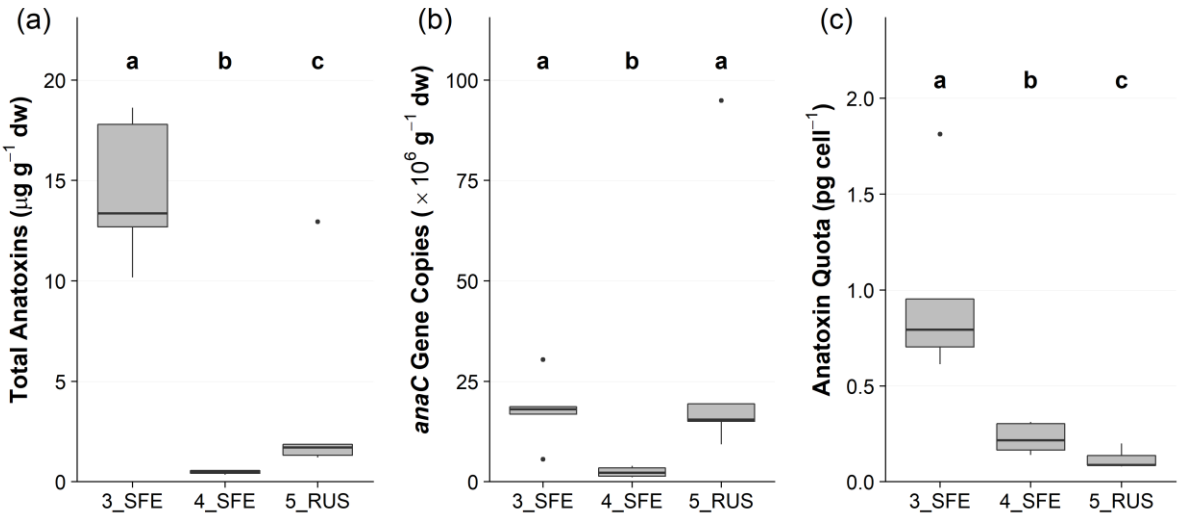

Figure 3.4. Anatoxin concentrations, *anaC* gene copies and anatoxin quota among three sites on the Eel and Russian rivers. (a) Summed anatoxin concentrations; (b) *anaC* gene copies; and (c) anatoxin quota; from five attached periphyton samples collected from two sites on the Eel River and one site on the Russian River. dw = dried weight. Note the different *y*-axis scales. See Figure 3.2 for interpretation of boxplots. A Kruskal-Wallis test and pairwise Wilcoxon rank sum test with a Benjamini-Hochberg adjustment were used to identify sites that were significantly different from one another ($p < 0.05$), denoted by the letter above the plot.

Among the five samples collected from each of three rocks at 4_SFE, total anatoxins varied by 7-fold. There were no obvious patterns among mats and no significant differences between anatoxin gene copies or quota ($p > 0.05$; Figure 3.5a-c). Individual samples with high anatoxin concentrations had comparable anatoxin quota upon normalisation to the abundance of toxic cells.

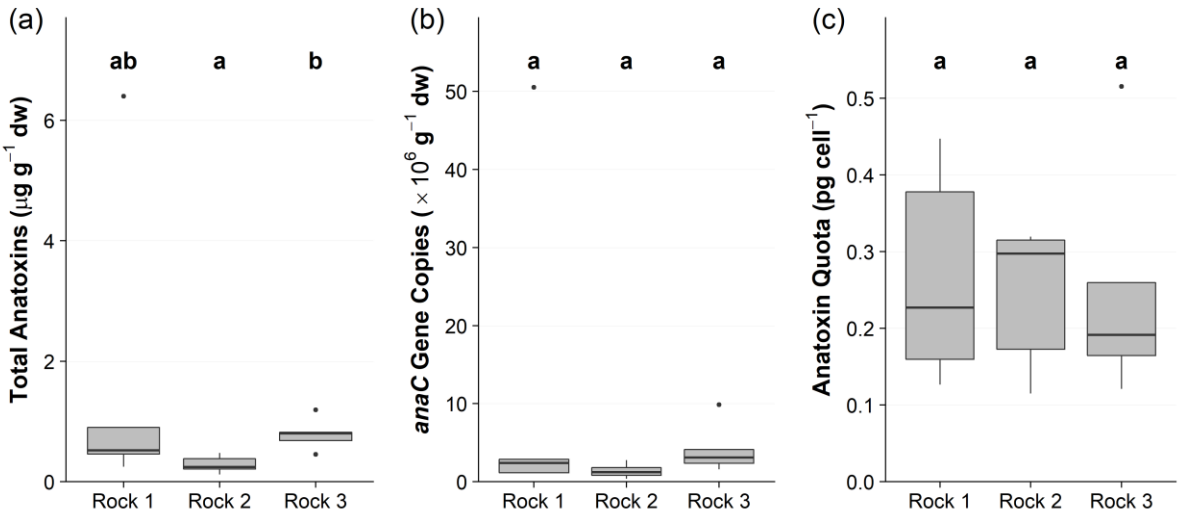

Figure 3.5. Anatoxin concentrations, *anaC* gene copies and anatoxin quota among fine-scale samples from three rocks at 4_SFE on the Eel river. (a) Summed anatoxin concentrations; (b) *anaC* gene copy concentrations; and (c) anatoxin quota; from five mat samples collected from each of three rocks at 4_SFE. Dw = dried weight. Note the different *y*-axis scales. See Figure 3.2 for interpretation of boxplots. A Kruskal-Wallis test and pairwise Wilcoxon rank sum test with a Benjamini-Hochberg adjustment were used to identify rocks that were significantly different from one another ($p < 0.05$), denoted by the letter above the plot.

3.5 Discussion

3.5.1 *Cyanotoxin producers*

The first aim of this study was to use molecular techniques to identify potential cyanotoxin producers in benthic mats from the Eel and Russian rivers. The *anaC* gene sequences confirm *Microcoleus* as the anatoxin producer, both in the *Microcoleus*-dominated mat samples and in the *Anabaena*-dominated samples. There was no evidence that *Anabaena* was the anatoxin producer in these samples. This is consistent with results from *Anabaena* cultures isolated from the Eel River, which have not produced anatoxins to date (Bouma-Gregson, personal communication). This study detected the *anaC* gene at sites where Bouma-Gregson et al. (2019) did not find anatoxin biosynthesis gene clusters in their metagenomes; however our results correspond with previous anatoxin detections in the upper reaches of the Eel watershed

(Bouma-Gregson et al. 2018). Bouma-Gregson et al. (2019) found that only a single *Microcoleus* species of four contained the anatoxin biosynthesis gene cluster, and this species was restricted to sites downstream of those sampled during the current study. This incongruity highlights the difficulty of obtaining representative samples when benthic cyanobacterial mats exhibit large spatial and temporal changes in the abundance of toxic genotypes.

Sequencing identified a nodularin producer with the gene most closely resembling that from *Nodularia spumigena*. This species prefers brackish water, though it has been reported in freshwater lakes in Europe based on morphometric identification (Akcaalan et al. 2009). The high sequence homology between *N. spumigena* and the nodularin producer in this study indicates further work is required to determine whether *N. spumigena* is the producing taxa. This would reveal either that the observed similarity is the result of a lack of sequences from freshwater nodularin producers in the reference database, or perhaps a novel nodularin producer is present. Sequences for *mcyE* were not detected as part of the screen, likely due to low abundance compared with nodularin sequences. Despite this, the presence of microcystins indicates that a yet-to-be-identified microcystin producer is also present in the Eel River.

3.5.2 *Toxin concentrations*

Toxin concentration data were consistent with patterns observed in previous studies (Bouma-Gregson et al. 2017, Bouma-Gregson et al. 2018), with anatoxin concentrations five times greater than microcystin/nodularin concentrations. Solid phase adsorption toxin tracking samplers (SPATTs) in the Eel River accumulated anatoxin-a and microcystin in 53% – 54% and 41% – 76% of samples for each toxin, respectively (Bouma-Gregson et al. 2018). Similar patterns of anatoxin-a and microcystin detections are found in benthic cyanobacterial mats dominated by *Anabaena* and *Microcoleus* (Bouma-Gregson et al. 2018). In contrast, microcystins were not detected in most samples in this study, and when they did occur, concentrations were below quantitation limits.

All samples were dominated by ATX and dhATX, which contrasts with observations elsewhere: dhATX, dhHTX and HTX typically dominate in New Zealand (McAllister et al. 2016, Wood and Puddick 2017), and in France ATX dominates (Echenique-Subiabre et al. 2018), although dhATX has also been detected (Mann et al. 2012). The biosynthesis of ATX or HTX was hypothesised to be encoded by a region of the *anaG* gene of the anatoxin

biosynthesis cluster, while *anaK* may be involved in the formation of dhATX (Méjean et al. 2014, Méjean et al. 2016). Bouma-Gregson et al. (2019) reported a missing region (ca. 300 base pairs) in the *anaG* gene and a complete lack of the *anaK* gene of the anatoxin biosynthesis cluster, a result obtained through metagenome sequencing of environmental *Microcoleus*-dominated mats from the Eel and Russian Rivers. Despite these missing regions, this study detected all four anatoxin congeners in some of the samples. The high proportion of dhATX indicates a need to use analytical methods that detect this congener (along with HTX and dhHTX) to prevent underestimation of the risk posed by benthic cyanobacteria in these systems.

The site with the highest concentrations of both anatoxins and nodularin was dominated by *Anabaena*, a finding that contrasts with that of Bouma-Gregson et al. (2018), who found no differences in anatoxin concentrations between *Anabaena* and *Microcoleus* dominated mats. Despite the dominance of *Anabaena*, the sequencing data from this study indicate that the detected cyanotoxins are being produced by other cyanobacteria (*Microcoleus* and *Nodularia*). Similarly, mats of the green alga *C. glomerata* at this site yielded concentrations of anatoxin and nodularin comparable with those found in the cyanobacteria-dominated samples. *Cladophora glomerata* is globally distributed and described as an ecosystem engineer due to its ability to modify ecosystems by providing habitat for associated complex microbial assemblages (Zulkifly et al. 2012). It is possible that *C. glomerata* provides habitat for a variety of cyanobacterial taxa, including *Microcoleus* and *Nodularia*. The presence of cyanotoxins at such high concentrations in this system raises questions about how extensive this phenomenon is in rivers with extensive benthic cyanobacterial proliferations, and whether *C. glomerata* and related taxa harbour toxic cyanobacteria and thus present a risk in systems without large, visible cyanobacterial proliferations. Investigation of these questions should be a priority for future research to assist with future risk assessments of cyanotoxin exposure.

3.5.3 *Spatial variability*

Spatial variability of anatoxin content occurred both among and within sites. The magnitude of this variability was relatively low, and upon normalisation to anatoxin quota, the variability in the anatoxin concentrations was reduced. This is consistent with other studies on *Microcoleus*-dominated mat samples, where large variation in anatoxin concentrations was reduced upon normalisation to anatoxin quotas (Wood and Puddick 2017, Kelly et al. 2018).

Anatoxin quotas in this study (0.12 pg cell^{-1} – 0.98 pg cell^{-1}) were similar to those observed in the Cardrona River in New Zealand (0.44 pg cell-1; Wood and Puddick 2017). Anatoxin quota can vary both within and between rivers, however, and anatoxin quotas in excess of 7.5 pg cell^{-1} have been reported (Wood and Puddick 2017, Kelly et al. 2018). Variability in anatoxin quota observed in *Microcoleus*-dominated mats is thought to be the result of different toxic genotypes with varying capacities of toxin production (Wood et al. 2012b, Wood and Puddick 2017). The results from the present study, similarly, indicate that anatoxin concentrations in benthic cyanobacterial mats from the Eel and Russian rivers are largely driven by the abundance of toxic cells. The low variability in anatoxin quotas at both site and within-rock scales observed in this study may, therefore, result from lower diversity in anatoxin-producing strains in the Eel and Russian rivers. Isolation and culture of *Microcoleus* strains would enable characterisation of toxic genotypes in order to confirm this.

3.6 Conclusions

The identification of *Microcoleus* and *Nodularia* as cyanotoxin producers in the Eel and Russian rivers in this study enables targeted sampling in future to isolate and culture these cyanotoxin producers. Dihydroanatoxin-a, homoanatoxin-a and dihydrohomoanatoxin-a were detected for the first time in Californian rivers and their concentrations indicate these congeners should be included in monitoring and risk assessments. Cyanotoxins produced by non-dominant taxa in mats, including both *Anabaena* and *Cladophora glomerata*, were present at high concentrations, which suggests these mats pose an exposure risk to cyanotoxins despite not being dominated by cyanotoxin-producing taxa. The presence of cyanotoxins in mats dominated by the green alga *C. glomerata* should be a priority for further research to investigate how widespread this phenomenon is.

CHAPTER 4

Phosphorus source and concentration have differential effects on alkaline phosphatase activity in *Microcoleus autumnalis* strains

4.1 Abstract

Toxic, benthic cyanobacterial proliferations have increased in frequency and severity globally and can have negative impacts on aquatic ecosystems, recreation and human health. *Microcoleus autumnalis* has been associated with numerous animal fatalities and is causing increasing concern. It tends to grow in systems with moderate dissolved inorganic nitrogen and very low dissolved reactive phosphorus. Acquisition of nutrients, particularly phosphorus, from organic sources may explain how *M. autumnalis* can reach the high biomass in these relatively nutrient-deplete environments. In the present study, the effect of phosphorus concentration and source on alkaline phosphatase activity was investigated in toxic and non-toxic *M. autumnalis* strains. Toxic strains exhibited significantly higher alkaline phosphatase activity than non-toxic strains ($p < 0.05$), and alkaline phosphatase activity increased in all strains under phosphorus-depleted conditions ($p < 0.05$). Alkaline phosphatase activity was also present in environmental *M. autumnalis* mats, though at lower levels than in laboratory experiments. The presence of alkaline phosphatase activity indicates that the acquisition of phosphorus from organic phosphorus sources may contribute to the ability of *M. autumnalis* to grow in systems with low dissolved reactive phosphorus.

4.2 Introduction

Cyanobacterial blooms are increasing in frequency and severity globally, and their effects on freshwater aquatic ecosystem functioning, recreational amenities and human health are causing concern (Paerl and Huisman 2009, Visser et al. 2016, Huisman et al. 2018). Many planktonic cyanobacterial taxa produce cyanotoxins (Carmichael 2001, Paerl and Fulton 2006). In contrast, little is known about the risks to ecology, recreation and human health posed by toxic, benthic cyanobacterial proliferations (Hamill 2001, Gugger et al. 2005, Wood et al. 2010b, Quiblier et al. 2013, Fetscher et al. 2015, Bouma-Gregson et al. 2017, Cantoral Uriza et al. 2017), and significant knowledge gaps remain regarding factors promoting growth and toxin production (Quiblier et al. 2013, McAllister et al. 2016). Proliferations dominated by the benthic cyanobacteria *Microcoleus autumnalis* (formerly *Phormidium autumnale*) and related taxa (e.g., *Phormidium* spp.) (Cadel-Six et al. 2007, McAllister et al. 2016, Bouma-Gregson et al. 2018, Echenique-Subiabre et al. 2018) produce anatoxins, which are potent neurotoxins (Teneva et al. 2005, Wood et al. 2007b, Heath et al. 2010, Wood et al. 2010a, McAllister et al. 2016).

Nutrient availability is a key driver of cyanobacterial growth and dominance across a range of ecosystems, with many blooms occurring in eutrophic waters (Paerl and Huisman 2009, O'Neil et al. 2012, Paerl and Otten 2013). Blooms and proliferations also occur in oligotrophic and mesotrophic waters, and this may be because some cyanobacteria fix atmospheric nitrogen and compete effectively for other nutrients, including phosphorus (Lehtimaki et al. 1997, Berman-Frank et al. 2003, Paerl and Fulton 2006, O'Neil et al. 2012). Dissolved organic phosphorus (DOP) compounds comprise a significant portion of the soluble phosphorus pool in many aquatic environments, and cyanobacteria are often capable of utilising them (Degerholm et al. 2006, Harke et al. 2012, O'Neil et al. 2012). In contrast to many planktonic cyanobacteria, *M. autumnalis*-dominated proliferations typically occur when water column dissolved reactive phosphorus (DRP) is less than 0.01 mg L^{-1} (Heath et al. 2015, McAllister et al. 2016, Wood et al. 2017a, McAllister et al. 2018a). As these biofilms develop, they form thick, cohesive mats, which are isolated from the overlying water column by a thin diffusive boundary layer, enabling within-mat conditions to differ from the overlying water (Jørgensen and Des Marais 1990). Within the mat matrix, DRP concentrations can be over 300-fold greater than in the overlying water column (Wood et al. 2015b), and this may be the result of a combination of desorption of phosphorus from fine sediment entrapped in the mat and the activity of enzymes

involved in nutrient cycling, such as alkaline phosphatases. Although the role of fine sediment as a source of phosphorus has received some attention (Wood et al. 2015b, Aristi et al. 2017), the contribution of alkaline phosphatase activity (APA) had not been well studied in *M. autumnalis*-dominated mats. Aristi et al. (2017) quantified phosphatase activity in environmental *M. autumnalis*-dominated mats but were unable to distinguish whether the phosphatase activity was produced by *M. autumnalis* or associated bacteria or diatoms. Alkaline phosphatase activity is maximal under alkaline conditions, with the optimal pH differing depending on the enzyme origin (Whitton 1991). The pH in *M. autumnalis*-dominated mats changes over diel cycles, with pH greater than 9 occurring during the day (Wood et al. 2015b) as a result of the depletion of bicarbonate during photosynthesis (Badger and Price 2003). This variation in the pH conditions within *M. autumnalis*-dominated mats may lead to cyclic activity in alkaline phosphatases in environmental mat samples.

Microcoleus autumnalis comprises both toxic and non-toxic strains whose relative abundance in environmental samples is variable (Kelly et al. 2018). Differences in the utilisation of nutrients have been identified between toxic and non-toxic strains of some cyanobacteria (e.g. *Microcystis* sp. and *Raphidiopsis raciborskii*; Davis et al. 2010, Burford et al. 2014, Willis et al. 2015). Non-toxic strains of *Microcystis* have higher growth rates than toxic strains under low nitrogen and phosphorus conditions; however, when nutrient levels are high, toxic strains exhibit higher growth rates (Vezie et al. 2002). Such differences in nutrient utilisation appear to influence the relative abundance of toxic and non-toxic strains in environmental samples containing *Microcystis* (Joung et al. 2011). Heath et al. (2016) demonstrated that non-toxic *M. autumnalis* exhibits higher growth rates than toxic strains under a range of high and low nitrogen and phosphorus treatments and reaches greater cell densities at stationary phase. They suggest this indicates non-toxic strains of *M. autumnalis* might outcompete toxic strains, but caution that studies are required to examine both toxic and non-toxic strains grown together.

The present study aimed to: (1) determine whether *M. autumnalis* produces alkaline phosphatase and if differences in APA were apparent between toxic and non-toxic strains; and (2) explore changes in APA in *M. autumnalis*-dominated mats across a diel cycle. It was expected that *M. autumnalis* would exhibit alkaline phosphatase activity and that toxic strains would have higher activity than non-toxic strains due to different optimal nutrient requirements. Changes in APA over a diel cycle were anticipated, with higher APA during the

day as a result of within-mat biochemical conditions, and lower APA at night when pH naturally reduces.

Laboratory experiments using four toxic and four non-toxic strains were undertaken under four different phosphorus treatments over 24 h. In addition, a field study was undertaken during a severe *M. autumnalis*-dominated proliferation in the Hutt River (Wellington, New Zealand). Samples were collected every two hours from one site over a 24-h period, with five additional sites along a downstream gradient sampled at a single time point. These data will contribute to our understanding of how severe proliferations of *M. autumnalis* occur in low phosphorus water bodies and provide some indication of the physiological differences in nutrient utilisation by toxic and non-toxic strains.

4.3 Materials and methods

4.3.1 *Culture conditions and sampling*

Four toxic and four non-toxic, non-axenic *M. autumnalis* strains were obtained from the Cawthron Institute Culture Collection of Micro-algae (CICCM), Nelson, New Zealand (Rhodes et al. 2016). Strains had been collected and isolated from various New Zealand locations between 2005 and 2008 (Table A3.1; Appendix 3; Heath et al. 2010). Cultures were grown to stationary phase in MLA media (Bolch and Blackburn 1996) on a 12:12 light-dark cycle at 18 °C prior to pre-treatment. Cultures were harvested using sterile tweezers, then rinsed and re-suspended in phosphate-free MLA media. The potassium concentration of the media was maintained by the addition of KCl when K_2HPO_4 was removed or reduced (Heath et al. 2014). Cultures were then incubated for 7 days on a 12:12 light-dark cycle at 17 °C to deplete internal stores of polyphosphates before treatments began (Wu et al. 2012). Following phosphate depletion, cultures were homogenised with an UltraTurrax T10 homogeniser (Ika, Staufen im Breisgau, Germany), and 1.5 mL of the homogenate pipetted into 40 mL of the relevant treatment media. Five replicate culture vessels for each strain and treatment combination were established. The four treatment media consisted of phosphate-free MLA media modified with the addition of 0.055 mL L^{-1} or 5 mL L^{-1} 0.04 M orthophosphate (K_2HPO_4; low and high treatments respectively) and 0.055 mL L^{-1} or 5 mL L^{-1} 0.04 M organic phosphorus (as β-glycerophosphate disodium salt; low and high treatments respectively;

Table 4.1). Measured orthophosphate and organic phosphorus concentrations in the lowest concentration treatment were unexpectedly low compared with the other treatments, possibly due to sorption to the walls of the vessel (Table 4.1). A 1 mL aliquot of this initial homogenate was preserved in Lugols iodine for later cell enumeration.

Cultures were incubated for 24 hours on a 12:12 light-dark cycle at 17 °C before being re-homogenised. A 10 mL sub-sample of homogenate was pipetted into a 15 mL centrifuge tube and centrifuged (3200 × *g*, 4 min) at room temperature. The supernatant was decanted into separate 15 mL centrifuge tubes and both these tubes and the tube containing the pellet were frozen (–20 °C) until APA analysis. Triplicate samples were harvested from each culture vessel.

Table 4.1. Treatment media for alkaline phosphatase activity laboratory experiment. Treatments consisted of MLA media modified to remove all phosphorus, with the subsequent addition of orthophosphate as K_2HPO_4 or organic phosphorus as β-glycerophosphate disodium salt. DRP = dissolved reactive phosphorus, DOP = dissolved organic phosphorus.

Treatment media	Orthophosphate concentration (mg L^{-1})	Organic phosphorus concentration (mg L^{-1})
High DRP, high DOP	5.2	4.0
High DRP, low DOP	4.8	0.2
Low DRP, high DOP	0.42	3.9
Very low DRP, low DOP	0.004	0.09

4.3.2 *Environmental sample collection*

Six sites on the Hutt River (Wellington) were chosen to sample *M. autumnalis*-dominated mats to determine APA in environmental samples (Table 4.2). In addition, samples were collected every 2 hours for 24 hours at one site (Silverstream, 41° 8' 24"S, 175° 0' 17"E). Samples were collected by lifting a rock from the water and scraping mat material (ca. 2 cm^2) into a sterile 2 mL cryotube. Samples were frozen immediately (–173 °C) and stored in a liquid nitrogen-filled dry shipper until transport to the laboratory. Water samples (ca. 50 mL) were collected at each site, syringe filtered (GF/C, 1.6 μm pore size, Whatman, England) and stored on ice

until transport to the laboratory, where the filtrate was frozen (–20 °C) for dissolved nutrient analysis.

Table 4.2. Environmental sampling locations on the Hutt River, Wellington, New Zealand. Sites are presented in order from upstream to downstream.

Site	Latitude	Longitude
Birchville	41° 5' 27" S	175° 5' 50" E
Maoribank Corner	41° 6' 37" S	175° 5' 41" E
Poet's Park	41° 6' 59" S	175° 2' 48" E
Silverstream	41° 8' 24" S	175° 0' 17" E
Boulcott	41°11' 46" S	174° 55' 16" E
Melling	41° 12' 5" S	174° 54' 39" E

4.3.3 *Laboratory analysis*

Environmental samples were thawed on ice and a sub-sample (ca.10 mg wet weight) weighed into a 15 mL centrifuge tube. Samples were then immediately processed for APA.

An additional sub-sample (ca. 200 mg wet weight) was weighed into the first tube of a PowerSoil® DNA Isolation Kit (Qiagen, CA, USA), and the DNA extracted according to the manufacturer's directions. The DNA was screened for inhibitors using an internal control inhibition assay (Haugland et al. 2005). All samples exhibited inhibition and were diluted 1:10 with RNA/DNA-free water (Thermo Fisher Scientific, MA, USA) and reanalysed. The total number of cyanobacterial 16S rRNA gene copies was determined using a quantitative PCR (qPCR) assay. The assay consisted of 5 μL Kapa SYBR Green Super Mix, 0.4 μL each of forward and reverse primers (cyano-real 16sF/R; Al-Tebrineh et al. 2010), 2 μL BSA (Sigma, USA), 1.2 μL DNA/RNA free water (Thermo Fisher Scientific) and 1 μL template DNA. A five-point standard curve was prepared as described in Kelly et al. (2018) and samples were run in triplicate with the standard curve and negative controls in each qPCR run. The cycling profile was: 95 °C for 3 min, followed by 40 cycles at 95 °C for 3 s and 60 °C for 20 s. A melt curve was run at the end of the cycling profile and consisted of 54 °C for 1 min followed by a ramp from 60 °C to 95 °C in 1 °C increments at 5 s intervals.

4.3.4 *Nutrient analysis*

Media samples and water samples from the laboratory and field experiments were analysed at Hill Laboratories (Hamilton, New Zealand). Samples were processed with a Lachat Quickchem® flow injection analyser (FIA+8000 Series, Zellweger Analytics Inc., League City, TX, USA) using 4500 methods (APHA 2005) for dissolved reactive phosphorus (DRP) and total phosphorus (TP). The limits of quantitation were 0.004 mg L^{-1} for DRP and TP. Dissolved organic phosphorus was estimated as TP minus DRP.

4.3.5 *Alkaline phosphatase activity*

The APA was measured using a methylumbelliferyl fluorometric assay modified for use with *M. autumnalis* samples (Marx et al. 2001, DeForest 2009). The 15 mL tubes were defrosted at room temperature and 2 mL of 50 µM Tris buffer containing 1 mM $MgCl_2$ (pH 10.3) and 250 µL of 100 µM 4-methylumbelliferyl phosphate was added to each tube, and the time recorded. Tubes were gently agitated and incubated at room temperature in the dark (1 hr). The reaction was terminated by the addition of 300 µL of 1 mM NaOH and the tubes centrifuged (3200 × *g*, 3 mins). Standards of 4-methylumbelliferone (MUB) were prepared at the same time as the sample incubation as follows: a 100 µM stock solution was prepared prior to the assay and frozen in aliquots until use. The 100 µM stock of MUB was pipetted into a centrifuge tube and Tris buffer added to make up 2.25 mL at the following concentrations: 30.5 µM, 10.5 µM, 5.5 µM, 0.5 µM and 0.1 µM. Each standard was incubated in the dark (1 hr) with the samples, after which 300 µL of 1 mM NaOH was added. Following centrifugation (3200 × *g*, 3 min), 250 µL of each sample and standard were pipetted into four technical replicate wells in a black 96-well microplate. Fluorescence was measured 10 min after the NaOH addition using a microplate reader (Enspire Multimode Plate Reader, Perkin Elmer, MA, USA) with an excitation wavelength of 365 nm and emission wavelength of 450 nm. Blanks for all microplates consisted of eight replicate wells containing 250 µL Tris buffer (pH 10.3).

4.3.6 *Statistical analyses*

All analyses were conducted in the R statistical package (http://www.r-project.org/). Concentrations of MUB were log-transformed to generate a linear regression between MUB and fluorescence. The log-concentrations of the unknown samples were calculated from the

regression equation and the exponential taken to convert to fM MUB. The fM MUB hydrolysed per hour (the incubation time) was calculated by taking the mean of the four technical replicates on each microplate. For the laboratory experiment, each replicate was normalised to the initial biomass of the cultures to give the APA per cell for each strain, and the mean for each treatment-strain combination calculated. The field APA was normalised to the number of copies of cyanobacterial 16S rRNA using equations 5 and 6:

$$16\text{S rRNA copies } (\text{mg}^{-1}) = \frac{(\text{copies per } \mu\text{L} \times C \times D \times E)}{\text{weight of starting material (mg)}}, \quad (5)$$

where *C* is the volume of the qPCR reaction, *D* is the dilution factor and *E* is the DNA elution volume in µL.

$$\text{APA } (\text{fM MUB 16S rRNA copy}^{-1}\ \text{hour}^{-1}) = \frac{[\text{APA}]\ \text{fM MUB hour}^{-1}\text{mg}^{-1}}{(16\text{S rRNA copies})\ \text{mg}^{-1}}, \quad (6)$$

where the APA is expressed as femtomoles of methylumbelliferone (fM MUB) produced per mg wet weight per hour, divided by the number of 16S rRNA copies per mg wet weight.

The APA results in the laboratory experiment were analysed with a linear mixed effects model to determine the effect of treatment and toxigenicity as fixed factors. Strain was included as a random (intercept) factor. Models were fitted in R using the "lme4" package (Bates et al. 2014), with the significance of the fixed effects assessed using the "lmerTest" (Kuznetsova et al. 2017) package with the Satterthwaite method. The normality of residuals and homogeneity of variance of the model were tested using quantile-quantile plots coupled with Shapiro-Wilk's test and Levene's Test (Zuur et al. 2010). Residuals were non-normal and there was heteroscedacity of variances even after data transformation; thus, a robust linear mixed effects model was used as this is insensitive to violations of these assumptions. Robust linear mixed models (rlmm) were fitted by restricted maximum likelihood using the rlmer function in the R package "robustlmm" (Koller 2016). Treatment and toxigenicity were included as crossed fixed factors, and strain as a random intercept factor. The effect of factors and interactions on APA were deemed significant when $t > 2$, utilising the t-as-z approximation (Luke 2017). To account for anti-conservativeness in the t-as-z approximation, results were compared to the original linear mixed effects model with the Satterthwaite method.

The APA of field samples was tested for normality using quantile-quantile plots and the Shapiro-Wilks test. APA was not normally distributed, and was square-root transformed for analysis. Statistically significant differences in APA at each time point and each site were assessed separately with one-way ANOVA. Where significant differences were identified, a Tukey's honest significant difference pairwise post-hoc test was undertaken to determine differences between pairs of samples.

4.4 Results

4.4.1 *Laboratory experiments*

Toxic strains exhibited higher mean APA than non-toxic strains (24.8 fM MUB cell^{-1} hour^{-1} and 12.2 fM MUB cell^{-1} hour^{-1}, respectively) regardless of treatment (Figure 4.1, $p < 0.05$, rlmm). There was also a significant interaction between the effects of the low DRP/low DOP treatment, the low DRP/high DOP treatment and toxigenicity, with non-toxic strains increasing APA more under these treatments relative to their baseline when compared to toxic strains ($p < 0.001$, rlmm; Figure 4.1). Toxic strains exhibited greater APA than non-toxic strains and the robust linear mixed effects model (rlmm) using the t-as-z approximation showed that the estimated effect was significant ($p < 0.05$); however, it was marginally non-significant using lmer ($p = 0.054$; Table A3.2; Appendix 3). Extracellular APA was detectable in the supernatant but was below quantitation limits.

In the phosphorus starvation (low DRP, low DOP) treatment, APA was higher than the phosphorus replete treatment (high DRP, high DOP) for all strains (Figure 4.2). Robust linear mixed effects modelling demonstrated that the effect of this was more pronounced in the non-toxic strains, which exhibited low APA (9.77 fM MUB cell^{-1} hour^{-1}) in phosphate replete conditions but increased APA (by 7.97 fm MUB cell^{-1} hour^{-1}) under phosphorus starvation ($p < 0.001$, rlmm; Table 4.3). Toxic strains exhibited higher APA under phosphorus replete conditions (23.16 fM MUB cell^{-1} hour^{-1}); however, the magnitude of APA increase under phosphorus starvation was lower (3.97 fM MUB cell^{-1} hour^{-1}, $p < 0.001$, rlmm; Table 4.3) than in the non-toxic strains.

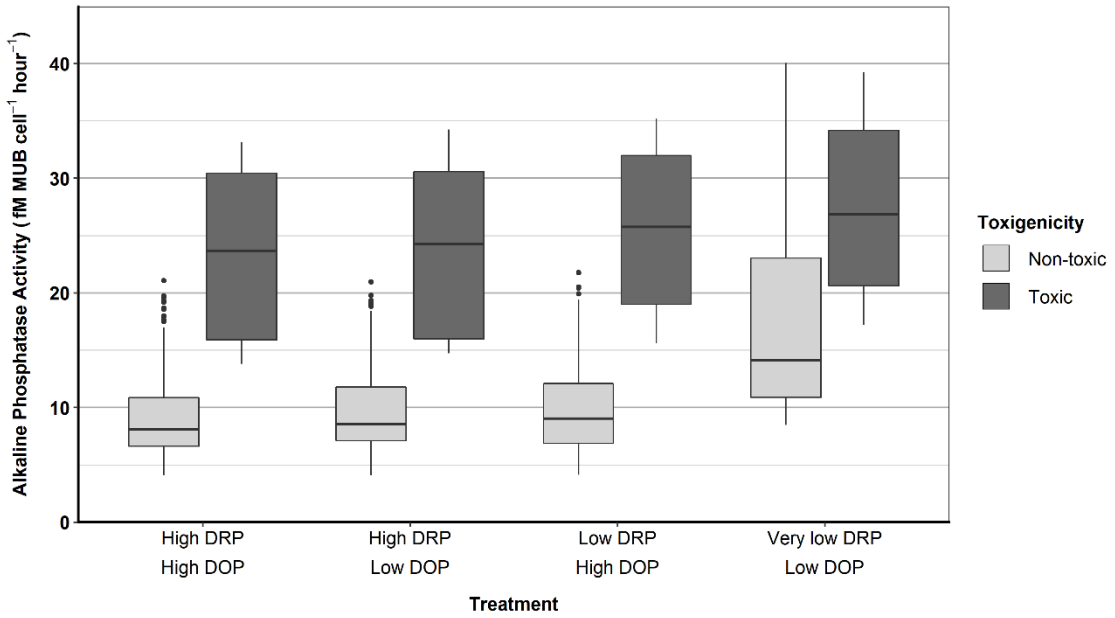

Figure 4.1. Alkaline phosphatase activity in toxic and non-toxic *Microcoleus autumnalis* under different phosphorus concentration regimes. Data are the average of four toxic and four non-toxic strains. Alkaline phosphatase activity (femtomoles of methylumbelliferone produced per cell per hour) in the very low DRP, low DOP treatment was significantly different from the high DRP, high DOP treatment ($p < 0.001$, robust linear mixed effects model with t-as-z approximation) and toxic strains exhibited higher APA ($p < 0.05$). Lines within the boxes are medians, the ends of boxes are quartiles and whiskers extend to the lowest or highest data point ≤ 1.5 × interquartile range. Black dots are outliers.

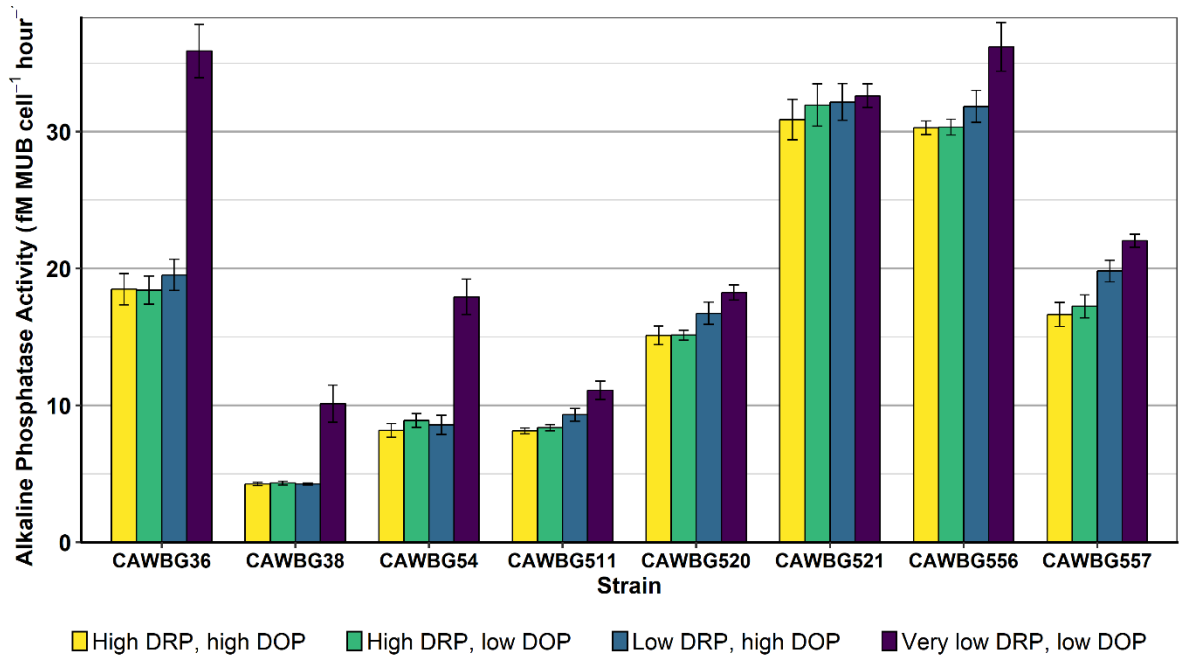

Figure 4.2. Alkaline phosphatase activity by strain and toxigenicity under different phosphorus regimes for four non-toxic (strains CAWBG36 to CAWBG511) and four toxic strains (CAWBG520 to CAWBG557) of *Microcoleus autumnalis*. There was considerable variability from strain to strain. DRP = dissolved reactive phosphorus, DOP = dissolved organic phosphorus. Filled bars show the mean APA for the respective strain-treatment combination, with error bars representing the standard deviation of the mean.

3.1.2 Strain-to-strain variability

Among the non-toxic strains, CAWBG36 had markedly higher APA than the other non-toxic strains in all treatments (Figure 4.2). Strains CAWBG521 and CAWBG556 exhibited higher APA than CAWBG520 and CAWBG557 in the toxic strains. Variability among strains explained 98% of the difference in APA after accounting for the effects of toxigenicity and treatment in the rlmm model and 94% of the difference in the lmer model.

4.4.2 *Environmental alkaline phosphatase activity*

All environmental samples exhibited APA, with activity ranging from 0.5 fM MUB cell^{-1} hour^{-1} to 10.9 fM MUB cell^{-1} hour^{-1}. The APA in environmental samples was low (all samples < 11 fM MUB cell^{-1} hour^{-1}) compared to the laboratory APA samples (mean 18.52 fM MUB cell^{-1} hour^{-1}; Figure 4.3a-b). One-way ANOVA analysis identified no significant difference in APA over the 24-h sampling period ($p > 0.05$). There was a significant difference in APA between sites on the river ($p < 0.05$; Figure 4.3a), and this was due to a significant difference between the most upstream site (Birchville) and the most downstream site (Melling; Tukey's post-hoc test). There were no significant differences in APA among the other sites ($p > 0.05$), though the general trend was for lower APA in upstream sites. APA was highly variable at all time points (Figure 4.3b).

River water column TP, DRP and DOP were below quantitation limits (0.004 mg L^{-1}) at all sites.

Table 4.3. Robust linear mixed effect model estimates of the fixed effects on alkaline phosphatase activity. The intercept represents the high dissolved reactive phosphorus (DRP), high dissolved organic phosphorus (DOP) treatment for the non-toxic strains and the estimates of all other effects are subsequently derived from this baseline. Significant estimates ($p < 0.05$) are in bold.

Fixed effects	Estimate	Standard Error	t value	*p* value (t-as-z)
Intercept	9.77	4.51	2.16	**0.030**
High DRP, High DOP				
Treatment B	0.26	0.22	1.16	0.245
High DRP, Low DOP				
Treatment C	0.64	0.22	2.88	**0.004**
Low DRP, High DOP				
Treatment D	7.97	0.22	35.72	**< 0.001**
Low DRP, Low DOP				
Toxic strains	13.39	6.38	2.10	**0.036**
High DRP, High DOP				
Treatment B: Toxic strains	0.09	0.32	0.29	0.776
High DRP, Low DOP				
Treatment C: Toxic strains	1.31	0.32	4.15	**< 0.001**
Low DRP, High DOP				
Treatment D: Toxic strains	–4.00	0.32	–12.68	**< 0.001**
Low DRP, Low DOP				

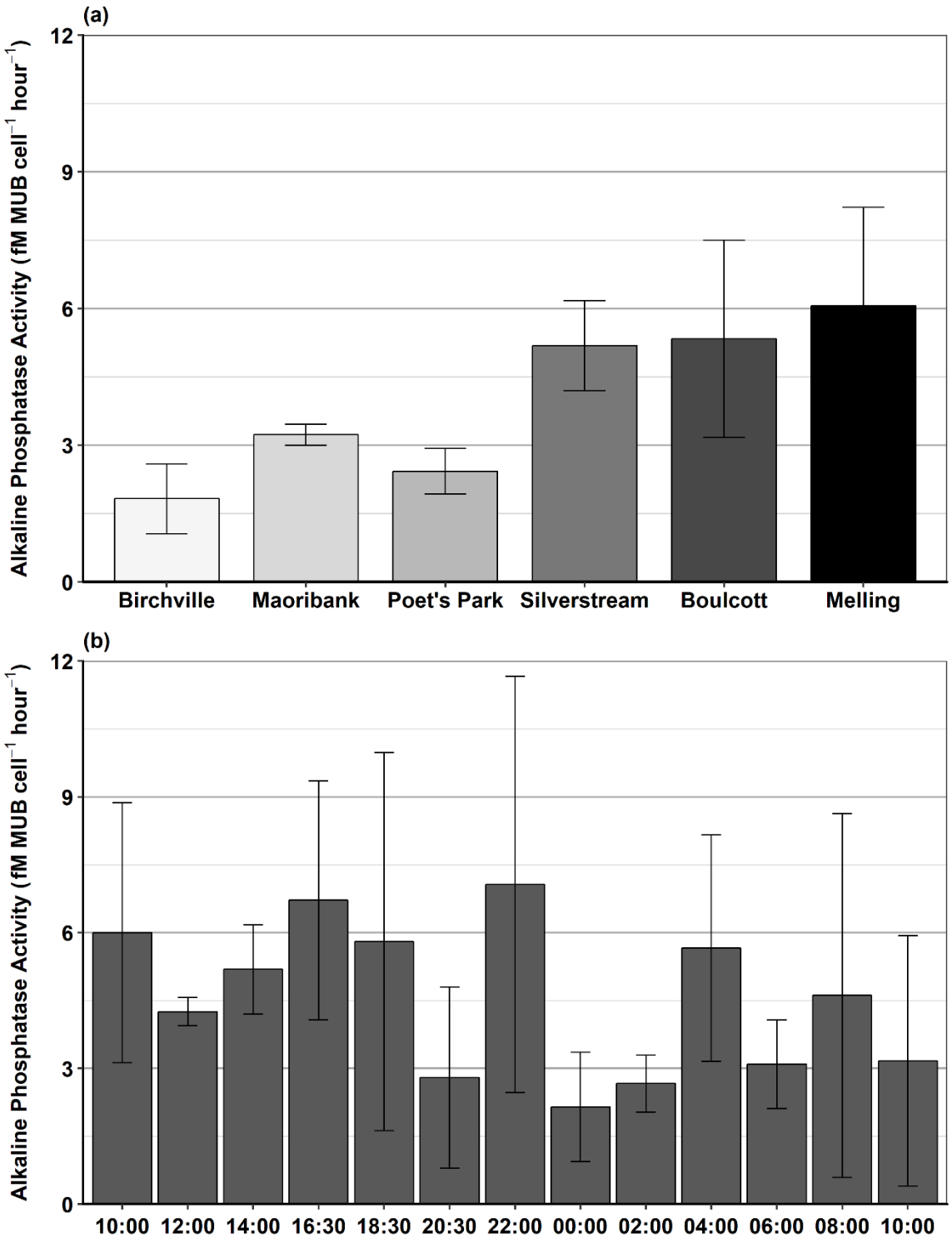

Figure 4.3. Alkaline phosphatase activity of environmental mats. (a) Alkaline phosphatase activity of *M. autumnalis*-dominated mats at six sites on the Hutt River (n = 3; the Silverstream samples were part of the time series and the data used here were from the sampling at 14:00 to compare with the other sites). A significant difference in APA ($p < 0.05$) between Birchville and Melling was detected (one-way ANOVA, Tukey HSD). (b) alkaline phosphatase activity of *M. autumnalis*-dominated mats collected at Silverstream over 24 h. Filled bars show the mean APA for the respective strain–treatment combination, with error bars representing the standard deviation of the mean.

4.5 Discussion

4.5.1 *Alkaline phosphatase activity in laboratory*

Alkaline phosphatase activity occurred in all *M. autumnalis* cultures and all treatments, indicating that *M. autumnalis* is responsible for at least some of the APA observed in a previous study with environmental samples (Aristi et al. 2017). This contrasts with the metagenomic data of (Bouma-Gregson et al. 2019), who reported sequences for acid phosphatases and glycerolphosphodiesterases, but not alkaline phosphatases (*phoA* and *phoD*), although *phoX* was present. The *phoX* alkaline phosphatase gene has been documented as more widely distributed in marine cyanobacteria than *phoA* or *phoD* (Sebastian and Ammerman 2009). It has also been reported in a range of freshwater cyanobacteria (Lin et al. 2018). Metagenome sequencing on New Zealand *M. autumnalis* has identified putative *phoX* and *phoD* genes (Handley and Tee, Auckland University, Wood, Cawthron; unpublished data).

Increased alkaline phosphatase activity was induced by phosphorus limitation in this study, consistent with Whitton et al. (1991), who found that phosphomonoesters supported growth in 50 strains from 10 cyanobacterial genera, and demonstrated that APA was upregulated under phosphate limitation and almost undetectable in phosphorus-replete media. In the present study, APA decreased in the treatment with low DRP and elevated DOP, which suggests inhibition of APA by phosphate generated from the hydrolysis of the DOP during the 24-h exposure period. *Raphidiopsis raciborskii* can utilise organic phosphorus compounds and upregulates APA genes in response to phosphorus starvation (Bai et al. 2014). *Raphidiopsis raciborskii* are capable of growing in low DRP waters and employ flexible phosphorus uptake strategies, where phosphates are stored as polyphosphates during pulses of nutrient input (Burford et al. 2018). Given *M. autumnalis* also grows in waters with low DRP (McAllister et al. 2016), it is possible that they employ similar strategies for growth when phosphorus supply is low and variable.

The differences in APA between toxic and non-toxic strains across treatments support the hypothesis that their nutrient requirements or acquisition ability differs. The higher APA in toxic strains indicates that they have a higher requirement for phosphorus than non-toxic strains. Some non-toxic *M. autumnalis* strains have higher growth rates than toxic strains in culture-based experiments (Heath et al. 2016). Toxic strains of both *Microcystis* spp. and *R.*

raciborskii can also have greater nutrient requirements for optimal growth (Davis et al. 2010, O'Neil et al. 2012, Burford et al. 2018); thus, differences in APA might represent strategies by those strains to utilise phosphorus more efficiently. Increased nutrient inputs can favour the growth of toxic strains of *Microcystis* spp. and in environmental studies, nutrient enrichment results in the increased dominance of toxic *Microcystis* strains (Davis et al. 2009). It has not been possible to test the relative abundance of toxic and non-toxic *M. autumnalis* strains until recently due to a lack of molecular tools (Kelly et al. 2018). Thus, direct competition experiments have not yet been undertaken, and this should be a priority in future studies.

Considerable strain–strain variation in APA is apparent, regardless of whether strains were toxic or non-toxic, or which nutrient treatment they were subject to. This is consistent with other cyanobacteria (e.g. *Raphidiopsis raciborskii*; Wu et al. 2012, Burford et al. 2018) and highlights the difficulty of using small numbers of strains in the laboratory to predict environmental outcomes where large assemblages of different strains coexist. *Microcoleus autumnalis* exhibits strain–strain variation in the amount and composition of anatoxins produced (Wood et al. 2012b), further demonstrating the need to be aware of the limitations of studies using a small number of strains in modelling environmental outcomes.

4.5.2 *Environmental alkaline phosphatase activity*

Environmental samples also contained APA, albeit at lower levels than in the laboratory strains. This difference may be the result of several factors: (1) phosphorus levels within *M. autumnalis*-dominated mats can be 300-fold higher than in the overlying water column (Wood et al. 2015b), thus the cells within the mats are not under phosphorus limiting conditions and do not need to utilise APA; and (2) the cultures utilised in this study, while isolated from environmental *M. autumnalis* mats, had been maintained in culture in MLA media (Bolch and Blackburn 1996) for a number of years and may have become adapted to high-nutrient media. Environmental *M. autumnalis*-dominated mats are a complex microbial community (Brasell et al. 2015), containing many other taxa that may be capable of producing APA. In these samples, it is not possible to measure the APA produced solely by *M. autumnalis*; however, any APA occurring in the mat matrix is likely to increase the availability of DRP for any organism in the matrix.

There were no temporal patterns in APA in environmental *M. autumnalis*-dominated mats. It is possible that APA does have a temporal trend that is controlled not by the amount of alkaline phosphatase enzyme, but by the pH within the mat. *M. autumnalis*-dominated mats can experience large diurnal changes in both pH (from ~7 overnight to > 9 during the day) and dissolved oxygen levels (Wood et al. 2015b), due to photosynthesis. These large diurnal changes may allow *M. autumnalis*-dominated mats to utilise both alkaline and acid phosphatases across day–night cycles, without large changes in the amount of the respective enzyme. Our study measured APA at a single pH, which would mask changes in APA from environmental samples in the case that APA is regulated by pH rather than the enzyme concentration within the mats. It is also possible that temporal trends are apparent, but across mat succession and development, rather than diel cycles. *Microcoleus autumnalis*-dominated mats have a distinct accrual cycle characterised by the attachment of filaments to the substrate, patch expansion and mat development, followed by detachment via sloughing or hydrological disturbance (McAllister et al. 2016). The arrival of nutrient cycling bacteria typically occurs during the patch expansion phase of the accrual cycle (McAllister et al. 2016); thus, the ability to make use of alternate nutrient sources may be more important for *M. autumnalis* in the early stages of mat development. Following the establishment of a mat matrix, the overlying water column is a poor predictor of DRP concentrations within the mat. This is due to geochemical conditions promoting the release of phosphorus from sediments and the isolation of the mat from the overlying water column. Both of these factors promote high within-mat DRP concentrations and may reduce dependence on APA later in mat succession.

4.5.3 *Conclusions*

Microcoleus autumnalis exhibits alkaline phosphatase activity, and this is dependent on the availability of dissolved reactive phosphorus. Although APA levels among strains are quite variable, toxic strains exhibit higher APA than non-toxic strains. Alkaline phosphatase activity was also present in environmental samples, though at lower levels than laboratory strains, and no short-term diurnal patterns were observed. This may be the result of high within-mat phosphorus concentrations due to geochemical release of phosphorus from sediments.

CHAPTER 5

An ecotoxicological assessment of anatoxin congeners on the New Zealand mayfly (*Deleatidium* spp.*)*

5.1 Abstract

Toxic benthic cyanobacterial proliferations may pose a significant risk in freshwater ecosystems. In New Zealand, the only anatoxin-producing benthic species reported to date is *Microcoleus autumnalis*. Management of *M. autumnalis* proliferations has primarily focused on mitigating the health risks associated with human and canine contact with the cyanobacteria. A major concern at the ecological level is the potential for lethal and sub-lethal effects on macroinvertebrates that may feed on toxic mats or use them as a refuge. In the present study, *Deleatidium* spp. larvae were exposed to different variants of purified anatoxins (anatoxin-a, dihydroanatoxin-a and a mixture of homoanatoxin-a/dihydrohomoanatoxin-a). There was no significant mortality of *Deleatidium* spp. larvae, even at doses far exceeding likely environmental exposures (300 μg L^{-1} to 600 μg L^{-1}). *Deleatidium* spp. larvae exposed to high doses of dihydroanatoxin-a had measurable concentrations of the toxin in their tissues 24 hours post-exposure. The results of this study indicate that anatoxins from benthic *M. autumnalis* proliferations are unlikely to result in mortality of *Deleatidium* spp. larvae in the environment, but uptake of anatoxins in their tissues may represent a pathway for anatoxin transfer up trophic levels.

5.2 Introduction

Proliferations of the toxic, benthic cyanobacterial genus *Microcoleus* spp. are increasing in frequency, duration and severity (Quiblier et al. 2013, McAllister et al. 2016). When *Microcoleus* spp. proliferate, they typically dominate the periphyton community (Brasell et al. 2015, McAllister et al. 2016), and can produce potent neurotoxins collectively known as anatoxins. The four anatoxin variants (anatoxin-a [ATX], dihydroanatoxin-a [dhATX], homoanatoxin-a [HTX] and dihydrohomoanatoxin-a [dhHTX]) are secondary amine alkaloids that act as post-synaptic acetylcholine receptor agonists (Devlin et al. 1977, Wiegand and Pflugmacher 2005, Dittmann et al. 2013). In vertebrates, anatoxins bind irreversibly to the nicotinic acetylcholine receptor, leading to overstimulation of muscle cells due to membrane depolarisation, which eventually results in respiratory arrest and death (Wiegand and Pflugmacher 2005, Dittmann et al. 2013). Although the neurotoxic properties of anatoxins have been known since the 1970s (Devlin et al. 1977, Petersen et al. 1984), the effects of anatoxins on aquatic flora and fauna are poorly understood.

Anatoxins inhibit photosynthesis and reduce pigment production in some macrophytes (e.g. Lemna minor and Cladophora fracta; Mitrovic et al. 2004, Kaminski et al. 2016) , though these effects occur at anatoxin concentrations higher than those typically measured in environmental samples. In some chlorophytes (e.g., *Selenastrum capricornutum*), anatoxin exposure in concert with microcystins has been shown to promote growth (Chia et al. 2019). Some freshwater fish (e.g., *Oncorhynchus mykiss* and *Cyprinus carpio*) accumulate anatoxins following exposure to anatoxin-contaminated water (Osswald et al. 2007a, Osswald et al. 2011). There is a scarcity of information on the effects of anatoxins on invertebrates. Osswald et al. (2008) showed that marine mussels can accumulate anatoxins and Macallan et al. (1988) showed anatoxin-a can bind to receptors from locust tissue. Invertebrates have nicotinic acetylcholine receptors (nAChRs) in their nervous systems (Macallan et al. 1988), and since anatoxins interact with these (Macallan et al. 1988), acute toxicity effects may be evident through inhibition of the major excitatory system. Crude cyanobacterial extracts containing anatoxins have been utilised in invertebrate toxicity assays, resulting in considerable mortality (Anderson et al. 2018). A single study examining the acute toxicity of purified anatoxin-a identified much lower toxicity than crude cyanobacterial extracts in *Chironomus* spp. larvae (Toporowska et al. 2014). To our knowledge, this is the only study to examine the acute effects

of purified anatoxins on benthic macroinvertebrates. This critical gap is a concern as invertebrates comprise an important component of aquatic ecosystems.

Mayfly larvae of the genus *Deleatidium* are ubiquitous in stony streams and rivers in New Zealand (Jowett and Richardson 1990, Scrimgeour 1991) and can be found in areas where *Microcoleus* spp. dominate periphyton. As *Deleatidium* spp. graze on the epilithic layer (Scrimgeour 1991), they are likely to be exposed to anatoxins from *Microcoleus* spp. *Deleatidium* spp. are also prey for native and introduced fish, invertebrate and bird species (Scrimgeour 1991) and are an important indicator of river health (Stark 1993). Ecotoxicity assays provide the most convenient and effective means to test the sensitivity of organisms like *Deleatidium* spp. to toxins at environmentally relevant concentrations.

The present study aimed to investigate the acute toxicity of ATX, dhATX and HTX/dhHTX on *Deleatidium* spp. larvae at environmentally relevant concentrations over a six-day exposure period.

5.3 Methods

5.3.1 *Study organism*

Native New Zealand mayfly larvae of *Deleatidium* spp. were collected from the Brook Stream in Nelson (41.31 °S, 173.29 °E) approximately 24 h prior to the commencement of the experiment. Kick nets were placed in the river and the substrate upstream agitated. The contents of the kick nets were transferred to 10 L buckets filled with river water, then transferred to the laboratory within 30 min. On arrival in the laboratory, mid-instar larvae of the mayfly *Deleatidium* spp. (ca. 4 mm – 6 mm in length, excluding cerci) were transferred to a clean bucket containing river water that had been filtered through a 20 μm mesh. Larvae with darkened wing-pads were discarded as they could emerge over the course of the experiment. The *Deleatidium* spp. larvae were then maintained in the bucket at 15 °C and continuously aerated until the commencement of the experiment (within 24 h).

5.3.2 *Preparation of test solutions*

Pure ATX fumarate was commercially available and was purchased from Abcam (Cambridge, UK) and re-suspended in 0.1% acetic acid to a nominal concentration of ca. 20 mg L^{-1}. The concentration of ATX in this solution was then determined by liquid chromatography tandem-mass spectroscopy (LC-MS/MS), as described below.

As dhATX and HTX/dhHTX are not available commercially, these compounds were purified from environmental material prior to the commencement of the experiment. The dhATX material and a mixture of 25% HTX / 67% dhHTX / 8% dhATX were purified from combined *M. autumnalis* bloom material collected from the Wai-iti River (Tasman, New Zealand; 41.36 °S, 173.09 °E), Hutt River (Wellington, New Zealand; 41.13 °S, 175.00 °E) and Cardrona River (Otago, New Zealand; 44.69 °S, 169.17 °E). Freeze-dried *M. autumnalis*-dominated mat material (Gamma 1-16 LSC freeze-drier; Martin Christ Gefriertrocknungsanlagen, Germany) was extracted in 0.1% acetic acid (v/v) by homogenising using a kitchen blender (high speed for 30 sec) and an Ultra-turrax disperser (17,500 rpm for 2 min; IKA, Rawang, Malaysia). The extract was left for 2 h to allow the dense foam that formed to settle. The foam was removed using a plastic spatula, and the liquid was strained overnight through butter muslin cloth. To precipitate proteins, the filtered extract was supplemented with chloroform ($CHCl_3$) at a 3:1 ratio (extract / chloroform), shaken and left for 15 min to form a phase partition. The upper aqueous layer was decanted and retained. The lower $CHCl_3$ layer was washed with 0.1% acetic acid (v/v, using 50% volume of the original extract). After shaking and allowing to settle to form a phase partition, the two aqueous extracts were combined and vacuum-filtered (GF/F; Whatman, Maidstone, United Kingdom). The clarified extract was aliquoted into plastic bottles (ca. 500 mL each) and lyophilised (Gamma 1-16 LSC freeze-drier; Martin Christ Gefriertrocknungsanlagen, Germany).

The concentrated extract was re-suspended in 100 mL of 0.1% formic acid, adjusted to pH ≤ 3.5 using concentrated formic acid, and vacuum filtered (GF/F; Whatman). The filtered solution was extracted three times with dichloromethane (DCM) in a separating funnel to remove neutral and acidic organics. Severe emulsions generally formed and needed to be broken by adding ethanol. The aqueous solution was retained, NaCl was added (1% w/v, final concentration) and the solution was adjusted to pH 10.5 using a saturated Na_2CO_3 solution. The alkaline solution was vacuum filtered (GF/F; Whatman), transferred back into the

separating funnel and extracted three times with DCM to extract the anatoxins. Severe emulsions generally formed and were broken by adding ethanol. The three DCM-extracts were dried over coarse anhydrous Na_2SO_4 and combined, then the DCM was evaporated using a rotary evaporator. Because the use of ethanol to break emulsions limited the separation efficiency of the liquid–liquid partitioning, the resulting anatoxin samples were put through the liquid–liquid partitioning procedure above a second time. On this occasion, emulsions did not form and the addition of ethanol was not required, resulting in a cleaner separation.

The liquid–liquid partitioning samples were re-suspended in 0.1% acetic acid and fractionated on a C18 Sep-Pak column (25 mm × 180 mm; Superflash C18; Agilent Technologies). The sample was loaded onto the column in 0.1% acetic acid (v/v) and separated isocratically at a flow rate of 20 mL min^{-1} for 30 min before progressing to a mobile phase of 20% methanol + 0.1% acetic acid (v/v) in a linear gradient over 30 min. Fractions were collected in clean 15 mL plastic tubes and assessed by LC MS/MS to determine where the various anatoxin congeners eluted (dhATX at 4.5 min, dhHTX at 8 min –12 min and HTX at 9.5 min – 22.5 min). The fractions containing dhATX and dhHTX/HTX were pooled, supplemented to 0.1% acetic acid (v/v; final concentration) and progressively freeze-dried into smaller vessels.

The dhATX co-eluted with phenylethylamine and tyramine. These impurities were removed through more refined fractionation using the C18 chromatography system described above. This resulted in a yellow oil that was 99.2% dhATX and 0.8% tyramine when analysed by proton nuclear magnetic resonance spectroscopy (data not shown).

The anatoxin concentrations of the purified dhATX and the semi-pure mixture of dhHTX/HTX were determined by LC-MS/MS as described below. Aliquots of each solution were diluted in 0.1% acetic acid to yield nominal concentrations of 10 mg L^{-1} for dhATX and 10 mg L^{-1} total anatoxins for HTX/dhHTX (ca. 67% HTX, 24% dhHTX, with the remainder as dhATX). The HTX and dhHTX could not be separated using the present chromatography system and preparative high-performance liquid chromatography is likely to be required, which was beyond the scope of this study.

5.3.3 *LC-MS/MS analysis of anatoxins*

The concentration of anatoxins in *M. autumnalis* bloom samples, algal cultures, extracts and purification fractions were determined by LC-MS/MS. Analyses were performed on a Waters Xevo TQ-S mass spectrometer coupled to a Waters Acquity I-Class liquid chromatography system. Sample components (5 µL) were separated using a Thermo Hypersil Gold-aq column (1.9 µm; 50 mm × 2.1 mm) at 40 °C with a gradient of water plus 0.1% formic acid to acetonitrile plus 0.1% formic acid at a flow of 0.6 mL min^{-1}, as described in Wood et al. (2017b). Anatoxins were quantified in positive ion mode using multiple reaction monitoring channels for ATX, HTX, dhATX and dhHTX. A mixed five-point calibration curve (0.5 ng mL^{-1} –20 ng mL^{-1} in 0.1% formic acid) was prepared using a certified reference material for ATX (National Research Council, Canada) and a standard of dhATX calibrated by quantitative nuclear magnetic resonance (NMR) spectroscopy. The concentrations of ATX and HTX were determined using the ATX calibration curve and the concentrations of dhATX and dhHTX were determined using the dhATX calibration curve. When anatoxin concentrations were outside of the calibration range, samples were diluted with 0.1% formic acid and re-analysed. Anatoxin concentrations of experimental solutions from the ecotoxicology assay were measured at the commencement of the experiment (Table 5.1) and the most concentrated solutions were re-tested at the endpoint using a pooled sample from three separate replicates.

The experimental solutions comprised a control, vehicle control and five concentrations of each of the three congeners tested. Controls were 20 µm filtered river water from the collection source of the *Deleatidium* spp. larvae. The vehicle control consisted of 200 mL of 0.1% acetic acid neutralised to pH 6.5 by the addition of 0.1 M NaOH and 800 mL of 20 µm filtered river water. Test solutions comprised 200 mL of 5 × stock solution neutralised to pH 6.5 by the addition of 0.1 M NaOH and 800 mL of 20 µm filtered river water (Table 5.1). To take into account the (50:50) racemic mixture of ATX (nuclear magnetic resonance; Abcam, Cambridge, UK), measured concentrations of ATX were considered to be twice the effective dose (only the + isomer is considered to exhibit significant biological activity; Spivak et al. 1983, Wonnacott and Gallagher 2006).

5.3.4 *Experimental procedure*

The experiment consisted of two separate dose-response tests. In the first, *Deleatidium* spp. larvae were placed individually into 50 mL polypropylene jars containing 40 mL of the respective test solution. Lids were not put on the jars to enable sufficient oxygen diffusion throughout the experiment. The test jars were maintained in an environment-controlled room at 17 °C, on a 12:12 light-dark cycle at (22.5 ± 7.5) μmol m^{-2} s^{-1}, with jars distributed randomly.

Dissolved oxygen and conductivity were measured with a HQ-40d handheld multi-parameter meter (Hach, CO, USA). Measurements were taken daily in three random test jars per treatment-dose to minimise stress induced by making these measurements on the organisms.

There were 17 treatment-dose combinations comprised of a river water control, vehicle control and five concentrations of ATX, dhATX or HTX/dhHTX in solution (Table 5.1) and 20 replicates per combination. Each *Deleatidium* spp. larva was checked daily for survival by agitation of the test solution. A positive response was recorded if independent movement was observed. If no movement was observed, the replicate was left for ten minutes and re-checked. If, after the second check, no movement was observed, the *Deleatidium* spp. larva was recorded as dead. The end of the experiment was determined as the time point at which survival in the control or vehicle control fell below 80%.

Following the initial exposure experiment, an additional two exposure doses of dhATX (300 μg L^{-1} and 600 μg L^{-1} dhATX) were tested, as no significant responses were observed at the lower doses. Fresh *Deleatidium* spp. mayfly larvae were collected and maintained as described above for approximately 24 h prior to the commencement of the experiment. Test solutions were prepared using the same protocol as those in the initial experiment, with 200 mL of a 5 × stock solution in neutralised acetic acid, and 800 mL filtered river water (Table 5.1). Survival was monitored in the same way as the initial experiment with a river water control and vehicle control prepared as above.

At the end of the experiment, surviving larvae from each of the two controls and two dose-treatments (n = 15 for each dose-treatment) of the follow-up exposure experiment were combined within their treatment and transferred to fresh jars containing 50 mL of filtered

(20 µm) river water. Larvae were maintained for 24 h to purge any test solution from their gut contents. After the 24-h purging period, the water was tipped off and larvae frozen (–20 °C) and lyophilised (Gamma 1-16 LSC freeze-drier; Martin Christ Gefriertrocknungsanlagen, Germany). The lyophilised larvae were weighed into 1.8 mL eppendorf tubes, homogenised with a plastic tissue grinder and extracted in 1 mL of 0.1% formic acid (v/v). Samples were subjected to three freeze-thaw cycles with sonication between each and clarified by centrifugation (14,000 × *g*; 3 min), and the anatoxins were quantified using LC-MS/MS, as described above.

5.3.5 *Statistical analysis*

Survival was compared among treatment-dose combinations using Kaplan-Meier survival estimates. A log-rank test for the equality of survival distributions with Benjamini-Hochberg correction for multiple comparisons was then undertaken to compare the control, vehicle control, ATX, dhATX and HTX/dhHTX treatments from the first experiment. A second log-rank test was undertaken to compare the survival distributions of control, vehicle control, 300 µg L^{-1} and 600 µg L^{-1} dhATX treatments from the second experiment. All analyses were conducted in the R statistical package (http://www.r-project.org/), using the 'survival' (Therneau and Grambsch 2013) and 'survMisc' packages.

5.4 Results

Dissolved oxygen remained > 90% saturation over the duration of the experiment. Conductivity in the river water control was between 90 µS cm^{-1} and 100 µS cm^{-1} for both experiments. Conductivity ranged between 300 µS cm^{-1} and 350 µS cm^{-1} in the vehicle control and all treatments in the first experiment, and between 350 µS cm^{-1} and 380 µS cm^{-1} in the second. Both dissolved oxygen and conductivity measures remained within the tolerable range for *Deleatidium* spp. larvae (Hickey and Vickers 1992). Concentrations of all anatoxin congeners were stable for the duration of the experiment, with no decrease in concentration measured (data not shown). Mortality was between 15% and 55% at the conclusion of the experiment, with mortality of 5% and 30% in the river water control and vehicle control, respectively. There was no statistically significant difference between the controls and treatments ($p > 0.05$); therefore, it was not appropriate to undertake a dose-response analysis.

Table 5.1. Treatment solution nominal and measured anatoxin concentrations. Solutions consisted of filtered (20 μm) river water (800 mL) and 200 mL of a 5 × stock solution of the respective treatment/concentration. Reported measured concentrations were taken at the start of the experiment. Note that the anatoxin-a was a fumarate salt, which is why the nominal concentration is greater than the measured concentration. Additionally, the anatoxin-a was also a (50:50) racemic mixture of +/- isomers, of which only the + isomer exhibits significant biological activity; thus, the effective concentration is considered to be half the measured concentration.

Treatment	Nominal concentration of anatoxins (μg L^{-1})	Measured concentration of anatoxins (μg L^{-1})
River water control	0	0
Vehicle control	0	0
Anatoxin-a	400	255.3
	200	135.4
	100	79.4
	50	39.3
	1	0.7
dihydroanatoxin-a	600*	670.1
	300*	338.1
	150	164.1
	75	86.6
	37.5	40.1
	18.75	20.1
	0.375	0.4
homoanatoxin-a/ dihydrohomoanatoxin-a	150	240.8
	75	119.4
	37.5	58.2
	18.75	30.6
	0.375	0.6

* denotes treatments in a follow-up experiment using higher doses of dhATX than the initial experiment

No differences in survival were detected across doses for ATX, dhATX or HTX/dhHTX (Figure 5.1a-c and Table A4.1, Appendix 4). The 300 µg L^{-1} and 600 µg L^{-1} doses of dhATX did not result in mortality greater than the controls (Figure 5.1d; log-rank test, $p > 0.05$).

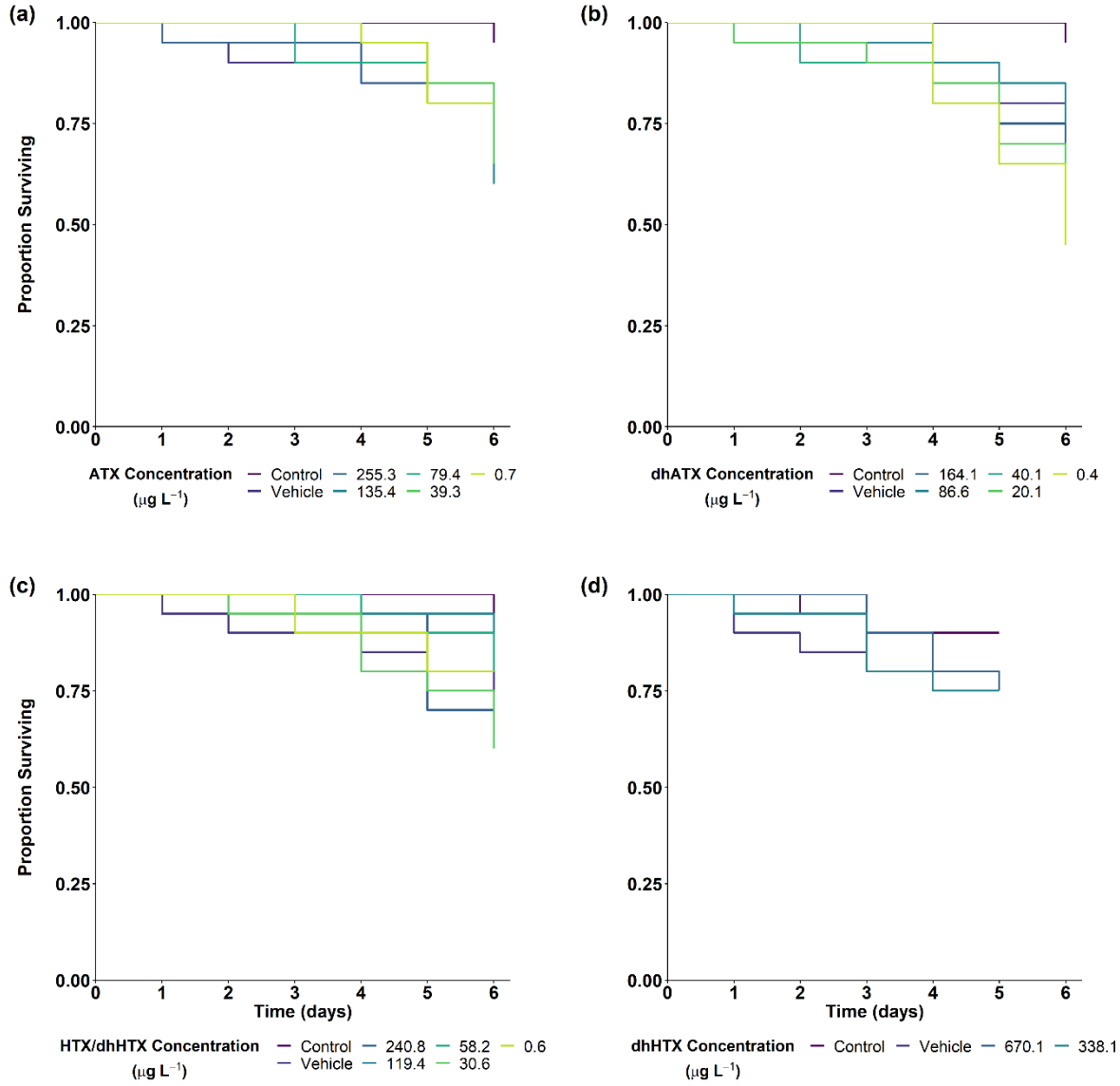

Figure 5.1. Kaplan-Meier plots of *Deleatidium* spp. larvae survival following exposure to anatoxins. (a) *Deleatidium* spp. exposed to five concentrations of ATX; (b) dhATX; (c) HTX/dhHTX mixture and (d) high doses of dhHTX in a second experiment. Note (a-c) share the same control and vehicle control measurement as these were from the same experiment. No significant differences were apparent between treatments or doses (log-rank test, $p > 0.05$).

When *Deleatidium* spp. larvae from the follow-up experiment were assessed for anatoxin content in their tissues, no dhATX was detected in larvae from the river water control or vehicle control. The larvae from the dhATX treatments both contained dhATX, with a concentration of 0.63 mg kg^{-1} dry weight (dw) and 1.16 mg kg^{-1} dw in the 300 μg L^{-1} and 600 μg L^{-1} treatments, respectively. During this assessment, there was interference present in the LC-MS/MS multiple-reaction monitoring channel used to measure ATX. While this did not influence the measurement of dhATX in this study, it indicates that further optimisation of the sample extraction protocol is required when measuring anatoxins from complex matrices such as macroinvertebrates.

5.5 Discussion

The lack of toxicity on *Deleatidium* spp. mayfly larvae for any of the tested anatoxin variants was unexpected. During the present study, at their maximum concentrations, the various anatoxin congeners were administered well in excess of that observed in the natural environment (6–30 times higher than the highest recorded concentrations in river water grab samples). To our knowledge, the highest recorded anatoxin concentration in a river water grab sample was 21.7 μg L^{-1} (Wood et al. 2011). As *Deleatidium* spp. are grazers, bulk water samples may not represent the most likely exposure route for anatoxins. While Wood et al. (2014a) measured the concentration of phosphorus and metals from water within the mat matrix, the authors are unaware of any studies that have measured the anatoxin concentrations from water within the matrix. Owing to the benthic growth of *Microcoleus*, it is not possible to relate concentrations of anatoxins measured from biomass (typically recorded as mg kg^{-1} dry weight) to a water volume. This makes comparisons between water concentrations and potential within-mat exposure difficult. The median concentration of anatoxin in *Microcoleus* biomass in New Zealand is less than 1 mg kg^{-1} (McAllister et al. 2016). Assuming a biomass-area relationship of 1 mg dry weight per cm^2, if the entire bottom of the test jar in our study (13.85 cm^2) was covered in that biomass, the concentration of anatoxin in the 40 mL test solution would be approximately 0.3 μg L^{-1}. This is well below the highest concentration tested in this study. At the highest measured concentration of anatoxin in *Microcoleus* mats (2,116 mg kg-1; Wood and Puddick 2017), applying the same calculation gives an estimated concentration of 732 μg L^{-1}, which is similar to the highest concentration tested in this study.

Previous work utilising crude cyanobacterial extracts containing anatoxins identified toxicity of these extracts to *Deleatidium* spp. mayfly larvae (Bridge 2013). In addition, crude extracts of *Microcoleus* sp. (described as *Phormidium* sp.) containing anatoxins were toxic to other invertebrate taxa commonly used in ecotoxicological studies (Anderson et al. 2018). Work to examine the acute effects of both toxic and non-toxic crude extracts from cultured *M. autumnalis* were undertaken in the early stages of the present study (Appendix 5) and indicated that crude extracts of strains unable to produce anatoxins were as toxic as those with anatoxins. On scaling this work up to a full assay, depleted dissolved oxygen in the test solutions, thought to be due to bacterial growth, resulted in significant mortality. Crude cyanobacterial extracts contain a complex mixture of metabolites produced by the cyanobacteria, and it is possible that observed mortalities in these other studies were in response to metabolites other than anatoxins. Unidentified secondary metabolites in crude extracts of *Raphidiopsis raciborskii* were responsible for significant lethal and developmental effects, even with strains incapable of cylindrospermopsin production (Berry et al. 2009). Environmental benthic cyanobacterial mats can also accumulate metals (Wood et al. 2014a) that are highly toxic to aquatic invertebrates, along with cyanobacterial metabolites. Identifying the toxicity of these crude extracts is a helpful first step in toxicity assessments; however, these should be followed with assessments of the purified toxin suspected to be the cause of mortality. This study has highlighted that anatoxins may not be driving mortality in these previous toxicology studies and indicates that further work on identifying the toxic compound/s in these extracts is needed.

While no lethal effects of anatoxins on *Deleatidium* spp. larvae were observed during this study, sub-lethal effects were not tested. Neonicotinoid insecticides, which have a similar (though not identical) mode of action to anatoxins in aquatic invertebrates, can reduce the ability of aquatic invertebrates to complete their lifecycle, as they can disrupt energy acquisition required for emergence (Palmquist et al. 2008). Anatoxins, as neurotoxins, may have similar effects on *Deleatidium* spp. larvae, reducing their ability to complete their lifecycle. Moreover, susceptibility to anatoxins may differ between stages of the lifecycle, as occurs with exposure to organopesticides (Stuijfzand et al. 2000). Temperature is also known to modulate the toxicity of the neonicotinoid imidacloprid in mayfly larvae, with increased temperatures resulting in greater metabolic rates and faster uptake of the toxin (Camp and Buchwalter 2016). *Deleatidium* spp. larvae in this study were exposed to anatoxins under a constant temperature of 17 °C, which does differ from natural diurnal fluctuations in the

environment. This genus tolerates constant temperatures of 20 °C, or a diurnal regime of 20 ± 5 °C without significant mortality, however, above these temperatures considerable mortality is typically observed within 96 h (Cox and Rutherford 2000). It is possible that synergistic effects also occur in *Deleatidium* spp. larvae in response to elevated temperatures and this should be investigated further. Future work should also aim to investigate sub-lethal effects of anatoxins on a range of life-stages of *Deleatidium* spp. larvae, including behavioural responses to anatoxin exposure and determining impacts on successful emergence.

In this study, larvae were identified to genus, but not species level, owing to the difficulty of distinguishing these species at the larval stage. Morphological taxonomic identification of late-instar *Deleatidium* spp. larvae from the collection site on the Brook Stream identified a single species: *Deleatidium myzobranchia*. There may be species-specific effects of anatoxins in *Deleatidium*, although this seems unlikely given that no acute toxicity was observed during this study. Nevertheless, the potential for species-specific effects should be considered in subsequent investigations, particularly of sublethal effects where different sensitivities may be more apparent.

Anatoxins bind irreversibly to the nicotinic acetylcholine receptor (nAChR), resulting in cell membrane depolarisation, and in vertebrates this typically leads to respiratory arrest and death. Invertebrates also possess nAChRs in their central nervous system and anatoxins may bind to these (Macallan et al. 1988). The invertebrate nAChR is a pentomeric cross-membrane protein, which acts as a ligand-gated ion channel and typically consists of 2 α-subunits, and a combination of β, γ, δ and ε subunits in different arrangements (Corringer et al. 2000). As a result, there are many possible configurations of the nAChR. Additionally, there are multiple variations of each of the subunits, resulting in different receptor subtypes. The arrangement of subunits and the composition of the nAChR subtypes influence the binding region of the nAChR, resulting in differential receptor binding affinities for sites on each nAChR type (Corringer et al. 2000). In mammals, different nAChR subtypes are found in various systems; for example, muscle-type nAChRs are comprised of two α-subunits, β, δ and either γ or ε, whereas neuronal-type nAChRs comprise only α and β subunits (Arias 1997). The lack of toxicity of anatoxins to *Deleatidium* spp. in this study may indicate the presence of different nAChRs, to which anatoxins exhibit differential receptor binding affinities. The function, structure and diversity of invertebrate nAChRs are very poorly understood, despite increased interest due to the use of neonicotinoid pesticides, which interact with nAChRs (Tomizawa and

Casida 2001). Ideally, receptors from the target organisms would be isolated and the kinetics of binding examined to provide more information on the binding affinity of anatoxin. Such characterisation, if combined with receptor-binding assays, would provide valuable information on the expected sensitivity of different organisms to anatoxins, enabling more accurate risk assessment and selection of appropriate organisms for toxicology to be made.

Aquatic organisms appear less sensitive to cyanotoxins than terrestrial organisms (Quiblier et al. 2013), which may be the result of frequent cyanotoxin exposure in aquatic organisms. Where *M. autumnalis*-dominated mats proliferate, *Deleatidium* spp. larvae can be found in and around the mats (Wood et al. 2014b), which would be unlikely if they were sensitive to anatoxins. Bivalves can accumulate cyanotoxins despite no evidence of lethal or sublethal effects (Saker et al. 2004, Ibelings and Chorus 2007). The presence of anatoxins in *Deleatidium* spp. larvae tissues without significant mortality in this study is consistent with those studies in bivalves. This study utilised whole *Deleatidium* spp. larvae for tissue toxin testing and tested dissolved toxins as a single exposure route, as a preliminary step to understanding the ecological impacts of anatoxins in benthic and aquatic ecosystems. Future work should include identifying whether anatoxins are localised to particular tissues in macroinvertebrates and examining the potential for bioaccumulation into higher trophic levels. Because the HTX/dhHTX could not be separated during toxin purification, some uncertainty about the toxicity of purified HTX and dhHTX remains. To our knowledge, this is the first detection of anatoxins in the tissues of *Deleatidium* spp. larvae, and further research is required to understand the potential for anatoxin transfer and bioaccumulation within aquatic food webs.

CHAPTER 6

General Discussion

6.1 Summary

Major gaps remain in our understanding of the drivers and effects of anatoxin production by benthic cyanobacteria in aquatic ecosystems. Increasing knowledge in this area is crucial to assist with risk assessments and management of benthic cyanobacterial proliferations. This thesis aimed to explore anatoxin variability and production in *Microcoleus autumnalis*-dominated mats and strains, and to examine the toxicological effects of anatoxins on the aquatic macroinvertebrate genus *Deleatidium*. The development and optimisation of a quantitative PCR (qPCR) assay for the determination of toxic and non-toxic *M. autumnalis*-strain abundance was integral to this work (Chapter 2).

Molecular techniques were used to investigate the abundance of toxic and non-toxic *M. autumnalis* and characterise benthic cyanotoxin producers (Chapters 2 and 3). Enzyme activity assays were used to identify potential drivers of changes in the relative abundance of toxic and non-toxic *M. autumnalis* (Chapter 4). Several important discoveries about anatoxin concentrations, and the abundance and physiology of toxic and non-toxic *M. autumnalis* were made in this work. Significantly, anatoxin concentrations in *M. autumnalis* mats were correlated with the abundance of toxic *M. autumnalis* strains (Chapter 2), and toxic and non-toxic strains differ in the production of alkaline phosphatase activity, which enables utilisation of organic phosphorus sources (Chapter 4). These findings corroborate research conducted on planktonic cyanobacteria, and extend it to benthic cyanobacteria, demonstrating that the abundance of toxic cyanobacteria is correlated with cyanotoxin concentrations (Davis et al. 2009), and that toxic and non-toxic strains respond differently to changes in environmental conditions, including nutrient availability (Vezie et al. 2002, Briand et al. 2008, Davis et al. 2009, Davis et al. 2010, Joung et al. 2011, Burford et al. 2014, Gobler et al. 2016).

A significant new discovery from this work was the identification of considerable cyanotoxin concentrations produced by non-dominant taxa in periphyton samples (Chapter 3). Molecular analyses of samples collected from California streams revealed that genes indicative of cyanotoxin production were most closely related to *Nodularia spumigena* and *Microcoleus*/*Oscillatoria* in samples dominated by *Cladophora glomerata* or *Anabaena* sp. *Cladophora glomerata* has previously been described as an ecosystem engineer due to the complex epiphytic microbial assemblages associated with them (Zulkifly et al. 2012). The most common epiphytic cyanobacteria on *C. glomerata* is *Chamaesiphon incrustans*; however, Oscillatoriales make up only 4% of the biomass in *C. glomerata* in the Eel River, CA (Furey et al. 2012). There is evidence that *C. glomerata* can take up anatoxins and microcystins (Pflugmacher et al. 2016). It is, therefore, unclear whether the nodularin and anatoxin detected in *C. glomerata* in this work is the result of production by epiphytic or co-occurring cyanobacteria, or accumulation of the toxins by *C. glomerata* from the water column.

An ecotoxicological assay examined anatoxin toxicity in the larvae of a New Zealand native mayfly (*Deleatidium* spp.). The finding that purified anatoxins do not result in mortality in *Deleatidium* spp. larvae, but that the toxins can be detected in the tissues of larvae exposed to them (Chapter 5), is a novel result. While crude cyanobacterial extracts containing anatoxins result in considerable mortality of macroinvertebrates (Anderson et al. 2018), it appears from this work that anatoxins were not likely to be the causative agent of mortality.

The findings from this thesis raise several important questions regarding the ecology of *Microcoleus*-dominated mats and their effects on aquatic organisms: (1) what factor/s influence the abundance of toxic/non-toxic genotypes in *Microcoleus*-dominated mats? (2) are benthic cyanotoxins underestimated? and (3) how does anatoxin production in these mats impact the benthic macroinvertebrate and wider stream community? These questions will be considered further here.

6.2 Ecology of *Microcoleus*-dominated mats

6.2.1 *How do biotic and abiotic factor/s influence the composition of toxic and non-toxic genotypes?*

The co-occurrence of toxic and non-toxic cyanobacterial strains is an important driver of overall toxin concentration and composition in cyanobacterial blooms and proliferations (Zurawell et al. 2005, Gobler et al. 2016). Toxic and non-toxic cyanobacterial strains can respond differently to various environmental conditions, which may result in changes in their relative abundance in the environment. Abiotic factors can differentially affect the growth and toxin production of cyanobacteria. In chemostat experiments with *Dolichospermum* (*Anabaena*), toxic strains grew faster than non-toxic strains at temperatures above 20 °C and with higher phosphate concentrations (Rapala et al. 1993). Similarly, toxic *Microcystis* strains have higher growth rates than non-toxic strains under increased nitrate conditions, which increases the relative abundance of toxic strains in the community when nitrate levels are high (Vezie et al. 2002, Davis et al. 2010, Gobler et al. 2016). High light levels favour toxic *Microcystis* strains over non-toxic strains; however, non-toxic strains exhibit higher growth rates under low light conditions (Deblois and Juneau 2012). Over the course of a *Microcystis* bloom, these differential responses to physicochemical factors may be responsible for observed changes in both toxin concentration and the composition of toxic and non-toxic strains, with high proportions of toxic strains (and toxin concentrations) early in the bloom when nutrient and light levels are high, followed by greater proportions of non-toxic strains as nutrients are depleted and self-shading begins to occur later in the bloom (Gobler et al. 2016). Biotic factors may also play a role in the composition of toxic and non-toxic cyanobacteria. Cyanophage activity has been correlated with a decline in toxic strains in *Microcystis* blooms (Yoshida et al. 2008), though causality was not determined. Some grazers and filter feeders also preferentially feed on non-toxic strains of *Nodularia* (e.g. Engström et al. 2001) and *Microcystis* (e.g. Liu et al. 2009). Preferential feeding may alter the composition of the cyanobacterial community by favouring the removal of non-toxic cyanobacteria, resulting in the relative abundance of toxic strains increasing.

The higher alkaline phosphatase activity (APA) observed in toxic strains of *Microcoleus. autumnalis* (Chapter 4) may indicate a higher phosphorus requirement in toxic strains over non-toxic strains. This is consistent with the observations of Heath et al. (2016), where different

nutrient regimes resulted in different growth rates for toxic and non-toxic *M. autumnalis* strains. Findings from this thesis are consistent with those from planktonic cyanobacteria (e.g. Microcystis; Gobler et al. 2016) and builds evidence that there is an energetic cost to toxin production. Thus, toxic strains exhibit greater growth rates under higher nutrient conditions relative to non-toxic strains (Heath et al. 2016). Microcystin, for example, is a large, N-rich compound; thus, the balance between the costs and benefits of production favour toxin production or the dominance of toxic strains when nitrate levels are high. In contrast, anatoxins are small molecules that are not particularly nitrogen-rich and may not be expected to have significant energetic or metabolic costs. When considering the biosynthetic pathway for anatoxin production, however, it becomes apparent that the enzymes and energy required for anatoxin biosynthesis are greater than would be expected just considering the resulting molecule (Méjean et al. 2014). This may explain why non-toxic strains seem to have lower nutrient requirements than toxic strains.

Considerable difficulties arise when trying to apply knowledge from laboratory studies to environmental settings. Multiple physiologically-distinct strains exist within each broader toxic or non-toxic classification (Wood et al. 2012b), resulting in wide variations in responses to physicochemical factors among strains. Marinho et al. (2013) highlighted that in competition experiments between *Microcystis* and *Raphidiopsis* (formerly *Cylindrospermopsis*), the outcome was dependent on the strain, rather than either genus inherently outcompeting the other. Benthic cyanobacteria pose additional challenges for understanding the composition of the cyanobacterial community due to the complexity of their population structure. In contrast to planktonic populations, which are more likely to undergo some mixing due to wind and currents, benthic cyanobacterial proliferations may be influenced by microhabitats generated by complex fluid dynamics at the benthic interface, combined with the lower mobility of benthic periphyton attached to the substrate. *Microcoleus*-dominated mats exhibit large changes in the proportion of toxic and non-toxic strains in very small spatial scales, as highlighted in Chapter 2.

The application of the qPCR from Chapter 2 to environmental systems in California (in Chapter 3) demonstrates the wider utility of the qPCR assay to determine anatoxin quotas in *Microcoleus* outside New Zealand. The anatoxin congeners identified in Chapter 3 were largely ATX and dhATX, which contrasts with New Zealand, where HTX, dhHTX and dhATX are the most abundant congeners (McAllister et al. 2016, Wood and Puddick 2017), but is

consistent with France where ATX and dhATX are detected (Mann et al. 2012). This contrast may reflect genetic or transcriptional differences in anatoxin production between New Zealand, French and Californian *Microcoleus*-dominated proliferations. Bouma-Gregson et al. (2019) constructed the anatoxin biosynthesis cluster from metagenomes of Californian *Microcoleus*-dominated mats, which highlighted missing regions (*anaK* and parts of *anaG*) that could be associated with the production of different congeners. In my work, there was some HTX and dhHTX at a single site; however, the lack of these congeners at other sites may indicate that *anaK* and *anaG* are required for the synthesis of the HTX and dhHTX congeners.

Thus far, investigation of factors influencing toxic and non-toxic strain abundance in *Microcoleus*-dominated mats has been limited by the lack of tools to enable abundance to be determined. The qPRC assay developed in Chapter 2 has helped resolve this limitation; however, a significant methodological hurdle remains in the ability to collect information on physicochemical parameters at relevant scales. In particular, within-mat physicochemical conditions can be drastically different to those in the overlying water column; e.g., phosphate concentrations can be over 320-fold greater inside mats compared to the overlying river water (Wood et al. 2015b). Sampling should, therefore, be conducted at fine spatial scales in an effort to measure the conditions directly experienced by the cyanobacteria within the mats. Overcoming this difficulty and extending sampling to a range of rivers with varying environmental parameters would provide valuable insight into understanding how physicochemical parameters influence the abundance of toxic and non-toxic strains within *Microcoleus*-dominated mats.

6.2.2 *Are cyanotoxins in periphyton being underestimated?*

Reports of toxic, benthic cyanobacteria have been increasing in frequency, from early reports in lakes in Scotland and Switzerland in the 1990s (e.g. Edwards et al. 1992, Mez et al. 1997), to reports from New Zealand and French rivers in the early 2000s (Hamill 2001, Cadel-Six et al. 2007, Wood et al. 2007b) and subsequent detections in the Netherlands (Faassen et al. 2012), USA (Fetscher et al. 2015) and Spain (Cantoral Uriza et al. 2017). As most of these investigations originated from animal toxicosis events, considerable research has recently been invested in understanding the drivers of toxic, benthic cyanobacterial proliferations and their toxin production as a result (see Quiblier et al. 2013 for review). Despite an increased focus on visible benthic cyanobacterial proliferations, the potential for other non-cyanobacterial taxa

(e.g., filamentous green algae) to provide habitat for cyanotoxin producers, and thus present a risk for cyanotoxin exposure, has not yet been systematically examined. While Foss et al. (2017) identified nodularin in benthic periphyton samples from streams in Pennsylvania (USA), they did not conduct any taxonomic analysis of the samples, so it is unclear whether the samples were dominated by cyanobacteria or other periphyton. Similarly, Foss et al. (2018) found both nodularin and anatoxin in benthic periphyton samples, but no effort was made to identify the producers of these toxins. Chapter 3 demonstrated that cyanotoxins can be present in *C. glomerata*-dominated mats at concentrations in excess of those in some cyanobacteria-dominated mats. Further investigation of this phenomenon is warranted given the potential harm if humans or animals come in to contact with this material.

6.3 Ecological effects of *Microcoleus*-dominated proliferations and associated toxins

6.3.1 *How does anatoxin production impact the benthic macroinvertebrate and wider stream community?*

Benthic macroinvertebrates are an important component of healthy stream ecology, which is why macroinvertebrate community indices are used as indicators of the health of rivers and streams (Stark 1993). Proliferations of *Microcoleus* can cover substantial areas of riverbed (McAllister et al. 2016), so benthic macroinvertebrates may be exposed either to anatoxin released from the mat material or to anatoxins retained within the mats. In Chapter 5, I found that purified anatoxins do not result in significant mortality of larvae of the mayfly *Deleatidium* spp. at concentrations considerably higher than those recorded in the environment. Despite this, crude extracts of cultured *M. autumnalis* at much lower anatoxin concentrations were toxic to three macroinvertebrates, including a mayfly larva (Anderson et al. 2018; Laura Kelly, unpublished observation). This is consistent with findings from other cyanotoxins, in which crude extracts are considerably more toxic than the purified toxins (Pietsch et al. 2001). As purified toxins exerted no effect in my study, it is possible that metabolites other than anatoxin may have been responsible for the mortality observed by Anderson et al. (2018), and further investigation is warranted using crude extracts of non-toxic *M. autumnalis* to elucidate those compounds that result in mortality. Macroinvertebrate communities can shift in response to the percentage cover of *Microcoleus*-dominated mats, with increasing species richness, but lower QMCI scores (Wood et al. 2014b). Despite this change, only one taxon (*Aoteapsyche*) was

negatively correlated with anatoxin concentrations; thus, there is insufficient evidence to suggest that anatoxins from *Microcoleus*-dominated mats drive shifts in macroinvertebrate community composition. Effects on the wider stream community are also unclear. Anatoxins can be detected in the tissues of *Deleatidium* spp. larvae exposed to high doses (Chapter 5), indicating a potential pathway for bioaccumulation or trophic transfer.

6.4 Priorities for future work

This thesis has provided new insights into the dynamics of toxic and non-toxic strains in *Microcoleus*-dominated mats, revealed cryptic toxin production in non-cyanobacterial dominated periphyton and the potential for transfer of anatoxin to higher trophic levels. The results of this thesis and the new technologies that are now available provide the basis for new directions for future research.

The qPCR assay developed in Chapter 2 opens a range of research directions that could not previously be explored. Key among these is the opportunity to investigate the influence of physicochemical parameters on the dominance of toxic and non-toxic strains in the field. A "big data" approach, with large-scale systematic sampling, will be required to provide sufficient information for modelling. An additional application of the qPCR assay is to transfer the assay to RNA and explore the regulation of the *anaC* gene both in field samples and in targeted laboratory studies. The utility of such a targeted assay is that it is low-cost compared to transcriptomics and would enable screening of samples to identify interesting samples for further analysis.

An important priority for future research is the application of 'omics' techniques to better understand anatoxin production and its role within the benthic microbial community. When applied to the anatoxin gene cluster, transcriptomics will provide opportunities to explore the regulation of toxin production, and this approach can be combined with laboratory-based manipulation of conditions to establish causality. Meta-transcriptomic techniques would be a useful addition to this work, by providing an insight into the microbial community function in *Microcoleus*-dominated mats. If combined with examining toxin concentrations, this could provide valuable insight into changes in community function associated with anatoxins. Metabarcoding of the microbial community in concert with data on anatoxin concentrations

and quota may also provide insight into the communities associated with toxic and non-toxic mats. Opportunities exist to investigate spatial and temporal variation in these communities at multiple scales to integrate knowledge of molecular scale processes with within-mat processes and whole ecosystem effects.

Following the discovery of significant cyanotoxin concentrations in *C. glomerata* materials, investigation of the potential for 'cryptic' cyanotoxin production and the potential risks this poses should be a priority. This is particularly important in areas that can experience both cyanobacterial and filamentous green algal proliferations, where public health warnings only focusing on cyanobacteria may lead to significant risk of contact with cyanotoxins and subsequent toxicosis.

Significant work remains to examine the ecotoxicology of anatoxins in aquatic organisms and investigate bioaccumulation potential. While I found no mortality from acute exposure to anatoxins in *Deleatidium* spp., chronic low-level exposure may have lethal or sublethal effects, which should be assessed, and in a range of taxa, to build a more complete picture of the effects of anatoxins on aquatic organisms. Assessment of anatoxin concentrations in the tissues of the larvae indicated that anatoxins are present in the tissues. Interference in the detection channels for anatoxin-a highlights a need to optimise toxin extraction methods for anatoxins from complex matrices such as animal tissues, and this work will be required to enable the assessment of bioaccumulation of anatoxins. Anecdotal reports of taste and odour compounds of cyanobacterial origin accumulating in trout indicate a pressing need to examine anatoxin accumulation, and lethal and sub-lethal effects of anatoxin exposure in higher trophic levels, particularly in freshwater fish.

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APPENDIX 1

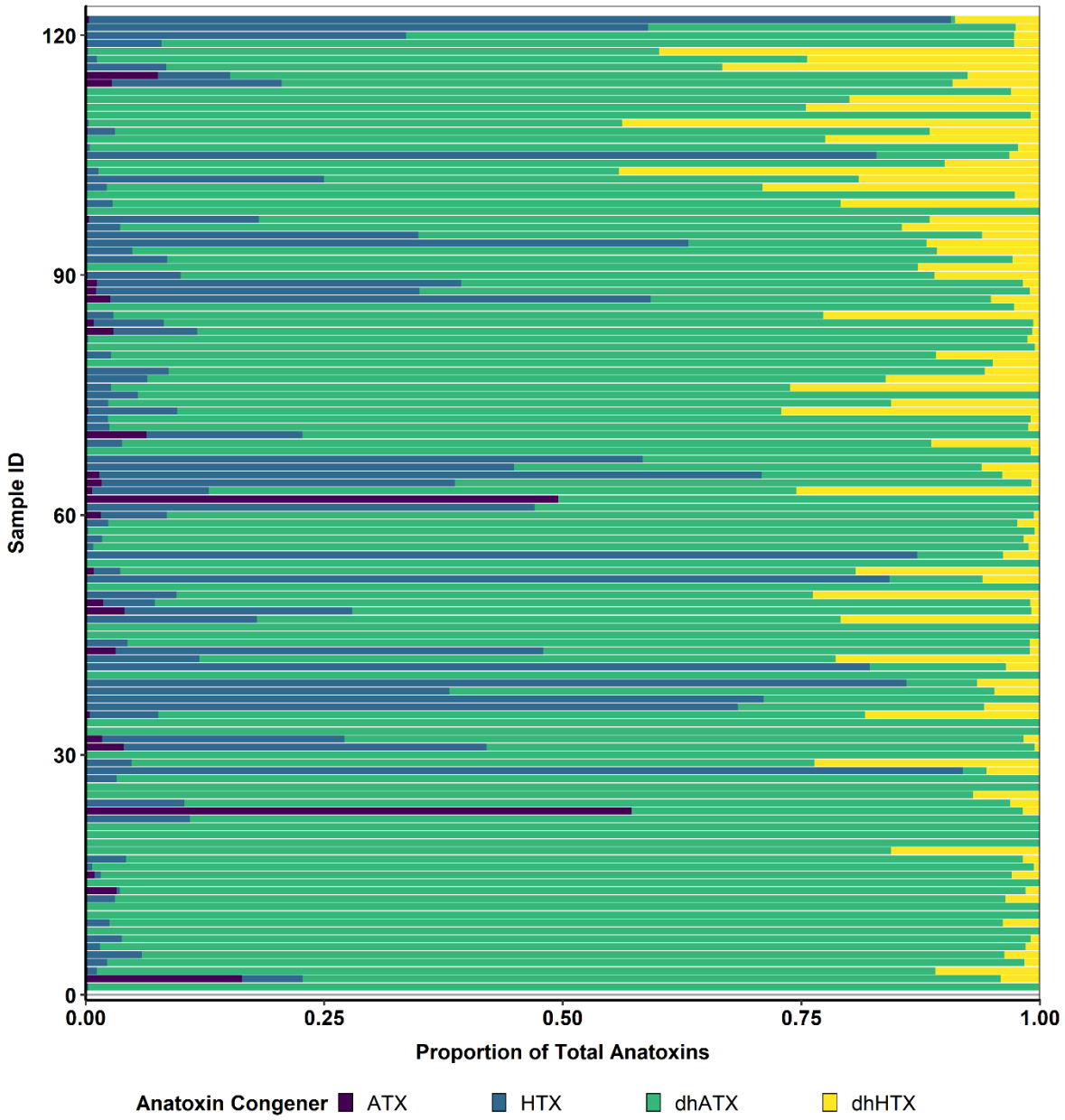

Figure A1.1. Proportions of each of the four anatoxin congeners for each of the environmental samples. Anatoxin-a = ATX, homoanatoxin-a = HTX, dihydroanatoxin-a = dhATX and dihydrohomoanatoxin-a = dhHTX. Sample IDs are in order from the lowest anatoxin quota (sample ID 1) to highest anatoxin quota (sample ID 122).

Table A1.1. Primer specificity was tested against DNA from a range of anatoxin-producing and non-anatoxin-producing cyanobacteria. The anatoxin-producing *Cuspidothrix* is the only known non-*Microcoleus* anatoxin producer in New Zealand.

Species	Culture Identification	Anatoxin producer	Ref
Aphanocapsa sp.	CAWBG585	-	
Aphanocapsa sp.	CAWBG615	-	
Aphanizomenon sp.	CAWBG588	-	
Aphanizomenon sp.	CAWBG594	-	
Aphanizomenon sp.	CAWBG01	-	
Cuspidothrix issatchenkoi	CAWBG02	+	(Wood et al. 2007a)
Dolichospermum circinale	CAWBG571	-	
Dolichospermum circinale	CAWBG601	-	
Dolichospermum lemmermanii	CAWBG572	-	
Dolichospermum lemmermanii	CAWBG593	-	
Dolichospermum planktonicum	CAWBG589	-	
Dolichospermum spiroides	CAWBG558	-	
Dolichospermum spiroides	CAWBG583	-	
Dolichospermum sp.	CAWBG608	-	
Leptolyngbya sp.	CAWBG586	-	
Leptolyngbya sp.	CAWBG700	-	
Microcystis sp.	CAWBG580	-	
Microcystis sp.	CAWBG561	-	
Nostoc sp.	S. Wood submitted	-	
Oscillatoria sp.	PCC 6506	+	(Méjean et al. 2010)
Oscillatoria sp.	PCC 9029	+	(Aráoz et al. 2005)
Planktothrix sp.	CAWBG616	-	
Raphidiopsis raciborskii	CAWBG598	-	
Raphidiopsis raciborskii	CAWBG614	-	
Scytonema sp.	CAWBG699	-	
Scytonema cf. *crispum*	CAWBG72	-	

Table A1.2. Sequences from *Microcoleus autumnalis* and *Oscillatoria* sp. used for primer and probe design. Sequences were used to identify a region of the *anaC* gene that is conserved within *M. autumnalis*, but not within *Oscillatoria*, to generate *M. autumnalis* specific primers.

Species	**Culture Identification**	**Genbank Accession number (16S rRNA gene)**	**Ref**
Microcoleus autumnalis	CAWBG 618	KX016036	(Wood et al. 2017b)
M. autumnalis	CAWBG 619	KX016037	(Wood et al. 2017b)
M. autumnalis	CAWBG 620	KX016038	(Wood et al. 2017b)
M. autumnalis	CAWBG 621	KX016039	(Wood et al. 2017b)
M. autumnalis	CAWBG 622	KX016040	(Wood et al. 2017b)
M. autumnalis	CAWBG 623	KX016041	(Wood et al. 2017b)
Oscillatoria sp.	PCC 10111	JF803654.1	(Rantala-Ylinen et al. 2011)
Oscillatoria sp.	PCC 10601	JF803652.1	(Rantala-Ylinen et al. 2011)
Oscillatoria sp.	PCC 6407	JF803648.1	(Rantala-Ylinen et al. 2011)
Oscillatoria sp.	PCC 9240	JF803651.1	(Rantala-Ylinen et al. 2011)
Oscillatoria sp.	PCC 9240 – strain 193	JF803653.1	(Rantala-Ylinen et al. 2011)

APPENDIX 2

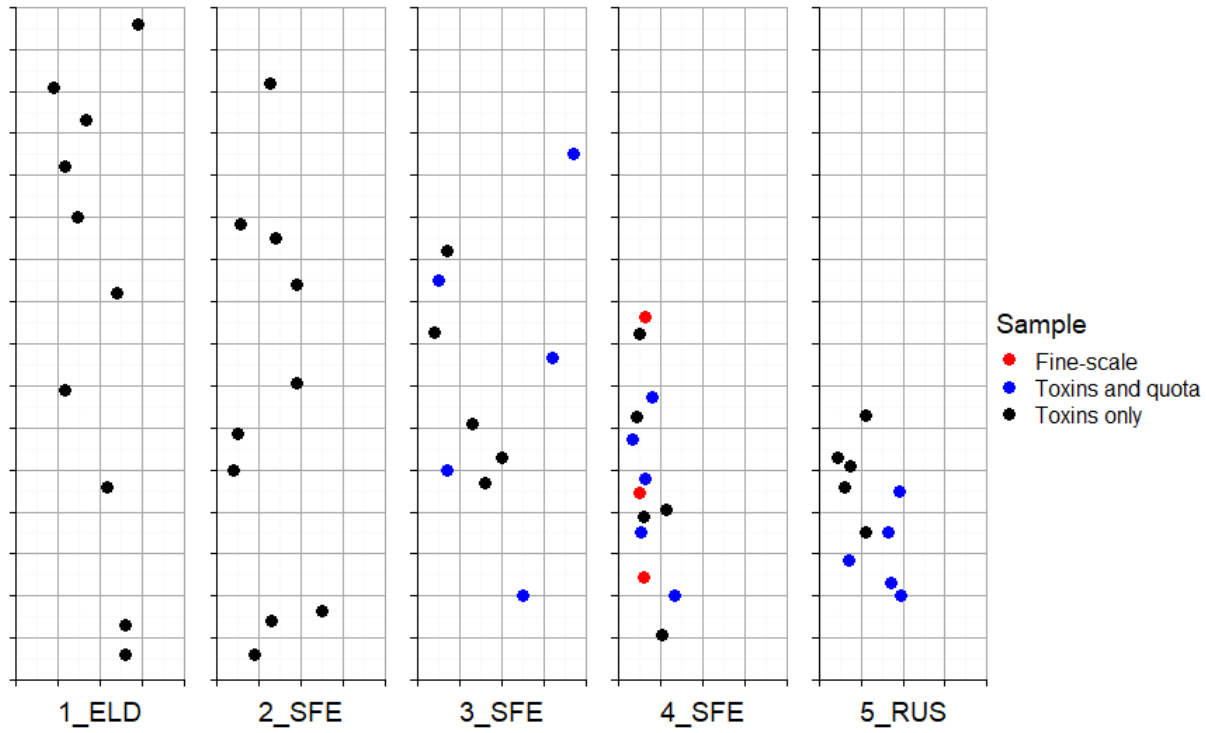

Figure A2.1. Spatial schematic of samples collected for analysis from the Eel and Russian rivers, California. Samples collected from the five sites were analysed for toxins, anatoxin quota and within-rock variation in anatoxin-quota. Squares in the grids represent 1 m^2. Fine-scale samples consisted of five samples collected from periphyton on a single cobble. ELD = Elder Creek; SFE = South Fork Eel River and RUS = Russian River.

Table A2.1. Primer and probe sequences for cyanotoxin gene screening. Primers were used for PCR screening for cyanotoxin genes and for ddPCR analysis of *anaC* gene copy number.

Toxin	Target Gene	Reaction	Primer and probe sequences	Reference
Anatoxins	*anaC*	PCR Sequencing	*anaC-gen-F* 5′-TCTGGTATTCAGTCCCCTCTAT-3′ *anaC-gen-R* 5′-CCCAATAGCCTGTCATCAA-3′	Rantala-Ylinen et al. (2011)
Anatoxins (*Oscillatoria*/*Microcoleus autumnalis*-specific)	*anaC*	ddPCR	*Phor-AnaC-F5* 5′-ACTAACCGAATCACTTCCACTT-3′ *Phor-AnaC-R5* 5′-CTCACCCACCTCACCTTTAG-3′ *Phor-AnaC-P5* 5′-TTCAGTATTAGCGCAGGCTTTGCC-3′	Kelly et al. (2018)
Microcystin Nodularin	*mcyE* *ndaF*	PCR Sequencing	*HEPF* 5′-TTTGGGGTTAACTTTTTTGGGCATAGTC-3′ *HEPR* 5′-AATTCTTGAGGCTGTAAATCGGGTTT-3′	Jungblut and Neilan (2006)
Cylindrospermopsin	*cyrJ*	PCR	*cynsulfF* 5′-ACTTCTCTCCTTTCCCTATC-3′ *cylnamR* 5′-GAGTGAAAATGCGTAGAACTTG-3′	Mihali et al. (2008)
Saxitoxin	*sxtA*	PCR	*Sxtaf* 5′-GCGTACATCCAAGCTGGACTCG-3′ *Sxtar* 5′-GTAGTCCAGCTAAGGCACTTGC-3′	Ballot et al. (2010)

APPENDIX 3

Table A3.1. *Microcoleus autumnalis* strains used in the study and the locations they were collected from. Strains were previously tested for the presence of two genes in the anatoxin gene cluster: *anaC* and *anaF*.

Strain	*anaC*†	*anaF**	LC-MS/MS**	Location initially collected from	
CAWBG36	-	nt	nt	Avalon Pond	Wellington
CAWBG38	-	nt	-	Wainuiomata River	Wellington
CAWBG54	-	nt	-	Rangataiki River	Bay of Plenty
CAWBG511	-	-	-	Hutt River	Wellington
CAWBG520	+	+	+	Hutt River	Wellington
CAWBG521	+	+	+	Hutt River	Wellington
CAWBG556	+	+	+	Waimakariri River	Canterbury
CAWBG557	+	+	+	Waimakariri River	Canterbury

+/-, detected/not detected; † (Kelly et al. 2018); *(Wood et al. 2012b); **(Wood et al. 2010a); nt indicates that samples were not tested for this attribute.

Table A3.2. Linear mixed effects model estimates of the fixed effects on alkaline phosphatase activity, with effects estimated using the Satterthwaite method. The intercept represents the high dissolved reactive phosphorus (DRP), high dissolved organic phosphorus (DOP) treatment for the non-toxic strains and the estimates of all other effects are subsequently derived from this baseline.

Fixed effects	Estimate	Standard error	Degrees of freedom	t value	*p* value
Intercept	9.76	3.99	6.04	2.44	0.050
High DRP, High DOP					
Treatment B	0.25	0.37	466	0.67	0.500
High DRP, Low DOP					
Treatment C	0.66	0.37	466	1.80	0.073
Low DRP, High DOP					
Treatment D	8.99	0.37	466	24.48	< 0.001
Low DRP, Low DOP					
Toxic strains	13.46	5.65	6.04	2.38	0.054
High DRP, High DOP					
Treatment B: Toxic strains	0.18	0.52	466	0.35	0.728
High DRP, Low DOP					
Treatment C: Toxic strains	1.24	0.52	466	2.40	0.017
Low DRP, High DOP					
Treatment D: Toxic strains	–4.95	0.52	466	-9.53	< 0.001
Low DRP, Low DOP					

APPENDIX 4

Table A4.1. *Deleatidium* spp. larvae survival in each of the treatment/concentration combinations at the conclusion of the experiment following six days of exposure ($n = 20$ for all treatment/concentration combinations).

Treatment	Measured concentration of anatoxins (μg L^{-1})	*Deleatidium* spp. survival (%)
River water control	0	95
Vehicle control	0	70
Anatoxin-a	255.3	70
	135.4	60
	79.4	70
	39.3	65
	0.7	80
dihydroanatoxin-a	670.1*	75
	338.1*	75
	164.1	60
	86.6	75
	40.1	50
	20.1	55
	0.4	45
homoanatoxin-a/ dihydrohomoanatoxin-a	240.8	60
	119.4	85
	58.2	80
	30.6	60
	0.6	80

* denotes treatments in a follow-up experiment using higher doses of dhATX than the initial experiment.

APPENDIX 5

Preliminary dose-finding experiments were conducted with crude extracts of environmental *Microcoleus* mats. Crude extracts were prepared by lyophilising environmental *Microcoleus* mats. These were then homogenised by grinding with a spatula. Subsamples were tested for the presence and concentration of anatoxins using liquid-chromatography tandem-mass spectroscopy (LC-MS/MS). Two crude extract solutions were prepared by weighing 18 g L^{-1} of lyophilised mat material into 1 L Schott bottles and 1 L of Milli-Q water added. The resulting solution was sonicated on ice for 30 m, then clarified by centrifugation at 3,200 × *g* for 10 m. The supernatant was carefully decanted into sterile 1 L Schott bottles. The two solutions were defined as non-toxic (not containing anatoxins) and toxic (containing anatoxins). Dilutions of the stock solutions were made with filtered (11 μm) river water to generate a concentration gradient for the dose-finding experiment from 0.395 g L^{-1} to 15.4 g L^{-1} (Table A5.1). The experimental set-up consisted of 40 mL of the relevant test solution in a 70 mL plastic jar. Each jar contained a single *Deleatidium* spp. larva, with five replicates per treatment-dose combination. The jars were maintained in a climate-controlled cabinet at 15 °C on a 12:12 light:dark cycle. The larvae were monitored daily for survival. The data were visualised using Kaplan-Meier survival analysis.

The dose-finding experiment resulted in 100% mortality of *Deleatidium* spp. larvae at crude extract concentrations of 2.5 g L^{-1} both in the non-anatoxin containing (Figure A5.1 A) and anatoxin containing (Figure A5.1 B) solutions. Dissolved oxygen concentrations and the conductivity of test solutions were stable and remained within the physiological tolerances of *Deleatidium* spp. for the duration of the dose-finding experiment.

Table A5.1. Treatment-dose combinations of the preliminary dose-finding ecotoxicity experiment. Extracts were prepared from environmental *Microcoleus* mats that had been lyophilised and extracted via sonication.

Treatment	Dose	Crude extract concentration (mg L^{-1})
River water control	NA	0
Non-toxic crude extract	Dose 1	0.395
	Dose 2	0.988
	Dose 3	0.247
	Dose 4	6.177
	Dose 5	15.436
Toxic crude extract	Dose 1	0.395
	Dose 2	0.988
	Dose 3	0.247
	Dose 4	6.177
	Dose 5	15.436

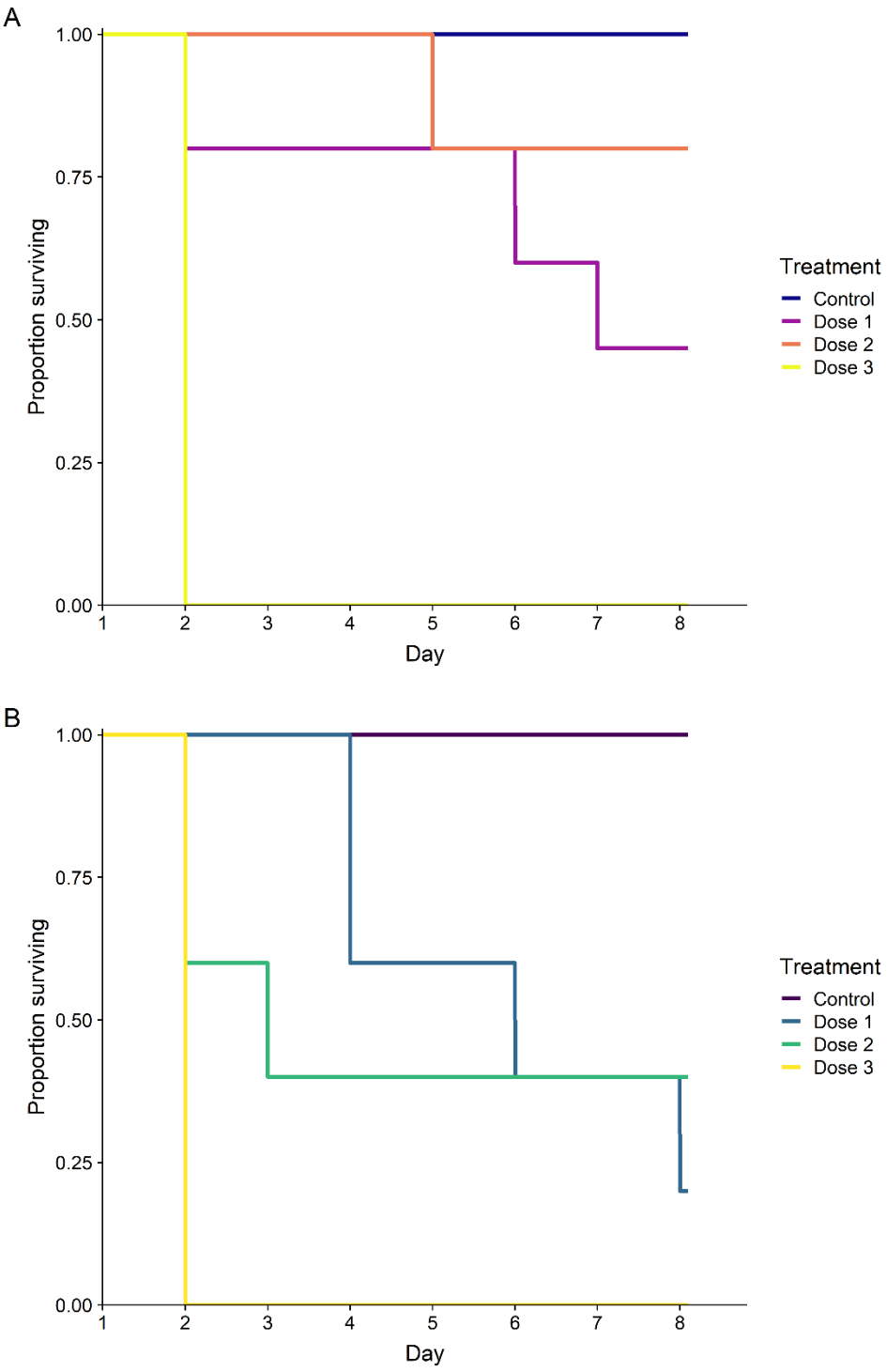

Figure A5.1. Kaplan-meier survival curves for preliminary dose-finding experiment. A: Crude extracts of *Microcoleus* environmental mats with no anatoxins measurable by LC-MS/MS; B: Crude extracts of *Microcoleus* environmental mats with measureable anatoxins. Controls were filtered river water for both A and B. Doses 1, 2 and 3 represent concentrations of mat material in filtered river water of 0.395, 0.988 and 2.45 g L^{-1}. Note doses 4 and 5 are not shown on these graphs but resulted in the same curve as dose 3 for both non-toxic and toxic treatments.

Following the dose-finding experiment, a range of concentrations of crude extract were chosen to conduct a full-scale experiment with the highest dose at 2.5 g L^{-1} to ensure the range captured 100% mortality. The treatment-dose combinations are outlined in table A5.2. All extracts were prepared as in the preliminary experiment. Twenty jars, each with a single *Deleatidium* larva were established for each treatment-dose combination and their survival monitored as above. Dissolved oxygen concentrations in the test media declined considerably throughout the experiment in a dose-dependent manner, with concentrations reducing to below physiological tolerances for *Deleatidium* spp. Test solutions became cloudy and it is thought this was the result of bacterial growth in the test solutions. Additional experimentation was conducted using crude extracts obtained through bulk-culturing toxic and non-toxic strains of *Microcoleus* in the laboratory. Trials included the addition of antibiotics, additional filtration steps (0.22 μm) of the crude extracts and river water, and addition of oxygen via bubbling with an aquarium pump. None of these prevented the depletion of dissolved oxygen. It is possible that other compounds (e.g. sugars) present in the crude extract provide optimal conditions for bacterial growth. It was not possible to completely sterilise the solutions as bacteria are introduced with the *Deleatidium* spp. larvae, therefore the main experiment was conducted with purified toxins.

Table A5.2. Treatment-dose combinations of the preliminary ecotoxicity experiment. Extracts were prepared from environmental *Microcoleus* mats that had been lyophilised and extracted via sonication.

Treatment	Dose	Crude extract concentration (mg L^{-1})
River water control	NA	0
Non-toxic crude extract	Dose 1	0.125
	Dose 2	0.25
	Dose 3	0.50
	Dose 4	1.00
	Dose 5	1.75
	Dose 6	2.50
Toxic crude extract	Dose 1	0.125
	Dose 2	0.25
	Dose 3	0.50
	Dose 4	1.00
	Dose 5	1.75
	Dose 6	2.50