Mat forming toxic benthic cyanobacteria in New Zealand

Species diversity and abundance, cyanotoxin production and concentrations

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A thesis submitted in part fulfilment of the requirements for the degree of Master of Science in Ecology and Biodiversity,

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August 2009

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Abstract

Recent research has shown that toxic cyanobacteria are more widespread in New Zealand water bodies than previously thought. However, that work has largely focused on planktonic species. Toxin production associated with benthic cyanobacteria is less widely understood despite benthic mat-forming cyanobacteria being prevalent throughout New Zealand rivers. Little is known on species responsible for toxin production, their distribution, frequency and factors triggering toxin production. This study is divided into two phases; (1) a phylogeographic study of benthic cyanobacteria from multiple rivers across New Zealand and (2) an in-depth study of spatial and temporal variability of toxic benthic cyanobacteria in two rivers.

Benthic cyanobacterial mats were collected from 22 different waterbodies around New Zealand between January 2005 and December 2008 and their anatoxin content determined using liquid chromatography-mass spectrometry. Thirty seven isolates were obtained from these samples and a polyphasic approach was used to identify them. Liquid chromatography-mass spectrometry and targeted PCR were used to determine if the isolates were producing anatoxins and microcystins.

Phormidium autumnale was the dominant cyanobacterium within the isolates, although molecular and morphological data indicated the existence of multiple strains within this species. Two isolates of *Ph. autumnale* produced anatoxin-a and formed their own clade based on partial 16S rRNA gene sequences. These data indicate that benthic *Ph. autumnale* mats are composed of multiple morphospecies and anatoxin production is dependant on the presence of anatoxin-producing genotypes. Microcystin production was confirmed in a potentially novel isolate, closely related to *Planktothrix* spp. This species was responsible for the death of a dog in the Waitaki river (South Island, New Zealand).

Cyanobacterial abundance, diversity and toxin production were monitored fortnightly in an in-depth study of the Hutt and Wainuiomata rivers (Wellington, New Zealand), over a 12 month period. Environmental parameters were correlated with cyanobacterial abundance and anatoxin production at eight locations along the rivers to elucidate possible causal factors.

Cyanobacterial proliferations and associated anatoxin production were spatially and temporally variable across eight different sampling sites. Both river flow and temperature had a significant effect on cyanobacterial abundance. Multiple physicochemical factors including nutrients and periphyton growth appear to be interacting to influence cyanobacterial abundance. The presence and concentration of anatoxin-a and homoanatoxin-a and their degradation products dihydro-anatoxin-a and dihydro-homoanatoxin-a were also highly variable across all sites and over time. Anatoxin concentration did not correlate with any physicochemical parameters.

The results of this study have demonstrated that the prevalence of freshwater cyanotoxin-producing benthic cyanobacteria is widespread in New Zealand and that toxin concentration is variable and unpredictable. This research will assist water managers in addressing the complex management issues associated with benthic cyanobacterial proliferations.

Contributions and publications

This thesis has been written in article format for the rapid submission to international journals. The results of this thesis have contributed to four manuscripts. The proposed manuscripts are:

- **Heath, M.W.,** Wood, S.A., Ryan, K.G. 2009. Polyphasic assessment of freshwater benthic mat forming cyanobacteria in New Zealand. Target journal: FEMS Microbiology.

This manuscript is a direct outcome of the results given in Chapter 2 of this thesis. I undertook all the work detailed in this chapter. My supervisors provided me with assistance and direction on laboratory techniques and commented on several drafts of the manuscript.

- **Heath, M.W.,** Wood S.A., Ryan, K.G. 2009 Spatial and temporal variability in *Phormidium* abundance and anatoxin production in the Hutt River, New Zealand. Target Journal: Applied and Environmental Microbiology.

This manuscript is a direct outcome of the results given in Chapter 3 of this thesis. I undertook all field and laboratory work detailed in this chapter. My supervisors provided me with assistance and direction on laboratory techniques and commented on several drafts of the manuscript.

- Wood, S.A., **Heath, M.W.,** McGregor, G., Holland, P.T., Munday, R., Ryan, K.G. 2009. Identification of a benthic microcystin producing *Planktothrix* sp. and an associated dog poisoning in New Zealand. Target Journal; Toxicon.

This article describes a new cyanobacteria species that was identified during my study. I contributed to this manuscript in the following ways: isolation and culturing, morphological identification, phylogenetic analyses and a section of writing. The work I undertook on this species is included in Chapter 2 (Morphotype D). Following my descriptions, further analyses were undertaken by co-authors.

- Wood S.A., **Heath, M.W.,** Ryan, K.G. 2009. Fine scale variability of anatoxin production in benthic cyanobacteria; implication for monitoring and management. Target Journal Microbial Ecology.

No data for this manuscript was sourced from my thesis. The idea for this paper however was developed as a result of the findings of Chapter 2 and 3 presented in this thesis. I contributed to this manuscript by assisting with field work, preparation of samples and the writing of the manuscript.

Acknowledgements

There are many people whom I must thank for their support, time, assistance and friendship throughout my thesis.

I would like to acknowledge my supervisors Ken Ryan and Susie Wood who allowed me to take on my project of choice. I will be forever grateful for their continued support and assistance, hasty draft returns and positive attitudes. I hope we can move forward into future collaborative research.

I wish to thank Juliet Milne, Summer Warr, Alton Perrie, Laura Keenan and Ted Taylor from the Greater Wellington Regional Council for their advice and assistance. I would also like to thank the Greater Wellington Regional Council for awarding me a science and research grant.

I have also been fortunate to be funded by Victoria University of Wellington's Strategic Research Grant and the Andy Ritchie Memorial Trust.

I was very privileged to spend significant time at the Cawthron Institute (Nelson). I acknowledge the Bio-toxin Laboratory, Phytoplankton Laboratory and Andy Selwood, Roel Van Ginkel, Michael Boundy and Aaron Quarterman for their technical assistance.

I also thank Glen McGregor from the Queensland Department of Natural Resources Mines for his assistance with species identification.

Special mention to the Ryan lab group, the help and support from the team has been great.

The following individuals must be thanked for their support and contribution: Ronan O'Toole, Stephen Hartley, John Haywood, Paul Marsden, Chris Thorn, Mary Murray, Sandra Taylor, Cameron Jack, Murray Williams, Jonathan Punddick, Lisa Bryant, Rochelle Wells, Andrew Martin, Gary Milbanke, Joanna Buswell, Eve Butler, Miles Benton, Ray Blick, Heather Murray, Jessie Prebble, Fiona Hodge and Sebastian Hernandez.

Lastly my parents Stephen and Gina and my very close extended family that could not be more supportive. I hope they understand how much they mean to me and how appreciative I am of everything they have done to help. I would like to thank them for their love, support and friendship, the opportunities they have provided and the interest they have taken in my research.

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Introduction and Objectives

In many parts of the world cyanobacterial blooms have been recognised for the potential risk they pose to animal and human health (Chorus and Bartrum 1999). These risks have been traditionally associated with planktonic cyanobacterial blooms that occur in still or slow flowing water-bodies, highlighted by the fatal intoxication by microcystin of 50 dialysis patients in Caruaru, Brazil (Azevedo *et al.* 2002). As a result of such tragedies, significant scientific investigation and attention has been devoted to research on planktonic cyanobacteria. In contrast there have been very few studies on benthic mat-forming cyanobacteria, despite being linked to dog and stock deaths worldwide (Edwards *et al.* 1992; Mez *et al.* 1997; Hamill 2001; Gugger *et al.* 2005).

Cyanobacteria are photosynthetic prokaryotic organisms that form an integral part of many aquatic ecosystems. These organisms produce a variety of secondary metabolites; the most common of these metabolites are the cyanotoxins. Although the natural function of cyanotoxins is unclear, they are very hazardous to terrestrial mammals. The mechanisms of toxicity for cyanotoxins are very diverse, ranging from hepatotoxicity and neurotoxicity to dermatotoxicity. Planktonic cyanobacteria occur commonly in natural environments, however, without anthropogenic influences, cyanobacteria blooms and their associated cyanotoxin production is rare. Increasing eutrophication of freshwater ecosystems has resulted in a marked increase in planktonic cyanobacteria blooms.

The diversity and growth of cyanobacteria and the formation of blooms are influenced by a variety of physical, chemical and biological factors. In planktonic cyanobacteria these variables have been extensively investigated (Oliver and Gnaf 2000). This has resulted in models that can predict phytoplankton dynamics, cyanobacterial bloom formation and the effect of remedial actions (e.g., DYRESM http://www.cwr.uwa.edu.au/services/models.php?mdid=2). In addition, there have been many studies investigating cyanotoxin regulation in different planktonic cyanobacteria. For example, studes by Rapala *et al.* (1993) and Rapala and Sivonen (1998) showed that in *Anabaena spp*. anatoxin-a concentration increases up to seven fold when grown at optimal temperatures for growth.

In the last decade, benthic cyanobacteria, traditionally regarded as innocuous, have been shown to be responsible for numerous animal poisonings worldwide (Mez *et al.*

1997; Baker *et al.* 2001; Hamill 2001; Gugger *et al.* 2005; Izaguirre *et al.* 2007). Toxic benthic cyanobacteria proliferations are becoming more prevalent. This represents a very serious animal and human health risk and unlike their planktonic counterparts benthic cyanobacteria have received little scientific attention. The physical, chemical and biotic factors contributing to benthic mat proliferation and toxin regulation are unknown. In most instances there is a very poor understanding of species diversity and their toxin producing capabilities.

The first documented case of animal poisoning from benthic cyanobacteria in New Zealand was in Southland on the Mataura River 1998 where six dogs were reported to have died from suspected anatoxin poisoning (Hamill 2001). Since these first reported deaths, numerous dog deaths have been reported from all over New Zealand (Hamill 2001; Wood *et al.* 2007b). Wood *et al.* (2007) confirmed anatoxin-a and homo-anatoxin-a as the toxins responsible for five dog deaths on the Hutt River in 2005. In New Zealand, there are clearly gaps in information on recent distributions and species composition of benthic mat-forming cyanobacteria, the variants and levels of cyanotoxins present in these mats and changes in cyanotoxin levels over time. Little is known of the physicochemical parameters contributing to cyanobacterial mat proliferations and associated toxin production. This study has begun to investigate some of these gaps in the scientific knowledge and has provided important data for future research. In addition, it is anticipated that the present study will provide information that will be useful to water managers, such as local authorities, drinking water plant managers and to government agencies. Results and conclusions have already been incorporated into the national guidelines for monitoring cyanobacteria in New Zealand (Ministry for the Environment and Ministry of Health, 2009); testament to the importance of this research.

During the present study, cyanobacterial mats were obtained from multiple freshwater bodies throughout New Zealand (refer to Appendix 1 for photographs of algae, including benthic cyanobacteria sampled/observed during this study). Cyanobacterial species and the levels and types of cyanotoxins in the samples were identified. An in-depth case study of the Hutt and Wainuiomata rivers investigated the spatial and temporal variation in cyanobacterial mat coverage and anatoxin production in concert with physicochemicalparameters.

This thesis is divided into two main sections and these are presented as manuscripts for submission to research journals:

1. Polyphasic assessment and cyanotoxin production ability of fresh-water benthic mat forming cyanobacteria in New Zealand (Chapter 2).
2. Spatial and temporal variability in *Phormidium* abundance and anatoxin production in the Hutt River and Wainuiomata River, New Zealand (Chapter 3).

Chapter 2 reports on a polyphasic examination of proliferating benthic cyanobacteria species diversity in mats isolated New Zealand rivers and their toxin producing capabilities. It responds to the following objectives:

- To determine which species are responsible for benthic cyanobacterial proliferations in New Zealand rivers and how these vary genetically and morphologically.
- To establish which species are producing cyanotoxins, what types and variants of cyanotoxins are being produced and their relative concentrations.

Chapter 3 is a case study of the Hutt and Wainuiomata rivers and examines benthic cyanobacterial proliferations and anatoxin concentrations over time at eight sites across two catchments. It responds to the following objectives:

- To assess the spatial and temporal distribution of benthic cyanobacterial mat coverage and anatoxin production.
- To assess which physicochemicalparameters leading to increases in cyanobacterial mat coverage and anatoxin concentrations.
- To assess the ability of predictive models to ascertain the presence or absence of benthic cyanobacteria.

The investigations undertaken to meet these objectives have produced valuable findings that add considerably to the current knowledge of benthic cyanobacteria both in New Zealand and worldwide. This work importantly, has developed a baseline dataset for future research.

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2

Polyphasic assessment of toxic and non-toxic freshwater benthic mat forming cyanobacteria in New Zealand

2.1 Abstract:

Mat-forming benthic cyanobacteria are widespread throughout New Zealand rivers and have been increasingly linked to animal poisonings. Analysis of benthic mats from around New Zealand has shown marked variations in the presence and quantities of cyanotoxins. In this study potentially toxic benthic proliferations of cyanobacteria were collected from 22 rivers and lakes around New Zealand. Each environmental sample was screened for anatoxins using liquid chromatography-mass spectrometry (LC-MS). Thirty seven cyanobacterial strains were isolated and cultured from the environmental samples. A polyphasic approach was used to identify each isolate; this included genotypic analyses (16S rRNA gene sequences) and morphological characterisation. Each culture was analysed for anatoxins using LC-MS and screened for microcystin production potential using targeted PCR with subsequent confirmation undertaken using LC-MS. *Phormidium autumnale* was found to be the dominant cyanobacterium in mat samples. Polyphasic analyses revealed multiple morphospecies within the *Ph. autumnale* clade and highlighted the difficulties of identifying Oscillatoriaceae members to species level, based solely on morphology or molecular data. Only one morphological variant (comprising the two strains CYN52 and 53) of *Ph. autumnale* was found to produce anatoxins. These strains formed their own clade based on partial 16S rRNA gene sequences. These data indicate that benthic *Ph. autumnale* mats are composed of numerous morphological variants and toxin production is dependant on the presence of toxin producing genotypes. Microcystin ([ASP-3]deMe MC-LR) production was confirmed in a culture (VUW25) of a potentially novel cyanobacterium, closely related to *Planktothrix* spp. This species was linked to the first confirmed dog death from a microcystin-producing benthic cyanobacterium in New Zealand. No other cyanotoxins were identified in this investigation. A number of other cyanobacteria were identified from the genera *Phormidium*, *Oscillatoria* and *Nostoc* and from the family Pseudanabaenaceae, including the first identification of *Ph. murrayi* outside of Antarctica.

Keyword: Benthic cyanobacteria, anatoxin, microcystin, *Phormidium*, *Phormidium autumnale*

2.2 Introduction

The first report of toxin production in planktonic cyanobacteria was published in 1878 (Francis 1878). Since then, multiple incidents of animal and human poisonings have been linked to planktonic cyanobacteria and the toxins are now well-characterised (Sivonen and Jones 1999). In contrast there has been little information available on toxic benthic cyanobacteria. However, over the past two decades, an increasing number of toxin-producing freshwater benthic cyanobacteria species have been documented (Edwards *et al.* 1992; Baker *et al.* 2001; Gugger *et al.* 2005; Mohamed *et al.* 2006; Fiore *et al.* 2009). The cyanotoxins produced by these micro-organisms pose a serious threat to animal and human health and have resulted in multiple animal poisonings (Hamill 2001; Krienitz *et al.* 2003; Gugger *et al.* 2005; Cadel-Six *et al.* 2007). In New Zealand, reports of dog poisonings linked to benthic cyanobacteria have increased in the last 10 years. Since the first dog fatalities were documented in 1998 (Hamil 2001), there have been more than 30 reported deaths (Wood *et al.*, 2007, Heath, unpubl. obsn.).

Globally, anatoxins (neurotoxins) and microcystins (hepatotoxins) are the two most common cyanotoxins produced by benthic cyanobacteria (Mez *et al.* 1997; Sivonen and Jones 1999; Cadel-Six *et al.* 2007; Izaguirre *et al.* 2007; Wood *et al.* 2007). Anatoxins are powerful neuromuscular blocking agents that act through the nicotinic acetylcholine receptor, while microcystins inhibit protein phosphatases causing liver necrosis (MacKintosh *et al.* 1990; Carmichael 1994). Recently, benthic cyanobacteria that produce cylindrospermopsins and saxitoxins have been identified (Carmichael *et al.* 1997; Seifert *et al.* 2007). Saxitoxins are fast acting neurotoxins that inhibit nerve conduction by blocking sodium channels; these toxins are common in marine dinoflagellates where they are known as paralytic shellfish poisons (PSPs), (Adelman *et al.* 1982). Cylindrospermopsins are potent inhibitors of protein and glutathione synthesis acting on the liver and kidneys (Terao *et al.* 1994; Falconer *et al.* 1999). There have been no reported cases of animal toxicosis from benthic cyanobacteria producing saxitoxins and cylindrospermopsins. Benthic *Phormidium* and *Oscillatoria* species have most commonly been linked to cyanotoxin production (Krienitz *et al.* 2003; Gugger *et al.* 2005; Cadel-Six *et al.* 2007; Izaguirre *et al.* 2007; Wood *et al.* 2007). However, cyanotoxins have also been found in benthic species of *Spirulina* (microcystins and anatoxins), *Fischerella* (microcystins), *Lyngbya* (saxitoxins and cylindrospermopsins), *Aphanothece* (microcystins) and *Nostoc* (microcystins)

(Carmichael *et al.* 1997; Krienitz *et al.* 2003; Dasey *et al.* 2005; Seifert *et al.* 2007; Fiore *et al.* 2009).

In New Zealand rivers, benthic mat-forming cyanobacteria are found in a wide range of water quality conditions (Biggs and Kilroy, 2000). The most common mat-forming genus in New Zealand is *Phormidium* (Ministry for the Environment and Ministry for Health, 2009). During optimal conditions, *Phormidium* forms expansive black/brown/green leathery mats over wide areas of river substrate. In the 2005/06 summer, Wood *et al.* (2007) identified the causative cyanobacterium of multiple dog deaths in the Hutt River (Wellington, New Zealand) as *Ph. autumnale*. This organism is the only benthic species known to produce anatoxin-a (ATX) and homoanatoxin-a (HTX) in New Zealand. Routine testing of *Phormidium* mats from around New Zealand has shown marked variations in the presence of anatoxins, in the anatoxin variants produced, and/or in their concentrations (S Wood, pers. comm.). There is uncertainty as to whether this variability is due to the presence of different species or strains within the mats and variations in their ability to produce anatoxins. The correct and early identification of cyanobacterial species and confirmation of those species that produce toxins will provide guidance that can be used in developing management and mitigation programmes aimed at protecting animal and human health.

In this study, 32 potentially toxic benthic proliferations of cyanobacteria were collected from 22 rivers and lakes around New Zealand. Each environmental sample was screened for anatoxins using liquid chromatography mass spectrometry (LC-MS). Individual isolates from each sample were cultured and anatoxins analysed using LC-MS. Each culture was screened for microcystin production potential using PCR and positive cultures were further assessed using LC-MS. Isolates from the pure cultures were identified where possible to the species level using morphology and phylogenetic analyses. This study is the first to describe the diversity and toxin production of benthic cyanobacteria in New Zealand

2.3 Materials and Methods

2.3.1 Site description and sample collection

Between 2005 to 2008, benthic cyanobacterial mats were collected from 22 New Zealand rivers and lakes experiencing cyanobacterial proliferations. Sampling sites from which

cyanobacterial strains were successfully isolated are shown in Figure 2.1 Cyanobacterial mats were predominantly found on rocky substrates but were also collected from fine substrate (0.2-0.02 mm) in the Whakatikei, Rangitaiki and Waitaki rivers. Cyanobacterial samples were collected by scraping mats into sterile plastic screw-cap bottles (50 ml, Biolab, New Zealand). All samples were placed on ice for transport. On arrival at the laboratory, samples were frozen (-20 °C) for later culturing and toxin analysis. Sub-samples (10 ml) were preserved using Lugol's Iodine for morphological identification.

2.3.2 Strain isolation and culture conditions

Frozen samples were thawed and cyanobacterial strains were isolated by streaking on solid MLA medium (Bolch and Blackburn, 1996). One half of the Petri dish containing the streaked material was covered in black PVC plastic. This assisted in isolating single filaments as some benthic cyanobacteria are motile and move towards light. After approximately 2 weeks when filaments had moved across the Petri dishes, single filaments were isolated by micro-pipetting and transferred to 24-well plates containing 500 µl MLA medium per well. Filaments were washed repeatedly and incubated under standard conditions (100 ± 20 µmol photons.m^{-2}.s^{-1}; 16:8h light:dark; 18 ± 1 ºC, Contherm, 190 RHS, New Zealand). Cycloheximide (100 µg/ml) was used in selected cultures to reduce eukaryotic growth (Ferris and Hirsch 1991; Bolch and Blackburn 1996; Urmeneta *et al.* 2003). Successfully isolated clonal strains were maintained in 50 ml plastic bottles (Biolab, New Zealand) under the same conditions.

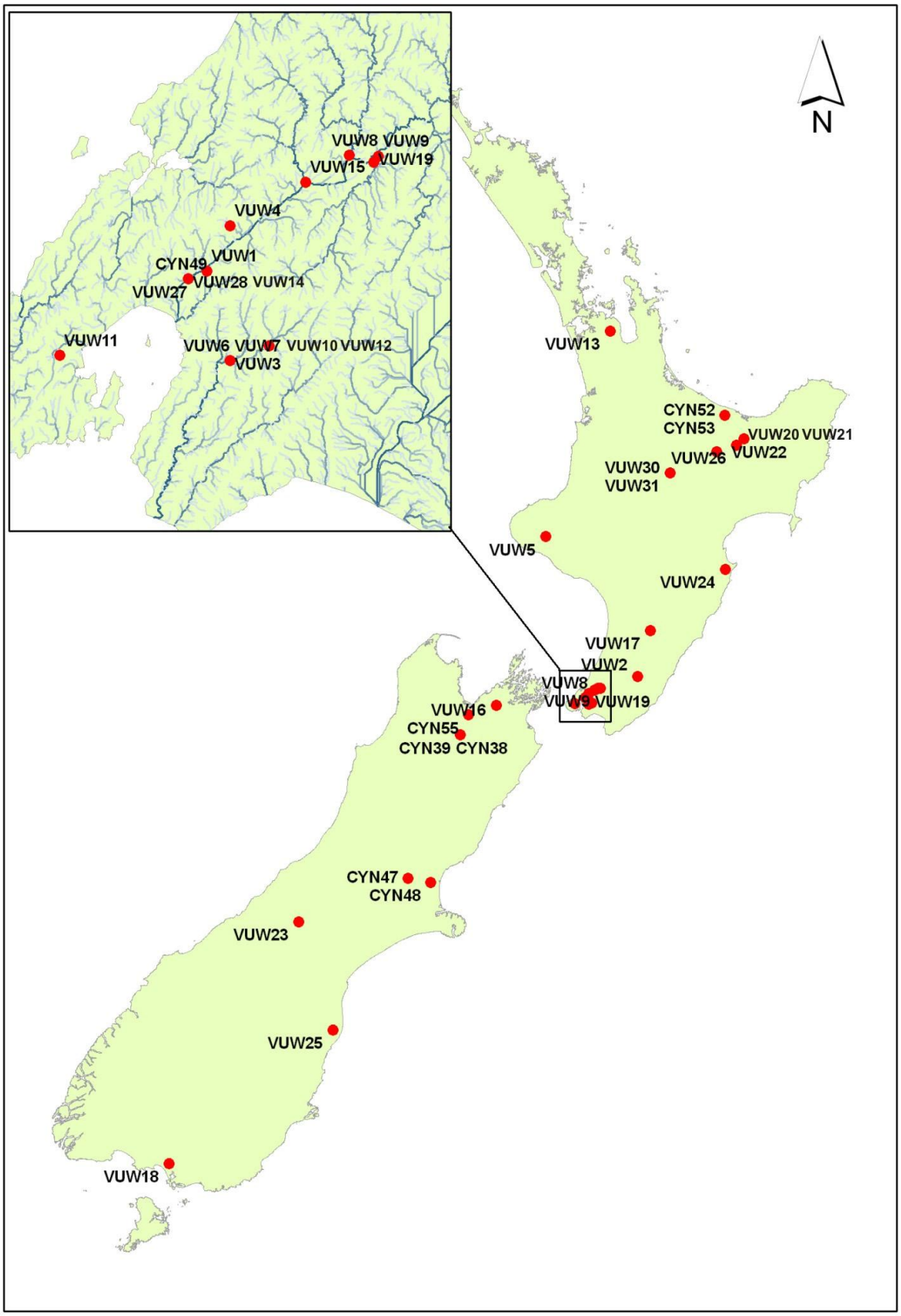

Figure 2.1, Source locations of successfully isolated cyanobacterial strains.

2.3.3 Morphological identification

Sub-samples of each cyanobacterial strain (in stationary phase) were identified by microscopy (Zeiss Photomicroscope II, Germany). Photomicrographs were taken using a digital camera (Canon Powershot S3IS) and further processed in Photoshop 7.0 (Adobe, USA). Species identifications were made primarily by reference to Komárek and Anagnostidis (2005) and McGregor (McGregor 2007). Thirty microscope measurements of vegetative cell lengths and widths were made for each sample, and detailed observations of phenotypic characteristics were noted for 15 filaments in each sample.

2.3.4 Molecular identification, isolation of DNA.

Sub-samples (500 µl) from each culture were centrifuged using an Eppendorf (USA) micro-centrifuge (15 000 × *g*, 1 min) and the supernatant removed by sterile pipetting. DNA was extracted from the pellets using a PureLink^{TM} Genomic DNA kit (Invitrogen, CA, USA) according to the gram negative bacteria protocol supplied by the manufacturer.

2.3.5 Polymerase Chain Reaction (PCR)

PCR amplifications of a segment of 16S rRNA gene were performed in 50 µl reaction volumes containing between 100–200 ng of DNA, 240 mM of each primer (27F and 809R, Jungblut and Neilan 2005; Geneworks, Australia)**,** 200 mM deoxynucleoside triphosphates (Roche Diagnostics, New Zealand), 1U Platinum Taq DNA polymerase (Invitrogen, New Zealand), 4 mM $MgCl_2$ (Invitrogen, New Zealand) and 0.6 mg of non-acetylated bovine serum albumin (Sigma, New Zealand). Thermal cycling conditions were; 94 ºC for 2 min followed by 55 ºC for 45 s, 50 ºC for 30 s, 72 ºC for 2 min, repeated for 30 cycles with a final extension of 72 ºC for 7 min. PCR was run on an Eppendorf Master-Cycler. PCR products were visualised on a 1.5% agarose gel, purified using a High Pure PCR product purification kit (Roche Diagnostics) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequences generated during this work were deposited in NCBI Genbank database (refer to Table 2.1 for accession numbers).

2.3.6 Sequence alignment and phylogenetic analysis

All 16S rRNA gene sequences were aligned using Clustal W in MEGA 4 (Tamura *et al.* 2007). Pair-wise distances were calculated using the Jukes-Cantor method and pairwise deletion used to account for sequence length variation or gaps. The phylogenetic tree was

constructed using a neighbour-joining algorithm (Saitou and Nei 1987) and Tamura-Nei distance estimates. Bootstrap analyses of 1000 iterations were performed to identify the node support for the consensus tree.

2.3.7 Microcystin potential

To assess the microcystin-producing potential of each culture, amplification of a region of the *mcy*E gene was undertaken using the methods of Jungblut and Neilan (2005). PCR was carried out as described above using the HEPF and HEPR primers (Jungblut and Neilan 2005). PCR products were visualised on a 1.5% agarose gel. Amplicons of the expected length were purified and sequenced bi-directionally as previously described using the HEPF and HEPR primers. Single sequence was deposited in NCBI Genbank database under accession number GQ451434.

2.3.8 Detection of anatoxins

Sub-samples of all cyanobacterial strains were lyophilized (FreeZone6, Labconco, USA). Lyophilised material (100 mg) was resuspended in 10 ml of double distilled water (DDW) containing 0.1% formic acid and sonicated (Cole Parmer 8890, Biolab, NZ) for 15 min. Samples were centrifuged at 4000 × *g* for 10 min. The procedure was repeated a second time using 5 ml DDW and the supernatants combined. Prior to undertaking all extractions, this procedure was optimised using 1 mg of lypholized material known to contain anatoxins (Hutt River, 06/01/08). A series of five extractions using 100% methanol and DDW were undertaken. The optimum extraction was found to be two extractions using DDW, which extracted over 85% of the available anatoxins (Appendix 2).

All cyanobacterial strains were analysed for ATX, HTX and their degradation products using LC-MS. Anatoxins were separated by LC (Acquity UPLC, Waters Corp., MA) using a 50 ´ 1 mm Acquity BEH-C18 (1.7 µm) column (Waters Corp., MA). The mobile phase A (water) and mobile phase B (acetonitrile) both containing 0.1% formic acid and were used at a flow of 0.3 ml/min^{-1}, isocratic for 1 min at 100 % A followed by a rapid gradient from 100% A to 50% A / 50% B over 2 min. Injection volume was 5 µl. The Quattro Premier XE mass spectrometer (Waters-Micromass, Manchester) was operated in ESI+ mode with capillary voltage 0.5 kV, desolvation gas 900 l/h^{-1}, 400 ^{o}C, cone gas 200 l/h^{-1} and cone voltage 25 V. Quantitative analysis was by multiple reaction monitoring (MRM) using

MS-MS channels set up for ATX (166.15 > 149.1; Rt 1.0 min), HTX (180.2 > 163.15; Rt ca. 1.9 min), dihydroanatoxin-a (168.1 > 56; Rt 0.9 min), dihydrohomoanatoxin-a (182.1 > 57; Rt ca. 1.9 min), epoxyanatoxin-a (182.1 > 98) and epoxyhomoanatoxin (196.1 > 140; Rt ca. 1.9 min). The instrument was calibrated with dilutions in 0.1% formic acid of authentic standards of anatoxin-a (A.G. Scientific, CA).

2.3.9 Detection of microcystins

Cultures which showed amplicons of the correct size for the *mcy*E gene were tested for microcystins using LC-MS. Samples were analysed for the microcystin variants MC-RR, MC-didesmethyl-RR, MC- demethyl-RR, MC-LR, MC-YR, MC-desmethyl-LR, MC-FR, MC-WR, MC-AR, MC-LA, MC-LY, MC-LW and MC-LF and for nodularin. Microcystins were separated by LC (Alliance 2695, Waters Corp., MA) using a 150 × 2 mm Luna C18 5 μm column (Phenomenex, CA) with water/methanol/acetonitrile gradient containing 0.15% formic acid (0.2 ml/min^{-1}, 10 μl injection). The Quattro Ultima TSQ mass spectrometer (Waters-Micromass, Manchester) was operated in ESI^{+} mode with multiple reaction monitoring (MRM) using MS-MS channels set up for 13 microcystins and nodularin. The m/z 135 fragment from the protonated molecular cation was selected for each toxin (the doubly charged molecular species for MC-RR; the singly charged molecular species for all other toxins).

2.4 RESULTS

2.4.1 Environmental samples and strain isolation

The majority of the 32 cyanobacterial mats sampled were collected from black/green/brown leathery mats consistent with that described for *Phormidium* (Biggs and Kilroy 2000; Wood *et al.* 2007). Only two other mat types were encountered and collected; green gelatinous *Nostoc* colonies and one brittle brown filamentous mat. Preliminary microscopic observation of the leathery mats confirmed they were comprised almost entirely of filamentous *Phormidium* species. Representatives of *Oscillatoria* were observed in two of the *Phormidium* mats. Additionally, Pseudanabaenaceae were observed in at least 10 of the samples, but these species proved difficult to isolate and culture. The brittle brown mat was a proliferation of two species from Pseudanabaenaceae. Of the *Nostoc* colonies observed, only one could be cultured and described. This strain of *Nostoc* was

found growing with an *Oscillatoria* species; together they were the only species collected from geothermal water. Thirty-seven unicellular cyanobacterial strains were successfully isolated and on-grown. Within all mat samples, a mixture of diatom species were also found. *Melosira, Cymbella, Frustulia* and *Gomphonema* were the most prominent genera.

2.4.2 Microscopic characterisation of isolates

Morphological characteristics including cell dimensions, apical cell profile, cross wall configuration and reproductive structures for each isolate are given in Table 2.1. All isolates were found to have traits in common with Oscillatoriales, with the exception of VUW31, which was placed in the Nostocales.

Cells from the majority of isolates were characterised by being isodiametric or slightly shorter than wide. Cells were generally 4-8.5 µm wide. Trichomes were motile, straight with well defined apical cells with a calyptra, (Figure 2.2, *a-h*). Those isolates sharing these morphological characteristics were identified as *Phormidium autumnale* (Agardh) Trevisan ex Gomont 1892 (Komarek and Anagnostidis 2005; McGregor 2007; Wood *et al.* 2007) and assigned to Morphotype A (Table 2.1, Figure 2.2, 1-h). Twenty four strains VUW1-5, 7, 9, 11, 14, 16-24, CYN47-49, CYN52-53 and 55 were included under this designation. Morphological variation was observed among strains in apical cell morphology, cell-wall granulation, sheath presence or absence and thickness but further classification was not possible.

Stains VUW8, 10 and 12 were morphologically similar to strains from Morphotype A, but with a more discoid cell structure (width 8.4-13.2 µm; length 1.8-4.8 µm), a distinctive rounded to hemispherical calyptra and cross walls not completely forming before commencement of next cell division (Figure 2.2, *i*). Isolates were identified as belonging to the genus *Oscillatoria* possessing distinctly thickened apical cell with calyptra, no constrictions at cross walls and incomplete cross wall formation during cell division (main generic feature) (Komarek and Anagnostidis 2005). These three strains were designated to Morphotype B. The only other strain identified as *Oscillatoria* was VUW30. This strain (Morphotype C) was distinctively granular, generally straight, possessing discoid cell structure (width 8.4-15.6 µm; long 2.2-3.8 µm) and was slightly constricted at cross walls (Figure 2.3-*a*).

Morphotype D (Figure 2.3, *b*) was represented by strains CYN38 and 39, which were assigned to *Phormidium murrayi* [(West and West 1911)] (Anagnostidis & Komarek 1988, Comte *et al.* 2007) with narrow, isodiametric cells (width 3.6-4.2 µm; length 2.4-4.2 µm), a prominent sheath that was sometimes absent and conical/rounded apical cells with no calyptra.

Morphotype E was represented solely by strain VUW25 and also possessed characteristics consistent with *Phormidium* (Figure 2.3, *c*). This strain was assigned to *Phormidium* group 5 (Komarek and Anagnostidis 2005; McGregor 2007). Trichomes had slight constrictions and granulation at cross walls (defining characteristics) and cells were isodiametric or slightly shorter than wide (width 5.4-6.6 µm; length 3-5.4 µm); apical cells were rounded. There was no visible sheath.

Strain VUW13 (Morphotype F) unfortunately stopped growing in culture before a full taxonomic classification could be given; however preliminary identification assigned this to the Phormidiaceae. It was distinctive from other collected morphotyes.

Single strains from the family Pseudanabaenaceae were assigned as morphotypes G, H, I, J, all differed slightly in morphology. Strain VUW6 (Morphotype G, Figure 2.3, *d*) and VUW28 (Morphotype J) were assigned to genus *Leptolyngbya*. Morphotype G was found in tightly tangled mats. Trichomes were slightly constricted at cross walls. Cells were isodiametric (width 1.8-2.4 µm; length 1.2-2.4 µm). Apical cells were rounded/conical and sheath was absent. Strain VUW28 (Morphotype J) formed a dense tangled mat, possessing no aerotopes, having slight constriction, sheath sometimes present and conical apical cell. Strain VUW15 (Morphotype H) was the only species isolated from the brittle brown mat. The strain was found to form benthic mats, comprising aerotopes at the septa, with slight constriction at cross walls and cells 2-3 times longer than wide. Strain VUW26 (Mophotype I; Figure 2.3, *e*) was found to be from the *Pseudanabaena* genus, defined by very brittle filaments and cell wall constriction. It formed a very loose ‘soup’ of filaments.

Finally strain VUW31 was the only representative of *Nostoc*. Possessing distinctive akinetes and heterocytes this strain was identified as *Nostoc muscorum* and designated as Morphotype K (Figure 2.3, *f*).

2.4.3 Genotypic analyses and comparison with morphological designations

The 16S rRNA gene sequences (647 bp) for all 37 cultures with the exception of culture VUW30 were used to construct a neighbour joining tree, to determine the phylogenetic relationship between isolates and other cyanobacterial 16S rRNA gene sequences obtained from GenBank (Figures 4 and 5).

Twenty-seven of the cultured isolates clustered together with 100% bootstrap support (Figure 2.5). Using BlastN, all 27 sequences in this group matched at greater than 98% sequence homology with *Ph. autumnale* representatives from GenBank (All GenBank representatives were from peer reviewed publications). The 27 isolates, however, shared a sequence similarity <97%. They consisted of all strains from Morphotype A and B. Within this clade there were a number of slight genotype variations resulting in the formation of a number of sub-clades, and these aligned with the slight morphological differences observed in this group (Figure 2.5). Interestingly strains from Morphotype B were found in a larger clade including strains from Morphotype A.

Strains CYN38 and 39 (Morphotype D) which shared identical sequences clustered together in a subgroup with *Ph. murrayi* (DQ493872 and AY493627) and *Microcoleus glaciei* (formerly *Ph. murrayi,* AF218374) with 99% bootstrap support and greater than 97% sequence similarity (Table 2.1; Figure 2.4). Other strains on the sister branch were phylogenetically more distant, sharing < 93% similarity.

In contrast to the morphological analysis, strain VUW25 (Morphotype E) clustered with three *Planktothrix* sequences; *Pl. rubescens* (AB045925 and AJ132252) and *Pl. agardhii* (AB045923), (Figure 2.4), with 98% bootstrap and sequence similarity. The nearest *Phormidium* clade was distant, sharing less than 91% sequence similarity.

Strain VUW13 (Morphotype F) clustered most closely with *Microcoleus, Symploca* and *Lyngbya* species; however this was supported with only a 64% bootstrap and less than 95% sequence similarity (Table 2.1; Figure 2.4). The nearest *Phormidium* strains (*Ph. murrayi*) exhibited less than 93% sequence similarity.

With the exception of strain VUW6, strains of the family Pseudanabaenaceae could not be further defined by phylogenetic analysis (Figure 2.4). Strain VUW6 (Morphotype G) was

found to cluster with *Leptolyngbya frigida* sequences (AY493611) and *Pseudanabaena tremula* (AF218371). The remaining three strains (VUW15, 26 and 28; Morphotype H, I and J) clustered together with GenBank sequences *Arthronema gygaxiana* (AF218370), *Oscillatoria limnetica* (AJ007908), *Pseudanabaena* sp. (AM259269), and *Limnothrix redekei* (AB045929 and AJ580007) with 99% bootstrap support and greater than 98% sequence similarity.

Lastly, strain VUW31 (Morphotype K) was found to cluster with other heterocytous species and had 99% node support with *Nostoc moscorum* (AM711524).

2.4.4 Cyanotoxin analysis

Anatoxins. LC-MS analysis identified anatoxins in seven of the 32 mats (Table 2.1). All seven mats were dominated by *Phormidium*. Interestingly, ATX was detected in only two of the 37 strains (CYN52 and CYN53), while HTX and its degradation products were not detected. Both strains produced ATX at 1000 mg/kg freeze dried (FD) weight. Strains CYN52 and CYN53 were both isolated from the same environmental sample from the Rangataiki River and identified by morphology and phylogenetic analysis as *Ph. autumnale*. These two strains also formed their own clade in the phylogenetic tree sharing 100% sequence similarity (Figure 2.5).

Microcystins. The microcystin-producing potential for each strain was determined by amplification of a segment of the *mcy*E gene (~340 bp). Amplification of this region was only successful for culture VUW25. The gene segment was submitted to BlastN and matched with 94% sequence homology to a *mcy*E segment from *Pl. agardhii* (AJ441056). Microcystins analysis using LC-MS identified the microcystin variant [ASP-3]deMe MC-LR (~1 mg/kg FD weight).

Table 2.1. Origin, culture number, morphological traits, toxin content and nearest GenBank (16S rRNA gene sequence) identification match for all 37 isolates. Ph, *Phormidium*, Ty, *Tychonema*, Pl, *Planktothrix*, Mi, *Microcoleus*. +/- = Detected/not detected, NT = Not tested, dhATX = Dihydroanatoxin, dhHTX Dihydrohomoanatoxin

Culture no. and location	Cell width(µm)	Cell length (µm)	Developed apical cell	Cross walls	*mcy*E gene detected	Anatoxin, production mg/kg dry weight	Environmental, ATX/HTX production µg/kg wet weight or mg/kg DW	Accession no.	ID of nearest match (accession no.)	% ID
Morphotype A										
VUW1, Avalon duck pond	6.0 - 7.8	2.4 - 5.4	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451411	*Ph. autumnale*; DQ493873	98%
VUW2, Lake Henley	6.6 - 7.8	2.4 - 3.6	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451409	*Ph. autumnale* ; DQ493873	98%
VUW3, Wainuiomata River	4.8 - 8.4	1.8 - 3.6	Conical/Capitate/Calyptra		-	-	-	GQ451402	*Ph. autumnale* ; DQ493873	98%
VUW4, Hutt River	7.2 - 7.8	4.2 - 6.6	Conical/Capitate/Calyptra	Granular	-	-	HTX 4 mg/kg DW dhATX 53 mg/kg DW dhHTX 9 mg/kg DW	GQ451410	*Ph. autumnale* ; DQ493873	98%
VUW5, Waingongoro River	4.8 - 6.0	2.4 - 3.6	Conical/Capitate/Calyptra	Granular	-	-	-	-	*Ty. bourrellyi*; AB045897 *Ph. autumnale* ; EF654081	98% 98%
VUW7, Wainuiomata River	6.0 - 6.6	3.0 - 4.8	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451400	*Ph. autumnale* ; DQ493873	98%
VUW9, Hutt River	7.8 - 9.6	3.6 - 5.4	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451417	*Ph. autumnale* DQ493873	98%
VUW11, Pembroke Road	6.0 - 7.2	2.4 - 4.8	Capitate/calyptra		-	-	NT	GQ451408	*Ph. autumnale* ; DQ493873	98%
VUW14, Hutt River	7.2 - 8.4	2.4 - 3.6	Conical/calyptra	Granular	-	-	HTX 9 mg/kg DW dhATX 325mg/kg DW dhHTX 95 mg/kg DW	GQ451399	*Ph. autumnale*; DQ493873	98%
VUW16, Pelorous River	6.6 - 7.8	2.4 - 4.2	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451398	*Ph. autumnale* ; DQ493873	98%
VUW17, Mangatinoka Stream	6.0 - 7.8	3.0 - 4.2	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451407	*Ph. autumnale* ; DQ493873	98%
VUW18, Makarewa River	6.0 - 7.2	2.4 - 3.6	Conical/Capitate/Calyptra		-	-	-	GQ451403	*Ph. autumnale* ; DQ493873	98%
VUW19, Mangaroa River	6.0 - 7.2	3.0 - 4.8	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451416	*Ph. autumnale* ; DQ493873	98%
VUW20, Rangataiki River	6.6 - 8.4	3.6 - 4.8	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451415	*Ph. autumnale* ; DQ493873	99%
VUW21, Whakatane River	7.2 - 8.4	3.0 - 5.4	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451432	*Ph. autumnale* ; DQ493873 *Ty. bourrellyi*; AB045897	98% 98%
VUW22, Waimana River	7.2 - 8.4	3.0 - 6.0	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451406	*Ph. autumnale* ; DQ493873	98%
VUW23, Godley River	6.6 - 8.4	3.0 - 4.8	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451422	*Ph. autumnale* ; DQ493873 *Ty. bourrellyi*; AB045897	98% 98%
VUW24, Tukituki River	6.0 - 9.6	3.0 - 4.8	Conical/Capitate/Calyptra	Granular	-	-	-	GQ451426	*Ph. autumnale* ; DQ493873 *Ty. bourrellyi* ; AB045897 *Ph. autumnale* ; EF654081	98% 98% 98%
CYN47, Ashley River	6.0 -7.2	3.0 - 6.0	Conical/Capitate/Calyptra	Granular	-	-	ATX/HTX	GQ451401	*Ph. autumnale* ; DQ493873	98%

CYN48, Ashley River	6.0 -7.2	3.0 - 6.0	Conical/Capitate/Calyptra	Granular	-	-	ATX/HTX	GQ451404	*Ph. autumnale* ; DQ493873	98%
CYN49, Hutt River	5.4 - 6.6	2.4 - 4.2	Conical/Capitate/Calyptra	Granular	-	-	ATX/HTX	GQ451405	*Ph.autumnale* ; DQ493873	98%
CYN52, Rangataiki River	4.2 - 6.0	3.0 - 5.4	Conical/Capitate/Calyptra		-	ATX (1000 mg/kg)	ATX, 200ug/kg WW	GQ451424	*Ty. bourrellyi* ; AB045897	99%
CYN53, Rangataiki River	4.2 - 6.0	3.0 - 5.4	Conical/Capitate/Calyptra		-	ATX(1000 mg/kg)	ATX, 200ug/kg WW	GQ451413	*Ty. bourrellyi* ; AB045897	99%
CYN55, Roding River	8.4 - 9.6	5.4 - 6.6	Conical/Capitate/Calyptra	Granular	-	-	NT	GQ451414	*Ph. autumnale* ; DQ493873	99%
Morphotype B										
VUW8, Akatarawa River	9.6 - 13.2	1.8 - 4.2	Conical/Capitate/Calyptra		-	-	Trace levels	GQ451420	*Ph. autumnale* ; DQ493873	99%
VUW10, Wainuiomata River	8.4 -12	2.4 - 4.8	Thicken/Calyptra	Granular	-	-	NT	GQ451419	*Ph. autumnale* ; DQ493873	99%
VUW12, Wainuiomata River	9.6 - 12	2.4 - 4.2	Thicken/Calyptra	Granular	-	-	-	GQ451418	*Ph. autumnale* ; DQ493873	99%
Morphotype C										
VUW30, Waikato River	9.0 - 15.6	1.2 - 4.2	Rounded	Granular Slightly restricted	-	-	-	-		
Morphotype D										
CYN38, Red Hills tarn	3.6 - 4.2	2.4 - 4.2	Rounded/Conical	Slightly Granular	-	-	NT	GQ451428	*Ph. murrayi* ; DQ493872	98% 98%
CYN39, Red Hills tarn	3.6 - 4.2	2.4 - 4.2	Rounded/Conical	Slightly Granular	-	-	NT	GQ451429	*Ph. murrayi* ; DQ493872	98%
Morphotype E										
VUW25, Waitaki River	5.4 - 6.0	3.6 - 4.8	Rounded	Slightly restricted/Granular	+	-	-	GQ451423	*Pl. agardhii* ; AB045923 *Pl. rubescens* ; AJ132252	98% 98%
Morphotype F										
VUW13, Mangataurhiri Stream	3.6 - 4.8	3.6 - 4.8	Rounded		-	-	-	GQ451421	*Symploca* spp. ; EU249122 *Mi.paludosus* ; EF654090 *Mi. chthonoplastes* ; EF654045	94% 93% 93%
Morphotype G, H, I & J										
VUW6, Wainuiomata River	1.8 - 2.4	1.2 - 2.4	Rounded	Slightly restricted	-	-	-	GQ451430	*Pseudanabaena tremula* ; AF218371 *Leptolyngbya frigida* ; AY493611	93% 93%
VUW15, Whakatikei River	1.8 - 2.4	2.4 - 4.2	Rounded/Conical?		-	-	Trace levels	GQ451431	*Oscillatoria limnetica* ; AJ007908. *Limnothrix redekei* ; AJ580007 *Pseudanabaena* ; AM259269	99% 99% 99%
VUW26, Whakatane River	1.2	1.2 - 3.6	Calyptra	Constricted	-	NT	-	GQ451427	*Pseudanabaena* spp. ; AM259268 *Arthronema gygaxiana* ; AF218370	97% 97%
VUW28, Hutt River	1.2 - 1.8	1.2 - 3.6	Rounded/Conical	Slight constriction	-	NT	NT	GQ451412	*Pseudanabaena* spp. ; AM259268	97%
Morphotype K										
VUW31, Waikato River	3.0 - 3.6	3.6 - 4.2		Restricted	-	-	-	GQ451425	*Nostoc muscorum*; AM711524	99%

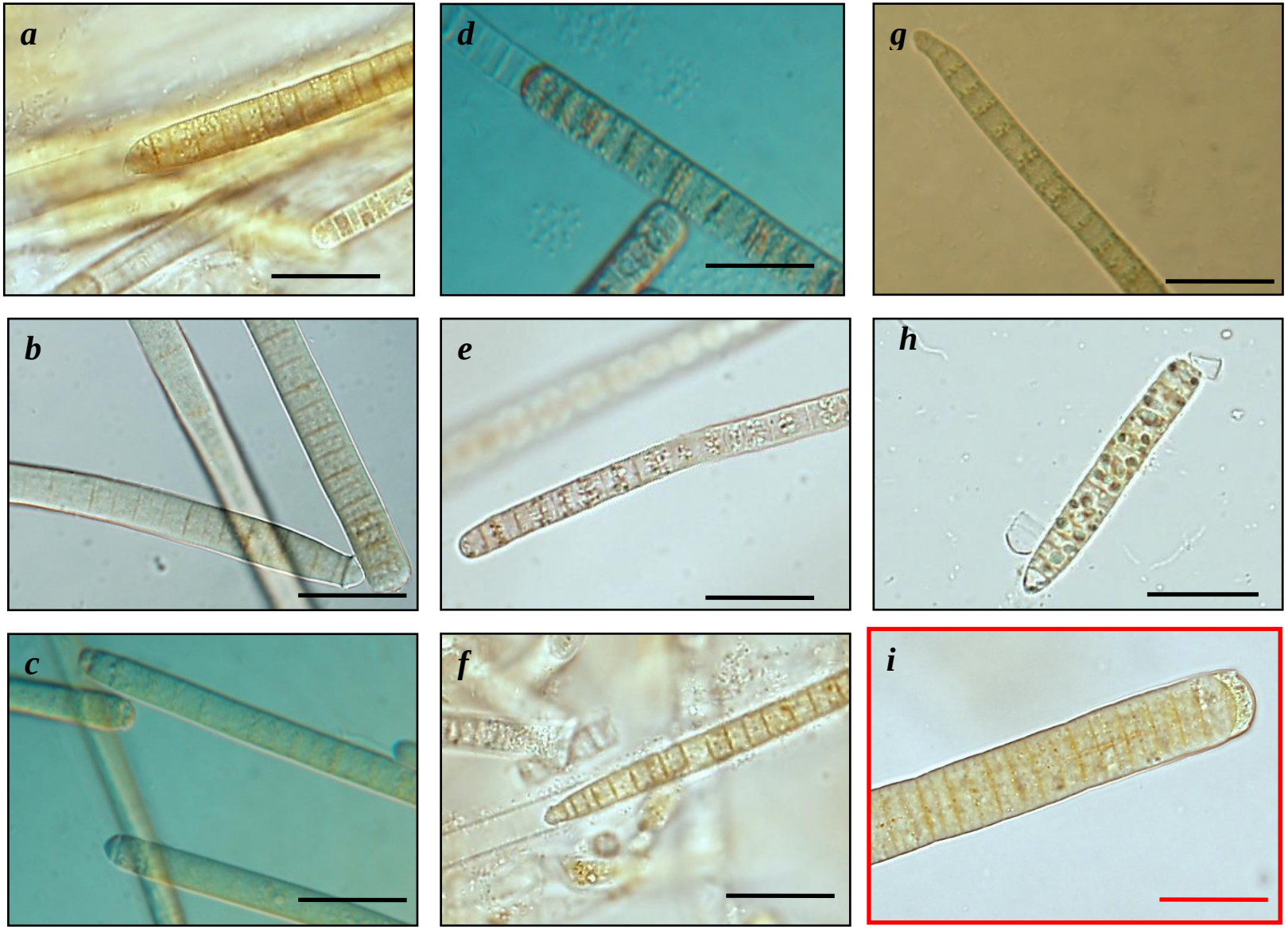

Figure 2.2: Photomicrographs of nine different *Phormidium autumnale* strains, *a-h* Morphotype A and *i* Morphotype B. (*a*) VUW18 Makarewa River, (*b*) VUW9 Hutt River, (*c*) VUW19 Mangaroa River, (*d*) CYN55 Roding River, (*e*) CYN53 Rangataiki River, (*f*) VUW1 Avalon Duck Pond, (*g*) VUW17 Mangatinoka Stream, (*h*) VUW24 Tukituki River and (*i*) VUW10 Wainuiomata River (red border, only representative from Morphotype B). All are described in Table 2.1. Scale bars 20µm.

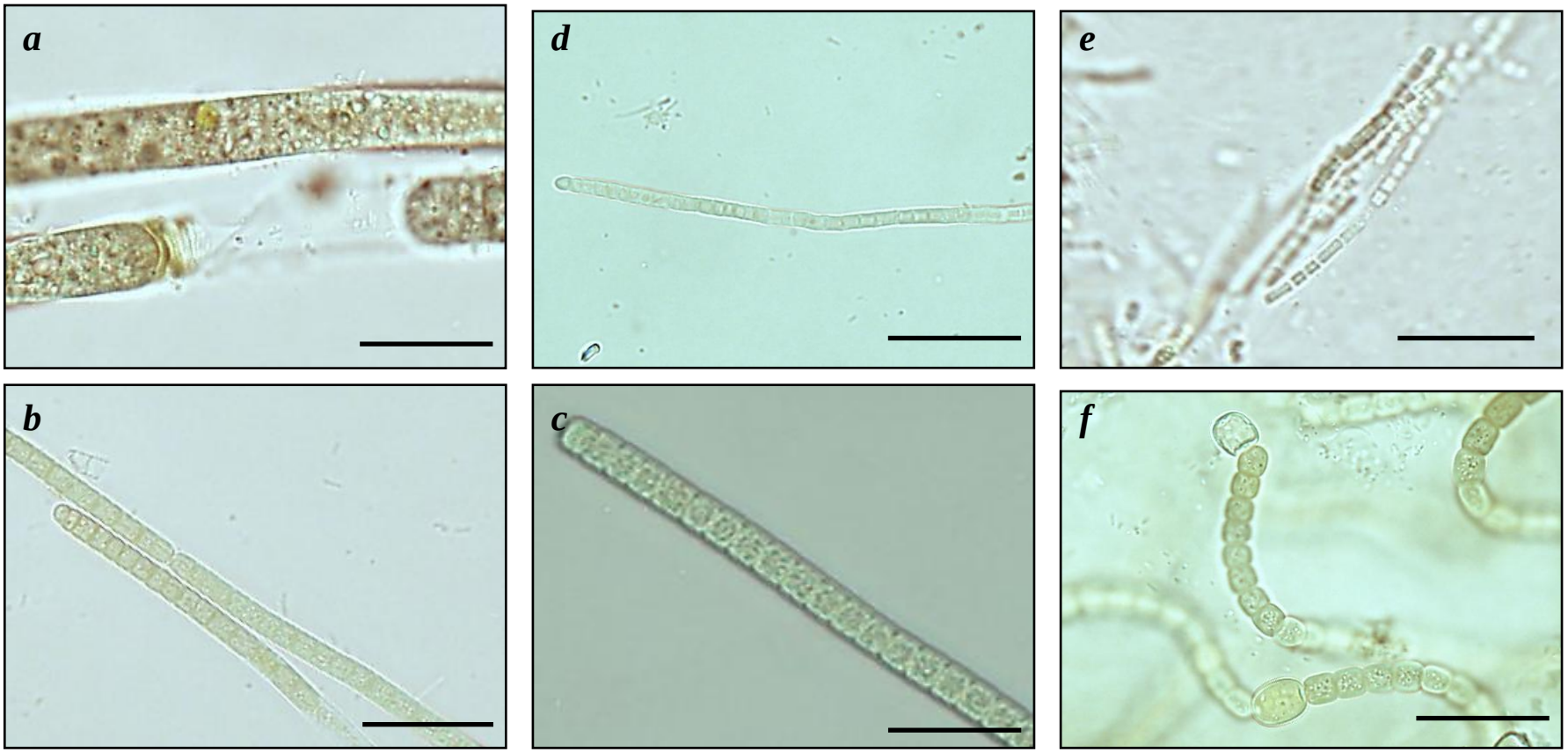

Figure 2.3: Photomicrographs of six strains from contrasting genera. (*a*) VUW30 Waikato River (Morphotype C), (*b*) CYN38 Red Hills Tarn (Morphotype D), (*c*) VUW25 Waitaki River (Morphotype E), (*d*) VUW6 Wainuiomata River (Morphotype G), (*e*) VUW26 Whakatane River (Morphotype I) and (*f*) VUW31 Waikato River (Morphotype K). Scale bars 20 µm.

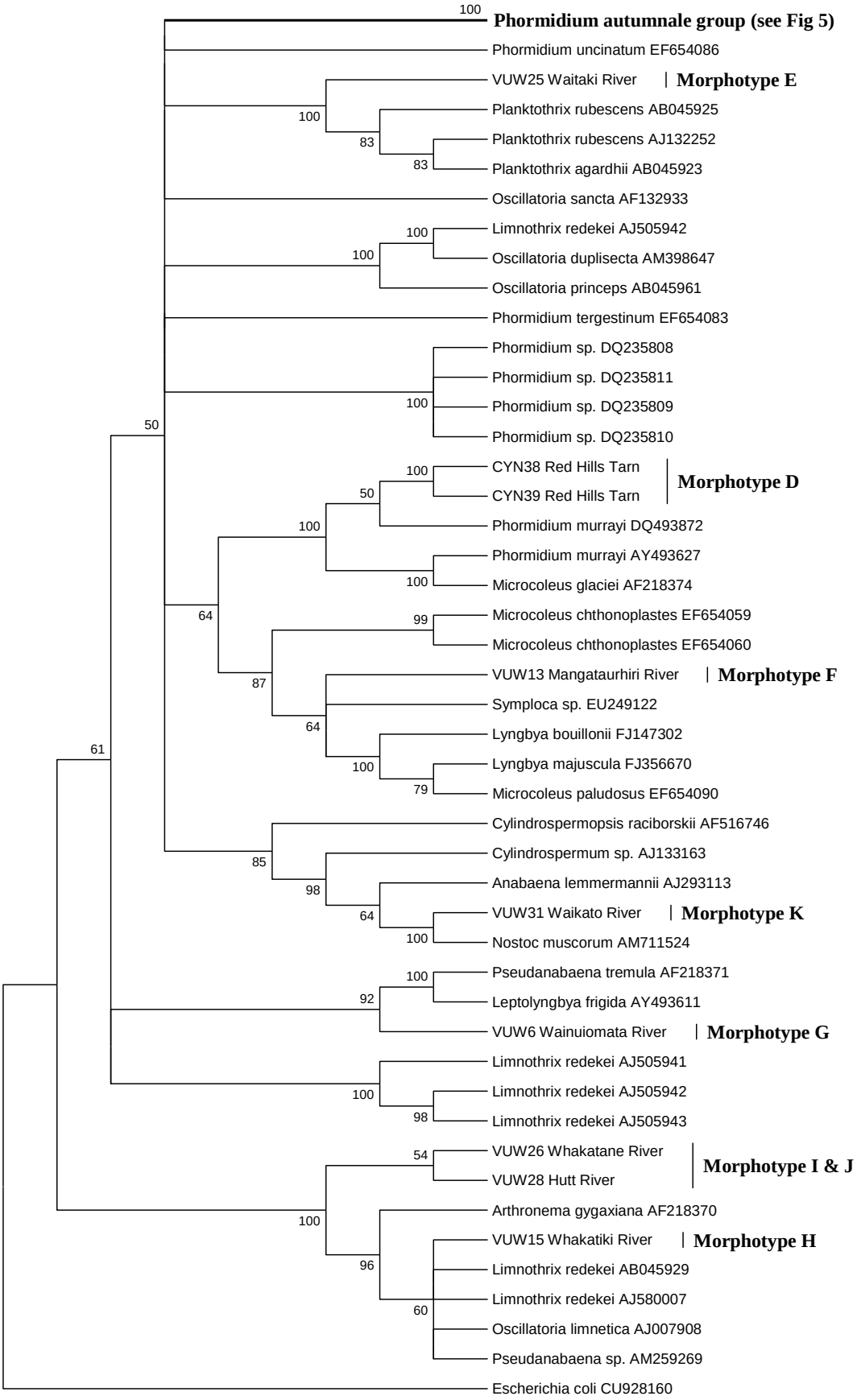

Figure 2.4. Phylogenetic tree based on the 16s rRNA gene sequences (647 bp) and obtained by the neighbour-joining method. Bootstrap values >50% are noted at the nodes. The different morphotypes in this study are noted in bold. The *Phormidium autumnale* section of the tree is shown in Figure 2.5.

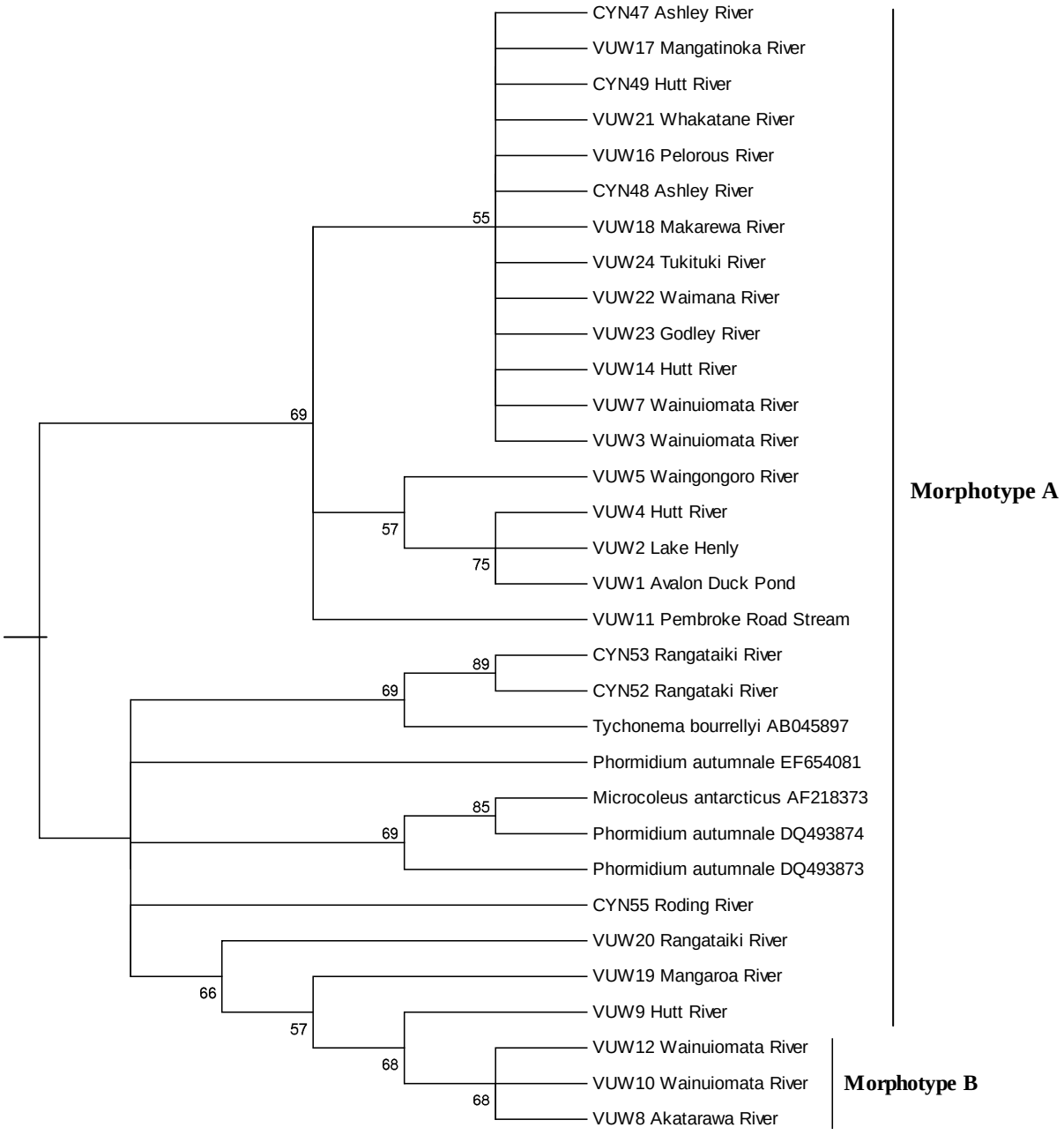

Figure 2.5. Phylogenetic tree of the *Phormidium autumnale* group based on the 16s rRNA gene sequences (647 bp) and obtained by the neighbour-joining method. Bootstrap values >50% are noted at the nodes. The different morphotypes in this study are noted in bold. The remainder of the tree is shown in Figure 2.4.

2.5 Discussion

Morphological investigation revealed the presence of 11 different morphotypes all from the Oscillatoriales and one (Morphotype K) from the Nostocales.

2.5.1 Morphotype A and B

Phormidium autumnale is known to have a cosmopolitan distribution and has been identified in many different habitats (Komarek and Anagnostidis 2005; Palinska and Marquardt 2008). It has unusually broad morphological and physiological characteristics (Palinska and Marquardt 2008). These features were also observed in this study. Morphotype A consisted of 24 isolates that were identified as *Ph. autumnale* by polyphasic assessment. These 24 isolates could not be further separated based on morphological criteria. Considerable morphological variation was however observed between isolates, but this variation was confined within the broad *Ph. autumnale* definition (Komarek and Anagnostidis 2005). Cell granulation, apical cell profile and sheath presence were all found to vary. Potentially this variation can be explained by environmental and culture conditions. Sheath production, traditionally used for systematic classification of Oscillatorialean (Anagnostidis and Komarek 1988; Komarek and Anagnostidis 2005; Palinska and Marquardt 2008), has been shown to be subject to the direct effects of both environmental and culture conditions (Rippka *et al.* 1979; Whitton 1992). Apical cell structures (in particular the calyptra), vary in trichomes of different age and are rarely seen in culture (Komarek and Anagnostidis 2005). The observed variation seen in this study is in contrast to Palinska and Marquardt (2008) who demonstrated that 10 *Ph. autumnale* subspecies showed relatively similar morphology in culture.

Intra-specific identification was therefore not possible in this group by morphology. However, the morphological heterogeneity observed in Morphotype A was represented by a number of genotype variations after phylogenetic analysis. These genotype variations resulted in the formation of 10 different clades indicating the presence of 10 distinct subspecies within this morphotype. This is consistent with Comte *et al.* (2007) who were unable to separate *Ph. autumnale* strains Arct-Ph5 and Ant-Ph68 based on morphological characters but found them to be two subspecies by genetic analysis (16S rRNA gene sequences). Strains CYN52 and 53 formed their own clade in Morphotype A and were the

only toxin producers in this lineage, indicative of their divergence from other subspecies in this group.

Unexpectedly, the three strains of Morphotype B, identified as *Oscillatoria* by morphology, formed their own clade within this *Ph. autumnale* lineage. Morphotype B has disc-like cells and a hemispherical calyptra. These features contrast with those of Morphotype A. Palinska and Marquardt (2008) found that cell size varies not only between strains but also within strains of *Ph. autumnale*. This was also found in the current study where Morphotype B possessed cell widths ranging between 8.4-13.2 µm. This is larger and with greater variation than that previously described for this species and differs markedly from Morphotype A. Apical cell structure (calyptra) furthermore has been recognised as a stable morphological structure used for distinguishing species (Komarek and Anagnostidis 2005). In this study, two distinct calyptra types were observed Morphotype A and Morphotype B (Figure 2.3). According to Komarek and Anagnostidis (2005) this suggests separation into two species.

The *Ph. autumnale* lineage consisted of all isolates from Morphotype A and B. Additionally GenBank representatives *Microcoleus* spp. and *Tychonema bourrellyi* were included in this clade (Figure 2.5). This clade was supported by 99% bootstrap support; however, together they shared less than 97% sequence homology. Previous studies have used sequence homologies of more than 97.5% as a threshold for bacterial species definition, while 95% has been used as a genus barrier for the 16S rRNA gene (Stackebrandt and Goebel 1994; Casamatta *et al.* 2005; Palinska and Marquardt 2008). This standard led Palinska and Marquardt (2008) to conclude that their two morphologically similar *Ph. autumnale* groups that shared less than 97% sequence homology may be two different species of the same genus. Thus, the 10 different clades observed in this study may represent a number of different species of the same genus. Caution however must be taken in separating species by phylogenetic analyses when using only the 16S rRNA gene. Further phylogentetic analyses of this clade using more molecular makers, such as the intergenic spacer region (ITS) between the 16S and 23S rRNA genes, is recommended and would assist in the systematic classification of this group.

GenBank additions *Microcoleus antarcticus* and *T. bourrellyi* also clustered in the *Ph autumnale* lineage. This is consistent with previous research (Willame *et al.* 2006; Comte *et al.* 2007; Palinska and Marquardt 2008). Palinska and Marquardt (2008) demonstrated the strong morphological similarities between these genera, and concluded the phenotypic criteria is too uncertain and overlapping for a clear distinction between *Ph. autumnale, T. bourrellyi* and *Microcoleus* species.

2.5.2 Morphotype D

Strains CYN38 and 39 were both identified as *Ph. murrayi* by morphologic and phylogenetic analysis. This morphotype comprises long narrow trichomes, isodiametric cells and a prominent sheath that separates it from Morphotype A and B. In the phylogenetic analysis, the two strains were found to cluster in a subgroup supported by 99% bootstrap support with three Antarctic *Ph. murrayi* strains (DQ493872 and AY493627 and *M. glacei* formerly *Ph. murrayi*, AF218374). To my knowledge this is the first recorded case of *Ph. murrayi* being isolated and described outside of Antarctica and hence dispels previous views that this morphotype is endemic to Antarctica (Casamatta *et al.* 2005). Furthermore, it raises interesting questions about the dispersal, habitat and the distribution of this species.

Recently there has been conjecture over the classification of the *Ph. murrayi*, which may cluster in the *Microcoleus* genus and not *Phormidium* (Casamatta *et al.* 2005; Comte *et al.* 2007). The sister branch to the *Ph. murrayi* clade in this study's tree comprises species from *Microcoleus* and *Lyngbya* but none from *Phormidium*. GenBank sequence homologies for morphotype D were found to align more closely with *Microcoleus* species than *Phormidium* species. These results are consistent with previous investigations (Casamatta *et al.* 2005; Taton *et al.* 2006; Comte *et al.* 2007) that show *Ph. murrayi* aligning in clades other than *Phormidium*. Casamatta *et al.* (2005) re-assigned *Ph. murrayi* to *M. glaciei* on the basis of their findings. In accordance with Comte *et al.* (2007), the genetic coincidence of our strains with those of the genus *Microcoleus* needs further clarification; however this result has now been consistent in three separate studies. Therefore this author accepts Casamatta et al, (2005) new classification of this species in the *Microcoleus* genus.

2.5.3 Morphotype E

Strain VUW25 assigned to Morphotype E is a taxonomic enigma; identified as *Phormidium* by morphology and *Planktothrix* by phylogenetic investigation. The *Planktothrix* genus description is relatively new (Anagnostidis and Komarek 1988) and includes planktonic species previously assigned to *Oscillatoria* but which contain aerotopes (Anagnostidis and Komarek 1988). This genus was subject to a comprehensive polyphasic review by Suda *et al.* (2002), where taxonomic adjustments were made that addressed the confusion in inter- and intra-specific taxonomy. Following the phylogenetic analysis in this study, strain VUW25 was found to cluster into *Planktothrix* group I (see Suda 2002). Group I consists of two different species *Pl. rubescens* and *Pl. agardhii* that differ from each other in their phycobilin pigment composition. Interestingly strain VUW25 only shared a sequence homology of 98% with members from group I, however, the 58 strains in group I of Suda (2002) shared a 99.2% sequence similarity (16S rRNA gene sequence). BlastN revealed the next closest matches (16S rRNA gene sequence) were *Pl. mougeotii* (group III) and *Pl. pseudagardhii* (group II) sharing less than 96% sequence similarity. The closest *Phormidium* sequence homology was less than 91%. Strain VUW25 was the only culture in which a 340 bp segment of the *mcy*E gene was successfully amplified. The *myc*E sequence shared a 94% sequence homology to *Pl. agardhii* (AJ133185), where as *mcy*E sequences from *Phormidium* species were found to share less than 87% sequence similarity (AY817166-8). LC-MS analyses revealed the production of microcystin. Both *Pl. rubescens* and *Pl. agardhii* are well-known microcystin producers (Henriksen 2001; Ernst *et al.* 2006).

This evidence suggests strain VUW25 is potentially a new species of *Planktotrix*; however this strain does not possess aerotopes and does is not planktonic; the fundamental criteria used to separate *Planktothrix* from *Oscillatoria* (Anagnostidis and Komarek 1988; Suda *et al.* 2002; Komarek and Anagnostidis 2005; McGregor 2007). Further analysis is needed to confirm the correct taxonomic classification for this strain.

2.5.4 Morphotypes C, F, G, H, I, J and K.

Strain VUW30 (Morphotype C) was identified as *Oscillatoria* sp. however this was solely based on morphological characterisation. Strain VUW13 (Morphotype F) identified by morphology as *Phormidium* aligns with non-*Phormidium* genera (*Lyngbya*, *Symploca* and

Microcoleus) by phylogenetic analysis. Casamatta *et al.* (2005) suggest that *Phormidium* species aligning in this non-*Phormidium* group are most likely to be from a different genus. Morphotype G, H, I and J were identified as members of the Pseudanabaenaceae. The genera from this family are not yet clearly supported by molecular analyses (Komarek and Anagnostidis 2005). This is consistent with the results seen in this study, with a number of different genera found in the same clade. Further morphological classification using a greater phenotypic criterion is needed in the analysis of these morphotypes. Strain VUW31 (Morphotype K) was the only Nostocales isolated in this study. Polyphasic investigation identified this as *Nostoc muscorum.*

2.5.5 Toxin Production

Cadel-Six *et al.* (2007) in their study of anatoxin producing benthic cyanobacteria, found that *Phormidium* strain Fil.2Da FY produced ATX even though it was isolated from an apparently non-toxic sample. They concluded this result may be due to the both toxic and non-toxic representatives of the same phenotype occurring at a single site and fortuitously they were able to isolate a filament that proved to be a toxin producer. In this study, *Ph. autumnale* showed a large radiation of subspecies, and it is possible many of these subspecies are present in a single mat proliferation. Therefore the chance of obtaining a toxin-producing strain is dependent on the filament isolated and its relative abundance in the mat. This is consistent with Vezie et al; (1998) who found blooms of planktonic microcystin-producing cyanobacteria are comprised of toxic and non-toxic strains.

LC-MS screening of the environmental mat samples identified ATX and/or HTX in nine samples, all of which were dominated by *Ph. autumnale*. However, ATX was only detected in CYN52 and 53 (Morphotype A). These two strains were both isolated from a cyanobacterial mat that had been collected in response to a dog neurotoxicosis on the Rangataiki River.

In routine testing of *Phormidium* mats around New Zealand it has been found that the occurrence, concentration and variants of anatoxins are unpredictable (S Wood, pers. comm.). This study demonstrated that not all strains of *Ph. autumnale* are capable of ATX/HTX production. The inability to isolate ATX/HTX producing strains from mats that were shown to contain ATX/HTX, coupled with the identification of a number of different

genotypes of *Ph. autumnale* provides further evidence to support the hypothesis that mats are comprised of multiple subspecies.

Previous research on planktonic cyanobacteria has shown that cyanotoxin concentrations can increase with favourable physicochemical factors, but generally by no more than a factor of five (Rapala *et al.* 1993; Rapala and Sivonen 1998; Sivonen and Jones 1999). This is significantly less that the variation in ATX/HTX concentrations observed in environmental samples collected within New Zealand (refer to chapter 2). In this study, HTX was not produced by any of the isolates even though it was detected in the initial screening of mats (Table 2.1). The presence of ATX or HTX within a mat therefore indicates the presence of morphospecies that are capable of producing it. Further research into mat community assemblage and diversity is required to test the validity of these explanations.

VUW25 was found to contain the *mcy*E gene and produced microcystin variant [ASP-3]deMe MC-LR. Although microcystin production by benthic cyanobacteria in New Zealand has been previously identified (Hamill 2001; Wood 2004), this is the first time the causative cyanobacterium has been isolated and the microcystin variant identified. This cyanobacterium was isolated from an environmental sample collected at the mouth of the Waitaki River where a dog died rapidly after ingesting cyanobacterial mat material. This is the first confirmation of a dog death from microcystins in New Zealand, although benthic species that produce microcystins are known to have killed animals elsewhere (e.g., Mez *et al.* 1997).

2.6 Conclusions

The results of this study revealed that *Ph. autumnale* is the predominant cyanobacterium in benthic mat proliferations in New Zealand rivers. Polyphasic analysis shows that these mats are comprised of a number of different *Ph. autumnale* subspecies and that only certain subspecies produce anatoxins. The anatoxin-a producing *Ph. autumnale* strain identified in this study was unique at the 16S rRNA gene sequence level. Isolation of further toxic strains would be required to assess whether anatoxin production is limited to only this strain across New Zealand and to investigate if this difference could be used in the development of a molecular based diagnostic tool. The marked morphological variation

observed within *Ph. autumnale* highlights the difficulties of identifying cyanobacteria from the Oscillatoriaceae to species level based solely on morphology. The use of a polyphasic approach is recommended. However, the number of 16S rRNA gene sequences currently deposited in worldwide databases is limited and larger scale systematic projects will be required to fully resolve current taxonomic uncertainties. This study led to the identification of a potentially novel cyanobacterium, closely related to *Planktothrix* sp, that produces microcystins. It was linked to the first confirmed dog death from a benthic microcystin-producing species in New Zealand. This reemphasises the paucity of information currently available on the diversity and toxin capabilities of New Zealand benthic cyanobacteria.

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3

Spatial and temporal variability in *Phormidium* abundance and anatoxin production in the Hutt and Wainuiomata Rivers, New Zealand

3.1 Abstract

Toxic benthic cyanobacteria proliferations in freshwater systems are becoming more prevalent worldwide. As a result animal poisonings are being reported with greater regularity. Few studies have investigated spatial distribution of freshwater mat-forming cyanobacteria, their toxin production and concentrations and how they change over time. As a result, little is understood of the physicochemical parameters affecting their distribution, growth and toxin production. The Hutt and Wainuiomata rivers (Wellington, New Zealand) are renowned for their anatoxin producing *Phormidium* blooms. These two relatively pristine rivers were monitored either weekly or fortnightly for benthic cyanobacterial growth, anatoxin production and a variety of physciochemical parameters over the course of a year (2008). Cyanobacterial proliferations and associated anatoxin production were found to be spatially and temporally variable across the eight different sampling sites. Anatoxin (anatoxin-a and homoanatoxin-a and their degradation products dyhydroanatoxin-a and dyhydrohomoanatoxin-a) presence, and concentrations were also found to be highly variable across all sites and over time. Higher cyanobacterial abundances (75% coverage) however, were observed in the summer months correlating significantly with temperature. Increases in river flow played a significant role in the removal of benthic mats. Anatoxin production was also generally restricted to those warmer summer months; however anatoxin production and concentration was not correlated to cyanobacterial abundance. The concentrations of water-soluble nutrients were not significantly correlated with benthic cyanobacterial abundance. The ability of benthic non-heterocytous *Phormidium* to source nutrients from bottom substrate is likely to give this organism a competitive advantage in low nutrient (oligotrophic) environments.

Keyword: anatoxin, benthic cyanobacteria, nitrogen, *Phormidium*, phosphorus and river flow

3.2 Introduction

Toxic cyanobacterial proliferations causing animal fatalities have been documented in fresh and brackish waters worldwide (Lawton and Codd 1991; Kuiper-Goodman *et al.* 1999; Azevedo *et al.* 2002). These fatalities have commonly been linked to the ingestion of toxic planktonic cyanobacteria (Carmichael 1994; Negri *et al.* 1995; Saker *et al.* 1999). Recently there have been an increasing number of incidents of animal toxicosis linked to benthic cyanobacteria (Edwards *et al.* 1992; Henriksen *et al.* 1997; Mez *et al.* 1997; Hamill 2001; Gugger *et al.* 2005; Cadel-Six *et al.* 2007; Wood *et al.* 2007b). The physical, chemical and biological parameters leading to planktonic cyanobacterial blooms have been extensively studied (e.g., Oliver and Gnaf 2000), as have variables regulating the amounts of toxin produced by planktonic species (Sivonen and Jones 1999). In contrast, research on benthic cyanobacteria has resulted largely in response to animal toxicosis and focused on characterising the toxin, its symptoms and the causative cyanobacterium (Cadel-Six *et al.* 2007; Wood *et al.* 2007b). There is a limited understanding of mechanisms leading to benthic cyanobacterial proliferations and the influences of environmental variables on regulating toxin production in these species.

Benthic cyanobacteria produce most of the known cyanotoxins e.g., microcystins, (Mez *et al.* 1997), saxitoxins (Carmichael *et al.* 1997) and cylindrospermopsins, (Seifert *et al.* 2007b). In New Zealand, anatoxin-a (ATX) and homoanatoxin-a (HTX) are the most commonly detected cyanotoxins. These are powerful neuromuscular blocking agents acting through the nicotinic acetylcholine receptor. In affected animals they can cause convulsions, coma, rigors, cyanosis, limb twitching, hypersalivation and/or death. Research on the regulation of anatoxin-a in planktonic species has revealed toxin production varies among species and with different physicochemical factors e.g., temperature, light and phosphorus (Rapala *et al.* 1993; Rapala and Sivonen 1998). Currently, no research has looked at anatoxin regulation and production in benthic mat-forming cyanobacteria despite the evident risk posed to human and animal health.

In November 2005 at least five dogs died rapidly after contact with water from the Hutt River (lower North Island, New Zealand). Dense mats of *Phormidium autumnale* were found in the river and anatoxin-a and homoanatoxin-a were identified in the mats and dog stomach contents (Milne and Watts 2007; Wood *et al.* 2007b). Increased monitoring of cyanobacteria abundance in subsequent summers found extensive coverage of *Phormidium* throughout the middle and lower reaches of the river. Three further cyanobacteria related

dog deaths were recorded in January of 2008. The Hutt River stretches 54 km south through the Hutt Valley and discharges into Wellington Harbour (Figure 3.1). It runs through several populated areas and has over 1 million recreational visits each year. In its upper reaches the river is used to provide the wider Wellington metropolitan area with up to 40% of its potable water. *Phormidium* mats are also present in other rivers in the region (Milne and Watts 2007; Wood *et al.* 2007b). The Wainuiomata River is known for high levels of benthic cyanobacteria in its middle reaches, however at its head waters this river has some of the most pristine water in New Zealand. The Wainuiomata river to the east of the Hutt River provided an ideal comparative study site (Figure 3.1).

In this study, six sites along the Hutt River and its tributaries and two sites on the Wainuiomata River were surveyed fortnightly (weekly during periods of high mat abundance) for one year to assess the percentage of cyanobacterial mat coverage at each site. Samples were collected for species identification and ATX and HTX analysis. Monthly water samples were taken for nutrient analysis at each site while river flow and temperature were logged continuously at different locations along each river. Percentage cover and community composition data were used in concert with physicochemical measurements to elucidate parameters resulting in cyanobacterial mat proliferation and ATX and HTX production.

3.3 Methods

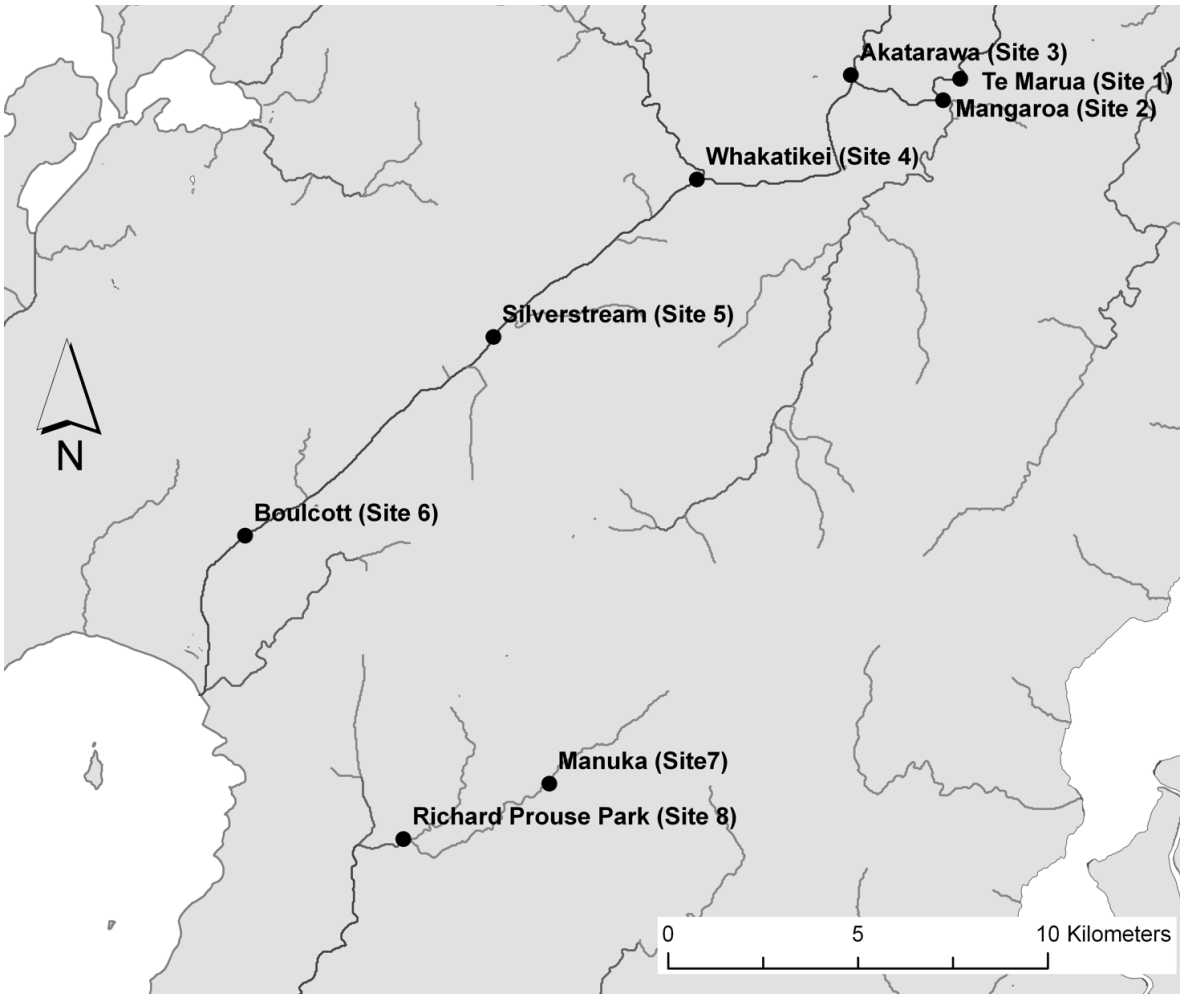

Figure 3.1. Locations of sampling sites. Sites 1-6 are located in the Hutt River catchment, and sites 7 and 8 in the Wainuiomata River catchment, Wellington, New Zealand. Note; sites 2-4 are all tributaries of the Hutt River.

3.3.1 Site descriptions

All sites are monitored by Greater Wellington Regional Council (GWRC) as part of the Rivers State of the Environment (RSoE) monitoring programme. Site 1 is the furthest upstream on the Hutt river (Figure 3.1). It is located a short distance from where the river emerges from its gorge, and its upstream catchment includes the forested area of Kaitoke regional park as well as some rural land use. Although the surrounding margins are well-forested, the sampling site was not shaded due to the large areas of rock cover either side of the river. This site was found to have the highest water quality in the Wellington region from using a Water Quality Index (WQI) to summarise water quality based on six variables: visual clarity (black disc), dissolved oxygen (% saturation), dissolved reactive phosphorus, ammoniacal nitrogen, nitrate nitrogen and faecal coliforms (Perrie 2007). The Mangaroa River site (site 2) is a tributary of the Hutt River and is located at the Hutt River confluence (Figure 3.1). Despite the site being well shaded the WQI rated water quality as

poor (Poor: median values for <3 of the 6 variables comply with guideline values) (Perrie 2007). The Mangaroa river experiences an average river flow of 3 m^3/s. Site 3 is located on the Akatarawa River at the Hutt River confluence (Figure 3.1). The site has a well-developed riparian margin, offering some shading. This site had excellent (excellent = median values for all 6 variables comply with guideline values) water quality as judged by the WQI (Perrie 2007). The Whakatikei River site (site 4) is situated at the Hutt River confluence (Figure 3.1). This site is well shaded rating excellent for water quality by the WQI (Perrie 2007). The Silverstream site (site 5) is located on the middle reaches of the Hutt River (Figure 3.1), and is heavily modified with boulder walls employed to stop erosion and flooding (Appendix 1, photo 32) This has resulted in uniform hydraulic characteristics. The site has a very thin riparian margin and was also rated as excellent by the WQI (Perrie 2007). The river at this location has an average river flow of 25m^3/s. The Boulcoutt site (site 6) is located on the lower reaches of the Hutt River (Figure 3.1), and has undergone extensive flood protection modification with the implementation of stop banks and intense gravel extraction in this heavily populated region. This modification has also resulted in this site having uniform hydraulic characteristics. The site was also rated as excellent by the WQI for 2003 to 2006 (Perrie 2007). The Manaka Track site (site 7) is located in the upper reaches of the Wainuiomata River which is a water catchment reserve where public access is prohibited (Figure 3.1). This site is well-shaded and located in the foothills of the Rimutaka Range. This portion of the Wainuiomata River is crystal clear and provides the Wellington metropolitan area with 15% of its drinking water. This site rated as good (median values for 5 of the 6 variables comply with guidelines) by the WQI (Perrie 2007) .The Richard Prouse Park site (site 8) is located in the middle reaches of the Wainuiomata river (Figure 3.1). The site is located alongside a sport playing field with no riparian margins and therefore experiences no shading. This site is not a RSoE monitoring site and therefore has not been characterised using the WQI. All eight sites of the Hutt and Wainuiomata catchments have a coarse greywacke substrate; a particularly hard sedimentary rock.

3.3.2 Data and sample collection

At each site, weekly cyanobacterial mat and periphyton cover was measured in three 1 m^2 quadrats that were randomly positioned within a larger 10 m^2 quadrat within a run (a swiftly flowing region of river with a relatively smooth surface). Monthly coverage measurements were also collected at the Hutt river for the first 6 months for both pool (a deep, slow moving region of a river, usually with fine substrate, often containing eddies)

and a riffle (shallow water, where the surface is broken into ripples or waves by totally or partially submerged obstructions). Extensive flow modification in the lower reaches of the Hutt river resulted in only run habitat being used for coverage measurements at sites 5 and 6. Changes in river flow and level during the sampling period at site 3 meant that the pool habitat was very hard to distinguish from run habitat. Cyanobacterial mat coverage data was collected only for run habitats at the Wainuiomata sites, due to the difficulty in differentiating between pools, riffles and runs in the upper reaches of this small river.

Cyanobacterial mats were collected at each site for anatoxin testing and morphological identification. Samples were collected by scraping mat material into sterile plastic screw-cap bottles (50 ml, Biolab, New Zealand). All samples were placed on ice for transport. On arrival at the laboratory, samples were frozen (-20 °C) for toxin analysis or preserved using Lugol's Iodine for morphological identification. Continuous river flow data and temperature for sites 1 to 7 were obtained from GWRC. Continuous readings for site 4 were obtained upstream of the sampling site by 10km, while continuous readings for sites 5 and 6 are measured in between them. Greater Wellington measures water level at these locations according to national hydrometric standards, and converts the water level to flow data using rating curves. At sites 1-3 and 5-6, water temperature was monitored continuously by GWRC using either Campbell C107 or Unidata LM34 temperature sensors. Water samples were collected monthly at every site, with the exception of site 8, to assess nitrate-N, nitrite-N, total nitrogen, total Kjeldahl nitrogen, dissolved reactive phosphorus and total phosphorus, this totalled 15 samples for each site. Samples for dissolve nutrients were filtered through 45 µm Whatman GF/C glass microfibre filters. Samples were frozen at -20˚C until further analyses.

3.3.3 Species identification, composition and abundances

Sub-samples of the preserved field samples were homogenised and allowed to settle in a cavity slide (1 ml) where species identification, abundances and composition were determined under an Olympus (CKX41) inverted microscope. Species Identifications were made primarily by reference to Komárek and Anagnostidis (2005), McGregor (2007) and Biggs and Kilroy (2000).

3.3.4 Extraction and detection of anatoxins

Frozen mat material from each site and sampling date was lyophilized (FreeZone6, Labconco, USA). Lypholized material (100 mg) was resuspended in 10 ml of double distilled water (DDW) containing 0.1% formic acid and sonicated (Cole Parmer 8890, Biolab, Auckland, NZ) for 15 min. Samples were centrifuged at 4000 × *g* for 10 min. The procedure was repeated a second time using 5 ml DDW and the supernatants combined (see Appendix 2 for optimisation).

All samples were analysed for ATX, HTX and their degradation products dihydroanatoxin (dhATX), dihydrohomoanatoxin (dhHTX), epoxyanatoxin-a (epATX) and epoxyhomoanatoxin-a (epHTX) using Liquid chromatography-mass spectrometry (LC-MS). Anatoxins were separated by LC (Acquity UPLC, Waters Corp., MA) using a 50´ 1 mm Acquity BEH-C18 (1.7 µm) column (Waters Corp., MA). Mobile phase A (water) and mobile phase B (acetonitrile) both containing 0.1% formic acid, were used at a flow of 0.3 $ml.min^{-1}$, isocratic for 1 min at 100 % A followed by a rapid gradient from 100% A to 50% A / 50% B over 2 min. Injection volume was 5 µl. The Quattro Premier XE mass spectrometer (Waters-Micromass, Manchester) was operated in ESI+ mode with capillary voltage 0.5 kV, desolvation gas 900 $l.hr^{-1}$, 400 ^{o}C, cone gas 200 $l.hr^{-1}$ and cone voltage 25 V. Quantitative analysis was by multiple reaction monitoring (MRM) using MS-MS channels set up for ATX (166.15 > 149.1; Rt 1.0 min), HTX (180.2 > 163.15; Rt ca. 1.9 min), dhATX (168.1 > 56; Rt 0.9 min), dhHTX (182.1 > 57; Rt ca. 1.9 min), epoxyATX (182.1 > 98) and epoxyHTX (196.1 > 140; Rt ca. 1.9 min. The instrument was calibrated with dilutions in 0.1% formic acid of authentic standards of anatoxin-a (A.G. Scientific, CA).

3.3.5 Nutrient analyses

Filtered nutrients were analysed on a Lachat QuickChem FLOW Injection Analyser (FIA + 8000 Series, Zellweger Analytics, Inc.) and a Konelab Aquakem 600 Discrete Analyser (Thermo Scientific). Nitrite-N was analysed using automated AZO dye colorimetry, and total oxidised nitrogen (nitrate-N + nitrite-N) was analysed using automated cadmium reduction following the methods of the American Public Health Association (Eaton *et al.* 2005). Total Kjedahl nitrogen was digested in sulphuric acid and analysed using phenol/hypochlorite colorimetry (Eaton *et al.* 2005). Nitrate was calculated by subtracting nitrite-N from total oxidised nitrogen (nitrate-N + nitrite-N), while total nitrogen was calculated by the addition of total kjedahl nitrogen, nitrite-N and nitrate-N. Total

phosphorus was digested with acid persulphate before using ascorbic acid colorimetry (Eaton *et al.* 2005). Dissolved reactive phosphorus was analysed using molybdenum blue colorimetry (Eaton *et al.* 2005).

3.3.6 Statistics

Spearman's non-parametric rank correlation coefficients were used to measure the correlation between physicochemical parameters and cyanobacterial mat cover for all sites. No nutrient data was collected for site 8; therefore no correlations could be conducted for this site. At site 2 considerable diatom coverage was observed; this was included in Spearman's correlations for this site. Additionally, the data from all six Hutt river sites were pooled (n=90) and a Spearman's correlation conducted to establish overall trends potentially contributing to cyanobacterial abundance. Correlations were considered significant at the 95% (P<0.05) confidence interval. River flow was found to be a significant parameter leading to the removal of benthic cyanobacteria and considerably altering other physicochemical parameters, therefore distorting those possible parameters that could be controlling cyanobacterial growth. To control for this, Spearman's correlation was also performed on a subset of the data where cyanobacteria were present, therefore eliminating the effect of river flow. Temperature, the resulting significant parameter, was entered into a step-wise linear regression model to ascertain its contribution to variation in cyanobacterial mat abundance. Spearman's correlation was used to examine possible correlations between anatoxins concentration and physicochemical parameters. To control for the effect of river flow Spearman's analysis was undertaken only for those data points where anatoxins were detected.

Stepwise logistic regression was used to predict the probability of the presence or absence of cyanobacteria from the pooled data of all six Hutt river sites. All nutrients, river flow and temperature were entered into the model. Binary predicatives were improved by decreasing the default of 0.5 probability to a cut value equal to the prevalence of presences in the data, i.e. to 0.33. Thereby, decreasing the probabilities of false negatives (cyanobacteria predicted to be absent when in reality they were present).

For water temperature and river flow (m^3/s), a five-day average prior to sampling was used to correlate to each sampling point. Standardisation was achieved by dividing the five-day average river flow by the yearly average river flow. The combined total of ATX, HTX,

dhATX and dhHTX were used. All data was analysed using *Statistical Analysis Software*, version 16.0. (SPSS 2007).

3.4 Results

3.4.1 Cyanobacterial mat abundance in different habitats

At sites 1-4 where percentage cover of cyanobacterial mats was monitored in a pool, run and riffle, mats were observed in all three habitats, with the exception of site 1 that had experienced no cyanobacterial mat growth in the pool habitat (Figure 3.2). Mat abundance varied at each site within the three different river environments. In general pools had the lowest percentage cover of cyanobacteria, with only site 3 having consistent growth. The highest percentage cover of cyanobacterial mats was observed at site 2 in a pool environment on the 8th of December 2007, where cover was 90%. Cyanobacterial percentage cover tended to be highest in riffles and runs across all sites (Figure 3.2). Riffles were generally found to have higher percentage cover than runs, although at site 3, runs had higher cover than riffles in approximately 50% of the observations. Furthermore, with the exception of the pool observations in December 2007 and January 2008 at site 3, on multiple occasions cyanobacterial mats were present only in riffles at sites 1, 3 and 4 and once at site 2. The highest riffle and run coverage were 75% (23rd of January 2008) and 70% (6th of January 2008) respectively, both observed at site 2.

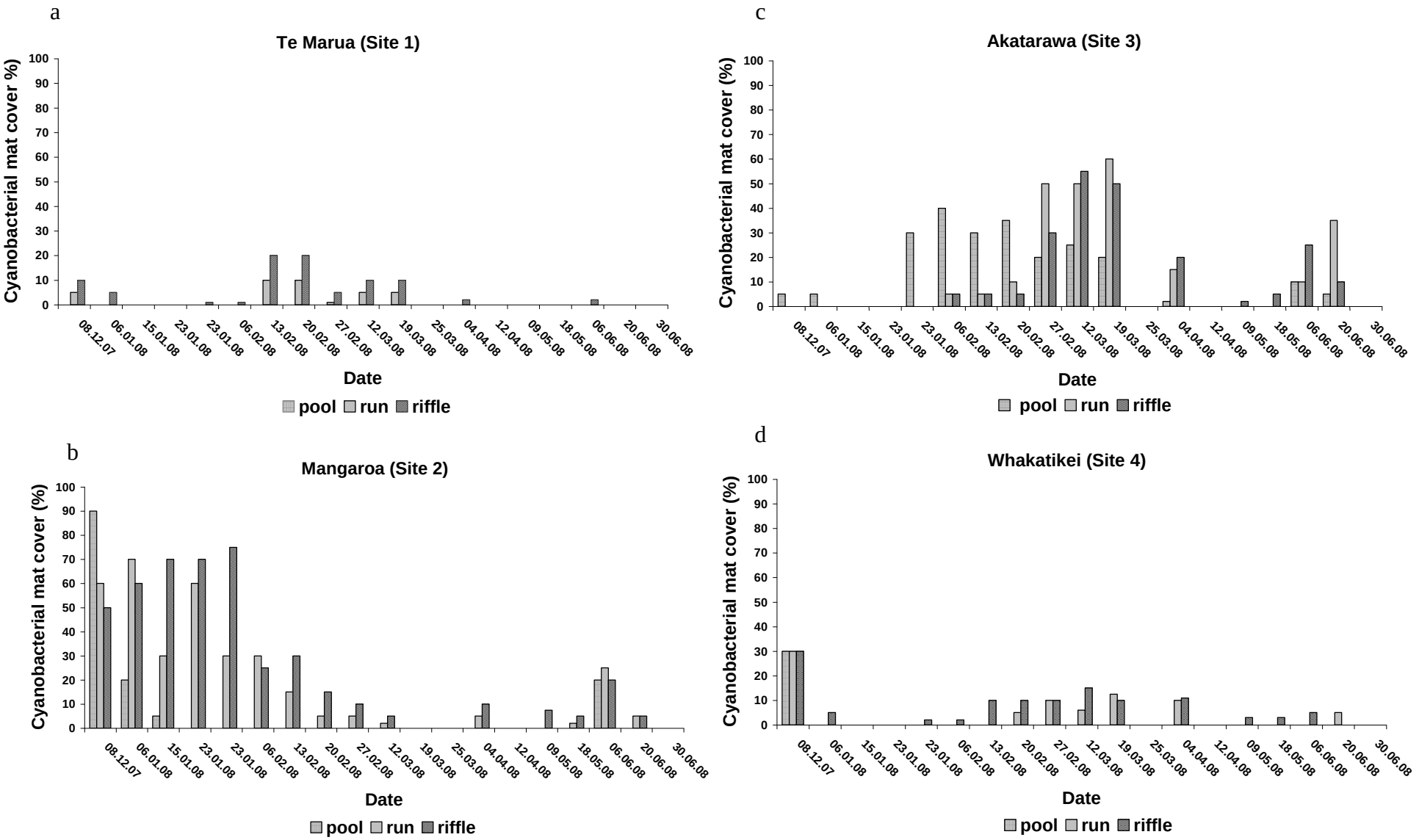

Figure 3.2 a, b, c & d: Cyanobacterial mat cover for four sampling sites on the Hutt River, New Zealand in three different river habitats; pool, run and riffle. a – site 1 (Te Marua), b- site 2 (Mangaroa), c- site 3 (Akatarawa), d- site 4 (Whakatikei)

3.4.2 Temporal and spatial variability in cyanobacterial mat abundance

Cyanobacterial mats were observed at all eight study sites. The dominant genus in these mats was *Phormidium*. Occasionally, species from the Pseudanabaenaceae were observed in low concentrations in the samples. Although infrequently observed growing in the winter months (between May and October 2008), cyanobacterial mat growth was generally restricted to summer months (Figure 3.2 a, b, c & d). This correlated with periods of stable river flow and warmer water temperatures

Percentage cyanobacterial mat cover was recorded at over 50% at four of the six (sites 2, 3, 5 and 6) Hutt river sites, with the highest abundance (70%) observed at sites 2 and 5 on 6th of January 2008 and 27th of February 2008 respectively. At site 1 cyanobacterial mats were observed from initial sampling to the 6th of January 2008 (Figure 3.3). Mats were not observed again until late February 2008, when the maximum percentage cover was less than 10%. Site 2 had extensive cyanobacterial mat cover at the time of initial sampling (peaking at 70% on the 6th January 2008), this steadily decreased until there was no cyanobacteria observed on the 26th of March 2008 (Figure 3.4). Cyanobacterial growth was observed up to 25% in May and June 2008 and then no cover was observed until November 2008. No cyanobacterial mats were observed at site 3 in January and only minimal growth (<15%) in February (Figure 3.5). In March 2008 cyanobacterial mat cover peaked at 60%, however, this was observed to be patchy in April and May 2008 before consistent growth was again observed from September to December 2008 (Figure 3.5). Site 4 experienced peak cyanobacterial cover in December 2007 (30%); patchy and low abundance cyanobacterial cover was then observed throughout January to March 2008 (<10%) (Figure 3.6). Low cyanobacterial mat cover was observed once in April and June 2008 respectively with no further growth until December 2008. Consistent cyanobacterial cover was observed at site 5 from December 2007 to the end of March 2008, for the majority of this time percentage cover was <40% (Figure 3.7). With the exception of cyanobacterial cover observed in June 2008, there was no more recorded until November 2008. Growth at site 6 with the exception of December 2007 and early January 2008 where cyanobacterial abundance peaked at 60%, was patchy and at low abundances (<10%) from late January 2008 to June 2008 and then October to December 2008 (Figure 3.8). The most upstream site on the Wainuiomata river (at Manuka Track, site 7), experienced no cyanobacterial cover until February 2008; consistent coverage was then observed until the end of March 2008 where it peaked at 15% (Figure 3.9). No further cyanobacterial coverage was observed at this site. Cyanobacterial cover at site 8 was patchy with only consistent cover observed from late January 2008 to early March 2008. No cyanobacterial mat cover was observed after June 2008 (Figure 3.10)

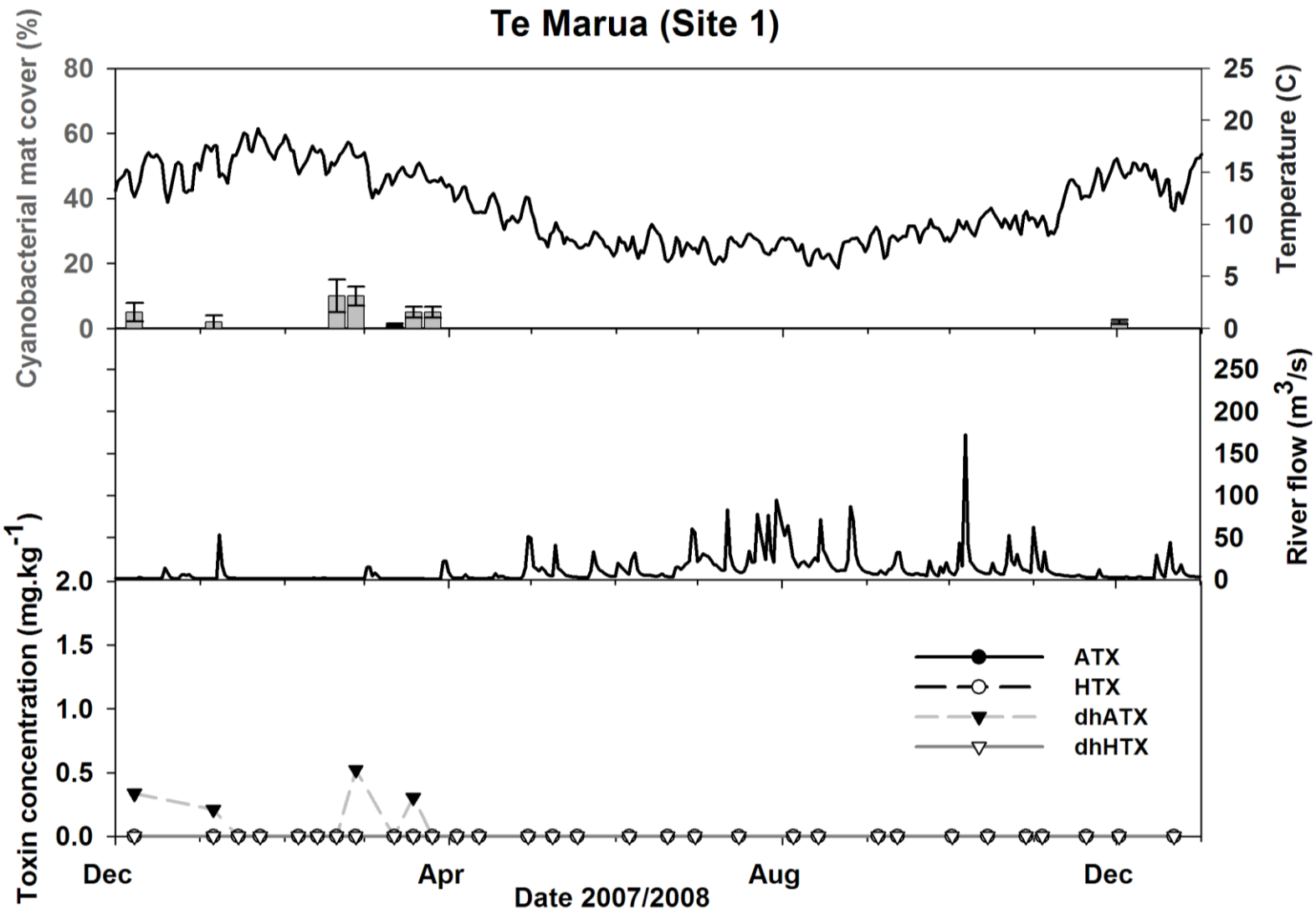

Figure 3.3: Percentage cyanobacterial mat coverage, temperature, river flow and anatoxin concentrations for Te Marua (site 1), Hutt River, New Zealand, between the 1st of December 2007 and the 1st of January 2009. Error bars represent standard error.

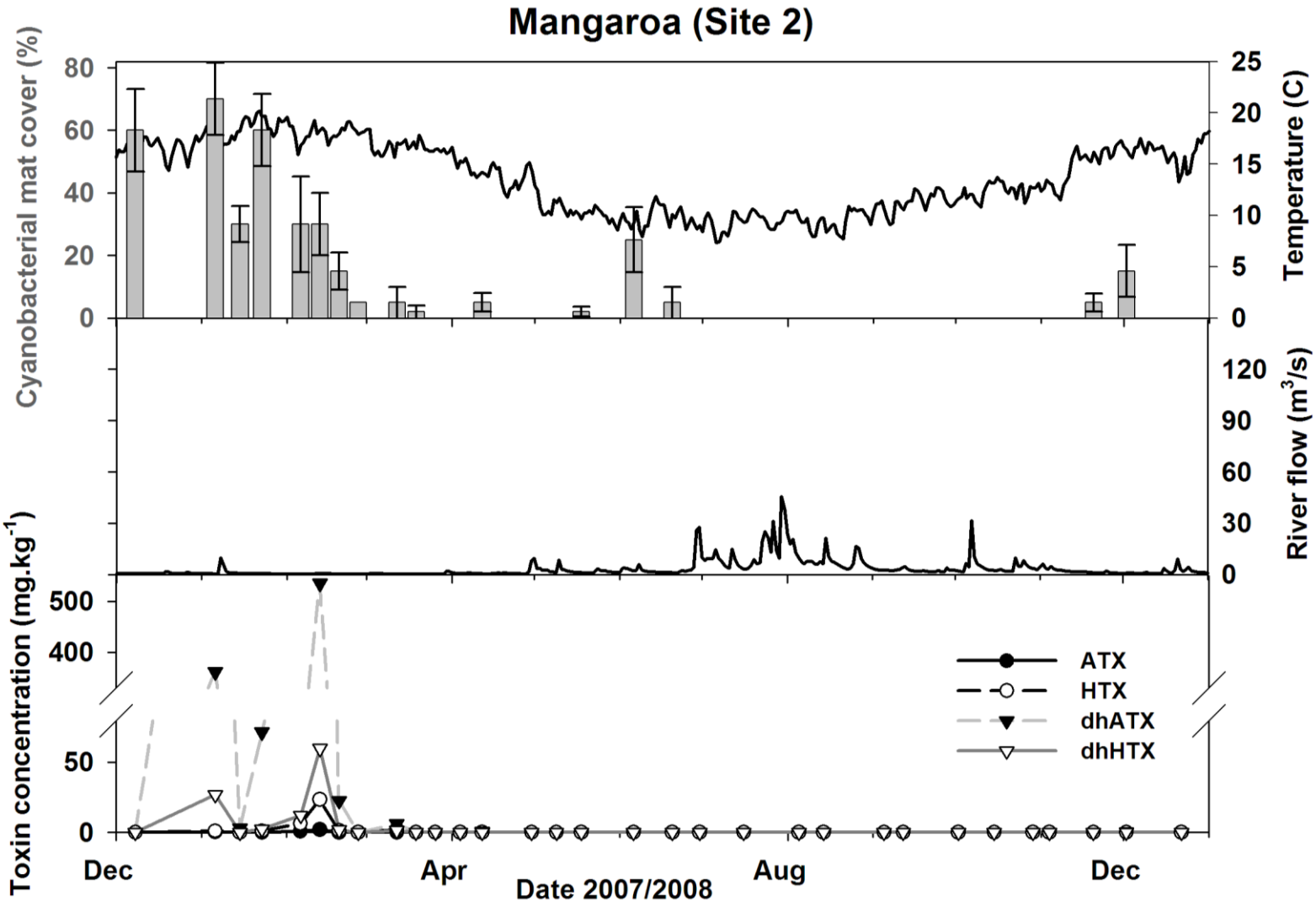

Figure 3.4: Percentage cyanobacterial mat coverage, temperature, river flow and anatoxin concentrations for Mangaroa (site 2) Hutt River, New Zealand, between the 1st of December 2007 and the 1st of January 2009. Error bars represent standard error.

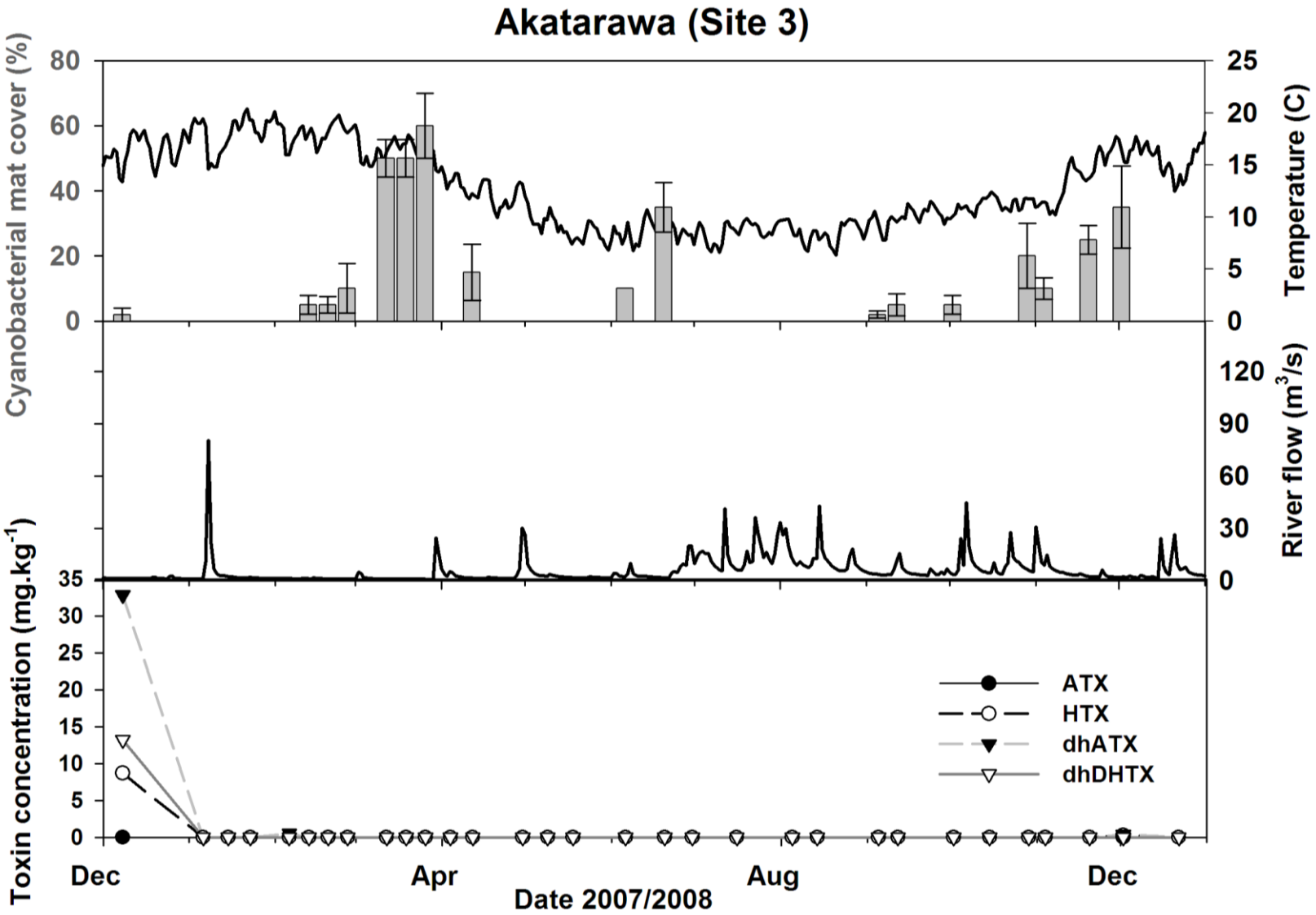

Figure 3.5: Percentage cyanobacterial mat coverage, temperature, river flow and anatoxin concentrations for Akatarawa (site 3) Hutt River, New Zealand, between 1st of December 2007 and the 1st of January 2009. Error bars represent standard error.

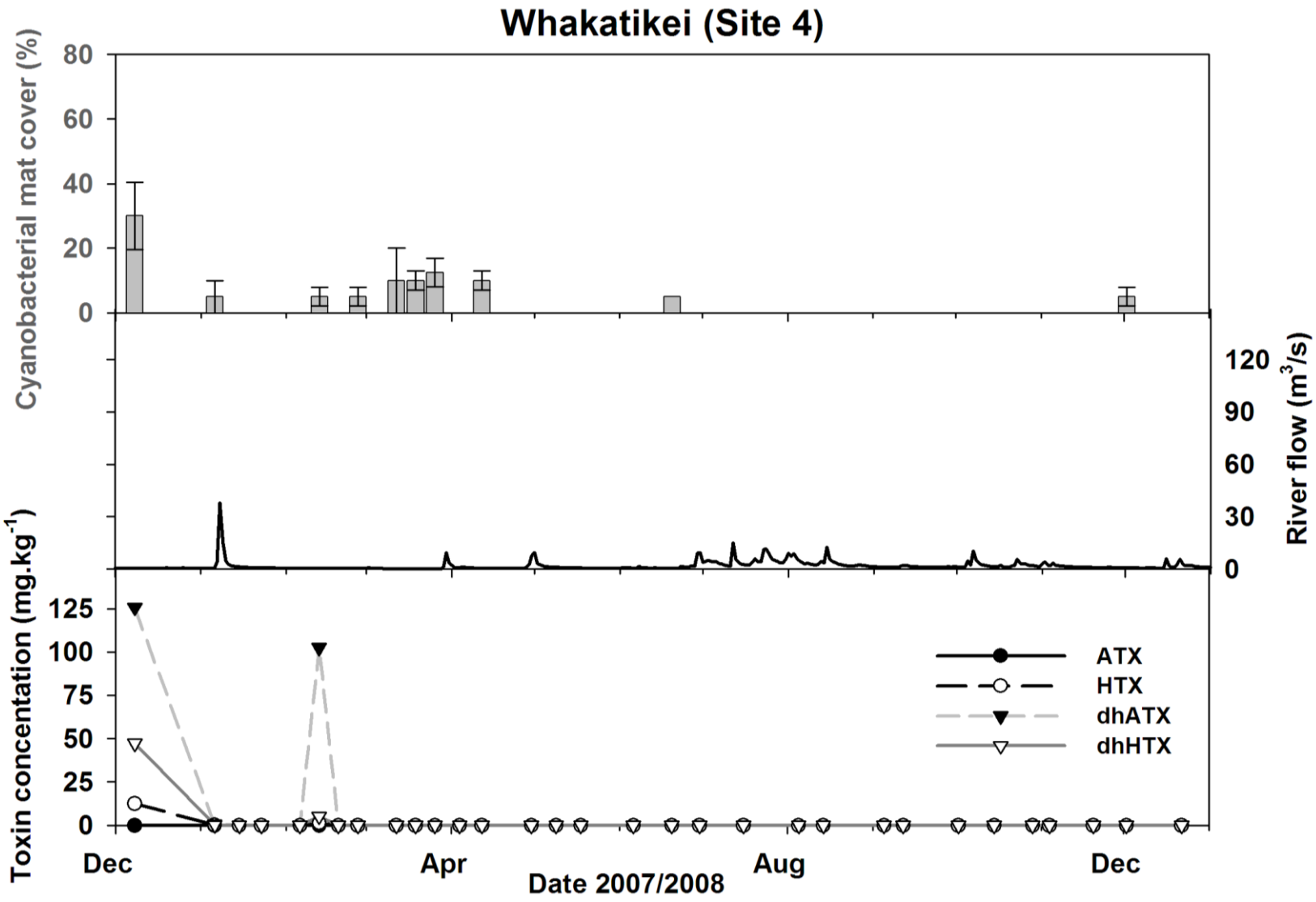

Figure 3.6: Percentage cyanobacterial mat coverage, river flow and anatoxin concentrations for Whakatikei (site 4), Hutt River, New Zealand, between the 1st of December 2007 and the 1st of January 2009. No temperature was recorded for this site. Error bars represent standard error.

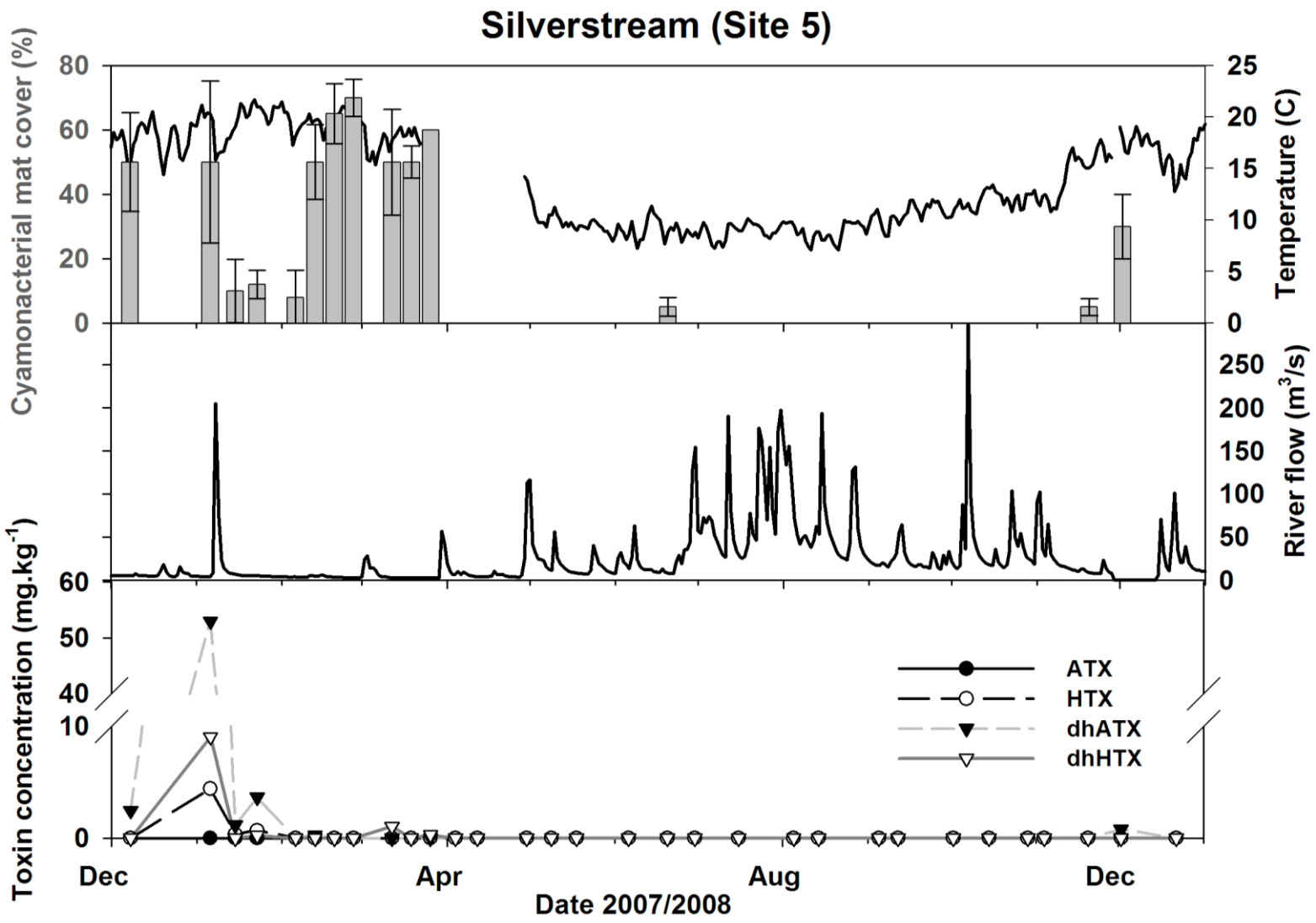

Figure 3.7: Percentage cyanobacterial mat coverage, temperature, river flow and anatoxin concentrations for Silverstream (site 5), Hutt River, New Zealand, between the 1st December 2007 and the 1st of January 2009. Error bars represent standard error.

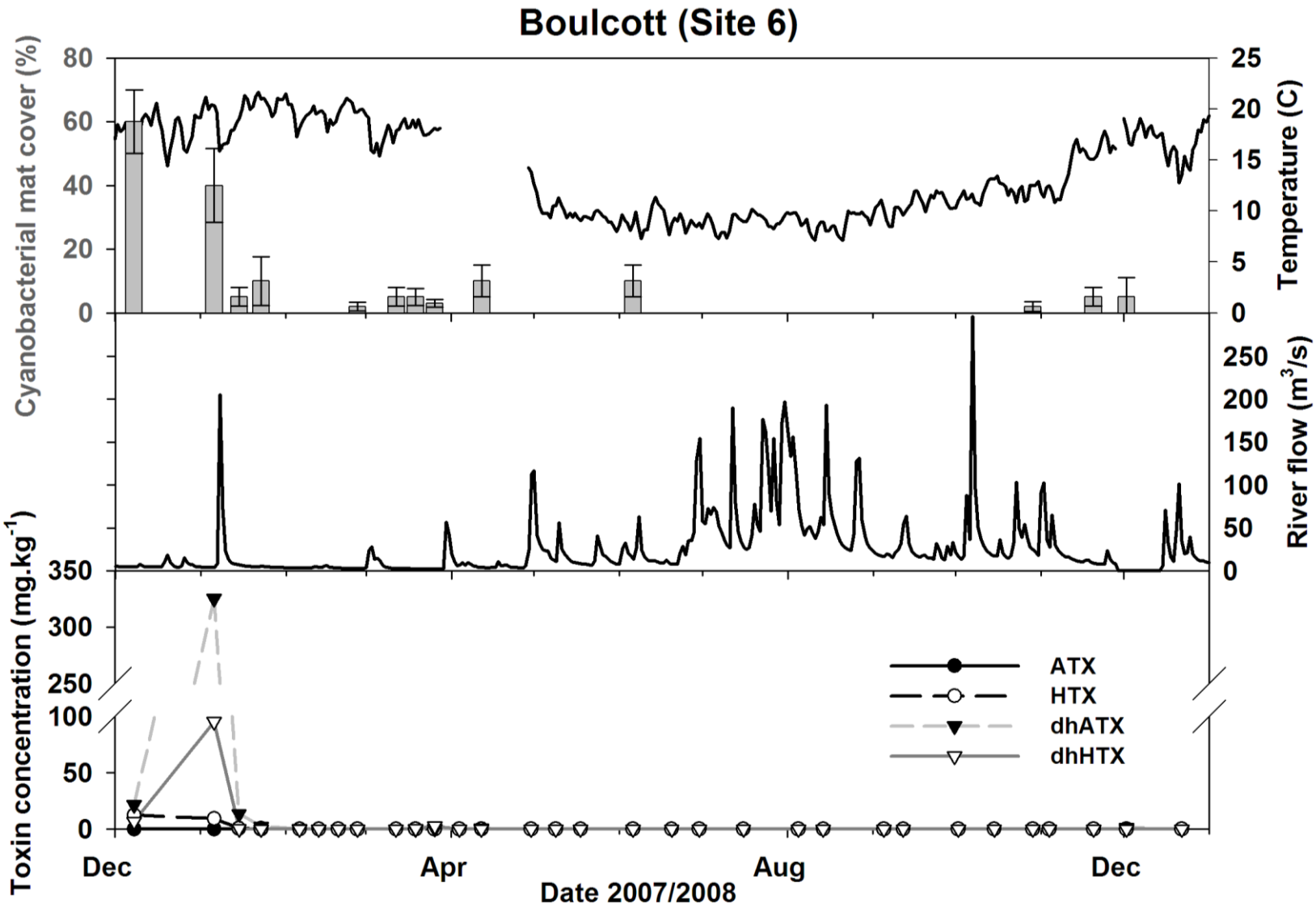

Figure 3.8: Percentage cyanobacterial mat coverage, temperature, river flow and anatoxin concentrations for Boulcott (site 6), Hutt River, New Zealand, between the 1st of December 2007 and the 1st of January 2009. Error bars represent standard error.

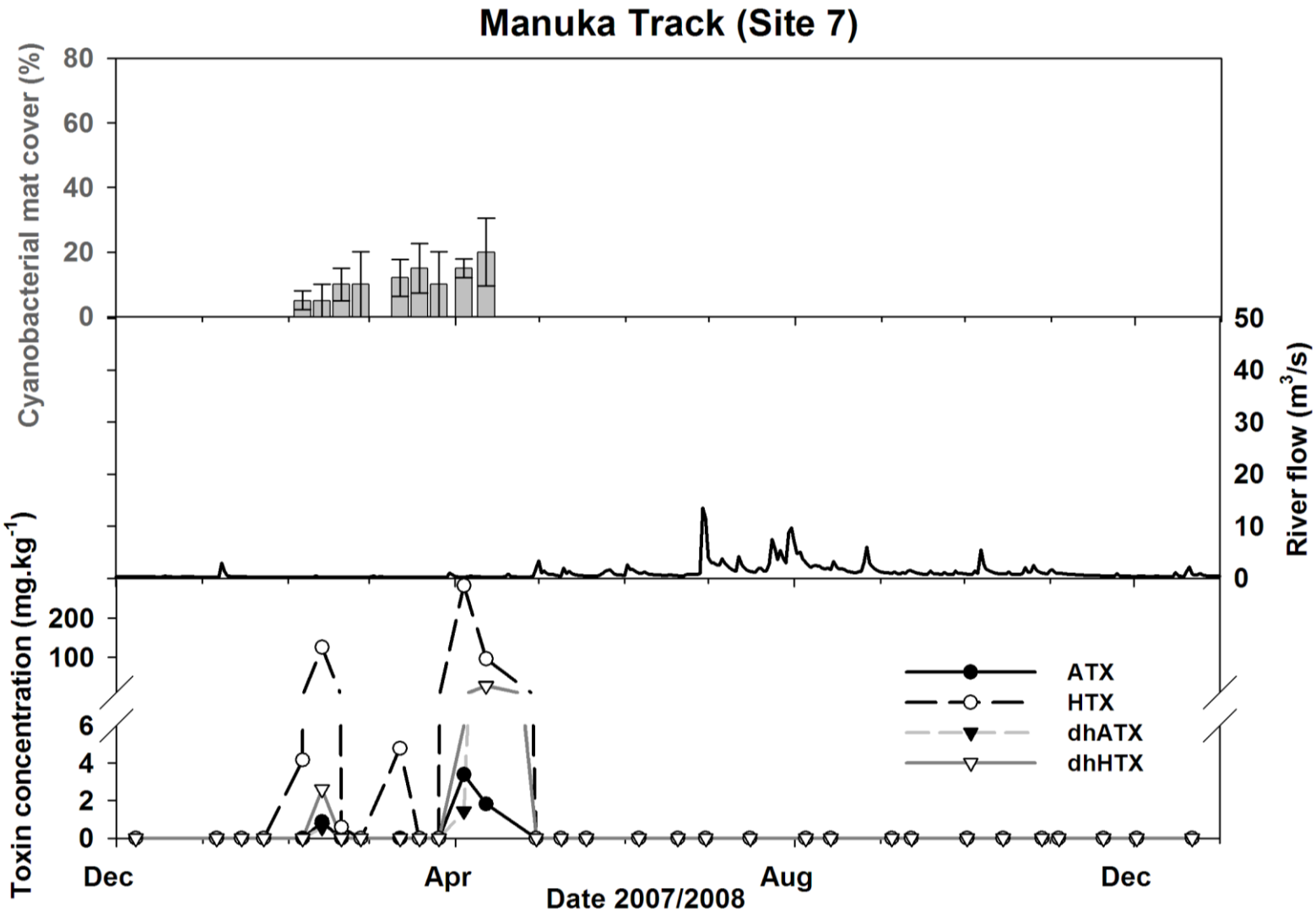

Figure 3.9: Percentage cyanobacterial mat coverage, temperature, river flow and anatoxin concentrations for Manuka Track (site 7), Wainuiomata River, New Zealand, between the 1st of December 2007 and the 1st of January 2009. Error bars represent standard error.

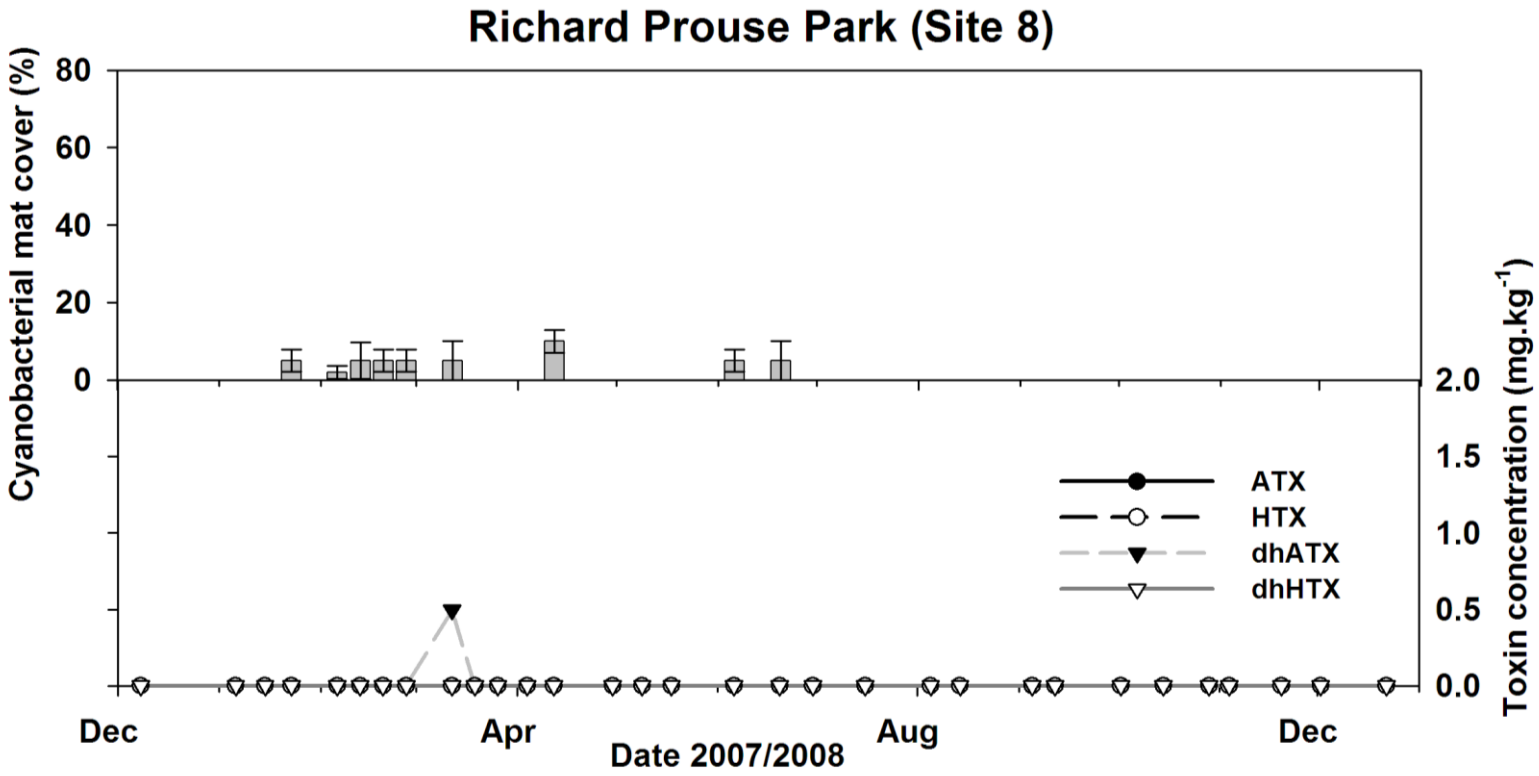

Figure 3.10: Percentage cyanobacterial mat coverage and anatoxin concentrations for Richard Prouse Park (site 8), Wainuiomata River, New Zealand between the 1st of December 2007 and the 1st of January 2009. River flow and temperature were not measured. Error bars represent standard error.

3.4.3 Physicochemical correlations for pooled Hutt river data

Spearman's rank correlation of pooled data from all six Hutt river and tributary sites revealed a strong relationship between temperature and river flow with cyanobacterial mat abundance ($P<0.01$), while total phosphorus was also significant ($P<0.05$; Table 3.1). Other physicochemical factors total nitrogen, nitrite-N, nitrate-N, total kjeldahl nitrogen and dissolved reactive phosphorus showed no correlation with cyanobacterial mat abundance.

Table 3.1: Spearman's rank correlations showing the relationship between cyanobacterial mat cover and a selection of physicochemicalfactors. Pooled data from six sampling sites on the Hutt River, New Zealand. n=90.

Temp	Flow	Dissolved Reactive Phosphorus	Total Phosphorus	Nitrate-N	Nitrite-N	Total Kjeldahl Nitrogen	Total Nitrogen
0.477**	-0.433**	-0.130	-0.220*	-0.004	0.075	-0.003	0.010

** Correlation significant at the $P=0.01$ level * Correlation significant at the $P=0.05$ level

3.4.4 Predicting the presence of cyanobacterial mats in the Hutt river

A stepwise logistic regression model was used to predict the percentage probability of the presence or absence of benthic cyanobacterial mats. River flow was identified as the only significant variable ($P<0.001$; data not shown) and consequently was the only parameter included in the logistic model (data not shown). At a cut value of 0.33, the model successfully predicted 76.7% of the observed results, correctly predicting 75% of the time cyanobacterial were absent mats and 80% of the time they were present. Using this model the probability of cyanobacterial mats (P) occurring for any given river flow can be calculated (Figure 3.11). The model shows when river flow is average (=1 in the model), the probability of cyanobacterial mats occurring was 13% while at a river flow value of 0.5 the probability is 43%.

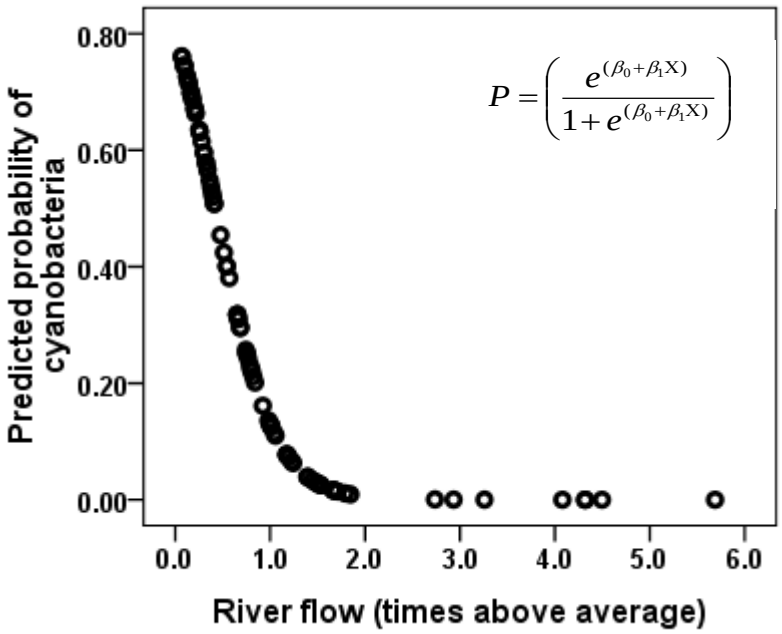

Figure 3.11: Logistic model of the predicted probability of cyanobacterial mats being present in the Hutt River (New Zealand) given different magnitudes of river flow. The model was derived from observed field data. Equation used to determine the probability of the occurrence of cyanobacteria (P) given a known level above the average river flow. $\beta_0 = \pm 0.483$, $\beta_1 = \pm 0.819$, x= river flow.

3.4.5 Physicochemical correlations for pooled Hutt river presence data

When zero percentage cover values were excluded, Spearman's rank correlation found temperature was the only significant physicochemicalvariable (P<0.01; Table 3.2); no other significant correlations were observed. Step-wise regression analysis showed that temperature accounted for 23% of the observed variability in cyanobacterial mat abundance (P<0.001).

Table 3.2: Spearman's correlations showing relationship between cyanobacterial mat cover and a selection of physicochemicalfactors. Pooled data from all six sampling sites on the Hutt River, New Zealand, where cyanobacterial mats were present (n=30).

Temp	Flow	Dissolved Reactive Phosphorus	Total Phosphorus	Nitrate-N	Nitrite-N	Total Kjeldahl Nitrogen	Total Nitrogen
0.509**	-0.362	-0.199	0.108	0.194	-0.007	0.082	0.113

** Correlation significant at the P=0.01 level * Correlation significant at the P=0.05 level

3.4.6 Correlations for individual sites

With the exception of site 2, individual sites were found to conform to those trends observed for pooled data. Cyanobacterial mat abundances were positively correlated with temperature and negatively correlated with river flow. Site 2 however, experienced extensive diatom (*Melosira varians*) proliferations. *Melosira varians* proliferations were found from late January to June 2008 and again in November and December 2008 (Appendix 1; Fig. 3 is of *M. varians*). Peak percentage coverage (80%) occurred on the 12th of April 2008 and coverage was observed above 50% from February to April 2008 and in June 2008 (Figure 3.12). The high *M. varians* percentage coverage appeared to correspond with low cyanobacterial mat coverage.

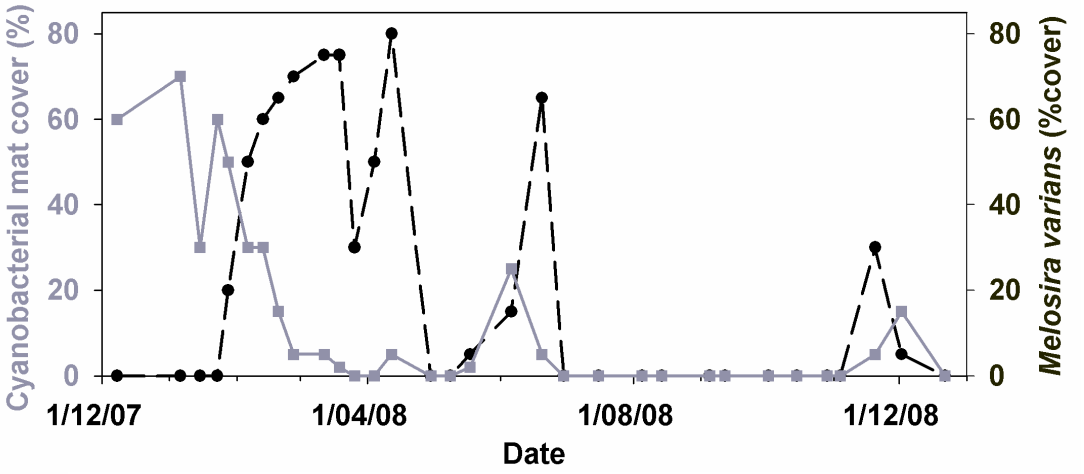

Figure 3.12, Cyanobacterial mat percentage cover and *Melosira varians* percentage cover at site 2 (Mangaroa), Hutt River, New Zealand.

Spearmans correlation showed *M. varians* percentage coverage was significantly negatively correlated with river flow, dissolved reactive phosphorus, total phosphorus and total kjeldahl nitrogen (Table 3.3). These three nutrients also shared a significant positive correlation with river flow. Interestingly, as well as temperature, *M. varians* percentage coverage was positively correlated with total nitrogen and nitrate although this was not significant by Spearman's correlation. When *M. varians* absence data points were excluded from the analysis (data not shown) total nitrogen and *M. varians* abundance were found to correlate at the 5% level (n=6). The relationship of this correlation is represented in Figure 3.13.

Table 3.3: Spearman's rank correlations showing cyanobacterial mat cover relationship with *Melosira varians* (Diatom) relationship with a selection of physicochemical factors. All data from the Mangaroa River (tributary of Hutt River, New Zealand) sampling site (n=15).

Site 2 (Mangaroa)

	Temp	Flow	Dissolved Reactive Phosphorus	Total Phosphorus	Nitrate-N	Nitrite-N	Total Kjeldahl Nitrogen	Total Nitrogen	Diatom
Flow	-0.758**	-	0.797**	0.857**	-0.265	-0.363	0.564*	-0.229	-0.735**
Diatom	0.474	-0.735**	-0.656**	-0.595*	0.331	0.132	-0.529*	0.394	-

** Correlation significant at the P=0.01 level * Correlation significant at the P=0.05 level

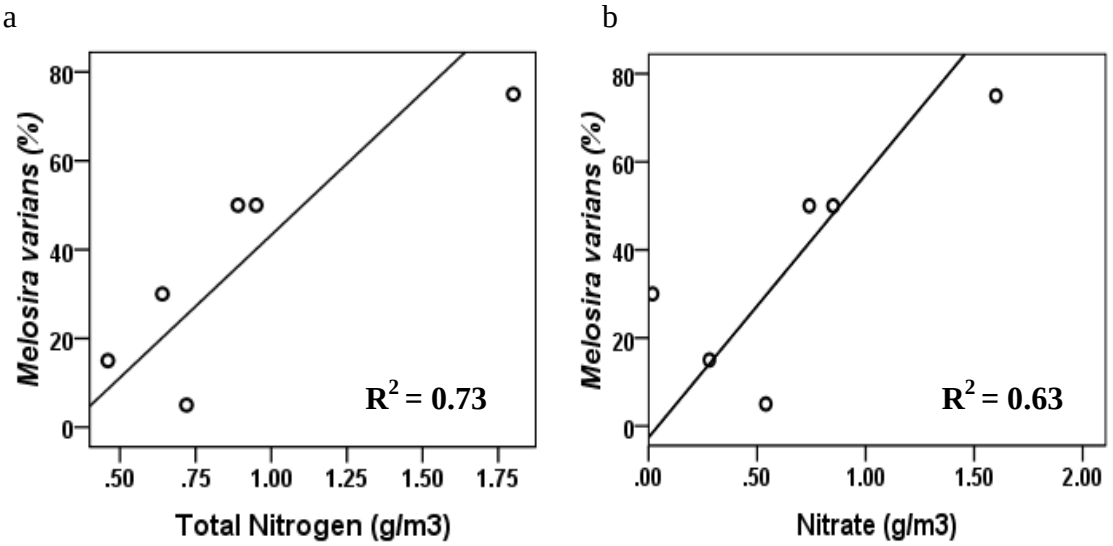

Figure 3.13: Scatter plots of *Melosira varians* percentage cover against total nitrogen (a) and nitrate (b) respectively observed at site 2 (Mangaroa, Hutt River, New Zealand). A linear regression is also plotted.

At the Manuka Track (site 7; data not shown) on the Wainuiomata river cyanobacterial mat cover was also found to have a negative correlation with river flow (P<0.05). None of the remaining physicochemical parameters had any correlation. No temperature measurements were obtained for site 7.

3.4.7 Anatoxin production and concentrations

Anatoxin-a and HTX as well as their degradation products dhATX and dhHTX were detected across all sites on the Hutt river and its tributaries (Figure 3.3-3.8). No epATX or epHTX were detected. At Hutt river sites the highest concentrations of ATX (1.7 mg.kg^{-1}), HTX (23.5 mg.kg^{-1}) and dhATX (535 mg.kg^{-1}) were all observed at site 2 on the 13th of February 2008, while the highest concentration of dhHTX (95.4 mg.kg^{-1}) was detected at

site 6 on the 6th of January 2008. Anatoxin-a was only detected at sites 2 and 6, while HTX was observed at all sites, with the exception of site 1. The most prolific compound dhATX was found at all sites, while dhHTX was observed at all sites except site 1. Anatoxins were detected only in the summer and spring, i.e., December 2007 to April 2008 (Figure 3.3-3.8).

At all Hutt river sites degradation products dhATX and dhHTX were always found in much higher concentration than their parent compounds, with dhATX constantly found in higher concentrations than dhHTX. Anatoxin concentrations were highly variable, both within and between sites (Figure 3.3-3.8). However, some temporal similarity was observed, with maximal anatoxin occurring at site 3 and 4 on the 6th of December 2007, at site 2, 5 and 6 on the 6th of January 2008 and site 1, 2 and 4 on the 12th of February 2008 (Figure 3.3-3.8). At the Wainuiomata river, no anatoxins were detected at site 8 with the exception of the 14th of March where dhATX (0.5 $mg.kg^{-1}$) was detected (Figure 3.10). In contrast ATX, HTX and degradation products were all detected at site 7 (Figure 3.10). The highest concentrations of ATX (3.4 $mg.kg^{-1}$) and HTX (283 $mg.kg^{-1}$) at this site were both recorded on the 4th of April 2008 at site 7 (Figure 3.10).

The Manuka Track (site 7) furthermore, was the only site in this study where the parent toxins (ATX and HTX) were found in higher concentrations than there degradation products. Of interest were the sometimes rapid temporal changes in anatoxin concentrations despite there being little change in cyanobacterial percentage cover. For example, at site 2 on the 6th of February 2008 combined anatoxin concentration increased from 201 $mg.kg^{-1}$ to 620 $mg.kg^{-1}$ on the 13th of February 2008, without any change in cyanobacterial mat abundance (Figure 3.4). At site 7 on the 13th of February 2008 the combined anatoxin concentration was 129.8 $mg.kg^{-1}$, but one week later on the 20th of February only HTX was detected at 0.6 $mg.kg^{-1}$ (Figure 3.10). Concentration of anatoxins did not appear to be correlated to the percentage of cyanobacterial mat cover. For example, at site 2 on the 23rd of January there was a combined toxin concentration of 74.8 $mg.kg^{-1}$ that increased to 201 $mg.kg^{-1}$ on 6th of February despite a 50% decrease in cyanobacterial mat cover (Figure 3.4).

3.4.8 Factors contributing to anatoxin abundance

Spearman's rank correlation was used to detect possible correlations between anatoxin production and physicochemical parameters (Table 4). Anatoxin concentrations were found to be significantly correlated with the presence of cyanobacterial mat cover (P<0.001, n=90), temperature (P<0.001, n=90) and river flow (P=0.001, n=75). In order to control for the effect of river flow, Spearman's correlation was conducted on anatoxin presence data only (n=19). In this analysis no significant correlations were observed, including temperature and cyanobacterial mat cover, that were previously found to be significant, however, a very weak relationship between cyanobacterial mat cover and dissolved reactive phosphorus and total phosphorus was observed (Table 4).

Table 3.4: Spearman's rank correlations showing the relationship of anatoxin concentrations with a selection of physicochemical factors. Initially all data was pooled from six sampling site on the Hutt River (n=90). The second analysis used only toxin presence data which was also pooled from all six sampling sites on the Hutt River (n=19)

Mat	Temp	Flow	Dissolved Reactive Phosphorus	Total Phosphorus	Nitrate-N	Nitrite-N	Total Kjeldahl Nitrogen	Total Nitrogen
Pooled quantitative data set								
0.592**	0.595**	-0.338**	-0.094	0.050	-0.115	-0.038	0.002	0.002
Presence data only								
-0.132	0.100	0.030	0.342	0.344	0.081	0.130	0.038	0.071

** Correlation significant at the P=0.01 level * Correlation significant at the P=0.05 level

3.5 Discussion

This research, to my knowledge, is the first to describe benthic cyanobacterial abundance and anatoxin concentration over the entire length of a river. Previous investigations of freshwater mat-forming cyanobacteria have taken point samples of mat material on single occasions for toxin testing and species identification (Mez *et al.* 1997; Hamill 2001; Cadel-Six *et al.* 2007; Izaguirre *et al.* 2007).

3.5.1 Cyanobacterial mat abundance in different habitats

Cyanobacterial mat abundance was measured in three distinct habitat types (pool, run, and riffle) at sampling sites 1, 2, 3 and 4 on the Hutt river and its tributaries. Cyanobacterial mat abundance varied between each habitat type with riffles generally observed to have

higher abundances than runs, while cyanobacterial mat prevalence was lowest in the pool habitat. Cyanobacteria are aerobic photoautotrophs relying on light energy to conduct photosynthesis (Mur *et al.* 1999). In a previous investigation, photosynthesis and cyanobacteria biomass have been shown to be highest at the water surface (Grey *et al.* 1990). Light intensity is significantly reduced and spectral composition are is changed with depth due to the absorption and scattering of particles and coloured components in the water column (Kirk 1994; Oliver and Gnaf 2000). The ability of benthic organisms to perform photosynthesis is reduced with depth. Thus, the ability of benthic cyanobacteria to perform photosynthesis and growth is dependent on their depth in the water column.

The stability of bottom substrate may also be a contributing parameter in habitat preference and therefore growth. Field observations of benthic cyanobacteria substrate preference suggested cyanobacterial mats are more likely to be found on larger and more stable rocks. Due to the different water velocity observed in each habitat, riffle and run were observed to have larger more stable substrate than pool, which generally had a finer substrate. In a previous investigation of benthic cyanobacterial proliferations, coarse cobble substrate has been found to be prerequisite for growth of cyanobacteria (Biggs 1990).

Consistent growth was seen in the pool at site 3, and on six different occasions in higher abundance there than in run and riffle habitat. This pool had a large flat rock in the middle that at its highest point protruded from the surface of the water (Appendix 1, Fig. 5). The bedrock had eroded such that it was an underwater ‘table’. The majority of cyanobacterial growth occurred on this bedrock table. This acted to lower the water level and offered a large stable substrate, and this may have allowed greater photosynthesis. Furthermore, with the low flows observed in summer this site that had originally been identified as a pool habitat, became more run like, and thus more favourable for cyanobacterial mat growth.

3.5.2 Cyanobacterial mat abundance and physicochemical parameters

The data from the Hutt and Wainuiomata rivers showed that cyanobacterial abundance is highly variable between sites and over a temporal scale. Cyanobacterial abundance was found to vary markedly among all eight sample sites. Despite this variation, a seasonal factor contributing to cyanobacterial mat abundance was apparent. Peak cyanobacterial mat abundances for all sites were observed in summer (December to March).

Cyanobacterial mats were less prevalent in the winter months, and this is consistent with Biggs and Price (1987) who found the summer periphyton crops were significantly higher than winter crops when assessing algal proliferation in New Zealand rivers. They attributed this is to a higher frequency of winter flushing river flows (Biggs and Close 1989; Biggs 1990; Clausen and Biggs 1997)

In this study, river flow shared a strong negative correlation with cyanobacterial abundance. High river flows, with their ability to pick up small rocks and pebbles, flush and sandblast attached mat material from the bottom substrate (Biggs and Close 1989). River flow has been previously described as a major controlling factor for benthic cyanobacteria in river systems (Biggs 1990; Milne and Watts 2007; Wood *et al.* 2007b) and similarly in lake systems (Johnson and Castenholz 2000; Dasey *et al.* 2005) and marine environments (Thacker and Paul 2001) where turbulence occurs in the form of wave action. The ability of river flow to control cyanobacterial mats has led local government agencies in the Wellington region to adopt this as one of the factors used to predict cyanobacterial mat abundance (Milne and Watts 2007). Two weeks without a river flow of three times the median, is being used as an early warning indicator of the strong likelihood of cyanobacterial growth. In this study, logistic regression was used to predict the probability of cyanobacterial mats occurring, and river flow was the only significant parameter in the logistic model controlling cyanobacterial mat abundance. The corresponding logistic model revealed that when river flow was three times the average over a five day period, cyanobacterial mats were not likely to occur. If the mean river flows over the five days prior to sampling was equal to the annual mean flow the probability of cyanobacterial mats being present is 13%. These results support GWRC's use of river flow as predictor of the presence of mat-forming benthic cyanobacteria.

Water temperature was shown to have a strong positive relationship with cyanobacterial mat abundance. Using Spearman's correlation, direct relationships can not be inferred; thus whether cyanobacterial mat cover is responding directly to water temperature or whether the correlation is coincidental needs further investigation. For the initial pooled data, river flow and water temperature were both found to be significant parameters (data not shown); therefore it remains unclear whether cyanobacterial mat growth was responding to the low river flow in the summer and/or the higher temperatures. Temperature was validated as a significant factor when river flow was controlled for by looking at cyanobacterial presence

data alone. This was consistent with Biggs (1990) who previously has elucidated temperature as a significant parameter in *Lyngbya* proliferations in New Zealand. Temperature was the only significant parameter leading to cyanobacterial mat growth in this investigation. A follow up linear regression analysis revealed water temperature accounted for 23% of the observed variation in cyanobacterial mat coverage. This is also consistent with previous planktonic cyanobacteria research, where optimal temperatures maximise growth (Robarts and Zohary 1987; Song *et al.* 1998). Caution should be taken in concluding water temperature is a key parameter in cyanobacterial mat growth because parameters such as light intensity and UV radiation may also be co-correlated with water temperature.

No other physicochemical parameters showed correlation with cyanobacterial growth. Research on planktonic and benthic cyanobacteria in marine, lake and culture environments has shown nutrients such as phosphorus and nitrogen to be key parameters responsible for growth (Kuffner and Paul 2001). Total phosphorus shared a negative correlation with cyanobacterial growth, however no such correlation was observed when cyanobacterial presence data was examined, i.e. eliminating the influence of river flow. Previous investigations of phosphorus have shown it to be a key nutrient responsible for the growth of benthic mat-forming cyanobacteria (Kuffner and Paul 2001; Ahern *et al.* 2008). Benthic cyanobacteria however, have been previously identified proliferating in New Zealand in sites with relatively low abundance of nutrient correlations (Biggs and Price 1987; Biggs 1990). Furthermore, these benthic cyanobacteria may have the ability to fix their own nitrogen (Pankratova *et al.* 1998), therefore enhancing their ability to persist in environments with limiting concentrations of chemically contained N. It has been suggested that essential nutrients may be sourced from the greywacke bottom substrate, and therefore are undetectable by nutrient analysis of overlying water. Biggs (1990) found that *Lyngbya* proliferations were highly associated with hard sedimentary rocks, which provide a source of phosphorus. Another possible nutrient source may come from the build up and collection of sediment under the cyanobacterial mat. In the field, numerous observations of a thin sediment layer were observed between the cyanobacteria and the rocky substrate. It is possible that cyanobacteria out-compete other periphyton species in oligotrophic conditions, with their proposed ability to fix nitrogen and/or source nutrients from their substrate. Future investigation with greater resolution in a laboratory or field

setting will help to elucidate critical nutrient thresholds and their sources for benthic cyanobacterial growth.

3.5.3 Diatom competition

At site 2 (Mangaroa), proliferations of *M. varians* appeared to displace cyanobacterial mats at certain times of the year. Spearman's correlation revealed a positive relationship between *M. varians* abundance and cyanobacterial mat cover; however this is misleading because river flow was not taken into consideration. Unfortunately, presence data alone could not be examined due to low sample size. When the overall trends in cyanobacterial and *M. varians* abundance are observed however, a clear negative relationship was revealed. *Melosira varians* abundance, like cyanobacterial abundance, had a significant negative correlation with river flow, i.e. with increased river flow *M. varians* abundance decreased. *Melosira varians* abundance also had significant negative correlations with total phosphorus, dissolved reactive phosphorus and total kjeldahl nitrogen, however these parameters also had a positive correlation with river flow. Following rainfall, nutrients are washed into the river system; hence at high flows there are high levels of nutrients (in particular phosphorus, therefore river flow is affecting levels of nutrients (White 1972; Biggs 1990).

Interestingly, total nitrogen and nitrate were found to have a weak positive correlation with *M. varians* growth, although this was not significant. When presence data was considered, both total nitrogen and nitrate-N were found to share strong linear relationships (R^2=0.73 and 0.63 respectively) with *M. varians* growth. Total nitrogen was found in much higher concentration at site 2 than at any other site (Appendix 3). Total nitrogen and nitrate-N had negative relationships with river flow, thus they were highest in times of low and stable flow. *Melosira varians* growth therefore occurred when nitrogen levels were high and river flow was low, while cyanobacterial growth occurred when nitrogen levels were low and likely at insufficient levels for *M. varians* growth. When nitrogen levels were high, *M. varians* smothered and out-competed cyanobacteria. This is consistent with the investigation of Vis et al; (2008) who showed a shift in composition from chlorophytes to cyanobacteria along a gradient of decreasing nitrate, while Biggs and Price (1987) demonstrated *M. varians* occurrence required higher enrichment levels than *Phormidium.*

3.5.4 Spatial and temporal variation in anatoxin production

The presence, variants and concentrations of anatoxin varied dramatically between sites and over time. To my knowledge this has not been demonstrated previously for benthic cyanobacteria. In New Zealand, water managers and authorities have used abundance as a proxy for anatoxin concentration in their management of benthic cyanobacterial blooms because of their known ability to produce anatoxin (Hamill 2001; Milne and Watts 2007; Wood *et al.* 2007b). Spearman's rank correlation confirmed this variability showing there is no correlation between mat abundance and anatoxin concentration for Hutt and Wainuiomata river sites, after river flow was controlled for. Anatoxins are not produced in every mat. This is consistent with Chapter 2 and previous investigation where cyanobacterial mats were found to be made up of multiple strains/genotypes of *Ph. autumnale* which consist of toxic and non-toxic strains (Cadel-Six *et al.* 2007). Therefore the large variations observed in anatoxin concentrations can be explained by the presence of toxic and non-toxic *Ph. autumnale* strains in any given mat.

With the presence of toxic and non-toxic strains in cyanobacterial mats, it is not surprising that no other physicochemicalparameters were found to be significant. There is currently no method to assess the proportion of anatoxin producing strains within a mat. Future research looking at anatoxin regulation in anatoxin-producing cultures (Chapter 1) may enable links between different physicochemicalparameters and anatoxin production to be elucidated. The recent discovery of a putative gene involved in anatoxin production (Cadel-Six *et al.* 2009) may provide the ability to use quantitative molecular techniques to monitor levels and expressions of this gene and allow more in-depth investigations to determine whether physicochemicalparameters are affecting anatoxin producing strains in field environments.

Dihydro degradation product concentrations were considerably higher than their parent compounds. Anatoxin is unstable especially in sunlight and at high pH, whereas the dihydro degradation products are more stable (Smith and Lewis 1987). On some sampling occasions degradation products were the only compounds detected. The detection of these compounds may provide information of previous or nearby toxic mats and they should be monitored in routine cyanotoxin analysis. The exception to this was site 7 where degradation products were in considerably lower concentrations than their parent compounds; this may have been due to the shaded nature of this site.

No obvious differences were observed between the Wainuiomata river and Hutt river. However, at site 7 HTX was the most dominant variant. The greater production of HTX than ATX is likely to be the result of HTX producing strains of cyanobacteria being dominant at this site.

3.6 Conclusion

The results of this study revealed that *Ph. autumnale* mats are spatially and temporally variable in the Hutt and Wainuiomata rivers. Furthermore, anatoxin concentrations were also found to vary on a weekly time-frame and among sampling sites. In contrast to previous reports, anatoxin concentrations did not correlate with cyanobacterial abundance. River flow was shown to control cyanobacterial abundance, and water temperature was elucidated as a parameter affecting the percentage of cyanobacterial mat coverage. This is likely to be as a result of faster growth rates at higher temperatures. The ability of *Phormidium* to obtain essential nutrients for growth from sedimentary rock substrate or from sediment trapped below mats and nitrogen via nitrogen fixation may give it a competitive advantage in the oligotrophic conditions in these rivers. Further investigations are required to confirm this. No physicochemicalparameters were found to have a significant effect on anatoxin concentration. Future investigations using quantitative molecular techniques (targeting enzymes involved in anatoxin production) have the potential to further elucidate physicochemicalparameters that regulate anatoxin production and to allow the detection of toxic strains within a sample. This ultimately will assist in the development of predictive models that will help in the early warning of cyanobacterial mat proliferation and anatoxin production.

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4

Discussion and Conclusions

This research was initiated due to the limited knowledge available on the diversity of toxic benthic freshwater cyanobacteria, both in New Zealand and worldwide. At the onset of this study limited information was available regarding the diversity, distribution and factors triggering proliferation of benthic freshwater cyanobacteria in New Zealand. Prior to this work, the only research investigating cyanotoxin production from benthic cyanobacteria has generally been opportunistic, occurring after animal poisonings. No study worldwide has looked at toxin production and benthic cyanobacterial distribution over temporal and spatial scales. As a result, little was known of the regulation of toxin production and the physicochemical parameters that lead to benthic cyanobacterial proliferations. This research has addressed the paucity of information regarding toxic benthic cyanobacterial proliferations and as a result more accurate assessments can now be made concerning the potential health risk posed to animal and human users of these water bodies. For example, high toxin concentrations are not dependent on high cyanobacteria coverage; run and riffle habitats are potentially more dangerous than pool habitats; high toxin concentration is observed in the summer months; river flow can be used to predict the presence of benthic cyanobacteria.

In Chapter 2, it was found that *Phormidium* spp. are the most prevalent species in benthic cyanobacterial mats in rivers across New Zealand. During the period of this study, reports of benthic cyanobacteria proliferations have been numerous, with sampled water-bodies only forming a portion of those experiencing benthic cyanobacterial blooms. Of the 37 isolated strains, 29 were identified as *Phormidium* and 27 of these were identified using morphological techniques as *Ph. autumnale*. *Phormidium autumnale* isolates were observed to have a wide range of morphological characters. This is consistent with previous investigations of the *Ph. autumnale* group (Komarek and Anagnostidis 2005; Willame *et al.* 2006; Comte *et al.* 2007; Palinska and Marquardt 2008), and it is likely this group actually consists of a number of different species (Willame *et al.* 2006; Comte *et al.* 2007; Palinska and Marquardt 2008). Phylogenetic analysis in Chapter 2 also revealed the *Ph. autumnale* group had a number of different clades. The substantial phylogenetic and morphological evidence presented, suggests that this group should be split into at least two different species. The two species would be split based on the observed difference in Chapter 2 between Morphotype A and Morphotype B. Such characterisation is beyond the scope

of this thesis, but will be undertaken at a later date using additional genetic markers and hybridization experiments.

Species from the *Nostoc* and *Planktothrix* genera and the Pseudanabaenaceae family were also isolated. Morphotype D consisted of two identical isolates (CYN 38 and 39) which were both identified as *Phormidium murrayi* using similarity analysis with existing Genbank sequences and morphological descriptions. To my knowledge, this is the first time this species has been identified outside Antarctica, and this observation dispels the suggestion that this species is endemic to Antarctica (Casamatta *et al.* 2005). Further detailed phylogenetic comparisons indicated that this culture and "*Ph. murrayi"* sequences from GenBank were found to align closely with the *Microcoleus* genus consistent with previous phylogenetic analyses (Casamatta *et al.* 2005; Taton *et al.* 2006; Comte *et al.* 2007). Based on these observations this species should be renamed to *Microcoleus glacei*, in light of the overwhelming molecular evidence now available regarding the taxonomic classification of this species. Morphotype E (VUW 25) is potentially a new species of *Planktothrix*. This species morphology is similar to that of the *Phormidium* genus, highlighted by its benthic nature and lack of aerotopes; however its genotype matches species of the *Planktothrix* genus with 98% sequence similarity (16S rDNA). *Planktothrix* spp. are planktonic and posses aerotopes. This isolated strain produced microcystin variant [ASP-3]deMe MC-LR responsible for the death of a dog on the Waitaki river. This finding highlights the lack of research on toxic benthic cyanobacteria, and the importance of this current investigation to identify toxin producing species in order to reduce health risks.

Chapter 2 demonstrated that *Ph. autumnale* is the main species responsible for the thick benthic mats observed in rivers around New Zealand. It highlighted the difficulties of relying solely on morphological features to identify these organisms to species level and emphasized the need to combine phylogenetic techniques and morphological analysis. Furthermore Chapter 2 has shown that a community of species exist in any one mat, and Chapter 3 revealed that not all of these produce cyanotoxins. It is therefore paramount in studies of cyanobacteria mats to undertake a polyphasic approach to species identification. Gaining knowledge of the species in these mats was essential for studying the concentration and regulation of toxic benthic

cyanobacterial proliferations in Chapter 3. The baseline species identification data gained in Chapter 2 will also allow for future assessments on changes in species composition that may occur as a result of environmental or climatic change.

Cyanotoxins were identified in eight of the cyanobacterial mats collected from around New Zealand (Chapter 2). All of these mats were dominated by *Ph. autumnale* (Morphotype A & B) and anatoxins were detected, with the exception of the mat from the Waitaki river where microcystin was detected. After isolation and culturing from these environmental samples, only three isolates were found to produce cyantoxins. Identical strains CYN52 and CYN53 produced ATX and both were isolated from the Rangitikei river. The *Planktothrix* strain isolated from the Waitaki River produced microcystin variant [ASP-3]deMe MC-LR. Because only three toxin-producing strains were isolated from eight mats that contained toxins, it was concluded that cyanobacterial mats in New Zealand are comprised of toxic and non-toxic strains. This was confirmed by the phylogenetic analysis (16S rDNA) which revealed the two anatoxin producing strains formed their own clade. The *Planktothrix* sp. also formed its own clade. This information was important to better understand the dynamics and variability of toxin concentrations and variants observed in Chapter 3 of this thesis.

Cyanobacterial mat cover and anatoxin production varied over time and between different sites in the case study of the Hutt and Wainuiomata river catchments (Chapter 3). Despite this observed variation, a seasonal factor contributing to cyanobacterial mat abundance and anatoxin concentration was evident. Cyanobacterial mat cover and anatoxin concentration for all sites peaked in the summer months (December to March). If New Zealand's climate continues its current trend towards warmer, drier, longer summers then the risk of increased toxin production in our rivers is likely to increase in the future. This is conducive to benthic cyanobacterial growth, mat coverage is therefore expected to increase and last longer.

Anatoxin production in this investigation did not correlate with cyanobacterial mat cover. Benthic cyanobacterial mats, are comprised of a number of different strains, and anatoxin concentration is dependant on the relative proportion of toxin and non-toxin producing genotypes within a mat. Recreational users are therefore potentially exposed to the high levels of risks from anatoxins at low levels of mat coverage. This

is valuable information for water managers and authorities in accessing the risk of a given water-body from benthic cyanobacteria.

Cyanobacterial mat abundance varied between each of the three habitat types sampled, with riffles generally observed to have higher abundances than runs, while cyanobacterial mat prevalence was lowest in pools. Light levels diminish through the water column and as a consequence, the ability of benthic cyanobacterial mats to perform photosynthesis may decrease with depth, due to the absorption and scattering of particles and coloured components in the water column (Kirk 1994). It is therefore likely that the riffle provides the most suitable habitat as this has the highest light levels. In addition, cyanobacterial mats appeared to be found on larger and more stable rock substrate. Due to the different water velocity observed in each habitat, riffle and run habitats have larger more stable substrates than pool habitats, which generally have a finer substrates. This research therefore suggests that the pool habitat could possibly be used for recreational use, even when run and riffle habitats are experiencing cyanobacterial mat proliferations. Caution and research is still required, given it is not yet known how much intracellular toxin is released into the water column.

River flow is a significant parameter in the removal of benthic cyanobacterial mats. Using stepwise logistic regression, the parameter “river flow” the only significant parameter in the model predicted 80% of the observed cyanobacterial mat proliferations. This result allows a baseline model to be used for the prediction of cyanobacteria, future research will be able to optimize and build on this model. Temperature was the only significant parameter contributing to 23% of the observed variation in cyanobacterial mat coverage, when river flow was controlled for and analysed using stepwise linear regression. Water-soluble nutrients were not significantly correlated with benthic cyanobacterial abundance. The assumed ability of benthic non-heterocystous *Phormidium* to fix nitrogen and source nutrients from bottom substrate is likely to give this organism a competitive advantage in oligotrophic environments and this should be addressed further in future studies. At Site 2 increases in water soluble nitrogen led to increases in the abundance of *Melosira varians,* which out-competed the benthic cyanobacteria. With nitrogen levels increasing in water-ways across the globe, due to eutrophication as a result of

anthropogenic causes, it can be assumed that this observation may forecast future species compositions. *Melosira varians* a nitrogen dominant species potentially could out-compete benthic cyanobacteria that currently grow in more pristine conditions. This may potentially mean that the toxic benthic cyanobacteria in this study will not be present in the future; however the risks associated with eutrophication are well publicized. For example, deoxygenated waters, death of river inhabitants, greater primary growth, the establishment of different toxic algal species and poor water quality.

These results from Chapters 2 and 3, have substantially increased knowledge of species responsible for toxin production and led to insights into factors triggering benthic cyanobacterial proliferations. The research has also highlighted areas requiring future research.

4.1 Research applications and future directions:

This research has established a diversity baseline of benthic cyanobacterial proliferations in New Zealand, with the isolation and identification of 37 strains representing 11 different morphotypes. This culture collection has been cryopreserved, and stored at the national culture collection of micro-algae, Cawthron Institute, Nelson (http://www.cawthron.org.nz/seafood-safety-biotechnology/micro-algae-culture-collection.html). This culture collection and associated data can now be used as a resource for future research.

With the establishment of this benthic cyanobacteria culture collection, future investigative culture experiments can be conducted. This will help in the further refinement of accurate predictive models that this research has begun to develop. No water soluble nutrients were found to correlate with cyanobacterial growth, in this investigation. Future culture experiments will be able to quantify the effect of chemical (e.g., nutrients and trace metals) and physical factors (e.g. light, temperature, pH) on benthic cyanobacterial growth. Results from these culture experiments could be used to predict times of high risk for different cyanobacteria species. A description of ideal growth conditions could then be incorporated into predictive and early warning systems.

Culture experiments performed on toxin producing strains can be used to determine optimal environmental conditions that enhance or trigger toxin production and release of toxin from cells. This will allow models to be developed that predict times of highest toxin concentration and therefore greatest health risk. These culture studies would also establish the ratios of extra-cellular and intra-cellular toxins and at what stage of the growth cycle cyanotoxins are released.

Toxin strains now in culture will aid in the development of rapid molecular tools for anatoxin producing strains as an alternative to the costly and time consuming methods currently used (e.g. HP-LC, LC-MS). A rapid molecular test could differentiate toxic and non-toxic strains based on observed genotype variations. The rapid identification of anatoxin producing species now looks to be a reality with the recent discovery of a putative gene involved in anatoxin production (Cadel-Six *et al.* 2009). Validation experiments using cultures from this study are currently underway to establish the effectiveness of this putative gene in the rapid identification of anatoxin producing species in New Zealand. Furthermore, if this method proves successful, it will allow New Zealand water authorities and managers to take immediate advantage of this relatively cheap technology. This will allow water manager to identify anatoxin producing species and therefore health risks at a number of different sites in a given river, catchment or region, which is currently not possible due to the cost associated with current methods. This putative gene could also be used to examine the activation of anatoxin production (using gene expression studies) over different time periods in a field environment and this could be correlated with changes in physicochemical parameters.

In Chapter 3 water-soluble nutrients were found to have no affect on benthic cyanobacterial mat cover. Potential sources of nutrients include sedimentary rock and a silt layer observed to build up underneath of the thick *Phormidium* mats. Further field studies investigating sources of nutrients outside of the water column will assist in addressing the influence different nutrients have on cyanobacterial mat proliferations and toxin production. Confirmation of the ability of non-heterocytous *Phormidium* to fix atmospheric nitrogen has been shown in a previous investigation (Pankratova *et al*. 1998). Confirmation of New Zealand *Phormidium* spp. ability to

source nutrients could be ascertained using *in situ* assays on either the established cultures or in the environment and nitrogen fixation by testing for the presence of *nif*H gene involved in synthesis of nitrogenase (Zehr and McReynolds 1989).

In this study, the degradation products dhATX and dhHTX were often found in higher concentrations than the parent compounds. However, at site 7 where the river is well shaded in comparison to all other sites, low levels of degradation products were observed. Further studies could investigate the stability of ATX/HTX and how rapidly it degrades in sunlight/river water (Smith and Lewis 1987). Little is known of the effect that mat drying has on the concentration of anatoxins (e.g., when river levels drop exposing cyanobacteria mats to the air; Appendix 1, photo 30) (Milne and Watts 2007; and Wood *et al*. 2007). This information would be useful for establishing animal and human health risk. Additionally, there is little information on whether anatoxins are maintained within cells during the growth cycle. Studies using the toxic strains established in this study could be used to address this knowledge gap (Ministry for the Environment and Ministry of Health 2009). This research is vital to the management of toxic benthic cyanobacteria. Healthy cyanobacterial mats may pose little risk (to humans) if the majority of cyanotoxins are intercellular and thus are not released into the water. However certain environmental conditions may cause mats to release cyanotoxins into the water thus creating a higher risk to human health.

The results documented in Chapters 2 and 3 demonstrated that both toxic and/or non-toxic genotype may exist within cyanobacterial mats. Additionally, anatoxin concentrations were not related to the abundance of *Phormidium*. Therefore, future research looking at the fine scale variability of anatoxins within a sampling site is needed to establish a methodology for collecting representative samples for use in risk assessments. Using current methods, where a single mat sample is collected at a site, there is a strongly likelihood that this mat's toxin content is not representative of the whole site. In the worse possible scenario, the mat collected could be non-toxic while the surrounding mats are highly toxic, this could potentially lead water authorities to make ill-informed decisions about the health risks at any given site.

No studies worldwide have investigated the affects of cyanotoxins on river ecosystem health. Future research should investigate the affects anatoxin has on food webs. For

example, do macro-invertebrates that graze on cyanobacteria accumulate cyanotoxins, and can these toxins be transferred through the food chain. This toxin transfer has not been observed in benthic cyanobacteria, but has been observed with microcystin produced by planktonic cyanobacteria (Vasconcelos 1999; Sipia *et al.* 2002; Wood *et al.* 2006).

4.2 Conclusions:

This thesis highlights the wide distribution of benthic cyanobacteria throughout New Zealand. At present cyanotoxin production is variable and cannot be predicted. This research begins to investigate the regulation of cyanotoxins and cyanobacteria cover and future research should build on these findings. This research has ramifications for all water users in predicting and managing health risks from toxic benthic cyanobacteria.

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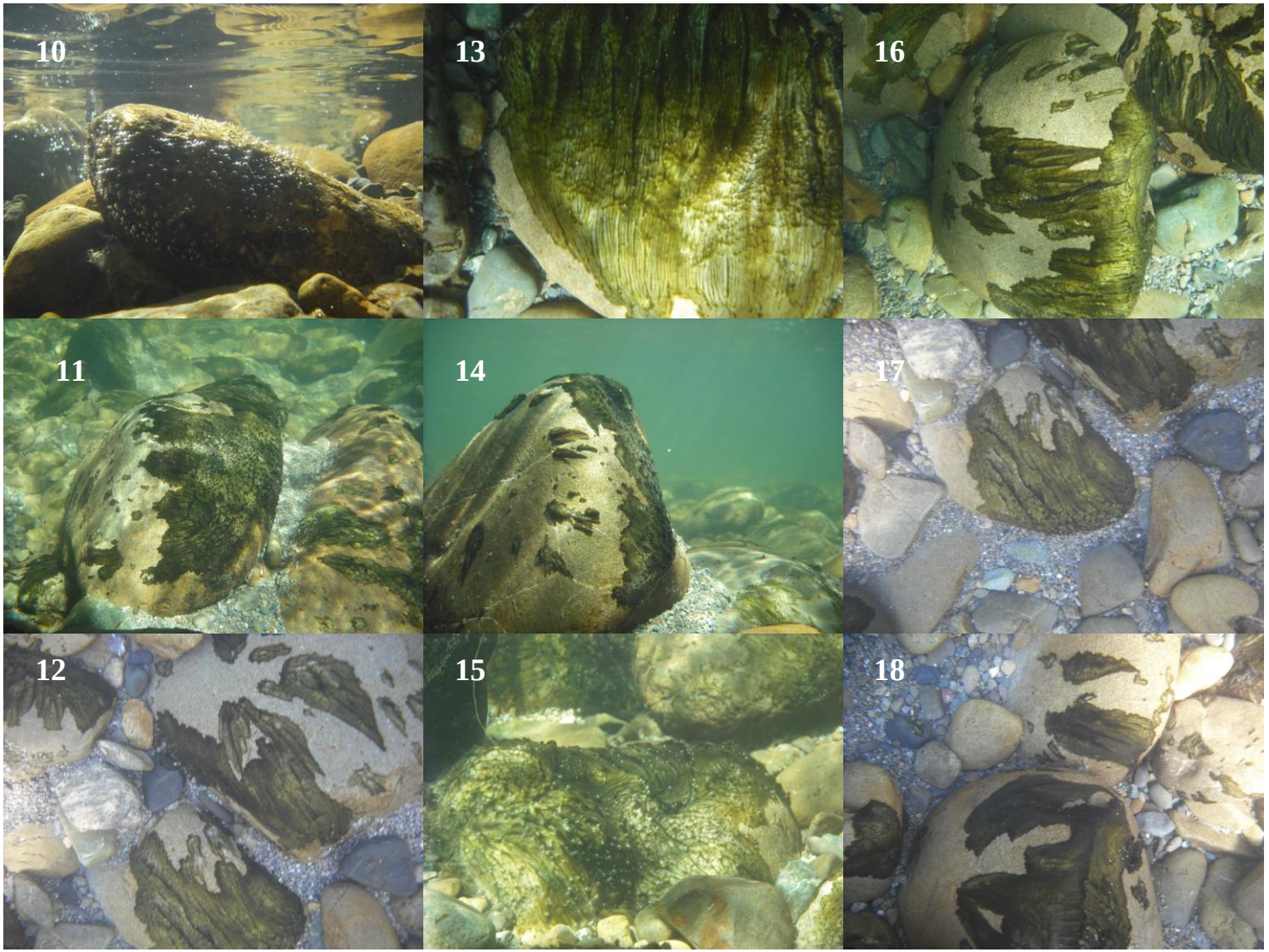
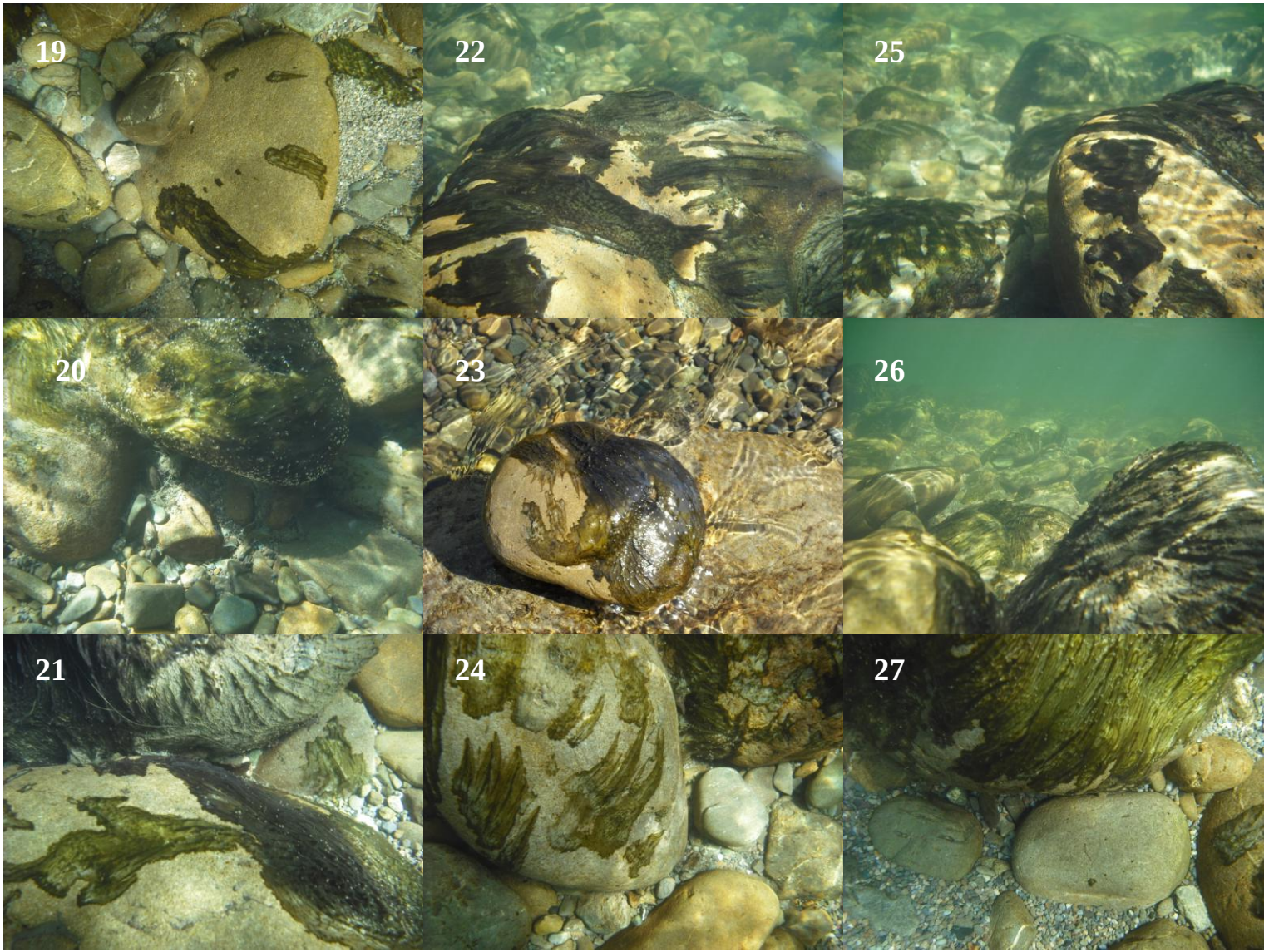
Appendix 1: Examples of mat-like growths of algae and of warning signs.

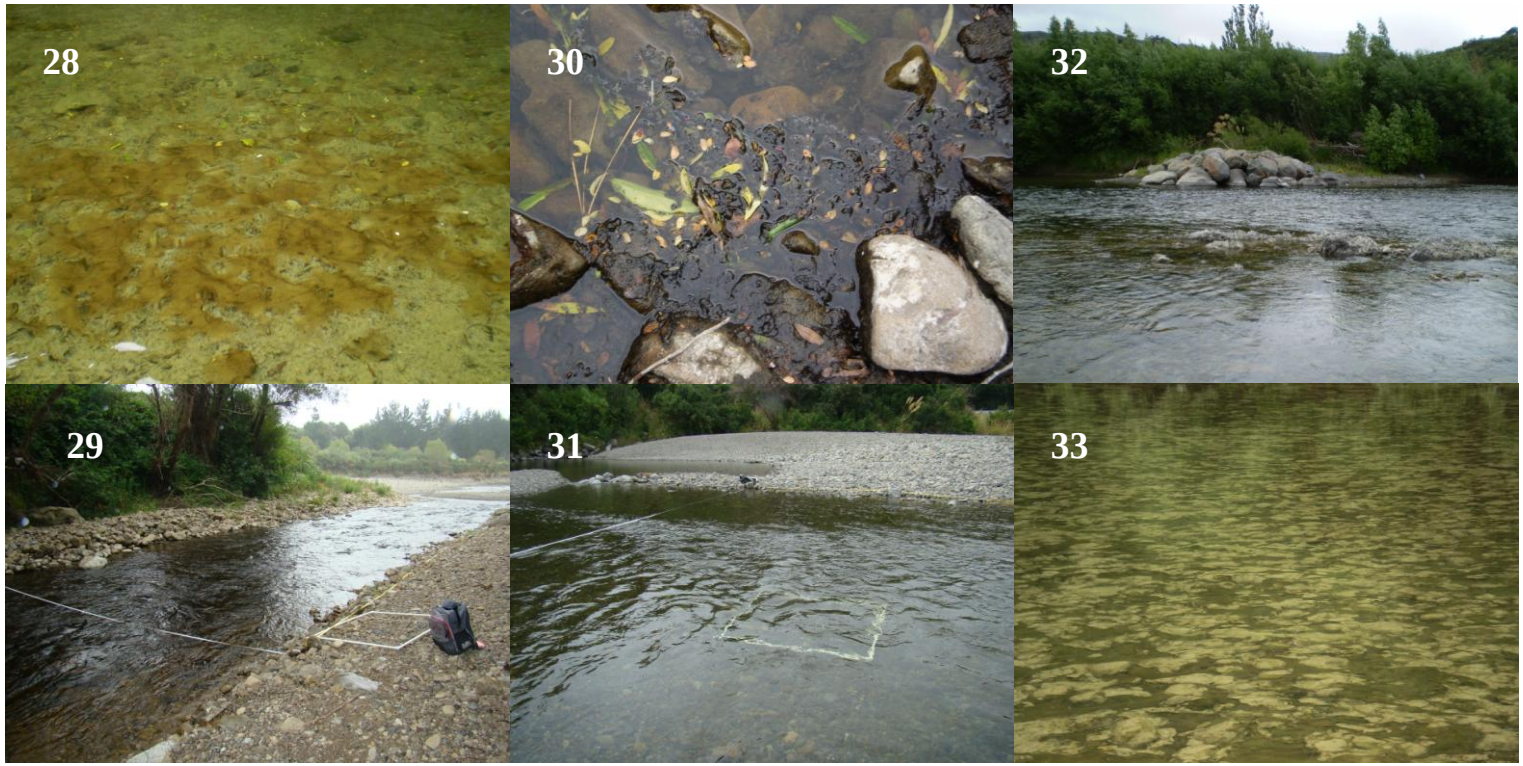
1, Rocks densely covered with *Phormidium* mats. 2, *Phormidium* mat cover across the Hutt River. 3, Rock covered in *Melosira varians.* 4, rocks densely covered with *Phormidium* mats. 5, Large flat rock located in site 3 pool. 6, rock covered in *Phormidium* mat. 7, rock which has had a portion of *Phormidium* mat removed off. 8 & 9 Warning signs erected along the Hutt River.



10-18, In situ *Phormidium* located 500m upstream of site 5, Hutt River.



19-27, In situ *Phormidium* located 500m upstream of site 5, Hutt River.



28, Pseudanabaenaceae mats growing on silt substrate just below site 4, Whakatikei River. 29, site 2 sampling grid Mangaroa River 30, detached mat material building-up on the rivers edge, Hutt River. 31, site 1 sampling grid, Hutt River. 32, rock wall at site 5, Hutt River. 33, cyanobacterial mat cover at site 6 growing on top of a silt layer created by earthworks upstream, Hutt River.

Appendix 2. Anatoxin extraction optimisation

The optimisation of the extraction procedure for anatoxins showed that double distilled water (DDW) extracted higher concentrations of anatoxins than methanol. Using DDW 85% of the available toxin was extracted after two extractions compared to 78% using methanol.

	Double Distilled Water containing 0.1% formic acid			Methanol containing 0.1% formic acid		
Extraction number	ATX (ng/ml)	HTX (ng/ml)	Cumulative Extraction (%)	ATX (ng/ml)	HTX (ng/ml)	Cumulative Extraction (%)
1	ND	1.31	66	ND	0.59	67
2	ND	0.39	85	ND	0.1	78
3	ND	0.11	91	ND	0.08	87
4	ND	0.13	97	ND	0.06	94
5	ND	0.05	100	ND	0.05	100

Efficiency of double distilled water and methanol in extracting anatoxins from lypholized *Phormidium* material. ND = Not detected, limit of detection = 0.02 ng/ml.

Appendix 3. Nutrient data for sites along the Hutt and Wainuiomata Rivers see Chapter 3 for details: All measurements made as g/m^3.

Site Name	Date	Total Nitrogen	Total Phosphorus	Nitrite-N	Nitrate-N	Total Kjeldahl Nitrogen	Dissolved Reactive Phosphorus
Site 1 (Te Marua)	6 December 2007	0.15	0.015	0	0.091	0.1	0
Site 1 (Te Marua)	10 January 2008	0.18	0.0062	0	0.034	0.15	0
Site 1 (Te Marua)	11 February 2008	0.26	0.0095	0	0.21	0.1	0
Site 1 (Te Marua)	5 March 2008	0.16	0.0052	0.0031	0.0589	0.1	0
Site 1 (Te Marua)	3 April 2008	0.13	0.01	0	0.067	0.1	0
Site 1 (Te Marua)	12 May 2008	0.16	0.009	0	0.078	0.1	0
Site 1 (Te Marua)	5 June 2008	0.18	0.01	0	0.1	0.1	0
Site 1 (Te Marua)	30 June 2008	0.26	0.026	0	0.11	0.15	0
Site 1 (Te Marua)	5 August 2008	0.19	0.0085	0	0.11	0.1	0
Site 1 (Te Marua)	2 September 2008	0.19	0.0068	0	0.15	0.1	0
Site 1 (Te Marua)	2 October 2008	0.19	0.0062	0	0.07	0.12	0.0046
Site 1 (Te Marua)	4 November 2008	0.11	0.0052	0	0.059	0.1	0.0078
Site 1 (Te Marua)	20 November 2008	0.16	0.0075	0.2	0.123	0.1	0
Site 1 (Te Marua)	1 December 2008	0.13	0.0055	0.0024	0.0846	0.1	0
Site 1 (Te Marua)	22 December 2008	0.17	0.0073	0.0024	0.0386	0.13	0

Site Name	Date	Total Nitrogen	Total Phosphorus	Nitrite-N	Nitrate-N	Total Kjeldahl Nitrogen	Dissolved Reactive Phosphorus
Site 2 (Mangaroa)	6 December 2007	0.82	0.008	0.0026	0.6874	0.13	0
Site 2 (Mangaroa)	10 January 2008	0.49	0.019	0.0022	0.2878	0.2	0.01
Site 2 (Mangaroa)	11 February 2008	0.89	0.012	0.004	0.736	0.15	0.005
Site 2 (Mangaroa)	5 March 2008	1.8	0.011	0.0037	1.5963	0.18	0
Site 2 (Mangaroa)	3 April 2008	0.95	0.015	0.002	0.848	0	0.0072
Site 2 (Mangaroa)	12 May 2008	0.53	0.026	0.003	0.267	0.25	0.01
Site 2 (Mangaroa)	5 June 2008	0.46	0.022	0.002	0.278	0.18	0.0044
Site 2 (Mangaroa)	30 June 2008	1.3	0.096	0.002	0.798	0.54	0.014
Site 2 (Mangaroa)	5 August 2008	0.86	0.034	0.0034	0.5866	0.27	0.0085
Site 2 (Mangaroa)	2 September 2008	0.76	0.021	0.002	0.608	0.15	0.01
Site 2 (Mangaroa)	2 October 2008	0.58	0.016	0.0028	0.3072	0.26	0.0064
Site 2 (Mangaroa)	4 November 2008	0.62	0.022	0.0024	0.4476	0.17	0.012
Site 2 (Mangaroa)	20 November 2008	0.64	0.004	0.002	0.016	0	0
Site 2 (Mangaroa)	1 December 2008	0.72	0.014	0.0031	0.5369	0.18	0.0051
Site 2 (Mangaroa)	22 December 2008	0.49	0.022	0.0022	0.2578	0.23	0.011

Site Name	Date	Total Nitrogen	Total Phosphorus	Nitrite-N	Nitrate-N	Total Kjeldahl Nitrogen	Dissolved Reactive Phosphorus
Site 3 (Akatarawa)	6 December 2007	0.13	0.008	0	0.014	0.12	0
Site 3 (Akatarawa)	10 January 2008	0.18	0.01	0	0.098	0.1	0
Site 3 (Akatarawa)	11 February 2008	0.18	0.0055	0	0.14	0.1	0
Site 3 (Akatarawa)	5 March 2008	0.1	0.0052	0	0.027	0.1	0
Site 3 (Akatarawa)	3 April 2008	0.16	0.006	0	0.11	0.1	0
Site 3 (Akatarawa)	12 May 2008	0.15	0.007	0	0.099	0.1	0
Site 3 (Akatarawa)	5 June 2008	0.36	0.0065	0	0.3	0.1	0
Site 3 (Akatarawa)	30 June 2008	0.34	0.012	0	0.22	0.12	0
Site 3 (Akatarawa)	5 August 2008	0.3	0.0085	0	0.2	0.1	0
Site 3 (Akatarawa)	2 September 2008	0.14	0.0068	0	0.11	0.1	0
Site 3 (Akatarawa)	2 October 2008	0.11	0.0052	0	0.049	0.1	0
Site 3 (Akatarawa)	4 November 2008	0.14	0.0052	0	0.1	0.1	0
Site 3 (Akatarawa)	20 November 2008	0.1	0.004	0	0.018	0.1	0
Site 3 (Akatarawa)	2 December 2008	0.11	0.004	0	0.0033	0.1	0
Site 3 (Akatarawa)	22 December 2008	0.19	0.0063	0	0.1	0.1	0

Site Name	Date	Total Nitrogen	Total Phosphorus	Nitrite-N	Nitrate-N	Total Kjeldahl Nitrogen	Dissolved Reactive Phosphorus
Site 4 (Whakatikei)	6 December 2007	0.11	0.007	0	0.022	0	0
Site 4 (Whakatikei)	10 January 2008	0.17	0.015	0	0.097	0	0.0062
Site 4 (Whakatikei)	11 February 2008	0.12	0.066	0	0.046	0	0.0041
Site 4 (Whakatikei)	5 March 2008	0.13	0.01	0	0.052	0	0.004
Site 4 (Whakatikei)	3 April 2008	0.22	0.015	0	0.16	0	0.0052
Site 4 (Whakatikei)	12 May 2008	0.18	0.012	0	0.12	0	0.0069
Site 4 (Whakatikei)	5 June 2008	0.76	0.01	0	0.69	0	0.025
Site 4 (Whakatikei)	30 June 2008	0.48	0.016	0.0033	0.3067	0.16	0.0069
Site 4 (Whakatikei)	5 August 2008	0.33	0.012	0	0.26	0	0
Site 4 (Whakatikei)	2 September 2008	0.2	0.004	0	0.15	0	0.0057
Site 4 (Whakatikei)	2 October 2008	0.12	0.012	0	0.047	0	0.006
Site 4 (Whakatikei)	4 November 2008	0.14	0.0082	0	0.081	0	0.0061
Site 4 (Whakatikei)	20 November 2008	0.1	0.0075	0	0.56	0.1	0.0044
Site 4 (Whakatikei)	1 December 2008	0.15	0.012	0	0.063	0	0
Site 4 (Whakatikei)	22 December 2008	0.19	0.011	0	0.059	0.13	0.0073

Site Name	Date	Total Nitrogen	Total Phosphorus	Nitrite-N	Nitrate-N	Total Kjeldahl Nitrogen	Dissolved Reactive Phosphorus
Site 5 (Silverstream)	6 December 2007	0.28	0.015	0	0.2	0	0
Site 5 (Silverstream)	10 January 2008	0.27	0.013	0.0021	0.1479	0.12	0.01
Site 5 (Silverstream)	11 February 2008	0.36	0.0095	0.0023	0.2577	0.11	0
Site 5 (Silverstream)	5 March 2008	0.33	0.0082	0	0.19	0.14	0
Site 5 (Silverstream)	3 April 2008	1	0.01	0	0.99	0	0
Site 5 (Silverstream)	12 May 2008	0.32	0.015	0.003	0.217	0	0.0051
Site 5 (Silverstream)	5 June 2008	0.33	0.016	0.0031	0.2069	0.12	0
Site 5 (Silverstream)	30 June 2008	0.83	0.048	0.0036	0.4564	0.37	0.0042
Site 5 (Silverstream)	5 August 2008	0.57	0.018	0	0.44	0.13	0
Site 5 (Silverstream)	2 September 2008	0.44	0.018	0	0.31	0.14	0
Site 5 (Silverstream)	2 October 2008	0.26	0.011	0	0.15	0.11	0.014
Site 5 (Silverstream)	4 November 2008	0.36	0.016	0.0026	0.2374	0.12	0.009
Site 5 (Silverstream)	20 November 2008	0.29	0.0045	0.2	-0.02	0.1	0
Site 5 (Silverstream)	1 December 2008	0.24	0.0045	0	0.15	0	0
Site 5 (Silverstream)	22 December 2008	0.48	0.028	0.0047	0.2053	0.27	0.0062

Site Name	Date	Total Nitrogen	Total Phosphorus	Nitrite-N	Nitrate-N	Total Kjeldahl Nitrogen	Dissolved Reactive Phosphorus
Site 6 (Boulcott)	6 December 2007	0.24	0.044	0	0.18	0.1	0
Site 6 (Boulcott)	10 January 2008	0.26	0.012	0	0.14	0.12	0
Site 6 (Boulcott)	11 February 2008	0.36	0.016	0	0.21	0.16	0
Site 6 (Boulcott)	5 March 2008	0.35	0.0052	0.0023	0.1677	0.18	0
Site 6 (Boulcott)	3 April 2008	0.41	0.009	0	0.33	0.1	0
Site 6 (Boulcott)	12 May 2008	0.3	0.015	0.0024	0.1976	0.1	0.005
Site 6 (Boulcott)	5 June 2008	0.28	0.01	0	0.15	0.13	0
Site 6 (Boulcott)	30 June 2008	0.6	0.076	0	0.35	0.25	0.0056
Site 6 (Boulcott)	5 August 2008	0.42	0.02	0	0.32	0.1	0
Site 6 (Boulcott)	2 September 2008	1.5	0.0088	0	1.5	0.1	0.0046
Site 6 (Boulcott)	2 October 2008	0.22	0.0062	0	0.14	0.1	0
Site 6 (Boulcott)	4 November 2008	0.25	0.0092	0	0.2	0.1	0.0043
Site 6 (Boulcott)	20 November 2008	0.23	0.004	0	0.18	0.1	0
Site 6 (Boulcott)	2 December 2008	0.25	0.0095	0	0.17	0.1	0
Site 6 (Boulcott)	22 December 2008	0.28	0.012	0.0047	0.1053	0.17	0

Site Name	Date	Total Nitrogen	Total Phosphorus	Nitrite-N	Nitrate-N	Total Kjeldahl Nitrogen	Dissolved Reactive Phosphorus
Site 7 (Manuka Track)	6 December 2007	0.11	0.76	0.0023	0.0197	0	0.0055
Site 7 (Manuka Track)	8 January 2008	0.41	0.04	0	0.11	0.31	0.011
Site 7 (Manuka Track)	4 February 2008	0.1	0.012	0	0.061	0	0.0098
Site 7 (Manuka Track)	3 March 2008	0.21	0.018	0	0.097	0.11	0.0097
Site 7 (Manuka Track)	1 April 2008	0.28	0.02	0	0.14	0.14	0.0099
Site 7 (Manuka Track)	5 May 2008	0.18	0.013	0	0.095	0	0.008
Site 7 (Manuka Track)	3 June 2008	0.2	0.024	0	0.11	0	0.0095
Site 7 (Manuka Track)	1 July 2008	0.17	0.024	0	0.11	0	0.0053
Site 7 (Manuka Track)	4 August 2008	0.27	0.012	0	0.23	0	0.0093
Site 7 (Manuka Track)	1 September 2008	0.24	0.015	0	0.21	0	0.0067
Site 7 (Manuka Track)	1 October 2008	0.1	0.012	0	0.04	0	0.01
Site 7 (Manuka Track)	3 November 2008	0.11	0.012	0	0.045	0	0.0079
Site 7 (Manuka Track)	20 November 2008	0.1	0.012	0	0.027	0	0.0076
Site 7 (Manuka Track)	1 December 2008	0.1	0.024	0	0.008	0	0.012
Site 7 (Manuka Track)	22 December 2008	0.11	0.013	0	0.003	0.11	0.005