Investigating Cyanotoxin Production by Benthic Freshwater Cyanobacteria in New Zealand

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Francine Mary Jorna Smith

Department of Chemistry

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For Mum

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Abstract

Cyanobacteria can form nuisance proliferations and produce large concentrations of toxins that pose a health hazard. This thesis investigates cyanotoxin production by New Zealand benthic cyanobacteria. Cyanobacteria were sampled from lakes, reservoirs, streams, and rivers. Thirty-five strains were isolated into culture and screened for genes involved in the biosynthesis of common cyanotoxins. Positive results were confirmed and cyanotoxin concentrations quantified using analytical chemistry techniques.

Genes involved in anatoxin-a/homoanatoxin-a biosynthesis were detected in nine out of ten *Phormidium* cf. *uncinatum* strains isolated from a single mat. Anatoxin-a was confirmed in these strains by LC–MS/MS at concentrations from 0.3 to 6.4 mg kg^{-1}. One strain also produced homoanatoxin-a. Anatoxin-a variation between strains may explain the wide range in anatoxin-a concentrations previously observed in New Zealand.

The *sxtA* gene involved in saxitoxin biosynthesis was identified in *Scytonema* cf. *crispum* strains. Saxitoxin was confirmed in strains and environmental samples by Jellett PSP Rapid Test and HPLC–FD. Gonyautoxins, neosaxitoxin, and decarbamoyl derivatives were also detected. This study is the first identification of these compounds in *Scytonema* and in New Zealand cyanobacterial strains. These strains were isolated from recreational and pre-treatment drinking water reservoirs, highlighting the risk benthic cyanobacteria pose to human and animal health.

Experiments were undertaken using cultures of *Phormidium* and *Scytonema* to determine how growth influences cyanotoxin production. The effects of iron and copper stress on *P. autumnale* were also investigated. High iron concentrations disrupted attachment mechanisms. Iron and copper had a significant effect on growth, without significantly affecting anatoxin-a production. However, the maximum anatoxin-a quota was consistently observed during early exponential growth. *Scytonema* cf. *crispum* produced higher saxitoxin quota throughout exponential growth than during the stationary phase. Both the *Phormidium* and *Scytonema* growth experiments indicate that high toxin quota can be expected early in benthic mat development, making early detection of these proliferations important.

Abbreviations

2,4-DAB	2,4-diaminobutyric acid
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6,-dienoic acid
AIC	Akaike Information Criteria
ana	genes involved in anatoxin-a and homoanatoxin-a biosynthesis
ANOVA	analysis of variance
aoa	genes from *Aphanizomenon ovalisporum* involved in cylindrsopermopsin biosynthesis
BG11	BG-11 culture medium
$BG11_0$	BG-11 culture medium without $NaNO_3$
BSA	bovine serum albumin
bp	base-pairs
BMAA	β-*N*-methylamino-L-alanine
CAWBG	cyanobacterial (blue-green) strain from CICCM
CICCM	Cawthron Institute Culture Collection of Micro-algae
C-toxins	*N*-sulfocarbamoyl toxins, this includes toxins C1 and C2
CYN	cyanobacterial strain from CICCM
cyr	genes involved in cylindrospermopsin biosynthesis
dcGTX	decarbamoyl gonyautoxin
dcneoSTX	decarbamoyl neosaxitoxin
dcSTX	decarbamoyl saxitoxin
df	degrees of freedom
DIN	dissolved inorganic nitrogen
DRP	dissolved reactive phosphorus
DWSNZ	Drinking Water Standards for New Zealand
ELISA	enzyme-linked immunosorbent assays
GTX	gonyautoxin
HPLC–FD	high performance liquid chromatography with fluorescent detection
HPLC–PDA	high performance liquid chromatography with photodiode array detection

HPLC–UV	high performance liquid chromatography with ultraviolet detection
ICP–MS	inductively coupled plasma mass spectroscopy
i.p.	intra-peritoneal administration
LC–MS/MS	liquid chromatography tandem mass spectrometry
LD_{50}	lethal dose required to kill 50% of the population
LOD	limit of detection
LW	*Lyngbya wollei* saxitoxin variant
mcy	genes involved in microcystin biosynthesis
MEGA	Molecular Evolutionary Genetics Analysis software
MegaBlast	Basic Local Alignment Search Tool for highly similar sequences
ML	maximum likelihood
MLA	MLA culture medium
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
nda	genes involved in nodularin biosynthesis
neoSTX	neosaxitoxin
NES	National Environmental Standards for sources in drinking water
NO_3-N+NO_2-N	total nitrogen from nitrate and nitrite sources
NRPS	non-ribosomal peptide synthetase
PCR	polymerase chain reaction
PKS	polyketide synthase
PP	protein phosphatase
P-PO_4	total phosphorus from phosphate sources
PMAV	provisional maximum allowable value
PSP	paralytic shellfish poisons
RO	reverse osmosis
STX	saxitoxin
sxt	genes involve in saxitoxin biosynthesis
UPLC	ultra performance liquid chromatography
WHO	World Health Organisation

1 Introduction

1.1 Why Study Cyanotoxin Production in Benthic Cyanobacteria?

> *In February 2009, a young beagle called "Charlie" ingested part of a cyanobacterial mat from the Selwyn River. The dog's owner rushed Charlie to the vet after he started to convulse. Another two dogs were treated by the same vet after consuming toxic cyanobacteria. One dog died, Charlie and the other dog survived* (Hartevelt, 2009).

It is widely recognised that cyanobacteria can produce potent toxins that pose a health hazard (Codd et al., 2005a; Falconer and Humpage, 2005; Steffensen et al., 1999; Stewart et al., 2006). Benthic algae, including cyanobacteria, can be broadly defined as algae associated with the bottom of an aquatic habitat (Stevenson, 1996). Humans and other animals are exposed to benthic cyanobacteria every summer. Many people enjoy recreational activities at New Zealand lakes and rivers where they may encounter benthic cyanotoxin producers. In addition, much of this country's drinking water supply for humans and stock is sourced from surface water, a potential habitat for cyanobacteria. Reports of dog fatalities connected with ingestion of benthic toxic cyanobacteria are quite common (Section 1.5.1). Concern about exposure to cyanotoxins in drinking and recreational water has led to the development of national guidelines and standards (Section 1.5.2).

Initial studies into benthic cyanotoxin producers in New Zealand arose after dog fatalities occurred along the Waikanae and Mataura Rivers (Hamill, 2001). Analogous incidents have been reported in Europe (Edwards et al., 1992; Faassen et al., 2012; Gugger et al., 2005; James et al., 1997) and North America (Puschner et al., 2008; Puschner et al., 2010). Dogs have been described as having a fatal attraction to toxic cyanobacterial mats (Codd et al., 1992). Similar to international studies, most research into New Zealand cyanobacteria and cyanotoxin production to date has focused on planktonic species (Pridmore and Etheredge, 1987; Reynolds et al., 1987; Wood et al., 2006). Recent research has highlighted cyanotoxins are produced by benthic cyanobacteria in rivers throughout the country (Hamill, 2001; Heath et al., 2010; Wood et al., 2010a; Wood et al., 2010b). Toxin production in benthic cyanobacterial mats from lakes has also been reported (Wood et al., 2012).

Cyanotoxins can affect the safety of recreational water and drinking sources for both humans and other animals. Limited knowledge is available about the benthic cyanotoxin-producers in New Zealand and how to manage these proliferations (Section 1.5). Currently it is unclear why cyanotoxins are produced (Section 1.4). Greater understanding of what induces this production is required so that more effective resource management plans can be established to protect human and animal health. Only a few studies have investigated the impact of environmental stressors on New Zealand benthic cyanobacteria. Understanding the relationships between environmental stressors and cyanotoxin production will ultimately lead to more effective management strategies.

This project aims to expand the knowledge of benthic cyanotoxin production in New Zealand. Information obtained can assist resource management decisions to reduce the impact of cyanobacteria on human and animal health. This chapter provides background information into cyanobacteria (Section 1.2) and their toxins (Section 1.3); an introduction to environmental stressor studies (Section 1.4); and an overview of cyanotoxin production, occurrence, problems, and management of these hazards in New Zealand (Section 1.5).

1.2 Cyanobacteria

The photosynthetic Gram-negative bacteria termed Cyanophyta or Cyanobacteria are colloquially referred to as blue-green algae. This name can be misleading as cyanobacteria: are prokaryotic, whereas, all other algae are eukaryotic; and range in colour from blue-green to red-violet (Mur et al., 1999). Cyanobacteria consist of a remarkable group of organisms with both bacterial and algal characteristics (Bartram et al., 1999).

Cyanobacteria can be traced back at least two billion years (Knoll, 2008). These organisms performed an important role in the oxygenation of the atmosphere and are still significant primary producers in many aquatic environments (Knoll, 2008). As one of the first organisms to colonise barren land, cyanobacteria are a crucial part of many ecosystems, providing nutrients for many other organisms (Mur et al., 1999). Some cyanobacteria are able to fix nitrogen from the atmosphere (Bartram et al., 1999).

The phylum Cyanobacteria consists of a very versatile group of organisms, which survive under a wide range of environmental conditions including variable light intensity, pH, salinity and temperature (Whitton and Potts, 2000). This versatility allows cyanobacteria to

grow in diverse habitats including terrestrial, marine, brackish, and freshwater environments. The competitive advantages of some cyanobacteria, including buoyancy regulation, low energy requirements, and ability to grow in diverse environmental conditions, can result in large proliferations in the form of extensive blooms, mats, and scums (Codd et al., 2005a; Falconer and Humpage, 2005; Mur et al., 1999). Planktonic cyanobacterial proliferations can appear rapidly, within hours, often due to the concentrating of cells due to failure of buoyancy regulation or wind accumulation (Oliver and Ganf, 2000).

Benthic or planktonic proliferations can cause odour and other aesthetic problems, harm aquatic ecosystems, and block filters and water intake pipes (Bartram et al., 1999; Paerl and Huisman, 2008; Pitois et al., 2000; Wood et al., 2010a). Some cyanobacterial proliferations pose a health hazard, producing high concentrations of potent toxins (Section 1.2; Bartram et al., 1999, Sivonen and Jones, 1999). Other cyanobacterial proliferations have been exploited by humans for diverse purposes including as food sources, biofertilizers, water treatment, and for medicinal properties (Gantar and Svircev, 2008; Thajuddin and Subramanian, 2005).

1.3 Cyanotoxins

Cyanobacterial toxins (cyanotoxins) are bioactive compounds produced by cyanobacteria that can pose a potentially fatal hazard to animals. People can be exposed via drinking water, diet, haemodialysis, recreational and occupational activities (Codd, 2000; Codd et al., 2005a; Gantar and Svircev, 2008; Rellán et al., 2009). Exposure to cyanotoxins usually occurs via skin contact, inhalation, and ingestion including bioaccumulation through the food web (Gantar and Svircev, 2008; Stewart et al., 2009). Parenteral exposure, including haemodialysis and accidental injection when handling toxins, must also be considered in risk assessments as this toxicological pathway is highly efficient (Codd et al., 1999; Stewart et al., 2009).

Cyanobacteria from terrestrial, brackish, marine, or freshwater habitats can produce cyanotoxins (Sivonen and Börner, 2008). Periphytic and planktonic cyanobacteria are most commonly associated with cyanotoxin production. These cyanotoxins are problematic in freshwater recreational areas and drinking water sources around the world (Bartram et al., 1999; Codd et al., 2005b; Stewart et al., 2006). There have also been a few reports of

cyanotoxin-producing epiphytic cyanobacteria associated with submerged macrophytes in the benthic environment (Mohamed and Al Shehri, 2010; Wilde et al., 2005). Only a limited number of terrestrial cyanotoxin-producers are known (Gehringer et al., 2012; Prinsep et al., 1992).

Cyanotoxins are often classified according to their modes of toxicity: hepatotoxic, neurotoxic, cytotoxic, and irritants (Codd et al., 2005b). The former three cyanotoxin classes are of utmost concern as they can be acutely toxic to humans and other animals, with well-documented poisoning events that have caused fatalities (Azevedo et al., 2002; Chorus, 2001; Codd et al., 2005a; Kuiper-Goodman et al., 1999). Less is known about the chronic effects of these cyanotoxins, although evidence suggests that some may be genotoxic and carcinogenic (Falconer and Humpage, 2005; Kuiper-Goodman et al., 1999; Žegura et al., 2011).

The cyanobacterial hepatotoxins of greatest concern worldwide are microcystins and nodularins (Section 1.3.3). Cylindrospermopsin is the most well recognised cyanobacterial cytotoxin. It can affect the liver making this toxin also hepatotoxic (Section 1.3.2). Anatoxin-a, anatoxin-a(s), and saxitoxin are the common neurotoxins identified in freshwater cyanobacteria (Section 1.3.1 and Section 1.3.4). This study focuses on the cyanotoxins of most concern to New Zealand (see Section 1.5). These cyanotoxins are well recognised throughout the world and have been associated with human and other animal fatalities (Codd et al., 2005b; Steffensen et al., 1999; Stewart et al., 2006).

1.3.1 Anatoxins

There are two classes of anatoxins: anatoxin-a and anatoxin-a(s). Despite the similarity in their names, these toxins have very different molecular structures (Figure 1.1). Anatoxin-a is a small bicyclic alkaloid, whereas anatoxin-a(s) is a natural organophosphate (Sivonen and Jones, 1999). Homoanatoxin-a is an analogue of anatoxin-a containing an additional methylene group in the side chain. A few other analogues including precursors and degradation products have been isolated or synthesised (Namikoshi et al., 2003; Namikoshi et al., 2004; Selwood et al., 2007; Wonnacott and Gallagher, 2006). Anatoxin-a and homoanatoxin-a are the primary neurotoxic variants produced by cyanobacteria (Wonnacott and Gallagher, 2006). Other naturally occurring analogues of anatoxin-a tested

for toxicity are non-toxic (Namikoshi et al., 2003). No analogues of anatoxin-a(s) are known (Sivonen and Jones, 1999).

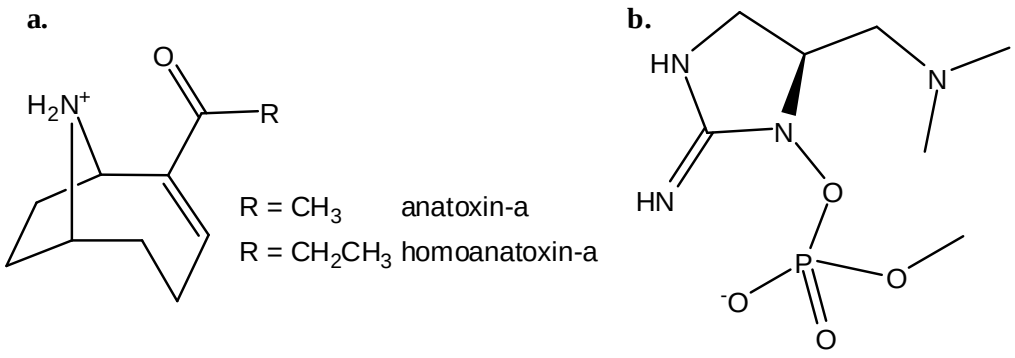

Figure 1.1 Structure of a) anatoxin-a and homoanatoxin-a and b) anatoxin-a(s).

Anatoxin-a and anatoxin-a(s) disrupt the acetylcholine signal pathway, which is involved in muscle stimulation (Kuiper-Goodman et al., 1999). Both classes of anatoxins can cause death via asphyxia from respiratory paralysis.

Anatoxin-a and homoanatoxin-a contain charged polar groups in a similar spatial arrangement to the corresponding functionalities in the neurotransmitter molecule acetylcholine (Figure 1.2). These neurotoxins bind to acetylcholine receptors triggering muscle contraction or other neural signal responses, but cannot be broken down by acetylcholine esterase, causing over-stimulation (Carmichael, 1994). The stereochemistry of anatoxin-a is essential in binding to the acetylcholine receptor. The synthetic enantiomer, (–)-anatoxin-a, is much less potent than the natural enantiomer, (+)-anatoxin-a (Aráoz et al., 2010; Wonnacott and Gallagher, 2006). Reduction of the alkene or carboxyl group also reduces the potency of anatoxin-a (Wonnacott and Gallagher, 2006).

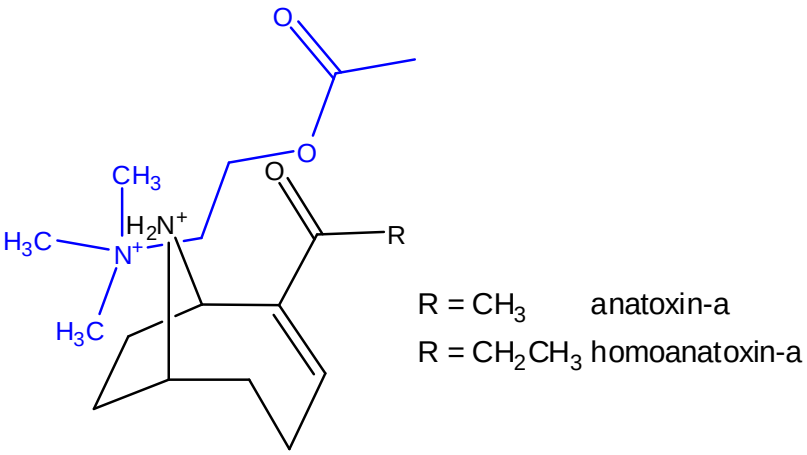

Figure 1.2 Structure of anatoxin-a (black) compared with acetylcholine (blue).

In contrast, anatoxin-a(s) blocks acetylcholine esterase inhibiting the breakdown of acetylcholine (Carmichael, 1994). Therefore acetylcholine continues to stimulate muscle contraction by continuing to bind to the acetylcholine receptor. This toxicity mechanism for anatoxin-a(s) is similar to the mechanism for organophosphate insecticides (Kuiper-Goodman et al., 1999).

Anatoxin-a production by cyanobacteria has been identified worldwide. This neurotoxin has been associated with animal fatalities in Africa, Europe, North America, and New Zealand from ingestion of benthic cyanobacteria (Aráoz et al., 2010; Osswald et al., 2007). It can also be produced by planktonic genera (Osswald et al., 2007). Anatoxin-a and homoanatoxin-a were isolated from axenic *Anabaena* and *Oscillatoria* strains, verifying the cyanobacterial origin of these metabolites (Aráoz et al., 2010).

Anatoxin-a(s) production is only associated with *Anabaena* species (Sivonen and Jones, 1999). This neurotoxin has been implicated in animal fatalities in Denmark (Henrikson et al., 1997; Onodera et al., 1997a) and North America (Cook et al., 1989; Mahmood et al., 1988). Of direct concern to human health, anatoxin-a(s) was confirmed in a Brazilian drinking water reservoir (Dörr et al., 2010; Molica et al., 2005). The presence of anatoxin-a(s) was also reported for another Brazilian drinking water reservoir, based on acetylcholine esterase inhibition. Further analyses were not completed to confirm the presence of this neurotoxin. This cyanotoxin is not identified as frequently as the

cyanobacterial neurotoxins anatoxin-a and saxitoxin, most probably due to the difficulties in confirming the detection of anatoxin-a(s) (Dörr et al., 2010).

Molecular genetics and detection methods for anatoxin-a and related compounds are discussed in Chapter 3, Section 3.1.1.

1.3.2 Cylindrospermopsin

Cylindrospermopsin (Figure 1.3) is a tricyclic alkaloid linked to uracil via a bridging carbon (Sivonen, 2008). This bridging carbon can be hydroxylated to form either cylindrospermopsin or the epimer 7-epicylindrospermopsin, or non-hydroxylated to form deoxy-cylindrospermopsin (Pearson et al., 2010; Sivonen and Börner, 2008). Cylindrospermopsin-producing cyanobacteria have been identified world-wide (Kinnear, 2010).

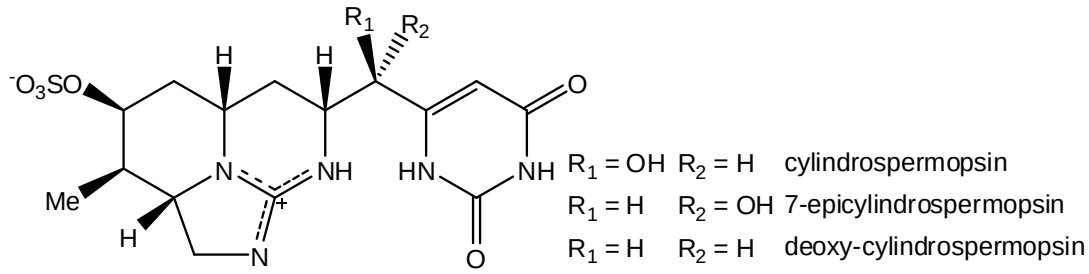

Figure 1.3 Structure of cylindrospermopsin and analogues.

Cylindrospermopsin can cause necrosis in liver, kidney, spleen, and other tissues (Falconer and Humpage, 2005; Sivonen and Börner, 2008). While the main mechanism of toxicity is irreversible inhibition of protein synthesis, other potential effects include glutathione synthesis inhibition, genotoxicity, and carcinogeniticty (Humpage, 2008; Žegura et al., 2011). Unlike many other cyanotoxins, high concentrations of extracellular cylindrospermopsin are often detected in environmental samples, making this cyanotoxin more readily bioavailable to aquatic organisms (Kinnear, 2010).

An outbreak of human hepatoenteritis occurred amongst the community on Palm Island, Queensland, Australia in November 1979 (Griffiths and Saker, 2003; Hawkins et al., 1985). One hundred and thirty-eight children and ten adults were hospitalised (Byth, 1980).

A dense cyanobacterial bloom in the drinking water reservoir was treated with $CuSO_4$, potentially causing mass release of intracellular cyanotoxins. Cylindrospermopsin producing *Cylindrospermopsis raciborskii* was later identified in this cyanobacterial bloom (Hawkins et al., 1985). Cylindrospermopsin has also been associated with cattle fatalities in Queensland (Saker et al., 1999).

Molecular genetics and detection methods for cylindrospermopsins are discussed in Chapter 3, Section 3.1.2.

1.3.3 Microcystin and Nodularin

Microcystin and nodularin are seven- and five-membered cyclic peptides respectively (Figure 1.4). Over 90 structural variants of microcystin have been described (Stewart and Falconer, 2008). The most commonly identified variant is microcystin-LR, which corresponds to the variant with amino acids L-leucine and L-arginine at positions 2 and 4 respectively (Gago-Martinez, 2007). The commonly identified microcystin analogues have variable L-amino acids at positions 2 and 4, whereas positions 3 and 7 frequently vary between methylated and demethylated forms (Sivonen and Börner, 2008). In contrast only a few analogues of nodularin occur (Beattie et al., 2000; Namikoshi et al., 1994; Saito et al., 2000; Sivonen and Jones, 1999). Both microcystin and nodularin have been isolated from axenic cyanobacterial strains, demonstrating that these hepatotoxins are cyanobacterial in origin (Sivonen and Jones, 1999).

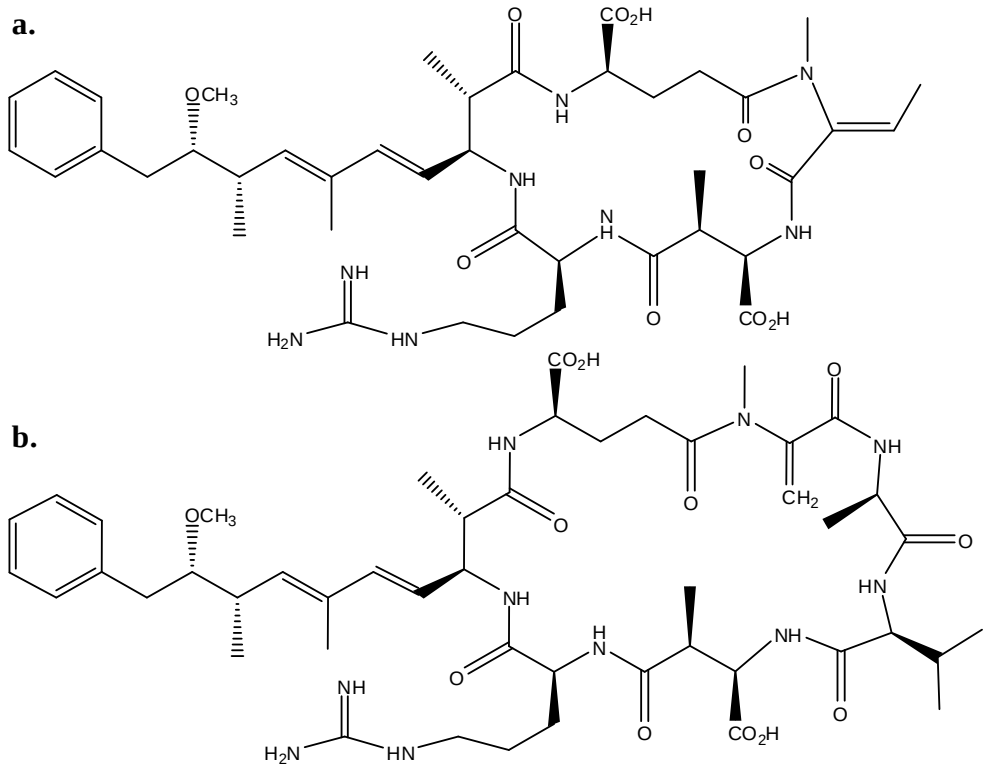

Figure 1.4 Structure of a) nodularin, and b) microcystin-LR.

In vertebrates, these cyclic peptide hepatotoxins are actively transported into the liver where they can cause severe damage to this organ (Wiegand and Pflugmacher, 2005). The main mechanism of toxicity in vertebrates is the inhibition of protein phosphatases (PP) 1 and 2A (Gago-Martinez, 2007; Gulledge et al., 2002; Wiegand et al., 2002). These hepatotoxins contain a few amino acids, uncommon in other natural products. These amino acids include 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6,-dienoic acid (Adda), ᴅ-erythro-β-methyl-isoaspartate, and either *N*-methyl-dehydroalanine in microcystins or *N*-methyl-dehydrobutyrine in nodularin (Welker, 2008; Wiegand and Pflugmacher, 2005). The methyl-dehydroalanine group of microcystins can covalently bond to cysteine subunits of these protein phosphatases. Inhibition of PP1 and PP2A is achieved by Adda interfering with the hydrophobic groove of the catalytic site (Wiegand and Pflugmacher, 2005). Without Adda these hepatotoxins are inactive. Linearized peptides or the enantiomer of Adda are less potent inhibitors of protein phosphatases (Gulledge et al., 2002). Structural analogues vary in toxicity. Both microcystins and nodularins are usually highly toxic, only a few non-toxic variants are known (Sivonen and Jones, 1999). Microcystin-LR is one of

the more toxic variants with an intra-peritoneal administration (i.p.) LD_{50} in mice of 25–50 μg kg^{-1} (Gago-Martinez, 2007). While other common microcystins can be similar in potency, or up to ten-fold less toxic (Gago-Martinez, 2007; Stewart and Falconer, 2008). The i.p. LD_{50} for nodularin is 60 μg kg^{-1} in mice (Carmichael et al., 1988). Studies have suggested that chronic exposure to microcystin-LR, and potentially other microcystins, may be carcinogenic (Pearson et al., 2010; Žegura et al., 2011).

Microcystin is the most frequently identified cyanotoxin globally. The majority of research has focused on this cyanotoxin, in particular production by *Microcystis*. Microcystins are known to be produced by all cyanobacterial orders: Chroococcales, Oscillatoriales, Nostocales, and Stigonematales (Jungblut and Neilan, 2006). Microcystin producing cyanobacteria have been identified in planktonic, benthic, and terrestrial habitats (Codd et al., 1999). Microcystins have been associated with both human and other animal poisonings including fatalities (Codd et al., 2005a). The most notorious incident of human fatalities involved over 50 haemodialysis patients in Brazil after the water supply was not treated correctly to remove microcystins during a cyanobacterial bloom (Azevedo et al., 2002; Pouria et al., 1998).

Until recently nodularin has only been detected in the cyanobacterium *Nodularia.* A *Nostoc* species that forms a terrestrial symbiont is now known to produce nodularin and the variant with L-homoarginine at amino acid position 2 (Gehringer et al., 2012). Recently nodularin has also been identified in benthic cyanobacterial mats, from lakes without *Nodularia*, however the nodularin producer has not been identified (Wood et al., 2012). In addition a nodularin variant, motuporin, has been isolated from a marine sponge *Theonella swinhoei*. The origin of the nodularin from *T. swinhoei* is not clear, and could potentially be from symbiotic cyanobacteria (Sivonen and Jones, 1999). Nodularin has been associated with animal fatalities (Carmichael et al., 1988).

Molecular genetics and detection methods for microcystins and nodularins are discussed in Chapter 3, Section 3.1.3.

1.3.4 Saxitoxins

Saxitoxins are fast-acting neurotoxic alkaloids produced predominantly by some freshwater cyanobacteria and marine dinoflagellates (Sivonen and Jones, 1999). In the marine environment, saxitoxins are commonly referred to as paralytic shellfish poisons (PSP). About 60 analogues of saxitoxin are known (Wiese et al., 2010). Saxitoxin variants recorded in cyanobacteria include decarbamoyl derivatives (dc), gonyautoxins (GTX); neosaxitoxin (neoSTX), *N*-sulfocarbamoyl toxins (C-toxins), saxitoxin (STX), and a class of toxins produced by *Lyngbya wollei* (Humpage et al., 2010). The most common saxitoxin variants are presented in Figure 1.5 and Table 1.1.

Saxitoxins inhibit nerve conduction by blocking sodium, potassium, and calcium ion channels (Llewellyn, 2006). Human poisonings are well-documented and usually occur after consuming contaminated seafood, often shellfish, in which saxitoxins have accumulated (Etheridge, 2010; Llewellyn, 2006). Paralytic shellfish poisonings from saxitoxins can be fatal to humans. Symptoms of poisoning are often experienced within a short time after ingesting contaminated food and death may occur within a few hours (Llewellyn, 2006). Saxitoxin is the most potent variant found in cyanobacteria with an i.p. LD_{50} of about 8–10 $\mu g\ kg^{-1}$ in mice (Llewellyn, 2006; Wilberg and Stephenson, 1960).

Shellfish accumulate saxitoxins through filter-feeding on toxic dinoflagellate phytoplankton. Saxitoxins can also accumulate through the food web through ingestion of toxic algae by non-filter feeders, including some crustaceans and fish, thereby presenting an additional risk to human health risk (Llewellyn, 2006; Wiese et al., 2010). Saxitoxins have been detected in the tissue and liver of the freshwater fish, tilapia (*Oreochromis niloticus*), from an aquaculture farm in Brazil (Galvão et al., 2009). *Anabaena spiroides* was identified as a possible source of saxitoxin (Galvão et al., 2009).

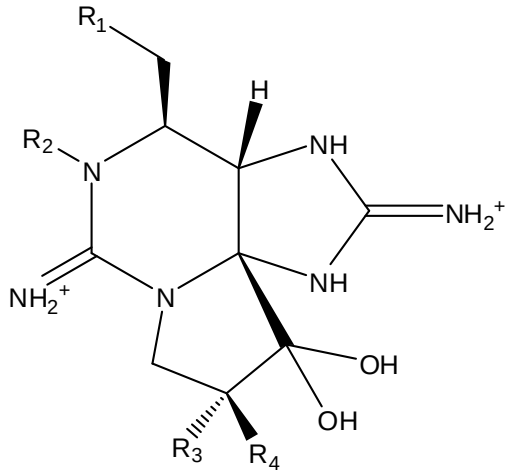

Figure 1.5 General structure of saxitoxin and common analogues found in cyanobacteria. See Table 1.1 for analogues.

Table 1.1 Specific variation of saxitoxin and common analogues found in cyanobacteria. The general structure is given in Figure 1.5.

Analogue	**R1**	R2	**R3**	**R4**
Carbamate toxins				
STX	CO_2NH_2	H	H	H
neoSTX	CO_2NH_2	OH	H	H
GTX1	CO_2NH_2	OH	OSO_3^-	H
GTX2	CO_2NH_2	H	OSO_3^-	H
GTX3	CO_2NH_2	H	H	OSO_3^-
GTX4	CO_2NH_2	OH	H	OSO_3^-
***N*-sulfocarbamoyl toxins**				
GTX5	$CO_2NHSO_3^-$	H	H	H
GTX6	$CO_2NHSO_3^-$	OH	H	H
C1	$CO_2NHSO_3^-$	H	OSO_3^-	H
C2	$CO_2NHSO_3^-$	H	H	OSO_3^-
C3	$CO_2NHSO_3^-$	OH	OSO_3^-	H
C4	$CO_2NHSO_3^-$	OH	H	OSO_3^-
Decarbamoyl toxins				
dcSTX	OH	H	H	H
dcneoSTX	OH	OH	H	H
dcGTX1	OH	OH	OSO_3^-	H
dcGTX2	OH	H	OSO_3^-	H
dcGTX3	OH	H	H	OSO_3^-
dcGTX4	OH	OH	H	OSO_3^-

C = *N*-sulfocarbamoyl toxins (C-toxins); GTX = gonyautoxin; neoSTX = neosaxitoxin; and STX = saxitoxin. Decarbamoyl derivatives are specified by the prefix dc. Table adapted from Kellmann and Neilan (2007).

In Australia, saxitoxins produced by cyanobacteria have been linked with sheep mortalities (Negri et al., 1995). Saxitoxins were also the only neurotoxin identified in *Anabaena circinalis* from the Murray-Darling river system (Humpage et al., 1994) where an extensive bloom in 1991 resulted in over 1600 stock deaths (Blue-Green Algae Task Force, 1992; Bowling and Baker, 1996).

The detection of saxitoxins in drinking water sources in Australia (Hoeger et al., 2004), Brazil (Molica et al., 2005), and the Waikato River in New Zealand (Kouzminov et al., 2007), demonstrates an additional pathway through which the public may be exposed to saxitoxins. Cyanobacteria were the likely source for the saxitoxin but this was not confirmed. In the Australian pre-treatment drinking water reservoir there was a correlation between the population of *Anabaena circinalis* and saxitoxin production (Hoeger et al., 2004). Molica et al. (2005) suggested that saxitoxin production found in the Brazilian drinking water reservoir was from *Cylindrospermopsis raciborskii*. The source of the Waikato River saxitoxin was thought to be an *Anabaena* sp. (Kouzminov et al., 2007).

Molecular genetics and detection methods for saxitoxins are discussed in Chapter 3, Section 3.1.4.

1.3.5 Other Cyanobacterial Toxins

Neurotoxins produced by marine cyanobacteria include kalkitoxin, antillatoxin, and jamaicamides (Aráoz et al., 2010). Neurotoxic amino acids, β-*N*-methylamino-L-alanine (BMAA) and its isomer 2,4-diaminobutyric acid (2,4-DAB), are widely produced by cyanobacteria (Banack et al., 2011; Cox et al., 2005). BMAA has been associated with motor-neuron disorders, in particular Amyotrophic Lateral Sclerosis and Alzheimer's disease (Aráoz et al., 2010; Banack et al., 2011). Biomagnification of BMAA from cyanobacterial symbionts through the food web has been linked with human fatalities (Cox et al., 2003).

Amongst the irritant cyanotoxins are the dermato- and gastrotoxins. Lipopolysaccharides are a common irritant that form part of the cell wall in Gram-negative bacteria, in which all cyanobacteria are classified (Sivonen and Jones, 1999). Lyngbyatoxins, aplysiatoxins, and debromoaplysiatoxin are produced by some benthic cyanobacterial species (Sivonen and

Jones, 1999). These cyanotoxins can cause dermal and gastrointestinal inflammation. The latter two are also potent tumour promoters (Sivonen and Jones, 1999).

Other cyanobacterial bioactive metabolites, including less well-known toxins, are reviewed elsewhere (Harada, 2004; Rastogi and Sinha, 2009; Sivonen and Börner, 2008; Van Wagoner et al., 2007).

1.4 Environmental Stressors

Understanding the influence of environmental stressors on cyanobacterial growth and cyanotoxin production is essential for effective water management (Kardinaal and Visser, 2005; Paerl et al., 2011; Tyler et al., 2009). Furthermore, investigating cyanotoxin production in response to environmental stressors may assist in identifying the role of cyanotoxin production in cyanobacteria (Sivonen and Börner, 2008). The function of cyanotoxins has been speculated to include defence mechanisms, nutrient/other metabolic storage reserves, antifouling agents, or to reduce abiotic stress (Codd, 1995; Sivonen and Börner, 2008). Further research is needed to identify why cyanobacteria produce cyanotoxins.

A number of environmental parameters have been investigated in relation to cyanotoxin production and growth, in particular that of microcystin-producing cyanobacteria (Sivonen and Jones, 1999). Physical parameters commonly studied include temperature, pH, light intensity, and salinity. Chemical factors studied include nitrogen, phosphorus, and micronutrients. In general these environmental stressors only affect cyanotoxin production by a factor of up to 3-fold (Sivonen and Jones, 1999). The highest cyanotoxin concentrations are usually detected under conditions of optimal growth (Kaebernick and Neilan, 2001). Inhibition of cyanotoxin production has occasionally been demonstrated. Saker and Griffiths (2000) observed the inhibition of cylindrospermopsin production when *Cylindrospermopsis raciborskii* was exposed to an increase in temperature from 25 to 35 °C. The ability to produce cylindrospermopsin was restored with a decrease in temperature.

Environmental stressor studies have been extensively reviewed elsewhere (Kardinaal and Visser, 2005; Neilan et al., 2012; Sivonen and Jones, 1999). In general the triggers for growth are well-known, however the stressors directly related to cyanotoxin production are

not well understood. The majority of these studies focus on microcystin production by planktonic species. An environmental study investigated microcystin production in benthic mats in relation to a number of physical and chemical parameters (Mez et al., 1998). Physical parameters including light, strong wind, rain, snowfall, and high iron concentrations were identified as probable factors influencing metabolic pathways and microcystin production (Mez et al., 1998).

Susceptibility to environmental stressors also varies between different strains of cyanobacteria. For example, non-toxic *Microcystis aeruginosa* strains were more resistant to nonlyphenol exposure, a persistent organic pollutant, than microcystin-producing strains (Wang et al., 2007). Further examples of variability in growth and cyanotoxin production in response to stress are described in Chapter 4, Section 4.1.2.

1.5 New Zealand Cyanotoxin-Producing Cyanobacteria

In total, 87 genera of cyanobacteria have been recorded in New Zealand, compared with about 215 genera described worldwide (Broady and Merican, 2012). Cyanobacteria can be terrestrial, planktonic, or members of mat, crust, and scum communities. Shifts in algal species composition within these communities can indicate changes in water quality, with extensive proliferations indicative of water quality deterioration (Biggs and Kilroy, 2000; Heisler et al., 2008). Benthic algae, found attached to or associated with the substrata of aquatic environments, are key primary producers in many freshwater environments (Stevenson, 1996). New Zealand cyanobacterial benthic taxa are not well-known (Broady and Merican, 2012). In New Zealand rivers and streams, the length of time between flood events is perhaps the dominant factor influencing periphyton biomass (Biggs and Kilroy, 2004). Large proliferations usually do not occur in rivers and streams that have been disturbed by floods within the previous 30 days (Biggs and Kilroy, 2004).

1.5.1 Cyanotoxins

Historically records of cyanobacteria in New Zealand are irregular and generally occur once aesthetic problems arise in lakes (Pridmore and Etheredge, 1987). A nationwide survey of 227 different water bodies, undertaken from 2001 to 2004, revealed that cyanotoxin-producing cyanobacteria were more extensive in New Zealand than originally documented (Wood et al., 2006). These results indicated that cyanobacteria pose a widespread health risk to humans and other animals including stock and dogs.

Anatoxin-a, cylindrospermopsin, microcystin, nodularin, and saxitoxin have been identified in New Zealand cyanobacterial proliferations (Wood et al., 2006). Nodularin was first elucidated from extracts of planktonic *Nodularia spumigena* from Lake Ellesmere/Te Waihora and Forsyth/Te Wairewa (Carmichael et al., 1988; Rinehart et al., 1988). Microcystin, the most frequently identified cyanotoxin worldwide, is also commonly identified in New Zealand (Wood et al., 2006). Anatoxin-a degradation products were first identified in benthic cyanobacterial samples from *Oscillatoria*-like species from the Waikanae River (Hamill, 2001). Confirmation of anatoxin-a, isolated from Lake Hakanoa was the first case of this neurotoxin identified from *Aphanizomenon issatschenkoi* in the Southern Hemisphere (Wood et al., 2007a). Cylindrospermopsin and deoxy-cylindrospermopsin in New Zealand were first detected in *Cylindrospermopsis raciborskii* (Wood and Stirling, 2003). Saxitoxins were found in drinking water supplies from the Waikato River, however the cyanotoxin-producer source was not identified (Kouzminov et al., 2007). Anatoxin-a(s) has not been detected (Ministry for the Environment and Ministry of Health, 2009).

Occasionally sheep, cattle, and dog fatalities have been linked with brackish or freshwater algal blooms, particularly associated with Lakes Ellesmere and Forsyth (Hogan, 1993). *Anabaena*, *Aphanizomenon*, and *Nodularia* were dominant within these blooms.

In New Zealand, benthic toxic cyanobacteria are potentially more problematic than toxic planktonic cyanobacteria. People and other animals, particularly dogs, can be exposed to large proliferations of benthic cyanobacteria recreationally. Anatoxin-a, microcystin, nodularin, and saxitoxins have been identified in benthic cyanobacteria in lakes and rivers (Wood et al., 2006; Wood et al., 2010a; Wood et al., 2012). Anatoxin-a was identified as the probable cause of dog fatalities after a series of poisoning incidents from 1998 to 2000 (Hamill, 2001). Since then detection of benthic cyanotoxin producers has become a frequent occurrence. Twelve dog fatalities were associated with benthic mats during the two summers from December 1998 to April 2000 (Hamill, 2001). Another series of dog fatalities, associated with the Hutt River during November and December 2005, contributed to the identification of homoanatoxin-a in New Zealand (Wood et al., 2007b). Microcystin from a benthic oscillatorialean species has also been implicated with dog fatalities (Wood et al., 2010a). These cases highlight the risk benthic cyanobacteria pose to

animal health, and have caused regional councils and territorial agencies to monitor benthic cyanobacteria to protect the public.

A precautionary approach is advised with any cyanobacterial proliferations as all cyanobacteria produce irritants and unknown cyanotoxin-producers could still be identified (Ministry for the Environment and Ministry of Health, 2009). Green algae and diatoms tend to form the major component of New Zealand periphyton communities, whereas cyanobacteria and red algae appear to be less dominant (Biggs, 1990). Periphyton surveys indicate that *Calothrix, Schizothrix, Lyngbya, Nostoc, Oscillatoria,* and *Phormidium* are the dominant benthic cyanobacterial species in rivers and streams (Biggs and Kilroy, 2000). The latter four of these genera are well recognised as potential cyanotoxin-producers.

1.5.2 Water Quality Standards and Guidelines

In New Zealand, the Ministry of Health and the Ministry for the Environment have set standards and provide guidelines for monitoring cyanobacteria and their toxins in drinking water and recreational areas. Provisional maximum allowable values (PMAV, Table 1.2) are provided for cyanotoxins in *Drinking Water Standards for New Zealand* (DWSNZ, Ministry of Health, 2008). When cyanotoxin concentrations in drinking water rise above 50% PMAV, monitoring twice a week is required to ensure these levels do not fluctuate above the PMAV (Ministry of Health, 2008).

Table 1.2 Provisional maximum allowable values for cyanotoxins in the Ministry of Health Drinking Water Standards for New Zealand 2005 (Revised 2008).

Toxin	**PMAV (mg L^{-1})**	**Comments**
Anatoxin-a	0.006	
Anatoxin-a(s)	0.001	
Cylindrospermopsin	0.001	
Homoanatoxin-a	0.002	
Microcystin	0.001	expressed as microcystin-LR equivalents
Nodularin	0.001	
Saxitoxin	0.003	expressed as saxitoxin equivalents

The *National Environmental Standards for Sources of Human Drinking Water* (NES) came into effect in June 2008 as part of the Resource Management Act 1991 (Ministry for the Environment, 2007). This standard was established to minimise drinking water source contamination. The NES requires regional and territorial authorities to assess how activities at or near a drinking water source will affect the quality of drinking water when issuing activity permits (Ministry for the Environment, 2007). For surface water sources this could include taking into consideration the effects of activities on cyanobacterial mat formation that can lead to cyanotoxin production.

The *New Zealand Guidelines for Cyanobacteria in Recreational Fresh Waters* provides advice for managing recreational areas where the public may be exposed to cyanobacteria (Ministry for the Environment and Ministry of Health, 2009). These guidelines emphasise the risks related to planktonic cyanobacteria in lakes and periphytic cyanobacteria in rivers. The guidelines outline roles and responsibilities of regional resource managers and health agencies. Known cyanotoxin producers in New Zealand are listed. These cyanotoxin-producers are *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Phormidium*, and *Planktothrix*. Of these species, *Nostoc*, *Oscillatoria*, *Phormidium*, and *Planktothrix* have been found in benthic habitats. In addition these guidelines recognise *Cylindrospermum*, *Fischerella*, and *Lyngbya wollei* as cyanotoxin-producers from benthic habitats internationally (Ministry for the Environment and Ministry of Health, 2009). Tools for recognising, monitoring, sampling, and reporting potential hazards to the public are also provided (Ministry for the Environment and Ministry of Health, 2009).

1.5.3 Management of Benthic Cyanotoxin Producers in Canterbury

Although periphytic cyanobacterial mats naturally exist in rivers and streams in Canterbury, the first mats containing cyanotoxins were not found until March 2007 (Environment Canterbury, 2008). These were brought to the attention of the Environment Canterbury, the regional council, after a dog became ill from ingesting a brownish-black algal mat from the Ashley River. Analysis revealed both anatoxin-a and homoanatoxin-a in cyanobacteria collected from two sites along the river (Environment Canterbury, 2008). As part of the recreational water quality monitoring programme, Environment Canterbury monitors for benthic cyanobacterial proliferations and takes precautions to advise the public of potential hazards as they arise.

When potentially toxic proliferations are identified, the District Health Board, City and Regional Council advise the public of the potential risks through media releases and erecting information signs near the site. Environment Canterbury, along with other regional councils around New Zealand, routinely monitors water quality in surface and ground water. In Canterbury, water quality often decreases as it flows from the mountains towards the ocean due to increased land use for agriculture and industry resulting in accumulation of contaminants (Meredith and Hayward, 2002). Contaminants in lakes and rivers can be a potential stressor to cyanobacteria and other organisms. Water quality can be degraded by a variety of contaminants including microorganisms, nutrients, trace elements, and organic compounds. Other stressors include pH, temperature, and turbidity. These physical, biological, and chemical parameters are included in monitoring plans.

1.6 Project Aims

This interdisciplinary project investigates cyanotoxin production in New Zealand benthic freshwater cyanobacteria. This study had the following aims and objectives:

- To sample benthic mat-forming cyanobacteria in South Island water bodies and establish a culture collection for use in future experiments (Chapter 2).

- To use morphological and molecular phylogenetic techniques to characterise the cyanobacterial cultures and field specimens (Chapter 2).

- To screen the culture collection for cyanotoxin production potential using a variety of molecular genetic, biochemical, and analytical chemistry techniques (Chapter 3).

- To monitor variability of cyanotoxin production during growth in batch cultures (Chapter 4–5).

- To investigate the effects of metal stressors on growth and cyanotoxin production (Chapters 4).

2 Identification, Description, and Distribution of Benthic Taxa used in this Study*

2.1 Introduction

Cyanobacteria have evolved into a structurally diverse group of microorganisms and can be unicellular, colonial, or filamentous (Mur et al., 1999). Filamentous cyanobacteria can differentiate specialised cells, called heterocytes and akinetes, and can be unbranched or exhibit true or false-branching (Komárek et al., 2003).

There are four orders of cyanobacteria:

Chroococcales: Unicellular, single or in colonies. True filaments absent.

Oscillatoriales: Cells forming filaments, without true branching or specialised cells.

Nostocales: Cells forming filaments, containing specialised cells, without true branching.

Stigonematales: Cells forming filaments, with specialised cells and true branching.

2.1.1 Benthic Cyanobacteria

General comments

Benthic cyanobacteria include periphyton, which grow attached to substrata, and metaphyton, which grow loosely associated with substrata but are not attached (Stevenson, 1996). Both can form dense intertwined mats. These proliferations were selected as the focus of this study. They can cause aesthetic problems, entrap sediment, impair ecosystems, and produce potent cyanotoxins (Biggs, 2000; Pitois et al., 2000; Wood et al., 2010a). Periphyton proliferations can occur in low flow, poor quality water with moderate to high concentrations of nutrients (Biggs and Kilroy, 2000). Both periphyton and metaphyton mats can trap bubbles of oxygen causing the mats to rise to the surface and

* Part of this chapter has been presented in the following publications:

Smith, F. M. J.; Wood, S. A.; van Ginkel, R.; Broady, P. A.; Gaw, S., First report of saxitoxin production by a species of the freshwater benthic cyanobacterium, *Scytonema* Agardh. *Toxicon* **2011**, *57*, 566-573.

Smith, F. M. J.; Kelly, D.; Wilks, T.; Broady, P. A.; Gaw, S., *Distribution of Scytonema (Cyanobacteria) and associated saxitoxins in recreational lakes in Canterbury*; R11/36; Environment Canterbury Technical Report: **2011**, p 18.

Smith, F. M. J.; Wood, S. A.; Wilks, T.; Kelly, D.; Broady, P. A.; Williamson, W.; Gaw, S., Survey of *Scytonema* (Cyanobacteria) and associated saxitoxins in the littoral zone of recreational lakes in Canterbury (New Zealand). *Phycologia* **2012**, doi:10.2216/11-84.1.

accumulate as nuisance proliferations along the shoreline (de Winton and Schwarz, 2004). These proliferations are of particular concern as they present potential hazards to humans and other terrestrial animals. Exposure to these mats can occur through shoreline use as drinking water intake sites, as sites where dogs can scavenge, and as access points of recreational users.

New Zealand freshwater benthic cyanobacteria

The majority of research on freshwater benthic algae in New Zealand has been targeted at periphyton in rivers and streams (Biggs and Kilroy, 2004). Few studies have investigated benthic cyanobacteria and algae in lakes (Wood et al., 2012). *Phormidium* is a common cyanobacterium that forms expansive benthic mats on the substrata of many rivers (Biggs and Kilroy, 2000; Heath et al., 2010). *Phormidium* can dominate in mesotrophic rivers (Biggs and Kilroy, 2004), particularly in late summer after extended periods of low flow (Biggs and Kilroy, 2000; Wood et al., 2007b).

Phormidium and *Oscillatoria*-like species that produce anatoxin-a are well documented in rivers and have caused dog fatalities (Hamill, 2001; Wood et al., 2007b). In the Waitaki, a microcystin-producing benthic cyanobacterium that forms *Phormidium*-like mats was associated with a dog fatality (Wood et al., 2010a). The microcystin producer was suggested to be *Planktothrix* (Wood et al., 2010a). Therefore rivers where benthic *Phormidium*-like proliferations occur were targeted in this study.

Saxitoxin was detected in *Scytonema* strain UCFS10 (Chapter 3; Smith et al., 2011b). Little is known about the current distribution of *Scytonema* spp. in the Canterbury region. Terrestrial and freshwater *Scytonema* spp. have previously been identified throughout New Zealand (Broady and Merican, 2012; Gaylarde et al., 2006; Hawes and Schwarz, 1996; Hawes and Smith, 1994). A survey was conducted with three key aims: 1) to determine the distribution of *Scytonema* in Canterbury; 2) to assess the presence of intracellular STX production and if this varied across sites (Chapter 3); and 3) to assess if the distribution and presence of STX was associated with particular environmental conditions (e.g. lake trophic status, salinity). The distribution of *Scytonema* is discussed in this chapter.

2.1.2 Taxonomy

Nomenclature

Identification of cyanobacteria can be difficult due to the ever-changing taxonomic understanding of this diverse group of organisms (Komárek, 2010b). Concepts of genera have been revised leading to merger or subdivision of many taxa. The taxonomic revisions by Drouet and colleagues (including Drouet, 1968, 1973; Drouet and Daily, 1973) have significantly condensed the number of species by merging taxa with similar characteristics. This approach has subsequently been shown to be based on incorrect assumptions (Whitton, 2008). Taxonomic classification requires constant re-evaluation to keep taxonomic records in conformity with the most recent data (Hoffmann et al., 2005). Therefore it is essential when attributing names to cyanobacterial taxa to state the sources used in making identifications and to attach the authorities to each species.

Morphology

Genera should be clearly defined based on distinct phenotypic features (Komárek and Anagnostidis, 2005). Infrageneric diversity into species is a useful concept but criteria cannot be standardised across all genera (Komárek, 2010b). Morphological identification of species is further complicated by the plasticity of some morphological characteristics under different environmental conditions. This plasticity includes colour, presence and absence of sheaths, and cell dimensions (Baker and Bold, 1970; Biggs and Kilroy, 2000; Mateo et al., 2011; Rippka et al., 1979). Therefore some morphological features are not always suitable for identification. If a specimen cannot be confidently assigned to a species then it can often be stated as being most similar to a species by comparison with descriptions in the literature. In this case, the notation cf. (Latin *confertim* to compare with), or aff. (Latin *affinis* related to) can be used (Komárek and Anagnostidis, 1999). These notations suggest that the observed species matches most, but not all, of the diagnostic criteria for the reference species. Species described with this notation may be an un-described species or one described in the more extensive literature that has not been accessed.

Molecular phylogeny

Molecular genetics has become a key tool in solving taxonomic problems. Cyanobacterial genera that are defined using morphological characteristics are usually well-supported by molecular phylogenetic assessment (Hoffmann et al., 2005; Komárek, 2010b). However, a few genera are not monophyletic and require taxonomic revision (Hoffmann et al., 2005).

Comparison of 16S rRNA gene sequences is the standard approach for assessment of phylogenetic relationships amongst species and genera (Komárek, 2010b). There are difficulties in use of this approach. Any researcher can bank their sequences in public genetic databases. The analysis of the quality of the sequence information is dependent solely on that researcher's analysis, and is not checked by a third party. Sequence lengths and regions of sequences analysed are variable and need to be aligned with other sequences for useful data analysis (Tindall et al., 2010). Correct identification of taxon is dependent on the knowledge of the researcher at the time of banking the sequence. Incorrect names are often used in GenBank (Komárek, 2010b), mistaken names in this database are rarely corrected. Caution is required with phylogenetic data to avoid an "invalid name creep", where incorrectly identified taxa are commonly cited and used to base phylogenetic decisions (Turner, 2010).

Polyphasic approach

Modern cyanobacterial taxonomy attempts to conserve the classical system with alterations based on information from both molecular and phenotypic markers (Hoffmann et al., 2005; Komárek, 2010b; Whitton, 2008). Polyphasic approaches require molecular and morphological evaluation including description of stable phenotypic traits and ecological features (Komárek, 2010b).

2.1.3 Aims and Objectives

This part of the study had the following aims and objectives:

- To collect and culture benthic cyanobacteria from selected sites from the South Island, New Zealand including both rivers and lakes.
- To investigate the distribution of *Scytonema* in Canterbury recreational lakes.
- To identify cyanobacteria in environmental samples and cultures based on morphology and molecular phylogenetic analysis.
- To cryopreserve and deposit the cultures in the Cawthron Institute Culture Collection of Micro-algae (CICCM).

2.2 Methods

2.2.1 Sampling

Site selection

Benthic cyanobacteria were collected from a range of South Island water bodies, targeting lakes and rivers with high recreational use. Lower reaches of rivers with low flows were selected where benthic cyanobacteria were known to proliferate. GPS co-ordinates were recorded for each site (Table 2.1).

Table 2.1 Dominant benthic cyanobacteria identified, collected, and used as a source of inoculum for cultures. All sites are from South Island water bodies.

Locality	Dominant genera collected	Latitude/Longitude*	Collection date
Lake/Dam/Reservoir			
Lake Alexandrina	*Scytonema*, *Tolypothrix*	43°56'11"S, 170°27'38"E	27.10.10
Lake Benmore	*Scytonema*	44°31'58"S, 170°04'07"E	28.10.10
The Gryones	*Scytonema*	43°27'02"S, 172°36'18"E	May 2009
Lake Hawdon	*Scytonema*	43°06'03"S, 171°50'60"E	20.10.10
Lake Middleton	*Nostoc*, *Scytonema*	44°16'28"S, 169°51'05"E	28.10.10
Lake Ohau	*Calothrix*	44°16'12"S, 169°50'56"E	28.10.10
Lake Ruataniwha	*Scytonema*, *Tolypothrix*	44°16'32"S, 170°04'11"E	28.10.10
Lake Ruataniwha	*Scytonema*	44°16'42"S, 170°04'58"E	28.10.10
Lake Sarah	*Scytonema*	43°03'03"S, 171°46'43"E	20.10.10
A South Island reservoir	*Scytonema*	no data†	Feb 2010
Stream/River			
Ashley River	*Phormidium*	42 16' 00"S, 172 34' 17"E	10.02.09
Bowenvale Reserve	*Phormidium*	43°35'51"S, 172°39'08"E	June 2009
Hinewai Reserve	*Anabaena*	43°48'36"S, 173°01'56"E	May 2009
Kaiapoi River	*Phormidium*	43°22' 25" S 172°38' 03"E	06.01.2010
Selwyn River	*Phormidium*	43°41'46"S, 172°24'50"E	Jan/Feb 2009
Waiau River	*Phormidium*	42°39'20"S, 173°01'56"E	09.02.09
Waimakariri River	*Phormidium*	43°25'09"S 172°38'01"E	April/May 2011

*GPS data is based on the World Geodetic System 1984.

† No GPS data supplied because the manager of this pre-treatment drinking water reservoir requested that the exact location remained confidential.

Rivers and streams

Initial periphyton sampling sites were selected at rivers where Environment Canterbury routinely monitors water quality. Some of these sites are periodically known to produce *Phormidium* mats, which sometimes produce anatoxin-a. Periphyton mats were collected from the Selwyn, Waiau, and Ashley Rivers (Table 2.1). Additional *Phormidium* samples were collected from a recreational area of the Kaiapoi River. Filamentous periphyton field specimens obtained from streams in Bowenvale and Hinewai recreational reserves were provided by Paul Broady. The Waimakariri tributary environmental sample was provided by Environment Canterbury. A 1 cm^2 segment of the Waimakariri environmental sample was used as inoculum to isolate ten strains within a single mat.

Lakes and reservoirs

Cyanobacteria were also collected from lakes and reservoirs. These sites included a New Zealand pre-treatment drinking water reservoir, the recreational lake at The Groynes reserve, and additional lakes as part of the *Scytonema* distribution survey. The initial sample from The Groynes was provided by Paul Broady. The sample from a South Island pre-treatment drinking water reservoir and details on the site were provided by the reservoir manager. An additional 55 sites from 34 high use recreational lakes were included as part of the *Scytonema* survey (Table 2.2). GPS co-ordinates, limnological data, and site information, including photographs, were recorded at each survey site on a data sheet (Appendix 8.1).

Table 2.2 Canterbury recreational lakes surveyed for *Scytonema*. Uses of water and surrounding land are also provided.

Source	Site	Longitude/Latitude*	Water use	Land use
Coopers Lagoon	1	43°52'09"S/172°18'05"E	fishing	pasture
Kaiapoi Lakes	1	43°21'30"S/172°39'54"E	picnic spot, scenic/ aesthetic values	urban/lifestyle
	2	43°21'36"S/172°39'49"E		
Kelland Pond	1	44°17'38"S/170°04'28"E	aesthetic values	native
Lake Alexandrina	1	43°58'09"S/170°26'39"E	boating, fishing	high country/ pasture
	2	43°56'11"S/170°27'38"E		
Lake Aviemore	1	44°36'08"S/170°16'50"E	contact recreation	pasture
Lake Benmore	1	44°22'30"S/170°11'33"E	hydropower, boating, fishing, swimming	alpine/pasture/ high country
	2	44°31'57"S/170°04'10"E		
	3	44°31'59"S/170°04'07"E		
Lake Camp	1	43°36'42"S/171°02'57"E	boating, fishing, jet-ski	high country/ pasture
Lake Clearwater	1	43°36'27"S/171°02'41"E	boating, fishing	high country/ pasture
Lake Coleridge	1	43°20'33"S/171°32'42"E	fishing, boating, power station	pasture
	2	43°17'22"S/171°31'29"E		
Lake Ellesmere	1	43°44'24"S/172°36'37"E	fishing, boating	pasture
	2	43°47'54"S/172°22'22"E		
	3	43°47'40"S/172°39'49"E		
Lake Emma	1	43°37'40"S/171°06'01"E	fishing, shooting birds	high country/ pasture
Lake Evelyn	1	43°15'07"S/171°32'08"E	fishing	high country
Lake Forsyth	1	43°47'25"S/172°45'30"E	fishing, boating, aesthetic	pasture
Lake Georgina	1	43°19'09"S/171°34'16"E	aesthetic	pasture
Lake Grasmere	1	43°03'55"S/171°46'19"E	fishing	high country pasture
Lake Hawdon	1	43°06'03"S/171°50'59"E	fishing	native/pasture
Lake Heron	1	43°29'28"S/171°09'23"E	boating, fishing	high country/ pasture
	2	43°29'35"S/171°10'11"E		
Lake Hood	1	43°58'11"S/171°45'46"E	boating, swimming, fishing	urban
	2	43°57'54"S/171°46'24"E		
Lake Lyndon	1	43°17'39"S/171°42'33"E	fishing	high country/ pasture
	2	43°17'58"S/171°42'33"E		
Lake McGregor	1	43°56'16"S/170°28'01"E	boating, fishing	high country/ pasture
	2	43°56'20"S/170°28'17"E		
Lake Middleton	1	44°16'28"S/169°51'05"E	contact recreation	native
Lake Ohau	1	44°16'27"S/169°56'07"E	hydropower, fishing	native
	2	44°16'12"S/169°50'56"E		
Lake Opuha	1	44°00'19"S/170°52'02"E	boating, fishing	pasture
	2	43°59'26"S/170°51'47"E		

Table 2.2 continued.

Source	Site	Longitude/Latitude*	Water use	Land use
Lake Pearson	1	43°05'40"S/171°46'46"E	fishing, boating (non-powered)	high country/ pasture
Lake Pukaki	1	44°11'23"S/170°08'28"E	hydropower, aesthetic, fishing	alpine/pasture/ high country
	2	44°07'01"S/170°12'33"E		
Lake Roto Kohatu	1	43°27'47"S/172°34'37"E	jet boating	urban/lifestyle
	2	43°27'53"S/172°34'34"E		
	3	43°27'54"S/172°34'24"E		
Lake Rotorua	1	42°24'30"S/173°35'00"E	boating, bird watching	native/pasture
	2	42°24'32"S/173°34'39"E		
	3	42°24'38"S/173°35'01"E		
Lake Ruataniwha	1	44°16'42"S/170°04'58"E	rowing, swimming	alpine
	2	44°16'31"S/170°04'11"E		
Lake Sarah	1	43°03'05"S/171°46'43"E	fishing	high country/ pasture
	2	43°03'03"S/171°46'43"E		
Lake Selfe	1	43°14'22"S/171°31'09"E	fishing	high country/ pasture
Lake Taylor	1	42°46'38"S/172°14'57"E	fishing, kayaking, swimming	native/pasture
Lake Tekapo	1	43°58'23"S/170°30'29"E	hydropower, boating, fishing, aesthetic	alpine/pasture
	2	44°00'08"S/170°28'58"E		
St Annes Lagoon	1	42°46'48"S/173°15'57"E	picnic spot, scenic/ aesthetic values	pasture
Styx Mill Reserve	1	43°27'59"S/172°36'27"E	aesthetic values	urban/lifestyle

*Individual site co-ordinates are recorded using the World Geodetic System 1984.

Sample collection

Cyanobacterial mats were observed by macroscopic identification with reference to the periphyton photoguide by Biggs and Kilroy (2000). Grab samples of periphyton were collected and stored with habitat water in polycarbonate containers (60 mL) in river water until isolation. Autoclave-sterilised spatulas were used to scrape periphyton from rock surfaces. Dominant benthic cyanobacteria in environmental samples (Table 2.1) were identified to generic level using light microscopy (Section 2.2.3) and used as inoculum for cultures (Section 2.2.2). The dominant cyanobacteria identified were *Anabaena*, *Calothrix*, *Nostoc*, *Phormidium*, *Scytonema*, and *Tolypothrix*.

Scytonema survey

To survey for *Scytonema* and potential cyanotoxin production by this genus, a range of high recreational use, fresh and brackish water lakes were selected by Environment Canterbury. Survey sites within lakes were selected to coincide with entry/exit points and calm areas with macrophytes, where *Scytonema* and other metaphytic algae tend to grow.

Methods for sampling metaphyton are not well-established. For the *Scytonema* survey, sampling methods were adapted from the literature (Goldsborough, 2001; Ministry for the Environment and Ministry of Health, 2009).

At each site, up to 500 m of the shoreline was visually surveyed for metaphyton or periphyton proliferations. Waders were used to survey up to 5 m from the shore. Grab samples of periphyton and metaphyton were collected using either a telescopic hooked pole (Hosseini and van der Valk, 1989), kick net, or by hand. These samples were preserved with Lugol's iodine solution and stored in the dark until identification. Field microscopes (Peak Pocket Microscope, Peak Optics, Japan and McArthur Field Microscope, Kirk Technology, England) were used to make preliminary identifications of dominant species. Where *Scytonema*-like species were observed in high abundance, two additional subsamples (60 mL) were collected and kept chilled. In the laboratory, one sample was frozen (–20 ºC) for toxin analysis and the second sample collected with 20 mL habitat water incubated at 18 °C and used as a source of inoculum for cultures. A water sample (800 mL) was also collected at these sites for extracellular toxin analysis. These water samples were pre-filtered through 80 µm nylon mesh into glass Schott bottles and kept cool during transport. In the laboratory, the water samples were filtered (GF/C, Whatman) and frozen at –20 °C until analysis.

2.2.2 Isolation and Culture Maintenance

Isolation

For most studies, clonal or unialgal cultures are suitable (Bolch and Blackburn, 1996). Some cyanobacterial species grow better in non-axenic cultures (Baker and Bold, 1970). The bacteria are thought to provide a range of essential metabolites, including vitamin B_{12} (Croft et al., 2005), and other factors that assist algal growth (Morris et al., 2008). Vitamins should be routinely used in culture media where strains contain an essential requirement for these nutrients (Rippka, 1988). *Prochlorococcus* is one example of a cyanobacterium that

is difficult to maintain in axenic conditions. Morris et al. (2008) hypothesised that one way heterotrophic bacteria support *Prochlorococcus* growth is by reducing oxidative stress. Cultures in this study were isolated to form non-axenic unialgal strains.

Filaments of dominant cyanobacteria in environmental samples were streaked onto either BG11, $BG11_0$ (Rippka et al., 1979), or MLA (Bolch and Blackburn, 1996) agarised culture media (1% w/v agar).

Cycloheximide (filter sterilised 100 µg L^{-1}) was used to suppress growth of eukaryotes in the initial environmental samples (Bolch and Blackburn, 1996). Nystatin (filter sterilised 100 µg L^{-1}) was also used because cycloheximide is not effective for all stages of the fungal life cycle (Bolch and Blackburn, 1996). Nystatin is not very water soluble and was prepared in DMSO. The final concentration of nystatin and DMSO in the media were 100 µg L^{-1} and <1%, respectively. Nylon filters (0.20 µm, Corning Incorporated) were used to sterilise the nystatin mixture to prevent damage to filters by DMSO.

After approximately 4 weeks incubation (18 or 25 ± 2 °C; 16:8 h or 12:12 h light:dark; 15–65 µEin m^{-2} s^{-1}) single trichomes were isolated to establish non-axenic clonal cultures. Single trichomes of oscillatorialeans were removed from surfaces of agarised cultures under a dissector microscope and transferred into liquid media (40 mL). Single trichomes of *S.* cf. *crispum* were isolated under the dissector microscope, pulled through 1% agar with sterile forceps to remove epiphytes prior to inoculation onto solid or into liquid $BG11_0$. Other nostocaleans were sonicated using an Ultra-Turrax (IKA Laborteknik, Germany), and filament fragments were isolated under an inverted microscope (Olympus IX70 or CK2). These fragments were transferred to 24 well multi-well plates containing $BG11_0$.

Cryopreservation

Maintaining cultures long term is laborious and can result in loss of morphological and genetic integrity (Day and Brand, 2005; Wood et al., 2008). Cyanobacterial populations continuously change with gene transfer and adaptation to environmental conditions (Komárek, 2010a). Cultures can lose characteristics: including production of cyanotoxins (Gallon et al., 1994; Wood et al., 2008; Yin et al., 1997); the ability to attach to surfaces of the culture vessel (Cadel-Six et al., 2009); production of gas vesicles or buoyancy ability

(Lehtimäki et al., 2000; Repka et al., 2004); and heterocyte production (Dias et al., 2002; Rippka et al., 1979). Cryopreservation allows long term storage of cultures, minimising the chance of genetic modifications and reducing the possibility of microbial contamination due to handling error (Day and Brand, 2005).

In this study, all cultures were maintained in liquid media prior to cryopreservation. Unialgal strains, checked for purity under the light microscope (Olympus BX50 or BX51), were selected for cryopreservation using rack and controlled freezing methods (Wood et al., 2008). Twelve straws of each strain were cryopreserved in 15% DMSO. Two straws were thawed using the methods by Wood et al. (2008) to test survival of the strains after 24–48 hours cryopreservation treatment. All cryopreserved strains were banked in the Cawthron Institute Culture Collection of Micro-algae (CICCM, Nelson, New Zealand; http://cultures.cawthron.org.nz).

2.2.3 Taxonomy

A polyphasic approach was used in this study to identify cyanobacteria brought into culture.

Morphology

Field and cultured specimens were identified using Olympus light microscopes (BX50 and BX51) at 400–2000× magnification. Morphological features, including cell and trichome size and shape, were used for identification with reference to taxonomic keys and descriptions in the literature. The taxonomic guides and descriptions by Komárek and Anagnostidis (1989, 1999, 2005) and Komárek (2003) were chosen as the primary reference material for morphological identification. Australian guides to freshwater cyanobacteria (Baker and Fabbro, 2002; McGregor, 2007) were also used. The Nostocales part of the Cyanoprokaryota guide in *Süßwasserflora von Mitteleuropa* has not yet been published. Therefore the classical approach of Geitler (1932) was used for identification of *Scytonema* species. Classical taxonomic descriptions of Geitler still stand as authoritative sources for filamentous cyanobacteria (Whitton, 2008). In the *Scytonema* survey, *Scytonema* were identified as closely as possible to species whereas other algae were identified to genus.

Molecular Phylogeny

Subsamples of each culture (Section 2.2.2) were transferred into 2 mL tubes and centrifuged (3000 × *g*, 1 min) to remove excess media, or for *Scytonema* species a subsample was taken using sterile forceps for genetic analysis. DNA was extracted using the PureLink™ Genomics (Invitrogen), MoBio PowerSoil™ or MoBio Powerbiofilm™ (MoBio Laboratories Inc) DNA Isolation Kits, as per the manufacturer's instructions. If the sample did not homogenise with vortex an additional step, using a bead beater (90 sec), after step 5 of the PowerSoil™ protocol was used to complete homogenisation. All DNA samples were eluted in 50 µL of the elution solution provided by the manufacturer and Quantified on a TLS Implen Nanophotometer. The extracted DNA was stored frozen.

Partial 16S–23S rRNA gene segments were amplified and sequenced to yield an approximately 1400 bp 16S rRNA gene segment to assist confirmation of morphological analysis. PCR amplification was carried out in a 200 µL reaction tube containing approximately 30 ng of DNA, 480 nM of forward and reverse primers (Geneworks, Australia), 1.2 µg non-acetylated bovine serum albumin (BSA, Sigma), and either 25 µL *i-Taq* 2 × PCR master mix (Intron, Gyeonggi-do, Korea) or 200 µM dNTPs (Invitrogen), 1 × *Taq* PCR buffer (Invitrogen), and 0.2 µL of Platinum *Taq* DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen). Primers were either 27F/809R (Jungblut et al., 2005) or 740F/1494R (Frazão et al., 2010; Neilan et al., 1997). For some species 23S30R (Rueckert et al., 2007) was used as the reverse primer for amplification when the 16S rRNA reverse primers PCR were unsuccessful. Thermal cycling conditions were 94 °C for 2 min followed by 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, repeated for 30 cycles with a final extension at 72 °C for 7 min. PCR reactions were undertaken on either an iCycler thermal cycler (Biorad, USA) or a DNA Engine thermal cycler (Biorad, USA). PCR products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining and UV illumination. Amplicons of the correct size were purified using either a High Pure PCR (Roche Diagnostics) or AxyPrep PCR (Axygen Biosciences, California, United States) product purification kits. Sequencing was usually bi-directional using the primers given above and either the 3730×l DNA Analyser or the 3130×l Genetic Analyser (Applied Biosystems, USA), and using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing was carried out by Canterbury Sequencing and

Genotyping, and Otago Genetic Analysis Services. All sequences were deposited in GenBank, accession numbers are provided in Section 2.3.3 and Table 2.4.

The 16S rRNA gene sequences were compared with sequences from the NCBI GenBank database (Benson et al., 2008). Sequences, identified through MegaBlast, with highest sequence similarity and > 90% coverage to the strains in this study were selected to construct the phylogenetic trees. Uncultured strains were not included in the MegaBlast search. Sequences were aligned using ClustalW in MEGA (version 5; Tamura et al., 2011), visually examined for misalignment and corrected if required. Phylogenetic trees were created using the Maximum Likelihood (ML) method. The ML algorithm was based on the best fitting model for each dataset: the Kimura 2-parameter model (Kimura, 1980) with 1000 bootstrap replicates.

2.3 Results

2.3.1 Distribution of Scytonema Morphospecies

Three *Scytonema* morphospecies (Section 2.3.3) were identified as part of the *Scytonema* survey. *Scytonema* was identified in samples from 10 of the 34 lakes (Figure 2.1 and Table 2.3). Genera similar in morphology to *Scytonema* including *Coleodesmium*, *Tolypothrix*, and *Dichothrix* were present in some of these lakes. Cyanobacteria and eukaryotic algal genera identified are presented in Appendix 8.2.

Table 2.3 Characteristics of sites where *Scytonema* was found.

***Scytonema* morphotype**	**Source***	**Type†**	**Trophic state†**	**Substrata†**
S. cf. *chiastum*	Lake Hawdon	glacial	oligotrophic	sediment/stones
	Lake Clearwater	glacial	eutrophic	stones
	Lake Ohau	glacial	oligotrophic	stones
S. cf. *crispum*	Lake Alexandrina	glacial	mesotrophic	sediment/ macrophytes
	Lake Benmore (Site 1)	reservoir	oligotrophic	sediment/ macrophytes
	Lake Benmore (Site 3)	reservoir	oligotrophic	stones/macrophytes
	Kelland Pond	nd	nd	nd
	Lake McGregor	glacial	nd	sediment/stones/ macrophytes
	Lake Ruataniwha (Site 1)	reservoir	nd	sediment/ macrophytes
	South Island reservoir	nd	nd	nd
	The Groynes	urban	supertrophic	macrophytes/sediment
S. cf. *fritschii*	Lake Middleton	nd	oligotrophic	nd
	Lake Ruataniwha (Site 2)	reservoir	nd	sediment
	Lake Sarah	glacial	mesotrophic	sediment

* GPS values for each site are given in Table 2.2.

† nd = no data.

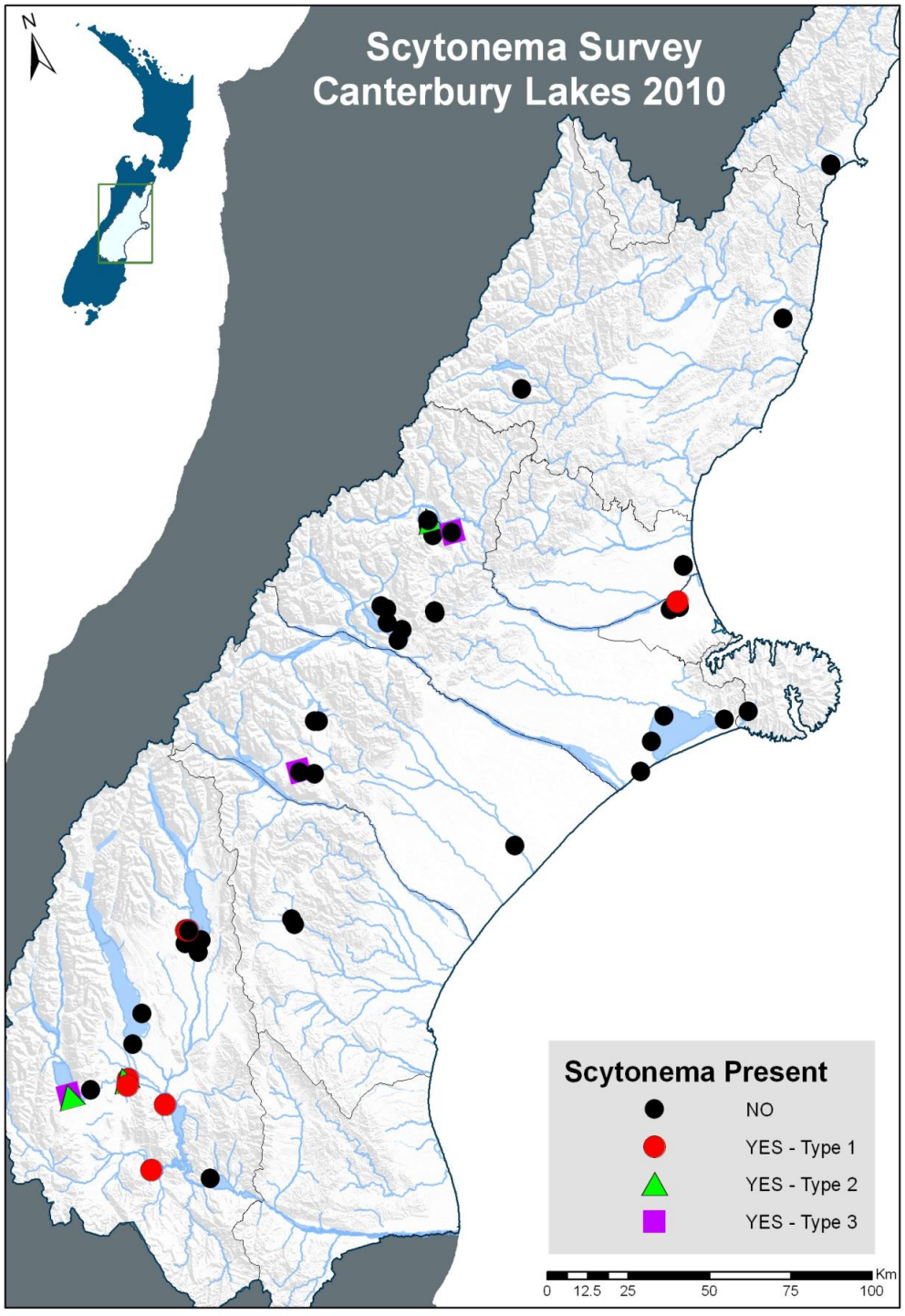

Figure 2.1: Distribution of *Scytonema* species. Type 1: *S.* cf. *crispum*; Type 2: *S.* cf. *fritschii*; and Type 3: *S.* cf. *chiastum*. Reproduced from Smith et al. (2011a), with permission from Environment Canterbury.

2.3.2 Cyanobacteria Isolated into Clonal Culture

Thirty-five strains were successfully isolated into culture during this project. These were 14 Nostocales, 20 Oscillatoriales, and 1 Chroococcales (Table 2.4). Their identification is presented in Section 2.3.3. Cultures of Nostocales were maintained in $BG11_0$ (Rippka et al., 1979). Strains UCFS9 and UCFS10 were trialled in full strength BG11 for one month, but this caused loss of their characteristic heterocytes. Strains UCFS19 and UCFS21–22 initially isolated and characterised from Lakes Hawdon and Ruataniwha were lost after power cuts caused by the February 2011 Canterbury earthquake. All other cultures were cryopreserved and maintained in the CICCM (Table 2.4). All strains were viable after 24–48 hours cryopreservation.

Table 2.4 Identifications of strains to morphospecies. Collection locality, Cawthron Institute Culture Collection of Micro-algae strain number, and GenBank accession numbers are also included.

Morphological identification	Collection locality	Strain	CICCM	Accession number
Order: Chroococcales				
Family: Synechococcaceae				
cf. *Cyanobium diatomicola*	Selwyn River	UCFS8	CAWBG525	JX088094
Order: Oscillatoriales				
Family: Phormidiaceae				
Phormidium cf. *uncinatum*	Waiau River	UCFS4	CAWBG527	JX088095
Phormidium cf. *uncinatum*	Ashley River	UCFS5	CAWBG528	JX088096
Phormidium cf. *uncinatum*	Waiau River	UCFS7	CAWBG529	JX088097
Phormidium cf. *uncinatum*	Bowenvale Reserve	UCFS12	CAWBG523	JX088098
Phormidium cf. *uncinatum*	Kaiapoi River Tributary	UCFS13	CAWBG71	JX088099
Phormidium cf. *uncinatum*	Waimakariri	CYN103	CYN103	JX088073
Phormidium cf. *uncinatum*	Waimakariri	CYN104	CYN104	JX088074
Phormidium cf. *uncinatum*	Waimakariri	CYN105	CYN105	JX088075
Phormidium cf. *uncinatum*	Waimakariri	CYN106	CYN106	JX088076
Phormidium cf. *uncinatum*	Waimakariri	CYN107	CYN107	JX088077
Phormidium cf. *uncinatum*	Waimakariri	CYN108	CYN108	JX088078
Phormidium cf. *uncinatum*	Waimakariri	CYN109	CYN109	JX088079
Phormidium cf. *uncinatum*	Waimakariri	CYN110	CYN110	JX088080
Phormidium cf. *uncinatum*	Waimakariri	CYN111	CYN111	JX088081
Phormidium cf. *uncinatum*	Waimakariri	CYN112	CYN112	JX088082
cf. Wilmottia sp.	Bowenvale Reserve	UCFS11	CAWBG522	JX088100

Table 2.4 continued.

Morphological identification	Collection locality	Strain	CICCM	Accession number
Family: Pseudanabaenaceae				
Geitlerinema sp.	Waiau River	UCFS3	CAWBG533	JX088104
Leptolyngbya sp.	Waiau River	UCFS2	CAWBG532	JX088103
Pseudanabaena sp. 1	Selwyn River	UCFS1	CAWBG530	JX088101
Pseudanabaena sp. 2	Waiau River	UCFS6	CAWBG531	JX088102
Order: Nostocales				
Family: Microchaetaceae				
Tolypothrix sp.	Lake Ruataniwha	UCFS24	CAWBG76	JX088105
Family: Nostocaceae				
Anabaena sp.	Hinewai Reserve	UCFS9	CAWBG526	JX088106
Nostoc sp.	Lake Ruataniwha	UCFS25	CAWBG77	JX088107
Nostoc sp.	Lake Middleton	UCFS27	CAWBG79	JX088108
Family: Rivulariaceae				
Calothrix sp. 1	Lake Ruataniwha	UCFS26	CAWBG78	JX088109
Calothrix sp. 2	Lake Ohau	UCFS28	CAWBG80	JX088110
Family: Scytonemaceae				
Scytonema cf. *chiastum*	Lake Hawdon	UCFS19	N/A*	JN565280
Scytonema cf. *crispum*	The Gryones	UCFS10	CAWBG524	HM629428
Scytonema cf. *crispum*	South Island Drinking Reservoir	UCFS15	CAWBG72	JN565279
Scytonema cf. *crispum*	Lake Alexandrina	UCFS16	CAWBG73	JN565276
Scytonema cf. *crispum*	Lake Benmore	UCFS17	CAWBG74	JN565277
Scytonema cf. *crispum*	Lake Ruataniwha	UCFS21	N/A*	JN565278
Scytonema cf. *fritschii*	Lake Ruataniwha	UCFS22	N/A*	JN565281
Scytonema cf. *fritschii*	Lake Sarah	UCFS23	CAWBG75	JN565282

* N/A not applicable, strain not cryopreserved.

2.3.3 Descriptions of Morphospecies

cf. *Cyanobium diatomicola* (Geitler) Komárek et al. 1999

Literature: Komárek and Anagnostidis (1999) p. 41 fig. 9.

Locality/isolate source: Strain UCFS8 was isolated from Selwyn River (Table 2.4).

Cultures: Macroscopic growth in cultures dark blue-green. Cells bright blue-green, 0.5–1.8 μm wide by 1–3.5 μm long, usually 1–3× as long as wide, solitary, in pairs or occasionally as short pseudofilaments (Figure 2.2).

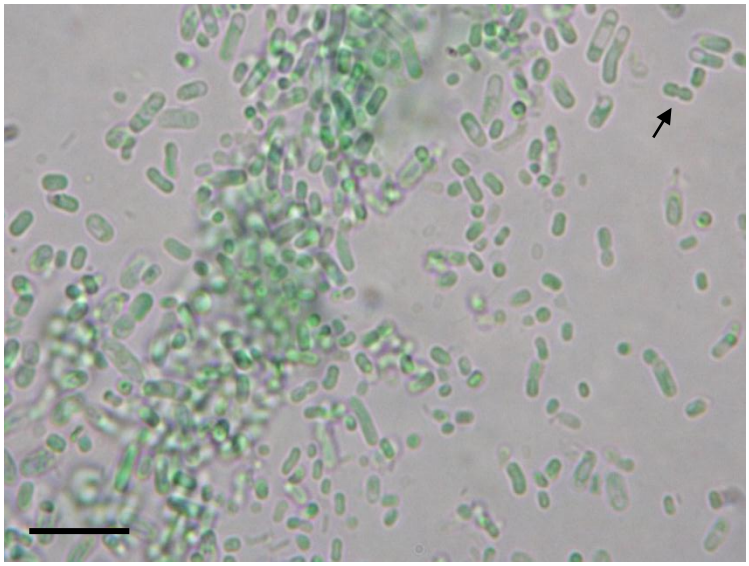

Figure 2.2 cf. *Cyanobium diatomicola*. Strain UCFS8 illustrating cells of varied length with some dividing in a single plane. Scale = 10 µm.

The chromatogram obtained from the first half of the 16S rRNA sequence of UCFS8 was noisy in the forward and reverse direction. Subsequent attempts at attaining a better resolved sequence, including re-extraction of DNA from the strain, were unsuccessful. The 708 bp sequence (accession number JX088094; Table 2.4) obtained for the second half of the 16S rRNA gave a well-resolved chromatogram. Therefore, only this half of the 16S rRNA sequence was used in analysis. Strains identified as *Cyanobium* sp. (AM710375, AM710376, and HM217058) and *Synechococcus* sp. (AY151247 and AY151249) were homologous (100% sequence identity) to UCFS8 over this 16S segment. MegaBlast searches also gave high sequence similarity for an *Aphanothece* sp. (AJ639901) with ≥ 99% sequence similarity.

Taxonomic notes: Based on cell division in a single plane and cell length to width ratio, strain UCFS8 is placed in Aphanothecoideae Komárek and Anagnostidis 1995. This strain conforms to only *Cyanobium*. *Cyanobacterium* species have larger cell dimensions, than UCFS8. *Cyanobium*, has peripherally arranged thylakoids but transmission electron microscopy is required to confirm this pattern in this strain. Komárek and Anagnostidis (1999) noted that some *Synechococcus* species have probably been assigned incorrectly to *Cyanobium*. *Synechococcus* usually has a greater length to width ratio than *Cyanobium* and can be several times longer than wide. Strain UCFS8 was homologous to *Cyanobium* and

Synechococcus spp. over the short 16S rRNA gene segment. Both morphological and phylogenetic analyses support the identification of UCFS8 to this genus.

Phormidium cf. *uncinatum* Gomont ex Gomont 1892

Literature: Komárek and Anagnostidis (2005) p. 481, fig. 719; McGregor (2007) p. 60, fig. 11A, pl. 8L.

Locality/isolate source: *P.* cf. *uncinatum* strains were isolated from Ashley, Waiau, and Waimakariri Rivers, a stream in Bowenvale Reserve, and a tributary of Kaiapoi River (Table 2.4).

Field specimens: Mats dense dark-brown, leathery, attached to substrata. Filaments consist of a single trichome within a firm colourless sheath. Trichomes blue-green to brown, motile, gradually attenuated towards apical cells, commonly forming hormogonia, not constricted at cross-walls. Cells generally shorter than wide with densely arranged granules at cross-walls. Apical cells rounded and occasionally capitate; calyptrate when mature.

Cultures: As described for field specimens. Old mats detach from surfaces of culture containers. Cells isodiametric or shorter than wide, 5.8–12.5 μm wide by 1.2–7.8 μm long (Figure 2.3 and Table 2.5).

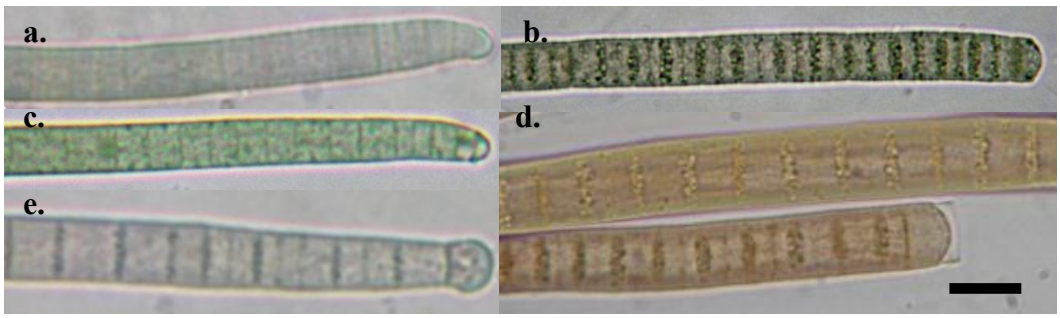

Figure 2.3 *Phormidium* cf. *uncinatum* strains, illustrating wide variability in colour, granulation, and cell dimensions. Trichome tips calyptrate: a) UCFS7, b) UCFS4; trichomes without calyptra: c) UCFS12, d) CYN104; trichome capitate with calyptra: e) CYN106. Scale = 10 μm.

Table 2.5 Cell dimensions of *Phormidium* cf. *uncinatum* strains compared with *P. autumnale* and *P. uncinatum* as described by Komárek and Anagnostidis (2005).

	cell width (µm)	cell length (µm)	cell width (µm) 1 4 7 10 13	length:width 0 0.4 0.8 1.2
P. autumnale *	(3.5) 4–7	2–4 (5)		
P. uncinatum *	(4) 5.5–9 (9.5)	2–6		
UCFS7	5.3–7.0	1.4–4.3		
UCFS12	5.4–7.7	1.2–3.5		
UCFS13	6.7–8.2	1.8–4.8		
CYN110	5.8–8.5	3.1–7.8		
UCFS4	5.2–10.9	2.2–6.7		
UCFS5	5.1–10.5	2.5–5.9		
CYN112	6.2–9.3	2.6–5.7		
CYN109	6.6–10.5	3.2–7.7		
CYN106	8.2–9.8	3.4–7.2		
CYN111	8.9–10.2	4.0–7.3		
CYN108	9.2–10.6	2.4–4.8		
CYN104	8.2–12.2	2.5–6.5		
CYN105	8.9–11.2	3.4–6.4		
CYN103	7.6–12.5	3.0–5.8		
CYN107	8.8–12.2	3.2–6.6		

* Description as in Komárek and Anagnostidis (2005).

Molecular phylogeny: Partial 16S rRNA sequences (1328–1356 bp) were obtained for all strains. Sequences were deposited in GenBank, accession numbers for these strains are provided in Table 2.4 and Figure 2.5. In general these strains shared high homogeneity (≥ 98%). MegaBlast searches revealed high sequence similarity with *Microcoleus* spp., *Oscillatoria* sp., *P. autumnale,* and *Tychonema* species (Figure 2.4). These species formed a single clade with 100% bootstrap support (Clade I, Figure 2.4). All *P.* cf. *uncinatum* strains in this study fell within this well-defined clade (Figure 2.5).

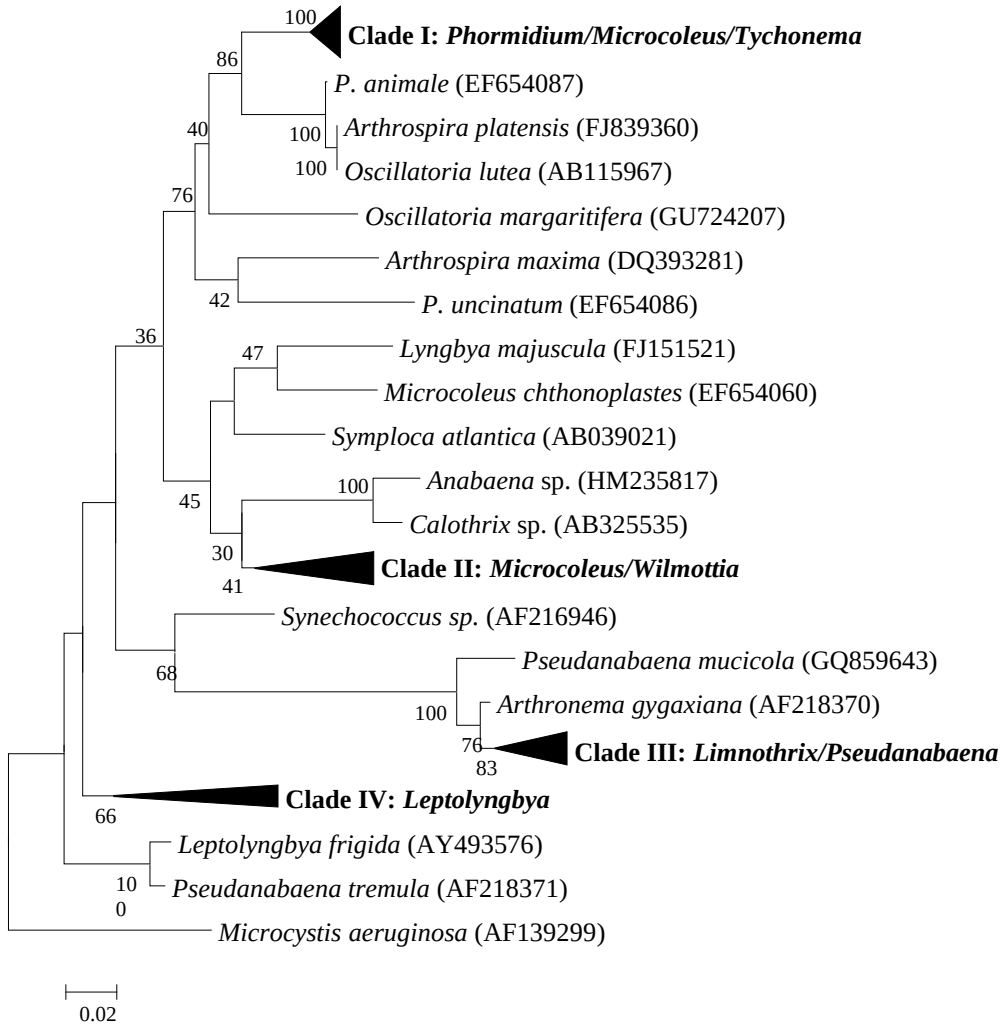

Figure 2.4 Maximum likelihood (ML) tree for Oscillatoriales 16S rRNA, based on 1277 nucleotide positions. Bootstrap support (1000 replicates) is provided near the node, only values ≥ 30% are reported. Clades containing strains from this study are in bold. GenBank accession numbers are provided in parentheses. Subtrees, labelled clades I–IV, are collapsed. These clades are expanded in Figure 2.5, Figure 2.7, and Figure 2.11. Scale = 0.02 substitutions per site.

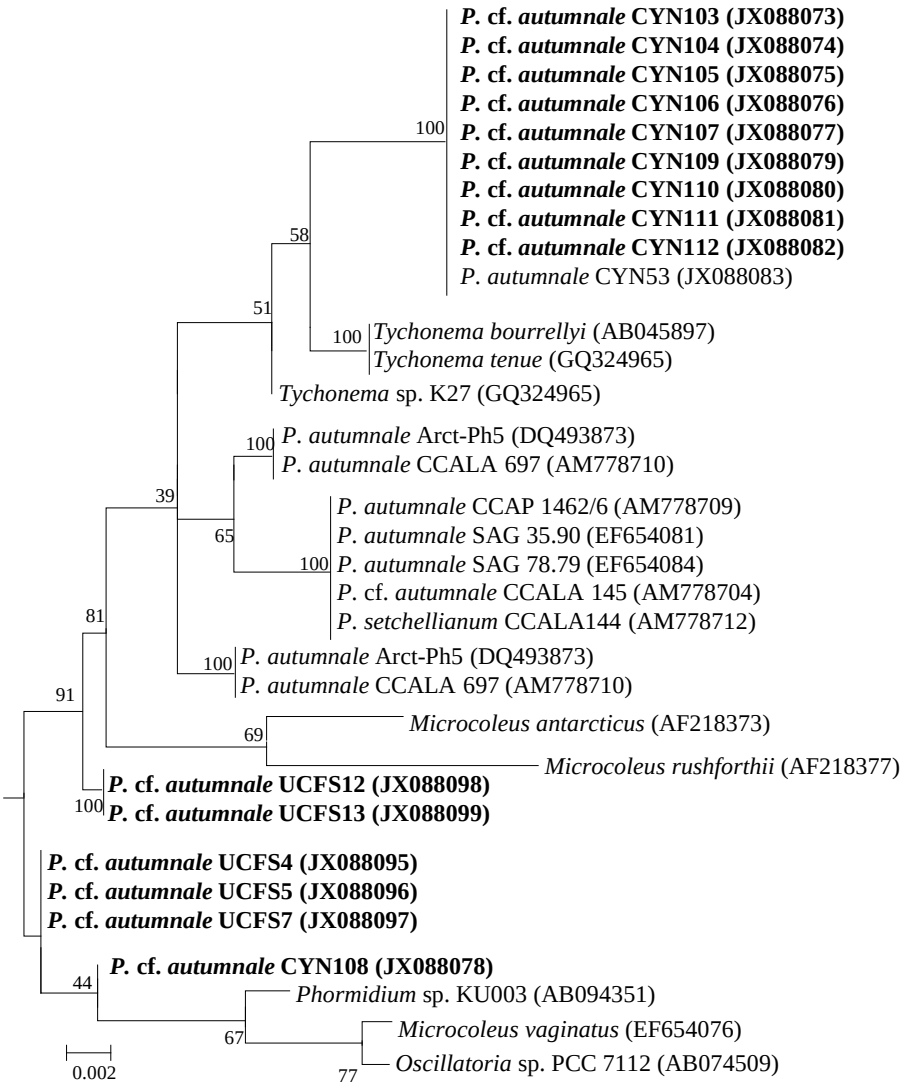

Figure 2.5 Clade I: *Phormidium/Microcoleus/Tychonema*, 16S rRNA sub-tree of the Oscillatoriales ML tree provided in Figure 2.4. Strains from this study are in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is provided near the node, only values ≥ 30% are reported. Scale = 0.002 substitutions per site.

Strains CYN103–107 and CYN109–112 were homologous over their 16S rRNA 1329 bp segment. Sequence data for *P. autumnale* strain CYN53 provided by Susie Wood (Cawthron Institute) was also identical. These sequences shared highest sequence similarity (≥ 99%) with *Tychonema* (GQ324965, AB045897, and GQ324973) and *P.* cf. *uncinatum* strains UCFS4–5, UCFS7, and UCFS12–13.

The 16S rRNA gene segment for strain CYN108 shared high sequence homogeneity (≥ 99%) with *Microcoleus vaginatus* strains (e.g. EF654076), *Oscillatoria* strains PCC7112 (AB074509), and strain 327/2 (FJ461751), and *Phormidium* strains: KU003 (AB094531), *P. autumnale* (FN813344), *P.* cf. *uncinatum* strains UCFS4–5, UCFS7, and UCFS12–13.

Strains UCFS4–5 and 7 were homologous over the partial 16S rRNA gene segment (1356 bp). These strains shared high sequence homogeneity (≥ 99%) with *P. autumnale* strains: Arct-Ph5 (DQ493873), Ant-Ph68 (DQ493874), SAG 35.90 (EF654081), and SAG 78.79 (EF654084). This high sequence homogeneity (≥ 99%) was also found for *Tychonema tenue* (GQ324973), *Tychonema bourrellyi* (AB045897), *Tychonema* sp. K27 (GQ324965), and *P.* cf. *uncinatum* strains CYN108 and UCFS12–13.

Strains UCFS12–13 were homologous over the partial 16S rRNA gene segment (1329 bp). These strains shared high sequence homogeneity (≥ 99%) with the same species as strains UCFS4–5 and UCFS7.

Taxonomic notes: *Phormidium uncinatum* Gomont ex Gomont was the closest in morphology and habitat to the 15 strains in this study. It is cosmopolitan, identified in streams and rivers, and has wider cell dimensions than *P. autumnale* [Agardh] Trevisan ex Gomont (McGregor, 2007). The upper end of the range in trichome width of strains CYN103–112 and UCFS4–5 was somewhat greater than *P. uncinatum* as described by Komárek and Anagnostidis (2005; see also Figure 2.3). Cells of *P. uncinatum* are described as rarely isodiametric, with cells usually 1/3–1/2 as long as wide (Komárek and Anagnostidis, 2005; McGregor, 2007) whereas the present strains varied from 1/4 as long as wide to slightly longer than isodimetric. The cell lengths of UCFS7 and UCFS12–13 were sometimes smaller than described in the literature. Trichome widths of *P. autumnale* are reported as much smaller than most of the *P.* cf. *uncinatum* strains in this study. However, the dimensions of UCFS7 fell within the description of both *P. autumnale* and *P. uncinatum* (Komárek and Anagnostidis, 2005). *Phormidium subfuscum* Kützing ex Gomont has closer cell dimensions, (5.5) 8–11.5 μm wide by 2–8 μm long (Komárek and Anagnostidis, 2005), to the *P.* cf. *uncinatum* strains in this study. This species was also similar to *P. uncinatum* in trichome morphology: not constricted, granulated at cross-walls, shorter than wide to isodiametric. However *P. subfuscum* was ruled out as a possibility

because this species is characteristically found in calcareous rivers and streams (Komárek and Anagnostidis, 2005). Whitton (2011) combines *P. autumnale, P. subfuscum,* and *P. uncinatum* as the species *P. autumnale*.

All *P.* cf. *uncinatum* strains in this study fell within the well-defined 16S rRNA *P. autumnale* clade (Figure 2.5). The *P. autumnale* clade was recently confirmed by morphological and ultrastructure data (Comte et al., 2007; Palinska and Marquardt, 2008). Strain *P. uncinatum* (EF654086) fell outside this clade (Figure 2.4). Phylogenetic analysis of the *P.* cf. *uncinatum* strains in this study shows that these strains are more closely related to *P. autumnale* than *P. uncinatum* (EF654086). In the current study the 15 *P.* cf. *uncinatum* strains shared close homology (>99%) with species of *Microcoleus* and *Tychonema*. This observation has previously been observed for 16S rRNA sequences of *P. autumnale* strains (Comte et al., 2007; Heath et al., 2010; Palinska and Marquardt, 2008). Strains CYN103–107 and CYN109–112 from the Waimakariri River were identical to the New Zealand anatoxin-a producing strain *P. autumnale* CYN53 over 1329 bp. However, CYN53 was somewhat smaller in size (4.2–6.0 μm wide by 3.0–5.4 μm long; Heath et al., 2010) than the Waimakariri strains (Table 2.1). CYN53 conforms closer to the dimensions of *P. autumnale*, but also fits in the lower range of *P. uncinatum* using the description by Komárek and Anagnostidis (2005). The strain UCFS7 could also fit either *P. autumnale* or *P. uncinatum* according to cell-dimensions. However as the 16S rRNA gene segments for UCFS7 were identical to the larger morphospecies of UCFS4–5, the strain UCFS7 is likely to be the same species.

Further studies using ITS regions could confirm whether these are the same or different species. The cosmopolitan species *P. autumnale* displays plasticity, with strains reported in literature varying from 1.6 to 13 μm wide by 1 to 13 μm long (Komárek, 2002). Therefore, if these *P. autumnale* strains have been correctly identified, the *P.* cf. *uncinatum* strains in this study may be one of these wide *P. autumnale* morphotypes. Komárek and Anagnostidis (2005) use a narrower definition of *P. autumnale*. Data in this study also illustrates a wide variability, with mean cell dimensions ranging from 6.2 to 10.9 μm wide by 2.4 to 5.4 μm long. Even strains isolated from a single mat were widely variable in cell dimensions, from ca. 8 μm wide in CYN 112 to ca. 11 μm wide in CYN107. Given the morphological and phylogenetic data, it is difficult to know whether these strains are all *P. uncinatum, P. autumnale,* or actually consist of different closely related species or

subspecies within the broadly defined *P. autumnale* group (Comte et al., 2007; Heath et al., 2010; Palinska and Marquardt, 2008). It has been recommended that this cosmopolitan species is comprehensively revised using modern taxonomic methods (Marquardt and Palinska, 2006; Palinska and Marquardt, 2008).

cf. *Wilmottia* Strunecký et al. 2011

Literature: Strunecký et al. (2011) p. 63, figs 3–4.

Locality/isolate source: Strain UCFS11 was isolated from a stream in Bowenvale Reserve (Table 2.4).

Field specimens: Filaments consisted of bright blue-green trichomes in a firm colourless sheath (Figure 2.6a). Trichomes not constricted at cross-walls. Cells isodiametric, ca. 7.5 μm wide by ca. 8 μm long. Apical cells rounded.

Cultures: Mats macroscopic bright green, attached to culture container surfaces during the growth phase. Trichome morphology was identical to field material. Culture morphology, described one year after isolation, differed from field material only in cell dimensions (Figure 2.6b). Cells approximately isodiametric 2.9–4.0 μm wide by 2.1–6.5 μm long.

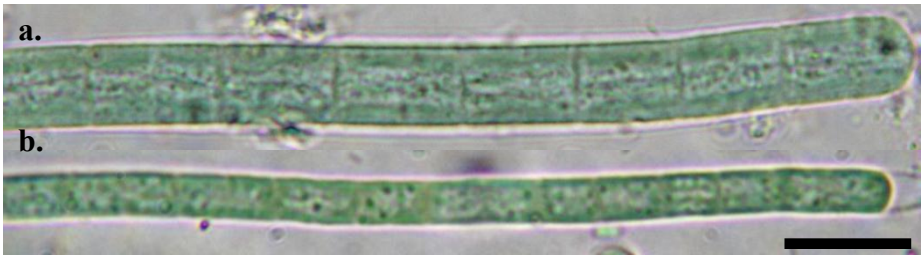

Figure 2.6 cf. *Wilmottia*: a) field material; b) narrower filament of strain UCFS11 Scale = 10 μm.

Molecular Phylogeny: A 1329 bp 16S rRNA gene segment was obtained for strain UCFS11 (accession number JX088100; Table 2.4). This sequence shared ca. 96% sequence similarity with *P. murrayi* (AY493598, AY493627, AY493626), *Microcoleus glaciei* (AF218374), and *Microcoleus paludosus* (EF654090). UCFS11 clustered near *Wilmottia murrayi* (= *P. murrayi*) strains on the 16S rRNA ML tree (Figure 2.7). These

Wilmottia strains formed a separate clade, also containing strains identified as *P. aerugineo-caeruleum* and *Microcoleus glaciei*, supported by 99% bootstrap support.

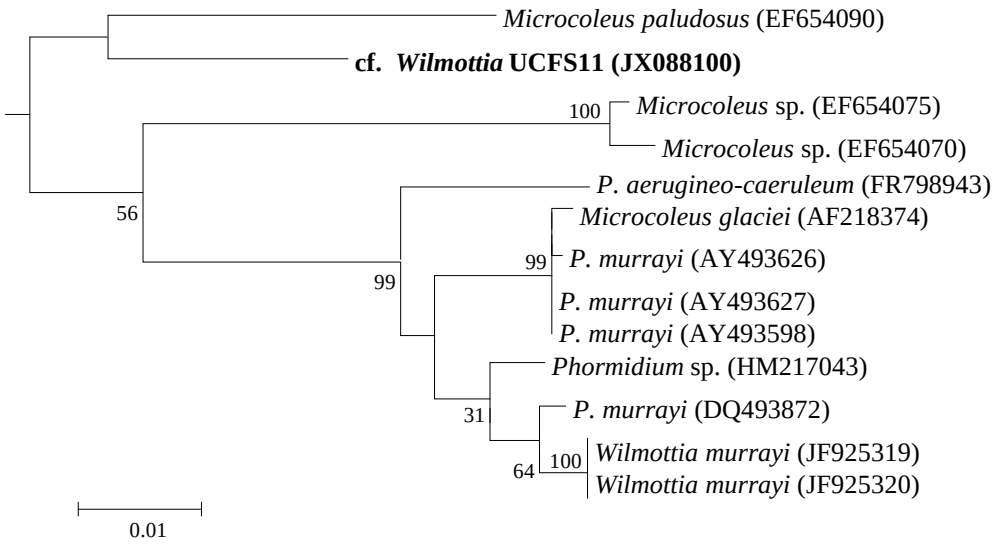

Figure 2.7 Clade II: *Wilmottia/Microcoleus*, 16S rRNA sub-tree of the Oscillatoriales ML tree given in Figure 2.4. Strains from this study are in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Scale = 0.01 substitutions per site.

Taxonomic notes: Recently the genera *Phormidesmis* (Turicchia et al., 2009) and *Wilmottia* (Strunecký et al., 2011) have been separated from *Phormidium*. However *Phormidesmis* has since been reclassified to the family Pseudanabaenaceae rather than Phormidiaceae (Komárek et al., 2009). Polyphasic studies including molecular phylogeny and morphology validate the separation of these genera. Strain UCFS11 did not cluster with other *Phormidium* strains in this study. It was morphologically similar to *Wilmottia*. The only described species, *W. murrayi*, is 3.6–5.4 μm wide by 3.4–7.2 μm long (Strunecký et al., 2011). The field specimens of cf. *Wilmottia* were wider than *W. murrayi*. Strain UCFS11 shared only 95% sequence homogeneity with *W. murrayi*. It is likely that this strain is another *Wilmottia* species.

Geitlerinema (Anagnostidis et Komárek) Anagnostidis 1989

Literature: Komárek and Anagnostidis (2005) p. 120, figs 126–154; McGregor (2007) p. 20, figs 4D–M, pl. 1I–L, 3D–H.

Locality/isolate source: Strain UCFS3 was isolated from the Waiau River (Table 2.4).

Cultures: Mats macroscopic bright blue-green. Trichomes long, blue-green, usually without constrictions (Figure 2.8). Trichomes attenuated and often bent towards the apical cell. Cells 0.8–2.0 μm wide by 1.1–5.7 μm long.

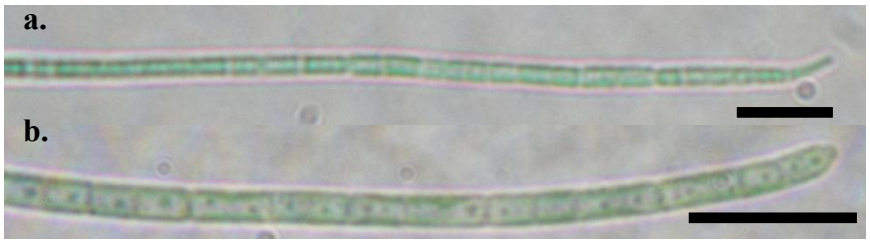

Figure 2.8 *Geitlerinema* strain UCFS3: a) characteristic bent tip of trichome; b) detail of cells within a trichome. Scale = 10 μm.

Molecular Phylogeny: The chromatogram obtained from the first half of the 16S rRNA sequence of UCFS3 had a weak signal in the forward and reverse direction. Subsequent attempts at attaining a better resolved sequence were unsuccessful. Only the well resolved second half of the 16S rRNA gene segment (750 bp, accession number JX088104; Table 2.4) was used in phylogenetic analysis for UCFS3. *Geitlerinema* strain UCFS3 shared high sequence similarity (≥ 99%) with *Geitlerinema* species. These were *G. carotinosum* (AY432710), *G.* cf. *pseudacutissimum* (EU196629), and *Geitlerinema* sp. (GU935346, GU935345, GU935347, and GU935348). These strains formed a single clade with UCFS3 supported by 100% bootstrap values (Figure 2.9).

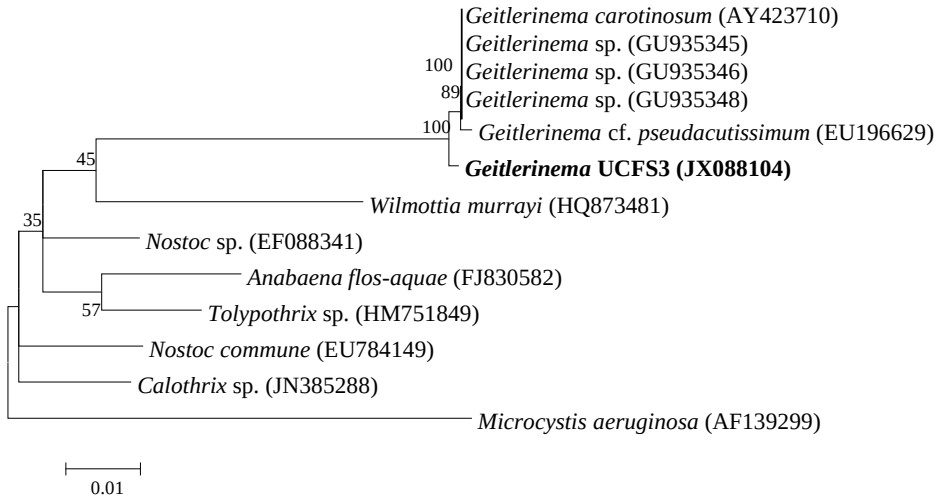

Figure 2.9 Maximum likelihood (ML) tree for *Geitlerinema* 16S rRNA, based on 695 nucleotide positions. Strain USFS3 from this study is in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Scale = 0.01 substitutions per site.

Taxonomic notes. High bootstrap value (100%) of the *Geitlerinema* clade supported the morphological identification of strain UCFS3 to this genus.

Leptolyngbya Komárek and Anagnostidis 1988

Literature: Komárek and Anagnostidis (2005) p. 174, figs 219–244; McGregor (2007) p. 26, figs 4D–M, pl. 2D–J, 3A–B.

Locality/isolate source: Strain UCFS2 was isolated from Waiau River (Table 2.4).

Cultures: Mats bright blue-green. Trichomes long and thin, enveloped in a tight clear sheath (Figure 2.10). Cells 0.7–1.2 μm wide, without constrictions. An accurate cell length was not able to be obtained.

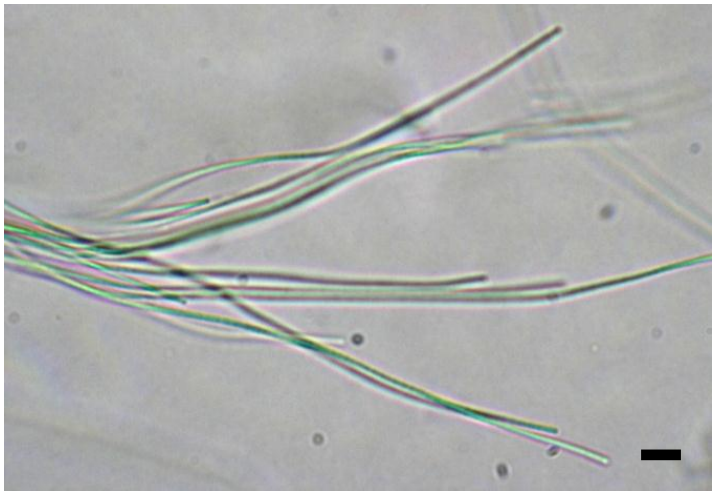

Figure 2.10 *Leptolyngbya* strain UCFS2. Narow trichomes, accurate cell lengths were unable to be measured. Scale = 5 μm.

Molecular phylogeny: *Leptolyngbya* strain UCFS2 shared high sequence similarity (≥ 99%) with *L. antarctica* (AY493590) over the 1380 bp 16S rRNA gene segment (accession number JX088103; Table 2.4). The next closest MegaBlast sequences were *Leptolyngbya* sp. (HM217074 and HM217045) with 94% sequence similarity. The *Leptolyngbya* clade was supported by 66% bootstrap support (Figure 2.4). *L. antarctica* (AY493590) clustered with UCFS2 with 100% bootstrap support (Figure 2.11b).

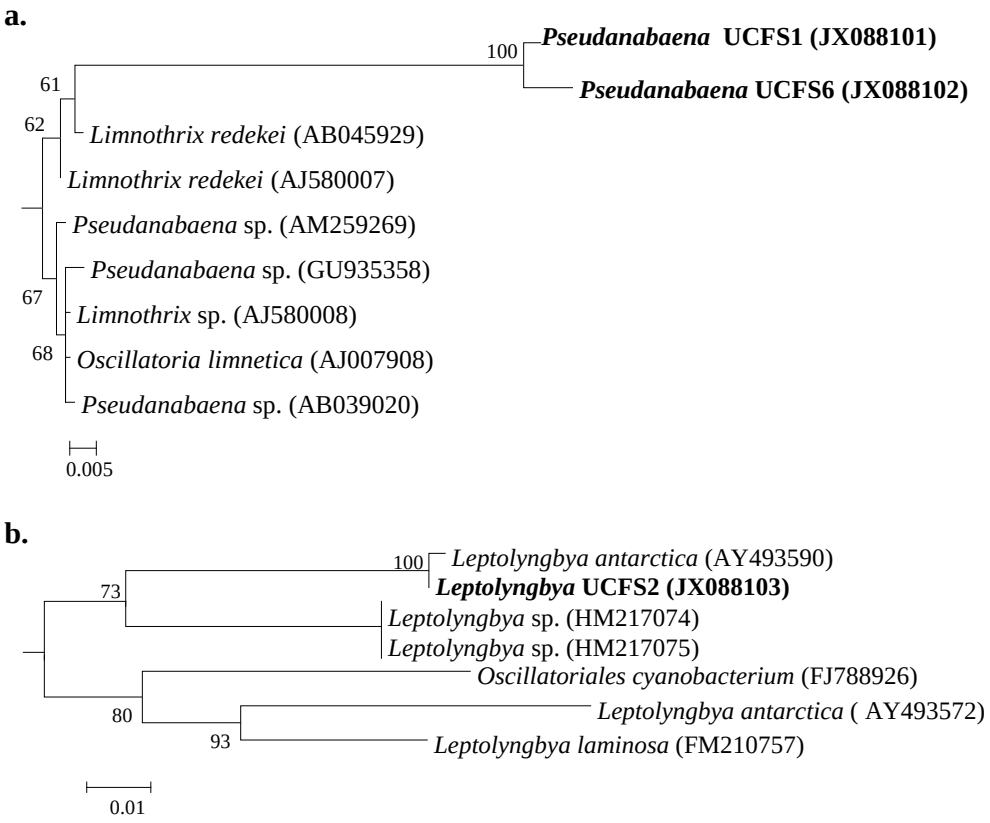

Figure 2.11 Pseudanabaenaceae Clades III and IV: a) *Pseudanabaena/Limnothrix* and b) *Leptolyngbya*, 16S rRNA sub-trees of the Oscillatoriales ML tree given in Figure 2.4. Strains from this study are in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Scale bars represent substitutions per site.

Taxonomic notes. Molecular phylogeny supported the morphological identification of strain UCFS2 to *Leptolyngbya.* Cell dimensions are required to identify this genus to the species level.

Pseudanabaena Lauterborn 1915

Literature: Komárek and Anagnostidis (2005) p. 70, figs 43–79; McGregor (2007) p. 35, figs 2E–I, 3A–B, pl. 4I–N.

Pseudanabaena sp. 1

Locality/isolate source: Strain UCFS1 was isolated from Selwyn River (Table 2.4).

Cultures: Mats macroscopic bright green. Trichomes bright blue-green, with distinct constrictions and hyaline bridges between cells (Figure 2.12a). Cells small, 1.0–1.8 μm wide by 1.1–4.6 μm long, usually longer than wide.

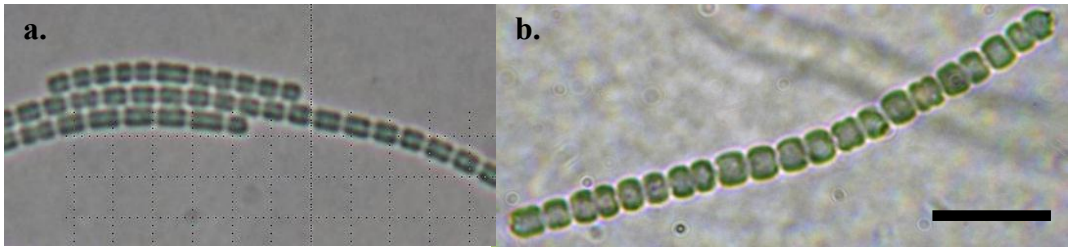

Figure 2.12 *Pseudanabaena*, illustrating the difference in cell dimensions: a) *Pseudanabaena* sp. 1, UCFS1 and b) *Pseudanabaena* sp. 2, UCFS6. Scale = 10 μm.

Pseudanabaena sp. 2

Locality/isolate source: Strain UCFS6 was isolated from Waiau River (Table 2.4).

Cultures: Mats green to dark brown. Trichomes blue-green, with distinct constrictions and hyaline bridges between cells, larger than *Pseudanabaena* sp. 1 (Figure 2.12b). Cells small, 1.5–3.0 μm wide by 1.0–2.4 μm long.

Molecular Phylogeny: Partial sequences of the 16S rRNA gene (ca. 1380 bp) were obtained for the two *Pseudanabaena* strains. Accession numbers are provided in Table 2.4 and Figure 2.11a). Strains UCFS1 and UCFS6 were ≥ 99% homologous. These formed a single clade supported by 100% bootstrap support (Figure 2.11a). GenBank strains with highest sequence similarity (94%) to UCFS1 were *Pseudanabaena* sp. (AM259269) and *Limnothrix redekei* (AJ58007 and AB045929). MegaBlast revealed highest sequence

similarity (93%) strains to UCFS6 were *Pseudanabaena* sp. (AM259269, AB039020, and GU935358), *Limnothrix redekei* (AJ58007 and AB045929), *Limnothrix* sp. (AJ580008), and *Oscillatoria limnetica* (AJ007908).

Taxonomic Notes: The two *Pseudanabaena* strains clustered with *Limnothrix* and *Pseudanabaena*, with 83% bootstrap values (Figure 2.4 and Figure 2.11a), supporting the identification to the Pseudanabaenaceae family. However, strains UCFS1 and UCFS6 shared only 93–94% sequence similarity with these other Pseudanabaenaceae species, suggesting these strains could be a different species to those currently in GenBank.

Tolypothrix Kützing ex Bornet et Flahault 1886

Literature: Komárek et al. (2003) p. 161, fig. 22.

Locality/isolate source: Strain UCFS24 was isolated from Lake Ruataniwha (Table 2.4).

Field specimens: Macroscopic blue-green to brown tufts. Filaments consist of a single trichome enveloped in a tight colourless sheath with occasional false-branching (Figure 2.13), which is characteristic of *Tolypothrix*. Trichomes usually not constricted at cross-walls. Vegetative cells blue-green. Heterocytes yellow, single or in chains of two to four at base of false-branches. Akinetes not observed.

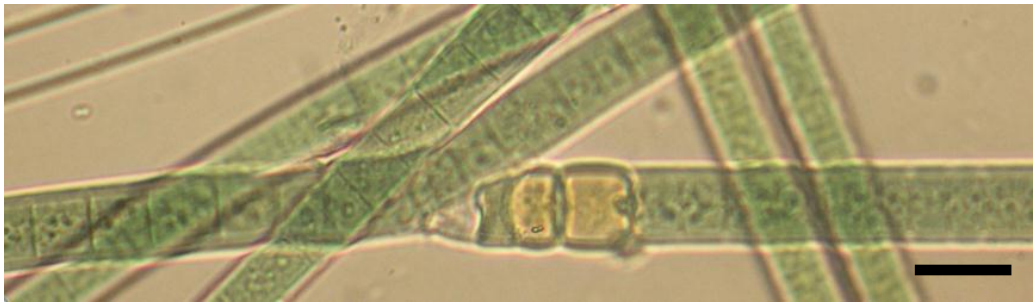

Figure 2.13 *Tolypothrix* illustrating characteristic heterocytes at the base of a false-branch. Scale = 10 µm.

Cultures: As described for field specimens. Sheaths colourless, 6.4–10.7 µm wide. Heterocytes 5.7–10.6 µm wide by 3.8–11.8 µm long. Vegetative cells 5.4–9.8 µm wide by 3.4–8.3 µm long.

Molecular phylogeny: The partial 16S rRNA of *Tolypothrix* sp. UCFS24 was 1340 bp long (accession number JX088105; Table 2.4). This sequence shared 99% sequence similarity with *Coleodesmium* sp. (AY493596) and *Tolypothrix distorta* (GQ287651). These three strains formed a single clade on the ML phylogenetic tree supported by a 94% bootstrap consensus value (Figure 2.14).

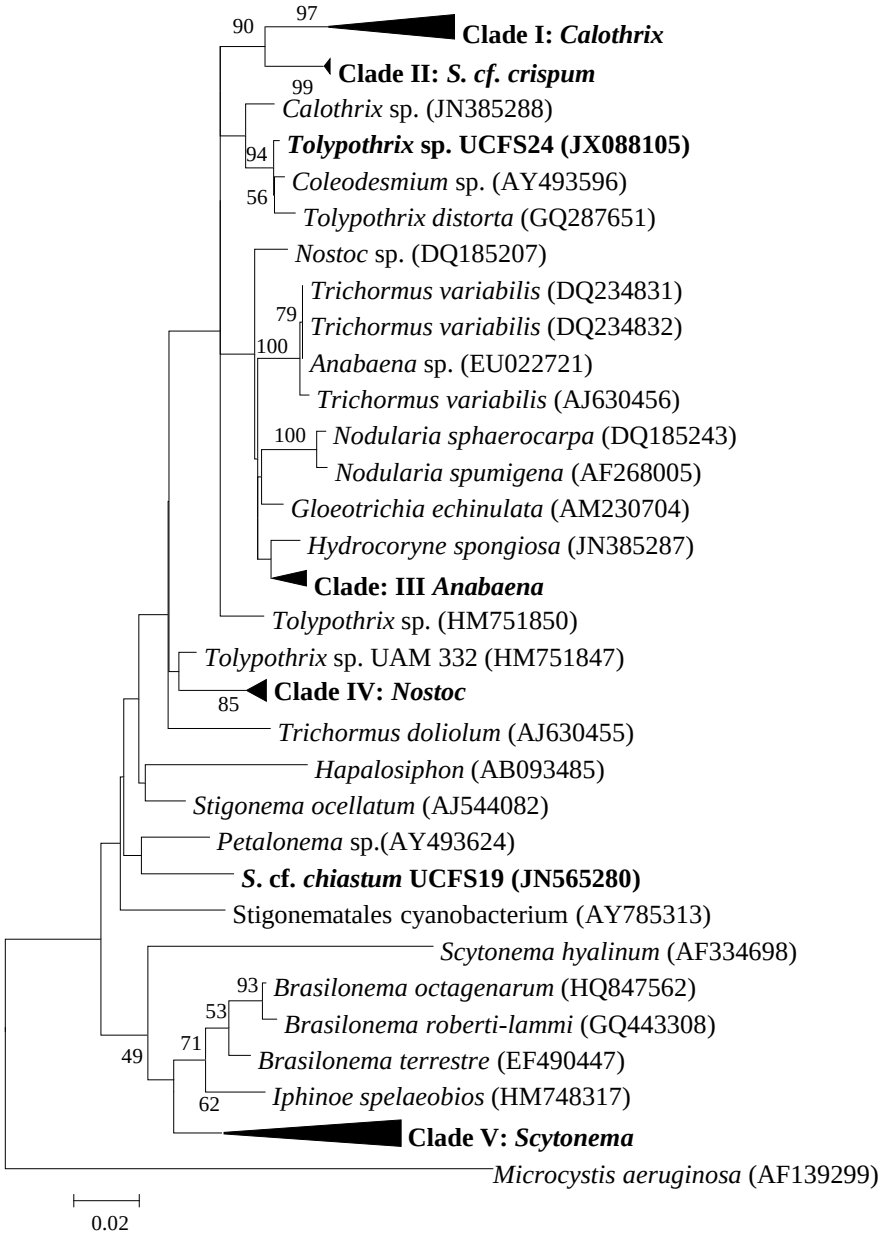

Figure 2.14 Maximum likelihood (ML) tree for Nostocales 16S rRNA, based on 1030 nucleotide positions. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Clades containing strains from this study are in bold. GenBank accession numbers are provided in parentheses. Subtrees, labelled clades I–V, are collapsed. These clades are expanded in Figure 2.16, Figure 2.19, Figure 2.24, and Figure 2.25. Scale = 0.02 substitutions per site.

Taxonomic notes: Phylogenetic analysis supported the morphological identification of *Tolypothrix* with high sequence similarity (99%) to *T. distorta* (GQ287651). This high sequence similarity was also shared with *Coleodesmium* sp. (AY493596). However, strain UCFS24 contains only one trichome per sheath, which does not conform to *Coleodesmium* that has several trichomes per sheath.

Anabaena Bory ex Bornet et Flahault 1886

Literature: Komárek et al. (2003) p. 169, fig. 28; Baker and Fabbro (2002) p. 32, fig. 7–8, pl. 40–61.

Locality/isolate source: Strain UCFS9 was isolated from a stream in Hinewai Reserve (Table 2.4).

Cultures: Mats blue-green, attached to culture container. Trichomes isopolar with deep constrictions at cross-walls (Figure 2.15), slightly attenuated towards mature apical cells. Vegetative cells bright blue-green, 3.3–4.9 µm wide by 2.4–6.4 µm long. Heterocytes intercalary, single, yellow-green, 4.1–8.1 µm wide by 4.5–8.4 µm long. Aged cultures contained bright blue-green granulated akinetes directly adjacent to heterocytes. Akinetes solitary or in pairs, 4.2–8.6 µm wide by 7.7–18.7 µm long.

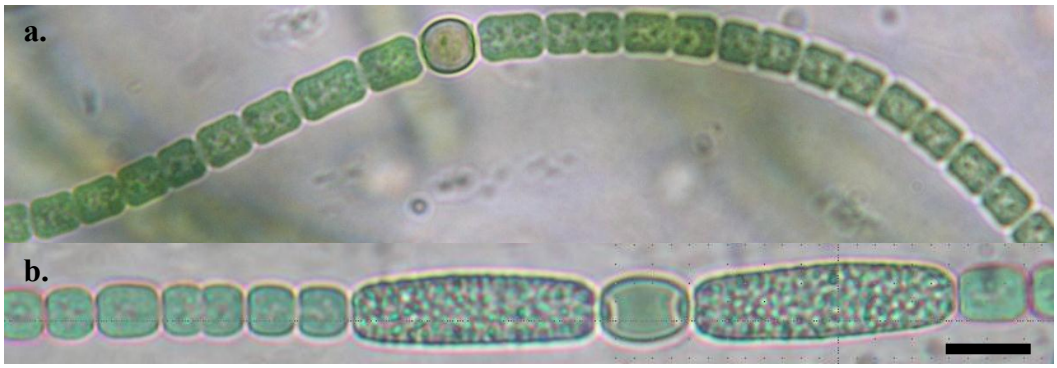

Figure 2.15 *Anabaena* strain UCFS10, illustrating trichome development: a) trichome with single heterocyte b) aged trichome with akinetes on each side of the heterocyte. Scale = 10 µm.

Molecular phylogeny: The partial 16S rRNA gene segment for *Anabaena* UCFS9 (accession number JX088106; Table 2.4) shared 98% sequence similarity with *A. cylindrica* (GQ443447 and AF247592), *A. augustumalis* (AJ630458), and *Hydrocoryne spongiosa* (JN385287). *Anabaena* UCFS10 formed a clade with these *Anabaena* species (Figure 2.14a and Figure 2.16).

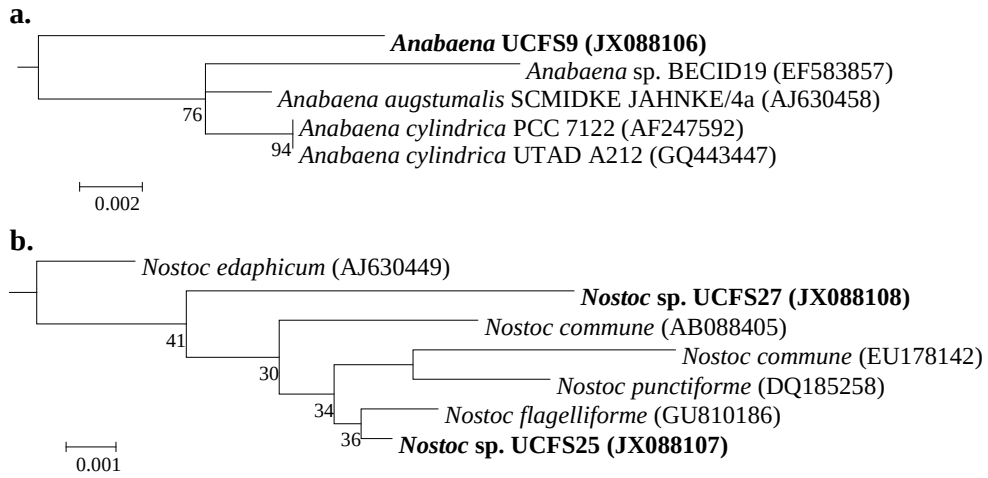

Figure 2.16 Nostocaceae Clades III and IV: a) *Anabaena* and b) *Nostoc*, 16S rRNA sub-trees of the Nostocales ML tree given in Figure 2.14. Strains from this study are in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Scale bars represent substitutions per site.

Taxonomic notes: Phylogenetic analysis supported morphological identification of strain UCFS9 as *Anabaena*. This strain had high sequence similarity (98%) with other *Anabaena* species and clustered with these *Anabaena* species in the phylogenetic tree.

Nostoc Vaucher ex Bornet et Flahault 1886

Literature: Komárek et al. (2003) p. 177, fig. 34; Baker and Fabbro (2002) p. 40, pl. 84–88.

Locality/isolate source: Strains UCFS25 and UCFS27 were isolated from Lake Ruataniwha and Lake Middleton (Table 2.4).

Cultures: Colonies macroscopic, colourless, gelatinous. Trichomes blue-green and isopolar, consisting of vegetative cells with regular intercalary heterocytes (Figure 2.17). Clear constrictions occurred at cross-walls. Vegetative cells 2.4–4.7 µm wide by 2.3–6.2 µm long. Heterocytes 3.3–7.2 µm wide by 3.6–7.2 µm long. Akinetes not observed.

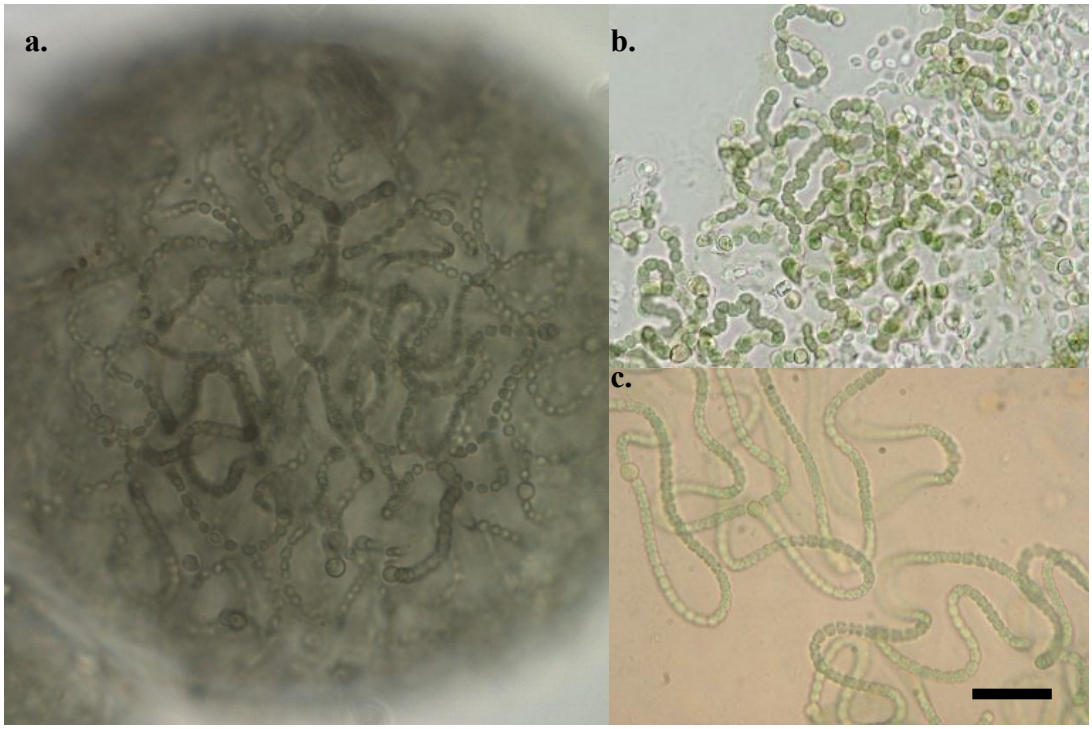

Figure 2.17 *Nostoc* strains in culture a) colony formation; b) strain UCFS25; c) strain UCFS27. Scale = 25 µm.

Molecular phylogeny: Partial sequences of the 16S rRNA gene (ca. 1380 bp) were obtained for the two *Nostoc* strains. Accession numbers are provided in Table 2.4 and Figure 2.16b). These strains, UCFS26 and UCFS28, were 98% homologous. These strains formed a single clade on the phylogenetic tree supported by a bootstrap value of 85% (Figure 2.14 and Figure 2.16b). The closest sequences in GenBank to UCFS25 were several *Nostoc* spp. with 99% sequence similarity. These strains were *N. punctiforme* (DQ185258), *Nostoc* spp. (AF027653, AM711531, EU022741, EU022711, EU022712, and EU022714), and cyanobiont *Nostoc* spp. (AF596246, AF506247, DQ185244, and EF174219). *Nostoc* UCFS27 shared 99% sequence similarity with *Nostoc commune*

(AB098071), *Nostoc* spp. (AF027653, AM711528, AM711539, AM711546, AY742449, EU022726, and EU022727), and cyanobiont *Nostoc* sp. (DQ185244).

Taxonomic notes: Phylogenetic analysis supported the identification of these strains as *Nostoc*. Both species have similar cell dimensions and were 98% homologous. Both strains shared high sequence similarity (99%) with a number of other *Nostoc* strains. Identification of morphospecies of *Nostoc* requires information about the macroscopic colony formation in the field.

Calothrix Agardh ex Bornet et Flahault 1886

Literature: Komárek et al. (2003), p. 164, fig. 25A.

Calothrix sp. 1

Locality/isolate source: Strain UCFS26 was isolated from Lake Ruataniwha (Table 2.4).

Cultures: Mats macroscopic, dark green. Filaments consisted of a single trichome enclosed in a wider colourless sheath, 4.7–13.1 µm wide. Occasionally false-branching present (Figure 2.18a, b, and c). Intercalary vegetative cells blue-green to green, 3.5–13 µm wide by 2.5–8.3 µm long. Heterocytes basal, 5.0–11.0 µm wide by 3.7–13.3 µm long. Akinetes not observed in culture. Apical cells 1.9–5.1 µm wide by 3.0 –10.8 µm long.

Figure 2.18 *Calothrix* strains in culture: a) strain UCFS26 false branching; b) strain UCFS26 terminal heterocyte and false branching; c) strain UCFS26 trichomes with terminal heterocyte; d) strain UCFS28 trichomes with terminal heterocytes. Scale = 10 µm.

Calothrix sp. 2

Locality/isolate source: Strain UCFS28 was isolated Lake Ohau (Table 2.4).

Cultures: Similar to *Calothrix* sp. 1 but smaller dimensions (Figure 2.18d). Sheaths 2.9–10.1 µm wide. Intercalary vegetative cells 2.5–7.1 µm wide by 2.3–9.6 µm long. Heterocytes basal, 3.6–9.6 µm wide by 3.7–7.4 µm long. Apical cells 1.1–5.2 µm wide by 2.3–7.5 µm long.

Molecular phylogeny: The partial 16S rRNA sequence of *Calothrix* UCFS26 (1385 bp) shared 99% sequence similarity with *Calothrix* sp. (H6847571 and HQ847581). *Calothrix* UCFS28 share 95% sequence similarity with *Calothrix* sp. (HQ847579, HQ847571, and HQ847580) and UCFS26 (1031 bp). These *Calothrix* spp. were part of a single clade supported by a 97% bootstrap value (Figure 2.14 and Figure 2.19). Accession numbers are provided in Table 2.4 and Figure 2.19).

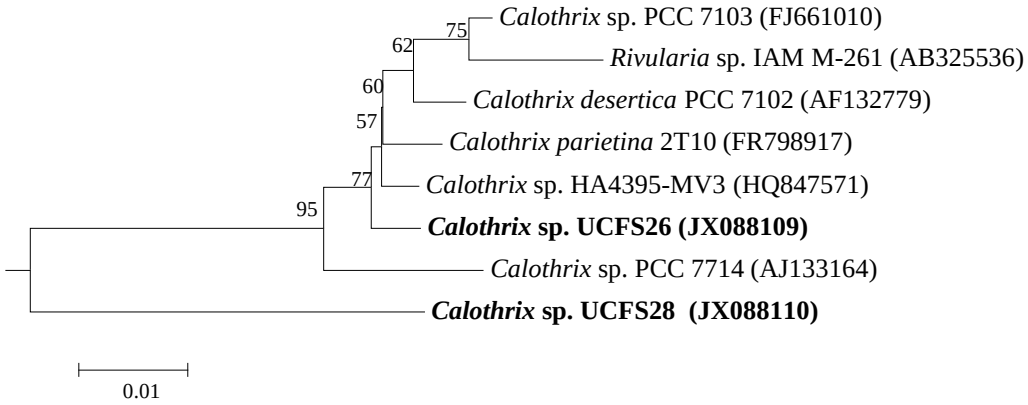

Figure 2.19 Clade I: *Calothrix,* 16S rRNA sub-tree of the Nostocales ML tree given in Figure 2.14. Strains from this study are in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Scale = 0.01 substitutions per site.

Taxonomic notes: Molecular phylogeny supported identification of these strains to *Calothrix*, forming a single clade with 97% bootstrap support (Figure 2.14 and Figure 2.19). Strain UCFS26 was supported by very high (99%) sequence similarity with other *Calothrix*. The closest sequences of strain UCFS28 were other *Calothrix* spp. with 95% sequence similarity.

Scytonema Agardh ex Bornet et Flahault 1886

Literature: Komárek et al. (2003), p. 158, fig. 20A; Geitler (1932) p. 740, figs 477-504.

Three morphospecies of *Scytonema* were identified: *S.* cf. *crispum*, *S.* cf. *chiastum*, and *S.* cf. *fritschii*. These morphospecies were macroscopically (Figure 2.20) and microscopically (Figure 2.21) distinct. All macroscopic growths were sampled and the morphospecies confirmed by light microscopy.

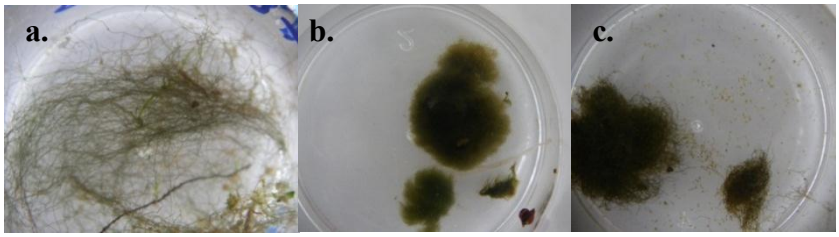

Figure 2.20 Macroscopic appearance of *Scytonema*: a) *S*. cf. *fritschii* from Lake Sarah; b) *S*. cf. *chiastum* from Lake Hawdon; and c) *S*. cf. *crispum* from Lake Alexandrina.

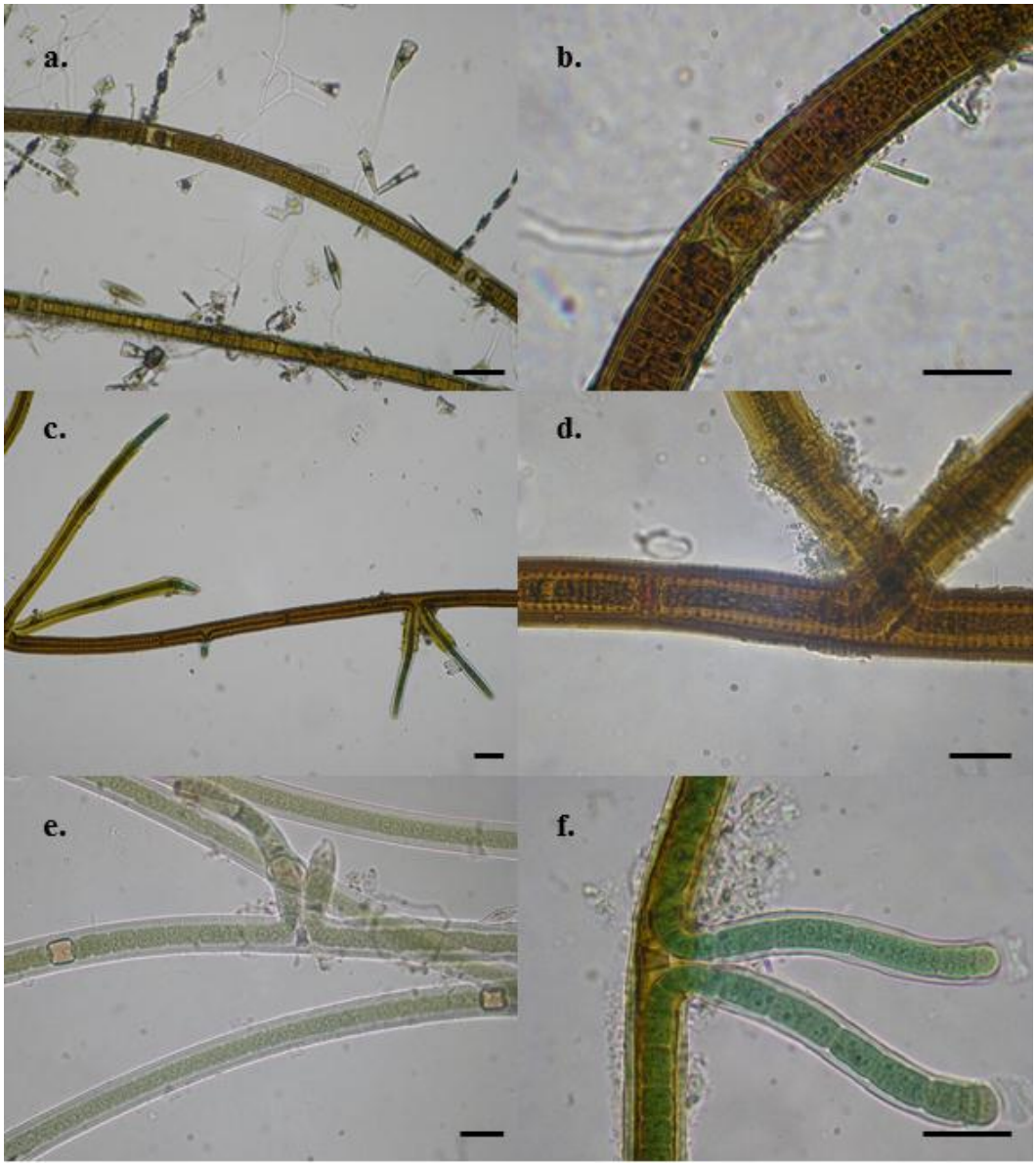

Figure 2.21 *Scytonema* morphospecies identified in the survey of Canterbury recreational lakes. *Scytonema* cf. *crispum* from Lake Benmore, preserved in Lugol's iodine: a) scale = 50 µm and b) 25 µm. *Scytonema* cf. *chiastum* from Lake Hawdon: c) unpreserved, scale = 50 µm and d) preserved in Lugol's iodine, scale = 25 µm. *Scytonema* cf. *fritschii*, unpreserved, scale = 25 µm: e) from Lake Sarah and f) from Lake Middleton.

Scytonema* cf. *crispum (C. Agardh) Bornet 1889

Literature: Geitler (1932) p. 748 fig. 477.

Locality/isolate source: Strains UCFS10, UCFS15–17, and UCFS21 were isolate from Lakes Alexandrina, Benmore, Ruataniwha, a lake at The Groynes, and a pre-treatment drinking-water reservoir (Table 2.4). This morphospecies was also identified in Kelland Pond and Lake McGregor environmental samples but not isolated into culture (Table 2.3).

Field specimens: Found in stagnant water. Formed thick, dark blackish-green mats loosely associated with aquatic plants and terrestrial plant debris (Figure 2.20 and Figure 2.22). Filaments long, isopolar, of indeterminate length, 24–40 µm wide and consisted of a single trichome surrounded by a firm, pale yellow-brown sheath that had parallel lamellations (Figure 2.23, Figure 2.21a and b). False-branching, a diagnostic characteristic of the genus *Scytonema*, uncommon but when present typified *Scytonema*-type branching (Figure 2.23). False-branching in *Scytonema* spp. usually occurs between vegetative cells when trichomes fragment and one or both ends of the trichome break through the sheath and continue to grow. Vegetative cells olive-green, sometimes brown, ca. 23 µm wide by ca. 7 µm long. Heterocytes yellow-brown, regularly spaced along the trichome, usually solitary but sometimes occurring in chains of two or three, ca. 24 µm wide by ca. 14 µm long. Apical cells rounded.

Figure 2.22 Macroscopic growth of *S.* cf. *crispum* entangled with macrophytes.

Figure 2.23 *Scytonema* cf. *crispum* field and culture samples a) false-branching in a field sample; b) culture with regularly spaced heterocytes along the trichome. Scale = 50 µm.

Cultures: Cell dimensions were slightly different from field material. Filaments 20–42 µm wide. Vegetative cells 18–35 µm wide by 4–15 µm long. Heterocytes 20–37 µm wide by 3–25 µm long.

Molecular phylogeny: Partial sequences of the 16S rRNA gene were obtained from all *Scytonema* cf. *crispum* strains. These sequences were deposited in GenBank under accession numbers HM629428 and JN565276–JN565279 (Table 2.4 and Figure 2.24). These strains formed a single clade with 99% bootstrap support and ≥99% sequence homogeneity (Figure 2.14 and Figure 2.24). The nearest *Scytonema* sp. to the *S.* cf. *crispum* strains was strain UCFS19 (94% sequence similarity), followed by the common *Scytonema* spp. clade (ca. 91% sequences similarity).

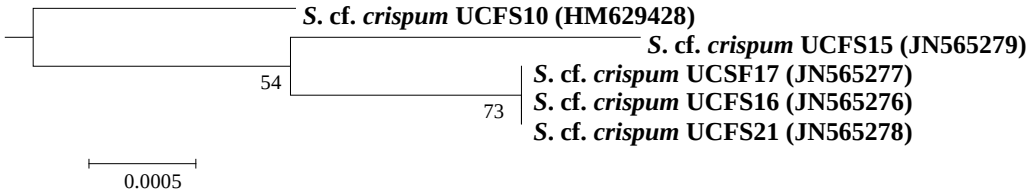

Figure 2.24 Clade II: *S.* cf. *crispum*, 16S rRNA sub-tree of the Nostocales ML tree given in Figure 2.14. Strains from this study are in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Scale = 0.0005 substitutions per site.

Taxonomic notes: The New Zealand environmental (Smith et al., 2011b) and culture specimens differed in filament width, which were slightly larger than *S. crispum* (16–36 µm wide; Geitler, 1932). Molecular phylogenies supported the identification of these strains to a single morphospecies. However the *S.* cf. *crispum* clade does not cluster with other *Scytonema* spp. (Figure 2.14).

Scytonema* cf. *chiastum Geitler (C. Agardh) Bornet 1925

Literature: Geitler (1932) p. 750, fig. 478.

Locality/isolate source: Strain UCFS19 was isolated from Lake Hawdon (Table 2.4). *Scytonema* cf. *chiastum* was also observed in Lake Clearwater and Ohau but not isolated into culture (Table 2.3).

Field specimens: A wide morphotype, ca. 34 µm wide, with characteristic *Scytonema*-like false-branching occurred frequently, usually as pairwise branches either crossing past each other or uncrossed (Figure 2.21c and d). Filaments consisted of a trichome of vegetative cells and intercalary heterocytes enveloped in a wider colourless sheath with parallel stratifications. Trichomes sometimes constricted at cell cross-walls.

Cultures: Morphospecies as described in field. Sheaths 22–43 µm wide. Vegetative cells 13–25 µm wide by 5–18 µm. Heterocytes 12–28 µm wide by 3–10 µm long.

Molecular phylogeny: The partial 16S rRNA sequence (accession number JN565280; Table 2.4) from *S.* cf. *chiastum* did not cluster near either of the above *Scytonema* clades (Figure 2.14). MegaBlast searches gave *Stigonema ocellatum* (AJ544082) as the closest species to *S.* cf. *chiastum* with 95% similarity. The closest *Scytonema* spp. to *S.* cf. *chiastum* was *S.* cf. *crispum* (UCFS10; HM629428) with only 94% similarity.

Taxonomic notes: *S. chiastum* was narrower (filaments usually straight, 25–27 µm wide) and contained false-branching that were usually crossed (Geitler, 1932). Morphologically this species fits the characteristic description of *Scytonema* (Geitler, 1932; Komárek et al., 2003). However phylogenetically *S.* cf. *chiastum* does not cluster with other *Scytonema* species in GenBank.

Scytonema cf. *fritschii* Ghose 1923

Literature: Geitler (1932) p. 755, fig. 480.

Locality/isolate source: Strains UCFS22 and UCFS23 were isolated from and Lake Ruataniwha and Lake Sarah respectively (Table 2.4). *Scytonema* cf. *fritschii* was also observed in Lake Middleton but not isolated into culture (Table 2.3).

Field specimens: A smaller morphotype (Figure 2.21e and f) than *S.* cf. *crispum.* Filaments consisted of at trichome of vegetative cells and intercalary heterocytes enveloped in a wider colourless to yellow sheath, ca. 17 µm wide, with parallel stratifications. False-branching occurred in pairs and was more frequent than that found in *S.* cf. *crispum*.

Cultures: Morphospecies as described in field. Sheaths 13–24 µm wide. Vegetative cells 5–12 µm wide by 4–13 µm long. Heterocytes 5–15 µm wide by 7.5–25 µm long.

Molecular phylogeny: The 16S rRNA gene sequences from *S.* cf. *fritschii* (accession numbers JN565281 and JN565282; Table 2.4) shared highest sequence homogeneity (93–94%) to *S. hofmanni* C. Agardh ex Bornet & Flahault (AM709637). *Scytonema* cf. *fritschii* was sister to a clade with *S. hofmanni* (AM709637) and two other unidentified *Scytonema* strains (AY069954 and AB093483; Figure 2.14 and Figure 2.25).

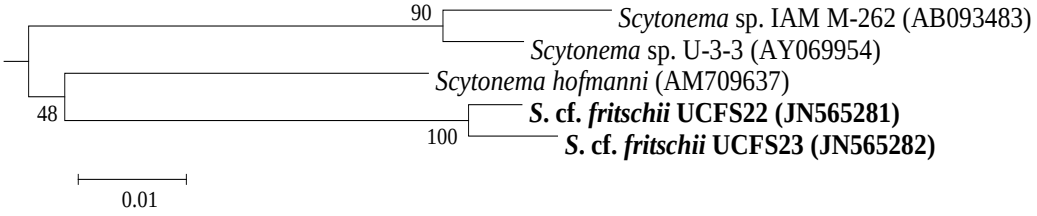

Figure 2.25 Clade V: *Scytonema*, 16S rRNA sub-tree of the Nostocales ML tree given in Figure 2.14. Strains from this study are in bold and GenBank accession numbers are provided in parentheses. Bootstrap support (1000 replicates) is given near the node, only values ≥ 30% are reported. Scale = 0.01 substitutions per site.

Taxonomic notes: This morphospecies generally had wider trichomes than *S. fritschii* (7–8 µm wide in Geitler's description). The description of the heterocytes for this species by Geitler (1932) is unclear. Morphological and molecular phylogenetic analyses support the identification of *S.* cf. *fritschii* strains to the *Scytonema* genus.

2.4 Discussion

2.4.1 General Comments on Distribution

Periphyton in rivers

Phormidium Kützing ex Gomont was the dominant genus collected from rivers and streams in this study (Table 2.1). This result is unsurprising since *Phormidium* has been commonly identified in New Zealand rivers (Heath et al., 2010; Wood et al., 2007b). It is widely distributed with species inhabiting both cosmopolitan and extreme environments (McGregor, 2007). *Phormidium* morphospecies are very difficult to identify with over 200 species described (Komárek and Anagnostidis, 2005).

Scytonema in lakes

Aquatic *Scytonema* species are commonly found in rocky littoral zones of lakes, and less commonly among water plants (Komárek, 1992). In this study morphological analysis identified three *Scytonema* morphospecies. The most frequently identified morphospecies was *S.* cf. *crispum*, which was metaphytic and commonly associated with aquatic plants (Table 2.3). *Scytonema* cf. *chiastum* and *S.* cf. *fritschii*, grew on sediment or stony substrata. *Scytonema* was observed in low abundance only at Lake Ohau, Lake McGregor and Kelland Pond; the small samples collected from these lakes were insufficient for saxitoxin analysis. Chapter 3 describes the results of the analysis for potential saxitoxin production of all other *Scytonema* environmental specimens (Table 2.3) and strains isolated into culture (Table 2.4).

Scytonema cf. *crispum* was not identified at Lake Aviemore during this survey. However, it is likely that this species may be elsewhere in this lake as *S.* cf. *crispum* was abundant upstream in the interconnected Lakes Benmore and Ruataniwha. *Scytonema* has also been reported previously in Lake Coleridge (Hawes and Schwarz, 1996) although the species was not identified. *Scytonema* species were not observed at any of the three Lake Coleridge sites surveyed in this study. This difference may be due to sampling location and depth. Hawes and Schwarz (1996) found *Scytonema* at a depth of 5 m, whereas depths exceeding two meters were not possible using waders and a telescopic pole in this study.

Scytonema cf. *crispum* was found as metaphyton amongst emergent macrophytes along sheltered shorelines in four recreational lakes in Canterbury: The Groynes, Lakes

Alexandrina, Benmore, Ruataniwha, and one drinking water reservoir in the South Island. *Scytonema* cf. *crispum* occurs in lakes with a range of water sources and trophic status: a eutrophic riverine urban lake, an oligotrophic reservoir (Lake Benmore), a mesotrophic glacial lake (Lake Alexandrina), and an oligotrophic drinking water reservoir (Smith et al., 2011b; Smith et al., 2012). The recreational lakes are used for a variety of activities including aesthetic values, hydropower, fishing, swimming, rowing, and boating.

Trophic status did not affect *S.* cf. *chiastum*, which was identified in both oligotrophic (Lakes Hawdon and Ohau) and eutrophic lakes (Lake Clearwater). Lakes where *S.* cf. *chiastum* occurred are predominantly used for hydropower, fishing, and boating.

Scytonema cf. *fritschii* was usually identified as blue-green/black tufts growing on, or floating close to, the sediment. The lakes, in which this species was identified, are used for fishing and swimming. These lakes were oligotrophic and mesotrophic.

Seasonal variation

In New Zealand, monitoring programmes focus on planktonic cyanobacteria in lakes and periphyton in rivers. These are the environments where most cyanotoxins have been identified (Heath et al., 2010; Ministry for the Environment and Ministry of Health, 2009; Wood et al., 2006). Periphytic *Phormidium* in rivers is seasonal and dependent on stable low-flow conditions (Wood et al., 2007b). In contrast, *S.* cf. *crispum* at The Groynes has been observed year-round (Smith et al., 2011b). Large proliferations of benthic cyanobacteria are also thought to occur year-round in sheltered areas of Myall Lake, Australia (Dasey et al., 2005). Methods for monitoring the abundance of phytoplankton in lakes and periphyton in rivers are well-established (Biggs and Kilroy, 2000; Graham et al., 2009; Hötzel and Croome, 1999; Ministry for the Environment and Ministry of Health, 2009). Metaphyton can be difficult to sample depending on how it is distributed in the water column (Goldsborough, 2001). Monitoring metaphytic growth can be problematic as many variables, including sample depth, macrophyte coverage/density, water clarity, and metaphyton density, make it difficult to estimate biomass. To our knowledge there are no standard methods developed for monitoring cyanobacterial metaphyton or littoral periphyton in lakes.

2.4.2 Polyphasic Identification

Molecular genetics, in particular using 16S rRNA gene segments, can distinguish many genera (Komárek et al., 2009). In this study, phylogenetic analyses usually supported morphological identification by placing strains as sister taxa, with ≥ 98% sequence similarity, to species identified to the same genus in other studies. *Calothrix* UCFS28, *Pseudanabaena* strains, *Scytonema* spp., and cf. *Wilmottia* UCFS11 were exceptions.

As the purpose of this study was to acquire a culture collection for subsequent research, not all strains isolated were compared with field specimens. Strains can display different dimensions in different culture media, and therefore also could differ from the size of field specimens (Mateo et al., 2011; Rippka et al., 1979). In this study only two strains were compared with the field specimens: *Scytonema* cf. *crispum* UCFS10 and cf. *Wilmottia* UCFS11. In *Scytonema* UCFS10 cells were generally larger than in environmental specimens, as was also the case for cells and heterocytes in *Anabaena spiroides* (Molica et al., 2005) and *Cylindrospermopsis raciborskii* (Hawkins et al., 2001). In UCFS11 the cell dimensions were smaller than in environmental specimens. Other studies have reported cell morphology, including dimensions, remained consistent between culture and environmental specimens (McGregor et al., 2011).

Phormidium cf. *uncinatum* strains were all grown in MLA media, yet cell dimensions were variable between strains, including those with identical 16S rRNA gene segments. Suggesting a wider genomic difference, not observed in the partial sequence (1329 bp) of this well-conserved gene. Two different 16S rRNA gene sequences were identified from the ten *P.* cf. *uncinatum* strains isolated from a single mat.

Scytonema is not well-defined in molecular phylogenies. More than 100 morphospecies are included within this genus (Komárek et al., 2003). Excluding the morphospecies from this study, only three *Scytonema* spp. have 16S rRNA sequences in GenBank: *S. crustaceum* (EF544991), *S. hofmanni* (AM709637, AB075996), and *S. hyalinum* (AF334698, AF334699, AF334700). Two of the morphospecies in this study, *S.* cf. *chiastum* and *S.* cf. *crispum* do not cluster with the main *Scytonema* clade. Species from this cluster shared low sequence homogeneity (ca. 92%) with strains of *S.* cf. *chiastum* and *S.* cf. *crispum*. These analyses suggest that this genus is not monophyletic and a detailed polyphasic evaluation of the genus is recommended (Smith et al., 2011b; Smith et al., 2012).

2.5 Conclusions

Thirty-five benthic strains were isolated from 16 New Zealand water bodies. In this study *Phormidium* was found to be the dominant mat-forming genus in rivers and streams, and fifteen strains were successfully isolated. These strains consisted of one morphospecies with a range of cell dimensions but similar apical cell morphology, granulation, and colour. Other Oscillatoriales isolated were *Geitlerinema*, *Leptolyngbya*, *Pseudanabaena*, and cf. *Wilmottia*. *Scytonema* spp. were targeted in lakes and reservoirs. Three *Scytonema* morphospecies were identified and eight strains were isolated into culture. Other Nostocales isolated were *Anabaena*, *Calothrix*, *Nostoc*, and *Tolypothrix*. Molecular phylogenetic analysis using a segment of the 16S rRNA gene was generally in agreement with morphological analysis. This analysis also identified different strains of *Phormidium* cf. *uncinatum* isolated from a single mat. The genus *Scytonema* was polyphyletic and requires taxonomic re-evaluation.

Cryopreservation is a useful method for long-term maintenance of isolated strains, this technique works well for cyanobacteria. Thirty-two strains were cryopreserved and banked in the Cawthron Institute Culture Collection of Micro-algae.

Scytonema morphospecies were present in a range of trophic states including mesotrophic, oligotrophic, and eutrophic lakes and reservoirs. *Scytonema* cf. *crispum* was usually identified in stagnant waters, and was found at The Groynes throughout the year. It is likely that other metaphyton and periphyton in sheltered areas of lakes and reservoirs are also present year-round.

3 Cyanotoxins in Benthic Cyanobacteria[†]

3.1 Introduction

Benthic cyanobacteria can produce a wide range of cyanotoxins (Section 1.3). The freshwater cyanotoxins of most concern in New Zealand are the neurotoxins anatoxin-a and saxitoxin (Section 1.3.1 and Section 1.3.4); the cytotoxin cylindrospermopsin (Section 1.3.2); and the hepatotoxins microcystin and nodularin (Section 1.3.3). Cyanotoxin concentrations cannot be simply inferred by the appearance of a particular cyanobacterial bloom (Elder et al., 1993). It is impossible to distinguish between toxic and non-toxic cyanobacterial strains using only light microscope techniques (Kurmayer and Christiansen, 2009). Cyanotoxin analysis is required as visual appearance alone cannot confirm toxicity. When assessing the health risk posed by proliferations of cyanobacteria, the potential for cyanotoxin production should be identified. Quantification of cyanotoxins in drinking or recreational use water bodies is essential to ensure maximum allowable values and guideline levels are not exceeded (Harada et al., 1999). Ideally fast, sensitive, accurate methods for the detection of cyanotoxins are required during risk assessments.

Methods for cyanotoxin detection include a wide range of techniques from biological assays to analytical chemistry techniques (Harada et al., 1999). Most detection methods screen for individual classes of cyanotoxins. Some LC–MS methods detect a range of classes of both hepato- and neurotoxins, however these approaches can have poor sensitivity due to the diverse nature of these molecules (Lawton and Edwards, 2008).

Advances in biochemical and genomic characterisation have assisted in understanding cyanotoxin biosynthesis (Kaebernick and Neilan, 2001; Méjean et al., 2009; Jamers et al., 2009). This knowledge has enabled the development of novel approaches for detecting cyanotoxin producers (Kurmayer and Christiansen, 2009). PCR-based methods are highly sensitive and can detect potentially toxic strains in environmental water samples before

[†] Part of this chapter has been presented in the following publications:

Smith, F. M. J.; Wood, S. A.; van Ginkel, R.; Broady, P. A.; Gaw, S., First report of saxitoxin production by a species of the freshwater benthic cyanobacterium, *Scytonema* Agardh. *Toxicon* **2011,** *57*, 566-573.

Smith, F. M. J.; Kelly, D.; Wilks, T.; Broady, P. A.; Gaw, S., *Distribution of Scytonema (Cyanobacteria) and associated saxitoxins in recreational lakes in Canterbury*; R11/36; Environment Canterbury Technical Report: **2011,** p 18.

Smith, F. M. J.; Wood, S. A.; Wilks, T.; Kelly, D.; Broady, P. A.; Williamson, W.; Gaw, S., Survey of *Scytonema* (Cyanobacteria) and associated saxitoxins in the littoral zone of recreational lakes in Canterbury (New Zealand). *Phycologia* **2012,** doi:10.2216/11-84.1.

blooms develop (Kurmayer and Christiansen, 2009). Studying the regulation of these genes may increase knowledge on the influence of environmental parameters on cyanotoxin production (Kaebernick and Neilan, 2001). Genes involved in cyanotoxin production generally form distinct contiguous clusters in the genome (Mihali et al., 2008).

A range of biochemical, analytical chemistry techniques and molecular genetics assays were used to screen for the commonly identified cyanotoxins in cyanobacterial strains and *Scytonema* field specimens from the South Island (Chapter 2). Anatoxin-a, cylindrospermopsin, microcystin, nodularin, saxitoxin (STX), and derivatives of these compounds were targeted in these analyses. Preliminary identification of potential cyanotoxin producers involved screening for specific genes encoding cyanotoxin biosynthesis. Detection and quantification methods, to confirm the presence of cyanotoxins, were chosen based on the methods currently used for routine analysis in New Zealand. These methods are recommended in *Drinking Water Standards for New Zealand* (DWSNZ) and *New Zealand Guidelines for Cyanobacteria in Recreational Fresh Waters* (Section 1.5.2; Ministry for the Environment and Ministry of Health, 2009; Ministry of Health, 2008).

3.1.1 Methods for Detecting Anatoxin-a Production

Methods for anatoxin-a analysis may target only anatoxin-a, or a combination of both anatoxin-a and its analogues (James et al., 2008). Ideally anatoxin-a analyses should identify anatoxin-a and the variant homoanatoxin-a, along with their major degradation products, dihydro- and epoxy- analogues. These metabolites are more stable than the parent compound (James et al., 2008). Although anatoxin-a, homoanatoxin-a, and some of their degradation products have all been synthesised (Osswald et al., 2007), anatoxin-a is the only commercially available analogue as this variant is identified with much greater frequency than homoanatoxin-a (James et al., 2008). Due to the high toxicity of anatoxin-a, accurate, sensitive detection methods are essential for monitoring (Osswald et al., 2007).

Molecular genetic assays for anatoxin-a genes

A putative gene cluster *anaA–anaH* encoding anatoxin-a and homoanatoxin-a production was elucidated by Méjean et al. (2009), including three modular type I polyketide synthase (PKS) genes. Cadel-Six (2009) designed primers that amplify a 412 bp segment of a modular PKS coding sequence, temporarily labelled ks2, occurring only in

anatoxin-a/homoanatoxin-a producing *Oscillatoria*. The partial ks2 PKS coding sequence was subsequently found to be part of the *anaF* gene (Méjean et al., 2009). The primers designed by Cadel-Six were not appropriate for detection of this PKS in anatoxin-a-producing *Aphanizomenon issatschenkoi* (Ballot et al., 2010a). However, the primers for identifying ks2 have successfully detected the *anaF* gene in anatoxin-a-producing *Phormidium autumnale* (Wood et al., 2010b). Ballot et al. (2010a) designed primers atxoaf and atxar to amplify the PKS encoding gene sequence for anatoxin-a in *Aphanizomenon*, which shared 89.9% sequence homogeneity with *Oscillatoria* amplicons of Cadel-Six et al. (2009). Rantala-Ylinen et al. (2011) recently designed a series of primers that amplify part of the *anaC* gene in anatoxin-a-producing cyanobacteria, including generic, *Oscillatoria*-specific, and *Anabaena*-specific genes. The *anaC* gene initiates anatoxin-a biosynthesis (Méjean et al., 2009).

Bioassays and analytical chemistry techniques for detection of anatoxin-a

Bioassays were the first methods used to detect anatoxin-a, initially known as Very Fast Death Factor because of its acute toxicity to mice (Devlin et al., 1977). Bioassays have been used to determine LD_{50} values for extracts from anatoxin-a-producing cyanobacteria (James et al., 2008). Rat, mice, brine shrimp, crustacean and insect larvae have all successfully been used in bioassays for the detection of anatoxin-a (James et al., 2008; Osswald et al., 2007).

Anatoxin-a is most commonly detected using either high performance liquid chromatography coupled to ultraviolet or fluorescent detection (HPLC–UV or HPLC–FD), or mass spectrometry (MS) techniques following a separation with gas or liquid chromatography (GC–MS or LC–MS) (Osswald et al., 2007). HPLC and MS based techniques allow detection of anatoxin-a, homoanatoxin-a, and their degradation products at low concentrations (Harada et al., 1999; Osswald et al., 2007). Detection of anatoxin-a using chromatographic or mass-spectrometry techniques can be problematic as phenylalanine has a similar mass (phenylalanine, 165.07898 Da, cf. anatoxin-a 165.11536 Da) and elutes at a similar retention time to anatoxin-a (Furey et al., 2005). Chemical reactions to solve miss-identification problems prior to analysis include selective methylation of phenylalanine and fluorimetric derivatisation of anatoxin-a (Furey et al., 2005). Another approach is to use improved mass spectrometry techniques including multiple tandem mass spectrometry (MS/MS) and high-resolution quadrupole time of flight

to distinguish between phenylalanine and anatoxin-a (Furey et al., 2005). The research presented in this chapter used LC–MS/MS for anatoxin-a detection because of its sensitivity, its short analysis time, and its ability to identify anatoxin-a, homoanatoxin-a, and their degradation products. LC–MS is also the preferred method of analysis for anatoxin-a in New Zealand (Ministry of Health, 2008).

3.1.2 Methods for Detecting Cylindrospermopsin Production

Genes involved in cylindrospermopsin biosynthesis

Mihali et al. (2008) identified a gene cluster encoding cylindrospermopsin bioysnthesis in *Cylindrospermopsis raciborskii* and proposed the mechanism for the corresponding biosynthesis via an intergrated polypeptide-polyketide pathway. The gene cluster consisted of 15 open reading frames *cyrA–O* (Mihali et al., 2008). Stücken and Jakobsen (2010) identified an analogous set of genes to *cyrA–K* in an *Aphanizomenon* species, however the genes *cyrL–O* were not identified in this species. A partial sequence from *Aphanizomenon ovalisporum* cylindrospermopsin biosynthesis gene cluster, *aoaA-aoaC*, was characterised, which is analogous to *cyrA–C*. Analysis of genes related to cylindrospermopsin biosynthesis in different genera has revealed high conservation, which was hypothesised as the reason there are only two known toxin analogues of cylindrospermopsin (Stüken and Jakobsen, 2010).

Molecular genetic assays for cylindrospermopsin genes

Schembri et al. (2001) designed two sets of primers associated with cylindrospermopsin production in both *Anabaena* and *Cylindrospermopsis*. One set of primers amplifies a region within a polyketide gene and the other primer set corresponds to a region within a polypeptide gene (Schembri et al., 2001). These peptide synthetase and PKS segments have subsequently been identified as part of *cyrB* and *cyrC* genes respectively (Rasmussen et al., 2008; Schembri et al., 2001; Stüken and Jakobsen, 2010).

Biochemical assays and analytical chemistry techniques for detection of cylindrospermopsin

Limited methods for detecting cylindrospermopsin have been published in comparison with other cyanotoxins (Lawton and Edwards, 2008). Initial analytical methods for the detection of cylindrospermopsin used high performance liquid chromatography with photodiode array detection (HPLC–PDA), which is suitable for detecting

cylindrospermopsin and its derivatives in routine analysis (Harada et al., 1999; Lawton and Edwards, 2008). Other methods for detecting cylindrospermopsin include enzyme-linked immunosorbent assays (ELISA) and LC–MS (Bláhová et al., 2009). HPLC–MS/MS has high sensitivity, with a limit of detection (LOD) of approximately 1 $\mu g\ L^{-1}$ (Eaglesham et al., 1999), and is now the preferred method for detection of cylindrospermopsin (Kinnear, 2010). Cylindrospermopsin is the only reference standard commercially available among the cylindrospermopsin variants (Pegram et al., 2008). The recommended methods for detection of cylindrospermopsin in DWSNZ are LC–MS and HPLC–PDA (Ministry of Health, 2008).

3.1.3 Methods for Detecting Microcystin and Nodularin Production

Genes involved in microcystin and nodularin biosynthesis

Biosynthesis occurs by an intergrated non-ribosomal peptide synthetase and polyketide synthase (NRPS/PKS) pathway that is regulated bi-directionally from a central promoter region (Pearson et al., 2010). The microcystin and nodularin gene clusters, *mcyA–J* and *ndaA–I*, usually comprise ten or nine open reading frames, respectively. The genes *mcyI* and *mcyF* can be absent in some microcystin producers, including *Planktothrix*, and instead contain *mcyT* resulting in only nine open reading frames (Christiansen et al., 2003).

Molecular genetic assays for microcystin and nodularin genes

Microcystin is produced by species in every order of cyanobacteria (Jungblut and Neilan, 2006). Early PCR detection of microcystin-producing gene segments in cyanobacteria usually focused on only one genus (Jungblut and Neilan, 2006). Jungblut and Neilan (2006) designed primers HEPF and HEPR as biomolecular markers to detect the aminomethyl transferase region of the *mcyE* or *ndaF* genes and predicted that these primers can detect these genes in any microcystin or nodularin producer. The enzymes *mcyE* and the nodularin-producing equivalent *ndaF* are a NRPS/PKS hybrid putatively involved in adding D-glutamic acid to the Adda complex, completing the biosynthesis of the Adda moiety (Pearson et al., 2010).

Biochemical assays and analytical chemistry techniques for detection of microcystin and nodularin

High similarity in the structural configuration due to highly analogous genes encoding the biosynthetic pathways of both microcystins and nodularins have allowed similar

approaches for the detection of these metabolites and their genes. Nodularin and a few of the common microcystin variants are available commercially as certified reference materials (Pegram et al., 2008). Many methods for detecting microcystin are also applicable to detecting nodularin (Harada et al., 1999; Lawton and Edwards, 2008). The most common method for detecting microcystins is HPLC–UV (Harada et al., 1999; Lawton and Edwards, 2008). Technological advances in LC–MS based techniques, in particular LC-MS/MS, are replacing HPLC–PDA as the method of choice because LC–MS/MS has greater sensitivity and selectivity (Lawton and Edwards, 2008). Matrix-Assisted Laser Desorption Ionisation mass spectrometry (MALDI) has also been used for rapid detection of all known and unknown microcystin variants in a sample (Erhard et al., 1997; Fastner et al., 2001). The protein phosphatase (PP) inhibition assays and ELISA are simple, very sensitive biochemical and immunological detection methods for detecting microcystins at concentrations lower than the WHO (World Health Organisation) guideline (Harada et al., 1999; Lawton and Edwards, 2008). Methods recommended for detection of microcystins and nodularin in the DWSNZ include HPLC with PDA or UV detectors, LC–MS, Adda–ELISA or PP2A inhibition assays (Ministry of Health, 2008).

3.1.4 Methods for Detecting Saxitoxin Production

Genes involved in saxitoxin biosynthesis

The biosynthesis encoded by the contiguous saxitoxin (STX) gene cluster (*sxt*) in *Cylindrospermopsis raciborskii* strain T3 has been revised by Kellmann et al. (2008). In their study, Kellmann et al. (2008) identified a previously described PKS-like gene *sxtA* consisting of four domains. Ballot et al. (2010b) designed primers, sxtaf and sxtar, based on a segment of the *sxtA* gene to identify STX production. *SxtA* is the enzyme that initiates STX biosynthesis by catalysing a Claisen-like condensation of arginine with acetate to form 4-amino-3-oxo-guanidinoheptane. This step occurs using the four domains of *sxtA* in a polyketide-like synthesis. *SxtA2* binds acetyl-coA to *sxtA3*, a ACPs-like domain. *SxtA1* methylates the acetyl-ACP to form propionyl-ACP. *SxtA4* carries out the Claisen condensation. However, samples in which there is a positive identification of the *sxtA* gene require further analysis to confirm STX production as several species that do not produce STX or its analogues also contain an *sxtA* gene segment (Ballot et al., 2010b). These non-

toxic strains may have lost part of the STX gene cluster causing mutation of *sxt* and loss of ability to produce saxitoxins.

Biochemical assays and analytical chemistry techniques for detection of saxitoxin

Saxitoxin standards have been developed and these certified reference material for major STX analogues are readily available through commercial sources. Extensive efforts have been invested in developing detection methods for STX and its variants because of the health risks these neurotoxins pose on the seafood industry (Llewellyn, 2006). These methods have recently been reviewed in Etheridge (2010) and Wiese et al. (2010). Chromatography techniques are used for the detection of STX including HPLC–FD, LC–MS, and capillary electrophoresis (Lawton and Edwards, 2008). HPLC–FD with either pre- or post-column oxidation is the analytical method most commonly used for detecting and quantifying STX variants (Harada et al., 1999; Lawton and Edwards, 2008). The commercially available Jellett PSP (paralytic shellfish poisons) Rapid Test (formally MIST Alert; Jellett et al., 2002) is an antibody-based assay that is now widely used for initial screening (Lawton and Edwards, 2008). The Jellett PSP Rapid Test detects all STX analogues, however the efficiency of detection varies among the different analogues (Jellett et al., 2002).

3.1.5 Aims and Objectives

This part of the study had the following aims and objectives:

- To determine which of three PCR primer sets was most applicable when screening strains for potential production of anatoxin-a/homoanatoxin-a.

- To screen cyanobacterial strains isolated from South Island water bodies (Chapter 2) for genes associated with anatoxin-a/homoanatoxin-a, cylindrospermopsin, microcystin, nodularin, and STX production.

- To determine, by LC–MS/MS, the concentrations of cyanotoxins (anatoxin-a, homoanatoxin-a, cylindrospermopsin, microcystin, and nodularin) in strains that were positive for genes involved in cyanotoxin production.

- To confirm STX production in any strains positive for genes associated with STX using a biochemical assay followed by HPLC–FD to determine concentration.

- To measure STX production by HPLC–FD in environmental samples identified as positive for cyanotoxins as determined by biochemical assays.

- To determine if anatoxin-a and homoanatoxin-a production varies between strains of *Phormidium* cf. *uncinatum* CYN103–112 (Section 2.3.3), isolated from a single mat.

3.2 Methods

3.2.1 Screening for Anatoxin-a/Homoanatoxin-a

Molecular genetic assays for genes involved in anatoxin-a/ homoanatoxin-a biosynthesis

DNA extracted from benthic cyanobacterial strains isolated from South Island water bodies (Chapter 2, Section 2.2.3) was screened for genes involved in anatoxin-a and/or homoanatoxin-a production. Three sets of primers were compared to assess which method would be most applicable to the range of species in the culture collection. 1) Strains UCFS1–13 were screened for the *anaF* gene involved in anatoxin-a and homoanatoxin-a production in *Oscillatoria* using the forward and reverse primers from Cadel-Six et al. (2009). These primers are presumed to be unsuitable for analysis of Nostocales (Ballot et al., 2010a). 2) Strains CYN103–112, UCFS9–13, UCFS15–17, UCFS19, and UCFS21–28 were screened for the *anaF* gene using atxoaf and atxar primers (Ballot et al., 2010a). 3) All strains (Section 2.3.2) were screened for the *anaC* gene using generic primers (Rantala-Ylinen et al., 2011).

All PCR reactions were carried out in 200 µL reaction tubes on either an iCycler or DNA Engine thermal cycler (Biorad, USA). Thermal cycling conditions were 94 °C for 4 min; followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min; then a final extension at 72 °C for 7 min. PCR reactions were visuallised on 1% agarose electrophoresis gel stained with ethidium bromide. The PCR reactions were prepared with approximately 15 ng of DNA, 0.48 µM of the forward and reverse primer (Geneworks, Australia), 200 µM dNTPs (Invitrogen), 1 × *Taq* PCR buffer (Invitrogen), 0.25 µL of Platinum *Taq* DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen), and 0.6 µg non-acetylated bovine serum albumin (BSA, Sigma) to give a final reaction volume of 20 µL. The PCR using atxoaf/atxar or generic *anaC* primers was modified by using 10 µL *i-Taq* 2 × PCR master mix solution (Intron, Gyeonggi-do, Korea) instead of *Taq* DNA polymerase, dNTPs, *Taq* PCR reaction buffer, $MgCl_2$, and BSA, giving a final volume was 20 µL. The *i-Taq* 2 × PCR master mix solution contains *i-Taq* DNA Polymerase, dNTPs, PCR reaction buffer, and a gel loading buffer. Using *i-Taq* 2 × PCR master mix solution reduces the possibility of contamination and potential pipetting errors from handling these components separately in PCR analysis. *Phormidium*

autumnale strain CYN53 (Cawthron Institute Culture Collection of Micro-algae, CICCM) was the positive control and Milli-Q water was the negative control.

The PCR for strains that were positive for the *anaF* gene segment, as determined by gel-electrophoresis, were repeated in a 50 μL reaction volume. Amplicons, of the correct size for the *anaF* gene segment, were purified using a High Pure PCR product purification kit (Roche Diagnostics). The purified PCR product was sequenced in the forward and reverse directions. Sequencing was carried out by Otago Genetic Analysis Services using an Applied Biosystems 3130×l Genetic Analyser and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequences were compared with similar sequences in the NCBI GenBank database (Benson et al., 2008). Highly similar sequences were imported into MEGA (version 5; Tamura et al., 2011), aligned, visually checked and corrected for misalignment, and used to infer a phylogenetic tree generated using the Maximum Likelihood (ML) method. The ML method was based on the best fitting model in MEGA for these data, the Kimura 2-parameter model with evolutionary invariable rates at some sites (Kimura, 1980). Reproducibility was tested using 1000 bootstrap replicates. The partial *anaF* gene segments were deposited in the NCBI GenBank database under the accession numbers provided in Section 3.3.1.

Liquid chromatography mass spectrometry analysis for anatoxin-a and homoanatoxin-a production

Extraction for liquid chromatography mass spectrometry analysis

Strains UCFS1–13 and CYN103–112 were selected for LC–MS/MS analysis. Samples were harvested at the stationary growth phase and lyophilised (FD-1, EYELA, Tokyo Rikaikai Co. Ltd, Tokyo, Japan) into preweighed Falcon tubes for anatoxin-a analysis to give approximately 4 mg sample dry weight. Milli-Q water (2 mL) acidified with 0.1% formic acid was added to each sample. The samples were vortexed, sonicated for 30 min on high, and centrifuged for 10 min at 3000 × *g*. Subsamples (1 mL) of the supernatant were pipetted into HPLC vials.

Liquid chromatography mass spectrometry analysis for anatoxin-a and homoanatoxin-a

Detection of anatoxin-a, homoanatoxin-a, and their dihydro- and epoxy- analogues was based on the LC–MS/MS method by Wood et al. (2011). Compounds were separated by liquid chromatography (Waters Acquity UPLC, Waters Corp., MA) on a BEH C18 column

(1.7 µm, 1 × 50 mm, Waters Corp., MA). Mobile phase A was Milli-Q water acidified with 0.1% formic acid and mobile phase B was acetonitrile acidified with 0.1% formic acid. The mobile phase flow rate was 0.30 mL min^{-1}. The gradient profile was isocratic for 1 min at 100% A; decreasing to 50% A and 50% B at 3 min; isocratic at 50% A and 50% B until 3.50 min; increasing to 100% A at 4 min; isocratic at 100% A until 5.50 min. The Quattro Premier XE triple quadrupole mass spectrometer (Waters-Micromass, Manchester) was operated in ESI+ mode with capillary voltage 0.5 kV, desolvation gas 900 L h^{-1}, 400 °C, cone gas 200 L h^{-1}, and cone voltage 25 V. The following MS/MS ions channels were set up anatoxin-a (166.15, 149.1 Da), homoanatoxin-a (180.2, 163.15 Da), dihydroanatoxin-a (168.1, 56 Da), dihydrohomoanatoxin-a (182.1, 57 Da), epoxyanatoxin-a (182.1, 98 Da), and epoxyhomoanatoxin-a (196.1, 140 Da). Certified anatoxin-a (A.G. Scientific) was serial diluted with 0.1% formic acid for instrument calibration. After calibration, select calibration standards were analysed every 5–8 samples to check instrument performance. The limit of detection (LOD) for anatoxin-a was approximately 0.2–0.5 ng mL^{-1} of injected sample. The LOD varies depending on whether interference peaks are present in the sample increasing the baseline. One of the calibration standards was also used as a quality control sample, injected in duplicate in each batch.

3.2.2 Screening for Cylindrospermopsin

Molecular genetic assays for genes involved in cylindrospermopsin biosynthesis

Primers M4/5 and M13/14 were used to screen for partial genes involved in cylindrospermopsin PKS and peptide synthetase respectively (Schembri et al., 2001). The PCR reaction was prepared with approximately 15 ng of DNA, 0.5 µM of the forward and reverse primer (Geneworks, Australia), 10 µL of *i-Taq* 2 × PCR master mix (Intron, Gyeonggi-do, Korea), the final volume of each reaction was 20 µL. Milli-Q water was used as a negative control for the cylindrospermopsin genes. A positive control was not available at the time this analysis was undertaken. Thermal cycling conditions were the same as for the anatoxin-a PCR (Section 3.2.1).

3.2.3 Screening for Microcystin/Nodularin

Molecular genetic assays for genes involved in microcystin/nodularin biosynthesis

HEPF and HEPR primers from Jungblut and Neilan (2006), which amplify a 472 bp product from *mcyE* or *ndaF* genes associated with microcystin and nodularin production respectively, were used to screen strains for the ability to produce these hepatotoxins. The PCR reaction was prepared with approximately 15 ng of DNA, 0.5 μM of the forward and reverse primer (Geneworks, Australia), 10 μL of *i-Taq* 2 × PCR master mix (Intron, Gyeonggi-do, Korea), and 0.4 μg BSA (Sigma), giving a final volume of 20 μL. Thermal cycling conditions were the same as the anatoxin-a PCR (Section 3.2.1). CYN60 (CICCM) was the positive control and Milli-Q water was used as a negative control.

3.2.4 Screening for Saxitoxins

Molecular genetic assays for genes involved in saxitoxin biosynthesis

The PCR reaction mixture for strains UCFS2–12 was prepared using approximately 10 ng of DNA, 0.48 μM of sxtaf and sxtar primers (Ballot et al., 2010b; Geneworks, Australia), 200 μM dNTPs (Invitrogen), 1 × *Taq* PCR buffer (Invitrogen), 0.25 μL of Platinum *Taq* DNA polymerase (Invitrogen), 2.5 mM MgCl2 (Invitrogen), and 0.6 μg BSA (Sigma). The final volume of each reaction was 25 μL. The reaction mixture for the remaining strains (UCFS1, UCFS13, UCFS15–17, UCFS19, UCFS21–28, and CYN103–112) was prepared with 10 μL of *i-Taq* 2 × PCR master mix (Intron, Gyeonggi-do, Korea), 0.6 μg BSA (Sigma), 0.2 μM of sxtaf and sxtar primers (Ballot et al., 2010b), and approximately 15 ng of template DNA. The thermal cycling conditions were 94 °C for 4 min; followed by 30 cycles of 94 °C, 55 °C, and 72 °C for 30 sec each; then a final extension at 72 °C for 4 min. CAWBG01 (CICCM) or UCFS10 were used as the positive control and Milli-Q water was used as the negative control.

The PCR for strains that were positive for the *sxtA* gene segment were repeated in a 50 μL reaction volume. These *sxtA* amplicons were purified using a High Pure PCR product purification kit (Roche Diagnostics). These PCR products were sequenced bi-directionally. Sequencing was carried out by either Canterbury Sequencing and Genotyping or Otago Genetic Analysis Services using the 3730×l DNA Analyser or the 3130×l Genetic Analyser (Applied Biosystems, USA) respectively. Both services used the BigDye Terminator v3.1

Cycle Sequencing Kit (Applied Biosystems, USA). These sequences were compared with sequences from the NCBI GenBank database (Benson et al., 2008). Sequences were aligned and phylogenetic trees created using the ML method. The ML method was based on the best fitting model in MEGA (version 5; Tamura et al., 2011). For these data, the Tamura 3-parameter model (Tamura, 1992) was the model of best fit. Reproducibility was tested using 1000 bootstrap replicates. The partial *sxtA* gene segments were deposited in the NCBI GenBank database. Accession numbers are provided in Section 3.3.4.

Biochemical assays for saxitoxin

Initial screening by PCR revealed the presence of an *sxtA* segment involved in STX biosynthesis in *Scytonema* cf. *crispum* UCFS10 (Section 3.3.4; Smith et al., 2011b). Strain UCFS10 and *Scytonema* field specimens collected for this project (Chapter 2) were analysed for the presence of saxitoxins using Jellett PSP Rapid Test kits (Jellett et al., 2002).

Cyanobacterial sample extraction for biochemical assays

A 0.5 g wet weight subsample each of strain UCFS10 and the environmental sample from the drinking water reservoir were added to 0.1% acetic acid in methanol (5 mL). Cells were homogenised (60 sec) using Ultra-Turrax (IKA Laborteknik, Germany), sonicated (20 min) and centrifuged (3000 × *g*, 5 min). The supernatant was dried under nitrogen at 35 °C and resuspended in 300 mL Milli-Q water with 0.1% formic acid. Field specimens, collected in the survey (Section 2.2.1), were lyophilised and weighed into 15 mL Falcon tubes. Milli-Q water (10 mL) was added to each sample, sonicated (10 min), centrifuged (3000 × *g*, 10 min), and the supernatant transferred to a scintillation vial. Methanol (10 mL) was added to the cell pellet in the Falcon tube, sonicated for a further 10 min, centrifuged a second time (3000 × *g*, 10 min), and the supernatants combined. The combined water and methanol supernatant samples were dried under nitrogen and reconstituted in 1 mL 3 mM hydrochloric acid. All algal samples were spin-filtered prior to analysis.

Water sample extraction for biochemical assays

Two water samples, from Lakes Benmore (Site 1) and Alexandrina (Site 2) where toxic *Scytonema* was abundant, were selected for extracellular toxin analysis. These samples were collected, filtered (GF/C, Whatman), and stored as described in Chapter 2, Section

2.2.1. Subsamples (100 mL) were acidified (0.1% formic acid), dried under rotary evaporation, and re-dissolved in 1 mL of 3 mM hydrochloric acid.

Biochemical assay for saxitoxin using the Jellett PSP Rapid Test

Aliquots of algal and water extracts were applied to Jellett PSP Rapid Test Kits (Jellett et al., 2002) according to the protocol supplied by the manufacturer. The LOD ranged from 0.02–0.15 mg kg^{-1} wet weight for the algal samples (Table 3.4) depending on the mass of lyophilised material. The LOD for water samples was 2 µg L^{-1}.

Analysis of saxitoxins by high performance liquid chromatography with fluorescent detection

HPLC–FD was used to identify and quantify STX variants. Only field specimens or strains isolated from environmental samples that were positive for saxitoxins when analysed by Jellett PSP Rapid Test were analysed by HPLC–FD.

Cyanobacterial sample extraction for high performance liquid chromatography with fluorescent detection analysis

Strains (UCFS10, UCFS15–17, and UCFS21) and environmental samples (The Groynes, Lakes Aviemore Site 1, Benmore Sites 1 and 3, and Ruataniwha Site 1) were analysed for intracellular saxitoxins. Samples were lyophilised (FD-1, EYELA, Tokyo Rikaikai Co. Ltd, Tokyo, Japan; or FreeZone, Labconco Corporation, Kansas City, MO, USA) in pre-weighed containers. Strain UCFS10 was re-analysed 1 year after the initial extraction following the same methods to test for changes in STX variants in culture over time.

To estimate the amount of STX produced per cell, an additional subsample of UCFS10 was washed in Milli-Q water to remove extracellular toxins before extraction. Filaments were homogenised (3 min) by Ultra-Turrax (IKA Laborteknik, Germany) in 5 mL Milli-Q water. The Ultra-Turrax probe was rinsed with 3 mL Milli-Q water, the rinsings and an additional 2 mL of Milli-Q water were added to the homogenised sample to give a final volume of 10 mL. A subsample (1 mL) was preserved with Lugol's iodine and enumerated using an inverted microscope (Olympus CK40) and Utermöhl settling chambers (Utermöhl, 1958). The remaining subsample (9 mL) was lyophilised for STX analysis.

Lyophilised samples were weighed and dissolved in 4 mL methanol acidified with 0.1% acetic acid and centrifuged (3000 × *g*, 5 min). Supernatants were reduced to dryness under

nitrogen (35 °C) and re-dissolved in 0.1 mM acetic acid (1–2 mL). The samples were vortexed and spin-filtered (3000 × *g*, 1 min Micro-Spin® Centrifuge Filter Tubes, Grace Davison Discovery Sciences, USA).

Liquid sample extraction for high performance liquid chromatography with fluorescent Detection analysis

Two water samples (100 mL filtered, GF/C, Whatman) from The Groynes and a four month old culture of UCFS10 were analysed to determine if extracellular saxitoxins were present. Each 100 mL water sample was reduced to 1 mL under rotary evaporation.

Sample oxidation prior to HPLC–FD analysis

As STX does not naturally fluoresce, oxidation is carried out so that STX and its variants can be detected by HPLC–FD. Oxidation by periodate and peroxide were used to detect hydroxylated and non-hydroxylated variants respectively. Both oxidation methods are used in these analyses so that all STX variants are identified. Because some metabolites can naturally fluoresce in a similar region to oxidised saxitoxins a non-oxidised sample was compared with the oxidised samples to check for false positives. The method was adapted based on the method by Lawrence et al. (2005). Samples were analysed the same day of oxidation.

Aliquots (100 µL of 20-fold diluted samples of lyophilised cyanobacterial sample, or 100 µL standard solution) were oxidised by adding the sample to 250 µL of 1 M NaOH and 25 µL of 10% H_2O_2 and mixed by vortex. After two minutes at room temperature 20 µL of glacial acetic acid was added, and the sample mixed by vortex, to quench the reaction. Non-oxidised samples for comparison were prepared by adding 100 µL sample to 295 µL Milli-Q water.

A periodate oxidation solution was prepared from equal aliquots of 0.03 M periodic acid, 0.3 M ammonium formate and 0.3 M $Na_2HPO_4.2H_2O$. The solution was adjusted to pH 8.15–8.25 with 1 M and 0.1 M sodium hydroxide. Aliquots (100 µL of 20-fold diluted samples of lyophilised cyanobacterial sample, or 100 µL standard solution) were added to 500 µL of the oxidant solution and mixed by vortex. After one minute at room temperature, 5 µL of glacial acetic acid was added and the sample mixed by vortex to quench the

reaction. Non-oxidised samples for comparison were prepared by adding 100 µL sample to 505 µL Milli-Q water.

High performance liquid chromatography with fluorescent detection analysis

Samples were analysed at the Cawthron Institute using a Kinetex C18 column (1.7 µm, 100 × 2.1 mm, Phenomenex) and HPLC–FD (Waters Acquity UPLC, Alliance 2695, Waters Corp., MA) following the methods of Lawrence et al. (2005). The mobile phase A (0.1 M ammonium formate adjusted to pH 6 with acetic acid) and mobile phase B (mixture of 95% mobile phase A and 5% acetonitrile) were used at a flow of 0.35 mL min^{-1}. The gradient profile was isocratic for 1 min at 100% A; declining to 95% A and 5% B at 2 min; decreasing to 40% A and 60% B at 4.5 min; returning to 100% A at 4.55 min. Fluorescent derivatives were analysed using a Waters Acquity fluorescence detector with excitation at 340 nm and emission at 395 nm. Three sets of standards were used in analysis Table 3.1. These standards were certified reference materials obtained from the National Research Council Canada, Institute for Marine Biosciences and diluted into 0.1 mM acetic acid prior to use. Three working standards were prepared by diluting each set of standard mixtures 50, 100, and 200-fold using 0.1 mM acetic acid. These working standards were used to prepare calibration curves of each analogue. Samples of known composition were oxidised in duplicate and run at the beginning and end of each sample batch. Samples spiked with 200 µL of one of the standard mixtures were analysed in each batch as part of quality control procedures.

Table 3.1 Standard mixtures of certified reference materials. Final concentration of STX variants prepared from certified reference materials used in each standard mixture for HPLC–FD analysis.*

Standard mixture	Analogue	Concentration (µg mL^{-1})	LOD ng mL^{-1}
Mix 1	GTX1/4	3.29	1.9
	neoSTX	2.07	0.8
Mix 2	dcGTX2/3	5.00	nd
	dcSTX	1.59	1.9
	GTX2/3	6.23	1.3
	STX	1.95	1.3
Mix 3	C1/2	7.08	6.2
	dcneoSTX	0.79	nd
	GTX5	2.47	7.9

* LOD reported as approximate STX.2HCl equivalents; nd no data. C = *N*-sulfocarbamoyl toxins; GTX = gonyautoxin; neoSTX = neosaxitoxin; and STX = saxitoxin. Decarbamoyl derivatives are specified by the prefix dc.

3.3 Results

3.3.1 Anatoxin-a

Molecular genetic assays for genes involved in anatoxin-a biosynthesis

The *anaC* and *anaF* gene segments were only detected in strains CYN103–107 and CYN109–112 (Table 3.2). Primers atxoaf/atxar by Ballot (2010a) worked well for strains CYN103–107 and CYN109–112 to detect the *anaF* gene segment. However, for the other strains the atxoaf/atxar primers often resulted in multiple bands of non-specific binding, making it difficult to determine whether the gene was present or not. Attempts at changing the annealing temperature and conditions did not improve the results and occasionally even the positive control displayed multiple bands. Ambiguous results were recorded as invalid. PCR analysis using the generic *anaC* primers was successfully applied in the analysis of all strains without any invalid results.

The atxoaf/atxar primers were used for sequencing the *anaF* amplicons. These anatoxin-a gene segments were identical and shared 99% sequence homogeneity with CYN53 (GU363536), a *P. autumnale* sequence isolated from the Rangataki River (Heath et al., 2010). However, this sequence shared only 73% coverage of the amplicons from in this study. An additional sequence of the *anaF* gene segment of CYN53 was provided by Thomas Palfroy and Susie Wood, Cawthron Institute, for phylogenetic comparison. This sequence and the other *anaF* sequences obtained in this study were deposited in GenBank under accession numbers JX088084– JX088093 (Figure 3.1). All other sequences used for phylogenetic comparison were obtained from GenBank. *Oscillatoria* PCC 6506 (FJ477836) contains 94% sequence homogeneity over the *anaF* gene segment amplified in this study. *Anabaena* sp. (JF803645) and *Anabaena flos-aquae* (AY210784) shared 91% homogeneity with the samples from this study over the same region. *Aphanizomenon issatschenkoi* (FN690757) was 89% similar over 96% of the amplified region in this study. The phylogenetic relationship of these sequences is described in a ML tree (Figure 3.1).

Table 3.2 Summary of genes identified in all strains isolated (Chapter 2).*

	Gene Identified						
Strain	***anaF*(Osc)**	***anaF*(Aph)**	***anaC***	**CYN-PKS**	**CYN-PS**	***mcyE/ndaF***	***sxtA***
CYN103	NT	+	+	–	–	–	–
CYN104	NT	+	+	–	–	–	–
CYN105	NT	+	+	–	–	–	–
CYN106	NT	+	+	–	–	–	–
CYN107	NT	+	+	–	–	–	–
CYN108	NT	–	–	–	–	–	–
CYN109	NT	+	+	–	–	–	–
CYN110	NT	+	+	–	–	–	–
CYN111	NT	+	+	–	–	–	–
CYN112	NT	+	+	–	–	–	–
UCFS1	–	NT	–	–	–	–	–
UCFS2	–	NT	–	–	–	–	–
UCFS3	–	NT	–	–	–	–	–
UCFS4	–	NT	–	–	–	–	–
UCFS5	–	NT	–	–	–	–	–
UCFS6	–	NT	–	–	–	–	–
UCFS7	–	NT	–	–	–	–	–
UCFS8	–	VOID	–	–	–	–	–
UCFS9	–	VOID	–	–	–	–	–
UCFS10	–	VOID	–	–	–	–	+
UCFS11	–	–	–	–	–	–	–
UCFS12	–	VOID	–	–	–	–	–
UCFS13	–	VOID	–	–	–	–	–
UCFS15	NT	VOID	–	–	–	–	+
UCFS16	NT	–	–	–	–	–	+
UCFS17	NT	VOID	–	–	–	–	+
UCFS19	NT	VOID	–	–	–	–	–
UCFS21	NT	VOID	–	–	–	–	+
UCFS22	NT	VOID	–	–	–	–	–
UCFS23	NT	VOID	–	–	–	–	–
UCFS24	NT	VOID	–	–	–	–	–
UCFS25	NT	VOID	–	–	–	–	–
UCFS26	NT	VOID	–	–	–	–	–
UCFS27	NT	VOID	–	–	–	–	–
UCFS28	NT	–	–	–	–	–	–

* *AnaF*(Osc) and *anaF*(Aph) are the detection of *anaF* using either *Oscillatoria* specific primers (Cadel-Six et al., 2009) or primers used to detect this gene in *Aphanizomenon* species (Ballot et al., 2010a) respectively. Generic primers (Rantala-Ylinen et al., 2011) were used to detect the *anaC* gene. CYN-PKS and CYN-PS are polyketide and polypeptide cylindrospermopsin producing gene segments identified in Schembri et al. (2001). *McyE*/*ndaF* are genes identified using primers from Jungblut and Neilan (2006). *SxtA* is the gene identified with primers from Ballot et al. (2010b).
+ present; – absent; NT is not tested. Tests that were invalid due to multiple non-specific binding are marked as VOID.

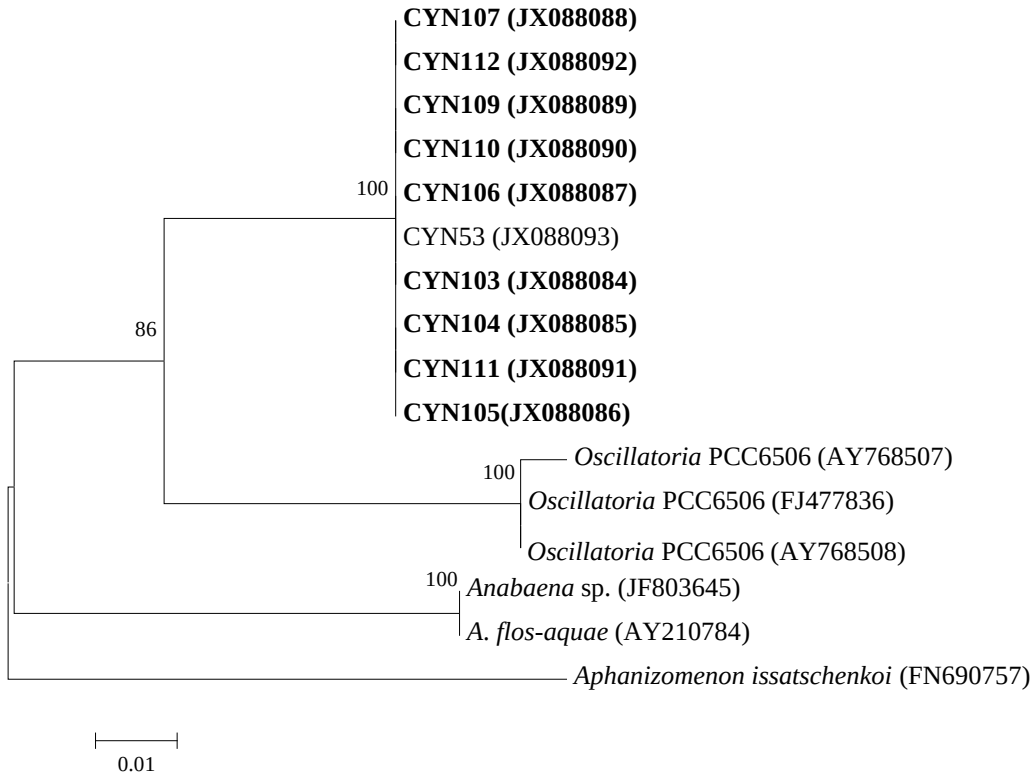

Figure 3.1 Maximum likelihood tree for the *anaF* gene, based on 404 nucleotide positions. Bootstrap support (1000 replicates) is provided near the node, only values ≥ 30% are reported. Clades containing strains from this study are in bold. GenBank accession numbers are provided in parentheses. Scale = 1% sequence divergence.

Liquid chromatography mass spectrometry analysis for anatoxin-a and homoanatoxin-a production

Strains positive for the *anaC* gene segment all contained anatoxin-a and dihydroanatoxin-a. CYN112 also contained homoanatoxin-a (Table 3.3). No epoxy-analogues were confirmed by LC–MS/MS. Anatoxin-a variants and degradation products were not detected in UCFS1–7, UCFS9–13, and CYN108, confirming the molecular genetic assays.

Table 3.3 Anatoxin-a and variants produced by strains CYN103–112, UCFS1–7, and UCFS9–13 determined by LC–MS/MS (mg kg^{-1} dry weight).

	Anatoxin-a	**Dihydro-anatoxin-a**	**Homoanatoxin-a**	**Dihydro-homoanatoxin-a**
CYN103	5.92	205.9	–	–
CYN104	5.79	156.1	–	–
CYN105	0.28	40.8	–	–
CYN106	0.47	56.4	–	–
CYN107	0. 93	9.1	–	–
CYN108	–	–	–	–
CYN109	3.25	128.4	–	–
CYN110	6.40	171.8	–	–
CYN111	1.08	71.9	–	–
CYN112	2.38	165.8	1.00	–
UCFS1	–	–	–	–
UCFS2	–	–	–	–
UCFS3	–	–	–	–
UCFS4	–	–	–	–
UCFS5	–	–	–	–
UCFS6	–	–	–	–
UCFS7	–	–	–	–
UCFS8	–	–	–	–
UCFS9	–	–	–	–
UCFS10	–	–	–	–
UCFS11	–	–	–	–
UCFS12	–	–	–	–
UCFS13	–	–	–	–

– not detected

3.3.2 Cylindrospermopsin

The PKS and peptide synthetase gene segments involved in cylindrospermopsin production were not detected in any of the strains (Table 3.2).

3.3.3 Microcystin and Nodularin

The *mcyE* or *ndaF* genes were not present in any of the strains (Table 3.2).

3.3.4 Saxitoxin

Molecular genetic assays for genes involved in saxitoxin biosynthesis

The gene *sxtA* involved in STX production was present in all *Scytonema* cf. *crispum* strains analysed (UCFS10, UCFS15–17, UCFS21; Table 3.2). The *sxtA* gene segment, from these *S.* cf. *crispum* strains, was amplified and sequenced in forward and reverse directions. These sequences were deposited in GenBank under accession numbers HM629429 and JQ182302– JQ182305. The *sxtA* gene segment in all *S.* cf. *crispum* strains shared high sequence homogeneity (≥ 97%). The amplified *sxtA* region of UCFS16–17 and UCFS21 was identical. UCFS10 shared 97% homogeneity with UCFS15 and 99% homogeneity with UCFS16–17 and UCFS21. The *sxtA* segment of strain UCFS15 shared 98% sequence similarity to UCFS16–17 and UCFS21. These gene segments shared high similarity to known *sxtA* genes from *Aphanizomenon* sp. NH-5 (95-6%, EU629175) and *Anabaena circinalis* (95%, EU629179). A phylogenetic tree (Figure 3.2) based on 554 nucleotide positions of the *sxtA* gene illustrated all *S.* cf. *crispum* strains clustered together.

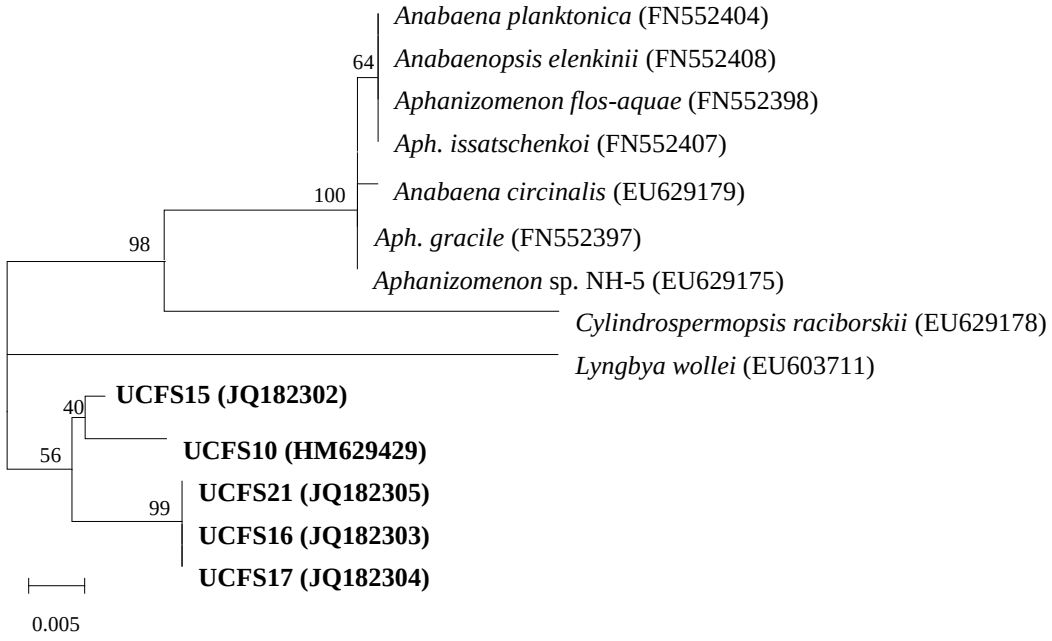

Figure 3.2 Maximum likelihood tree for the *sxtA* gene, based on 554 nucleotide positions. Bootstrap support (1000 replicates) is provided near the node, only values ≥ 30% are reported. Clades containing strains from this study are in bold. GenBank accession numbers are provided in parentheses. Scale = 0.5% sequence divergence.

Biochemical assays for saxitoxin

Strain UCFS10 was analysed by Jellett PSP Rapid Test instead of an environmental sample from The Groynes. The remaining ten algal field-samples (Section 2.3.1), nine from the *Scytonema* survey and one from the pre-treatment drinking water reservoir, were also analysed with the Jellett PSP Rapid Test for the presence of saxitoxins. The results from the analyses of cyanobacterial samples are presented in Table 3.4. The limits of detection are variable as the mass of cyanobacterial samples tested varied between 2–25 g wet weight cyanobacterial mat depending on the amount of sample collected at each site. Saxitoxins were detected in all *S.* cf. *crispum* algal samples tested. Saxitoxins were not detected in *S.* cf. *chiastum* or *S.* cf. *fritschii* algal samples, or in the two lake water samples collected in lakes with STX-producing *S.* cf. *crispum.*

Table 3.4 Analysis of *Scytonema* for saxitoxins by Jellett PSP Rapid Test.

Scytonema morphospecies	**Source**	**LOD (mg kg^{-1})**	**Result**
S. cf. *chiastum*	Lake Hawdon	0.15	–
	Lake Clearwater	0.10	–
S. cf. *crispum*	Lake Alexandrina	0.05	+
	Lake Benmore (Site 1)	0.02	+
	Lake Benmore (Site 3)	0.02	+
	Lake Ruataniwha (Site 1)	0.05	+
	South Island Reservoir	0.02	+
	The Groynes (strain UCFS10)	0.02	+
S. cf. *fritschii*	Lake Middleton	0.10	–
	Lake Ruataniwha (Site 2)	0.02	–
	Lake Sarah	0.15	–

– absent; + present; LOD limit of detection recorded as wet weight mat (mg kg^{-1}).

Analysis of saxitoxins by high performance liquid chromatography with fluorescent detection

Initial HPLC–FD analyses confirmed the presence of the STX variant in The Groynes algal sample (65.6 mg kg^{-1}) and in UCFS10 (119 mg kg^{-1}). No other STX variants were present in either UCFS10 or The Groynes algal sample. The STX quota for UCFS10 was 1.3 pg cell^{-1}. STX was present in the culture media of UCFS10 at 10 µg L^{-1}. Extracellular saxitoxins were not detected in The Groynes water sample. All values are reported as the STX.2HCl equivalent.

The four environmental samples from Lakes Alexandrina, Benmore (sites 1 and 3), and Ruataniwha contained a diverse mixture of variants including GTX 1–5, neoSTX, STX, dcSTX, and dcGTX 2/3 (Table 3.5). C-toxins or dcneoSTX were not detected. The reanalysis of culture UCFS10 (one year after isolation) also identified only STX (Table 3.6). In contrast a number of variants were detected in the other strains. The analogue dcGTX2/3 was the dominant STX variant in UCFS16–17 and UCFS21 (65, 74, and 65% of the total variants respectively). In these three strains a peak was observed in the HPLC chromatogram at the retention time of GTX5, but it was below the quantitation detection limit. GTX1/4, dcGTX1/4, and neoSTX could not be detected in these samples due to sample matrix effect and high concentrations of non-hydroxylated variants that may mask these variants. GTX 5 was the dominant variant in UCFS15 and the samples from Lake Benmore (Site 1 and 3), consisting of 49, 34, and 62% of the total variants respectively. STX was the dominant variant in the other two environmental samples (58% from Lake Alexandrina, 51% from Lake Ruataniwha).

Table 3.5 Saxitoxin variants detected by HPLC–FD in environmental samples of *Scytonema* collected from Lakes Alexandrina, Benmore, and Ruataniwha.

Saxitoxin Variant	**Alexandrina** (Site 2)	**Benmore** (Site 1)	**Benmore** (Site 3)	**Ruataniwha** (Site 1)
dcGTX 2/3	0.10	0.25	0.94	0.22
dcSTX	0.95	0.69	0.90	0.50
GTX 1/4	0.29	0.76	2.8	0.65
GTX 2/3	0.16	0.41	1.6	0.30
GTX 5	—	1.3	12	—
neoSTX	—	0.13	0.11	0.12
STX	2.1	0.37	0.77	1.9

Results are presented in mg kg^{-1} of freeze-dried material. STX, saxitoxin; dcGTX, decarbamoyl gonyautoxins; GTX, gonyautoxins; neoSTX, neosaxitoxin; dcSTX, decarbamoyl saxitoxin; — not detected.

Table 3.6 Saxitoxin variants detected by HPLC–FD detection in strains of *Scytonema* cf. *crispum.**

Saxitoxin Variant	UCFS10	UCFS15	UCFS16†	UCFS17†	UCFS21†
dcGTX 2/3	—	—	68	87	55
dcSTX	—	1.7	9.6	8.2	6.9
GTX 1/4	—	13	—	—	—
GTX 2/3	—	8.5	10	11	11
GTX 5	—	23	—	—	—
neoSTX	—	1.1	—	—	—
STX	7.4	0.77	16	11	12

*Results are presented in mg kg^{-1} of freeze-dried material. STX, saxitoxin; dcGTX, decarbamoyl gonyautoxins; GTX, gonyautoxins; neoSTX, neosaxitoxin; dcSTX, decarbamoyl saxitoxin; — not detected.

† A peak corresponding to the same retention time as GTX5 was observed in strains UCFS16–17 and UCFS21, however the concentration of this peak fell below the quantifiable detection limit.

3.4 Discussion

Application of PCR techniques to screen for genes associated with cyanotoxin biosynthesis have provided simple, inexpensive methods for detecting potential cyanotoxin producers in water, algal samples, and cultures (Kurmayer and Christiansen, 2009; Sivonen, 2008). However, positive identification of these genes must be complemented by alternate methods to safeguard against false positives possibly due to mutations in other DNA segments that regulate the corresponding biosynthetic pathway (Sivonen, 2008). PCR techniques in this study were used to screen all cyanobacterial strains isolated from South Island water bodies (Section 2.3.2). Genes associated with anatoxin-a or STX were identified in some of these strains, but genes associated with cylindrospermopsin, microcystin or nodularin were not detected. This preliminary screening prevented the need to analyse all strains using costly and time-consuming analytical chemistry techniques. Only those strains that had a positive result for cyanotoxin biosynthesis genes were further analysed to confirm and quantify cyanotoxin-producing ability.

3.4.1 Benthic Cyanotoxin Producers

Anatoxin-a and homoanatoxin-a

Animal poisoning events from contact with benthic periphytic cyanobacterial mats (Codd et al., 1992; Gugger et al., 2005; Krienitz et al., 2003; Mez et al., 1997; Sivonen et al., 1990; Wood et al., 2007b) are well-documented. *Phormidium autumnale* is a benthic mat-forming species that can produce either one or both of anatoxin-a and homoanatoxin-a (Heath et al., 2010). *Phormidium* spp. have been implicated in dog fatalities in France, the Netherlands, New Zealand, and the United States of America (Faassen et al., 2012; Gugger et al., 2005; Puschner et al., 2010; Wood et al., 2007b). In this study, the *P.* cf. *uncinatum* strains CYN103–107 and CYN109–112 produced anatoxin-a, while CYN112 also produced homoanatoxin-a.

Benthic anatoxin-a-producing *Phormidium* occurs in many New Zealand rivers and tends to proliferate in summer months when temperatures are elevated and river flows are stable (Wood et al., 2010b; Wood et al., 2007b). *Phormidium autumnale* is the predominant anatoxin-a producer in New Zealand rivers (Heath et al., 2010). Initial screening for genes involved in anatoxin-a and homoanatoxin-a production used primers by Cadel-Six (2009) designed for identifying a PKS segment ks2 associated with the *anaF* gene in *Oscillatoria.*

These primers work well for *Oscillatoria* but are not suitable for identification of anatoxin-a production in some species including *Aphanizomenon* (Ballot et al., 2010a). However, these primers work well for *Phormidium* species (Wood et al., 2010b). Anatoxin-a production was not detected in any of the strains UCFS1–13 analysed using these *Oscillatoria* specific primers described by Cadel-Six (2009). The primers by Ballot et al. (2010a) used to identify a similar region of the *anaF* PKS were trialled in this study, however these primers frequently produced non-specific binding with genes in non-anatoxin-a producing strains. These primers were successful at detecting anatoxin-a production in nine strains, CYN103–107 and CYN109–112. The amplified *anaF* regions in these strains were identical and shared high sequence homogeneity with *P. autumnale* sequence CYN53. All strains were analysed with a recently published generic primer that is designed to identify potential anatoxin-a production in both Nostocales and Oscillatoriales anatoxin-a producing species (Rantala-Ylinen et al., 2011). These primers target the *anaC* gene, which putatively initiates anatoxin-a synthesis by adenylation of proline (Méjean et al., 2009). These generic PCR primers identified the partial *anaC* gene segment in the same nine strains in which the *anaF* gene was identified. No other strains contained the *anaC* gene segment. There was no problem with non-specific binding using the primers by Rantala-Ylinen et al. (2011). LC–MS/MS analysis of UCFS1–13 and CYN103–112 confirmed that only the strains that were positive by PCR for the *anaC* gene produced anatoxin-a or homoanatoxin-a. The generic primers for *ana*C proved a useful tool to screen strains for the identification of anatoxin-a production potential in this study.

Wood et al. (2010b) found that environmental mats randomly taken from within a 10 m^2 area can contain both anatoxin-a-producing and non-toxic *Phormidium*. In the current study, ten *P.* cf. *uncinatum* strains were isolated from a single 1 cm^2 portion of a single cyanobacterial mat (Chapter 2) that produced high concentrations of homoanatoxin-a. Of these isolates, only strain CYN112 produced both anatoxin-a and homoanatoxin-a. This study is the second time a homoanatoxin-a producer has been isolated in New Zealand. On the previous occasion, the isolated strain lost the ability to produce both anatoxin-a and homoanatoxin-a in culture (Wood et al., 2008). Strain CYN112 has been cryopreserved to conserve homoanatoxin-a production, making it the first viable homoanatoxin-a producing cultured strain in New Zealand. Eight strains, CYN103–107 and CYN109–111, from the same mat as CYN112 produced only anatoxin-a. These strains shared identical 16S rRNA

gene segments (Section 2.3.3). Only one *P*. cf. *uncinatum* strain, CYN108, isolated from the mat was nontoxic. This strain was not identical to the 16S rRNA sequence of the anatoxin-a-producing strains. These data demonstrate, for the first time, the co-existence of anatoxin-a, homoanatoxin-a, and non-toxic *Phormidium* strains within a single benthic mat. This coexistence within a single benthic mat was suspected, as previous strains of *P. autumnale* isolated from mats containing anatoxin-a were nontoxic (Heath et al., 2010). Similarly, anatoxin-a/homoanatoxin-a and non-anatoxin-a producing strains of *Phormidium* have been isolated from floating scum samples from the same site of the River Tarn (Cadel-Six et al., 2007). Anatoxin-a and non-anatoxin-a producing strains also co-exist in strains of *Aphanizomenon issatschenkoi* isolated from Lake Stolpsee, Germany (Ballot et al., 2010a). Other *P*. cf. *uncinatum* strains in this study, UCFS4–5, UCFS7, and UCFS12–13, were non-toxic.

Anatoxin-a concentration varied between the nine anatoxin-a-producing *P*. cf. *uncinatum* strains (Table 3.3). CYN110 produced the highest concentration of anatoxin-a (6.4 μg g^{-1} culture dry weight). The variation in anatoxin-a production by different strains within a single mat may contribute to the large variability in detection of anatoxin-a, homoanatoxin-a, and their degradation products observed in cyanobacterial mat samples from New Zealand (Heath et al., 2010; Wood et al., 2011). CYN112 produced 2.38 and 1 μg g^{-1} culture dry weight anatoxin-a and homoanatoxin-a respectively and 165.8 μg g^{-1} dihydroanatoxin-a. Dihydroanatoxin-a (9.1–205.9 μg g^{-1}) was detected in all anatoxin-a-producing strains but was not proportional to anatoxin-a concentrations. The degradation products, dihydro- and epoxy- analogues of anatoxin-a and homoanatoxin-a, are particularly important to detect in environmental samples as these metabolites are more stable than the parent compound (James et al., 2008). Detection of these non-toxic degradation analogues can be used to provide evidence of a previous toxic event or nearby proliferations of anatoxin-a/homoanatoxin-a-producing species (Wood et al., 2011).

Cylindrospermopsin

None of the benthic strains isolated in this study contained the PKS or peptide synthetase genes involved in cylindrospermopsin production. Cylindrospermopsin and analogues of this cyanotoxin are known to occur in benthic *Lyngbya wollei* (Seifert et al., 2007) and *Oscillatoria* sp. (Mazmouz et al., 2010).

Microcystin and nodularin

Microcystin has previously been identified in benthic field samples of *Nostoc commune* (Wood et al., 2006) and benthic Oscillatoriales (Wood et al., 2010a) in New Zealand. Microcystins have also been associated with benthic cyanobacterial proliferations in Africa (Mohamed et al., 2006), America (Izaguirre et al., 2007), Australia (Dasey et al., 2005), and Europe (Aboal and Puig, 2005; Mez et al., 1997; Mez et al., 1998). In the current study, the *mcyE* gene involved in microcystin biosynthesis was not identified in any strain. The presence of a benthic nodularin producer has recently been recorded in a New Zealand lake by identification of the *ndaF* gene and detection of nodularin by LC–MS/MS from benthic field samples (Wood et al., 2012). The identity of the nodularin producer within the benthic mats is unknown (Wood et al., 2012). The *ndaF* gene involved in nodularin biosynthesis was not identified in any of the strains isolated during the current study.

Saxitoxin

Reports of STX-producing cyanobacteria are limited and much less frequent than those of either microcystin or cylindrospermopsin (Chorus, 2001; Sivonen and Jones, 1999). This study is the first report of STX production by a species of *Scytonema* and it is the first confirmation of a STX-producing cyanobacterium in New Zealand. It is surprising that STX production in *Scytonema* has not been previously reported as several species of *Scytonema* have been intensively studied due to their ability to produce a wide range of bioactive compounds. Examples include the cytotoxic and antifungal compounds tubercidin, scytophycins, and tolytoxins (Carmeli et al., 1990; Stewart et al., 1988), and cyanobacterin, which has antibiotic and algicidal properties (Mason et al., 1982).

The cyanobacterial STX gene cluster, *sxt*, has recently been identified (Kellmann et al., 2008). Amplification of part of the *sxtA* gene in UCFS10, UCFS15–17, and UCFS21 confirmed the potential for STX production in these strains. There is some variability in the *sxtA* gene segments in each of these strains (97–100% sequence homogeneity). Slight variation in the *sxtA* gene segments has also been reported for *Aphanizomenon gracile* strains (97–100% sequence homogeneity; Ballot et al., 2010b). The identification of *sxtA* in these strains supports the usefulness of PCR-based approaches for screening culture strains or environmental samples for STX production potential. Some strains that do not produce saxitoxins have tested positive for the *sxtA* gene (Ballot et al., 2010b), therefore results need to be confirmed using a chemical or immunological approach.

Previous studies on STX production in cyanobacteria have identified more than one STX variant in each genus (Table 3.7). Most of these STX analogues are less potent than STX, however these variants can be converted into more toxic variants in the human digestive track (Llewellyn, 2006; Wiese et al., 2010). Initially only the regular saxitoxin variant, STX, was identified in *S.* cf. *crispum* strain UCFS10 (Smith et al., 2011b). The re-analysis of UCFS10 confirmed only the STX variant in this strain, although this was detected in much lower concentrations, 7 mg kg^{-1} STX.2HCl equivalents, indicating the STX variant profile did not change over time. This decrease in STX production could be a reflection of sampling at different stages of the growth cycle. The concentration of STX, the most potent STX variant detected in *S.* cf. *crispum*, was generally higher in cultured strains than in environmental samples. The analysis of environmental samples containing *S.* cf. *crispum* revealed the presence of multiple variants (Table 3.5). This study is the first identification of these STX variants from cyanobacteria in New Zealand. The variant dcGTX was detected in only the environmental samples and strains isolated from Lakes Alexandrina, Benmore, and Ruataniwha. Variation in STX profiles among cultured strains of one species has been seen in *Cylindrospermopsis raciborskii* from Brazilian reservoirs (Castro et al., 2004; Lagos et al., 1999; Molica et al., 2002; Pomati et al., 2004a). STX and neoSTX were detected in strain T1 from a reservoir in Amparo (Lagos et al., 1999), whereas Molica et al. (2002) detected STX, neoSTX, dcSTX, and GTX6 in strain ITEP-018 from Tabocas Reservoir. Two strains isolated from the Billings Reservior at Taquacetuba produced either dcSTX, GTX2/3, and STX (strain C10; Castro et al., 2004), or GTX2/3 and STX (strain T2; Lagos et al., 1999). Whereas a third isolate from this site, strain T3, was reported to produce STX, C1, and C2 (Pomati et al., 2004a), STX, dcSTX, GTX5, and neoSTX (Kellmann et al., 2008), or STX, neoSTX, and dcneoSTX (Soto-Liebe et al., 2010). Similar variations have also been observed in other STX-producing cyanobacteria including *Anabaena circinalis* (Negri et al., 1997; Negri et al., 1995; Testé et al., 2002; Velzeboer et al., 2001) and amongst *Aphanizomenon* spp. (Ballot et al., 2010b; Liu et al., 2006; Pereira et al., 2004). Ballot et al. (2010b) reported the primary STX variant produced by environmental samples of *Aphanizomenon gracile* varied between Lakes Scharmützel, GTX5 dominant, and Melang, neoSTX dominant.

Table 3.7 Saxitoxin variants from cyanobacteria recorded in the literature.*

Genus	Saxitoxin derivatives reported in environmental blooms or in strains					Reference
	C-toxin	**GTX**	**neoSTX**	**STX**	**other**	
Anabaena	C1-2	dcGTX2-3, GTX1-6		dcSTX, STX		Humpage et al., 1994; Negri et al., 1997
Aphanizomenon		GTX1,3-6	neoSTX	dcSTX, STX		Ballot et al., 2010b; Ferreira et al., 2001; Pereira et al., 2000; Pereira et al., 2004
Cylindrospermopsis	C1-2	GTX2-3,5-6	dcneoSTX, neoSTX	dcSTX, STX		Lagos et al., 1999; Molica et al., 2002; Pomati et al., 2004a Kellmann et al., 2008
Lyngbya		dcGTX2-3, GTX1-6		dcSTX	LW1-6	Carmichael et al., 1997; Onodera et al., 1997b; Yin et al., 1997
Planktothrix				STX	4 possible variants	Pomati et al., 2000
Raphidiopsis				dcSTX, STX		Yunes et al., 2009
Scytonema		dcGTX2-3, GTX1-5	neoSTX	dcSTX, STX		This study

*Analogues have been grouped into C-toxin = *N*-sulfocarbamoyl toxins; GTX = gonyautoxin; neoSTX = neosaxitoxin; and STX = saxitoxin and other. Other includes the LW = *Lyngbya wollei* PSP specific to *L. wollei*; The 4 variants described in Pomati et al., 2000 were not identified. For analogues with multiple variants the number identifying variant is specified (e.g. C1–2, GTX1–6). Decarbamoyl derivatives are specified by the prefix dc.

The identification of STX in this study, from cultured strains and environmental samples of *S.* cf. *crispum*, from metaphyton (Chapter 2) highlights another benthic habitat where cyanobacteria may pose a risk to human and animal health. Saxitoxins have been identified in the freshwater benthic mat-forming species *Lyngbya wollei* (Carmichael et al., 1997). This species has both a benthic — either periphytic, growing on the substratum, or metaphytic, in loose association with the substratum — and a planktonic phase, consisting of dense floating mats (Bridgeman and Penamon, 2010). Saxitoxins have been identified in both benthic and planktonic samples of *L. wollei* (Carmichael et al., 1997). All other known cyanobacterial STX-producers, *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Planktothrix*, and *Raphidiopsis*, are planktonic.

Metaphytic STX-producing *Scytonema* has been observed throughout the year at The Groynes reserve (Smith et al., 2011b). Other benthic cyanobacteria in New Zealand, including benthic anatoxin-a producing *Phormidium*, generally only proliferate in summer (Wood et al., 2007b; Wood et al., 2010b). Therefore *Scytonema* needs to be monitored throughout the year. Dog poisonings from ingestion of cyanotoxin-producing mats that have accumulated along shorelines of lakes and rivers are well-documented (Edwards et al., 1992; Gugger et al., 2005; Hamill, 2001; Wood et al., 2007b; Wood et al., 2010a). *Scytonema* cf. *crispum* also accumulates along shorelines, and therefore may pose a risk to dogs, farm stock, and wildlife.

Recent studies have reported that saxitoxins produced by planktonic cyanobacteria can accumulate in freshwater fish (Galvão et al., 2009). Accumulation of STX is likely to depend on fish diet and whether they directly feed on STX-producing species or indirectly ingest STX through the food web. There are no data on whether any aquatic organisms, including fish and invertebrates, feed on *Scytonema*. Tissue samples from five brown trout from a lake at The Groynes with metaphytic *S.* cf. *crispum* were negative for saxitoxins (Environment Canterbury, unpublished data).

Toxins can be released during cell decomposition and lysis (Sivonen and Jones, 1999). Extracellular STX, 10.6 $\mu g\ L^{-1}$ STX.2HCl equivalent, was detected in a four month old strain of UCFS10 so there is potential for saxitoxins to be released in the natural environment. Water samples from The Groynes (Smith et al., 2011b), Lake Alexandrina, and Lake Benmore did not contain extracellular saxitoxins but these were just single

samples from each location where saxitoxin-producing *Scytonema* were collected. The extracellular STX in culture may be due to either active export of STX from cells or release of toxin during cell lysis. Because the recreational water bodies in this study are not used for drinking water it is unlikely that extracellular saxitoxins pose a risk to human health at these sites. However, STX-producing *S.* cf. *crispum* was identified in a pre-treatment drinking water reservoir (Smith et al., 2011b). This suggests that drinking water sources should be monitored for this cyanobacterium.

The increase in the number of identified toxic benthic cyanobacteria worldwide (Cadel-Six et al., 2007; Seifert et al., 2007) and related dog and bird poisonings (Codd et al., 1992; Gugger et al., 2005; Krienitz et al., 2003; Wood et al., 2007b) clearly demonstrates that these constitute a hazard to animal and human health. The identification of toxic benthic cyanobacteria in a drinking-water source in this study and in research elsewhere (Izaguirre et al., 2007; Mohamed et al., 2006) highlights the largely unconsidered risk they may pose to human health. Actively growing mats may pose little risk as the majority of cyanotoxins are likely to be intracellular and not released into the water (Sivonen and Jones, 1999). However, when mats die there could be large-scale release of cyanotoxins as has been observed previously in cylindrospermopsin producing *C. raciborskii* blooms and cultures (Griffiths and Saker, 2003; Hawkins et al., 2001). This cyanotoxin has been associated with human illness from ingestion of extracellular cylindrospermopsin from a drinking water reservoir (Griffiths and Saker, 2003; Hawkins et al., 1985). Sampling programmes should consider benthic as well as planktonic species during monitoring for cyanotoxins in drinking-water sources. Previous identification of STX in freshwater environmental samples indicated the presence of STX-producing cyanobacteria in New Zealand (Wood et al., 2006). However in this case the majority of samples were from planktonic blooms, *Scytonema* sp. was not reported, and the STX-producing cyanobacterium was not identified (Wood et al., 2006). There is little doubt that additional STX-producing species will be identified as further studies of New Zealand cyanobacteria are undertaken.

3.4.2 Future Studies

Previous studies have identified an anatoxin-a precursor, 11-carboxyl anatoxin-a, in *Aphanizomenon issatschenkoi* (Selwood et al., 2007). The homoanatoxin-a equivalent of this precursor could be screened for in CYN112 to aid understanding of the anatoxin-a and homoanatoxin-a biosynthetic pathway.

Further monitoring for saxitoxins near *Scytonema* growth is required to determine the risk of exposure to extracellular saxitoxins in the environment throughout the year and at different stages of the growth cycle. These extracellular toxins could be monitored using Solid Phase Adsorption Toxin Tracking (SPATT) (MacKenzie et al., 2004; MacKenzie, 2010). SPATT has been used to detect cyanotoxins that were released from the mats into the water column from benthic cyanobacteria in rivers (Wood et al., 2011) and lakes (Wood et al., 2012). SPATT has not been developed to monitor saxitoxins, thus some development work would be required before this technique could be applied to monitoring extracellular saxitoxins in these lakes.

Only *S.* cf. *crispum* was identified as a STX-producer in this study. *Scytonema* is a large genus containing over 100 species (Komárek et al., 2003) and may contain further STX-producers. Only three morphospecies have been tested; *S.* cf. *chiastum, S.* cf. *crispum,* and *S.* cf. *fritschii*. These morphospecies were distributed in three distinct clades as discussed in Section 2.3.3, supporting earlier suggestions that the *Scytonema* genus is not monophyetic and a polyphasic re-evaluation is required (Smith et al., 2011b).

3.5 Conclusions

Of the 35 strains studied in culture, nine were found to produce anatoxin-a/ homoanatoxin-a and five other strains produced saxitoxins. LC–MS/MS confirmed and quantified anatoxin-a production in these nine strains. STX production was confirmed by Jellett PSP Rapid Test and HPLC–FD. Production of cylindrospermopsins, microcystins, or nodularins was not detected in any strain.

Strain CYN112 is presently the only viable culture of a homoanatoxin-a producing species from New Zealand. It also produces anatoxin-a. This strain has been cryopreserved and banked in the Cawthron Institute Culture Collection of Micro-algae (CICCM, Nelson New Zealand; http://cultures.cawthron.org.nz; Section 2.2.2). Strains CYN103–112 were isolated form a single anatoxin-producing mat from Waimakariri River. Both toxic and non-toxic *P.* cf. *uncinatum* species were isolated. All these strains were morphologically similar, and CYN108 shared high 16S rRNA sequence similarity to the anatoxin-a-producing strains (98%, Section 2.3.3). Among the toxic species the concentration of toxins produced varied markedly. This variability may contribute to the large difference in anatoxin-a concentrations within and between mats sampled from rivers in New Zealand.

Three primers associated with anatoxin-a biosynthesis were trialled. The generic primers associated with the *anaC* gene were most applicable for the detection of potential ability to produce anatoxin-a or homoanatoxin-a. These primers identified the *anaC* gene in all anatoxin-a producing *P.* cf. *uncinatum* strains. False-positives, false-negatives or invalid results were not detected using this primer set.

Scytonema cf. *crispum* is the first positive identification of the species of a STX-producing cyanobacterium in New Zealand. HPLC–FD identified and quantified the saxitoxin variants in strains and environmental samples. This study is the first time that the variants STX, GTX1–5, neoSTX, dcSTX, and dcGTX2/3 have been reported in a cyanobacterium in New Zealand. There are likely to be other STX-producing cyanobacteria in New Zealand that have not been identified. *Scytonema* occurs year-round posing a potential hazard in drinking and recreational water sources. Metaphyton has been overlooked as a potential cyanotoxin producer source, monitoring programmes should include this habitat in their risk assessments.

4 *Phormidium autumnale* growth and anatoxin-a production under iron and copper stress[‡]

4.1 Introduction

Cyanobacteria are influenced by numerous fluctuating environmental factors. These environmental factors may have synergistic, antagonistic or additive effect on growth and metabolite production, including cyanotoxin biosynthesis (Bláha et al., 2009; Codd et al., 2005a; Sivonen and Jones, 1999). Chemical and physical parameters have been only weakly correlated with cyanotoxin production in environmental surveys, due to the complexity of interacting stressors (Rapala and Sivonen, 1998). In this study the effects of iron and copper concentrations on anatoxin-a production and growth in *Phormidium autumnale* were examined in batch culture.

4.1.1 Metal Uptake and Requirements in Algae and Cyanobacteria

Cyanobacteria and eukaryotic algae readily accumulate metals from their environment (Baptista and Vasconcelos, 2006; Gadd, 1988; Mehta and Gaur, 2005). Metal uptake mechanisms generally occur by a two-step process. The rapid passive adsorption onto the outer cell wall is followed by slower metabolically driven active transport into the cell (Fiore and Trevors, 1994; Gadd, 1988). This property has been exploited using living or dead microorganisms in bioremediation of metal-contaminated water bodies through adsorption, absorption, surface complexation, precipitation and ion exchange (Gadd, 2009; Singh et al., 1998). Cyanobacteria have been used to remove heavy metals from industrial waste streams (Gadd, 1988; Mehta and Gaur, 2005). The ability for algae and cyanobacteria to uptake metals depends on variables including taxa composition, biomass, structure of binding sites, pH, temperature, metal concentration, and metal speciation (Baptista and Vasconcelos, 2006; Mehta and Gaur, 2005; Rueter and Petersen, 1987).

Trace metals are essential cofactors in metabolic pathways, including carbon fixation, photosynthesis, and protein function (Baptista and Vasconcelos, 2006; Rueter and Petersen, 1987; Shcolnick and Keren, 2006). Iron complexes are fundamental to cyanobacterial photosynthetic electron transport pathways as well as other important pathways including

[‡] Part of this chapter has been presented in the following publication:

Smith, F., Effects of metal stressors on cyanotoxin production. *Chemistry Education in New Zealand* **2010,** *121*, 2-7.

nitrogen reduction (Baptista and Vasconcelos, 2006; Rueter and Petersen, 1987). Copper is an essential cofactor in some proteins in cyanobacteria (Baptista and Vasconcelos, 2006) including plastocyanin used in photosynthesis (Shcolnick and Keren, 2006). The bioavailability and regulation of these trace metals is essential to regulation of cellular processes including photosynthesis. By selecting between co-factors involved in these cellular processes, cyanobacteria can mitigate the effects of induced stress. This stress can be caused when trace metal availability is insufficient to support optimal growth. The electron carrier flavodoxin can be replaced by ferredoxin in some cyanobacteria when iron is limited (Latifi et al., 2009). Plastocyanin can be used instead of cytochrome c-533 in some algae and cyanobacteria when copper is limited (Shcolnick and Keren, 2006).

4.1.2 Effects of Metal Stressors on Algae and Cyanobacteria

Exposure to non-essential metals or elevated concentrations of essential metals can be highly toxic to cells (Baptista and Vasconcelos, 2006; Rueter and Petersen, 1987). These metal stressors can affect algal growth (Rueter and Petersen, 1987) and alter the algal metabolites produced (Jamers et al., 2009). Maldonado et al. (2002) demonstrated that neurotoxic domoic acid production in *Pseudo-nitzschia* spp. can be activated by metal stress including increase in copper or reduction in iron availability.

Iron co-ordinated metabolic pathways can produce harmful radicals. To minimise oxidative damage to the cell, iron must be carefully regulated within the cell (Baptista and Vasconcelos, 2006; Rueter and Petersen, 1987). The requirement for copper is far lower than that of iron in cyanobacterial cells, and copper can be toxic to cells at low concentrations. This property has been exploited to produce copper-based algicides to eradicate problematic algal blooms. However, when sufficient amounts of copper algicide are applied to kill-off a cyanobacterial bloom, a highly concentrated pulse of intracellular cyanotoxins may be released into the environment (Sivonen and Jones, 1999). Copper algicides also harm other aquatic biota important in the ecosystem including bacteria that may assist in cyanotoxin biodegradation (Edwards and Lawton, 2009).

Excess or deficient supply of trace elements can trigger defence systems to protect cells from the effects of these stressors. For example iron starvation can promote expression of iron-stress inducible proteins in cyanobacteria to help alleviate the effects of oxidative stress caused by deficient iron availability (Latifi et al., 2009). Iron and copper-zinc

complexes can form the catalytic centres of superoxide dismutase to assist in removing reactive oxygen species to prevent damage to the cell (Latifi et al., 2009). Cell death can occur when cyanobacteria cannot mitigate this oxidative stress (Latifi et al., 2009).

Tolerance to metal stressors varies between different algal species and strains (Thompson et al., 2011). A study focusing on several green algae taxa and the cyanobacterium *Oscillatoria* concluded that *O. tenuis* was more resistant to copper than the eukaryotic algae (Lembi, 2000).

4.1.3 Metal Stressors on Cyanotoxin Production

The majority of studies investigating the effects of metal stressor on cyanotoxin production are limited to research on microcystin production by *Microcystis aeruginosa*. This species is often used as the test organism because microcystin has been frequently identified in *M. aeruginosa* blooms worldwide (Sivonen and Jones, 1999). Other studies have also investigated cylindrospermopsin production by *Cylindrospermopsis raciborskii* and microcystin production by *M. novacekii* and *Microcystis* field populations (species not identified) in relation to metal stress (Amé et al., 2003; Amé and Wunderlin, 2005; Chuang et al., 2001; Li et al., 2009).

Arsenic

Arsenic is not an essential element. Arsenic in the form of arsenate is thought to be taken up by the phosphate transport system owing to its structural resemblance to phosphate (Gong et al., 2009). Therefore, the presence of arsenate under phosphate-limited conditions can be of concern. Gong et al. (2009) studied the effect of a range of biologically significant arsenic concentrations on microcystin production under various phosphate regimes. The stress the cultures were exposed to prior to the arsenate-stress was an important factor in the production of intra- and extracellular toxins. Intracellular microcystin production generally increased linearly with an exponential increase of arsenic concentration. However, when cultures were preconditioned to phosphate starvation no correlation was observed (Gong et al., 2009).

Iron and Manganese

Iron and manganese catalyse part of the photosynthetic electron transport pathway, and in the process create reactive oxygen species that can be detrimental to the cell (Shcolnick and

Keren, 2006). Studies investigating the effect of iron availability on microcystin production have reported contrasting results (Table 4.1). In some experiments, iron availability was modified by altering the concentration of iron in each culture medium, as well as by increasing concentrations of metal-chelators (Lukač and Aegerter, 1993; Martin-Luna et al., 2006; Utkilen and Gjølme, 1995). Contradictory results were reported in 1995 and 1996 for microcystin production in relation to iron stress for experiments undertaken by the same research group with the same culture medium and culturing conditions (Lyck et al., 1996; Utkilen and Gjølme, 1995). Bickel and Lyck (2001) reported lower microcystin concentrations at 0.5 µM and 1 µM iron compared to 10 µM iron. However, there was no comment about the decrease from 1 to 0.5 µM iron correlated to an increase of 1.97 to 7.83 ng microcystin μg^{-1} protein (Bickel and Lyck, 2001). Utkilen and Gjølme (1995) reported faster iron uptake over time with higher light intensity. Iron uptake was also notably higher in cyanotoxin producing strains than in non-toxic strains under the same light conditions (Utkilen and Gjølme, 1995).

Table 4.1 Summary of laboratory studies investigating the effect of iron deprivation on microcystin production.*

***Microcystis* source**	**Microcystin production correlation parameter(s)**	**Change in microcystin under iron deprivation**	**Iron concentrations investigated (µM) †**	**Culture conditions**	**Reference**
M. aeruginosa PCC 7806	dry weight	increase	0–100	BG11 medium batch culture	Lukač and Aegerter, 1993
M. aeruginosa CYA 228/1	dry weight protein content	decrease decrease	0.3–10	O2 medium continuous	Utkilen and Gjølme, 1995
M. aeruginosa CYA 228/1	dry weight protein content chlorophyll	inconsistent increase increase	0–10	O2 medium continuous	Lyck et al., 1996
M. aeruginosa CYA 228/1	dry weight protein content	decrease inconsistent	0.5–10	O2 medium continuous	Bickel and Lyck, 2001
Microcystis field population	dry weight	increase	data not presented	n/a (field sample)	Amé et al., 2003
Microcystis field population	cell count protein content	decrease inconsistent	1–10	Z8 medium semi-continuous	Amé and Wunderlin, 2005
M. aeruginosa PCC 7806	protein content	increase	0.18–30	BG11 medium batch cultures	Martin-Luna et al., 2006
M. aeruginosa Lake Dianchi	culture medium volume	decrease	4–16	BG11 medium batch cultures	Jiang et al., 2008
M. aeruginosa PCC 7806	protein content	increase	0–30	BG11 medium batch cultures	Sevilla et al., 2008
M. novacekii UAM 250	dry weight culture medium volume	decrease decrease	0–5	BG11 medium batch cultures	Li et al., 2009
M. aeruginosa PCC 7806	cell count	increase	0.01–1	Fraquil medium batch cultures	Alexova et al., 2011

* Microcystin production was reported normalised to various parameters to observe whether cyanotoxin production increases under different iron concentrations. The strain or culture used also varied between studies.

† Cultures with 0 µM Fe added may contain trace amounts of Fe impurities from other components in the culture medium.

Whether an increase or decrease in cyanotoxin production is observed with increasing iron concentration is dependent on many interacting factors. These factors include the species or strain selected for investigation, as well as growth conditions and the parameters chosen to normalise cyanotoxin concentration (Bickel and Lyck, 2001; Kaebernick and Neilan, 2001). Growth parameters include culture medium, light, temperature, and whether cultures are grown under batch or continuous conditions (Sivonen and Jones, 1999). Analysis parameters commonly chosen to normalise the measured cyanotoxin concentration include cyanotoxin concentration per cell, chlorophyll-a content, dry weight, and mass of protein (Table 4.1; Kaebernick and Neilan, 2001).

Chuang et al. (2001) analysed cylindrospermopsin production and growth in *Cylindrospermopsis raciborskii* under different iron and manganese concentrations. The cylindrospermopsin production rate constant was linearly correlated to the cell division rate constant (Chuang et al., 2001). Iron limitation reduced the growth rate, which had an indirect effect on cylindrospermopsin production. Significant changes in growth or cyanotoxin production were not observed under different manganese concentrations (Chuang et al., 2001). Similarly manganese had no effect on growth or microcystin production in *Microcystis aeruginosa* (Lukač and Aegerter, 1993).

Copper and Zinc

Few microcystin production studies have investigated copper and zinc. Copper and zinc bind *in vitro* to microcystin with intermediate strength formation constants at pH 7.5 (Humble et al., 1997). Gouvêa et al. (2008) investigated the effect of copper and zinc and UV irradiance on microcystin production. UV decreased microcystin production the most, an increase in copper caused a smaller decrease, and zinc had little effect (Gouvêa et al., 2008). In contrast, Lukač and Aegerter (1993) had earlier reported that microcystin yield increased under zinc limitation.

4.1.4 Experiment Overview and Selection Parameters

Environmental factors affecting anatoxin-a production are largely unknown. Osswald et al. (2007) suggested that anatoxin-a production depends on individual strains and is strongly influenced by light, temperature and nutrient concentration. The effect of metal stressors on the production of anatoxin-a has not been investigated. In this study the effect of copper and iron on anatoxin-a production by a common New Zealand benthic cyanobacterium,

Phormidium autumnale, was investigated. Cell counts were chosen to normalise anatoxin-a production instead of normalising cyanotoxin production to mass or other metabolites. Metabolites, including chlorophyll-a, carbohydrates, and proteins, may vary considerably with growth phase and not reflect the growth cycle.

Iron

High iron concentrations were selected to be comparable with the concentrations of iron reported in Canterbury streams (200–800 ppb; Harding and Winterbourn, 1995). These values are also similar to the mean iron concentration in rivers throughout the world (Livingstone, 1963). It was anticipated that iron limitation would be detrimental to iron growth. A lower iron concentration of 40 ppb was selected. This low concentration was within the same order of magnitude of several iron limitation stressor experiments conducted on microcystin production by *Microcystis* (Amé and Wunderlin, 2005; Bickel and Lyck, 2001; Jiang et al., 2008; Utkilen and Gjølme, 1995).

Copper

Copper is often applied at 10–1000 ppb Cu to problematic algal blooms as an algicide (McKnight et al., 1983). Although it was anticipated that an increase in copper concentration would be detrimental to cyanobacterial growth, the effects of this trace metal on anatoxin-a production are largely unknown. A non-lethal dose of copper in waterways may alter cyanotoxin production. In this study copper concentrations were selected to be non-lethal to cyanobacteria. These doses allowed cells to be enumerated and anatoxin-a production to be compared under different metal treatments. The upper copper concentration (250 ppb) was based on the order of magnitude of copper observed to approximately halve the growth of *Anabaena* (Surosz and Palinska, 2004) and *Oscillatoria* (Lembi, 2000). Copper stress responses have also been observed in *Microcystis* strains after only 24 hours exposure to 250 ppb copper (Wu et al., 2007). This high copper exposure is unlikely to occur in most freshwater sources. In New Zealand, concentrations of 0.04–1.74 ppb dissolved copper have been recorded in uncontaminated South Island Rivers (Kim and Hunter, 2001). Worldwide surface freshwater copper concentrations typically range between 0.2 and 30 ppb with a median value of 3 ppb (Bowen, 1979). The lower copper concentration used in this experiment was the standard concentration (2.5 ppb) in MLA culture medium (Bolch and Blackburn, 1996). This concentration is also

the New Zealand trigger value for copper when protecting 80% of freshwater species (ANZECC and ARMCANZ, 2000).

4.1.5 Aims of this Experiment

Anatoxin-a production was monitored throughout the growth cycle of *Phormidium autumnale* under various iron and copper regimes. This part of the study had the following aims and objectives:

- To develop a method for monitoring anatoxin-a production across the growth curve of a benthic cyanobacterium.
- To determine the effect of copper and iron on the growth of *P. autumnale* strain CYN52.
- To assess the correlation of anatoxin-a production with growth of strain CYN52.
- To examine variation in the anatoxin-a quota in relation to metal stress of strain CYN52.

4.2 Methods

4.2.1 Culturing and Experiment Design

Experimental set up

New Zealand *Phormidium autumnale* strain CYN52 was obtained from the Cawthron Institute Culture Collection of Micro-algae (CICCM, Nelson New Zealand; http://cultures.cawthron.org.nz). Strain CYN52, isolated from the Rangataki River, produces anatoxin-a but not homoanatoxin-a (Heath et al., 2010). This strain was grown in batch culture in 500 mL MLA culture medium (Bolch and Blackburn, 1996) to obtain sufficient wet weight inoculum for the experiment.

The experimental design was modified from Esson et al. (2011) who inoculated 15 culture containers with wet weight *Phormidium* and harvested in triplicate at 5 time-points. In the current experiment, MLA and five variations of this culture medium were prepared in 5 L acid-washed plastic carboys by modifying the $FeCl_3$ or $CuSO_4$ concentrations (Table 4.2). Aliquots (30 mL) of culture media were pipetted into pre-numbered, gamma-sterilised polystyrene culturing containers (70 mL, Labserv). Each container was inoculated with 7 mg (± 0.5 mg) wet weight CYN52. MLA control samples, consisting of culture media (30 mL) containing no inoculum, were incubated and harvested together with the culture samples.

Table 4.2 Nominal iron and copper concentrations series for modified MLA culture media used for each treatment in this experiment.

Treatment	Fe [ppb]	Cu [ppb]
$MLA_{1\times Fe=1\times Cu}$ *	400	2.5
$MLA_{0.1\times Fe}$	40	2.5
$MLA_{2\times Fe}$	800	2.5
$MLA_{10\times Fe}$	4000	2.5
$MLA_{10\times Cu}$	400	25
$MLA_{100\times Cu}$	400	250

* $MLA_{1\times Fe=1\times Cu}$ represents standard MLA medium without modifications.

Cultures and controls were incubated (Figure 4.1a) at 18 °C (± 1 °C), under 36 µEin m^{-2} s^{-1} of light (12 h light-dark cycle). To minimise variation in growth due to different light intensities to each culture container, these positions were randomised at each harvest date

according to a random number generator (www.random.org/sequences). The intensity of light available to each culture position is dependent on the number of cultures surrounding each position. Gaps are created in the culture position matrix when a sample is harvested, increasing the amount of light reaching neighbouring cultures. To avoid an increasing in light intensity from this situation, the culture position gaps were filled with culture container blanks containing 30 mL of water (Figure 4.1b and c).

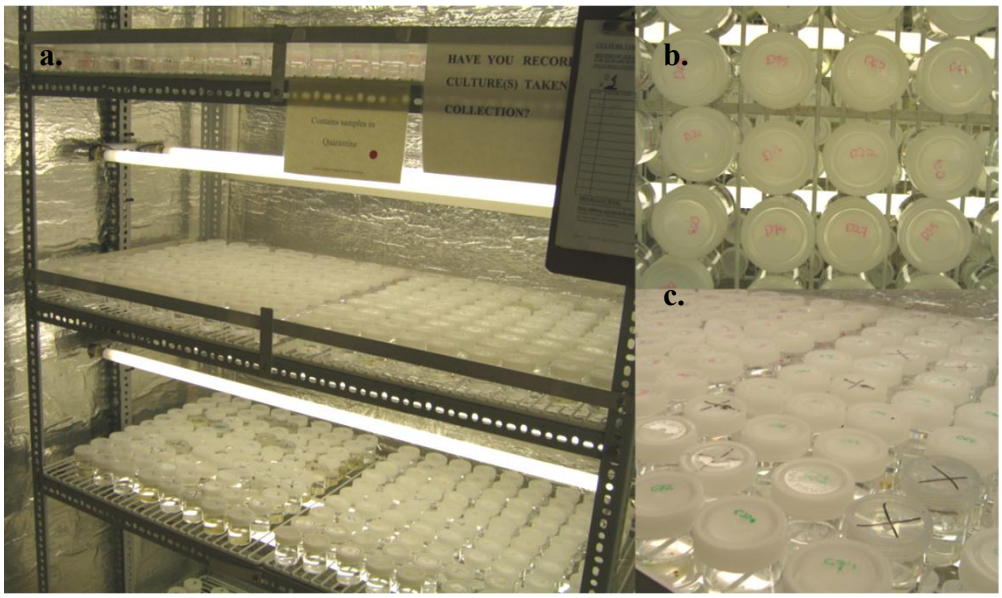

Figure 4.1 Incubation of cultures for the iron and copper stress experiment a) Inoculated cultures for each treatment arranged into randomised positions; b) Numbered culture containers for $MLA_{10\times Cu}$; c) Standard $MLA_{1\times Fe=1\times Cu}$ with blank containers, marked with a cross, to replace the harvested containers.

Harvest and sampling

Two sets of triplicate culture samples from each treatment were harvested for growth and cyanotoxin analysis at ten sampling points. Duplicate MLA control samples were harvested at five sampling points for each treatment. These control samples were used to test for abiotic related change in the medium composition over time. It was not feasible to sample for anatoxin-a and growth on every day of the seven week experiment. An additional two sampling points were included for the standard MLA treatment to gain extra data in the early stages of the growth profile (Table 4.3). The experiment ran for 49 days.

Table 4.3 Harvest regime for the iron and copper stressor experiment.*

Harvest day	MLA control samples	Culture samples†	Additional culture samples†
Day 0	✓	✓	
Day 3		✓	
Day 6	✓	✓	
Day 9		✓	
Day 10			✓
Day 12		✓	
Day 13			✓
Day 17	✓	✓	
Day 22		✓	
Day 32	✓	✓	
Day 41		✓	
Day 49	✓	✓	

* MLA control samples contain only culture medium of each treatment. Culture samples were inoculated with CYN52 for each treatment. Additional culture samples were inoculated for the standard MLA treatment to collect extra data for the anatoxin-a profile at the early stages of the growth profile.

† Each set of culture samples consists of three cultures for cyanotoxin and three cultures for growth analyses for each treatment.

The sampling process at each harvest day is presented in Figure 4.2. MLA was removed from one set of culture triplicates for extracellular anatoxin-a and culture media analysis as described in Section 4.2.2. The cyanobacterial mat remained in the culturing container and was processed for anatoxin-a analysis (Section 4.2.4). The remaining triplicate samples were processed for growth analysis as described in Section 4.2.3.

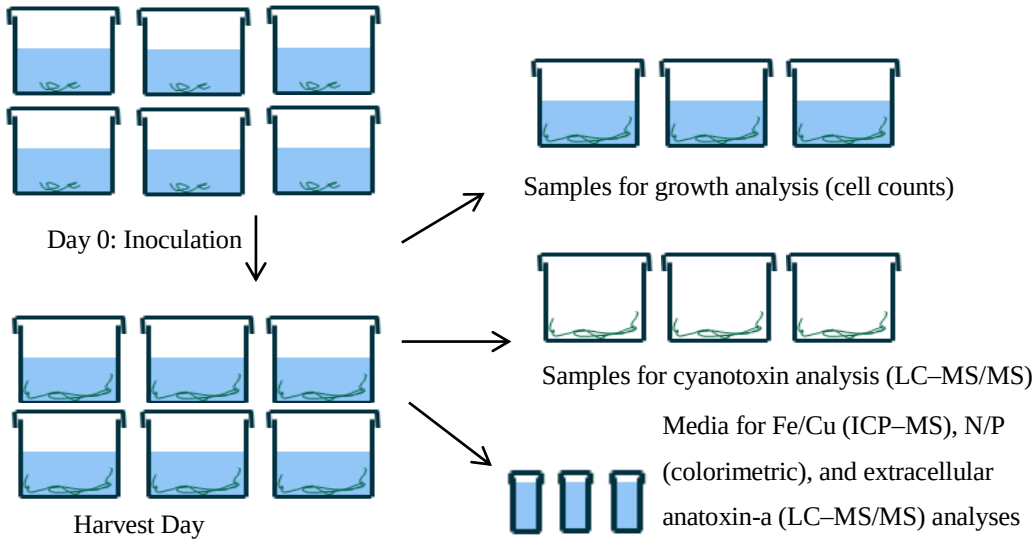

Figure 4.2 Analysis of harvested samples for each iron and copper treatment. Inoculated samples were incubated until harvest date and replicates were collected for cell concentrations, anatoxin-a concentrations and culture media analysis.

4.2.2 Analysis of Growth Medium

Subsamples of medium (10 mL), from each cyanotoxin analysis and MLA control sample, were collected for metal analysis. These samples were stored in acid-washed polypropylene test-tubes and acidified with HNO_3 (8 M, 500 µL). The acidified subsamples from $MLA_{2\times Fe}$ were diluted 4-fold with 2% HNO_3, prior to analysis. Samples were measured by Agilent 7500 cx. ICP–MS at the University of Canterbury. Duplicate and spiked samples were analysed every 10^{th} and 20^{th} ICP–MS samples respectively. Samples were spiked with 20 ppb iron or copper. A blank sample and standards ranging from 0.1–1000 ppb were used to calibrate the instrument at the start of every run. Standards were analysed every 20 samples. The mean percentage difference (n=26) in duplicates was 6.5% for Cu and 3.6% for Fe. Mean spike recoveries (n=4) were 89.5% and 82.4% for Cu and Fe respectively.

The pH of the remaining growth medium samples were measured (SevenEasy meter, Mettler Toledo) at each sampling point. Additional growth medium subsamples were taken for extracellular anatoxin-a (1 mL) and nutrient (10 mL) analyses. The nutrient and extracellular subsamples were filtered (GF/C, Whatman) and stored frozen (–20 °C) until analysis. Extracellular anatoxin-a samples were analysed as described in Section 4.2.4.

The nutrient subsamples were analysed for total nitrogen from nitrate and nitrite (NO_3-N+NO_2-N) and total phosphorus from phosphate (PO_4-P) using an automated analyser EasyChem Plus (Systea). This analyser uses the US EPA colorimetric methods for determination of nitrogen and phosphorus (US EPA Methods 353.1, 353.2, and 365.1). Phosphate and nitrate samples from the culture samples and growth medium control samples were diluted 100-fold prior to analysis. Working stock solutions of 10 ppm PO_4-P, 10 ppm NO_3-N, and 1 ppm NO_2-N were prepared daily for calibrating the instrument. Samples were run in duplicate with a Milli-Q blank between every sample. The mean of duplicate samples was reported. Two quality control samples were included in every batch. The mean percentage difference (n=75) between sample duplicates were 9.3% and 1.4% for combined nitrogen, from nitrate and nitrite sources, and phosphorus respectively.

4.2.3 Growth Analysis

Samples for cell counts were preserved in Lugol's iodine and stored in the dark until analysis. The samples were homogenised (2–5 min) by Ultra-Turrax (IKA Laborteknik, Germany). Homogenisation disintegrated the mucilage binding the mat and fragmented trichomes into a more uniform size. The Ultra-Turrax probe was rinsed twice with Milli-Q water (5 mL), and the rinsates added to the sample. A subsample, 2–5 mL depending on the concentration of cells, was transferred to an Utermöhl chamber (Utermöhl, 1958), and left to settle in the dark (2+ hours). If the concentration of cells was too dense to count, the sample was diluted between 2–14-fold and a subsample transferred to an Utermöhl chamber as described above.

All trichomes in the Utermöhl chamber were measured along one transect at 400× magnification on an inverted microscope (Olympus CK40 or ITM). If the number of trichomes counted was less than 60, two or more transects were counted so that ca. 100 trichomes were measured for each sample. Cell lengths (n=50+) were measured at 800–1000× magnification using an Olympus light microscope (BX51). These measurements were taken from the original homogenised cultures on Day 0, 3, 9, 17, 32, and 49. The mean cell length for each treatment medium was calculated and used to determine the number of cells per trichome measured in the Utermöhl chamber. From this calculation, the cell concentration in the original 30 mL culture was determined.

4.2.4 Anatoxin-a Analysis

Intracellular sample preparation

The cyanobacteria biomass from the cyanotoxin samples remaining after MLA removal was lyophilised, weighed, and stored frozen until analysis. Aliquots of acidified Milli-Q water (5 mL, 0.1% formic acid) were added to each lyophilised sample. Trichomes attached to the culture container wall were transferred into this volume and the sample was sonicated for 30 min. This sample was centrifuged (3000 × *g*, 5 min) and 1 mL of eluent was removed for intracellular toxin analysis.

Intra- and extracellular anatoxin-a analysis

The filtered 1 mL culture medium subsample (from Section 4.2.2) was transferred to an HPLC vial for extracellular toxin analysis. Intra- and extracellular samples were analysed at the Cawthron Institute for anatoxin-a, homoanatoxin-a, and their degradation products dihydroanatoxin-a and dihydrohomoanatoxin-a using the LC–MS/MS method described in Section 3.2.1. Sample blanks of MLA from control media samples were analysed in each batch as negative controls.

4.2.5 Statistical Analysis

Statistical analyses were carried out to test the null hypotheses for the three copper or three iron treatments:

H_{0A}: Exposure to different concentrations of metal stress has no effect on growth.

H_{0B}: Anatoxin-a production is proportional to growth.

H_{0C}: Exposure to different concentrations of metal stress has no effect on anatoxin-a production.

All statistical analyses were carried out using R version 2.13.1 (www.r-project.org).

Data transformation

Experimental design was balanced as all treatments consisted of three replicates. Anatoxin-a measurements were not correlated with one particular cell concentration because the three anatoxin-a replicates were different samples from the three cell concentration replicates. Therefore, anatoxin-a quota was calculated from the mean cell concentration for the corresponding treatment and day of each anatoxin-a data point. Raw data from growth and anatoxin-a production were transformed logarithmically prior to analysis to ensure residuals were normally distributed. Assumptions checks for normally distributed error were tested by plotting normal probability. Homoscedasticity and misspecification were assessed by plotting residuals against fitted values.

Analysis of Variance

Two-way analysis of variance (ANOVA) was used to determine significant relationships between metal concentration and either growth or anatoxin-a production over the course of the experiment. In these models both factors (days and metal concentration) are treated as categorical variables. *P*-values <0.05 were considered significant. To determine where significant differences occurred, 95% confidence intervals were calculated on the mean values for each treatment and day. These data were back-transformed and plotted to identify where data points were significantly different from one another. The 95% confidence intervals were plotted on the mean data point for each treatment and day. Significant differences between data points occur when the 95% confidence intervals do not overlap.

H_{0A}: exposure to different concentrations of metal stress has no effect on growth

Two-way ANOVA was carried out for growth data as described above. In order to describe the overall model for growth for all three treatment levels, a linear model was fitted to logarithmic and logistically transformed data. Akaike Information Criteria (AIC, Akaike, 1974) was used to compare statistical models for the same datasets. AIC evaluates models based on the log-likelihood of the data and how well the model parameters fit the data. The model providing the lowest AIC score best fits the dataset.

H_{0B}: anatoxin-a production is proportional to growth

If anatoxin-a production was proportionate to growth then anatoxin-a quota (anatoxin-a $cell^{-1}$) would be constant across all days. Under this hypothesis days would not be significant factor in describing a statistical model for anatoxin-a production using the two-way ANOVA method described above. To further test this hypothesis, linear models of the intercept were created using mean anatoxin-a production data and these models were compared using AIC.

H_{0C}: exposure to different concentrations of metal stress has no effect on anatoxin-a production.

Significant differences were observed by plotting the 95% confidence intervals as described above.

4.3 Results

4.3.1 Media Analysis

Iron concentrations decreased rapidly in cultures whereas concentrations remained fairly constant in controls, which contained no *Phormidium* (Figure 4.3). The largest decrease in iron was observed under nominal 800 ppb Fe conditions ($MLA_{2\times Fe;}$ Figure 4.3e). At this Fe concentration, the control and culture were initially measured at 876 ± 119 and 617 ± 276 ppb Fe, respectively. The $MLA_{2\times Fe}$ culture decreased to 212 ± 105 ppb Fe on Day 3. Copper concentrations of both cultures and controls remained constant throughout the experiment (Figure 4.4).

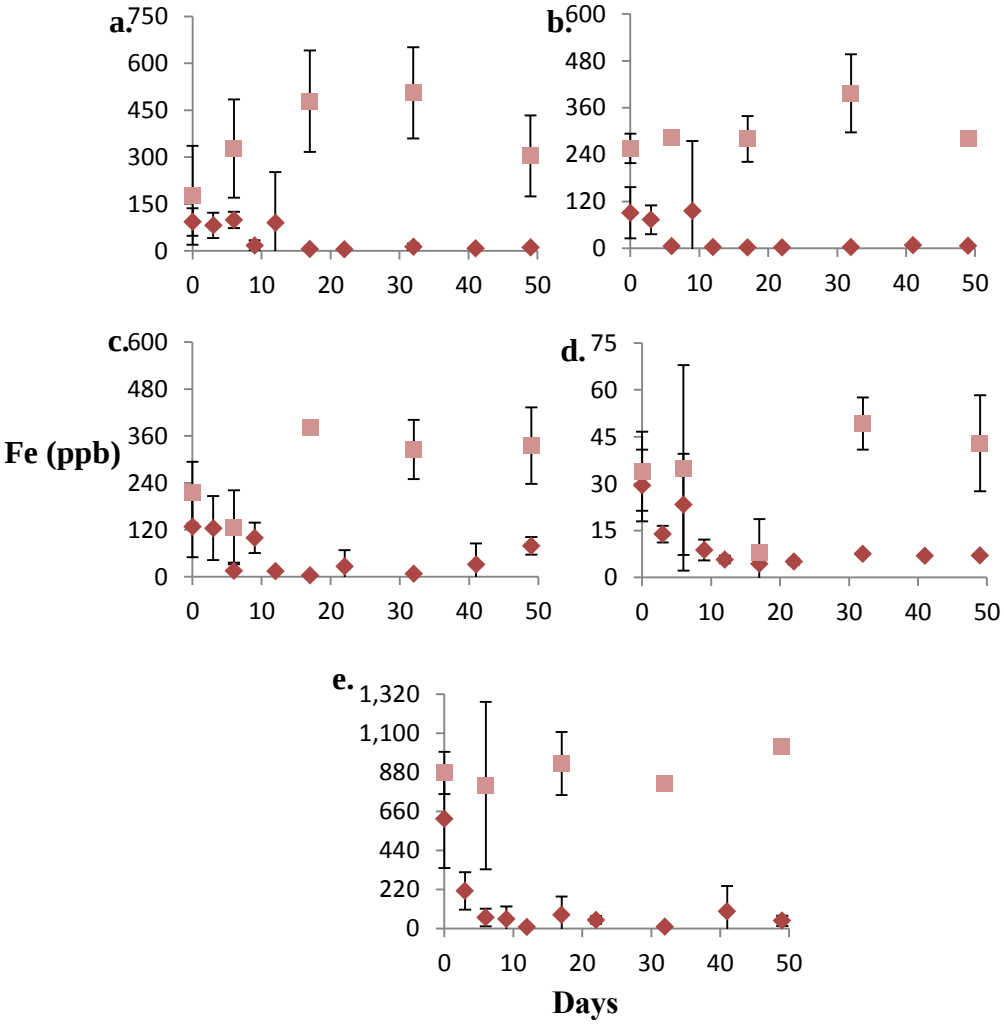

Figure 4.3 MLA iron concentration measured in growth experiments for strain CYN52. Iron concentrations contributed by the original media a) $MLA_{1\times Fe=1\times Cu}$, 400 ppb Fe, b) $MLA_{10\times Cu}$, 400 ppb Fe, c) $MLA_{100\times Cu}$, 400 ppb Fe, d) $MLA_{0.1\times Fe}$, 40 ppb Fe, and e) $MLA_{2\times Fe}$, 800 ppb Fe. ■ control (n=2+) and ◆ culture (n=3); error bars represent 95% confidence intervals.

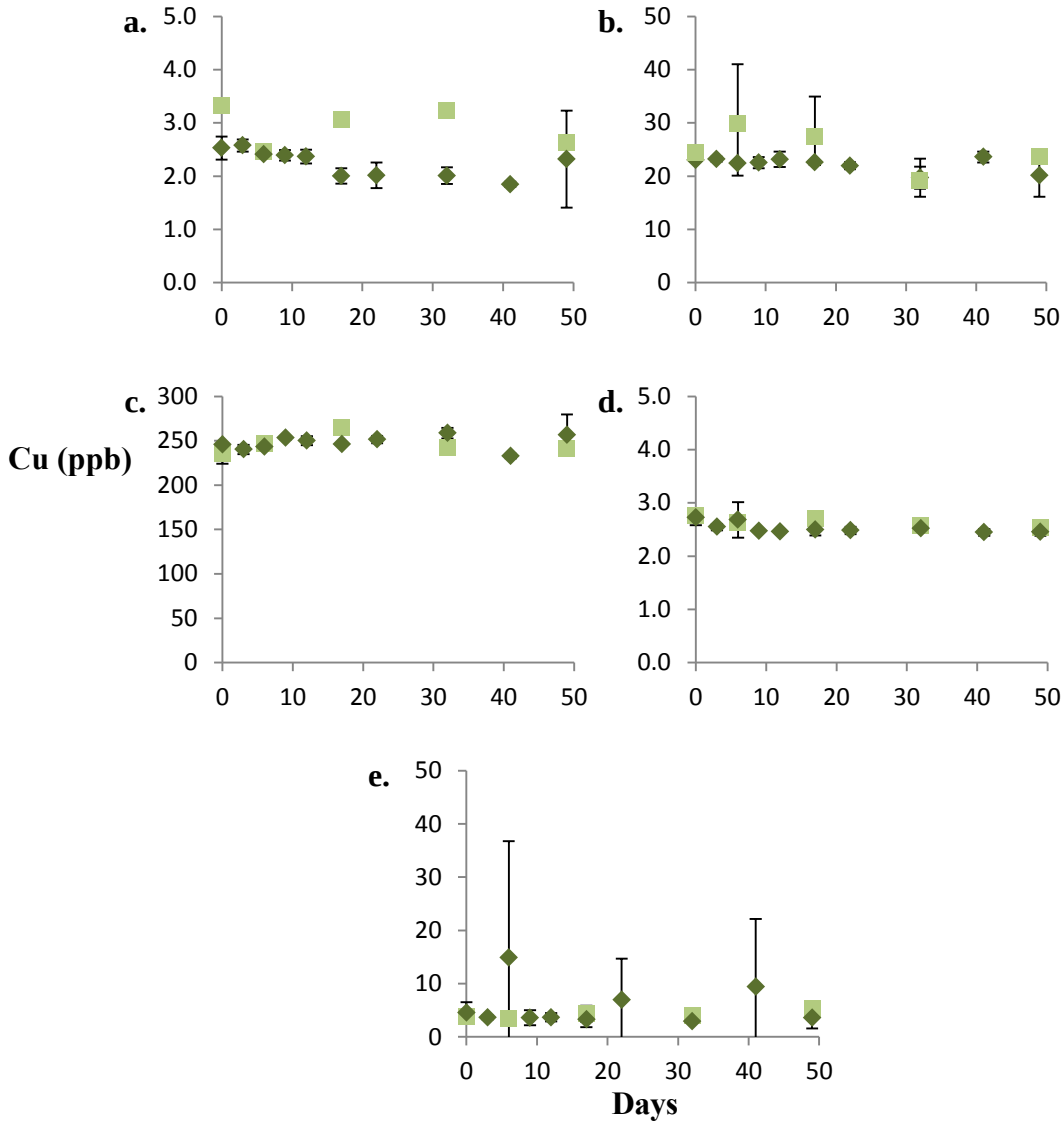

Figure 4.4 MLA copper concentration measured in growth experiments for strain CYN52. Copper concentrations contributed by the original media a) $MLA_{1\times Fe=1\times Cu}$, 2.5 ppb Cu, b) $MLA_{10\times Cu}$, 25 ppb Cu, c) $MLA_{100\times Cu}$, 250 ppb Cu, d) $MLA_{0.1\times Fe}$, 2.5 ppb Cu, and e) $MLA_{2\times Fe}$ 2.5 ppb Cu. ■ control (n=2+) and ◆ culture (n=3); error bars represent 95% confidence intervals.

The culture media pH values ranged between 8 and 9.8 (mean = 8.6). The pH of the culture media generally increased over time. The control media pH, with a mean pH of 8.2 over all treatments, was generally lower than culture media pH.

Nutrient concentrations remained relatively constant throughout the experiment in both control and culture medium (Figure 4.5, Appendix 8.3). The expected N values calculated for MLA culture medium was 28 ppm. This value is consistent with the observed NO_3-N +NO_2-N in MLA control samples (mean 28.5 ± 1.8 ppm). The expected PO_4-P concentration calculated for MLA was 5 ppm. There was an excess of P in this experiment due to an error preparing the stock solutions. This error resulted in a 14-fold increase in P with a mean of 70.5 ± 0.6 ppm detected in medium control samples. The sample means across all culture medium for PO_4-P and NO_3-N +NO_2-N were 69.8 ± 0.5 ppm and 24.6 ± 1.0 ppm, respectively.

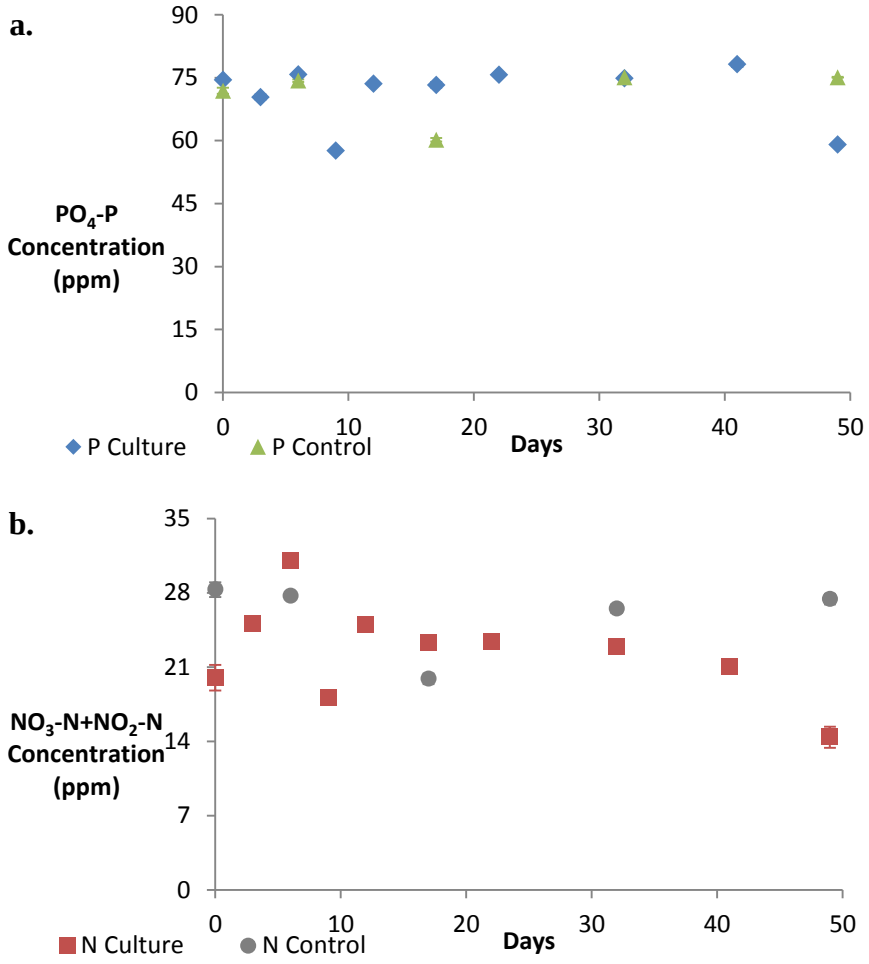

Figure 4.5 Nutrient concentrations in standard MLA. Control concentrations were recorded for MLA without any culture added. a) PO_4-P and b) nitrate and nitrite calculated as N (NO_3-N + NO_2-N). Error bars are standard error of the mean (n=2); all results are presented in Appendix 8.4.

4.3.2 Growth Analysis

The growth profile (Figure 4.6) of all cultures followed the typical stages of a lag phase, exponential phase, and stationary phase. During the stationary phase, trichomes usually started to pull away from the walls of the culturing container. This trend was not observed under the lowest and highest iron concentrations tested, $MLA_{0.1\times Fe}$ and $MLA_{10\times Fe}$ respectively, where these cultures struggled to survive. At high iron concentrations $MLA_{2\times Fe}$ and $MLA_{10\times Fe}$ trichomes did not firmly attach to the culturing container.

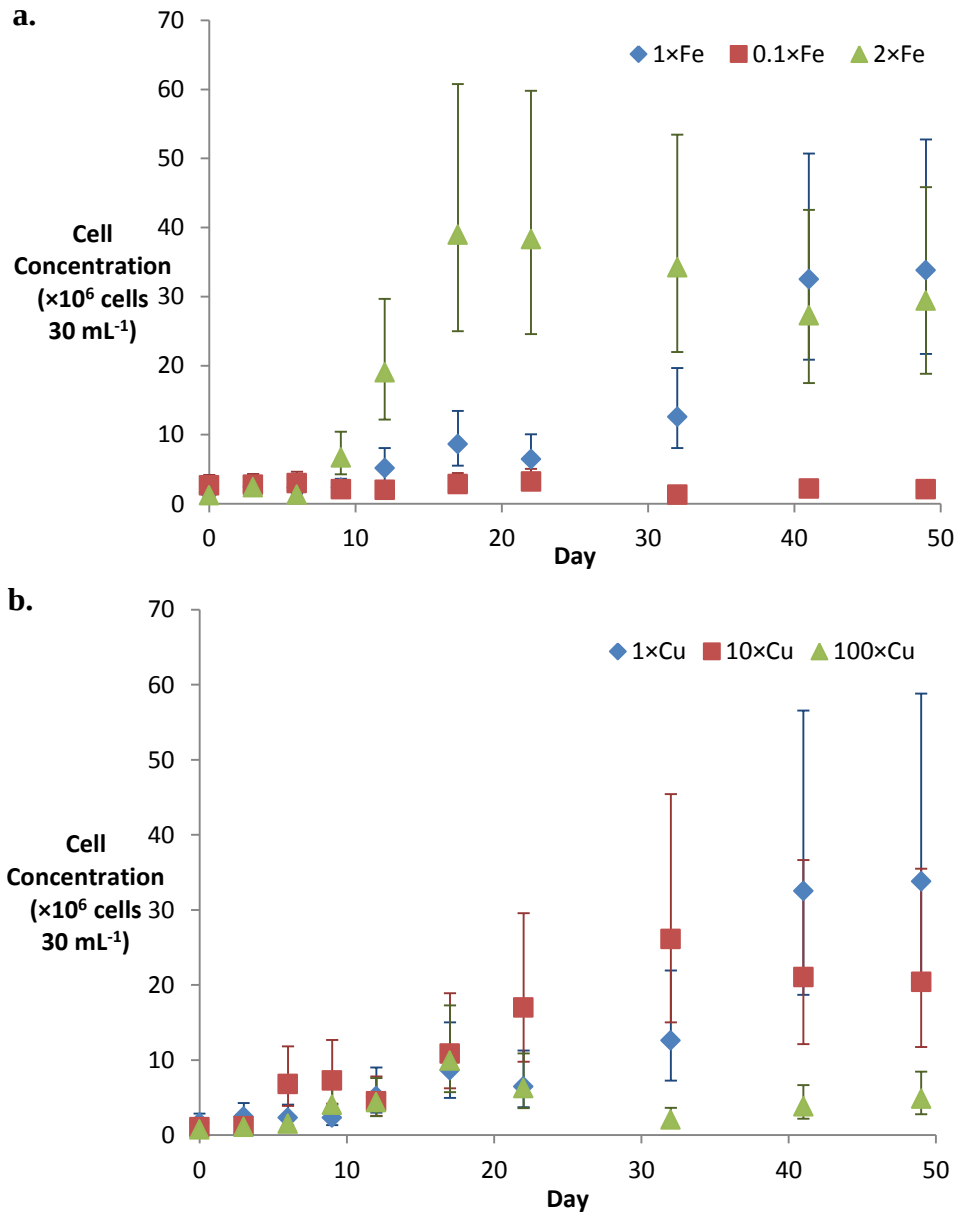

Figure 4.6 Growth profile for *Phormidium autumnale* (CYN52) under the three different iron and copper regimes over 49 days. Growth is recorded as concentration of cells per 30 mL culture. a) iron treatments; b) copper treatments. Error bars are 95% confidence intervals (n=3).

The hypothesis H_{0A}, that exposure to different concentrations of metal stress has no effect on growth, was tested. The factors metal treatment, day, and the interaction term (to test non-additive effects) were tested using two-way ANOVA (Table 4.4 and Table 4.5). For both copper and iron, the metal concentration and the day were significant (p <0.05) in describing the effect on growth.

Table 4.4 Analysis of variance for the effect of iron and days on growth. Days and iron concentration are treated as categorical factors.

	df †	***P*-value**
Fe	2	<0.0001*
Day	9	<0.0001*
Fe×Day	18	<0.0001*
Residuals	60	

* significant *p*-values, p <0.05 were considered significant; †degrees of freedom.

Table 4.5 Analysis of variance for the effect of copper and days on growth. Days and copper concentration are treated as categorical factors.

	df †	***P*-value**
Cu	2	<0.0001*
Day	9	<0.0001*
Cu×Day	18	<0.0001*
Residuals	60	

* significant *p*-values, p <0.05 were considered significant; †degrees of freedom.

Iron

In strain CYN52, the onset of exponential growth occurred earlier as iron concentrations increased from 40–800 ppb Fe ($MLA_{0.1\times Fe}$ to $MLA_{2\times Fe}$, Figure 4.6a). In $MLA_{0.1\times Fe}$, the cultures spread across the available substratum but did not increase significantly in cell numbers throughout the experiment. Cell concentrations in $MLA_{0.1\times Fe}$ after Day 30 were significantly lower than $MLA_{1\times Fe}$ or $MLA_{2\times Fe}$. In contrast, $MLA_{2\times Fe}$ grew more rapidly than the other cultures and had a significantly higher yield on Days 12, 17, and 22, than cultures in $MLA_{0.1\times Fe}$ or $MLA_{1\times Fe}$ media. The ability for mats to adhere to the culturing container walls was disrupted in $MLA_{2\times Fe}$. Strain CYN52 in $MLA_{2\times Fe}$ was able to increase in cell numbers, resulting in the steepest exponential phase and highest cell numbers, without firm

attachment to the culturing container throughout the experiment. Strain CYN52 in $MLA_{10\times Fe}$ also did not attach firmly to the culturing container walls. The majority of cultures in $MLA_{10\times Fe}$ did not survive past 14 days at which point the experiment was terminated for this treatment. Data from $MLA_{10\times Fe}$ was excluded from subsequent growth and anatoxin-a production analyses.

Copper

An increase in copper concentration, from 2.5 to 250 ppb, resulted in significantly lower cell concentrations at the end of the trial (Figure 4.6b). The growth profile of $MLA_{100\times Cu}$ was significantly different to $MLA_{1\times Cu}$. There was no significant difference in the overall growth trend of $MLA_{1\times Cu}$ and $MLA_{10\times Cu}$. Cell concentrations in $MLA_{100\times Cu}$ after Day 30 were significantly lower than $MLA_{1\times Cu}$ or $MLA_{10\times Cu}$. The categorical model was a better fit than the linear model for growth.

Growth models

Ideally a linear model would have been fitted to the growth data to describe the growth rate and overall trend. Linear models can compare the total effects of each treatment on growth. Linear models, explaining the overall trend of the data over time, were compared to a categorical model where days were treated as a factor. Data at high copper or low iron concentrations consisted of very linear growth over all data points. The other lower copper and higher iron data series tend to resemble a sigmoidal curve with an exponential growth phase plateauing at a stationary phase. Attempts using linear models based on logarithmic and sigmoidal curve did not fit the data very well as indicated by the Akaike Information Criteria (AIC, Table 4.6). The models that used day as categorical data rather than continuous data, gave the best fit.

Table 4.6 Comparison of different growth models using Akaike Information Criteria to evaluate the goodness of fit.*

Growth Model Test for Each Treatment	AIC
Iron Treatment	
Categorical model	-41.1
Logarithmic linear model	37.9
Sigmoidal linear model	51.5
Copper Treatment	
Categorical model	-1.6
Logarithmic linear model	48.2
Sigmoidal linear model	122.7

* For each dataset, a linear model (explaining the overall trend of the data over time) was compared to a categorical model (where days were treated as a factor) and intercept model (where time was not a variable).

4.3.3 Anatoxin-a Analysis

Extracellular anatoxin-a

Anatoxin-a was not detected in any of the culture media blanks. Every sampling point of treatment $MLA_{1\times Fe=1\times Cu}$ was analysed for extracellular anatoxin-a. Extracellular toxins were initially detected on Day 3 and were also identified in the media from Day 9 until the end of the experiment (Figure 4.7). The highest extracellular toxin concentration detected was 2.5 ± 1.9 ng mL^{-1} recorded on Day 17.

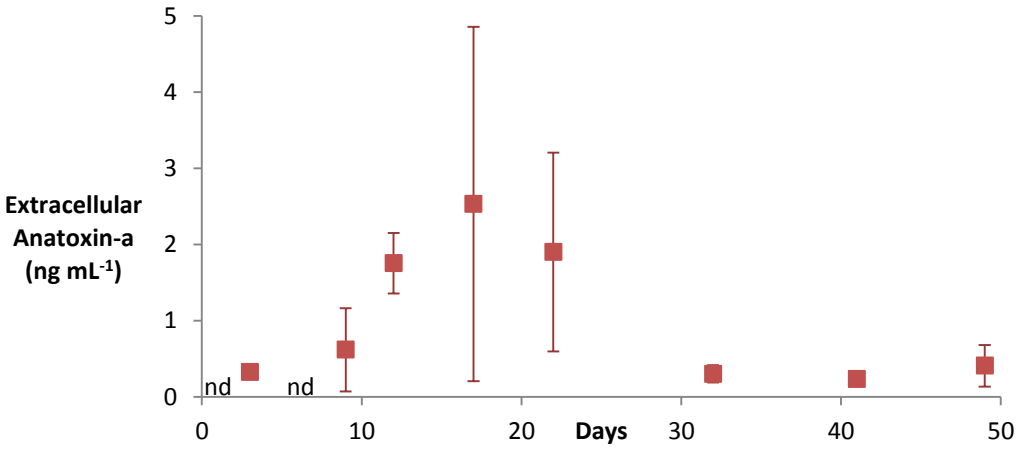

Figure 4.7 Extracellular anatoxin-a detected in cultures of *Phormidium autumnale* (CYN52) under standard $MLA_{1\times Fe=1\times Cu}$. Error bars represent 95% confidence intervals (n=3); nd, not detected.

In standard MLA, only very low concentrations of extracellular anatoxin-a were detected. Therefore, the modified MLA treatments were only analysed in the mid-exponential and stationary phase on Days 17, 32, and 49 (Figure 4.8). Treatment $MLA_{100\times Cu}$ had a significantly higher percentage of extracellular anatoxin-a on Day 32 and 49 (10.2 and 22.7% respectively). Extracellular toxins were not detected for $MLA_{0.1\times Fe}$ on Day 32.

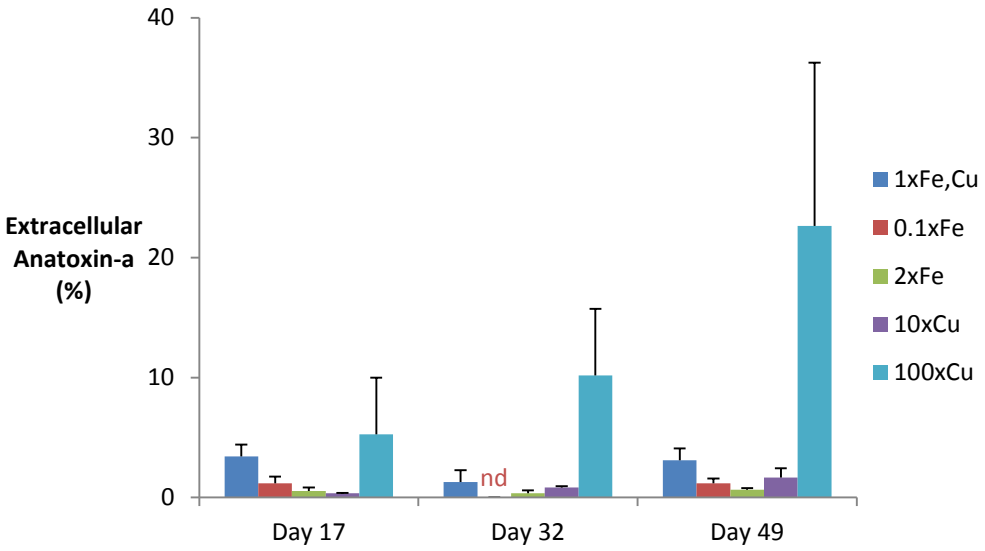

Figure 4.8 Extracellular anatoxin-a detected for each treatment during the late exponential and stationary phase. Data is recorded as percentage of extracellular anatoxin-a from total intra- and extracellular anatoxin-a. Error bars represent 95% confidence intervals; nd, not detected.

Intracellular anatoxin-a

Anatoxin-a (<0.01–0.55 pg $cell^{-1}$) was detected in all samples (Figure 4.9a and Figure 4.10a). Homoanatoxin-a was not detected. The degradation product dihydroanatoxin-a was detected in low concentrations (1–151 fg $cell^{-1}$) in cultures on some days (Figure 4.9b and Figure 4.10b). No degradation products were detected for some treatments and days: $MLA_{1\times Fe=1\times Cu}$ on Days 0, 6, and 9; $MLA_{100\times Cu}$ on Day 42, or for $MLA_{0.1\times Fe}$ on Days 0, 3, and 6.

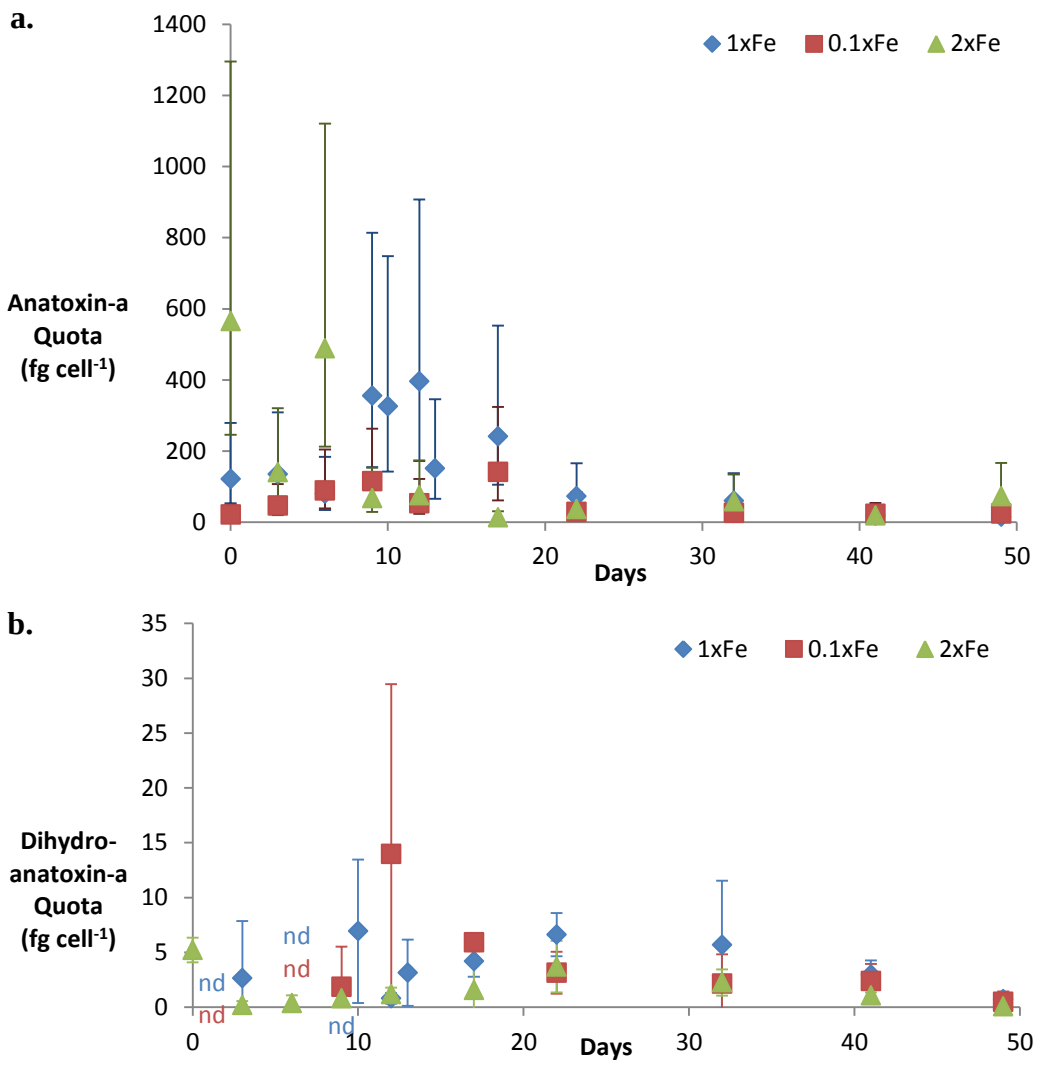

Figure 4.9 Intracellular anatoxin-a and degradation product dihydroanatoxin-a in *Phormidium autumnale* (CYN52), for the three different iron regimes. Anatoxin-a and dihydroanatoxin-a quota reported in fg cell^{-1}. Error bars represent the 95% confidence intervals of the mean (n=3); nd, not detected.

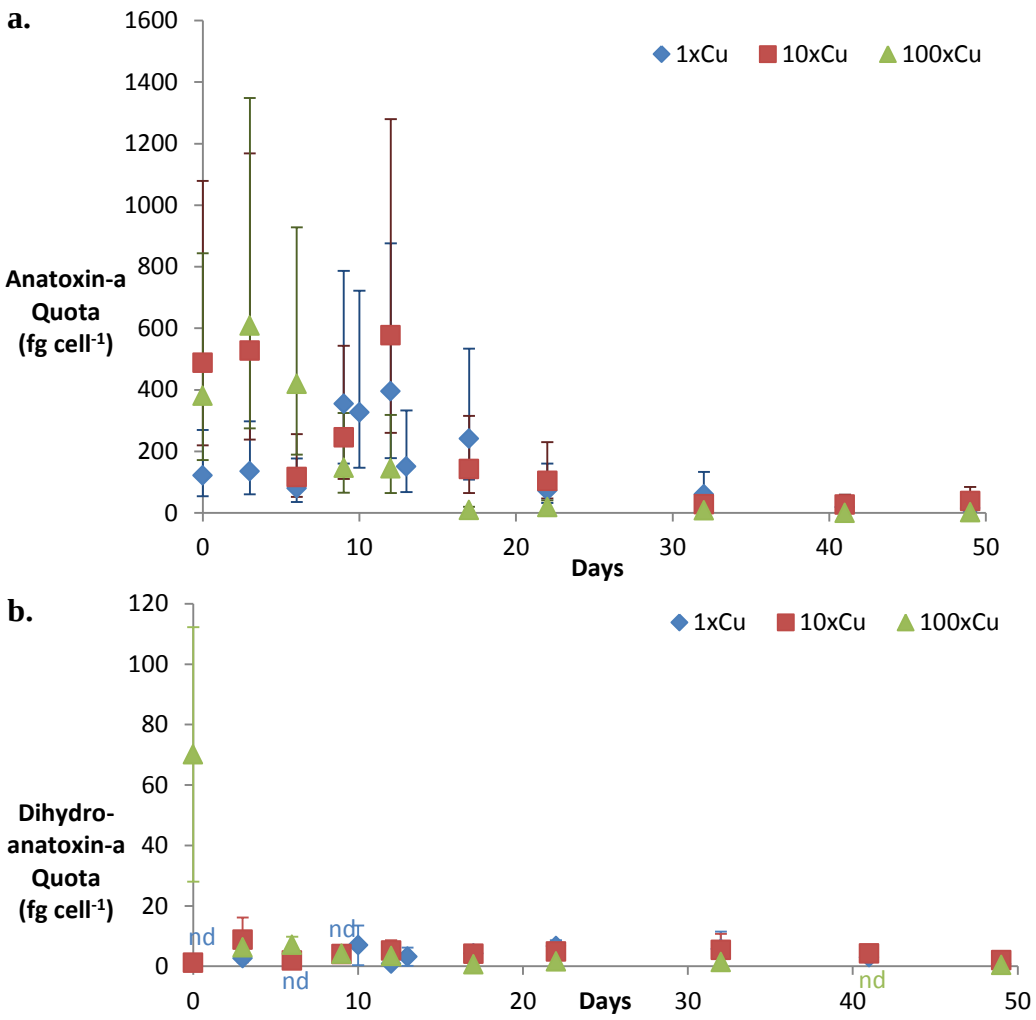

Figure 4.10 Intracellular anatoxin-a and degradation product dihydroanatoxin-a in *Phormidium autumnale* (CYN52), for the three different copper regimes. Anatoxin-a and dihydroanatoxin-a quota reported in fg cell^{-1}. Error bars represent the 95% confidence intervals of the mean (n=3); nd, not detected.

Three types of models were compared to describe anatoxin-a quota (anatoxin-a per mean cell concentration) in relation to growth. Linear models were used to explain the overall trend of the data over time. Categorical models treated days as a factor and allowed comparison between data points. Intercept models, where time is not a significant parameter, assumed constant anatoxin-a quota throughout the growth curve. Categorical models provided the best fit (Table 4.7). The AIC data suggests the intercept model does not fit the data as well as the categorical model and therefore anatoxin-a quota is not proportional to growth. This suggestion is supported by being significant (p <0.05) under

the two-way ANOVA model (Table 4.8 and Table 4.9). Therefore the null hypothesis H_{0B}, that anatoxin-a production is proportional to growth, was rejected.

Table 4.7 Comparison of different anatoxin-a quota models using Akaike Information Criteria to evaluate the goodness of fit.*

Growth Model Test for Each Treatment	AIC
Copper Treatment	
Categorical model	48.1
Logarithmic linear model	98. 8
Intercept model	221.6
Iron Treatment	
Categorical model	57.8
Logarithmic linear model	105.7
Intercept model	137.4

* For each dataset, a linear model (explaining the overall trend of the data over time) was compared to a categorical model (where days were treated as a factor) and intercept model (where time was not a variable).

Table 4.8 Analysis of variance for the effect of iron and days on anatoxin-a production. Days and iron concentration are treated as categorical factors.

	df †	*p*-value
Fe	2	<0.0001*
Day	9	<0.0001*
Fe×Day	18	<0.0001*
Residuals	60	

* significant *p*-values, $p < 0.05$ were considered significant; †degrees of freedom.

Table 4.9 Analysis of variance for the effect of copper and days on anatoxin-a production. Days and copper concentration are treated as categorical factors.

	df †	*p*-value
Cu	2	<0.0001*
Day	11	<0.0001*
Cu×Day	18	<0.0001*
Residuals	60	

* significant *p*-values, $p < 0.05$ were considered significant; †degrees of freedom.

In all media the maximum intracellular anatoxin-a quota occurred within the first 20 days (Table 4.10, Figure 4.9a and Figure 4.10a). The maximum anatoxin-a quota occurred latest for the lowest iron concentrations $MLA_{0.1\times Fe}$ (Table 4.10). These maxima usually coincided with spread of trichomes over all the surface area available. All anatoxin-a quota maxima were significantly higher than the data recorded for the same treatment in the last sampling points of the experiment from Days 22–49. After Day 20, cell quotas were less than 0.1 pg $cell^{-1}$, and this limit was maintained under all media conditions for the remainder of the experiment. Anatoxin-a maxima occurred around 0.53 pg $cell^{-1}$ for all media except $MLA_{0.1\times Fe}$ and $MLA_{10\times Fe}$, both which displayed little change in anatoxin-a concentration and growth (Table 4.10).

Table 4.10 Anatoxin-a quota maxima and day of maxima recorded

Treatment	**Highest anatoxin-a quota (pg $cell^{-1}$)**	**Day**
$MLA_{1\times Cu;\ 1\times Fe}$	0.54	Day 10
$MLA_{10\times Cu}$	0.55	Day 12
$MLA_{100\times Cu}$	0.49	Day 0
$MLA_{0.1\times Fe}$	0.15	Day 17*
$MLA_{2\times Fe}$	0.53	Day 6
$MLA_{10\times Fe}$	0.06	Day 3†

* No significant increase in cell numbers was observed for $MLA_{0.1\times Fe}$.

† Growth was inhibited in $MLA_{10\times Fe}$ and cultures struggled to survive, this treatment was terminated on Day 12.

Intracellular anatoxin-a under different iron treatments

The 95% confidence intervals for each iron treatment and day were used to identify significant differences (Figure 4.9). Significant differences in anatoxin-a production were clear for $MLA_{2\times Fe}$ and $MLA_{1\times Fe}$, which followed a normal growth curve (Figure 4.6a). The maximum anatoxin-a quota for the highest iron concentration ($MLA_{2\times Fe}$) occurred on Day 6, this value was significantly higher than all the other iron treatments on the same day. The maximum anatoxin-a quota for $MLA_{1\times Fe}$ was significantly higher than all anatoxin-a data between all iron treatments from Days 22–49. These $MLA_{2\times Fe}$ and $MLA_{1\times Fe}$ maxima were significantly higher than the data points for other iron media on adjacent days including $MLA_{0.1\times Fe}$ on Day 12 and $MLA_{2\times Fe}$ on Days 9 and 12.

The lowest iron concentration $MLA_{0.1\times Fe}$ had a maximum anatoxin-a quota of 0.15 pg anatoxin-a $cell^{-1}$ on Day 17. This value is within the 95% confidence interval of the maxima anatoxin-a quota recorded at the iron concentrations $MLA_{1\times Fe}$ and $MLA_{2\times Fe}$. However, this maximum anatoxin-a quota for $MLA_{0.1\times Fe}$ was significantly higher than the value recorded for $MLA_{2\times Fe}$ on Day 17. The anatoxin-a quota of $MLA_{0.1\times Fe}$ at Day 17 was significantly higher than the initial anatoxin-a concentration and the anatoxin-a quota for $MLA_{0.1\times Fe}$ recorded from Days 22–49. $MLA_{0.1\times Fe}$ was the only iron treatment that had an anatoxin-a maxima that fell outside of the 95% confidence interval for the initial starting point. This low value could be reflected by the low growth rate of this treatment, with little change in cell numbers throughout the experiment (Figure 4.6).

Intracellular anatoxin-a under different copper treatments

The maximum anatoxin-a quota was observed for $MLA_{1\times Cu}$ and $MLA_{10\times Cu}$ occurred on similar days (Table 4.10). In contrast, the anatoxin-a quota maximum for treatment $MLA_{100\times Cu}$ occurred on day 3. This observation may be explained by the limiting affect 250 ppb Cu had on growth. The maxima anatoxin-a quota recorded for each copper treatment were within the 95% confidence interval of each other. All anatoxin-a quota maxima for copper treatments were significantly higher than the anatoxin-a quota recorded for all treatments from Day 32–49 (Figure 4.9).

4.4 Discussion

4.4.1 Correlation of Anatoxin-a Production with Growth

Only anatoxin-a and the degradation product dihydroanatoxin-a were detected in cultures of CYN52. Homoanatoxin-a and its degradation products were not identified. Heath et al. (2010) also found that CYN52 produces only anatoxin-a, confirming the cyanotoxin profile for this strain.

Anatoxin-a production was not completely inhibited by any concentration of the metal stressors tested. Anatoxin-a production was not correlated to cell concentrations. The highest anatoxin-a quota was identified during the late lag-phase/early exponential growth phase (Table 4.10, Figure 4.9, and Figure 4.10). Selwood et al. (2007) observed a similar trend with the maximum anatoxin-a quota of *Aphanizomenon issatschenkoi*. This maximum occurred on Day 7, during the early exponential growth phase (Selwood et al., 2007). In contrast, Gupta et al. (2002) found that the anatoxin-a quota maximum for *Anabaena flos-aquae* occurred in the late exponential or early stationary phase. However, Gupta et al. (2002) measured anatoxin-a using bioassays not using mass-spectrometry as in the current *P. autumnale* study. Production of microcystins by *Microcystis* was reported to be proportional to cell concentrations in some studies (Briand et al., 2005).

The position where anatoxin-a production maxima occur in the growth cycle may vary under different culturing conditions and between different species (Rapala et al., 1993). Rapala et al. (1993) reported anatoxin-a production profiles varied between *Anabaena* spp. and *Aphanizomenon* sp. under various light, temperature, and nutrient conditions. Anatoxin-a maxima could occur in the early, mid, or late exponential phases. However, in their study, Rapala et al. (1993) reported anatoxin-a normalised to cell dry weight rather than cell concentrations making it difficult to compare with this *P. autumnale* study. Correlation of cyanotoxin to different parameters including, dry weight, chlorophyll-a content, and protein content, makes comparison between studies difficult because these parameters may not be linearly correlated with cell enumeration. An example of variation in cyanotoxin production depending on the selected method of normalisation was illustrated by Negri et al. (1997). In their paper, Negri et al. (1997), observed significant changes in *A. circinalis* saxitoxin production when normalised to dry weight, but the saxitoxin quota was not significantly different. Issues arise when using mass to normalise

toxin production throughout the growth cycle. These issues include mass variability due to the presence of bacteria (Baker and Humpage, 1994) and changes in carbohydrate storage as the culture ages (Utkilen and Gjølme, 1995). Carbohydrate variability can affect the mass of the cell without necessarily affecting cyanotoxin production (Utkilen and Gjølme, 1995).

Extracellular anatoxin-a degradation is not expected to be affected by changes in iron or copper in the surrounding medium. Stevens and Krieger (1991) observed that photolytic degradation of anatoxin-a occurred on a faster time-scale compared to non-photolytic degradation catalysed by iron or copper. For example, anatoxin-a in solution had a photolytic degradation half-life between 96 and 330 minutes. The non-photolytic degradation pathway half-life ranged between 3.8 and 10 days (Stevens and Krieger, 1991). The majority of anatoxin-a in the current *P. autumnale* study was found to be intracellular under standard MLA medium, which is consistent with anatoxin-a being mostly intracellular under beneficial growth conditions (Sivonen and Jones, 1999).

Under high copper concentrations (250 ppb), a significantly higher percentage of extracellular anatoxin-a to total anatoxin-a was detected late in the growth cycle, compared with other treatments (Figure 4.8). These high anatoxin-a concentrations could be due to cell lysis (Sivonen and Jones, 1999) or cell wall deformation by copper (Surosz and Palinska, 2004) allowing anatoxin-a to leak into the surrounding culture medium.

4.4.2 Effect of Copper and Iron on Anatoxin-a Production of Phormidium autumnale

The iron and copper conditions did not appear to change the maximum concentration of anatoxin-a produced. While iron and copper altered the growth profiles of CYN52, there was little change in the anatoxin-a profile. Most cultures had a maximum anatoxin-a quota of ca. 0.5 pg cell^{-1} (Table 4.10). The exception was $MLA_{0.1\times Fe}$ where there was no noticeable growth throughout the experiment. In this treatment, the maximum toxin concentration recorded only reached 0.15 pg cell^{-1}. Production of other cyanotoxins could be affected by iron deficiency in a different way. For example, Alexova et al. (2011) detected highest microcystin quota in *M. aeruginosa* for iron-deplete conditions. In this case, exponential growth of *M. aeruginosa* coincided with the maximum microcystin quota under all iron treatments. However, in this *P. autumnale* study the maximum anatoxin-a

production for $MLA_{0.1\times Fe}$ could not occur with the early exponential phase of growth as in the other treatments because no exponential growth phase was observed. These results indicate that exponential growth is important for maximum anatoxin-a production.

The culture condition and previous history of stress prior to additional stressor exposure affects the organism's stress response (Gong et al., 2009; Thompson et al., 2011). Thompson et al. (2011) demonstrated *Prochlorococcus* cell size decreased within 20 hours in iron-deplete conditions, whereas cell size did not increase when iron was restored. Gong et al. (2009) identified microcystin production by *Microcystis* could vary when exposed to arsenic depending on the history of previous phosphate stress. In the experiments in this iron and copper stressor study, culture inoculum was grown in the same media conditions (standard MLA) until stationary phase to avoid changes due to variation in culture history.

Role of anatoxin-a

The physiological or ecological role of anatoxin-a has not been identified (Kaebernick and Neilan, 2001; Osswald et al., 2007). The maximum anatoxin-a quota occurred early in the growth cycle for all cultures, usually corresponding with the spread of trichomes over all available substratum. This observation suggests that anatoxin-a may be involved in initial colonisation of the substratum. It is unlikely that anatoxin-a plays a role directly in substrata adhesion, as anatoxin-a is produced in both planktonic and benthic cyanobacteria (Sivonen and Jones, 1999). The cyanotoxin and growth correlation observed in this experiment suggests that anatoxin-a may indirectly assist in substrata adhesion or distribution of *Phormidium* across all available surface area. Anatoxin-a may be involved with intra- or inter-species interactions, including allelopathic or chemotaxic signalling between algae and bacteria, during this initial growth phase. Anatoxin-a could be used as a defence mechanism, the green alga *Chlamydomonas reinhardtii* is paralysed by exposure to anatoxin-a (Kearns and Hunter, 2001). However, extracellular toxin concentrations may not be strong enough to cause this chemotaxic effect, as the concentration of extracellular anatoxin-a is usually far lower than intracellular toxins (Sivonen and Jones, 1999). In this *P. autumnale* study low extracellular anatoxin-a values were also observed, with the exception of $MLA_{100\times Cu}$ which had a significantly higher percentage of extracellular anatoxin-a than the other treatments. Under standard MLA extracellular anatoxin-a was highest between Days 12 and 22 during the early exponential growth phase; supporting the suggestion that anatoxin-a is required in higher concentrations during this period.

4.4.3 Effect of Copper and Iron on the Growth of Phormidium autumnale

Both iron and copper had a significant effect on the growth of *P. autumnale* CYN52 (Figure 4.6, Table 4.4 and Table 4.5). High copper concentrations and low iron concentrations reduced growth. If iron concentrations were too high, growth was also inhibited. The two stressors, iron and copper, were added by changing the concentration of $FeCl_3.6H_2O$ and $CuSO_4.5H_2O$ in the culture medium. These salts are the same as those used to prepare standard MLA medium (Bolch and Blackburn, 1996). The concentration of SO_4^{2-} increases from 27.8–28.2 ppm when Cu is increased to 100× the standard MLA Cu concentration. The concentration of Cl^- varied between 7.18–7.67 ppm when iron is increased from 0.1–2× standard MLA Fe concentration. Therefore, concentrations of SO_4^{2-} and Cl^- were altered with Fe and Cu concentration. The overall effect of variation in anion concentrations is assumed to be negligible compared with those caused by changes in iron and copper concentrations.

Only one anatoxin-a producing strain of *P. autumnale* was analysed for the effects of iron and copper on growth. The effects of Fe and Cu on growth may be strain specific rather than applicable to all anatoxin-a producing *P. autumnale* strains. Rapala and Sivonen (1998) observed strain specific rather than cyanotoxin specific differences in growth when microcystin and anatoxin-a producing strains were exposed to different light and temperature gradients. Cyanobacterial strains that are well adapted to handle iron-limited stress are more likely to be sensitive to higher copper concentrations. This sensitivity is due to their increased ability to uptake trace metals at lower concentrations (Thompson et al., 2011). Observations in this study cannot be assumed to be true for other anatoxin-a producing *Phormidium* strains as each strain probably responds to stress in a slightly different way.

Iron and growth

Iron is an essential element, often involved in high energy pathways such as photosynthesis. Tight regulation of these pathways is needed to avoid formation of free-radicals that can be detrimental to the cell (Baptista and Vasconcelos, 2006; Rueter and Petersen, 1987). This experiment supports the importance of maintaining iron concentrations within cells. If the iron concentration is either too low (40 ppb, $MLA_{0.1\times Fe}$) or too high (4000 ppb, $MLA_{10\times Fe}$) growth was inhibited. This observation is probably due

to oxidative stress resulting from too much or too little available iron (Estevez et al., 2001; Latifi et al., 2009).

Under high iron concentrations ($MLA_{2\times Fe}$ and $MLA_{10\times Fe}$) cultures did not attach firmly to the culture container walls as observed in all other experiments. The concentration of iron in $MLA_{2\times Fe}$ (800 ppb Fe) used in the current experiment is environmentally relevant, and has been detected in Canterbury streams (Harding and Winterbourn, 1995). In these streams, high iron concentrations could inhibit benthic proliferations of *Phormidium* by preventing these species adhering to the substratum. $MLA_{10\times Fe}$ was not suitable for culturing CYN52 as no growth was observed for this treatment probably due to oxidative stress resulting from the high iron concentration. Estevez et al. (2001) also observed a reduction in growth attributed to oxidative stress induced by high iron concentrations in *Chlorella vulgaris* cultures. High iron concentrations caused a detrimental effect on *M. aeruginosa* growth, with poor or no growth observed at 62.5 and 100 µM Fe respectively (Lukač and Aegerter, 1993). Cultures adapted to iron-deplete medium decayed when transferred back to standard 28 µM Fe BG11 culture medium (Lukač and Aegerter, 1993).

Under a low Fe regime ($MLA_{0.1\times Fe}$) the cultures were distributed across the surface of the culture container but an exponential growth phase was not observed (Figure 4.6). This reduction in growth below 10 ppb has also been observed for other cyanotoxin-producing cyanobacteria, including *Cylindrospermopsis raciborskii* (Chuang et al., 2001) and microcystin-producing *Microcystis aeruginosa* (Alexova et al., 2011). In contrast to Lukač and Aegerter (1993) where 100 µM Fe inhibited growth, other *Microcystis* strains were reported to grow better under high iron concentrations (Xing et al., 2007). Both *M. aeruginosa* and *M. wesenbergii* strains grew under 100 µM $FeCl_3$ than 0.01 µM iron-deplete conditions (Xing et al., 2007). The reduction in growth under iron-deplete conditions may be explained by oxidative stress (Latifi et al., 2009; Xing et al., 2007). The *M. wesenbergii* strain grew better than *M. aeruginosa* under both conditions (Xing et al., 2007). Therefore, different species may grow better at certain iron conditions than others. *Phormidium autumnale* strain CYN52 does not grow well under very high (100 µM Fe) iron concentration. Oxidative stress on cyanobacteria from iron deficiency was reported for *Anabaena* sp., where increased concentrations of reactive oxygen species have been recorded under iron starved conditions (Latifi et al., 2005). The reduction in growth observed in CYN52 under low iron could be partially attributed to oxidative stress. Genes

triggered under oxidative stress have also been observed in *Anabaena* sp. under iron deficient conditions (Latifi et al., 2005). As oxidative stress was not observed under iron starvation on heterotrophic bacteria, Latifi et al. (2009) proposed that only photosynthetic bacteria are susceptible to oxidative stress via iron limitation.

Iron uptake

Iron was rapidly removed from the media after CYN52 was inoculated. The metal concentrations in the medium control samples were constant throughout the current experiment. These controls confirm that the metals were not removed from the media via sorption to the polystyrene containers during the experiment. The trend of a rapid decrease in iron after inoculation occurs for all iron treatments. CYN52 was unialgal but non-axenic, so this decrease is partially attributed to metal interactions with the *Phormidium* strain as well as any associated bacteria and viruses co-existing within the culture. Even low quantities of bacteria can accelerate iron removal from liquid cultures (Wightman and Fein, 2005). The metal interactions with cyanobacteria are likely to be a combination of adsorption onto the outer cell wall and absorption into cells of the organism. Initial microbial metal uptake mechanisms usually consist of rapid adsorption to the cell wall, followed by a slower metabolism dependent phase transporting the metal into the cell (Fiore and Trevors, 1994; Gadd, 1988). Singh et al. (1998) observed two stages for rapid uptake of iron by harvested dry weight *Microcystis* cultures. These stages were an initial rapid uptake within about 20 minutes of exposure to iron, followed by a slower uptake process over the next two and a half hours (Singh et al., 1998). The initial rapid uptake is assumed to be caused by passive surface adsorption mechanisms. Rapid adsorption to the surface would contribute to the significant decrease in iron concentrations observed in this experiment between the media controls and first set of cultures harvested. This trend was observed under all treatments with iron concentrations of 400 or 800 ppb. In $MLA_{1\times Fe=1\times Cu}$ the iron concentration in the culture media decreased from 400 ppb to under 25 ppb within two weeks after inoculation.

Culture medium pH will affect metal ion speciation. An increase in pH was observed, probably due to CO_2 removal during photosynthesis (Sunda et al., 2005). Freshwater culture media is susceptible to pH changes because it contains lower concentrations of carbonate and bicarbonate buffering ions that regulate marine media (Sunda et al., 2005). Increasing pH generally causes a reduction in metal ion bioavailability (Macek and

Mackova, 2011). The pH should not affect initial iron uptake since this rapid iron uptake occurred when culture medium pH were similar to that of the controls.

Copper and iron biosorption interaction

No change in copper concentration was observed under any of the concentrations tested compared with the controls. In contrast Singh et al. (1998) observed iron had an antagonist effect on the biosorption of copper. However, this difference was for very high copper and iron concentrations (20–50 ppm range), which are not likely to be encountered as a natural environmental stress for cyanobacteria. In this current *P. autumnale* study, an increase in copper concentration did not appear to change the biosorption of iron in CYN52. Singh et al. (1998) reported that copper did not affect the biosorption of iron, which agrees with this current iron and copper study.

Copper and growth

The increasing copper concentrations chosen were expected to have a detrimental effect on growth. Copper sulfate used as an algicide applied at concentrations between 10–1000 ppb is sufficient to eradicate most algae (McKnight et al., 1983). However, this experiment revealed that although copper concentrations of 250 ppb had a significant effect on growth (Figure 4.6) it did not kill all *Phormidium* cells. Applying copper algicides at a concentration of up to 250 ppb would be insufficient to kill off *P. autumnale* proliferations if the effect was similar to that observed in CYN52. Cyanobacteria can acclimatise and become resistant to copper stress (García-Villada et al., 2004).

Cyanobacteria can be more sensitive to copper toxicity than eukaryotic algae (Rueter and Petersen, 1987). Lembi (2000) in contrast, observed that *Oscillatoria* was more resistant to copper stress than mat-forming eukaryotic algae. There was no significant uptake of copper throughout the experiment as copper concentration remained consistent throughout the experiment with both cultures and the media controls.

4.4.4 Future Studies

The experimental design used in this study was modified from Esson et al. (2011). The further development of this their method during this work will contribute to greater understanding of the role of growth in relation to toxin production in benthic algae. Sivonen (1990) investigated gravimetric microcystin production in *Oscillatoria agardhii*

using a similar approach. However, in the *O. agardhii* study 3–6 samples were pooled for each data point. In the current *Phormidium* study three replicates were used for each data point, allowing estimation of errors for growth and cyanotoxin production. The use of cell concentration rather than biomass allows for a better estimation of growth. This method can be applied to any benthic algal species without disrupting the unique attachment and growth mechanisms of these species. Using this method other metabolites could also be monitored over the growth cycle of benthic species.

Improvements for future experiments

In CYN52 the concentrations of anatoxin-a were high enough to be easily detected throughout the growth cycle. Future studies using CYN52 could halve each sample at the harvest date for cyanotoxin production and cell concentrations. This procedure would allow these two parameters to be correlated to an individual sample rather than the mean cell concentration for a particular harvest date. This approach was used in the following chapter (Section 5.2.2)

Since this study was completed, it was noticed that some *Phormidium* trichomes stored in Lugol's iodine attach over time to the walls of the culturing storage containers. Therefore when cells are counted the calculated cell concentration could potentially be lower than the actual value. The amount of adsorption onto the container wall varies between strains (Heath and Wood, 2011). Addition of a small amount of Sigmacote™ (Sigma) to detach trichomes from the container walls was recommended. A selection of the CYN52 cell concentration samples were reanalysed after treatment with Sigmacote and no net change in cell numbers was observed. Future studies should monitor for adsorption onto the container walls during storage and treat with Sigmacote™ if required.

Suggestions for future experiments

The relationship between anatoxin-a production and homoanatoxin-a production has not been reported in a culture growth experiment. The method described here would allow homoanatoxin-a production to be investigated alongside anatoxin-a production to monitor variability in these cyanotoxins in benthic cyanobacteria. To minimise variation between strains, and compare anatoxin-a and homoanatoxin-a production, the cyanotoxin-producing strains need to be carefully selected from identical morphospecies with similar growth profiles.

Anatoxin-a quota maxima of CYN52 occurred within the first 3 weeks of the experiment. Future experiments on *Phormidium* could focus on only the first 3–4 weeks of the growth profile since the stationary phase is not required to observe anatoxin-a profile variation. Experiments focusing on the early growth phase when anatoxin-a is highest could identify the role of this metabolite in *Phormidium*.

Future studies on *Phormidium* strains under iron deficient conditions should investigate the production of reactive oxygen species and induction of genes related to oxidative stress. However, it is difficult to predict which genes are likely to be up regulated. Many genes are not well understood and some genes that can be triggered under iron stress may not be expressed (Thompson et al., 2011).

4.5 Conclusions

In this study a method was developed for monitoring cyanotoxin production and growth in benthic cultures. This method provides a useful tool for observing changes in cyanotoxin production, or other metabolites, throughout the growth cycle in benthic algae.

Iron and copper concentrations affected *P. autumnale* (CYN52) growth. Growth was inhibited under iron-deficient or excessive copper conditions. High iron disrupted the attachment mechanisms of CYN52. Waterways with high iron concentrations (~800 ppb or higher) may be unsustainable for *Phormidium* growth if this species cannot attach to the substratum.

Although iron and copper concentrations significantly influence growth of CYN52, no significant trend in anatoxin-a production under different iron and copper regimes was observed. In general there was little variability in maximum anatoxin-a production over the range of copper and iron concentrations examined. Extracellular anatoxin-a should be monitored in the environment if high copper concentrations occur. Maximum intracellular anatoxin-a quota coincided with the steep decrease in iron availability in the media. This decrease probably occurs by initial surface adsorption followed by slower absorption of iron into the cyanobacteria, or associated bacteria and viruses.

Anatoxin-a profiles in these experiments revealed that anatoxin-a quota were highest early in the growth phase. Anatoxin-a may play an important role in colonising space during these early phases.

5 *Scytonema* growth experiment

5.1 Introduction

Previous studies have demonstrated that production of saxitoxins can vary throughout the cyanobacterial growth cycle (Table 5.1). This variation has implications for monitoring and management. Yunes et al. (2009) reported saxitoxin (STX) and decarbamoyl saxitoxin (dcSTX) production were higher in the exponential rather than the stationary phase of growth. A similar finding was reported by Ibrahim (1990) for STX and neosaxitoxin (neoSTX). In contrast Negri et al. (1997) and Velzeboer et al. (2001) reported a linear correlation between total STX and growth in *Anabaena circinalis* strains. Furthermore, toxin quota of STX and its variants have also been reported highest during the early to late stationary phase, rather than the exponential phase (Carneiro et al., 2009; Dias et al., 2002). The two previous analyses of *Scytonema* cf. *crispum* strain UCFS10 have identified saxitoxin concentrations of 7.4 and 119 mg kg^{-1} when the strain was analysed a year apart for changes in saxitoxin profile (Chapter 3, Section 3.3.4). This variability suggests that saxitoxin production in *S.* cf. *crispum* may vary in relation to dry weight at different stages of the growth cycle.

Table 5.1 Parameters reported for literature saxitoxin growth and stressor studies.

Cyanobacterial Strain	Saxitoxin Measurements*	Growth Parameters*	Reference
Anabaena circinalis	gravimetric, toxin quota, volumetric	biomass, cell concentrations	Negri et al., 1997
A. circinalis	toxin quota, volumetric	cell concentrations	Velzeboer et al., 2002
A. circinalis	mouse neuroblastoma bioassay, volumetric	biomass, † cell concentrations, chlorophyll-a †	Testé et al., 2002
Aphanizomenon sp. *NH-5a*	gravimetric	biomass	Ibrahim, 1990
***Aphanizomenon* sp.** LMECYA 31	toxin quota, volumetric	cell density	Dias et al., 2002
Cylindrospermopsis raciborskii	saxitoxin per optical density	optical density	Pomati et al., 2003; Pomati et al., 2004b
C. raciborskii	saxitoxin per total protein content	optical density	Pomati et al., 2004a
C. raciborskii	gravimetric	optical density	Castro et al., 2004
C. raciborskii	toxin quota	cell concentrations	Carneiro et al., 2009
Lyngbya wollei	mouse bioassay	biomass, § chlorophyll-a, † phycobilin †	Yin et al., 1997
Raphidiopsis brookii	saxitoxin per biovolume	biovolume, chlorophyll-a, trichome count	Yunes et al., 2009

* Biomass = dry weight of cells; biovolume = volume of cells mL^{-1}; cell concentrations = cell enumeration mL^{-1} culture; gravimetric = STX cell dry $weight^{-1}$; mouse bioassay = mouse units g^{-1} dry weight; mouse neuroblastoma bioassay = ng STX equiv. mL^{-1}; toxin quota = STX $cell^{-1}$; volumetric = total μM STX variants $culture^{-1}$.

† These parameters were mentioned in the method, but the results were not reported in these papers.

§ Yin et al. (1997) recorded biomass as fresh and dry weight, but dry weight was consistently about 20% of the fresh weight so only dry weight was reported.

Comparison between cyanotoxin growth studies is challenging because of the different methods used to measure growth and report cyanotoxin concentrations. Cyanotoxin concentrations are often reported normalised to dry weight (Sivonen and Jones, 1999). Dry weight can vary considerably throughout the growth cycle and may not be a very good estimate of cell concentrations (Negri et al., 1997; Section 4.4.1). Cell counting is more labour intensive than using biomass, but provides a better estimate of growth. Previous growth and stressor studies on STX-producing cyanobacteria have reported STX normalised to various parameters (Table 5.1). Growth, of cyanobacterial STX-producers, has also been estimated from a range of parameters including biomass, biovolume, cell density, optical density, trichome count, and the pigments chlorophyll-a and phycobillins. Of these growth parameters, cell concentrations, and dry weight biomass are most frequently used (Table 5.1). Volumetric analysis (cyanotoxin concentration per volume of culture) has been most commonly used in growth and stressor experiments for saxitoxin-producing species (Negri et al., 1997; Testé et al., 2002; Velzeboer et al., 2001).

5.1.1 Aims and Objectives

In this study, STX concentration was monitored through a growth cycle. STX is reported as gravimetric (STX dry weight^{-1}), STX quota (STX cell^{-1}), and volumetric (total STX 30 mL culture^{-1}) to enable comparison with previous STX-producing cyanobacterial studies (Table 5.1).

This part of the study had the following aims and objectives:

- To measure *Scytonema* cf. *crispum* strain UCFS10 growth using changes in biomass and cell concentrations.

- To determine if saxitoxin production in strain UCFS10 varied with growth phase.

5.2 Methods

5.2.1 Culturing and Experiment Design

Culture, media, and experimental set up

Saxitoxin producing *Scytonema* cf. *crispum*, strain UCFS10, was used in this experiment. UCFS10 produces only the STX variant (Chapter 3, Section 3.3.4). UCFS10 was grown in two batch cultures of 100 mL $BG11_0$ liquid medium (Rippka et al., 1979). These cultures were combined to acquire the starting inoculum for the growth experiment.

Strain UCFS10 is metaphytic (Section 2.4.1), growing as dense floating benthic mats in culture. Measuring growth of *Scytonema* cultures by subsampling requires physical separation of these mats disrupting growth patterns. This disruption presents a problem similar to measuring periphytic *Phormidium* (Section 4.2.1). To overcome this problem a new method based on Section 4.2.1 and Esson et al. (2011) was developed. Aliquots (30 mL) of $BG11_0$ culture medium were pipetted into 65 pre-numbered, pre-weighed, gamma-sterilised polystyrene culturing containers (70 mL, Labserv). Inoculate from strain UCFS10, 70 ± 5 mg wet weight, were placed into 57 culturing containers using sterile forceps. The remaining containers were not inoculated and used as culture medium controls, which were incubated and harvested alongside the culture samples.

Cultures and culture medium controls were incubated at 18 °C (± 1 °C) under 37 µEin m^{-2} s^{-1} light (12 h light-dark cycle). Variation in light intensity was minimised throughout the experiment through culture position randomisation and filling gaps with culture container blanks as previously described (Chapter 4, Section 4.2.1). Positions were randomised every harvest date for the first 6 weeks and every second harvest date thereafter (Figure 5.1).

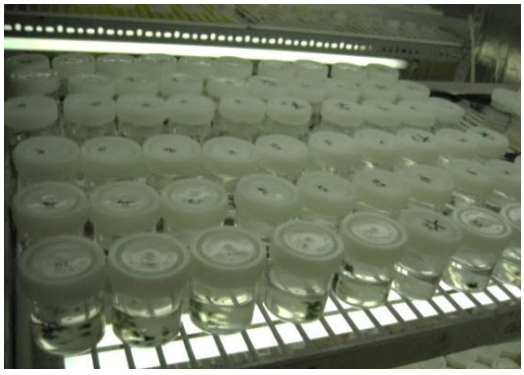

Figure 5.1 *Scytonema* growth experiment set up. Containers were arranged into randomised positions every 1–2 weeks.

5.2.2 Harvest and Sampling

Triplicate culture samples were harvested as described below, approximately weekly for 131 days. Duplicate culture medium control samples were harvested on Days 0, 42, 87 and 131. The media from culture samples and culture medium blanks were filtered (GF/C, Whatman) and the pH recorded (SevenEasy meter, Mettler Toledo). A subsample (1–2 mL) of each was frozen for subsequent extracellular STX analysis. The remaining filtrate from either the three cultures or two culture medium samples were combined and stored frozen for nutrient analysis (Section 5.2.3).

Samples were harvested by removing *Scytonema* filaments and dividing the sample in half for toxin analysis and cell counts as described below. Mechanical sample homogenisation, by Ultra-Turrax, sonication probe, or tissue grinder and pestle, caused cell lysis. To avoid this problem, the harvested filaments were rinsed with distilled water, transferred to a plastic petri-dish, and cut into small fragments using a scalpel blade. The fragmented filaments were transferred into a pre-weighed Falcon tubes (50 mL), rinsing the petri dish with RO water to ensure all fragments were transferred. The volume was topped up to 30 mL with RO water, mixed well, and a subsample (15 mL) was removed to determine cell concentrations. This subsample was preserved in Lugol's iodine solution and stored in the dark until analysis. The remaining 15 mL sample was lyophilised, weighed for growth analysis, and stored frozen until extraction for intracellular STX analysis (Section 5.2.4).

5.2.3 Nutrient Analysis

Nutrient samples were analysed by Flow Injection Analysis. Dissolved inorganic nitrogen was determined by combining the concentration of ammonia, nitrate, and nitrite following the methods outlined by the American Public Health Association (APHA et al., 2005a, 2005b). Dissolved Reactive Phosphorus (DRP) was also analysed following the methods outlined by the American Public Health Association (APHA et al., 2005c). These analyses were carried out at the Cawthron Institute by an IANZ (International Accreditation New Zealand) accredited laboratory. Calibration standards were run in every batch. Quality control methods included running Milli-Q blanks, re-checking standards, and analysing independent quality control samples.

5.2.4 Growth Analysis

Trichome fragments of *Scytonema* cf. *crispum* did not settle well in Utermöhl chambers. Therefore cell concentration of Lugol's preserved sample were analysed using a Sedgewick-Rafter chamber (McAlice, 1971). A subsample (1 mL) was placed in the counting chamber. All trichome fragments within two transects (equivalent to 100 µL) of the counting chamber were measured at 80× magnification using a light microscope (Olympus BX51). If cell concentrations were too dense, the sample was diluted between 2 to 8-fold with Lugol's iodine solution in RO water and recounted as described above. Mean cell length (n=50+) on Weeks 0, 9, and 18 was measured at 400–800× (Olympus BX50). The mean cell length was used to determine the number of cells in each trichome, which was then used to calculate the cells concentration of the original 30 mL culture.

5.2.5 Saxitoxin Analysis

Samples for intracellular STX analysis were extracted as described for lyophilised samples in Section 3.2.4. Extracellular STX and culture medium control subsamples (1 mL) were acidified with 0.025 M acetic acid (10 µL). Samples were oxidised and analysed by HPLC–FD as described in Section 3.2.4. Saxitoxins were reported in three ways: as total STX per culture (cf. volumetric analysis); as STX quota, which is STX normalised to cell concentration; or gravimetrically, which is STX normalised to dry weight. Extracellular saxitoxins toxins were reported volumetrically and as a percentage of total extra- and intracellular STX.

5.2.6 Statistical Analysis

Statistical analyses were carried out to test the following null hypotheses:

H_{0A}: STX production is proportional to the growth curve, where growth is measured by either cell concentrations or dry weight.

H_{0B}: STX quota are identical during exponential and stationary growth phases.

H_{0C}: STX quota is proportional to culture medium pH.

Statistical analysis was carried out using R version 2.13.1 (www.r-project.org). *P*-values <0.05 were considered significant.

Data transformation

Raw data from growth and STX quota and gravimetric STX were logarithmically transformed prior to analysis to ensure residuals were normally distributed. Assumption checks were carried out as described in Section 4.2.5.

H_{0A}: Saxitoxin production is proportional to growth

If STX production was proportionate to growth then and gravimetric STX should be constant across all days. This hypothesis was tested in two ways: analysis of variance (ANOVA) and model comparison.

ANOVA was used to determine if there was a significant relationship between STX production and time (in days) over the course of the experiment. In this model day was treated as a categorical value. To determine where significant differences occurred, 95% confidence intervals were calculated on the mean values for each day. These data were back-transformed and the 95% confidence intervals were plotted on the mean data for each day. Significantly different data occurs when the 95% confidence intervals do not overlap on the graph.

To further test this hypothesis, linear models of the intercept were created using mean STX production data. Various linear models (fitted to logarithmic and logistically transformed data, and with or without day as a factor) were trialled to assess different models of STX production in relation to growth. Akaike Information Criteria (AIC, Akaike, 1974) were used to compare statistical models for the same datasets. AIC evaluates models based on the log-likelihood of the data to determine the fit of model parameters. The lowest AIC score provided the model of best fit.

H_{0B}: Saxitoxin quota are identical for exponential and stationary growth phases

Subsets of STX quota data were analysed to verify if the exponential or stationary phases were proportional to cell concentrations. The STX quota associated with either of these phases, as determined by nutrient limitation and the growth curve, was tested by ANOVA. Categorical, continuous, and intercept models were compared using AIC as described above. The mean STX quota from the exponential growth and stationary phases were compared using Student's *t*-test.

H_{0C}: pH is significant in describing STX quota

Culture medium pH has been associated with STX production (Pomati et al., 2004b). To test the effect of pH on STX production, two-way ANOVA was used. Both pH and days, or pH alone were investigated in relation to STX quota.

5.3 Results

5.3.1 Media Analysis

Dissolved reactive phosphorus (DRP) decreased from a mean of 4.40 ± 0.17 ppm on days 0–35 to a mean of 0.08 ± 0.02 ppm on Days 98–131 (Appendix 8.4). This decrease was not observed in the controls where DRP remained fairly constant over the whole experiment (mean 4.75 ± 0.37 ppm). The DRP concentrations recorded in the controls and in the first 5 weeks of the culture samples were similar to the expected value of 4.60 ppm.

The nitrogen contributed from the medium was approximately 0.344 ppm. The initial dissolved inorganic nitrogen (DIN) concentrations were 0.321 and 0.328 ppm in the cultures and controls respectively (Appendix 8.4). In the control samples the nitrogen values decreased over time to 0.023 ppm. The amount of nitrogen observed in the cultures fluctuated throughout the experiment with a maximum value of 0.487 ppm and minimum value of 0.024 ppm.

The pH of cultures ranged from 7.6–10.6 (mean 9.4) throughout the experiment. The culture pH had reached 9.5 ± 0.1 by Day 57 increasing to a maximum pH of 10.52 ± 0.02 by Day 80. The culture then remained consistently between pH 9.9 and 10.2 until Day 125, dropping to pH 9.3 ± 0.1 on Day 131. The control medium pH remained constant with a mean of 7.8 ± 0.1.

5.3.2 Growth Analysis

The mean cell length was 6.1 ± 0.2 μm. Dry mass and cell concentrations were used to determine the growth profile of *Scytonema*. The cell concentration profile displayed typical lag phase, exponential growth, and stationary phase (Figure 5.2). The profile using dry mass was similar with a less pronounced exponential growth phase (Figure 5.2). Differences between replicates became larger as the mass or cell concentrations increased.

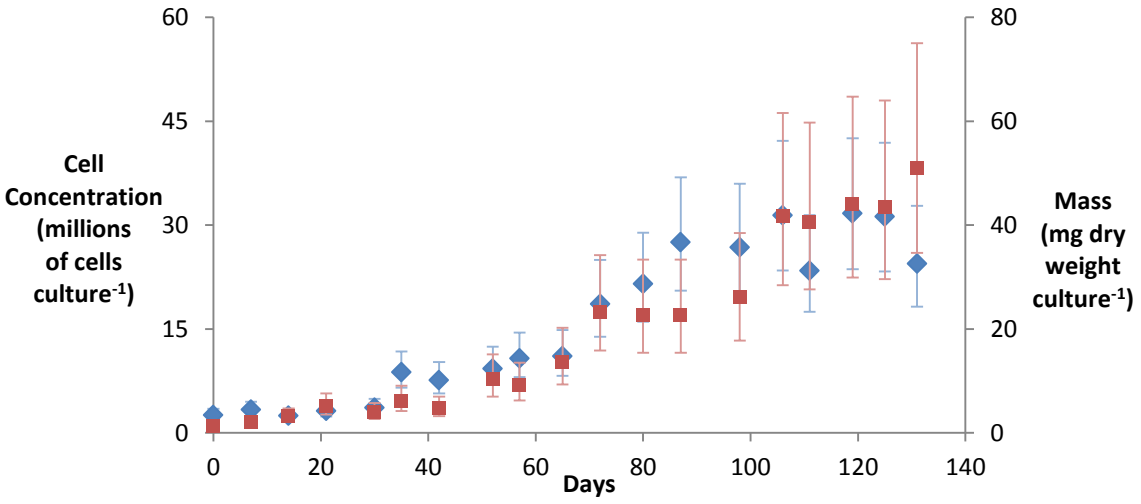

Figure 5.2 Growth profile of *Scytonema* cf. *crispum* UCFS10 in batch culture over 131 days. Cells per 30 mL of culture ◆ and mass per 30 mL culture ■. Error bars represent 95% confidence intervals (n=3).

5.3.3 Saxitoxin Analysis

Saxitoxin was determined as total STX in culture, STX normalised to cell concentrations to give STX quota, or normalised gravimetrically by dry weight. Total STX per culture (Figure 5.3) followed a similar trend to the growth curves (Figure 5.2).

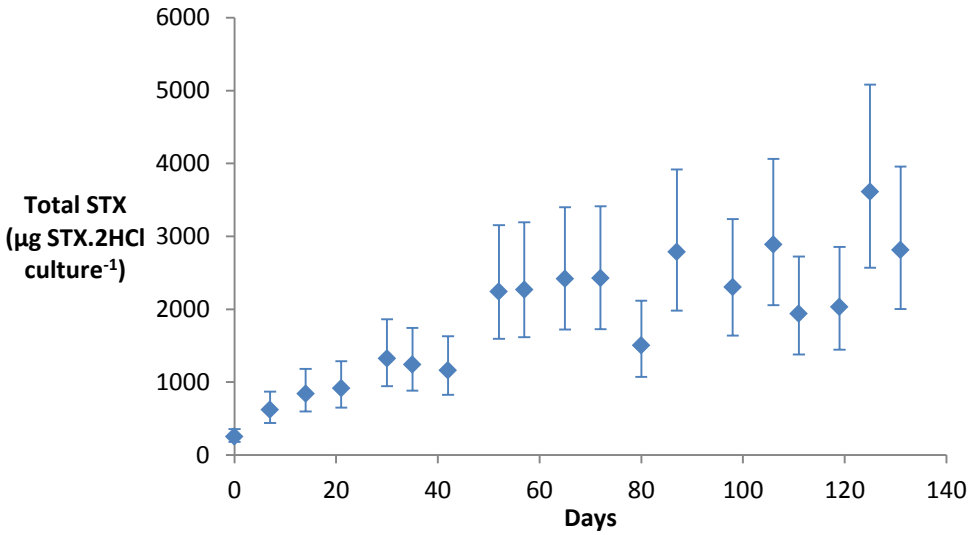

Figure 5.3 Total, intracellular and extracellular, saxitoxins per culture of *Scytonema* cf. *crispum* UCFS10 throughout the growth cycle. Error bars represent 95% confidence intervals (n=3).

Intracellular saxitoxin

Intracellular STX was reported as either STX quota or gravimetrically by normalising STX to either cell concentrations or biomass respectively. Both these parameters had similar STX profiles over the 131 days sampled (Figure 5.4).

The maximum intracellular STX quota (0.25 pg cell^{-1}, Figure 5.4) occurred on Day 14. This value was significantly higher than Day 0, 35, and 80–119. This maximum STX quota occurred in the late lag-phase or early exponential phase, when cell concentrations were significantly lower than Day 35 and later (Figure 5.2). Gravimetric STX, normalised to dry weight, was highest on Day 30 (230 ng mg^{-1}, Figure 5.4). This value was significantly higher than Day 72–119 and 131. This maximum STX production also corresponded to early in the growth cycle, where mass was significantly higher than Day 0 and still significantly lower than Day 52 onwards (Figure 5.2). Saxitoxin was not detected in any of the culture media blanks.

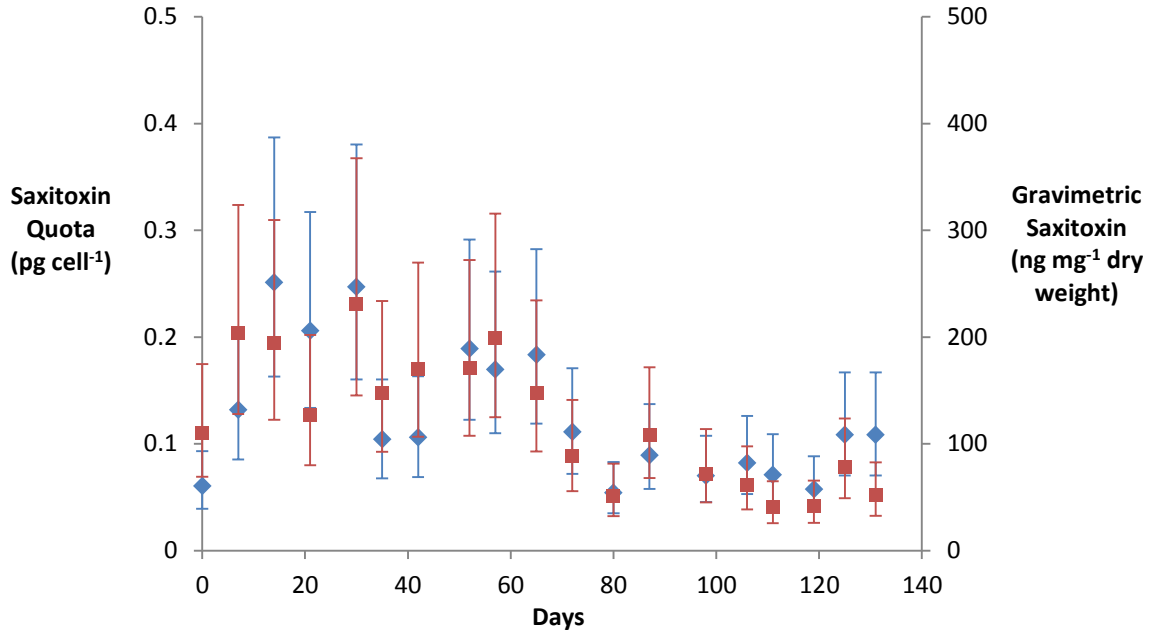

Figure 5.4 Intracellular saxitoxin production of *Scytonema* cf. *crispum* recorded as saxitoxin quota ◆ or gravimetric saxitoxin ■ throughout the growth experiment. Error bars = 95% confidence intervals (n=3).

Extracellular saxitoxin

The percentage of extracellular to total STX concentration in the culture decreased over the experiment (Figure 5.5). However, the concentration of extracellular STX did not vary markedly, remaining between 8–16 ng mL^{-1} of culture medium from Day 21 to Day 111. The highest proportion of extracellular STX (36% of total STX) was recorded on Day 0. However, this observation could be due to the set up method breaking trichomes, which may release STX into the surrounding medium. The extracellular STX percentage recorded on Day 0 was significantly higher than the percentage of extracellular STX recorded on Day 87 onwards. The percentage of extracellular STX remained elevated during the late lag phase and early exponential phase of the experiment. The second highest extracellular STX proportion occurred on Days 30 and 42 (30% of total STX). These days produced a significantly higher proportion of STX than Days 72, 87, and 106 onwards.

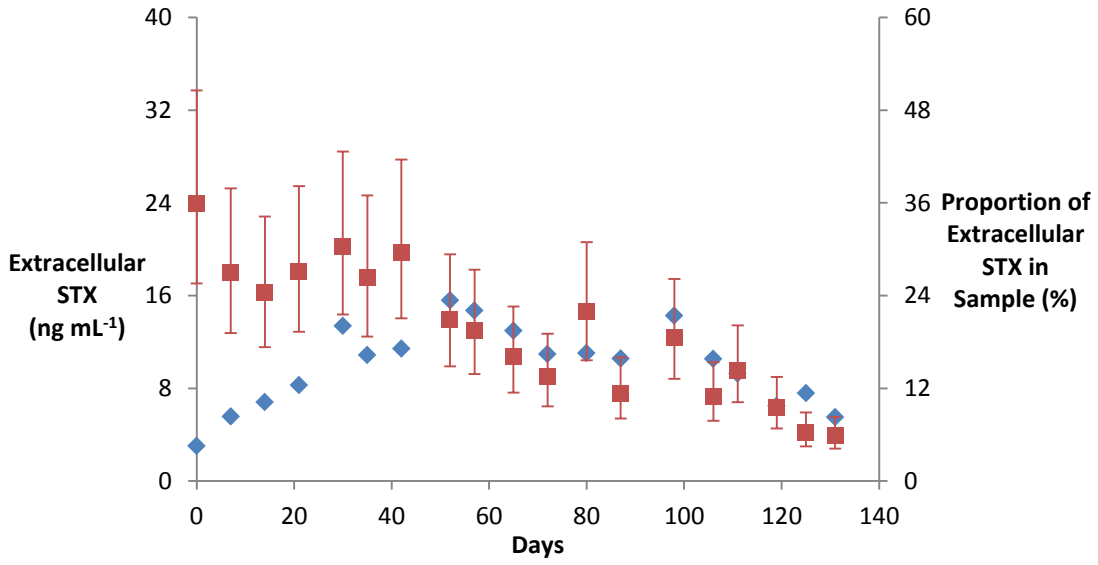

Figure 5.5 Extracellular saxitoxin concentration in media from *Scytonema* cf. *crispum* batch culture (◆, n=1); and as the percentage of extra cellular toxins from total saxitoxins in culture (■, n=3). Error bars = 95% confidence interval.

5.3.4 Statistical Analysis for Hypothesis Testing

H_{0A}: saxitoxin production is proportional to the growth as either cell concentration or dry weight

The null hypothesis H_{0A} that STX production is proportional to growth, described as either dry weight or cell concentration, was tested by ANOVA and AIC model comparison. Categorical and continuous models describing the relationship between STX and days were compared to determine if a general model was suitable for describing the STX trend using AIC (Table 5.2). These models were also compared to the intercept model, explaining only the mean STX data not controlled for day. The categorical model was the best fit to describe both STX quota and STX measured per dry weight. In both cases the intercept model had the highest AIC value, suggesting that this model was not a good fit. Therefore STX varies with cells and biomass throughout the growth curve. ANOVA further confirmed this theory, demonstrating that Day was a significant factor in determining STX production (p <0.05; Table 5.3 and Table 5.4). Therefore the null hypothesis that STX production is proportionate to the growth measured as cell concentrations or biomass is rejected.

Table 5.2 Comparison of different growth models using Akaike Information Criteria to evaluate the goodness of fit.*

Growth Model Test for Normalised Saxitoxin Production	**AIC**
Saxitoxin Quota Model	
Categorical model	-29.7
Linear model	-2.7
Intercept model	5.6
Saxitoxin per Dry Weight Model	
Categorical model	-21.8
Linear model	-14.5
Intercept model	20.7

* For each dataset, a linear model (explaining the overall trend of the data over time) was compared to a categorical model (where days were treated as a factor) and intercept model (where time was not a variable).

Table 5.3 Analysis of variance intracellular saxitoxin normalised to cell concentration. Day was a categorical factor.

	df †	***p*-value**
Day	18	<0.0001*
Residuals	38	

* significant *p*-values, $p < 0.05$ were considered significant; † degrees of freedom.

Table 5.4 Analysis of variance for intracellular saxitoxin normalised to dry mass. Day was a categorical factor.

	df †	***p*-value**
Day	18	<0.0001*
Residuals	38	

* significant *p*-values, $p < 0.05$ were considered significant; † degrees of freedom.

The significant differences in STX production can be seen in Figure 5.4 where the 95% confidence intervals do not overlap. Examining these significant differences in STX quota, it appears as if two distinct phases of STX production occur. The initial STX quota values between Day 7 and 57 are generally significantly higher than the STX quota after Day 98. This difference indicates that there may be two general phases of STX production over the growth cycle correlated with the exponential growth and stationary phase respectively.

H_{0B}: saxitoxin quota are identical for exponential and stationary growth phases.

This hypothesis was tested using ANOVA and AIC model comparison of the separate growth and stationary phases respectively. The stationary phase, as determined by the DRP limited phase of the growth cycle (DRP <1 ppm) and the growth profile (Figure 5.2), occurred from Day 72 onwards. Following the same reasoning, the exponential phase occurred prior to Day 72. Day 0 represents the day cultures were inoculated from the stationary phase of growth. Data from this day was excluded from the dataset because it may not reflect exponential growth. Day was a significant factor ($p < 0.05$) for the exponential growth phase (Table 5.5). ANOVA on the stationary phase STX quota data illustrated that day was not significant in describing STX quota (Table 5.6). This observation was confirmed by AIC where the intercept model, describing only the STX

quota not correlated to days was the best fit (lowest AIC score) for the stationary phase data (Table 5.7).

Table 5.5 Exponential phase saxitoxin quota ANOVA. Day was a categorical factor.

	df †	***p*-value**
Day	8	0.0338*
Residuals	18	

* significant *p*-values, $p < 0.05$ were considered significant; † degrees of freedom.

Table 5.6 Stationary phase saxitoxin quota ANOVA. Day was a categorical factor.

	df †	***p*-value**
Day	8	0.1978
Residuals	18	

† degrees of freedom, $p < 0.05$ were considered significant.

Table 5.7 Comparison of different growth models using Akaike Information Criteria to evaluate the goodness of fit.*

Growth Model Test for Normalised Saxitoxin Production	**AIC**
Saxitoxin Quota Exponential Phase	
Categorical model	-17.1
Linear model	-9.5
Intercept model	-11.4
Saxitoxin Quota Stationary Phase	
Categorical model	-11.9
Linear model	-11.8
Intercept model	-13.5

* For each dataset, a linear model (explaining the overall trend of the data over time) was compared to a categorical model (where days were treated as a factor) and intercept model (where time was not a variable).

The mean STX quota for the exponential and stationary phases were statistically different (Student's *t*-test, $p < 0.01$). Therefore, the null hypothesis H_{0B} that STX quota are identical during exponential and stationary phases is rejected.

H_{0C}: saxitoxin quota is proportional to culture medium pH

During the stationary phase the culture medium pH was high (pH 9.2–10.6, mean pH 10.0) compared with the exponential phase (pH 7.6–9.8, mean pH 8.7). This variation in pH may explain the differences in stationary and exponential STX quota. Two-way analysis of variance, with Day and pH as variables, was carried out on STX quota data. This analysis indicated that pH was not significant in explaining STX quota, however the effect of pH combined with days was significant (Table 5.8). Analyses of exponential or stationary phases only confirmed pH alone was not significant. In the stationary phase Day, pH, and the interaction factor were not significant (data not shown). The null hypothesis H_{0C} that pH is a significant factor in describing STX quota is rejected.

Table 5.8 Saxitoxin quota two-way ANOVA. Day was a categorical factor.

	df †	***p*-value**
Day	18	<0.0001*
pH	1	0.2341
Day×pH	18	0.0498*
Residuals	39	

* significant *p*-values, $p < 0.05$ were considered significant; † degrees of freedom.

5.4 Discussion

5.4.1 Growth

The method presented here can be used to monitor growth of *Scytonema* and other metaphytic cyanobacteria that form dense floating mats. Similar growth profiles were observed when estimating growth by cell enumeration or by dry weight biomass (Figure 5.2). Dry weight was a reasonable approximation for growth and normalising STX production in UCFS10. However, caution is required when dry weight is used to normalise toxin production. Changes in cellular components, including protein and carbohydrates, may not reflect cell division or lysis (Negri et al., 1997; Section 4.4.1). Therefore the more time-consuming cell enumeration method is generally recommended as it is more accurate measure of growth (Orr and Jones, 1998). Cell counting also provides a better insight into cell morphology variation over time (Hawkins et al., 2001).

Strain UCFS10 grows slowly and the complete growth cycle took over 4 months. By comparison, anatoxin-a-producing *Phormidium autumnale* strain CYN52 grows more rapidly and the stationary phase is reached within 42 days (Section 4.3.2). STX-producing cyanobacteria *Anabaena circinalis* and *Raphidiopsis brookii* reach the stationary phase within 2–3 weeks (Negri et al., 1997; Testé et al., 2002; Velzeboer et al., 2001) and 1–2 months (Yunes et al., 2009) respectively. Other *Scytonema* spp. also have a faster growth rate than *S.* cf. *crispum*. For example, Patterson & Bolis (1993) reported that *S. ocellatum* took approximately 40 days to reach the stationary phase.

Media and Growth

In the cultures, DRP concentrations decreased reasonably consistently over time whereas DIN levels fluctuated (Appendix 8.5). Highest DIN concentrations were observed in cultures when phosphorus was diminished. *Scytonema* contain intercalary heterocytes capable of nitrogen fixation (Komárek et al., 2003), therefore *Scytonema* are not limited to nitrogen concentration in growth media. When DRP is growth limiting, nitrogen-fixing cyanobacteria can cause high DIN concentrations in excess of biological requirements (Chorus and Mur, 1999), as was seen in this experiment. In this situation, nitrogen usually cannot be stored by cyanobacteria (Lawton et al., 1999), and must be excreted into the environment usually as ammonium (Gendel and Nohr, 1989).

DIN concentration decreased in controls, however no decrease in DRP was observed in these controls. This DIN decrease is probably due to uptake by bacteria that were observed in the control samples. Bacteria were present in controls despite culture medium sterilisation under standard autoclave procedures prior to the start of this experiment.

The general increase in pH over time can be attributed to a reduction in CO_2 in the media as it is utilised for photosynthesis. High levels of carbonate and bicarbonate ions buffer the media pH (Sunda et al., 2005). Freshwater media, including $BG11_0$, contains less of these buffering ions and is therefore predisposed to fluctuating pH levels as the media changes from culture utilisation (Sunda et al., 2005).

5.4.2 Saxitoxin and Growth Correlations

Intracellular

The mean STX quota during exponential growth was 168 ± 4 fg cell^{-1} and 81 ± 3 fg cell^{-1} during the stationary phase (Figure 5.4). Similar STX quota levels were described for *A. circinalis* (Negri et al., 1997; Velzeboer et al., 2001), which suggests that STX is produced at similar concentrations by both genera. These *A. circinalis* toxin quota included both STX and its variants, with between 60 to 215 fg saxitoxins cell^{-1} detected (Negri et al., 1997; Velzeboer et al., 2001). In contrast, Carneiro et al. (2009) reported maxima of only 4.58 fg neoSTX cell^{-1} and 0.48 fg STX cell^{-1} for *C. raciborskii*.

Growth and STX quota were not significantly correlated, despite similarities between cell concentration or biomass and total STX in each culture (Figure 5.2 and Figure 5.3). Results suggested STX is not produced at a constant rate throughout the growth cycle (Table 5.3 and Table 5.4). This suggestion contrasts with Negri et al. (1997) and Velzeboer et al. (2001), who both reported that growth and STX production were correlated. In *S.* cf. *crispum* study STX concentrations were only correlated to the number of cells during the stationary phase (Table 5.6). In *S.* cf. *crispum* strain UCFS10, STX was present at significantly higher concentrations (Student's *t*-test, $p < 0.01$) during the exponential growth than the stationary phase. These higher intracellular STX concentrations make this species more hazardous during exponential growth. Similarly, Yunes et al. (2009) also reported lower toxicity and STX concentrations normalised to biovolume for *R. brookii* in the stationary phase. The reverse situation, saxitoxins detected at higher quota levels during the

stationary phase, was reported for *Aphanizomenon* sp. (Dias et al., 2002) and *Cylindrospermopsis raciborskii* (Carneiro et al., 2009).

Variability of toxin quota throughout the growth cycle has also been reported for dinoflagellates. Saxitoxin production can be correlated or uncorrelated with growth depending on culturing conditions (Anderson et al., 1990). Concentration or toxicity of STX and variants per cell can peak in the early-mid exponential growth phase (Boczar et al., 1988) or late exponential/stationary phase (Hwang and Lu, 2000; Lim et al., 2006).

Similar variations in toxin quota with growth were also described for other cyanotoxin-producing strains with contradictory results reported. Saker and Griffiths (2000) reported *C. raciborskii* strains produced highest total cylindrospermopsin per dry weight during the exponential growth phase. Hawkins et al. (2001), however, reported the same species produced the highest cylindrospermopsin production on a dry weight basis during the stationary phase.

Pomati et al. (2004b) described an exponential relationship between STX production and higher pH values in *C. raciborskii* strain T3. Pomati et al. (2004b) reported a 10-fold increase in STX production corresponding to an increase from pH 9.5 to 10. In the current experiment, high pH values (pH 9.2–10.6), occurred during the stationary phase of UCFS10 when STX production was lowest. However, in the study by Pomati et al. (2004b) STX production was normalised to optical density and the variation in pH was applied to cultures in the same stage of growth. Unlike Pomati et al. (2004b) the pH variation in the current *S.* cf. *crispum* experiment was due to biological factors altering pH, allowing the cultures to adapt to pH variation. In the current study, pH was not a significant factor (Table 5.8), so it is unlikely to have had an effect on STX production. There was also no significant effect on STX quota from the interaction factor between pH and days during the stationary phase when pH was high. These results contradict Pomati et al.'s (2004b) theory that STX is involved in regulating pH homeostasis within the cell. If increasing pH does have increase intracellular STX production then that hypothesis may not be true for cultures that adapt to change in pH over time, or cell pH requirements for *S.* cf. *crispum* UCFS10 differ from *C. raciborskii* strain T3.

The amount of STX present in *S.* cf. *crispum* UCFS10 was not proportional to the number of cells during exponential growth. This observation could be due to the STX quota reflecting net STX, i.e. total STX produced less the amount used or degraded. These factors could vary with STX production and individual cell requirements over time. *Scytonema* cf. *crispum* grows much slower than other STX-producing cyanobacteria (Section 5.4.1). Therefore, it is more likely that net STX production depends on STX production, use and degradation inside the cells. STX production of *A. circinalis* is less likely to degrade over it's much shorter exponential growth phase, and net cyanotoxin production is more closely aligned with STX produced per cell.

Extracellular

Extracellular STX concentrations in *S.* cf. *crispum* UCFS10 cultures remained reasonably constant throughout the growth cycle (Figure 5.5). The initial high extracellular STX proportion (36 ± 15%) could be attributed to unintentional damage to cells through dividing UCFS10 into small portions of inoculate. This process may release higher levels of extracellular STX than under normal growth conditions. Saxitoxin and its derivatives are generally very stable in sterile fresh water at biologically relevant temperature (20–25 °C) and neutral to alkaline pH (7–10) with half-lives of approximately 3 weeks to 3 months (Jones and Negri, 1997; Pereira et al., 2002). In non-axenic conditions, degradation is faster with a half-life of 7 days for STX, and between 5 days and 4 weeks for other analogues at 20–25 °C (Jones and Negri, 1997; Pereira et al., 2002). After one week the initial release extracellular STX could still have an impact on total extracellular levels. However, the total concentration of extracellular STX increased consistently over the first few weeks from 3 ng mL^{-1} on Day 0 to 13.4 ng mL^{-1} on Day 30 (Figure 5.5), indicating extracellular STX was released throughout the exponential phase. Extracellular STX decreased to 6 ± 2% of total saxitoxins by Day 131 (Figure 5.5). This decrease in extracellular saxitoxin suggests that during stationary phase cell division and lysis little extracellular STX is released, suggesting the role of STX is predominantly intracellular. The percentage range of extracellular STX produced to total STX throughout the experiment is similar to that of *A. circinalis* (Negri et al., 1997, Velzeboer et al., 2001) and *C. raciborskii* (Pomati et al., 2004b). In another *C. raciborskii* study, extracellular neoSTX and STX concentrations fell below the detection limit (Carneiro et al., 2009). In *Aphanizomenon* sp. the percentage of

extracellular saxitoxins were similar to *S.* cf. *crispum* during the exponential phase, but rose to much higher levels (84%) under some conditions (Dias et al., 2002).

The highest proportion of extracellular STX compared to total intra- and extracellular STX occurred early in the growth cycle (Figure 5.5). In *A. circinalis*, *Aphanizomenon* sp. and *C. raciborskii* the opposite relationship between extracellular STX production and growth was reported (Castro et al., 2004; Dias et al., 2002; Negri et al., 1997). High extracellular STX production occurred in the late exponential and stationary phases (Castro et al., 2004; Dias et al., 2002; Negri et al., 1997). Similarly in cylindrospermopsin-producing *C. raciborskii*, a larger proportion of extracellular cyanotoxins occurred in the late exponential transition to the stationary phase (Hawkins et al., 2001; Saker and Griffiths, 2000). This difference in extracellular toxins behaviour may explain extracellular variation in cyanotoxin field observations. The lake water samples tested for saxitoxins near *Scytonema* cf. *crispum* proliferations were negative (Section 3.3.4). In contrast, cylindrospermopsin was detected near *C. raciborskii* blooms in Solomon Dam, Australia (Saker and Griffiths, 2000), and at a water storage facility at Hervey Bay, Australia (Chiswell et al., 1999). These data agree with culture studies where high percentages of extracellular cyanotoxins were released in the stationary phase of *C. raciborskii* (Hawkins et al., 2001; Saker and Griffiths, 2000) but not *S.* cf. *crispum* UCFS10 (Figure 5.5).

5.4.3 Future Studies

In this study, only UCFS10 was investigated to determine the variation in STX production over the growth cycle. Other *S.* cf. *crispum* strains should be investigated to identify if there is a change in the STX variants profile over time in strains capable of producing a number of STX analogues. Velzeboer et al. (2001) reported the dominant variant in STX profiles can change at different point of the growth cycle and production of STX variants may be completely suppressed under some conditions. In Section 3.3.4, *S.* cf. *crispum* strains had different saxitoxin profiles, and some variants of STX were only observed in environmental samples. Studying the growth cycle could confirm whether other strains completely suppress some STX variants as some stages of the growth cycle.

5.5 Conclusions

The method developed in this study was effective at monitoring *Scytonema* growth. It could be applied to any metaphytic cyanobacteria/algae where dense mats prevent traditional planktonic subsampling growth experiments.

No correlation was found between growth and STX production, determined as either cell-quota or gravimetrically. However, there was a correlation between cells concentrations and STX production during the stationary phase. During this phase STX-quota was around 81 fg $cell^{-1}$. As cells in the exponential growth phase had significantly higher STX concentrations, the toxicity of developing *S.* cf. *crispum* mats could pose a greater hazard than established mats where toxicity is lower. Extracellular STX concentrations were consistently present at low levels. The proportion of extracellular STX to total STX decreased throughout the experiment. Extracellular toxicity could also be more problematic for actively growing *S.* cf. *crispum*.

6 Conclusions and Recommendations

6.1 Summary and General Discussion

6.1.1 Identification of Benthic Cyanobacteria and Associated Cyanotoxins

The first aim of this thesis was to establish a culture collection of benthic cyanobacteria isolated from South Island freshwater bodies. One chroococcalean, 20 oscillatorialean and 14 nostocalean strains were isolated into culture. Subsequently, three *Scytonema* strains were lost after the February 2011 earthquake in Christchurch. The remaining 32 strains were banked in the Cawthron Institute Culture Collection of Micro-algae.

A polyphasic approach was used for identifying these strains. Generally morphological and phylogenetic analyses were in agreement at the genus level. *Scytonema* cf. *chiastum* and *S.* cf. *crispum*, however, did not cluster with the traditional *Scytonema* clade and instead clustered near other Nostocales: *Petalonema* and *Calothrix* respectively. The well-known difficulties in identifying *Phormidium* sp. were encountered during the polyphasic identification of 15 *P.* cf. *uncinatum* strains in this study. These strains were closer to *P. uncinatum* Gomont ex Gomont morphologically, but 16S rRNA identified these strains as closely related to strains identified as *P. autumnale* [Agardh] Trevisan ex Gomont. In addition, these strains were quite variable in cell dimensions, even among species with identical 16S rRNA sequences isolated from the same source material.

A variety of molecular genetic, biochemical, and analytical chemistry techniques were used to screen for cyanotoxin production. Genes involved in the production of cylindrospermopsins, microcystins, or nodularins were not identified in any strain isolated for this project. Genes associated with anatoxin-a/homoanatoxin-a biosynthesis were identified in nine out of ten *Phormidium* strains isolated from the single mat sample. Anatoxin-a concentrations in these strains ranged between 0.3 and 6.4 mg kg^{-1}. These variable results may explain the wide range in anatoxin-a concentrations previously measured in environmental samples in New Zealand. One strain also produced homoanatoxin-a (1 mg kg^{-1}). Five strains of *Scytonema* cf. *crispum* contained a saxitoxin biosynthesis gene. HPLC–FD identified saxitoxin and saxitoxin analogues in these strains including gonyautoxins, neosaxitoxin, and decarbamoyl derivatives. The concentration of saxitoxin identified in strains varied from 0.1 to 119 mg kg^{-1} freeze dried material. The

concentration of other saxitoxin analogues varied widely between strains. This study is the first identification of these compounds in New Zealand cyanobacterial strains. It is also the first identification of saxitoxins production by a *Scytonema* species.

Three PCR primer sets associated with the biosynthesis of anatoxin-a genes were trialled for detecting potential anatoxin-a/homoanatoxin-a producing ability. The ks2 primer set (Cadel-Six et al., 2009), designed for *Oscillatoria* strains, worked well for *Phormidium*. However, other studies have indicated that these primers are not applicable to some anatoxin-a producers. The atxoaf/atxar primer set (Ballot et al., 2010a) identified potential anatoxin-a production in *Phormidium* strains. The use of these PCR primers exhibited non-specific binding in the current study, making interpretation of the gel difficult. The generic *anaC* primers (Rantala-Ylinen et al., 2011) were the most applicable for identifying potential anatoxin-a biosythesis, giving positive and negative results identical to LC–MS/MS analyses of the corresponding strain.

Scytonema distribution in Canterbury recreational lakes was investigated. Three morphospecies were identified: *S.* cf. *chiastum*, *S.* cf. *crispum*, and *S.* cf. *fritschii*. Genetic screening and biochemical assays (Jellett PSP Rapid Test) identified the *sxtA* gene and saxitoxins only in environmental samples containing *S.* cf. *crispum* or cultures of this species. The saxitoxin-producing strains were isolated from sheltered areas of mesotrophic, oligotrophic, and eutrophic lakes and reservoirs. HPLC–FD further confirmed and quantified saxitoxin production in these samples. Variants GTX1/4, GTX5, and neoSTX were identified in some environmental samples but not in the corresponding strains. This variation between environmental samples and strains suggests that, similar to anatoxin-a production, saxitoxin production variability may also exist between *Scytonema* strains within a single mat. GTX1/4, GTX5, and neoSTX were all detected in the *S.* cf. *crispum* strains isolated from the drinking water reservoir, confirming the likely origin of these cyanotoxins is from this species.

6.1.2 Growth and Cyanotoxin Production Studies in Benthic Cyanobacteria

The methods developed in this thesis for monitoring growth of benthic species were applied to both periphytic and metaphytic cyanobacteria. These methods can also be utilised to monitor a broad range of algae, where traditional planktonic subsampling of

cultures is not effective. The initial method for cyanotoxin sampling was improved during the *Scytonema* experiment. These improvements enabled cyanotoxin quota to be directly correlated to individual cultures by subdividing each culture sample for growth and toxin analysis.

In the environmental stressor experiment, both iron and copper had a significant effect on growth. Low iron and high copper concentrations inhibited growth. However no significant change was detected in cyanotoxin quota. Anatoxin-a quota maxima were consistently identified in the late-lag to early exponential phase of *Phormidium autumnale* strain CYN52 growth. Therefore, early proliferations of *P. autumnale* may be more toxic than well-established mats. However, because of the high biomass of mats in the stationary phase of growth, considerable cyanotoxins can be present, particularly when dominated by cyanotoxin producing *Phormidium* strains. Usually it is the well-established mats that attract dogs and cause fatalities.

In the *Scytonema* cf. *crispum* UCFS10 growth experiment, cyanotoxin quota remained high across the exponential growth phase. A significant decrease in saxitoxin quota occurred during the late exponential and stationary phases. Therefore, proliferations of *S.* cf. *crispum* can be more toxic while establishing than older, well-formed mats.

6.2 Recommendations

6.2.1 Monitoring for Cyanotoxins in Benthic Species

Identification of toxic benthic cyanobacteria from water bodies used for recreation and a pre-treatment drinking water reservoir, highlights the risk benthic cyanobacteria pose to human and other animal health. Cyanobacterial monitoring programmes should include sampling and assessments of benthic environments. Metaphyton are often overlooked, but this study indicates that metaphytic cyanobacteria can also be toxic and should be included in future monitoring regimes developed by local councils and regulatory agencies. The calm, sheltered conditions of lakes, where metaphyton and periphyton can accumulate, provide fairly stable habitats for algae year-round. Therefore, information signs for benthic cyanotoxin-producers in lakes (Appendix 8.5; Smith et al., 2011a) should be permanently displayed in accordance with the persistent nature of these benthic proliferations.

Prior to this study, *Scytonema* was not known to be a saxitoxin-producer. Several of the other genera isolated in this study are known to produce cyanotoxins elsewhere. However, the strains under investigation were either nontoxic or may produce a toxin that was not screened for during this study. For example, a potent cytotoxin was recently identified in a *Limnothrix*-like or *Geitlerinema*-like species. The authors named this toxin "Limnothrixin" (Bernard et al., 2011; Humpage et al., 2012). When the structure of this toxin is elucidated, other Pseudanabaenaceae species including *Geilterinema*, *Pseudanabaena*, and *Leptolyngbya* strains isolated in this study should be screened for "Limnothrixin". As studies on benthic cyanobacteria increase it is likely that more toxin producers will be identified. All benthic cyanobacterial genera should be considered as potentially toxic until proven otherwise.

Benthic cyanobacterial mats are frequently identified in New Zealand rivers, and reports of dog fatalities are not uncommon. *Phormidium* spp. must be monitored as early as possible, since trichomes are most toxic during the early growth phase. *Phormidium* proliferations are dependent on low flows, posing a more seasonal problem than *Scytonema* cf. *crispum*.

6.3 Future Work

Many species of benthic algae have not been tested for cyanotoxin production. Consequently, they cannot be ruled out as potentially toxic species. Until this study, *Scytonema* was not known to produce saxitoxins, and there was no evidence for concern regarding these neurotoxins in New Zealand freshwater lakes. Large proliferations of cyanobacteria can contain substantial concentrations of cyanotoxins. Historically, cyanotoxin-producing species in New Zealand were only recognised after animal poisonings indicated their presence. Monitoring of prolific cyanobacteria must continue to identify problematic species and prevent unexpected outbreaks of cyanotoxin poisoning.

Scytonema cf. *crispum* is currently the only species of this genus proven to produce saxitoxin. There could be related species of *Scytonema* that also produce saxitoxins. The other species of *Scytonema* identified did not contain any of the cyanotoxins genes investigated, nor was the molecular phylogeny of these species closely related to *S.* cf. *crispum*. *Scytonema* is a large cosmopolitan genus and these studies indicate that it must be re-evaluated using polyphasic analyses, as it does not form a monophyletic clade. Other *Scytonema* species could also contain saxitoxins, and should be screened for cyanotoxins

when identified; particularly if they are closely related to *S.* cf. *crispum* in molecular phylogeny.

Further growth experiments investigating saxitoxin production by *S.* cf. *crispum* should select a species that produces a number of saxitoxin variants. Investigations on strains UCFS16, UCFS17, and UCFS21 will reveal whether some analogues identified in environmental samples were supressed at certain growth stages, or not produced at all in these particular strains. Future studies might also identify any changes in dominant saxitoxin analogues throughout the growth cycle.

Environmental stressors that directly influence anatoxin-a and homoanatoxin-a production were not identified in this study. Further studies on anatoxin-a and homoanatoxin-a production by *P. autumnale* are required to provide better tools for managing these species in New Zealand. The methods provided here can be adapted for investigating common stressors on *Phormidium.* Current work at Victoria University of Wellington is investigating the effects of nutrients on *P. autumnale* (Heath, 2012).

Most environmental stressor studies do not take into account a combination of different cyanotoxins produced or exposure to multiple environmental stressors. Hepatotoxic and neurotoxic cyanobacteria have been reported to co-occur in the same habitats (Graham et al., 2004; Sivonen et al., 1990). This co-occurrence of cyanotoxin-producers was true of the majority of lakes surveyed in mid-western States of America (Graham et al., 2004). The next step in investigations into benthic cyanobacteria should investigate the influence of multiple stressors on cyanotoxin production.

This thesis has highlighted that benthic cyanobacteria are an issue in a range of freshwater aquatic habitats. It has described possibilities for future research on benthic periphytic and methaphytic cyanobacteria. Finally this thesis has illustrated that the highest cyanotoxin quota occur at the beginning or during exponential growth phase in *P. autumnale* and *S.* cf. *crispum* respectively. For now benthic cyanobacteria still pose a health hazard in many rivers. Future research building on this thesis may lead to prevention, mitigation, or prediction of benthic cyanotoxic proliferations in New Zealand.

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8 Appendices

Appendix 8.1: Datasheet for *Scytonema* survey

Field sampling sheet

Sample Information:

Date: ...../...../.....Time: .....

Sample location (detailed – include GPS):.....
.....
Sampling method (no. of samples, composite or grab): .....
Depth of samples (m):.....Distance from shore (m): .....

Water Use:

Recreational activities (eg, swimming/sailing/fishing): .....
Other (please state):.....

Weather Conditions:

Weather at time of collection (circle): Clear Drizzling Light rain Moderate rain Heavy rain
Cloud cover: 1−8 (1 = clear):.....**Wind strength:** 1−8 (1 = calm): ..... **Wind direction**:.....
Weather conditions for 24 hours prior to sampling: .....

Algal Information:

What percentage of the water body does the bloom/mat/metaphyton cover: .....
What colour is the bloom/mat?..... Is there any distinctive smell? .....
Any water discolouration (what colour?): .....
Are there any signs of animal/human poisonings (eg, dead birds, fish, stock, rashes on swimmers):
.....
.....

Limnological Data:

Max. water depth:..... Ave. water depth: .... Secchi depth: .....
Area of water body: .....Water temperature: ....
Dissolved Oxygen:……..……mg/L……..……% pH.....
Water discolouration:……. Shade cover (%).....
Predominant catchment cover (eg, farmland): .....
What wildlife is present on or in water body (eg, trout/ducks):.....
.....
Bank Vegetations (this can be covered by photograph):……..

Photographs:

(Photograph ID and brief description, e.g. survey area, metaphyton mats) .....
.....
.....
.....
.....
.....

Samples Collected:

- ☐ Cyanobacterial identification (Lugol's preserved)
- ☐ Cyanobacterial identification (unpreserved, keep cool)
- ☐ Extracellular cyanotoxin identification (unpreserved, filter, freeze on return)
- ☐ Intracellular cyanotoxin identification (unpreserved, freeze on return)

Additional Comments:

……
……
……
……
……
……
……

Figure 8.1 Datasheet used in *Scytonema* survey, adapted from the *New Zealand Guidelines for Cyanobacteria in Recreational Fresh Waters* (Ministry for the Environment and Ministry of Health, 2009).

Appendix 8.2: Genera identified in *Scytonema* survey

Table 8.1 Genera identified in *Scytonema* survey.

Genera		Source	Coopers Lagoon	Kaiapoi Lakes	Kelland Pond	Lake Alexandrina	Lake Avienmore	Lake Benmore	Lake Camp	Lake Clearwater	Lake Coleridge	Lake Ellesmere	Lake Emma	Lake Evelyn	Lake Forsyth	Lake Georgina	Lake Grasmere	Lake Hawdon	Lake Heron
	Cyanobacteria	*Anabaena /Trichormus*		✓	✓	✓													
		Coleodesmium					✓												✓
		Dichothrix									✓					✓			
		Leptolyngbya									✓			✓	✓	✓			
		Nostoc				✓		✓											
		Lyngbya/Phormidium/Oscillatoria	✓				✓	✓			✓		✓			✓	✓	✓	
		Pseudanabaena						✓			✓								✓
		Scytonema			✓	✓		✓		✓								✓	
		Tolypothrix				✓	✓	✓											
		Chamaesiphon									✓		✓						
		unicellular cyanobacteria		✓						✓	✓		✓						
		unidentified filamentous		✓		✓					✓	✓	✓	✓				✓	
	Bacillariophyta	*Didymosphenia*					✓												
		Encyonema					✓	✓											✓
		Epithemia											✓					✓	✓
		Gomphonema/Gomphoneis	✓	✓		✓		✓			✓		✓	✓	✓	✓	✓		✓
		Melosira	✓																
		Tabellaria			✓		✓	✓		✓	✓			✓		✓			✓
		unidentified epiphytic diatoms	✓	✓	✓		✓			✓		✓	✓		✓	✓			
		unidentified diatoms	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Chlorophyta	*Bulbochaete*											✓	✓		✓			
		Chaetophora											✓						✓
		Cladophora													✓				
		Closterium																	
		Geminella																	
		Gloeocystis /unidentified colonial	✓																
		Klebsormidium					✓	✓					✓	✓		✓			
		Microspora				✓													
		Mougeotia				✓		✓	✓		✓					✓	✓	✓	✓
		Oedogonium						✓				✓							
		Scenedesmus	✓	✓								✓							
		Spirogyra	✓	✓		✓		✓								✓	✓	✓	✓
		Stigeoclonium									✓								
		Zygnema	✓	✓				✓			✓			✓		✓	✓	✓	
		unidentified green algae		✓		✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓
	Other	*Dinobryon*							✓	✓									
		Vaucheria										✓							

Table 8.1 continued.

Source	Genera		
	Cyanobacteria	**Bacillariophyta**	**Chlorophyta**	**Other**
Lake Hood	*Anabaena*/*Trichormus*, *Leptolyngbya*, *Lyngbya/Phormidium/Oscillatoria*, *Pseudanabaena*, unidentified filamentous	*Encyonema*, *Tabellaria*, unidentified epiphytic diatoms, unidentified diatoms	unidentified green algae	
Lake Lyndon	*Leptolyngbya*, unidentified diatoms			
Lake McGregor	*Coleodesmium*, *Lyngbya/Phormidium/Oscillatoria*, *Pseudanabaena*, *Scytonema*, unidentified filamentous	*Encyonema*, *Epithemia*, *Gomphonema*/*Gomphoneis*, unidentified epiphytic diatoms, unidentified diatoms	*Closterium*, *Gloeocystis*/unidentified colonial, *Mougeotia*, *Stigeoclonium*, unidentified green algae	
Lake Middleton	*Anabaena*/*Trichormus*, *Scytonema*, unidentified filamentous	*Gomphonema*/*Gomphoneis*, *Tabellaria*, unidentified epiphytic diatoms, unidentified diatoms	*Closterium*, *Gloeocystis*/unidentified colonial, *Mougeotia*	
Lake Ohau	*Coleodesmium*, *Scytonema*	*Didymosphenia*, *Tabellaria*, unidentified diatoms		
Lake Opuha	*Leptolyngbya*, *Lyngbya/Phormidium/Oscillatoria*, unidentified filamentous	*Tabellaria*, unidentified epiphytic diatoms, unidentified diatoms	*Gloeocystis*/unidentified colonial, *Mougeotia*, *Scenedesmus*, *Spirogyra*, *Zygnema*, unidentified green algae	
Lake Pearson	unidentified filamentous	unidentified epiphytic diatoms, unidentified diatoms	*Geminella*, *Gloeocystis*/unidentified colonial, *Klebsormidium*, *Mougeotia*, *Spirogyra*, *Zygnema*, unidentified green algae	*Dinobryon*
Lake Pukaki	no algae observed to collect			
Lake Roto				
Kohatu	*Lyngbya/Phormidium/Oscillatoria*, *Pseudanabaena*, unicellular cyanobacteria	*Encyonema*, *Epithemia*, *Gomphonema*/*Gomphoneis*, *Tabellaria*, unidentified epiphytic diatoms, unidentified diatoms	*Gloeocystis*/unidentified colonial, *Scenedesmus*, unidentified green algae	
Lake Rotorua	*Anabaena*/*Trichormus*, *Coleodesmium*, *Leptolyngbya*, unidentified filamentous	unidentified epiphytic diatoms, unidentified diatoms	*Oedogonium*, unidentified green algae	
Lake Ruataniwha	*Leptolyngbya*, *Lyngbya/Phormidium/Oscillatoria*, *Scytonema*	*Encyonema*, *Epithemia*, *Tabellaria*, unidentified diatoms	*Mougeotia*, *Spirogyra*, *Zygnema*	
Lake Sarah	*Lyngbya/Phormidium/Oscillatoria*, *Scytonema*, *Tolypothrix*, unicellular cyanobacteria, unidentified filamentous	*Epithemia*, *Gomphonema*/*Gomphoneis*, *Tabellaria*, unidentified epiphytic diatoms, unidentified diatoms	*Gloeocystis*/unidentified colonial, *Mougeotia*, *Spirogyra*, *Zygnema*, unidentified green algae	*Vaucheria*
Lake Selfe	*Lyngbya/Phormidium/Oscillatoria*, unidentified filamentous	*Didymosphenia*, *Encyonema*, *Epithemia*, *Gomphonema*/*Gomphoneis*, *Tabellaria*, unidentified epiphytic diatoms, unidentified diatoms	*Bulbochaete*, *Gloeocystis*/unidentified colonial, *Klebsormidium*, *Mougeotia*, *Oedogonium*, *Spirogyra*, *Zygnema*, unidentified green algae	
Lake Taylor	*Tolypothrix*, unidentified filamentous	*Gomphonema*/*Gomphoneis*, *Melosira*, unidentified diatoms	unidentified green algae	
Lake Tekapo	unidentified diatoms	unidentified diatoms	*Spirogyra*, unidentified green algae	
St Annes Lagoon	unidentified diatoms	unidentified epiphytic diatoms, unidentified diatoms	*Oedogonium*, unidentified green algae	
Styx Mill Reserve	*Anabaena*/*Trichormus*, *Lyngbya/Phormidium/Oscillatoria*, *Pseudanabaena*, unidentified filamentous	*Gomphonema*/*Gomphoneis*, *Melosira*, unidentified diatoms	unidentified green algae	

Appendix 8.3: Nutrient data from *Phormidium autumnale* growth experiment

Table 8.2 Nutrient analysis (PO_4-P) of culture media in Fe/Cu stressor experiment.

Day	$MLA_{1\times Fe=1\times Cu}$	$MLA_{10\times Cu}$	$MLA_{100\times Cu}$	$MLA_{0.1\times Fe}$	$MLA_{2\times Fe}$
0	74.5 ± 0.1	62.1 ± 0.3	66.2 ± 0.0	69.6 ± 0.3	81.2 ± 0.2
3	70.4 ± 0.6	41.5 ± 0.1	56.3 ± 0.7	72.3 ± 3.2	82.7 ± 0.6
6	75.8 ± 0.5	59.2 ± 0.2	62.5 ± 0.4	69.5 ± 0.7	84.7 ± 0.1
9	57.6 ± 0.3	61.2 ± 0.3	68.9 ± 1.0	71.6 ± 0.3	83.7 ± 0.2
12	73.6 ± 0.2	58.7 ± 0.8	68.5 ± 0.2	71.6 ± 0.3	81.3 ± 0.6
17	73.3 ± 0.7	60.1 ± 0.5	66.3 ± 0.5	71.5 ± 0.7	83.2 ± 0.9
22	75.7 ± 0.3	62.7 ± 1.2	61.6 ± 0.7	70.3 ± 0.4	84.0 ± 0.5
32	74.9 ± 0.4	65.3 ± 0.2	73.9 ± 0.1	72.7 ± 0.6	85.3 ± 1.2
41	78.2 ± 0.6	40.6 ± 0.7	71.9 ± 0.6	78. 5 ± 0.4	69.4 ± 0.2
49	59.1 ± 0.2	60.3 ± 0.1	73.2 ± 0.7	77.0 ± 0.4	76.3 ± 0.4

Table 8.3 Nutrient analysis (PO_4-P) of media controls in Fe/Cu stressor experiment.

Day	$MLA_{1\times Fe=1\times Cu}$	$MLA_{10\times Cu}$	$MLA_{100\times Cu}$	$MLA_{0.1\times Fe}$	$MLA_{2\times Fe}$
0	71.9 ± 0.7	59.9 ± 1.1	66.3 ± 1.0	70.2 ± 0.6	76.8 ± 0.1
6	74.3 ± 0.3	57.4 ± 1.0	67.8 ± 0.7	71.4 ± 0.4	82.7 ± 0.2
17	60.2 ± 0.4	60.1 ± 0.25	67.3 ± 0.7	72.5 ± 0.3	83.3 ± 1.1
32	75.1 ± 0.1	64.6 ± 0.2	69.1 ± 0.4	72.1 ± 0.0	84.0 ± 0.8
49	75.1 ± 0.1	54.7 ± 1.3	75.1 ± 1.6	65.4 ± 0.3	86.1 ± 0.8

Table 8.4 Nutrient analysis (NO_3-N +NO_2-N) of culture media in Fe/Cu stressor experiment.

Day	$MLA_{1\times Fe=1\times Cu}$	$MLA_{10\times Cu}$	$MLA_{100\times Cu}$	$MLA_{0.1\times Fe}$	$MLA_{2\times Fe}$
0	20.0 ± 1.2	26.3 ± 2.1	29.4 ± 0.3	26.9 ± 1.0	30.0 ± 0.5
3	25.1 ± 0.05	21.2 ± 0.6	22.3 ± 0.7	28.1 ± 0.1	33.8 ± 1.0
6	31.0 ± 0.6	24.3 ± 1.5	24.6 ± 0.3	29.0 ± 1.3	31.4 ± 0.5
9	18.1 ± 0.4	28.4 ± 0.0	30.3 ± 0.3	23.9 ± 0.3	31.4 ± 0.1
12	25.0 ± 0.4	27.9 ± 0.9	32.2 ± 0.7	28.6 ± 0.6	22.4 ± 0.2
17	23.3 ± 0.3	24.16 ± 1.3	27.8 ± 0. 5	38.8 ± 6.0	20.1 ± 0.1
22	23.4 ± 0.0	24.0 ± 0.2	27.0 ± 2.0	29.7 ± 5.8	16.8 ± 0.1
32	22.9 ± 0.4	21.3 ± 0. 5	21.1 ± 5.1	35.9 ± 9.6	20.15 ± 0.4
41	21.0 ± 0.3	12.9 ± 0.4	24.3 ± 0.1	25.2 ± 0.7	14.4 ± 0.3
49	14.4 ± 1.0	17.3 ± 0.4	25.0 ± 0.8	25.5 ± 0.7	12.9 ± 0.3

Table 8.5 Nutrient analysis (NO_3-N +NO_2-N) of media controls in Fe/Cu stressor experiment.

Day	**$MLA_{1\times Fe=1\times Cu}$**	**$MLA_{10\times Cu}$**	**$MLA_{100\times Cu}$**	**$MLA_{0.1\times Fe}$**	**$MLA_{2\times Fe}$**
0	28.3 ± 0.7	28.3 ± 0.3	28.7 ± 1. 8	27.5 ± 0.1	31.6 ± 0.5
6	27.7 ± 0.1	28.4 ± 0.1	30.4 ± 1. 5	28.8 ± 1.8	30.4 ± 4.7
17	19.9 ± 0.5	30.8 ± 1.3	31.6 ± 0.1	24.2 ± 2.6	31.6 ± 4.3
32	26.5 ± 0.4	30.8 ± 1.4	27.6 ± 1.1	22.4 ± 9.0	34.3 ± 3.4
49	27.4 ± 0.5	24.4 ± 0.5	28.8 ± 1.5	31.3 ± 7.1	31.6 ± 0.1

Appendix 8.4: Nutrient data from *Scytonema* growth experiment

Table 8.6 Nutrient analysis of culture media in *Scytonema* growth experiment.*

Day	DRP	NH_3-N	NO_3-N	NO_2-N	DIN
0	4.0	0.27	0.049	0.002	0.32
7	4.8	0.038	0.016	<0.001	0.054
14	4.6	0.024	0.002	0.003	0.029
21	4.6	0.039	0.031	0.004	0.074
30	4.0	0.016	0.007	0.001	0.024
35	3.2	0.032	0.008	<0.001	0.040
42	3.4	0.027	0.005	0.002	0.034
52	2.4	0.075	0.007	0.003	0.085
57	2.4	0.034	0.015	0.002	0.051
65	1.7	0.043	0.004	0.003	0.050
72	0.51	0.19	0.29	0.007	0.49
80	0.16	0.11	0.071	0.008	0.19
87	0.41	0.096	0.13	0.004	0.23
98	0.11	0.25	0.023	0.018	0.29
106	0.08	0.14	0.002	0.014	0.16
111	0.14	0.028	<0.002	0.024	0.052
119	0.07	0.11	0.022	0.011	0.14
125	0.04	0.097	0.026	0.009	0.13
131	0.09	0.16	0.19	0.018	0.37

* DRP, dissolved reactive phosphorus; NH_3-N, nitrogen concentration from ammonia; NO_3-N, nitrogen concentration from nitrate; NO_2-N, nitrogen concentration from nitrite; DIN dissolved inorganic nitrogen. All units in ppm.

Table 8.7 Nutrient analysis of control media in *Scytonema* growth experiment.*

Day	DRP	NH_3-N	NO_3-N	NO_2-N	DIN
0	3.7	0.29	0.037	0.001	0.33
42	5.1	0.078	0.063	0.006	0.15
87	5.4	0.026	0.027	0.005	0.058
131	4.8	0.012	0.010	0.001	0.023

† DRP, dissolved reactive phosphorus; NH_3-N, nitrogen concentration from ammonia; NO_3-N, nitrogen concentration from nitrate; NO_2-N, nitrogen concentration from nitrite; DIN dissolved inorganic nitrogen. All units in ppm.

Appendix 8.5: Information Sign

Figure 8.2 Public information sign from The Groynes recreational reserve, highlighting the health hazards associated with saxitoxin production in algae at these lakes.

Appendix 8.6: Research Outputs (to date) Arising from this Thesis

8.6.1 Publications

Smith, F. M. J.; Wood, S. A.; Wilks, T.; Kelly, D.; Broady, P. A.; Williamson, W.; Gaw, S., Survey of *Scytonema* (Cyanobacteria) and associated saxitoxins in the littoral zone of recreational lakes in Canterbury (New Zealand). *Phycologia* **2012,** doi:10.2216/11-84.1.

Smith, F. M. J.; Kelly, D.; Wilks, T.; Broady, P. A.; Gaw, S., *Distribution of Scytonema (Cyanobacteria) and associated saxitoxins in recreational lakes in Canterbury*; R11/36; Environment Canterbury Technical Report: **2011,** p 18.

Smith, F. M. J.; Wood, S. A.; van Ginkel, R.; Broady, P. A.; Gaw, S., First report of saxitoxin production by a species of the freshwater benthic cyanobacterium, *Scytonema* Agardh. *Toxicon* **2011,** *57*, 566-573.

Smith, F., Effects of metal stressors on cyanotoxin production. *Chemistry Education in New Zealand* **2010,** *121*, 2-7.

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Survey of Scytonema (Cyanobacteria) and associated saxitoxins in the littoral zone of recreational lakes in Canterbury (New Zealand)

FRANCINE M. J. SMITH[1]*, SUSANNA A. WOOD[2,3], TARYN WILKS[4], DAVID KELLY[4], PAUL A. BROADY[1], WENDY WILLIAMSON[5] AND SALLY GAW[1]

[1]Department of Chemistry and School of Biological Sciences, Canterbury University, Private Bag 4800, Christchurch, 8140, New Zealand
[2]Cawthron Institute, Nelson, New Zealand
[3]School of Biological Sciences, Waikato University, Hamilton, New Zealand
[4]Environment Canterbury, Christchurch, New Zealand
[5]Institute of Environmental Science and Research Ltd, Christchurch, New Zealand

SMITH F.M.J., WOOD S.A., WILKS T., KELLY D., BROADY P.A., WILLIAMSON W. AND GAW S. 2012. Survey of Scytonema (Cyanobacteria) and associated saxitoxins in the littoral zone of recreational lakes in Canterbury (New Zealand). Phycologia 51: 00–00. DOI: 10.2216/11-84.1

; The recent identification of saxitoxin-producing Scytonema cf. crispum triggered a survey of metaphyton and periphyton for Scytonema spp. in 34 high-use recreational lakes across Canterbury, New Zealand. Scytonema was observed in 10 of the lakes surveyed. Three morphospecies were identified: S. cf. crispum, Scytonema cf. chiastum and Scytonema cf. fritschii. Environmental samples containing Scytonema were analysed for saxitoxins using the Jellett rapid test for paralytic shellfish poisoning, and saxitoxin variants were identified in positive samples using high-performance liquid chromatography with fluorescence detection (HPLC–FD). Cultures were established from selected sites and their phylogeny investigated using partial 16S rRNA gene sequences. These cultures were also screened for a region of sxtA, a gene involved in saxitoxin production. Cultures containing the sxtA gene were analysed for saxitoxins with HPLC–FD. Saxitoxins were only identified in cultures of S. cf. crispum and environmental samples containing this species. HPLC–FD analysis of these environmental samples and cultures identified saxitoxin and the variants gonyautoxins (GTX1–5), neosaxitoxin, decarbamoyl saxitoxin and decarbamoyl gonyautoxins (dcGTX2/3). This was the first report of these saxitoxin variants from cyanobacteria in New Zealand. All cultures of S. cf. crispum contained the sxtA gene segment. The partial 16S rRNA gene sequence of Scytonema C. Agardh ex Bornet & Flahault cultures were compared with cyanobacterial sequences from GenBank, only S. cf. fritschii clustered amongst other Scytonema species. The identification of metaphytic saxitoxin-producing S. cf. crispum highlighted a new freshwater habitat where toxic cyanobacteria may need to be monitored.

KEY WORDS: Blue-green algae, Metaphyton, Paralytic shellfish poisoning toxins, PSP, sxtA gene, 16S rRNA

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First report of saxitoxin production by a species of the freshwater benthic cyanobacterium, Scytonema Agardh

Francine M.J. Smith [a,*], Susanna A. Wood [b], Roel van Ginkel [b], Paul A. Broady [c], Sally Gaw[a]

[a] Department of Chemistry, Canterbury University, Private Bag 4800, Christchurch 8140, New Zealand
[b] Cawthron Institute, Private Bag 2, Nelson 7001, New Zealand
[c] School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand

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abstract

Saxitoxins or paralytic shellfish poisons (PSP) are neurotoxins produced by some species of freshwater cyanobacteria and marine dinoflagellates. Samples collected from the metaphyton of a drinking-water supply's pre-treatment reservoir and a small eutrophic lake in New Zealand returned positive results when screened using a Jellett PSP Rapid Test Kit. The dominant species in the sample was identified as Scytonema cf. crispum. A non-axenic clonal culture (UCFS10) was isolated from the lake. The partial 16S rRNA gene sequence shared only a 91% or less sequence similarity with other Scytonema species, indicating that it is unlikely that this genus is monophyletic and that further in-depth phylogenetic re-evaluation is required. The sxtA gene, which is known to be involved in saxitoxin production, was detected in UCFS10. Saxitoxin concentrations were determined from the lake samples and from UCFS10 using pre-column oxidation high performance liquid chromatography with fluorescence detection. Saxitoxin was the only variant detected and this was found at concentrations of 65.6 mg g_1 dry weight in the lake sample and 119.4 mg g_1 dry weight or 1.3 pg cell_1 in UCFS10. This is the first confirmation of a saxitoxin-producing species in New Zealand and the first report of saxitoxin production by a species of Scytonema.

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Effects of metal stressors on cyanotoxin production

Francine Smith

University of Canterbury (email: *francine.smith@pg.canterbury.ac.nz*)

Abstract

Proliferation of toxic cyanobacteria in waterways is a significant global problem. Environmental stressors, including metal toxicity, can affect algal growth and metabolism. There is a need to understand the influence of environmental stressors on the formation and regulation of both cyanobacterial blooms and toxin production. This review will investigate the effects of metal stressors on cyanotoxin production. Studies in this field have mainly focused on essential metals, in particular iron. These studies report contradictory results regarding the effects of stressors on cyanotoxin production.

Introduction

Toxic cyanobacteria cause significant health risks in recreational and drinking waterbodies, both in New Zealand and internationally. Cyanobacteria ("blue-green algae") are a highly diverse group of photosynthetic prokaryotes, including unicellular, colonial, branched or unbranched filamentous species (Fig. 1). Some filamentous species contain heterocysts, which are specialised cells for nitrogen fixation, and resistant spores called akinetes. They can be very versatile, tolerating a wide variety of environmental conditions and occupy a diverse range of habitats including freshwater lakes and rivers, thermal pools, hot and polar desert soils and rocks, brackish and marine waters (Mur *et al*., 1999). Cyanobacterial proliferations can produce potent toxins (cyanotoxins), degrade aesthetic values of water and congest waterways. However, as primary producers, cyanobacteria are an important part of the ecosystem, providing oxygen and nutrients to many organisms. Hazardous algal blooms are frequently reported in waterbodies. Exponential growth of algae is generally attributed to eutrophication of waterbodies due to input of nutrients from intensive land use (Heisler *et al*., 2008; Chorus *et al*., 1999).

Cyanotoxins include neurotoxins, hepatotoxins, dermatotoxins and gastrotoxins. The neuro- and hepatotoxins are highly potent and most research has focused on these two classes. Most cyanotoxins are found intracellularly during the growth stage of the cyanobacterial bloom. Sivonen & Jones (1999) report that extracellular toxins are generally only concentrated in the environment during the stationary phase of the bloom owing to the dissolution or destruction of the cell membrane, i.e., cell lysis.

Cyanobacterial neurotoxins (Fig. 2) include anatoxins, anatoxin-a(s) and saxitoxins. Anatoxin-a(s), anatoxin-a and derivatives are unique to cyanobacteria (Carmichael, 1994). In contrast, saxitoxins are also produced by eukaryotic algae (Carmichael, 1994). Saxitoxin and neosaxitoxin block sodium channels in the nervous system, preventing signal propagation from neurons to muscles. Under normal conditions, acetylcholine binds to a receptor causing the muscle to contract. Acetylcholine is then broken down by acetylcholine esterase, preventing excessive muscle stimulation. Anatoxin-a and homoanatoxin-a bind to the acetylcholine receptor but are not broken down by the esterase, whereas anatoxin-a(s) inhibit acetylcholine esterase (Carmichael, 1994; Kuiper-Goodman *et al*., 1999). These neurotoxins can lead to death by asphyxia owing to muscle paralysis (Carmichael, 1994).

Cyanobacterial hepatotoxins include microcystins, nodularins, and cylindrospermopsins (Fig. 3). Nodularins and microcystins are five- and seven-membered cyclic peptides, respectively: both these toxins inhibit protein phosphatase 1 and 2A (Kuiper-Goodman *et al*., 1999). Cylindrospermopsin is a cyclic alkaloid that inhibits glutathione and protein biosynthesis (Kuiper-Goodman *et al*., 1999). Microcystin-LR, named for the leucine and arginine side chains, is the most commonly reported cyanotoxin worldwide and also the most frequently studied (Kuiper-Goodman *et al*., 1999).

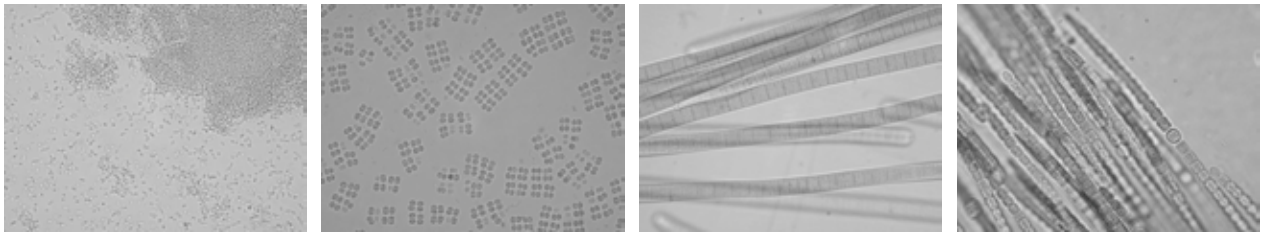

Fig. 1: *Morphological diversity of cyanobacteria increasing in complexity from left to right: unicellular, colonial, filamentous, filamentous with heterocysts. (Photomicrographs by F. Merican and F. Smith).*

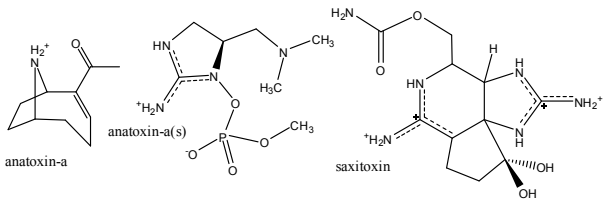

***Fig. 2:** Cyanobacterial neurotoxins.*

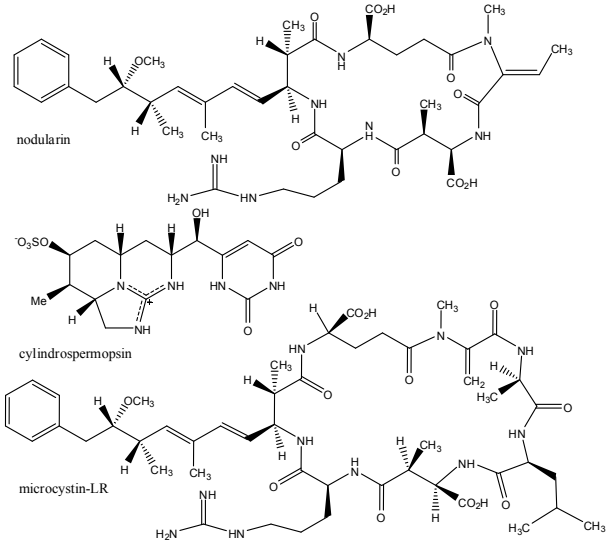

***Fig. 3:** Cyanobacterial hepatotoxins.*

Understanding the effects of stressors on cyanotoxin production is essential for regulating problematic algal blooms and for the management of recreational and drinking waterbodies. A variety of anthropogenic and environmental stressors, including nutrients (nitrogen and phosphorus), light, temperature, pH, salinity and metal stress, have been studied in relation to cyanotoxin production (Sivonen & Jones, 1999). This review focuses on how trace metals affect cyanotoxin production.

Stressors

Stressors can affect an algal bloom either directly, through regulating the metabolic pathways for toxin production, or indirectly by changing growth dynamics and altering the ratio of toxic to non-toxic species within a bloom (Amé & Wunderlin, 2005). Sources of anthropogenic stressors include industrial discharges, urbanisation, agriculture and horticulture.

Water treatment measures can also increase stressors within the aquatic environment. The use of iron sulphates to reduce phosphate levels can result in increasing iron concentrations (Li *et al*., 2009). Copper algicides, commonly used to control problematic blooms, are a potential stressor. Different algal species and strains require different quantities of copper algicides to kill off a bloom. However, even if enough copper is applied to destroy cyanotoxic blooms, this application can create a greater problem: in causing cells to lyse, copper releases large quantities of toxins into the environment, since the majority of cyanotoxins are found intracellularly (Sivonen & Jones, 1999). Copper algicides also have harmful effects on other aquatic biota, including bacteria that may assist in cyanotoxin biodegradation (Edwards & Lawton, 2009).

Microcystin production by *Microcystis* species is highly prevalent worldwide. Correspondingly, research into the influence of environmental factors on cyanobacterial growth and toxicity has focused predominately on this toxin and species (Sivonen & Jones, 1999). A limited number of studies has focused on other cyanotoxins, including nodularin, anatoxin, and cylindrospermopsin. A range of different species studied in relation to toxin production includes *Aphanizomenon*, *Oscillatoria*, *Planktothrix*, *Anabaena*, *Nodularia*, *Microcystis*, and *Cylindrospermopsis* (Sivonen & Jones, 1999). While optimal growth conditions appear to produce the greatest amount of toxins (Sivonen & Jones, 1999; Kaebernick & Neilan 2001) there are contradictory reports in the literature (Sivonen & Jones, 1999). It is difficult to compare stressor experiments carried out by various researchers owing to differences in experimental design, including toxin analysis, range of stressor analysed and culturing conditions (Kaebernick & Neilan, 2001; Bickel & Lyck, 2001).

Metal Stressors

Algae are able to absorb metals from the aquatic environment (Baptista & Vasconcelos, 2006). Essential trace metals, including iron, copper, nickel and zinc, are required in cellular processes, including nitrogen fixation, photosynthesis, and protein function (Baptista & Vasconcelos, 2006; Rueter & Petersen, 1987). However, non-essential metals or elevated concentrations of essential metals can be highly toxic to cells and can inhibit a diverse range of cellular processes (Baptista & Vasconcelos, 2006; Rueter & Petersen, 1987). Bioavailability of metals depends on a range of factors, including pH, the structure of the algal cell membrane and metal speciation (Baptista & Vasconcelos, 2006; Rueter & Petersen, 1987).

Lukač & Aegerter (1993) studied the effects of a range of B, Co, Cu, Mn, Zn, Fe, Al, Cd, Cr, Ni and Sn on microcystin production and growth in *Microcystis aeurginosa*. The majority of these trace elements had little or no effect on microcystin production. Iron and zinc had the greatest effect on toxin production as well as growth (Lukač & Aegerter, 1993). These are discussed in further detail in the following sections.

Iron

Iron is an essential element associated with many energy intensive cellular processes. It is a cofactor of electron transport pathways and enzymes. In cyanobacteria, iron complexes are essential for photosynthesis and nitrogen reduction processes (Baptista & Vasconcelos, 2006; Rueter & Petersen, 1987). As production of harmful radicals occurs during iron co-ordinated metabolic pathways, tight regulation of iron reactions within the cell to minimise the amount of oxidative damage is important (Baptista & Vasconcelos, 2006; Rueter & Petersen, 1987).

Iron deprivation has been reported to both increase and decrease cyanotoxin production (Table 1). In some experiments, iron availability was modified by altering the concentration of iron in the culture media, as well as by increasing concentrations of metal-chelators in the media (Lukač & Aegerter; Utkilen & Gjølme, 1995; Martin-Luna *et al*., 2006). Contradictory results were reported in 1995 and 1996 for microcystin production in relation to iron stress for experiments undertaken by the same research group with the same media and culturing conditions (Utkilen & Gjølme, 1995; Lyck *et al*., 1996). Bickel & Lyck (2001) reported a decrease in toxin production at lower concentrations of iron (0.5μM and 1μM) compared to 10μM iron. The focus of this study was the relationship between energy charge and microcystin production at three concentrations of iron or phosphorus. Bickel & Lyck (2001) did not comment on the reported increase in microcystin production from 1.97 to 7.83ng toxin μg^{-1} protein observed when iron concentration was decreased from 1 to 0.5μM iron. Utkilen & Gjølme (1995) observed a positive correlation of increasing light intensity with iron uptake over time. Iron uptake in toxin producing strains was notably stronger than in non-toxic strains under the same light conditions.

While iron stressor studies have mostly been undertaken on cultures of *Microcystis aeruginosa*, the effects of iron on cyanotoxin production has also been investigated in cultures of *Microcystis novacekii* (Li *et al*., 2009) and field populations dominated by *Microcystis* (Amé & Wunderlin, 2005; Amé *et al*., 2003). Less intracellular and extracellular microcystin was produced at low iron concentrations (0,

Table 1: *Laboratory studies into the effect of iron deprivation on cyanotoxin production.*

Cyanobacterial Strain	Toxin production correlation parameter(s)	Change in toxin production under Fe deprivation	Fe concentrations investigated (μM)	Culture Conditions	Publication
Microcystis aeruginosa PCC 7806	dry weight	increase	100, 62.5, 28, 12.5, 2.5, 0.5, 0.1, 0	BG11 medium Batch culture	Lukač & Aegerter, 1993
Microcystis aeruginosa CYA 228/1	dry weight protein content	decrease decrease	10, 0.8, 0.3	O2 medium Continuous culture	Utkilen & Gjølme, 1995
Microcystis aeruginosa CYA 228/1	dry weight protein content chlorophyll	inconsistent increase increase	10, 0	O2 medium Continuous culture	Lyck, et al. 1996
Microcystis aeruginosa CYA 228/1	dry weight protein content	decrease inconsistent	10, 1.5, 0.5	O2 medium Continuous culture	Bickel & Lyck, 2001
Microcystis field population	dry weight	increase	data not presented	n/a (field sample)	Amé, et al. 2003
Microcystis field population	cell protein content	decrease inconsistent	10, 1	Z8 medium Semi-continuous	Amé & Wunderlin, 2005
Microcystis aeruginosa PCC 7806	protein content	increase	30, 0.18	BG11 medium Batch cultures	Martin-Luna, et al. 2006
Microcystis aeruginosa PCC 7806	protein content	increase	30, 0	BG11 medium Batch cultures	Sevilla, et al. 2008
Microcystis novacekii UAM 250	dry weight volume of medium	decrease decrease	5, 0.5, 0.25, 0	BG11 medium Batch cultures	Li, et al. 2009

0.25 and 0.5µM) in a culture of *Microcystis novacekii* compared to the culture grown at 5µM iron (Li *et al*., 2009). Amé *et al*. (2003) observed a decrease in microcystin at high iron concentrations in a field study. Subsequently Amé & Wunderlin (2005) observed a different effect, an increase in microcystins at high iron concentrations, *in vitro* using field material dominated by wild *Microcystis* species in culture experiments. Amé & Wunderlin (2005) investigated only two iron concentrations (1 and 10µM). The effect of increasing the iron concentration from 1µM to 10µM caused a significant increase in total microcystins and proteins produced per cell, and an increase in growth rate. However, the ratio of microcystin to protein remained fairly constant at both iron concentrations (Amé & Wunderlin, 2005). Iron may be related to toxin concentration or growth parameters in algae. Changes in toxin concentration per cell observed in these experiments could be due to either a direct change in toxin regulation, or an indirect change by altering the ratio of toxic to non-toxic algae within the wild *Microcystis* culture (Amé & Wunderlin, 2005).

Microcystin Gene Cluster and Ferric Uptake Regulators

Molecular biology experiments may identify how iron proteins interact with the microcystin gene cluster. Microcystin biosynthesis is encoded by a hybrid NRPS-PKS gene cluster that transcribes bidirectionally from a promoter region between the *mcyD* and *mcyA* genes, and there is speculation that iron may regulate microcystin synthesis (Tillett *et al*., 2000). Ferric Uptake Regulators (FUR) are site-specific DNA-binding proteins associated with controlling iron availability within a cell and reducing oxidative stress (Hantke, 2001, Hernández *et al*., 2007). These FUR proteins are present in most prokaryotic organisms (Hantke, 2001, Hernández *et al*., 2007). Martin-Luna *et al*. (2006) screened the *mcy* gene cluster promoter regions of *Microcystis aeruginosa* for potential FUR-like sites. Two putative FUR binding sites, or FUR boxes, were identified in the bidirectional promoter region between *mcyD* and *mcyA* (Martin-Luna *et al*., 2006). Further potential FUR boxes have also been identified in other *mcy* genes (Martin-Luna *et al*., 2006). Binding assays suggested that FUR can bind to the bidirectional promoter region (Martin-Luna *et al*., 2006).

Sevilla *et al*. (2008) studied the effect of iron starvation on toxic and non-toxic *Microcystis* strains, monitoring *mcyD* transcription in the toxic strain using real-time reverse transcription (RT)-PCR. In agreement with Utkilen & Gjølme (1995), Sevilla *et al*. (2008) observed that the non-toxic strain was less tolerant of iron deprivation than the toxic strain. The toxic strain's intracellular-iron reserves were consumed within four days (Sevilla *et al*., 2008). RT-PCR experiments found that there was an induction of the *mcyD* transcription in iron-starved *M. aeruginosa* after four days when the intracellular iron stores had been exhausted (Sevilla *et al*., 2008). This up-regulation of *mcyD* transcription was consistent with an increased production of microcystin.

Copper and Zinc

Copper and zinc are essential elements and required in a variety of cellular processes including copper co-ordination in thylakoid membrane proteins and zinc as a co-factor in several enzymes (Baptista & Vasconcelos, 2006). A limited number of studies have investigated the effect of copper and zinc on microcystin production. Copper and zinc bind *in vitro* to microcystin with intermediate strength formation constants at pH 7.5 (Humble *et al*., 1997). Owing to this *in vitro* binding of copper and zinc to microcystin, Gouvêa *et al*. (2008) investigated these parameters in relation to microcystin production. In this study these authors also observed the effect of UV on microcystin production: while UV had the greatest effect on microcystin, an increase in copper also decreased the amount of toxin produced, zinc had minimal effect on microcystin production. Contrary to this finding, Lukač & Aegerter (1993) had earlier reported that under zinc limitation toxin yield increased.

Arsenic

Arsenic is not an essential element. Arsenic in the form of arsenate is thought to be taken up by the phosphate transport system owing to its structural resemblance to phosphate (Gong *et al*., 2009). Therefore, the concentration of arsenate available under phosphate-limited conditions can be of concern. Gong *et al*. (2009) studied the effect of a range of biologically significant arsenic concentrations on microcystin production under various phosphate regimes. Cultures were either pre-conditioned to phosphate starvation, or grown on phosphate-replete media before exposure to arsenate. These cultures were then exposed to arsenate at both phosphate-limited and phosphate-starved conditions. The cultures were tolerant of arsenate at all tested conditions (Gong *et al*., 2009). The stress the cultures were exposed to prior to the arsenate-stress was an important factor in the production of intra- and extra-cellular toxins. Cultures that were not preconditioned to phosphate starvation displayed a linear increase in intracellular microcystin with an exponential increase in arsenate concentrations (Gong *et al*., 2009). However, there were no significant changes in toxin produc-

tion for cultures preconditioned to phosphate starvation except at the highest arsenate concentration tested ($1x10^{-4}$ M). In these preconditioned cultures at $1x10^{-4}$ M arsenate concentrations, the concentrations of intracellular toxins were significantly lower compared to the control (Gong *et al*., 2009). Changes in extracellular toxins were generally insignificant except for the case of preconditioned cells to phosphate starvation that were exposed to arsenate under phosphate-limited conditions. In this case, there was a considerable increase from less than 20 μg L^{-1} to ~ 120 μg L^{-1} of extracellular microcystins in the culture media (Gong *et al*., 2009). Conditions of the cells before the stress is added affect how the culture responds to stress.

Discussion

Despite decades of research into environmental parameters and microcystin production, researchers have not established a correlation between metal stress and toxin production to help predict, prevent or minimise cyanotoxin occurrence. Results from metal stressor studies undertaken to date are contradictory. Environmental parameters have been shown to affect various species and strains differently (Baptista & Vasconcelos, 2006). In general, studies often report high toxin concentrations when environmental parameters for growth are favourable (Sivonen & Jones, 1999; Kaebernick & Neilan, 2001). However, no conclusive triggers have been identified from the environmental parameters studied to date (Kaebernick & Neilan, 2001).

It is important to understand how environmental parameters affect cyanotoxin production in order to manage waterbodies effectively. It is difficult to compare results from separate investigations due to the different experimental approaches that were used. The cyanobacterial strains, the condition of the culture prior to stress, variation in bacterial contamination, culture exposure time to the stressor, and quantities of stressor parameters studied are all likely to influence the results of exposure studies. Difference may also be attributed to analysis (including toxin analysis) and the growth parameters that toxin production is correlated with (including mass, chlorophyll-a, protein content and cell numbers). If the stressor affects the parameter chosen more than it affects toxin regulation there could be misleading results. To prevent this, toxin concentration should be compared to two or three growth parameters.

Experimental design should consider the stressor range as well as the initial condition of algae. The range of stressor quantities, including concentration, pH, salinity and irradiance, can be either broadly (Lukač & Aegerter, 1993; Gong *et al*., 2009) or narrowly studied (Amé & Wunderlin, 2005, Li *et al*., 2009). In some cases, conclusions have been reached after testing only two or three stressor quantities, or only whether the stressor was present or absent. The selected stressor quantities may not represent the degrees of stress that may occur in the environment. A range of several environmentally relevant degrees of stressors should be considered in experimental design. Previous stress conditions may have an important effect on the response of the organism to additional stress. Accordingly, both pre-stressed and non-stressed cultures should be included in the experimental design. Future experiments into the effects of stressors on cyanotoxin production require careful consideration of experimental design in order to form environmentally relevant conclusions that can assist in controlling and preventing hazardous algal blooms.

Cyanobacteria are ecologically and morphologically diverse. Extrapolating results of environmental effects of stressors from one species to predict the outcomes of the environmental parameters on another cyanobacterial species may be misleading, as effects can vary significantly amongst species. The majority of studies have focused on microcystin produced by axenic *Microcystis* strains with the addition of one or more stressors. Regulation of cyanotoxin production by heavy metal stress appears to have been investigated only in relation to microcystin production. However, in the environment there will be complex parameters involved in regulating a variety of stressors (Kaebernick & Neilan, 2001). Cyanobacteria species and strains can be either toxic or non-toxic (Sivonen & Jones, 1999). The influence of environmental factors on the whole community can alter the ratio of toxic to non-toxic algae. Studying the effects of multiple stress factors on a variety of species in relation to cyanotoxin production may provide a better overview of the effects of environmental stressors.

Conclusions

Metal stressors have not been comprehensively studied in relation to cyanotoxin production. The few studies in the literature have focused on microcystin production, predominantly by *Microcystis*; other toxins have been neglected. Whether the cyanobacteria have been pre-exposed to other stressors can considerably change the amount of intra- and extra-cellular toxins produced. Understanding the role of metal stressors from a water management perspective may assist in predicting and preventing toxic blooms.

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8.6.2 Seminars/Invited Talks

Smith, F., Investigating Cyanotoxin Production by Benthic Freshwater Cyanobacteria in NZ, Department of Chemistry Seminar, University of Canterbury, Christchurch, New Zealand. 27 July **2012.**

Smith, F.; Wood, S.; Broady, P.; Gaw, S.; Williamson, W.; Blunt, J.; Munro, M., Detection of cyanotoxins in New Zealand benthic cyanobacteria. Invited talk; Cawthron Institute, Nelson, New Zealand. 16 April **2012.**

Smith, F., Cyanobacteria in Selwyn River and Te Waihora/Lake Ellesmere. Waterways River Mouth Seminar; University of Canterbury, Christchurch, New Zealand. 5 August **2011**.

Smith, F.; Gaw, S.; Broady, P.; Blunt, J.; Munro, M.; Williamson, W.; Wood, S., Studies of toxin production in New Zealand benthic cyanobacterial mats. Invited talks; Environment Canterbury, Christchurch. 13 September **2010**; Environmental Science and Research Institute Ltd, Christchurch, New Zealand. 6 August **2010**.

Smith, F., Benthic cyanobacteria research during Cawthron Institute visit March–May 2010. Invited talk; Cawthron Institute, Nelson, New Zealand. 20 May **2010**.

Smith, F., Stressed out: Effects of metal stressors on cyanotoxin production. Department of Chemistry Seminar; University of Canterbury, Christchurch, New Zealand. 12 October **2009**.

Smith, F., What Triggers cyanotoxin production? Invited talk to the Environment Canterbury Councillors; Environment Canterbury, Christchurch, New Zealand. 10 December **2008**.

8.6.3 Conference Presentations

Smith, F.; Wood, S.; Broady, P.; Gaw, S.,; Blunt, J.; Munro, M., Detection of cyanotoxins in New Zealand benthic cyanobacteria. Society for Environmental Toxicology and Chemistry (SETAC) Australasia Conference; Brisbane, Australia. 5 July **2012**.

Smith, F.; Wood, S.; Wilks, T.; Kelly, D.; Broady, P.; Williamson, W.; Gaw, S., First report of saxitoxin from *Scytonema* (Cyanobacteria). New Zealand Institute of Chemistry (NZIC) Conference 2011; Hamilton, New Zealand. 29 November **2011**.

Smith, F.; Wood S.; Palfroy, T.; Broady, P., Gaw, S., Anatoxin-a and homoanatoxin-a variability in *Phormidium* (Cyanobacteria) strains isolated from a single mat sample. New Zealand Institute of Chemistry (NZIC) Conference 2011; Hamilton, New Zealand. 27 November–1 December 2011. (Poster presentation).

Smith, F.; Wood, S.; Gaw, S., Investigating metal stressors on anatoxin-a production in mat-forming *Phormidium autumnale*. New Zealand Freshwater Sciences Society Conference; Christchurch, New Zealand. 24 November **2010**.

Smith, F.; Wood, S.; van Ginkel, R.; Broady, P.; Gaw, S., First report of saxitoxin from a species of *Scytonema*. New Zealand Freshwater Sciences Society Conference; Christchurch, New Zealand. 22–26 November **2010**. (Poster presentation).

Smith, F.; Wood, S.: Gaw, S., Tracking anatoxin-a production in benthic mat-forming *Phormidium autumnale*. The 8th International Conference on Toxic Cyanobacteria; Istanbul, Turkey. 31 August **2010**.

Smith, F.; Wood, S.; van Ginkel, R.; Broady, P.; Gaw, S., First report of saxitoxin from a species of *Scytonema*. The 8th International Conference on Toxic Cyanobacteria; Istanbul, Turkey. 29 August–4 September **2010**. (Poster presentation).

Smith, F.; Wood, S.; Broady, P.; Gaw, S., Williamson, W.; Munro, M.; Blunt, J.; Polyphasic Studies of benthic Cyanobacteria in New Zealand. 18th Symposium of the International Association for Cyanophyte Research; České Budějovice, Czech Republic. 16–20 August **2010**. (Poster presentation).

Smith, F.; Merican, F.; Broady, P.; Gaw, S.; Blunt, J.; Munro, M.; Williamson, W., Characterisation of species diversity and toxicity of periphytic Cyanobacteria in tributaries of Lake Ellesmere. Vth Asian Pacific Phycological Forum: Algae in a changing world; Wellington, New Zealand. 10–14 November **2008**. (Poster presentation).