Investigation of Microcystin Processing, Production and Export by *Microcystis* sp.

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ABSTRACT

During this study, the effect of different processing methods on the microcystin quota (microcystins per cell) was investigated. Microcystins from two *Microcystis* strains and one *Planktothrix* strain were quantified using liquid chromatography-mass spectrometry (LC-MS) after processing via; (1) direct freezing (no prior cell concentration) and extraction by freeze-thaw cycles, (2) cell concentration by centrifugation and extraction in methanol and (3) cell concentration by filtration and extraction in methanol. Microcystin quotas were lower for samples concentrated by filtration compared with the other two methods.

In order to distinguish between extracellular microcystins actively exported from cells and those that are present as a result of cell lysis, a comparative microcystin export (CME) assay was developed using LC-MS to compare the proportion of extracellular microcystins with the proportion of extracellular non-ribosomal peptide which does not get actively exported. A culture-based experiment using the CME assay demonstrated the utility of this method and indicated that export from one of the *Microcystis* strains had occurred. With further validation experiments, the CME assay has the potential to be a valuable new tool for determining the occurrence of microcystin export.

An *in situ* experiment was carried out in Lake Rotorua, Kaikoura, New Zealand, using mesocosms to study the effect of *Microcystis* sp. cell density on microcystin production, while measuring a wide array of abiotic parameters (temperature, light intensity, pH, dissolved oxygen and nutrients). Lake water was added to 'control'

mesocosms in which cells were concentrated to different levels and added to ‘medium cell addition’ and ‘high cell addition’ mesocosms. The microcystin quota remained relatively constant throughout the study, despite induced changes in cell density and associated environmental parameters. These results indicate that cell density is not the only factor responsible for increased microcystin synthesis and that further investigation into the effect of stress inducing abiotic parameters such as pH and dissolved oxygen concentration may be required.

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None of us is as smart as all of us. ~Ken Blanchard

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ABBREVIATIONS USED

ACN, acetonitrile; **ADDA**, 3*S*-amino-9*S*-methoxy-2*S*,6,8*S*-trimethyl-10-phenyl-4,6-dienoic acid; **ATP**, adenosine-5'-triphosphate; **ABC**, adenosine triphosphate binding cassette; **Aeru**, aeruginosin; **Ala**, alanine; **NH_3**, ammonia; **ANOVA**, analysis of variance; **Arg**, arginine; **Asp**, aspartic acid; **bp**, base pairs; **Choi**, 2-carboxy-6-hydroxyoctahydroindole; **ca.**, circa or approximately; **CAPB**, cocamidopropyl betaine; **CME**, comparative microcystin export; **Da**, Dalton(s); **dm**, desmethyl; **Dha**, dehydroalanine; **Dhb**, dehydrobutyrine; **DNA**, deoxyribonucleic acid; **Ein**, Einstein (one mole of photons; ESI, electrospray ionisation; **ELISA**, enzyme-linked immunosorbent assay; **Extra/E**, extracellular; **Masp**, erythro-beta-methylaspartic acid; **EtOH**, ethanol; **EDTA**, ethylenediaminetetraacetic acid; **FOV**, field of view; **FA**, formic acid; **GF/C**, glass fibre filter; **Glu**, glutamic acid or glutamate; **HPLA**, hydroxyphenyllactic acid; **HPLC**, high performance liquid chromatography; **Hil**, homoisoleucine; **HCl**, hydrochloric acid; **Intra/I**, intracellular; **LDH**, lactate dehydrogenase; **Leu**, leucine; **LOQ**, limit of quantitation; **LC**, liquid chromatography; **MS**, mass spectrometry; ***m/z***, mass-to-charge ratio; **MALDI**, matrix-assisted laser desorption/ionisation; **MeOH**, methanol; **MC**, microcystin; ***mcy***, microcystin synthase gene; **Mdha**, *N*-methyldehydroalanine; **MRM**, multiple reaction monitoring; **NCBI**, National Centre for Biotechnology Information; **NAD/H**, nicotinamide adenine dinucleotide oxidised/reduced; **NRPS**, non-ribosomal peptide synthetase; **C_{18}**, octadecyl carbon chain; **Ω**, ohm(s) or electrical resistance; **ORF**; open reading frame; **PKS**, polyketide synthase; **Q/PCR**, quantitative/polymerase chain reaction; ***g***, relative centrifugal force; **RT-QPCR**,

reverse transcriptase-quantitative polymerase chain reaction; **r/RNA**, ribosomal/ ribonucleic acid; **CEN**, sample centrifuged, cell pellet freeze-dried and extracted in MeOH; **DF LN FD**, sample directly frozen in liquid nitrogen, freeze-dried and extracted in MeOH; **DF-20**, sample directly frozen at -20 ºC and extracted via freeze-thaw cycles; **DF-80**, sample directly frozen at -80 ºC and extracted via freeze-thaw cycles; **DF LN**, sample directly frozen in liquid nitrogen and extracted via freeze-thaw cycles; **FIL**, sample filtered, cells freeze-dried and extracted in MeOH; ***n*,** sample size; **s**, second(s); **MS/MS**, tandem mass spectrometry; **TOF**, time of flight; **NOx**, total oxidised nitrogen species; **UV**, ultraviolet; **Vis,** visible; **v/v**, volume per volume ratio.

CHAPTER 1: INTRODUCTION

1.1 Cyanobacteria and Toxin Production

Cyanobacteria are a phylum of photosynthetic prokaryotes that use solar radiation, water and carbon dioxide to produce carbohydrates, releasing oxygen as a by-product.[1] Cyanobacteria date back at least 3.5 billion years and this long evolutionary history is believed to be one of the reasons for their presence in almost every terrestrial and aquatic habitat including oceans, lakes, geothermal hot springs, desert soils and polar regions.[1]

Recent research has linked cyanobacterial dominance in freshwater environments to increased eutrophication and climate change,[2,3] as higher temperatures can accelerate cellular metabolism and, when supported by greater nutrient availability, can result in a rapid increase in cyanobacterial growth rates.[2] In addition, warming of surface waters can cause vertical stratification of lakes which is advantageous to some Cyanobacteria which have gas vesicles that create buoyancy. This can enable them to float on the surface where they can access light and atmospheric carbon dioxide for photosynthesis.[1,4] The formation of dense cyanobacterial surface blooms increases turbidity and blocks light from underlying aquatic plants and microalgae, negatively affecting the habitat and food sources for other aquatic organisms.[3]

Cyanobacteria are known to produce a diverse range of non-ribosomal peptides which, in terms of chemical structure, can be divided into linear (aeruginosins,

microginins), cyclic (anabaenopeptins, cyanopeptolins, microcystins, cyclamides) and multicyclic (microviridins) peptide classes.[5] For example, the linear peptide, aeruginosin is characterised by a derivative of hydroxy-phenyl lactic acid (Hpla) at the N-terminus, the amino acid 2-carboxy-6-hydroxyoctahydroindole (Choi) and an arginine derivative at the C-terminus (Figure 1.1).[6] Structural variants of aeruginosins have been isolated from the cyanobacterial genera; *Microcystis*, *Planktothrix* and *Nodularia*.[5]

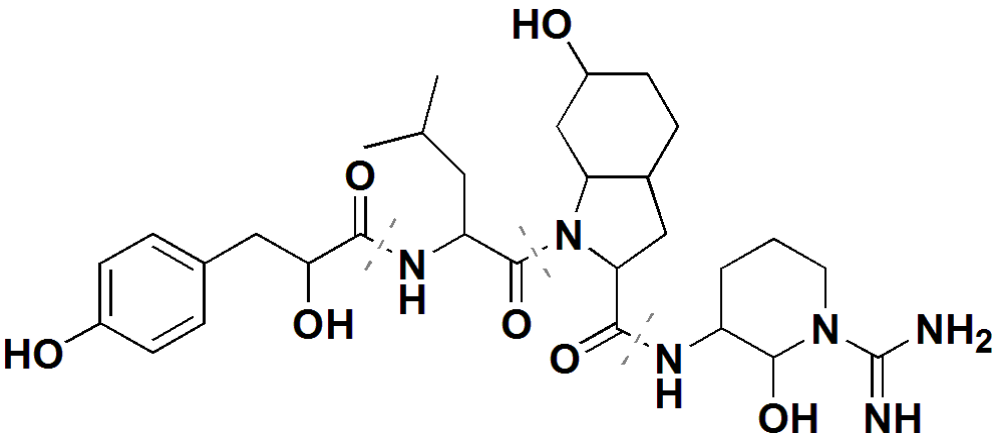


Figure 1.1: Structure of aeruginosin 602.

Microcystins are the most notorious non-ribosomal peptides as they are toxic and pose a significant risk to animal and human health.[7] These cyclic peptides are unable to pass through the membranes of most mammalian tissue cells but are actively transported into liver cells where they are concentrated.[8] Microcystins exhibit their toxicity in the liver by irreversibly inhibiting serine/threonine phosphatases 1 and 2A which are vital in many cell signalling cascades and result in hyper-phosphorylated proteins,[9] changes in cell shape and loss of cell-to-cell adhesion.[10,11] There is also growing evidence that microcystins may be actively transported through the blood-brain barrier, exerting toxic effects on mammalian neuronal cells.[12,13,14]

There have been numerous reports of livestock, wildlife and fish poisoning from microcystin exposure, usually when wind and wave action concentrate microcystin producing cells along the shoreline and animals drink this water.[3,15] A notable case of microcystin-induced hepatotoxicity in humans occurred in 1996 in Brazil, where fifty two dialysis patients died after being treated intravenously with microcystin-contaminated water.[16] In addition to the reported incidences of acute toxicity, chronic exposure to microcystins has the potential to cause cancer. The growth of large tumour nodules in the livers of mice occurred in studies after sub-lethal doses of microcystins were administered and exposure to microcystins has been linked to increased incidences of human liver cancer in China.[17,18]

1.2 Microcystin Structure and Biosynthesis

Microcystins are a family of cyclic heptapeptides produced non-ribosomally by many cyanobacterial genera including *Microcystis*,[19] *Anabaena*,[20] *Nostoc*,[21] *Oscillatoria*,[22] *Anabaenopsis*,[23,24] *Aphanocapsa*,[25] *Arthrospira*,[26] *Fischerella*,[27] *Hapalosiphon*,[28] *Phormidium*,[29] *Planktothrix*,[30] *Radiocystis*,[31] *Synechocystis*,[32] *Woronichinia*,[33] *Lyngbya*,[29] *Synechococcus*,[22] *Leptolyngbya*[34], *Limnothrix*[35] and *Cylindrospermopsis*.[36]

There are at least 130 different microcystin congeners with modifications reported for the amino acids at all positions[37] but the chemical structure is commonly characterised by the β-amino acid Adda (3*S*-amino-9*S*-methoxy-2*S*,6,8*S*-trimethyl-10-phenyl-4,6-dienoic acid) in position five, D-amino acids at position one, D-glutamate (D-Glu) at position six, D-erythro-β-methylaspartic acid (Masp) at position three and *N*-methyldehydroalanine (Mdha) at position seven (Figure 1.2). Positions two and four (X and Z respectively) are occupied by variable

L-amino acids and the different analogues of microcystins are named according to the amino acids incorporated at these positions. For example, microcystin-LR has leucine (L) at position two and arginine (R) at position four.[5] Modifications at any other amino acid position are placed in brackets preceding the congener name; for example, the desmethyl-Mdha variant of MC-LR is named [Dha[7]] MC-LR.[5]

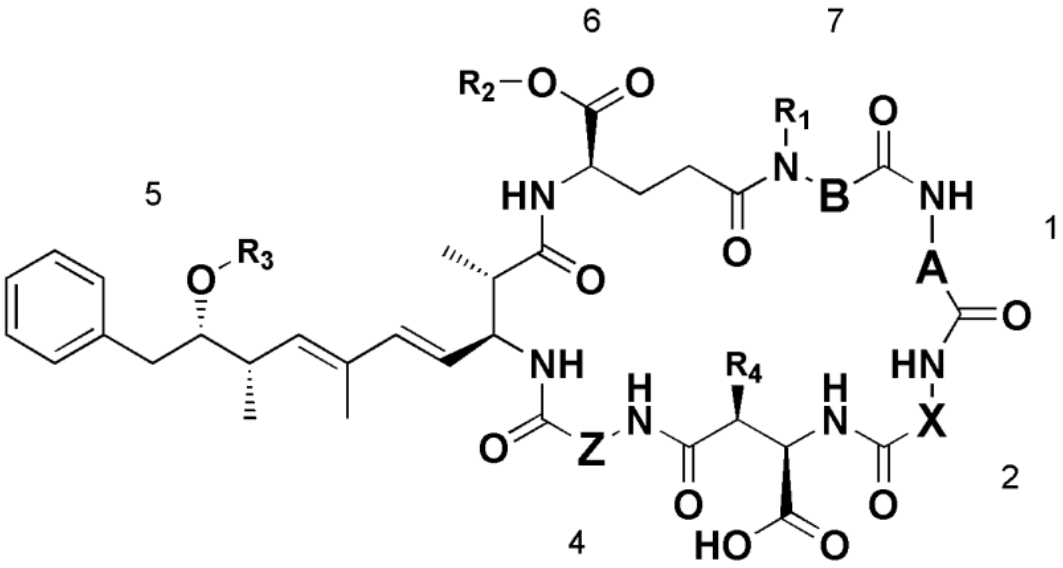


Figure 1.2: General structure of a microcystin.[7]

Microcystins are produced by a hybrid polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) called microcystin synthase.[7,38,39] This large enzyme complex is made of multiple enzyme modules, each carrying out a specific function.[39] Differences in the order of these modules, the number of catalytic domains and the type of catalytic domain can affect the final structure of the

peptide and results in the production of different microcystin congeners.[7] The catalytic domains also have varying levels of substrate specificity which allow a single strain of Cyanobacteria to produce more than one microcystin congener.[40]

The biosynthetic pathway for microcystins was elucidated from feeding experiments,[38] insertional mutagenesis[39] and sequence comparisons with the structure and function of known NRPS and PKS genes.[7] Feeding experiments deduced the origin of the carbon in the Adda and Masp units[38] while insertional mutagenesis of the NRPS gene cluster believed to code for microcystin synthase resulted in the inability of mutated cells to produce microcystins.[39] Sequence analysis of the microcystin synthetase gene cluster in *M. aeuruginosa* revealed the presence of two bi-directional operons (*mcyA-C* and *mcyD-J*) separated by a 750 base pair promoter region. The larger operon (*mcyD-J*) encodes PKS/NRPS modules responsible for the formation of Adda and its linkage to D-glutamate. The smaller operon (*mcyA-C*) encodes NRPS modules responsible for the extension of this intermediate to form a heptapeptide and subsequent cyclisation.[7]

The *mcy* gene cluster in *Planktothrix* has also been sequenced. In contrast to *M. aeruginosa*, the *mcy* genes were on the same strand and were transcribed as a single operon.[41] In addition, *Planktothrix* did not contain *mcyF* and *mcyI* open reading frames (ORF) found in *Microcystis* and had a unique ORF (*mcyT*) not found in *Microcystis*.[41]

1.3 Environmental Triggers of Microcystin Production

Although the genes responsible for microcystin production and the biosynthetic pathway have been elucidated, factors regulating microcystin production are still

largely unknown. Most studies have been culture-based, manipulating environmental factors typically associated with bloom formation such as temperature,[42-46] light intensity,[42,44-48] phosphorus,[42,44,45,49,50] nitrogen[42-45,49,51] and iron concentrations.[43,52,53] In general, a parabolic relationship between temperature and microcystin concentrations has been observed[42,43,45,46,50] with maximum levels at 25 ºC for *Anabaena* species,[45,46] and over a range of 18-25 ºC for *Microcystis* species.[42,43,45,50] Studies investigating the effect of light intensity showed the same parabolic curve with maximal microcystin levels varying between studies and between species.[42,44-48] The effects of nitrogen and phosphorus concentrations on microcystin production were highly variable among studies. Some studies have found that phosphorus concentration has no effect on microcystin production[44,52] while others have found that microcystin concentrations increased with elevated phosphorus levels.[42,45] Similarly, research investigating the effects of nitrogen concentration on microcystin production have yielded conflicting results with some studies showing greater microcystin production with increased nitrogen levels[44,49,52] and others demonstrating a decrease.[43] Studies investigating the effect of iron concentration on microcystin production have also yielded conflicting results. For example, one study found an increase in microcystin production with increased iron availability[52] while a contradictory study found that iron starvation triggered microcystin synthesis and an increase in transcription of the *mcyD* gene.[53]

These contradictory findings may be attributed to the use of different cyanobacterial species and strains, different culture conditions (batch versus continuous; axenic versus non-axenic) and particularly, different methods for extracting and quantifying microcystins.[54] For example, the use of a gravimetric

measurement (toxin per gram dry weight) to express toxin concentration may yield different results to using cell quotas (toxins per cell) as carbohydrates, proteins and extracellular polysaccharide production change the weight of a cell and may not be directly coupled to microcystin production.[51]

Most culture-based studies manipulating an environmental parameter have only yielded small changes (three to four-fold) in the concentration of microcystins detected.[55] One possible explanation for this was that growth limiting factors, such as nitrogen, phosphorus and temperature, indirectly affect microcystin production by influencing cell growth and division but do not directly affect the biosynthetic pathway for microcystins.[51] A study investigating the synthesis of MC-LR in *M. aeruginosa* in response to nitrogen found that while excess nitrate input increased the growth rate, it did not induce expression of the *mcy* operon and no change was seen in microcystin concentration per cell or the ratio of microcystins to total protein.[56]

1.4 Ecophysiological Roles of Microcystin Production

The gene cluster encoding microcystin synthase constitutes about 1.5% of the genome of Cyanobacteria,[57] suggesting a significant investment in energy and prompting much research into the ecophysiological role of microcystins. Microcystins have been proposed to play a role in defence against grazers,[58-60] gene regulation,[61] intraspecific regulation,[62] siderophoric scavenging[63] and protein modulation.[64] Several laboratory studies have found links between reduced feeding and growth in *Daphnia* and toxic Cyanobacteria.[58,60,65] A negative correlation between the amount of microcystins in *M. aeruginosa* and *Daphnia* growth and survival has been shown.[59] However, the same study also found that a

microcystin-free mutant strain of Cyanobacteria showed reduced feeding and growth by *Daphnia*.[59] While microcystins contribute to the lethal effects on daphnids and related zooplankton, other confounding factors cannot be excluded.[59] Variable factors such as colony size,[65] nutritional deficiency[66] and other toxic compounds present in cyanobacterial strains[60,67] are all possible additional contributors to the effect of Cyanobacteria on *Daphnia* growth. Furthermore, a phylogenetic analysis of microcystin synthetase genes indicated that the ability to produce microcystins evolved before predators that feed on Cyanobacteria, which indicates there may be other ecological roles for microcystins.[68]

It has been suggested that microcystins act as infochemicals involved in intra-specific communication (quorum sensing), their release into the environment being sensed by surrounding cells at a threshold concentration as an indication of an increase in cell death.[62] Once sensed by the remaining cells, the microcystins act to stimulate an increase in microcystin biosynthesis, which may have a secondary purpose to enhance defence capabilities or increase their competitiveness, either directly, or indirectly by altering the expression of other genes.[62]

Sequence analysis of an ORF (*McyH*) present in the *mcy* gene cluster[7] suggested it belonged to the adenosine triphosphate (ATP) binding cassette (ABC) transporter superfamily,[69] responsible for the ATP-dependent transport of a vast range of substrates across cellular membranes.[70] Presence of the ABC transporter gene suggests microcystins may be actively exported out of cells and that cell lysis may not be the only mechanism for the release of microcystins into the aquatic

environment.[7,69] Oscillations in which intracellular microcystin quotas were inversely proportional to equivalent extracellular microcystin quotas during a twelve: twelve hour light/dark cycle and a twenty four hour light cycle have been reported.[71] The decreases in intracellular microcystin quotas at specific times were characteristic of a controlled endogenous rhythm over time, in which microcystins were actively released into the surrounding medium as opposed to cell lysis.[71] Another study that measured the transcript levels of *mcyB* and *mcyD* showed that light quality (red light) had a major influence on microcystin synthetase production and suggested that toxins were produced constitutively under low and medium light intensities and then upregulated under certain threshold intensities.[72] The higher transcript level of *mcyB* and *mcyD* did not always reflect the observed intracellular toxin bioactivity, suggesting microcystin export under higher threshold light intensities or post-transcriptional/post-translational regulation of microcystin synthase activity.[72]

Microcystins entering the environment via a controlled mechanism known as active export, supports the hypothesis for additional or alternative roles in intercellular communication that could aid in adaptation to changing environmental conditions. For example, microcystins were shown to significantly enhance colony formation by causing upregulation of genes involved in polysaccharide formation.[73] This is advantageous as colonies of increased size have greater protection from grazers and have increased buoyancy, potentially allowing greater access to solar radiation.

A recent *in-situ* study found that microcystin synthesis is not always constitutive.[74] A twenty eight-fold increase in the intracellular microcystin quota

was observed in correlation with a 400-fold upregulation of *mcyE* expression. This coincided with a period of dramatic increase in cell density (due to gentle onshore winds) which suggested that *Microcystis* can 'switch on' microcystin production in response to an increase in cell density or a co-occurring environmental variable (for example, increased pH in scums). This study also found that as the intracellular level of microcystins increased, there was a corresponding escalation in extracellular concentrations, supporting the hypothesis that microcystins may act as an extracellular signalling molecule via active export.[74] Following on from these results, an experimental protocol was established using mesocosms to manipulate cell density in the lake environment.[57] This latter study confirmed a relationship between microcystin cell density and microcystin synthesis. However, the corresponding increase in extracellular microcystins observed in the previous study was not observed using the experimental protocol.[57] This led the researchers to postulate that the increased microcystin production may not be due to the increase in cell density, but could be related to changes in other environmental parameters such as pH, nutrients and oxygen saturation, which occur when surface scums form.[57]

It is possible that microcystins do not get actively exported out of the cell and, instead, play an intracellular role. In support of this, it has been found that after incorporating radioactive carbon into microcystin molecules, there were no significant losses of the intracellular pool of radiolabelled microcystins, either by export or breakdown.[75]

Evidence of a linear correlation between cell division and microcystin production suggested that microcystins play an important role in primary cellular metabolism

while non-microcystin containing Cyanobacteria may employ peptides of similar structure to carry out the same role that microcystins play in primary metabolism.[51] For example, photosynthetic pigments and accessories and iron chelating siderophores such as deferriferrichrome are cyclic peptides of similar structure which carry out an intracellular function.[51]

There is evidence that microcystins carry out an intracellular function in light adaptation processes.[76] Research has shown that in limited light conditions, *M. aeruginosa* mutants lacking the ability to produce microcystins had significantly lower chlorophyll-*a* content and a higher ratio of phycocyanin to chlorophyll-*a* when compared with the wild type. The higher ratio of phycocyanin to chlorophyll-*a* indicated a change in the photosystem I/photosystem II ratio which influences light adaptation mechanisms in Cyanobacteria.[76] One such light adaptation mechanism in which microcystins could play a role, is response to oxidative stress caused by high light exposure. A recent study found that microcystins can bind to cysteine residues in proteins to protect them from degradation under high ultra-violet (UV) exposure.[64] The intracellular role of microcystins in light adaptation processes is in accordance with their localisation in and around the thylakoid membranes[77,78] and the light regulated transcription of the microcystin synthetase gene cluster.[72]

Immuno-gold localisation studies have also found that a small proportion of microcystins was preferentially localised around the periphery of polyphosphate bodies, indicating a possible role in ligating excess metals and detoxification.[77,79,80] A recent study found that microcystins were localised in filament sheaths and mucilage of *Rivularia* colonies,[78] suggesting active export

and an extracellular role for microcystins. The lack of microcystin localisation around the membrane in previous studies could be attributed to the fact that cells in colonies were not examined or that microcystin export may only occur under specific conditions.

At present, there is no method to discriminate between microcystins which are actively exported from the cell and those that enter the environment through cell lysis. The active export of microcystins would provide evidence that microcystins do not solely occupy an intracellular function and may act as an intercellular signalling molecule.

Ultimately, an increased understanding of the triggers of microcystin production and of their ecophysiological role will contribute to the ability to predict periods of greatest health risk and alleviate the detrimental effects of toxic cyanobacterial blooms.

1.5 Methods for Microcystin Detection and Quantification

There are multiple methods that can be employed for the detection and quantification of microcystins. Their suitability depends on the availability of facilities and expertise, the type of information required and the level of selectivity and sensitivity required.[81] These methods can be divided into either biochemical or physicochemical categories based on the type of detection and information provided.[81]

Examples of the biochemical methods commonly used for microcystin detection include enzyme linked immunosorbent assay (ELISA) and the protein

phosphatase inhibition assay.[81] ELISA provides a highly sensitive method for the quantification of microcystins[82] based on the binding of specific antibodies to microcystin congeners.[83] However, studies have shown variable cross-reactivities between different microcystin congeners, resulting in a lack of correlation with the toxicity of samples.[84,85] The phosphatase inhibition reaction caused by microcystins can be used to accurately measure their concentration based on a calibration curve constructed using microcystin standards of known concentrations.[37] Formerly, ^{32}P radiolabelled substrates were used but the requirement for radioactively labelled substrates and expensive enzymes led to the development of more cost effective colorimetric and fluorescent detection methods instead.[81]

Physicochemical methods exploit aspects of chemical structure such as polarity, molecular weight and the presence of UV chromophores. Examples of common methods used for microcystin quantification include high performance liquid chromatography (HPLC) and mass spectrometry (MS).[81]

Microcystins and other small peptides are generally separated by HPLC using a reversed-phase C_{18} column. Peptides of differing polarities elute at different retention times,[37,86] while different mobile phases may be employed in order to optimise resolution of the analytes.[87] The most common method of detection for microcystins within the HPLC system is by UV/Vis absorbance.[87] Microcystins can be detected by the UV absorption of the Adda moiety at 238 nm.[81] The concentration of microcystin congeners can be determined according to the expected retention time and a calibration curve constructed using microcystin standards of known concentrations. However, other co-eluting components in the

sample may absorb at the same wavelength, contributing to the signal and resulting in inaccurate results.[88]

Mass spectrometry is an analytical technique that can detect the components of a sample based on their mass-to-charge ratio (*m/z*).[89] Mass spectrometers consist of a sample introduction method, an ionisation source, a mass analyser and a detector.[90] Different combinations and variants of each component result in mass spectrometers optimised for specific purposes. Examples of ionisation sources used to analyse peptides include matrix assisted laser desorption ionisation (MALDI) commonly coupled to a time of flight analyser (TOF)[91] and electrospray ionisation (ESI).[92] Both MALDI and ESI are relatively 'soft' ionisation techniques and do not result in destruction of the peptides being analysed.[89]

MALDI-TOF MS uses laser irradiation to ionise a matrix which is co-crystallised with the sample on a MALDI target.[91] The matrix, in turn, ionises the sample which can then be separated in a TOF mass analyser and detected on the basis of *m/z*.[91] MALDI ionisation produces predominantly singly protonated ions, resulting in a simplified spectrum.[91] The use of MALDI for quantitative analysis has been limited by poor shot-to-shot and sample-to-sample reproducibility due to local concentration variations in the crystal structure.[93,94] The use of an internal standard can overcome these problems, but selection of an appropriate standard is crucial for accurate sample quantification. The standard must have similar chemical properties, ionise with similar efficiency and have a different mass to the analyte of interest.[93]

ESI produces charged droplets of analyte and solvent by passing a liquid sample through a charged capillary. The solvent is evaporated from the charged droplets and converted to gas phase ions.[92] This method of ionisation often produces multiply charged ions which complicates spectra and can require cumbersome de-convolution.[92] ESI mass spectrometry requires prior separation by HPLC which increases analysis time but also provides additional chromatographic information[92] and thus, LC-MS is one of the most effective methods for characterisation and quantification of microcystins.

Mass spectrometers with multiple mass analysers in a series can perform tandem MS (MS/MS) which allows an ion of interest to be separated by the first mass analyser, fragmented and then separated by a second mass analyser. This gives structural information on a compound as it fragments in predictable positions and may be characterised by peaks of a common *m/z*.[89] In addition, multiple reaction monitoring (MRM) methods can also be utilised with MS/MS to improve the selectivity and sensitivity for quantification.[95] MRM methods involve fragmentation of the parent ion and selection of a fragment ion for quantification. The concentration of microcystins present can be determined by comparison with a standard curve constructed using microcystins at known concentrations.[37]

1.6 Overview and Objectives of this Study

The focus of this study is to contribute evidence towards understanding the factors regulating microcystin production by *Microcystis* species in their natural environment and to further elucidate the ecophysiological role of microcystins by developing a tool that will determine whether microcystins are actively exported out the cell and under what conditions.

Objectives:

1) To assess the influence of sample processing on microcystin quota, using combinations of various cell concentration, preservation and extraction methods, as well as the length of time between concentration and preservation.
2) To undertake a field study in Lake Rotorua (Kaikoura, South Island, New Zealand) using mesocosms to examine the relationship between cell density and microcystin production while measuring a number of other possible confounding environmental parameters.
3) To develop a comparative microcystin export (CME) assay to discriminate between extracellular microcystins that are actively exported and those that result from cell lysis.
4) To investigate (using the CME assay) whether an increase in cell density as a result of cell growth causes microcystin export in a laboratory environment.

CHAPTER 2: EFFECTS OF SAMPLE PROCESSING ON MICROCYSTIN QUOTA

2.1 Introduction

Microcystins are toxic compounds produced by Cyanobacteria which are present in freshwater lakes globally and pose significant risk to human and animal health.[7] Microcystins irreversibly inhibit serine/threonine phosphatase enzymes inducing hepatotoxicity.[9] In addition, long term exposure to microcystins has been linked to cancer tumour promotion[17,18] and microcystin-LR was placed into category 2B, 'probably carcinogenic for humans' by the World Health Organisation.[96]

The detrimental effects of toxin production by Cyanobacteria have resulted in decades of research into investigating environmental factors regulating microcystin production and its ecophysiological role. Culture-based studies that manipulate environmental factors typically associated with a bloom have shown contradictory results and only yielded small changes in microcystin quotas (microcystins per cell).[54] These findings might be due to the use of different methods for preserving, extracting and quantifying microcystins.

Despite there being several different combinations of concentration, preservation and extraction methods with variations of solvents, temperatures, times and volumes used, there is still no consensus on the most suitable microcystin processing method.[97] Cyanobacterial biomass may be concentrated by centrifugation, micro-filtration using glass fibre filter paper (GF/C) or by vacuum

filtration.[98] Methods for preservation include freezing at -20 ºC, -80 ºC or in liquid nitrogen and/or lyophilisation. Different extraction/cell disruption methods range from extraction in a solvent such as methanol or butanol,[88] freezing and thawing,[99] sonication[100,101] and by microwave oven.[102]

Most studies have focused on comparing the different extraction methods[98,102-106] and have overlooked how different cell concentration and preservation methods may influence microcystin quota. A recent *in situ* study showed that an increase in *Microcystis* cell concentration resulted in an eighteen-fold increase in microcystin quotas.[57] This highlighted the need to further investigate the effect of processing methods and the length of time between the concentration and preservation steps on the microcystin quota.

2.2 Results

Effect of cell processing methods on microcystin quota

The microcystin concentrations of two different *Microcystis* strains (CYN06 and CYN11) and one *Planktothrix* strain (CYN60) were quantified using high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after processing via; (1) direct freezing (no prior cell concentration) and extraction by freeze-thaw cycles, (2) cell concentration by centrifugation and extraction in methanol and (3) cell concentration by filtration and extraction in methanol. Triplicate subsamples for each treatment were analysed and the same experiment was repeated three times on different days.

The concentrations of microcystin produced by each strain varied significantly among the experimental sampling days and this was particularly apparent for

CYN60 (Figure 2.1). On account of this, the microcystin quotas for each strain were standardised against the control sample (samples directly frozen in liquid nitrogen, freeze-dried and extracted in methanol) taken for each strain on each day. This allowed comparisons to be made between different days and between different strains. The filtration method had a significantly lower ($P<0.0001$) microcystin yield that all other treatments (Figure 2.2). There were no statistically significant differences among the other treatments.

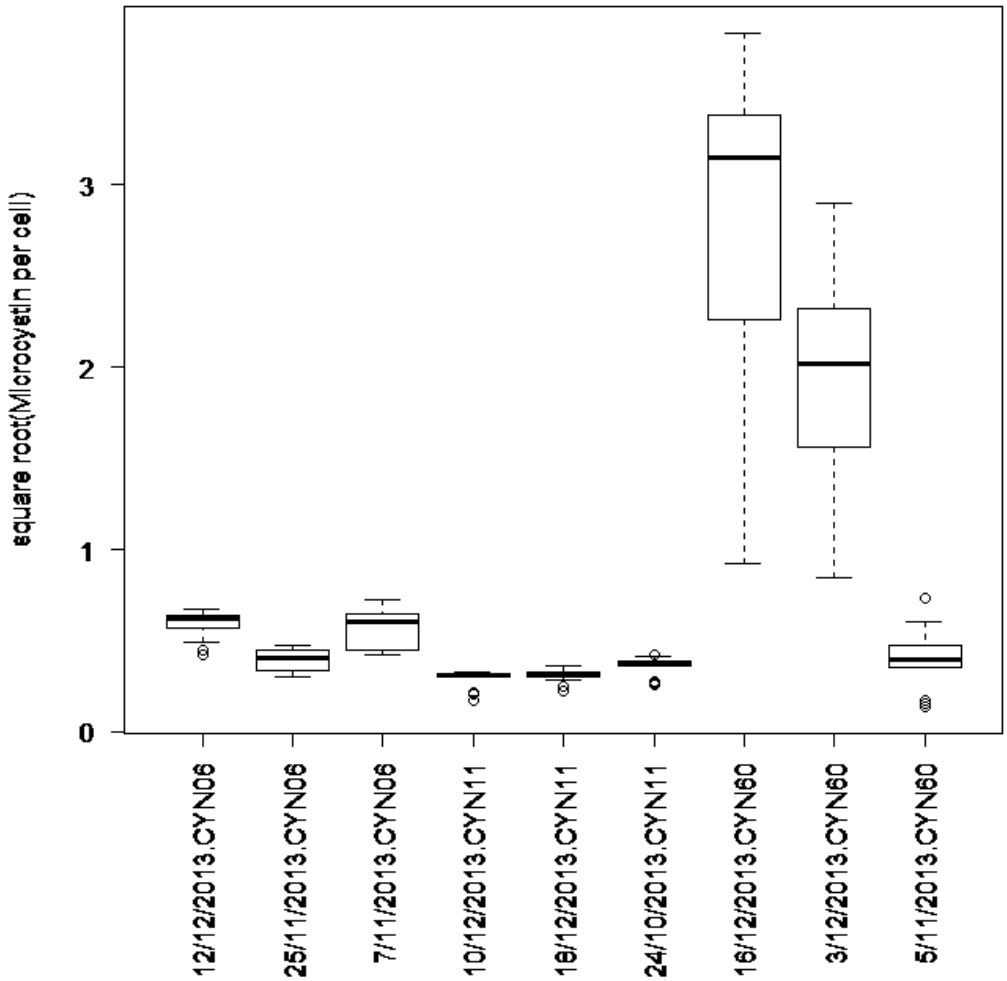


Figure 2.1: Microcystin quota (pg/cell) across all treatments for each strain on different experimental days. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outliers beyond this range.

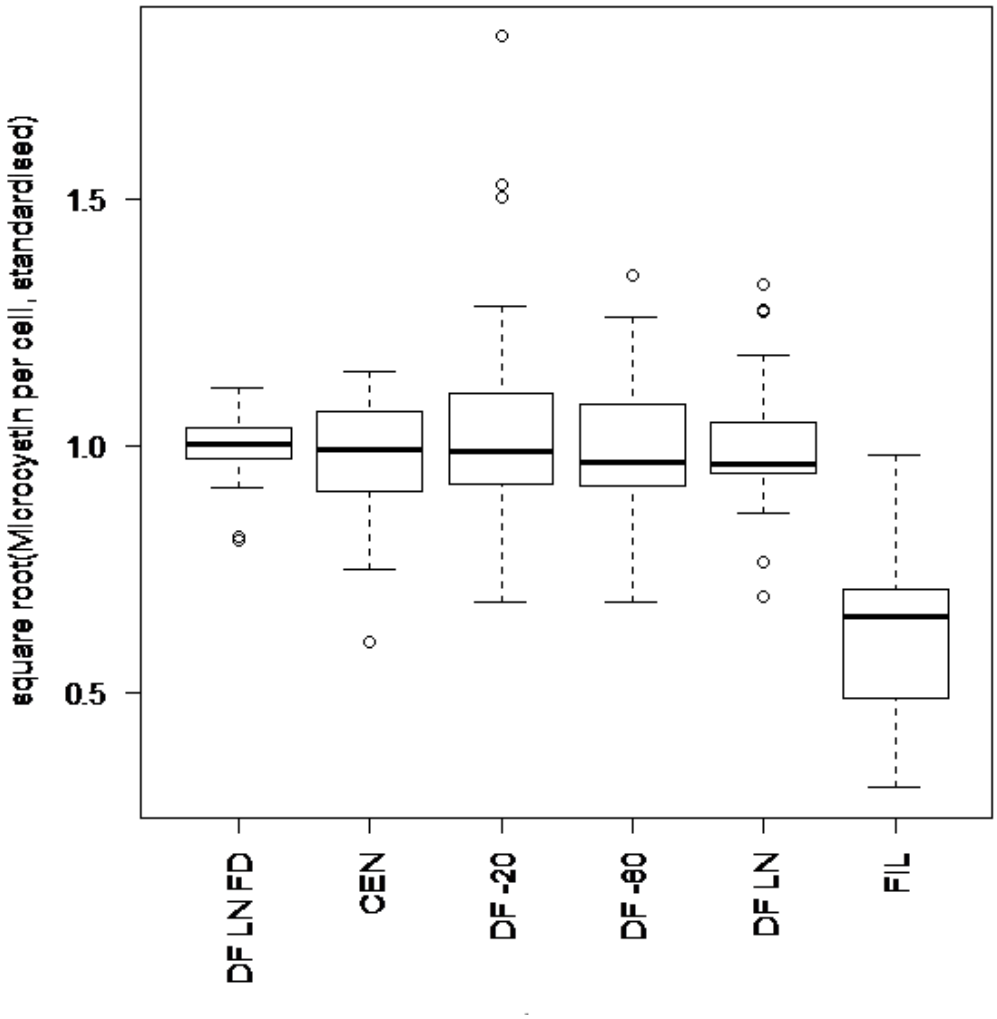


Figure 2.2: Standardised microcystin quota (pg/cell) for CYN06, CYN11 and CYN60. Solid black line shows median, box shows 1st and 3rd quartiles, whiskers extend to the last data point within 1.5 times the interquartile range if there is data that far from it. Open circles are outliers beyond this range. DF LN FD = sample directly frozen in liquid nitrogen, freeze-dried and extracted in MeOH; CEN = sample centrifuged, cell pellet freeze-dried and extracted in MeOH DF-20 = sample directly frozen at -20 ºC and extracted via freeze-thaw cycles; DF-80 = sample directly frozen at -80 ºC and extracted via freeze-thaw cycles; DF LN = sample directly frozen in liquid nitrogen and extracted via freeze-thaw cycles; FIL = sample filtered, cells freeze-dried and extracted in MeOH.

Temporal effect of centrifugation on microcystin quota

The microcystin concentration of a *Microcystis* strain (CYN11) was quantified using LC-MS/MS after; triplicate subsamples were centrifuged to concentrate cells. The supernatant was then either: (1) left in the tube with the pellet at room

temperature for zero, one, two, three, four and five hours after which the supernatant was removed for extracellular microcystin analysis and the pellet was frozen, freeze-dried and extracted in methanol for intracellular microcystin analysis, or (2) removed immediately and discarded whilst the pellet was left at room temperature for zero, one, two, three, four and five hours, before being frozen. The sample was then freeze-dried and extracted in methanol for intracellular microcystin analysis.

There were no significant differences in the intracellular (P = 0.22) and extracellular (P = 0.16) microcystin concentration in CYN11 after centrifugation and incubation at room temperature for various lengths of time (Figure 2.3).

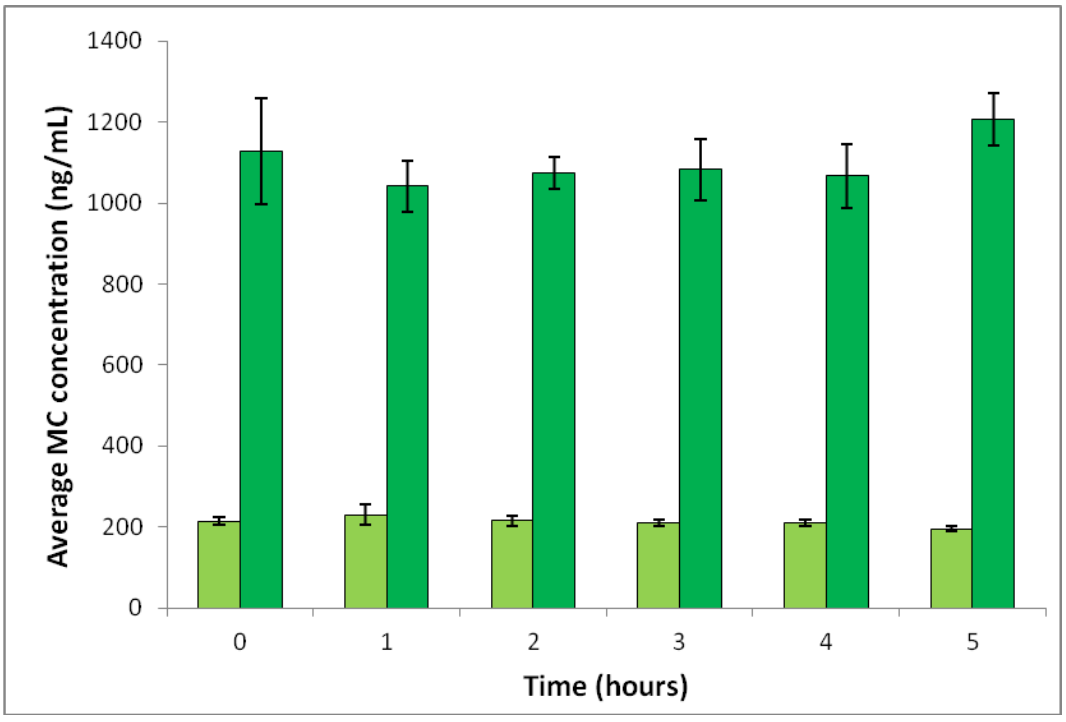


Figure 2.3: Average (n=3) intracellular (light green) and extracellular (dark green) microcystin (MC) concentration after centrifugation and incubation at room temperature for various lengths of time.

In the second experiment, the average microcystin concentration in CYN11 remained the same during the first hour of the experiment where the supernatant

was removed immediately (Figure 2.4). At two hours, there was a significant increase in microcystin concentration (P = 0.0007), which remained at that level until a significant decrease (P = 0.02) in microcystin concentration was observed at five hours.

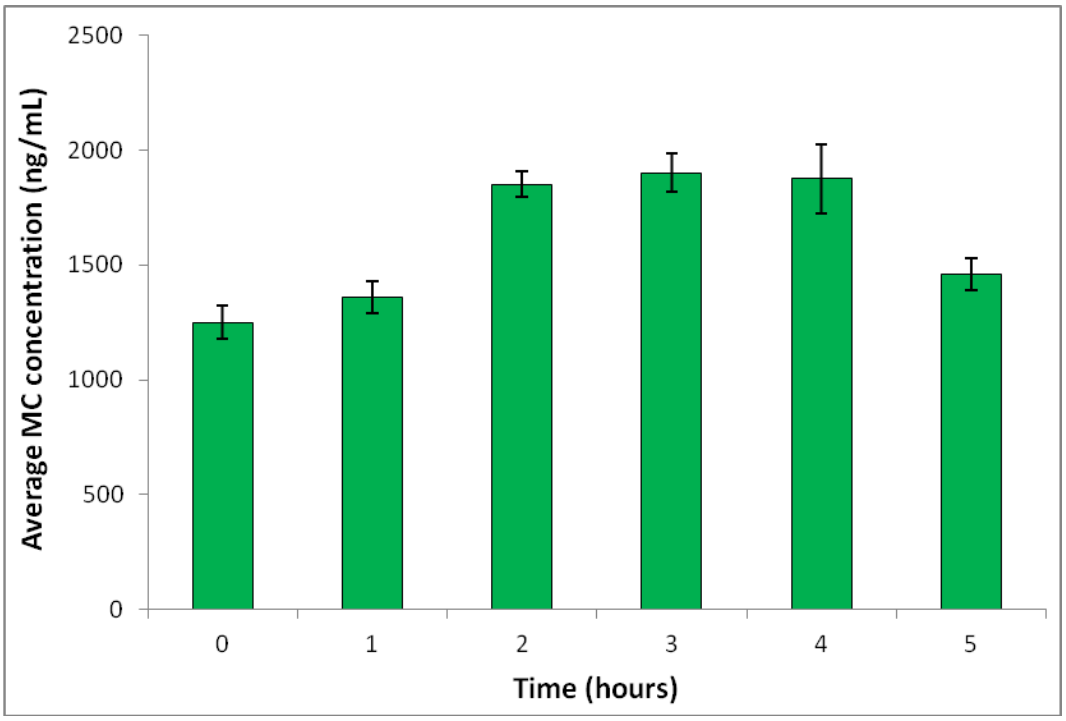


Figure 2.4: Average (n=3) intracellular microcystin (MC) concentration after centrifugation, removal of supernatant and incubation at room temperature for various lengths of time.

2.3 Discussion

Effect of cell processing methods on microcystin quota

Apart from filtration, all processing methods tested in this study resulted in similar microcystin quotas for all strains. Therefore, the choice of which processing method to utilise could vary depending on the equipment available during sampling, the time taken to process the sample and the amount of labour required. For example, for laboratory-based experiments, cell concentration by centrifugation may be used instead of filtration to obtain the intracellular fraction

of microcystins. Centrifugation is less applicable in the field, in which case, samples may be directly frozen in liquid nitrogen for total microcystin analysis and a separate sample filtered for extracellular microcystin analysis. The intracellular fraction of microcystins can be deduced by subtracting extracellular microcystins from total microcystins.

The significantly lower microcystin quotas detected in samples processed by filtration in all of the strains investigated could be a result of either of two scenarios. Whole cells could have passed through the GF/C filter and into the extracellular medium and/or cells could have become embedded in the filters, impeding cell lysis and reducing extraction efficiency. An investigation to determine whether cells were being passed through GF/C filters and entering the extracellular fraction was carried out (Appendix A). There was no significant difference between the extracellular microcystin concentration of samples processed by centrifugation (P = 0.16) and samples processed by filtration, despite a significantly lower intracellular concentration (P = 0.005) for samples processed by filtration compared with centrifugation (Appendix A). This indicated that cells were not passing through the filter into the extracellular fraction. Furthermore, there was no significant difference between the extracellular microcystin concentrations of samples processed by GF/C filtration and samples processed by GF/C filtration and a second filtration step (P = 0.13; Appendix A), demonstrating that the first GF/C filter was sufficient. The lower microcystin quota from sample processing by filtration was likely due to reduced extraction efficiency from cells becoming embedded in the filters and impeding cell lysis.

The larger variation observed in microcystin quotas between triplicate samples in CYN60 compared with the other strains could be attributed to sampling error. The filamentous nature of the strain and the formation of mats that adhere to the culture vessel[107] made obtaining representative subsamples for cell enumeration and microcystin quantification difficult.

Temporal effect of centrifugation on microcystin quota

In the first experiment, there were no changes in the intracellular or extracellular microcystin concentration after centrifugation and incubation with supernatant left in the tube over five hours. The second experiment showed a marked increase in microcystin concentration after centrifugation, supernatant removal and incubation for one hour. An increase in microcystin concentration due to cell division after centrifugation should be minimal as growth is slow in *Microcystis* with an average doubling time of about ten hours.[108,109] This suggested that increasing the cell density and/or associated environmental stressors may be influencing the final microcystin yield by upregulating microcystin production.[57] Removal of the supernatant would have created a harsh, dry environment, leaving cells more susceptible to changes in the environment and with no opportunity for concentrated solutes and compounds to diffuse into the surrounding supernatant.

2.4 Conclusion

Apart from filtration, all processing methods tested in this study resulted in similar microcystin quotas for each cyanobacterial strain tested. The most suitable processing method is likely to be the direct freezing of samples, followed by freeze-thaw extraction, as this method can be utilised in both field and laboratory environments and gave consistent microcystin quotas in all three strains tested. Leaving samples at room temperature for up to five hours after concentration did not appear to have a marked influence on the microcystin yield, however, if removing the supernatant after concentration, preservation shortly after is recommended.

CHAPTER 3: COMPARATIVE MICROCYSTIN EXPORT ASSAY DEVELOPMENT AND VALIDATION

3.1 Introduction

Over the past thirty years, many theories on the ecophysiological role of microcystins have been proposed, including but not limited to; defence against grazers,[59] gene regulation,[61] intraspecific regulation[62] and siderophoric scavenging.[63] It has been proposed that microcystins act as infochemicals involved in interspecific communication (quorum sensing), where their release into the environment is sensed by surrounding cells.[62] Upon recognition of the signal, the remaining cells then increase microcystin production which could potentially enhance defence capabilities or increase their competitiveness. This may occur directly through their toxicity or indirectly by altering the expression of other genes.[62]

The presence of an open reading frame (ORF) coding for an ATP binding cassette (ABC) transporter in the *mcy* gene cluster suggests that microcystins could be actively exported out of living cells.[7] Intercellular communication could aid in adaptation to changing environmental conditions. For example, it has been found that microcystins significantly enhance colony formation which provides a competitive advantage since colonies of increased size are more protected from grazers and are more buoyant, allowing better access to light.[73]

A recent *in situ* study made a significant discovery that microcystin synthesis is not always continuous.[74] Up to twenty eight-fold changes in intracellular microcystin concentrations were observed in correlation with a 400-fold upregulation of *mcyE* gene transcription after a dramatic increase in cell density (due to gentle onshore winds) within a five hour period. This study also found that as the intracellular level of microcystins increased, there was a corresponding increase in extracellular concentrations. This supports the hypothesis that microcystins may act as intercellular signalling molecules outside the cell that then induces upregulation of microcystin production under conditions associated with high cell density.[74]

To the best of my knowledge, there are currently no methods available to distinguish between extracellular microcystins which have been actively exported from those that are present as a result of cell lysis. Cyanobacteria also produce other non-ribosomal peptides such as cyanopeptolin, aeruginosin and microginin[5] which, like microcystins, can be detected by mass spectrometry.[91] These peptides will be termed auxiliary peptides for the purpose of this study.

Based on the assumption that auxiliary peptides are not actively exported out of cells and have similar degradation rates to microcystins, an assay was developed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). LC-MS/MS was used to detect peptides in a single *Microcystis* strain selected from four strains (Rotorua A, B, C and D strains) that were isolated from Lake Rotorua, Kaikoura and characterised. The assay, named the comparative microcystin export (CME) assay, was designed to determine the proportion of extracellular microcystins present as a result of active export. Theoretically, this

would be achieved by comparing the proportion of extracellular microcystins with the proportion of extracellular auxiliary peptides, as these would only be present due to cell lysis (Figure 3.1).

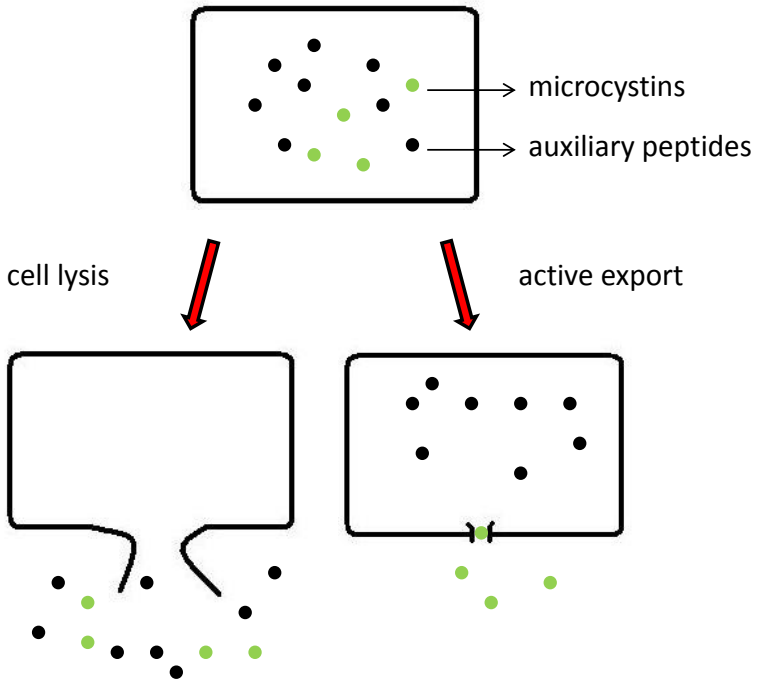


Figure 3.1: Illustration of the basis for the comparative microcystin export (CME) assay.

Evidence showing that the auxiliary peptide does not get actively exported from the cell was sought to validate the CME assay by measuring the proportion of auxiliary peptides present as a result of the level of induced cell lysis. Methods currently used to quantify the level of cell lysis in cyanobacterial cells include microscopy and cell staining,[110,111] flow cytometry,[112] measuring ion leakage[113-115] and ultra-violet (UV) absorption at 260 nm to indicate the level of cell lysis by the amount of DNA and RNA released from the cell.[116] However, flow cytometry can cause complications when using a colonial species of Cyanobacteria,[117] staining and microscopy can be time consuming, measuring ion leakage may be complicated by interference from the cell lysis agent at different concentrations

and measuring absorption at 260 nm may not be sufficiently specific as other compounds present under different conditions at different levels in the cell may also absorb UV light at 260 nm. A simple biochemical assay, the lactate dehydrogenase (LDH) assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega) was tested for its efficacy in measuring the degree of cell lysis in the selected Rotorua *Microcystis* strain of Cyanobacteria. LDH assays are commonly used on human cells[118,119] but no published research to date has used it on cyanobacterial cells. The assay quantitatively measures lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis. There are many different methods for inducing cell lysis,[98] however, there are few reports on chemical agents or detergents that induce a dose-dependent response in Cyanobacteria. Cocamidopropyl betaine (CAPB) has been found to elicit a dose- and time-dependent response on marine microalgae[113] and was selected to be trialled as the cytotoxic agent to be used with the CytoTox 96® Non-Radioactive Cytotoxicity Assay.

After development of the CME assay, a culturing experiment was used to begin validation of this fledging test. A *Microcystis* strain was cultured over several growth stages to verify the theory that extracellular microcystins will increase at a higher rate than extracellular auxiliary peptides, indicating active export. The active export of microcystins would provide evidence that microcystins do not occupy a solely intracellular function and could be an intercellular signalling molecule. After further validation, this will be a valuable tool to assist in unravelling the ecophysiological function of microcystins.

3.2 Results

3.2.1 Morphological Characterisation

Morphological characterisation was carried out on four cyanobacterial strains isolated from Lake Rotorua (Kaikoura, New Zealand) using microscopy to identify distinctive features. The four strains possessed morphological characteristics consistent with Chroococcales and the species *M. aeruginosa*.[120] The cells were sub-spherical in shape and densely aggregated in colonies (Figure 3.2). The colonies were spherical, irregular and at times clathrate (lattice-like). The mucilage was colourless, homogeneous and did not extend beyond the colony edges. Cells were brownish green, with aerotopes (gas vacuoles), 3.6-4.5 µm in diameter (Table 3.1). This is similar to that expected for cells belonging to the species *M. aeruginosa* (4-6 µm long).[120,121]

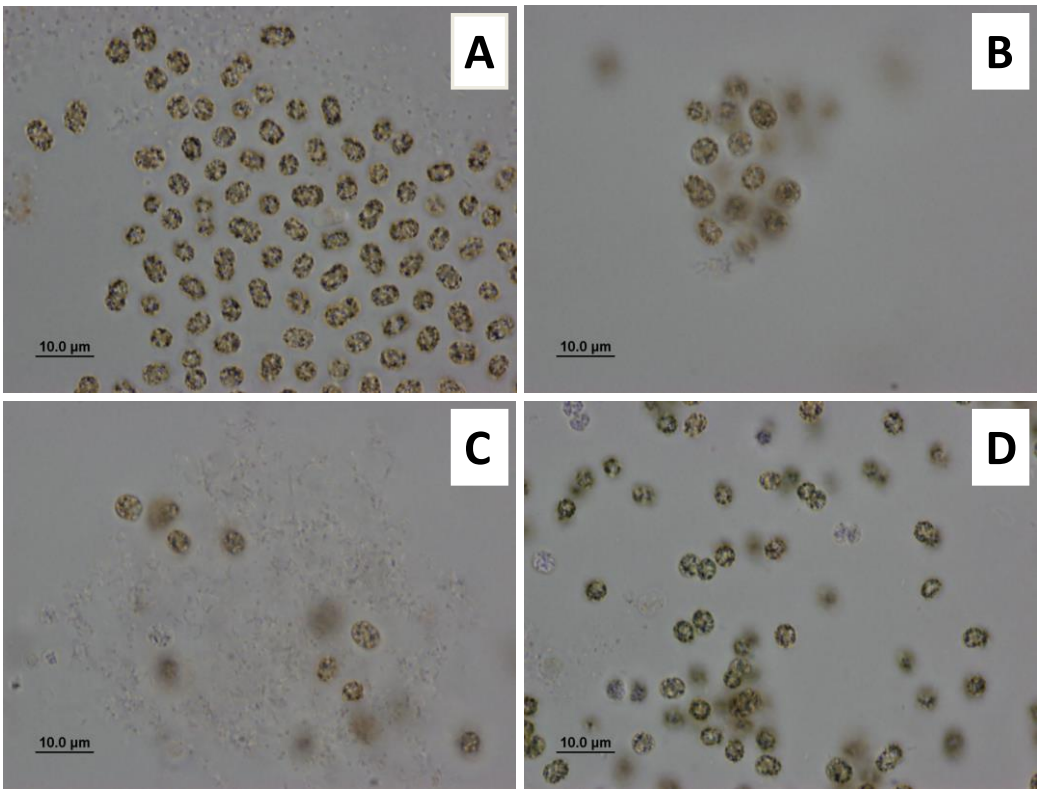


Figure 3.2: Photomicrographs of cyanobacterial strains isolated from Lake Rotorua (Kaikoura, New Zealand) (**A**) Rotorua strain A, (**B**) Rotorua strain B, (**C**) Rotorua strain C and (**D**) Rotorua strain D.

Table 3.1: Average diameter of Rotorua strains A, B, C and D (*n*=30).

Strain	Average diameter (µm)	Range (µm)
Rotorua A	4.3	3.6-5.6
Rotorua B	4.3	3.4-5.5
Rotorua C	4.5	3.5-6.0
Rotorua D	3.6	2.8-4.7

3.2.2 Molecular Characterisation

DNA sequence analysis was carried out in order to identify the nearest species match based on the 16S rRNA gene sequences of the four strains. The partial sequences of the 16S rRNA gene (1360 base pairs) of Rotorua A, C and D strains were identical. Rotorua strain B was identical to the other three strains apart from base position 101, where Rotorua strain B had a guanine instead of an adenine and base position 478, where a thymine was present instead of a cytosine. Comparison of the 16S rRNA gene sequences with 16S rRNA gene sequences from the NCBI Gene Bank database revealed that all strains had a high (>99%) sequence homology to that of *M. aeruginosa* (Table 3.2).

Table 3.2: Sequence homology between the 16S rRNA gene sequences of Rotorua strains A, B, C and D and 16S rRNA sequences from the NCBI GenBank database.

Strain	Accession no.	ID of nearest match; accession no.	% ID
Rotorua A	KF481973	*M. aeruginosa*; HE975020.	100
Rotorua B	KF597137	*M. aeruginosa*; AP009552	100
Rotorua C	KF597138	*M. aeruginosa*; HE975020	99
Rotorua D	KF597139	*M. aeruginos*a; HE975020	99

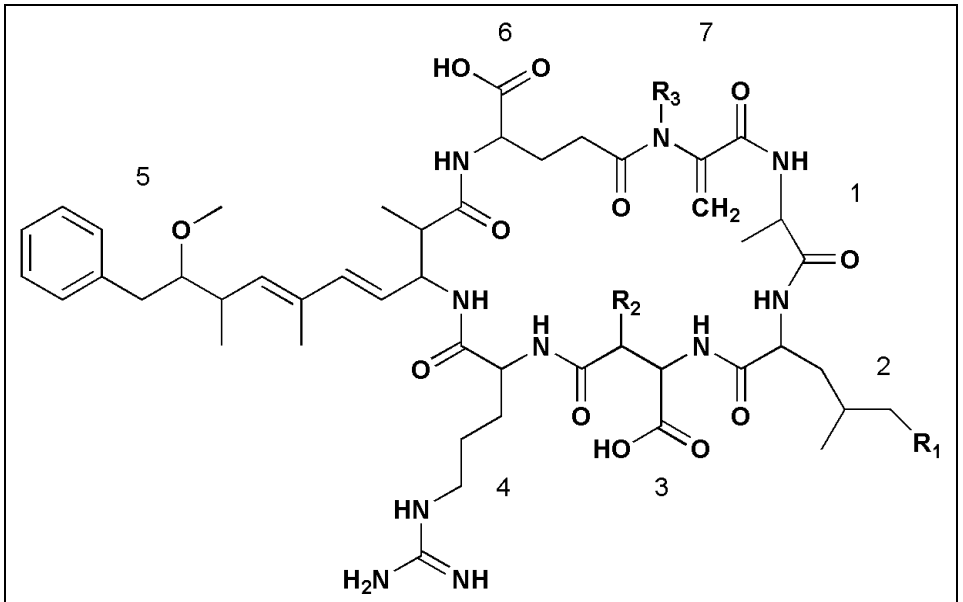
3.2.3 Chemical Characterisation

LC-MS was used to identify peptides present in the four strains by screening for ions of a mass-to-charge ratio (*m/z*) which corresponded to any known peptides and the structures were confirmed using MS/MS analysis. A thiol derivatisation technique using β-mercaptoethanol was also used to confirm the presence of a methyldehydroalanine (Mdha) or dehydroalanine (Dha) containing microcystin.

The predominant peak present in the liquid chromatograms of Rotorua A, B, C and D strains corresponded to *m/z* of 981.5 ion in the positive ion mode at a retention time consistent with that of the microcystin (MC) congener desmethyl MC-LR (ca. 5.8 min). A peak with *m/z* 967.5 ion was also detected at the expected retention time of didesmethyl MC-LR (ca. 6.1 min). Rotorua strains B and C also contained a minor peak with *m/z* of 995.5 which correlates with the commonly observed MC-LR.

Thiol derivatisation was used to determine whether the compounds observed in the Rotorua cyanobacterial strains were Mdha-/Dha-containing microcystins. Under alkaline conditions, the amino acids Mdha or Dha react readily with β-mercaptoethanol to form a derivatised product that has increased in mass by 78 Da, while derivatisation of microcystins containing Dhb moieties takes substantially longer.[122] This thiol derivatisation technique indicated that *m/z* 995.5, 981.5 and 967.5 ions were highly likely to be Mdha-/Dha-containing microcystins as a 78 Da mass increase was observed following a two hour incubation.

Fragmentation by tandem mass spectrometry of *m/z* 981.5 and 967.5 ions in Rotorua A strain indicated the presence of several characteristic daughter ions for [Dha^7] MC-LR and [D-Asp^3, Dha^7] MC-LR respectively (Figure 3.3, Table 3.3). Fragmentation of the $[M+H]^+$ 995.5 ion did not result in the characteristic daughter ions for MC-LR as expected but instead revealed a fragmentation pattern indicative of a new microcystin congener; [Dha^7] MC-HilR (Figure 3.3, Table 3.4 and Appendix B).



Microcystin	M_r (Da)	R_1	R_2	R_3
[Asp^3, Dha^7] MC-LR	966.5	-H	-H	-H
[Dha^7] MC-LR	980.5	-H	-CH_3	-H
MC-LR	995.5	-H	-CH_3	-CH_3
[Dha^7] MC-HilR	995.5	-CH_3	-CH_3	-H

Figure 3.3: Structures of microcystin congeners detected in Rotorua A strain.

Table 3.3: Fragment ions detected for *m/z* 981.5 and 967.5 microcystin ions in Rotorua A strain.

Fragment Assignment	**[Dha^7] MC-LR**	**[D-Asp^3, Dha^7] MC-LR**
M + H	981	967
M – $CH_2NHCN_2H_3$ + H	909	895
M – (Me)Asp + H	852	
M – Glu + H	852	838
(Me)Asp-Arg-Adda-Glu + H	728	714
Arg-Adda-Glu + H	599	599
(Me)Asp-Arg-Adda + H	599	
Dha-Ala-Leu-(Me)Asp-Arg + H	539	525
Dha-Ala-Leu-(Me)Asp-Arg + NH_4		542
Ala-Leu-(Me)Asp-Arg + H	470	
Arg-Adda + H	470	470
Adda'-Glu-Dha-Ala + H	432	432
Adda'-Glu-Dha + H	361	

Table 3.4: Fragment ions detected for *m/z* 995.5 microcystin ion in Rotorua A strain.

Fragment Assignment	**[Dha^7] MC-HilR**
M + H	995
M – H_2O + H	977
Masp-Arg-Adda-Glu + H	728
Masp-Arg-Adda + H	599
Arg-Adda-Glu + H	599
Dha-Ala-Hil-Masp-Arg + NH_4	570
Dha-Ala-Hil-Masp-Arg + H	553
Ala-Hil-Masp-Arg + NH_4	501
Arg-Adda + H	470
Adda-Glu-Dha-Ala + H	432
Adda-Glu-Dha + H	361

The LC-MS screen of the Rotorua A strain revealed a peak corresponding to $[M-H]^-$ of 601, indicating the presence of the non-ribosomal peptide aeruginosin.[123] Further MS/MS analysis in the negative ion mode resulted in several daughter ions indicative of aeruginosin 602 (Figure 3.4 and Table 3.5).

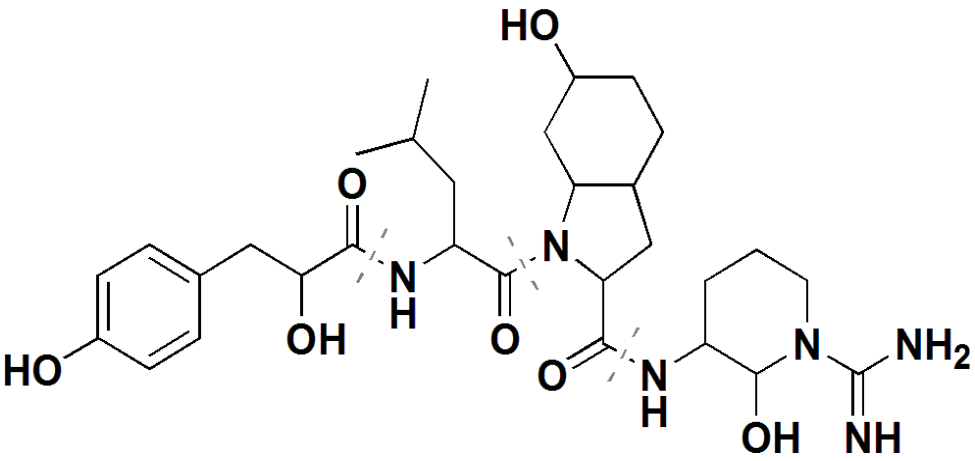


Figure 3.4: Structure of aeruginosin 602.

Table 3.5: Fragment ions detected for *m/z* 601 peptide ion in Rotorua A.

Fragment Assignment	**Aeruginosin 602**
M – H	601
M – NH_3– H	584
M – H_2O– H	583
M – CN_2H_2– H	559
M – H_2O-CN_2H_2– H	541

3.2.4 Development of the Comparative Microcystin Export Assay

Of the four *Microcystis aeruginosa* strains isolated and characterised, Rotorua strain A was selected to use for developing the CME assay as it contained [Dha]^7 MC-LR, [Asp^3, Dha^7] MC-LR, an unknown microcystin congener (putatively [Dha]^7 MC-HilR) and aeruginosin, a non-ribosomal peptide which meets the requirements for a comparative peptide as described in Section 3.1. A multiple reaction monitoring (MRM) method analysing [Dha^7] MC-LR ([M−H]$^-$ = 979.5), [D-Asp^3, Dha^7] MC-LR ([M−H]$^-$ = 965.5) and aeruginosin ([M−H]$^-$ = 60.5) was created using the collision amplitude of 0.80. The analysis of [Dha]^7 MC-HilR was excluded due to its low abundance and the requirement for a second injection in order to quantify the compound (Table 3.6).

Table 3.6: MRM method for the comparative microcystin export assay.

Retention time (min)	**Peptide**	**[M−H]$^-$**	**Quantitation Fragment**
0-4.10	Aeruginosin	601.5	541.5
4.10-14	[Dha^7] MC-LR	979.5	961.5
	[Asp^3, Dha^7] MC-LR	965.5	947.5

The optimal collision amplitude to produce aeruginosin 602 fragment ions in the highest abundance was determined. At all collision amplitudes tested, the dominant daughter ion for aeruginosin was at *m/z* 541 ($[M - H_2OCN_2H_2]^-$) and its abundance was similar for all collision amplitudes tested (Figure 3.5). Therefore, the collision amplitude of 0.80 was selected as this is the collision amplitude at which microcystins were also most abundant.[37]

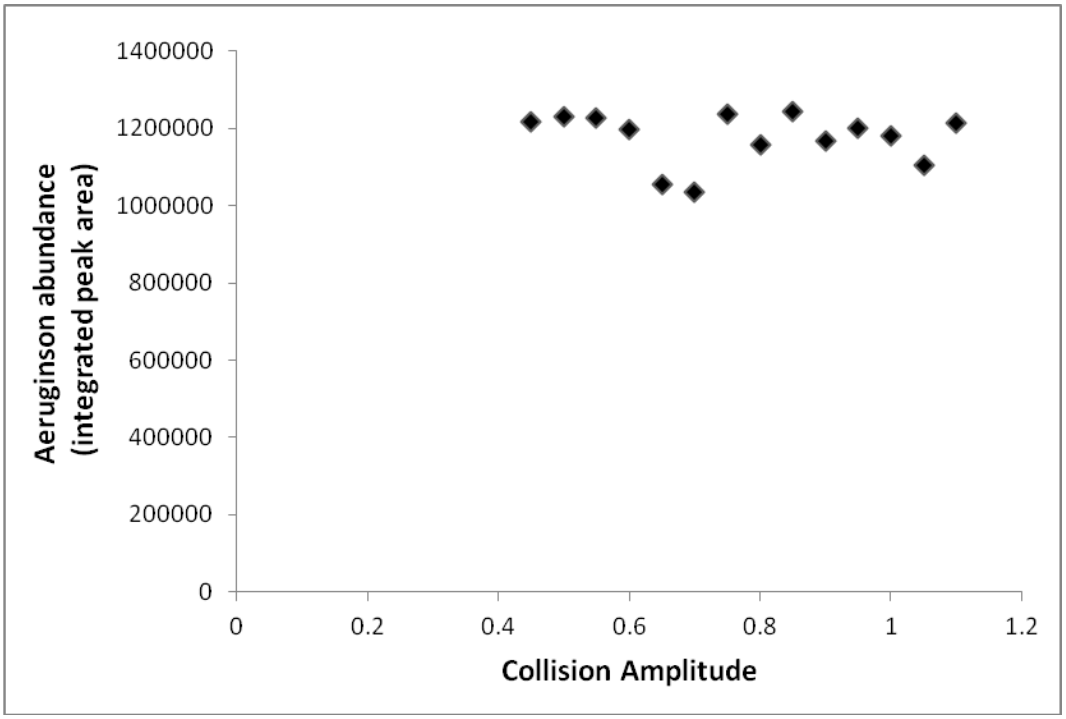


Figure 3.5: Abundance of aeruginosin at selected collision amplitudes.

A liquid chromatography gradient was developed which was able to separate [Dha^7] MC-LR, [D-Asp^3, Dha^7] MC-LR and aeruginosin 602 in the shortest time possible whilst maintaining repeatable chromatography (Table 3.7).

Table 3.7: High performance liquid chromatography gradient for the comparative microcystin export assay.[a]

Time (min)	%A (98:2 H_2O/ACN + 0.1% FA[b])	%B (2:98 H_2O/ACN + 0.1% FA[b])
0	90	10
1	90	10
1.5	80	20
7.5	0	100
9.5	0	100
10.5	90	10
14	90	10

[a] Flow rate = 250 µL/min; Column temperature = 40 °C.
[b] FA = Formic Acid

The repeatability of the MRM response for six injections of Rotorua A strain intracellular extract and extracellular fraction was acceptable, with a relative standard deviation ranging between 4.7 and 14.3% (Table 3.8).

Table 3.8: Repeatability of the multiple reaction monitoring method for the comparative microcystin export assay.

Peptide	Intracellular Sample (%RSD[a])	Extracellular Sample (% RSD[a])
Aeruginosin 602	14.3	5.5
[Dha^7] MC-LR	5.9	7.8
[Asp^3, Dha^7] MC-LR	4.7	4.5

[a] RSD = relative standard deviation

A linear peptide, angiotensin I, was considered for use as an internal standard, however, due to high variability between replicates and the possible degradation of angiotensin I in the cell extract (Appendix C), an internal standard was not used.

3.2.5 Validation of the Comparative Microcystin Export Assay

Positive Control: Induction of Cell Lysis

Evidence showing that the auxiliary peptide does not get actively exported from the cell was sought to validate the CME assay by measuring the proportion of auxiliary peptides present as a result of the level of induced cell lysis. The CytoTox 96® Non-Radioactive Cytotoxicity Assay was tested for its efficacy in measuring the level of cell lysis induced by the cell lysis agent, cocamidopropyl betaine (CAPB).

The concentration of cells lysed by freeze-thawing was linearly proportional (R^2 = 0.98) to the lactacte dehydrogenase (LDH) activity (absorbance at 490 nm) detected by the CytoTox 96 Non-Radioactive Cytotoxicity Assay from 77,000 cells/mL (Figure 3.6). Below this cell concentration, the LDH activity was too weak to be detected using the assay and the absorbance at 490 nm fluctuated between 0.048 and 0.084 (Figure 3.7).

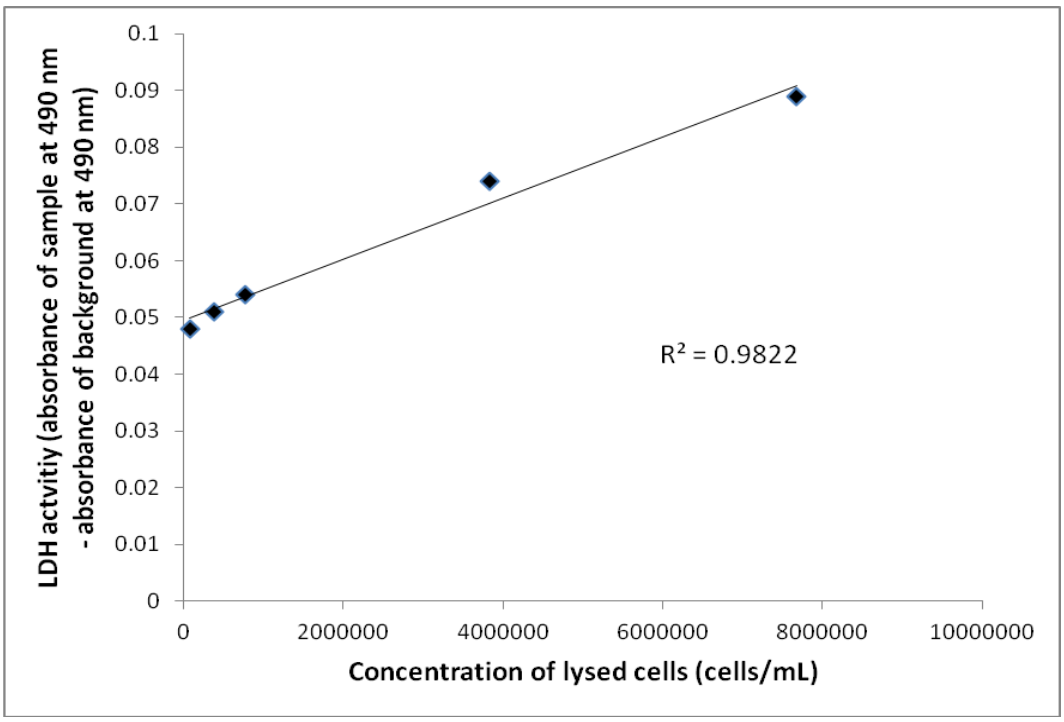


Figure 3.6: Linear response of LDH activity detected by CytoTox 96 Non-Radioactive Cytotoxicity Assay in relation to degree of cell lysis.

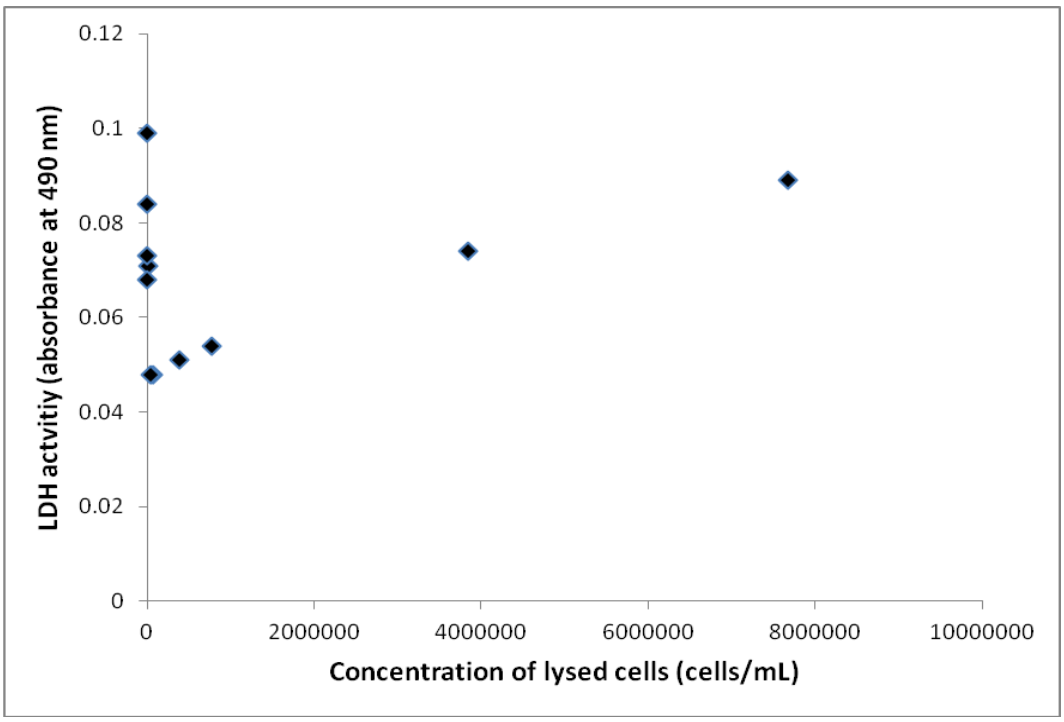


Figure 3.7: LDH activity detected by CytoTox 96 Non-Radioactive Cytotoxicity Assay in relation to degree of cell lysis.

A control sample containing culturing media, used to correct for background LDH activity absorbance and any other possible absorbing compounds at 490 nm, had an absorbance of 0.99. After correction for the background activity, the absorbance values were negative, indicating poor sensitivity for the CytoTox 96 Non-Radioactive Cytotoxicity Assay when used on *Microcystis* cells.

The cytotoxic compound, CAPB, appeared to induce cell lysis in a dose dependant manner (R^2= 0.94) after its incubation with Rotorua A strain for one hour (Figure 3.8). Maximum cell lysis was achieved after treatment with 175 mg/L of CAPB. The LDH activity remained consistent on exposure to higher CAPB concentrations (Figure 3.9). There was a high degree of variability between triplicate samples, indicating poor repeatability when using the CytoTox 96® Non-Radioactive Cytotoxicity Assay on Rotorua A with CAPB as the cytotoxic agent (Figure 3.9).

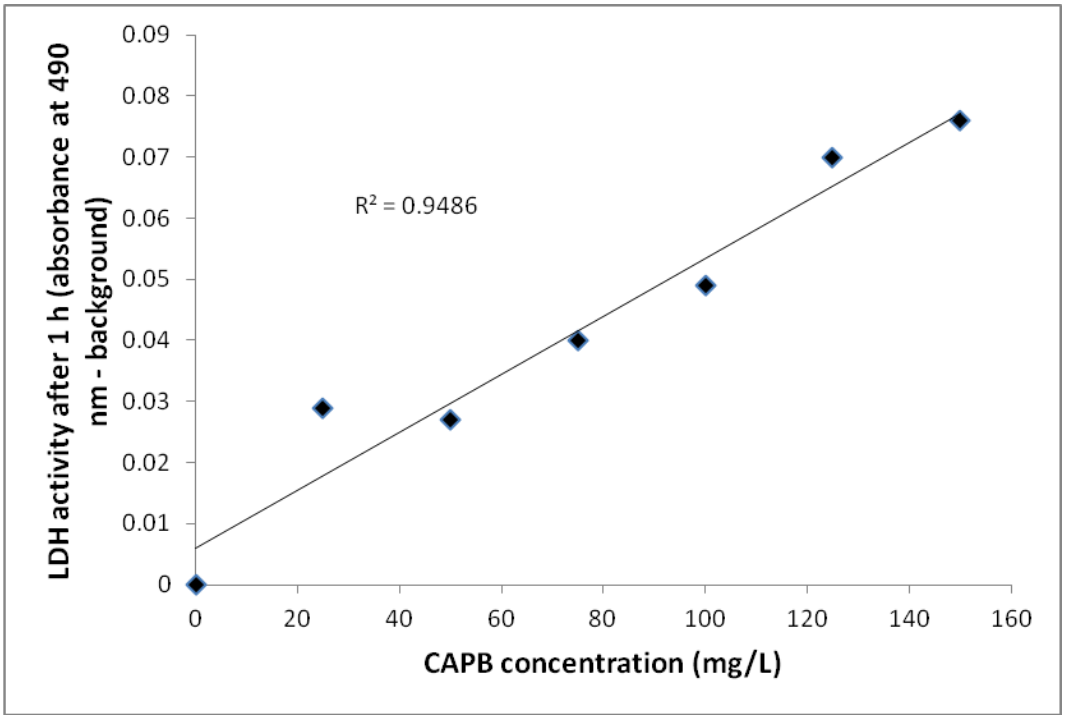


Figure 3.8: LDH activity after incubation with CAPB for one hour.

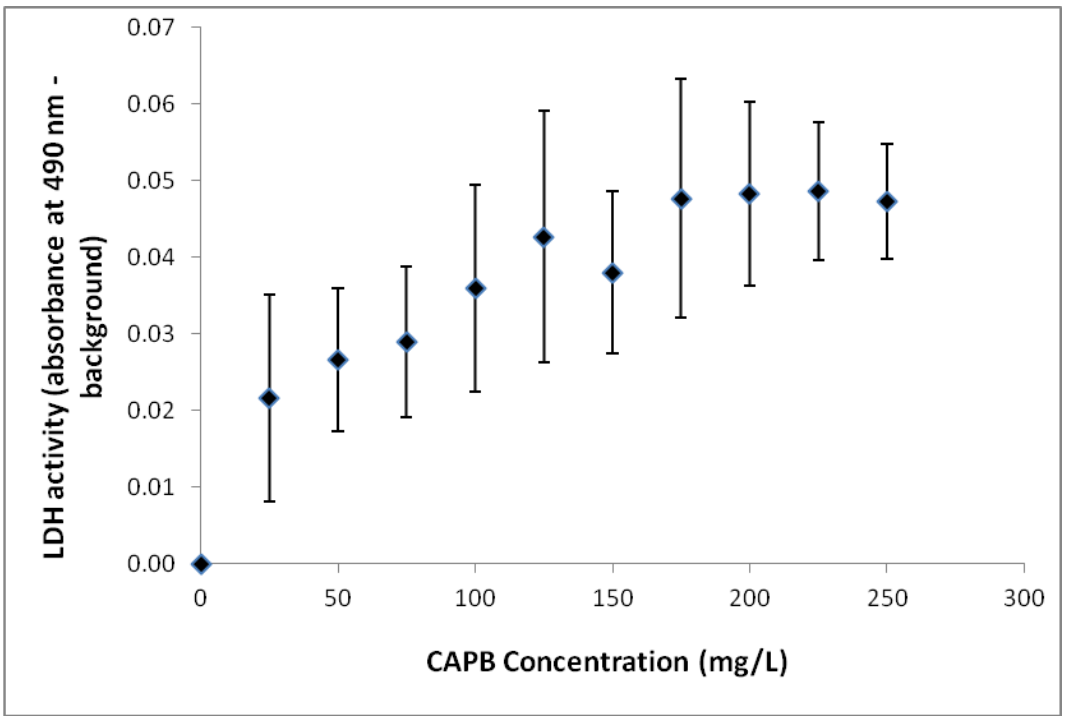


Figure 3.9: LDH activity detected by CytoTox 96 Non-Radioactive Cytotoxicity Assay after incubation with CAPB.

Due to poor repeatability, the CytoTox 96 Non-Radioactive Cytotoxicity Assay Cytotoxicity using CAPB was not suitable for use in conjunction with the CME assay as a positive control for method validation at the present time.

Negative Control: Natural Growing Conditions

The CME assay was used to determine whether active export of microcystins occurred over several growth phases of Rotorua A *Microcystis* strain over a culturing period of eleven days.

During this period, cell density increased from 1,000,000 cells/mL to 3, 500,000 cells/mL over the first four days, followed by a gradual decrease over the subsequent week (Figure 3.10).

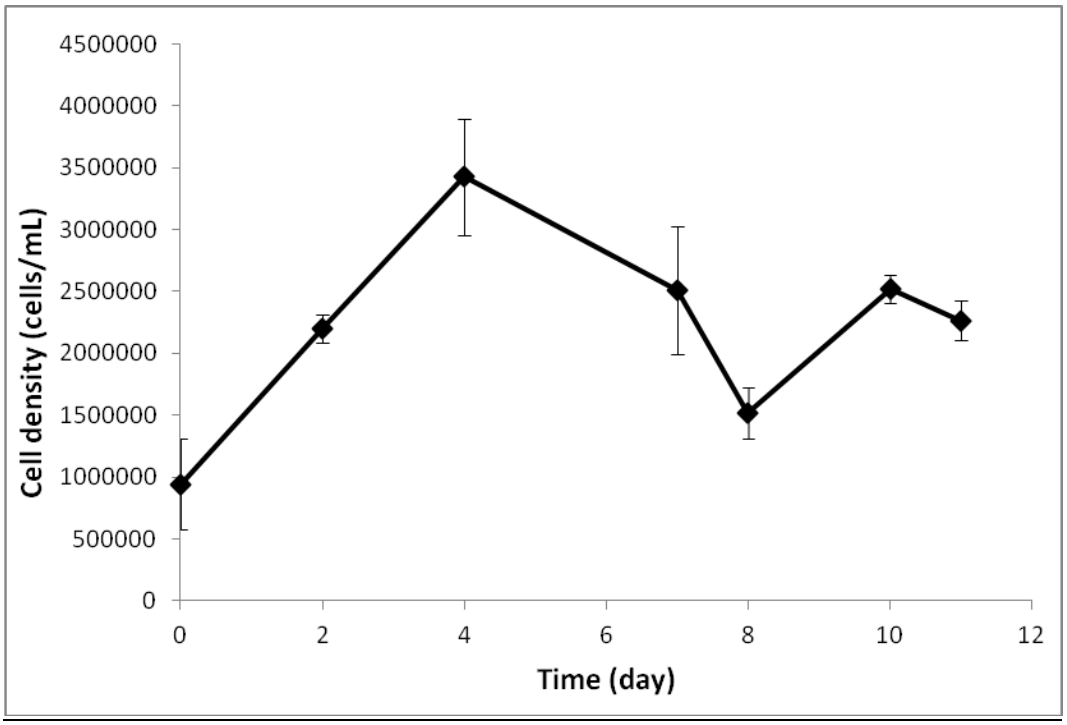


Figure 3.10: Cell density over the sampling period.

Both intracellular microcystin and aeruginosin quotas fluctuated at the same time and at similar rates (Figure 3.11). Extracellular aeruginosin remained fairly constant throughout the sampling period while, in contrast, extracellular microcystins increased over the duration of the experiment (Figure 3.12). The increase in the extracellular microcystin quota coincided with a decrease in cell density between days four and eight and an increase in the intracellular microcystin quota between days four and seven.

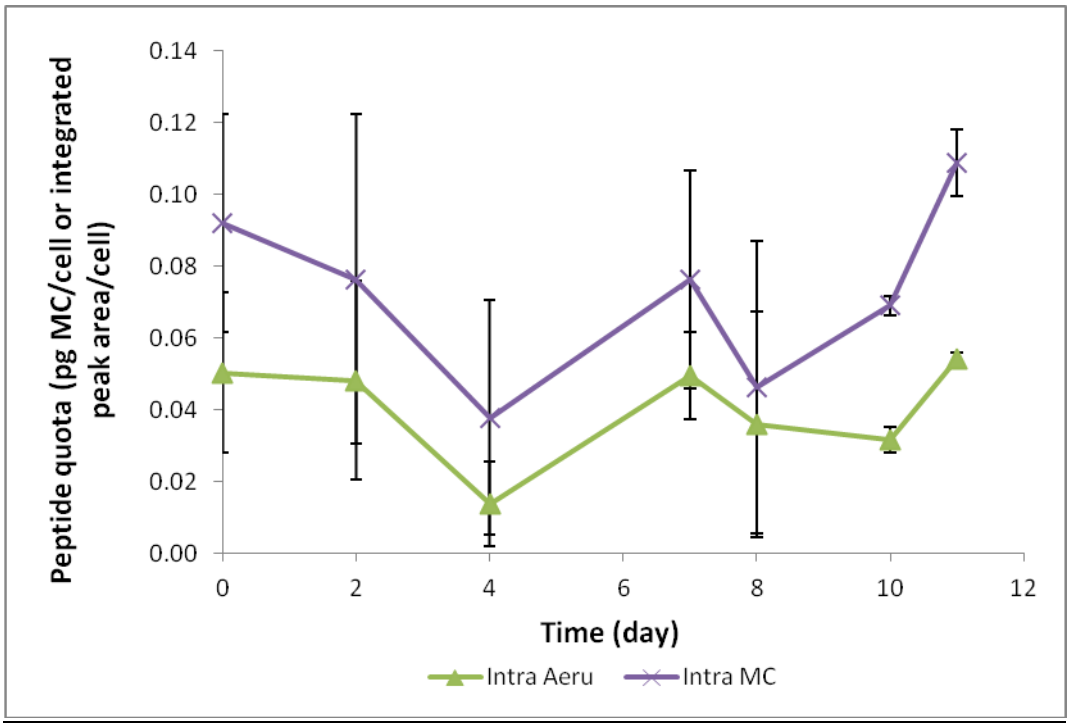


Figure 3.11: Intracellular (Intra) microcystin (MC) and aeruginosin (Aeru) quotas over the sampling period.

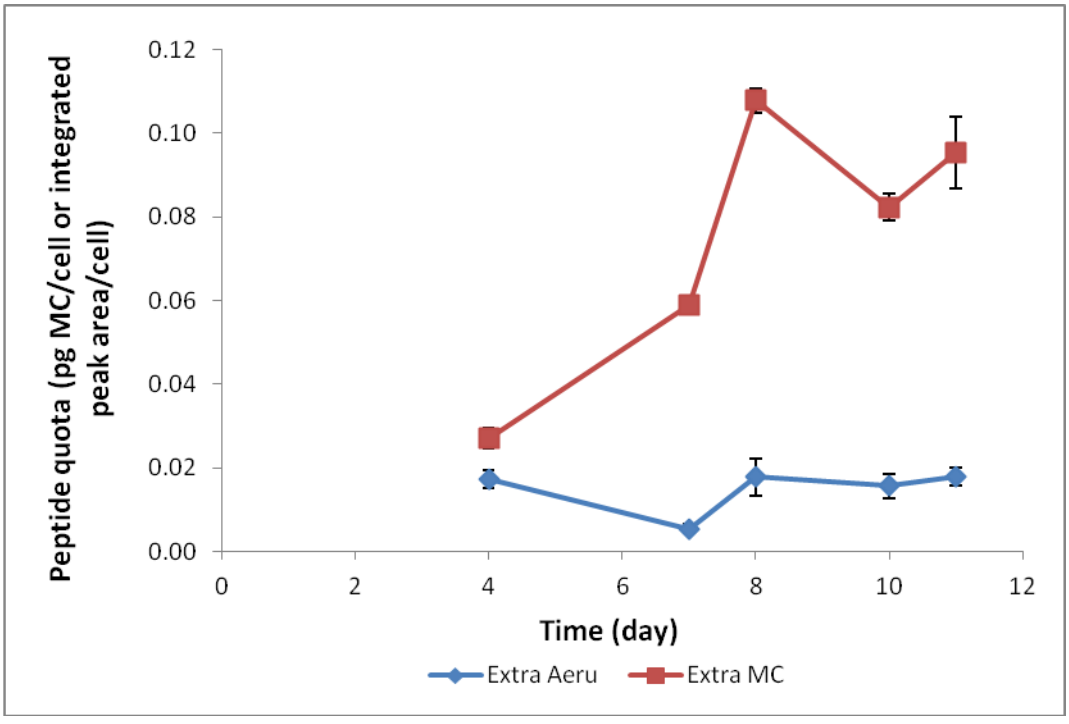


Figure 3.12: Extracellular (Extra) microcystin (MC) and aeruginosin (Aeru) quotas over the sampling period.

The decline in the EA/EM ratio between days four and seven represents the disproportionate increase in extracellular microcystin in comparison to extracellular aeruginosin (Figure 3.13).

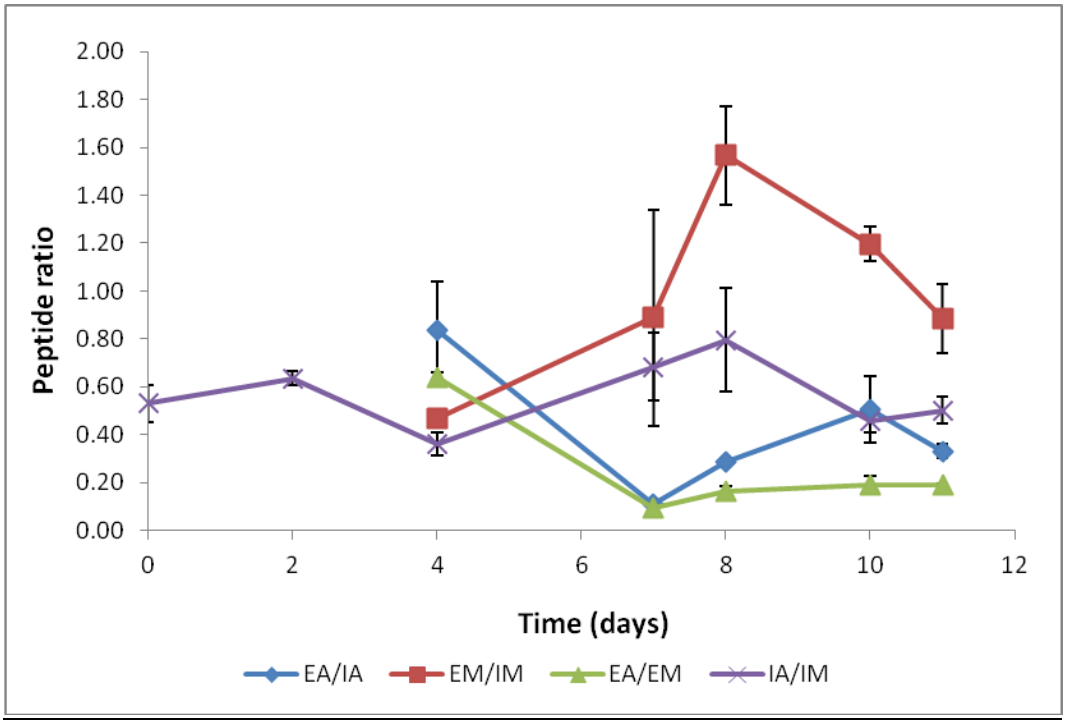


Figure 3.13: Intracellular (I) and Extracellular (E) microcystin (M) and aeruginosin (A) ratios over the sampling period.

3.3 Discussion

Morphological and DNA sequence analysis indicated the four strains isolated from Lake Rotorua were *Microcystis aeruginosa*. LC-MS/MS analysis showed that all strains contained [Dha^{7}] MC-LR and [D-Asp^{3}, Dha^{7}] MC-LR congeners while Rotorua A strain contained an additional new congener, putatively [Dha^{7}] MC-HilR. Rotorua A strain also contained a linear non-ribosomal peptide; aeruginosin 602, at sufficient levels and was therefore selected for CME assay development. Aeruginosin 602 was a convenient peptide for comparison as it was easily separated from the microcystins and present at quantifiable levels.

Experiments determining the degradation rates of aeruginosin and the microcystins analysed by this method would be required to confirm that different degradation rates have no influence on the ratio of extracellular microcystins to aeruginosin. A method that compared extracellular levels with multiple peptides present in many microcystin producing genera would have a wider applicability, however, difficulties in identifying unknown peptides in the strains and the lack of knowledge of the structure and behaviour of these peptides prevented further development at this point in time.

In order to prove the efficacy of the CME assay, evidence showing that aeruginosin does not itself get actively exported out of cyanobacterial cells is required. Cyanobacteria are known to contain LDH[124] and a *Microcystis* strain, PCC7806, was found to possess a NAD-dependent lactate dehydrogenase which catalyses the reduction of pyruvate to L-lactate.[125] Initial pilot tests demonstrated that an LDH assay may be a good indicator of cell lysis in Cyanobacteria, and could be used in parallel with LC-MS/MS analysis to determine whether extracellular aeruginosin levels are proportional to the degree of cell lysis induced. The CytoTox 96® Non-Radioactive Cytotoxicity Assay used during this study resulted in a linear detection of LDH activity in response to the concentration of cells lysed. Unfortunately, the kit had poor sensitivity and could not be used during the culturing experiment conducted. A different kit adapted for bacterial cells may be preferable to achieve higher sensitivity (for example, the CytoSelect™ LDH Cytotoxicity Assay Kit). CAPB was shown to be a good cytotoxic agent as it induced cell lysis in a dose dependant manner after incubation with cyanobacterial cells for one hour. It is possible that the low repeatability observed between triplicate samples was due to using a colonial

strain of *Microcystis* with colonies of different sizes affecting the cell lysis efficiency of CAPB. Another explanation could be that the assay was not sensitive enough for use with cyanobacterial cells, resulting in larger variations in absorbance values due to it being close to or below the limits of detection. Further optimisation and experiments are required to determine the applicability of this assay for indicating cell lysis using CAPB.

The laboratory experiment monitoring changes in extracellular and intracellular peptide quotas demonstrated the utility of the CME assay developed by showing that extracellular microcystins increased at a higher rate than extracellular auxiliary peptides. A doctoral thesis investigating peptides in *Microcystis* strains showed a balanced distribution of aeruginosin intracellularly and extracellularly and suggested that this finding along with the properties of the peptide (relatively small, linear and polar) may be indicative of active export of aeruginosin.[126] The current study found that the percentage of extracellular aeruginosin to total aeruginosin ranged between 0 and 56%. However, the relatively consistent levels of extracellular aeruginosin maintained throughout the experiment coupled with fluctuations in the intracellular aeruginosin quota, suggests that aeruginosin was likely not being actively exported out of the cell and that the percent extracellular aeruginosin was more a reflection of changes in intracellular aeruginosin quotas.

The disproportional increase in the extracellular microcystins compared with extracellular aeruginosin between days four and seven suggested the active export of microcystins over this period. The increase in extracellular microcystins coincided with an increase in intracellular microcystins, providing further evidence for the upregulation and active export of microcystins. However,

between days four and seven there was a decrease in cell density, suggesting either a delayed response and the requirement of time for microcystins to accumulate from the previous increase in cell density, or that cell density did not trigger the active export of microcystins. The increase in the concentration of extracellular microcystin quota was only four-fold within a four-day period which is low in comparison to previous in-lake observations (eight-fold within a two hour period).[74] However, the increase in extracellular microcystins in the field study was coupled to a mass aggregation event caused by wind and wave action, which was not simulated in this study.[74]

3.4 Conclusion

The CME assay developed provides a starting platform for further expansion and validation. A preliminary laboratory experiment carried out using the CME assay indicated that export from a *Microcystis* strain occurred under the gentle culturing conditions and thus demonstrated the utility of this new technique. Experiments determining the degradation rates of the extracellular microcystins and aeruginosin, and other culturing experiments conducted in parallel with a functioning cytotoxicity assay are still required for validation of the assay. Once optimised, the CME assay could provide a valuable tool for future culturing experiments and field work to determine whether microcystins are actively exported from microcystin producing Cyanobacteria and if so, under what environmental conditions.

CHAPTER 4: RELATIONSHIP BETWEEN *MICROCYSTIS* CELL DENSITY AND MICROCYSTIN PRODUCTION IN MESOCOSMS

4.1 Introduction

Despite extensive research, the factors regulating microcystin production are still largely unknown. Most of the studies carried out have been laboratory-based using a single strain of microcystin producing Cyanobacteria and manipulating only one physicochemical parameter such as nitrogen or phosphorus,[49-51,56] temperature[42-46] or iron availability.[53] Although many of these studies have demonstrated correlations between microcystin quota (concentration of microcystins per cell) and the altered environmental parameter, results between studies are often contradictory and experiments have only induced three- to four-fold changes in microcystin quotas.[55] The regulation of microcystin production could be the result of a complex interaction of abiotic and/or biotic factors that cannot easily be replicated in an artificial laboratory environment.

In 2009, a study was conducted in which samples were collected from Lake Rotorua, a small eutrophic lake in the South Island of New Zealand, during the formation and dispersal of a dense *Microcystis* scum.[74] This study found that when cell concentrations increased from 1,400,000 up to 5,400,000 cells/mL, there was a corresponding twenty eight-fold increase in microcystin quotas. Microcystin producing cells have only one copy of the microcystin synthase gene, thus quantitative polymerase chain reaction (QPCR) assays[127,128] were used to

determine the number of toxic cells (genotypes) by quantifying the concentration of *mcyE* copies in each sample. The results showed a high congruence with microscopically determined cell counts, indicating that almost all the cells in the population were toxic. The increase in cell quotas coincided with increased levels of *mcyE* gene transcripts which were determined using a reverse transcriptase-quantitative polymerase chain reaction (RT-QPCR) assay,[129] providing convincing evidence that changes in *Microcystis* cell concentrations influenced microcystin production. Furthermore, a corresponding increase in extracellular microcystin concentrations with increased microcystin quotas was observed, possibly indicating that microcystins were being actively exported out of the cell and could be functioning as signalling molecules.[74] In an extension of this study, an *in situ* experiment was carried out at Lake Rotorua using mesocosms to manipulate cell density and study the effect on microcystin production.[57] A marked increase in microcystin quotas (eighteen-fold) was observed when the cell density was increased from 500,000 cells/mL to 7,000,000 cells/mL over six hours. No relationship was observed between extracellular microcystins and cell density or between microcystin quota and the abiotic parameters measured during this study (temperature, light and nutrients). However, it is still possible that changes in these and other interacting parameters associated with changes in cell density (for example, pH, nutrients and dissolved oxygen) could be regulating microcystin production.

Previous studies have attributed differences in microcystin concentrations to differences in the abundance of microcystin and non-microcystin producing *Microcystis* genotypes.[130] Lake Rotorua is dominated by two cyanobacterial genera, *Microcystis* and *Anabaena*, with only *Microcystis* capable of producing

microcystins.[57] Lake Rotorua is an ideal study site as the microcystin concentrations are unlikely to be influenced by other non-microcystin producing genera (less than 3% of total cyanobacterial cells) and all *Microcystis* genotypes are toxic, allowing straightforward determination of microcystin quota.[57]

In the present study, an increased understanding of the mechanisms involved in triggering microcystin synthesis was sought by replicating a similar *in situ* experiment in Lake Rotorua using mesocosms to manipulate cell density, but assessing a wider spectrum of parameters simultaneously. The mesocosms, made from polyethylene bags attached to a float, provided the opportunity to study microcystin production in the natural environment but under controlled conditions.

4.2 Results

Lake water was added to the 'control' mesocosms while cyanobacterial cells in the lake water adjacent to the mesocosms were concentrated and added to the 'medium cell addition', 'high cell addition' and 'high cell addition-cell removal' mesocosms. Over the following 45 minutes, additional lake water was concentrated and added to 'the high cell addition' and 'high cell addition-cell removal' mesocosms to simulate the formation of a dense cyanobacterial scum. After three hours, cells were removed from 'high cell addition-cell removal' mesocosms. The 'high cell addition' mesocosms were mixed at intervals during this process to replicate the mixing occurring in 'high cell addition-cell removal' mesocosms. At various intervals, abiotic parameters such as temperature, light intensity pH and dissolved oxygen were recorded. Samples were taken for nutrient analysis, cell enumeration, QPCR assays and microcystin analysis.

4.2.1 Abiotic Parameters

Temperature

The temperature at the surface of the mesocosms remained relatively constant (15.9-20.6 °C) with little variation between duplicate mesocosms and between mesocosms of difference cell additions (Figure 4.1). There was a gradual rise in surface temperature throughout the day until a maximum of ca. 21 ºC was reached at 13:30, after which it declined gradually. The air temperature followed the same general trend, but was 2-3 ºC cooler than the water surface in the mesocosms. The temperature at 20 cm depth within the mesocosms was more stable than at the surface and slightly cooler, ranging between 14.6 and 17.1 °C. A sharp decrease in temperature at the surface of the 'high cell addition' and 'high cell addition-cell removal' mesocosms (2.5 and 2.9 °C respectively) and a less pronounced decrease in temperature (0.7 °C and 0.4 ºC respectively) at 20 cm depth were observed at 13:40. The decrease in temperature coincided with cell removal from 'high cell addition-cell removal' mesocosms and mixing of 'high cell addition' mesocosms.

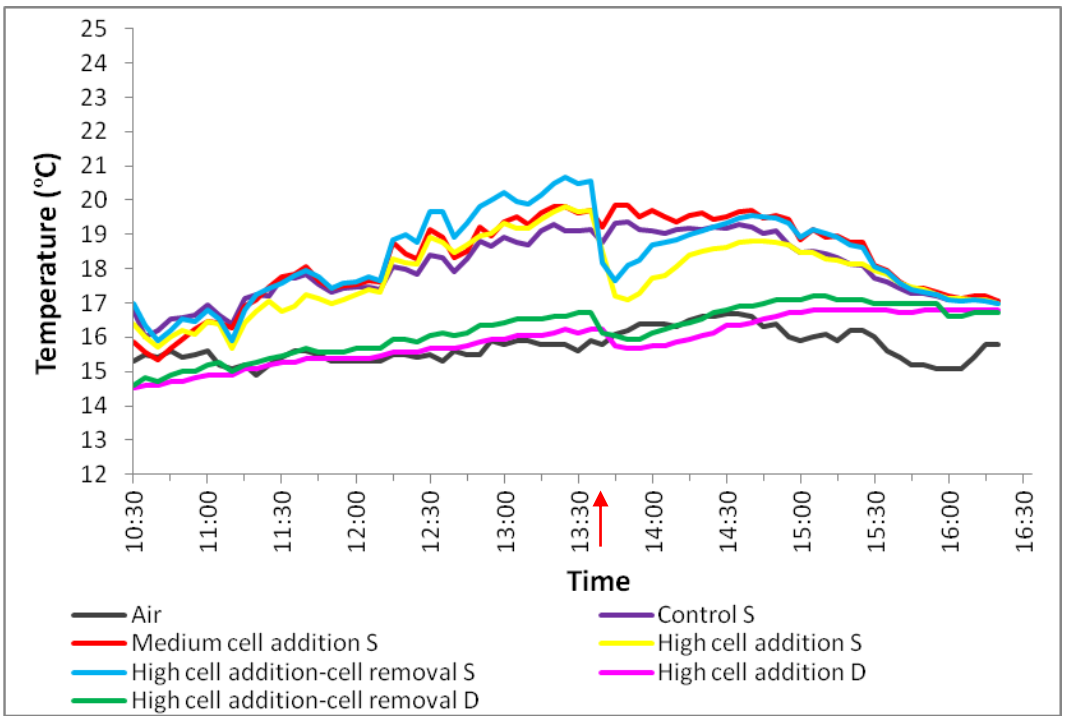


Figure 4.1: Average temperature (*n*=2) at the surface (S) of mesocosms and at 20 cm depth (D) of mesocosms over the sampling period. The arrow represents mixing in 'high cell addition' and 'high cell addition-cell removal' mesocosms.

Light Intensity

Frequent oscillations between 2,000 and 12,000 lux were observed at the surface of the mesocosms (Figure 4.2). Despite the high variability in light intensity detected with time, the same general trend as for temperature was observed for all mesocosms; a steady increase in light intensity until a maximum (between 74,400 and 115,700 lux for all mesocosms) at 13:30 before a gradual decline in light intensity. The light intensity at 20 cm depth was much lower (ca. five times less intense) and relatively stable in comparison to the light intensity at the surface of the mesocosms.

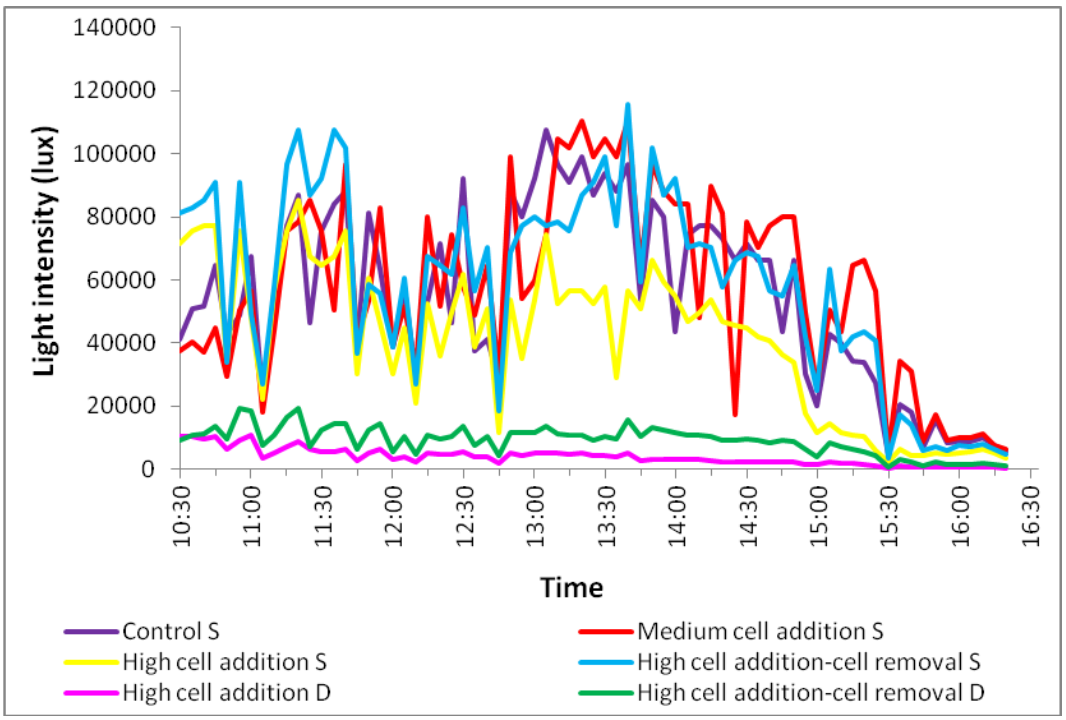


Figure 4.2: Average light intensity (*n*=2) at the surface (S) of mesocosms and at 20 cm depth (D) of mesocosms over the sampling period.

pH

The temperature-adjusted pH values steadily increased from 6.2 to 10.3 over the sampling period (Figure 4.3). The pH was more alkaline with increased cell addition. For example, at 13:20 (mid-way through the experiment), the average pH of the 'control' mesocosms was 9.0, while the average pH of the 'high cell addition' mesocosms was 10.4.

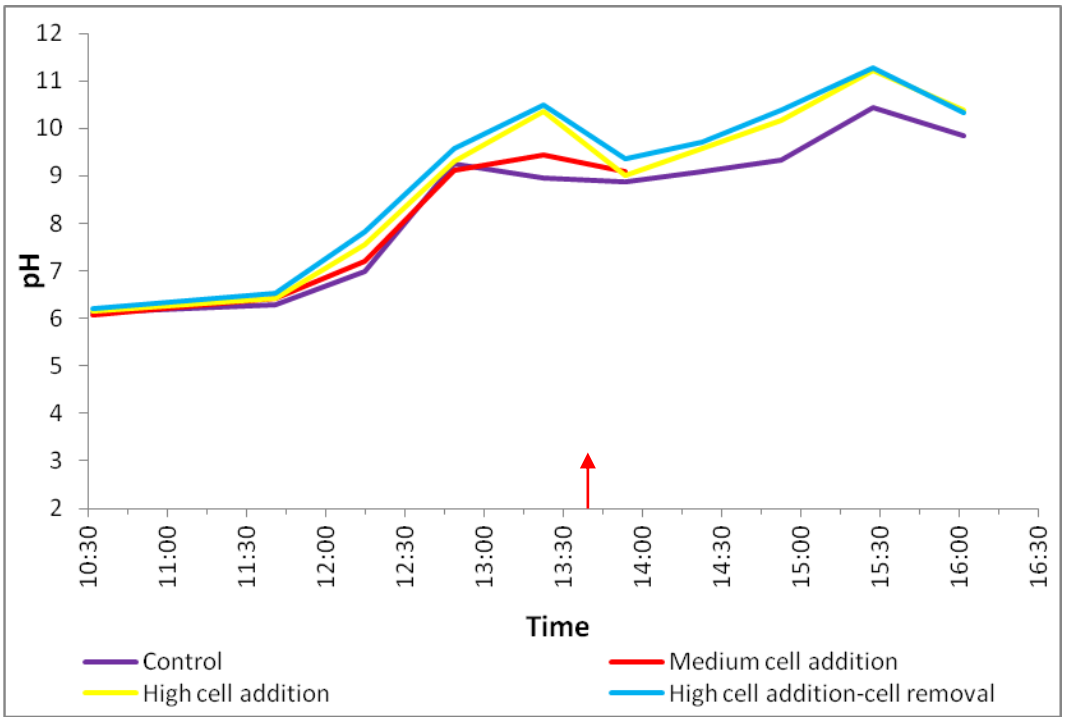


Figure 4.3: Average pH (*n*=2) at the surface of mesocosms over the sampling period. The arrow represents mixing in 'high cell addition' and 'high cell addition-cell removal' mesocosms.

Dissolved Oxygen

In general, the average temperature-adjusted dissolved oxygen at the surface of all mesocosms increased over time. The dissolved oxygen levels were much higher in the 'high cell addition' mesocosms compared with the 'control' mesocosms towards the end of the experiment at 15:50 (12.97 compared with 17.9 mg/L). A sharp decline in dissolved oxygen in the 'high cell addition' and 'high cell addition-cell removal' mesocosms was observed between 13:00 and 14:05 (ca. 3.6 mg/L and 2.56 mg/L respectively) which coincided with cell removal and mixing in those mesocosms.

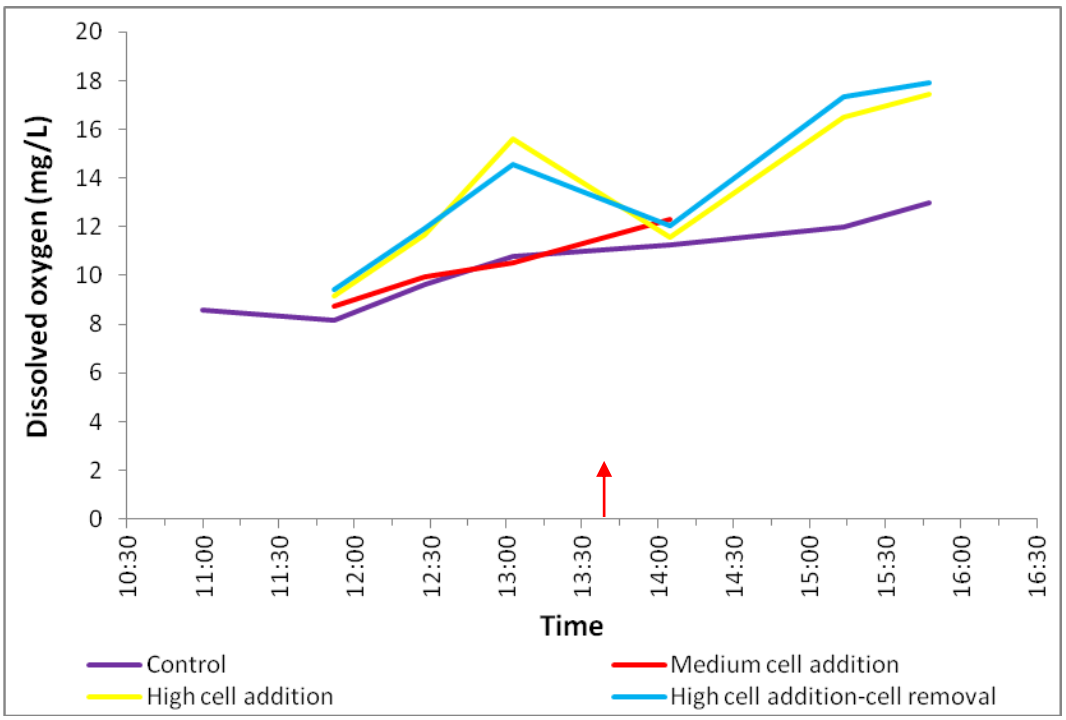


Figure 4.4: Average dissolved oxygen (*n*=2) at the surface of the mesocosms over the sampling period. The arrow represents mixing in 'high cell addition' and 'high cell addition-cell removal' mesocosms.

Dissolved and Total Nutrients

The levels of nitrate (NO_3-N) and dissolved reactive phosphorus (PO_4-P) remained relatively constant among the different mesocosms and over the sampling period apart from one outlier for PO_4-P at three hours (Figure 4.5 and Figure 4.6). This outlier was likely due to a sampling or analytical error as the same trend was not seen in both duplicates.

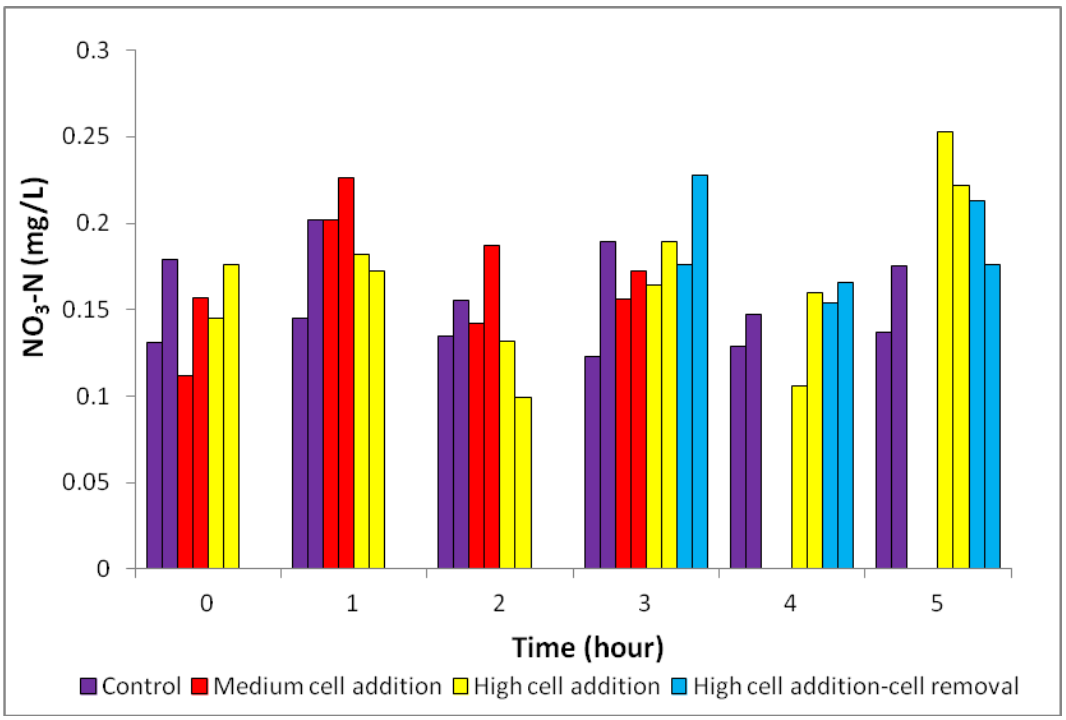


Figure 4.5: Nitrate-nitrogen (NO_3-N) in mesocosms over the sampling period.

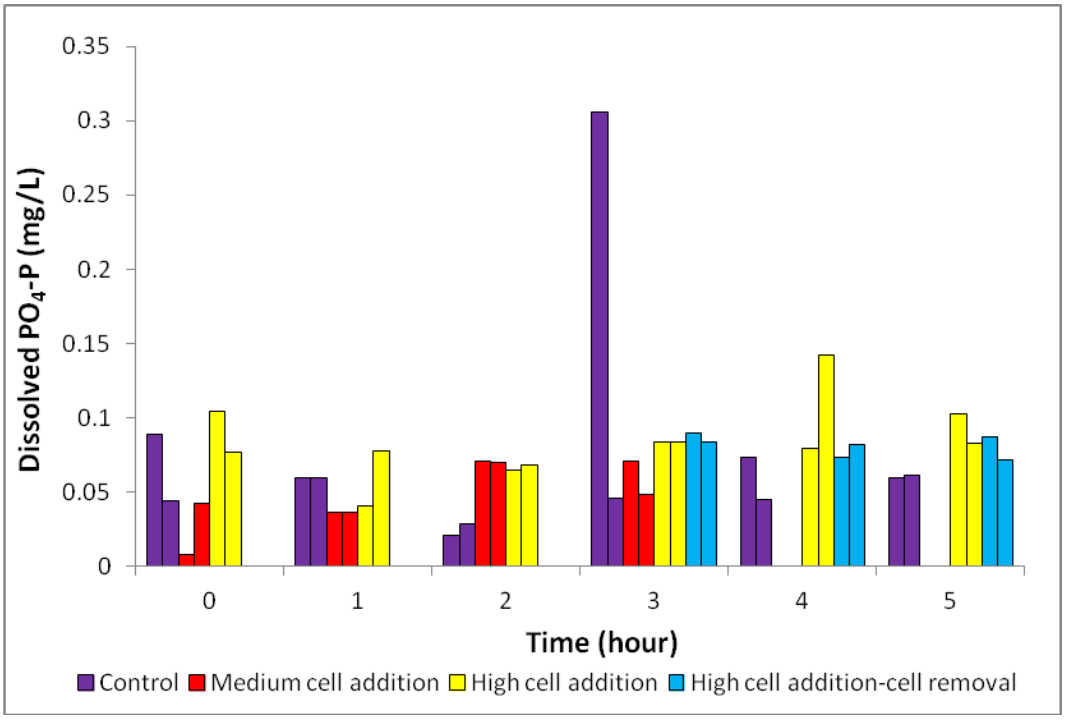


Figure 4.6: Dissolved reactive phosphorus (PO_4-P) in the mesocosms over the sampling period.

The ammonium (NH_4-N) concentration decreased in all of the mesocosms (Figure 4.7). A faster rate of NH_4-N concentration decrease was measured in the 'high cell addition' mesocosms (0.1 mg/L/h), followed by the 'medium cell addition' (0.06 mg/L/h) and then 'control' (0.04 mg/L/h). After cell removal at three hours, the dissolved NH_4-N concentration initially increased at four hours from 0.015 mg/L to 0.124 mg/L but decreased again to 0.013 mg/L at five hours.

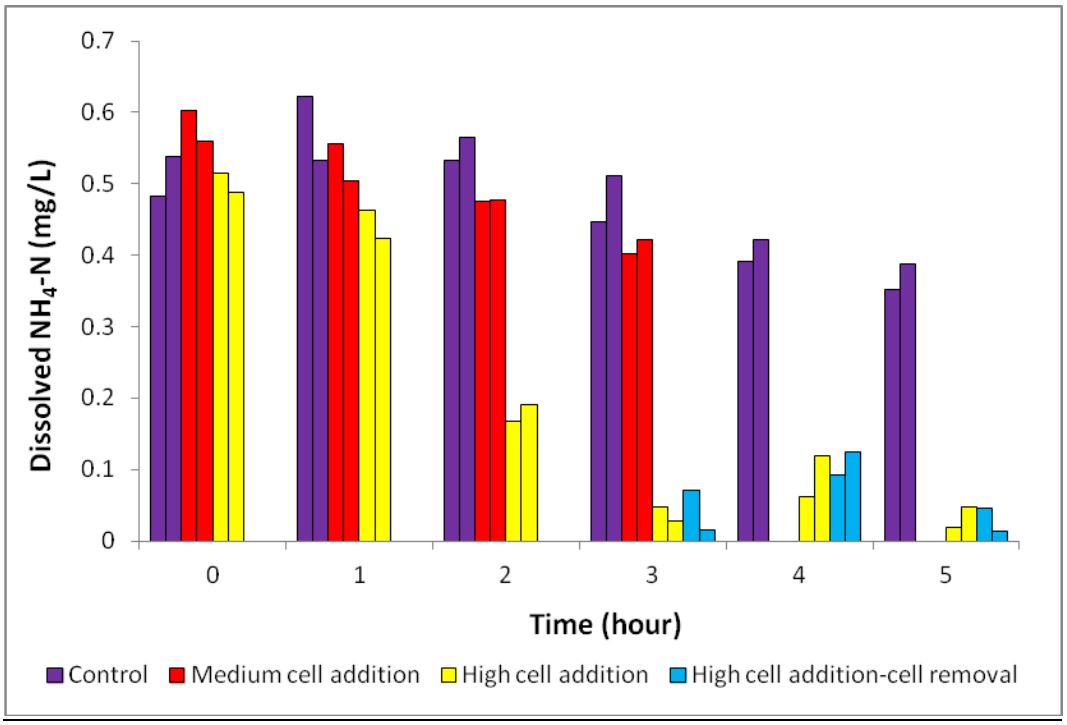


Figure 4.7: Ammonium-nitrogen (NH_4-N) in mesocosms over the sampling period.

A strong negative correlation was observed between cell density and NH_4-N for the different mesocosms (R^2 = 0.72-0.99; Figure 4.8).

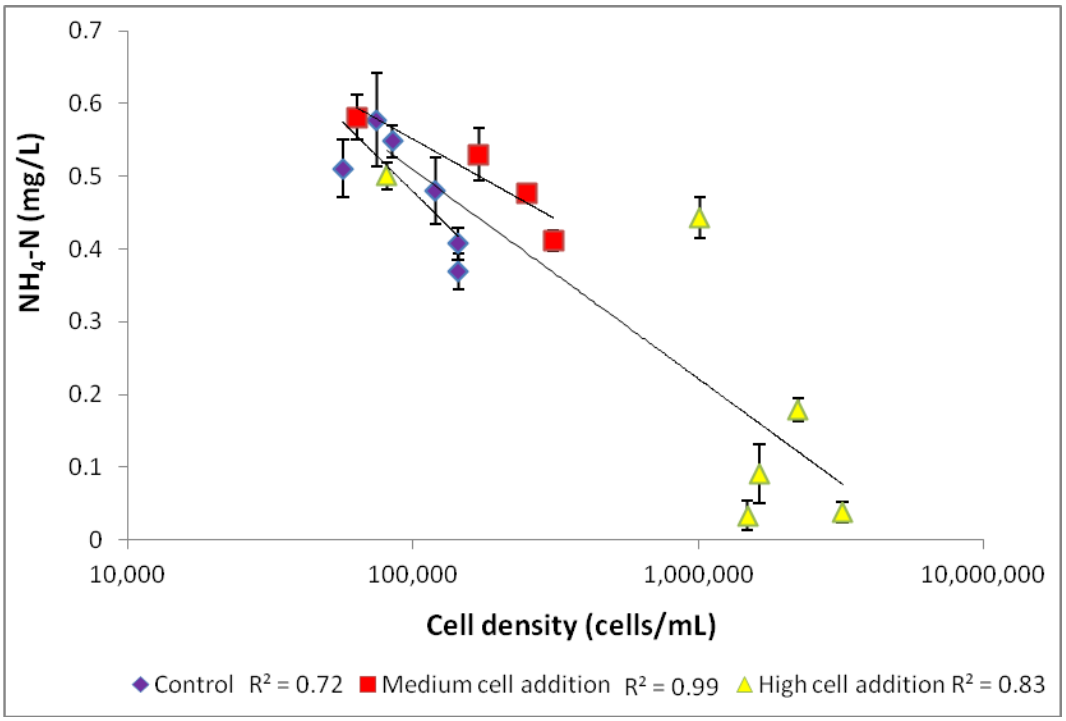


Figure 4.8: Relationship between average NH_4-N concentration and average cell density in mesocosms (*n*=2) (purple diamonds = control, red squares = medium cell addition, yellow triangles = high cell).

The total nitrogen concentration remained relatively constant at ca. 5 mg/L in both the 'control' and 'medium cell addition' mesocosms (Figure 4.9). The exception was the 'control' mesocosm at five hours, which was markedly higher than all other mesocosms including the duplicate 'control' mesocosm at 38.2 mg/L. This is most likely due to sampling or analysis error. The total nitrogen concentration in the 'high cell addition' mesocosms increased to higher levels over the first three hours until a maximum was reached (22.5 mg/L) and then slowly declined at four and five hours (13.5 mg/L). The total nitrogen concentration in the 'high cell addition-cell removal' mesocosms was similar to, and followed the same trend as, the 'high cell addition' mesocosms at three, four and five hours.

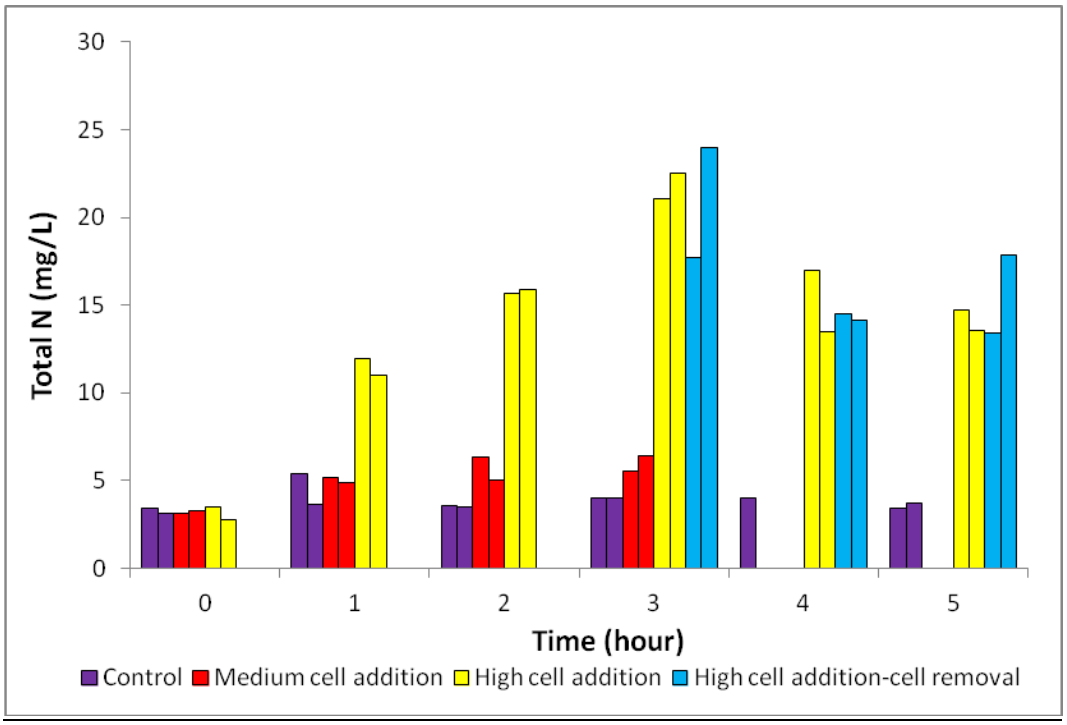


Figure 4.9: Total nitrogen in mesocosms over the sampling period.

The total phosphorus concentrations in the 'high cell addition' mesocosms were highest, followed by the 'medium cell addition' mesocosms and the 'control' mesocosms (Figure 4.10). The total phosphorus in the 'control' mesocosms remained relatively constant at ca. 0.5 mg/L while a small increase in total phosphorus concentrations was observed over time within the 'medium cell addition' mesocosms up to three hours. Between zero and one hour, the total phosphorus concentration in the 'high cell addition' mesocosms increased rapidly, followed by a slower increase up to three hours. At three hours, the total phosphorus in the 'high cell addition' and 'high cell addition-cell removal' mesocosms declined slowly.

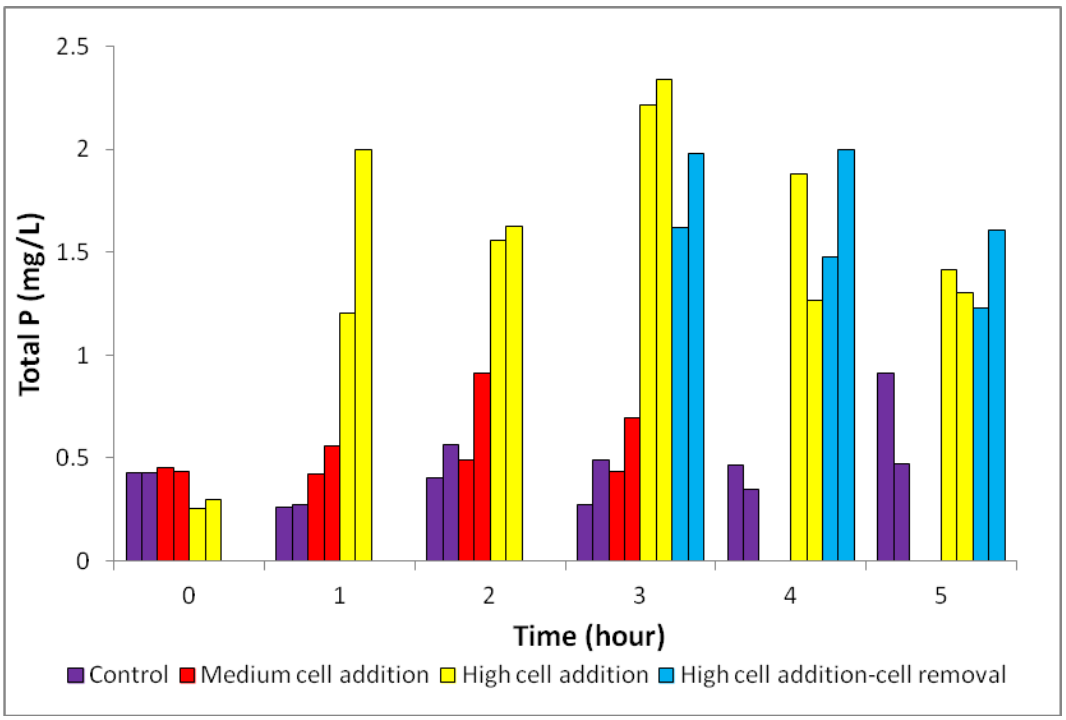


Figure 4.10: Total phosphorus (Total P) in mesocosms over the sampling period.

4.2.2 Cell Density

The cell density, determined by microscopy, in the 'control' and 'medium cell addition' mesocosms was similar and increased gradually over the five hour sampling period (Figure 4.11). In the 'high cell addition' mesocosms, cell numbers increased until a maximum of ca. 3,000,000 cells/mL after three hours. The density then dropped to ca. 1,500,000 cells/mL after five hours. At three hours, the cell density in the 'high cell addition-cell removal' mesocosm was reduced to ca. 1,500,000-2,000,000 cells/mL.

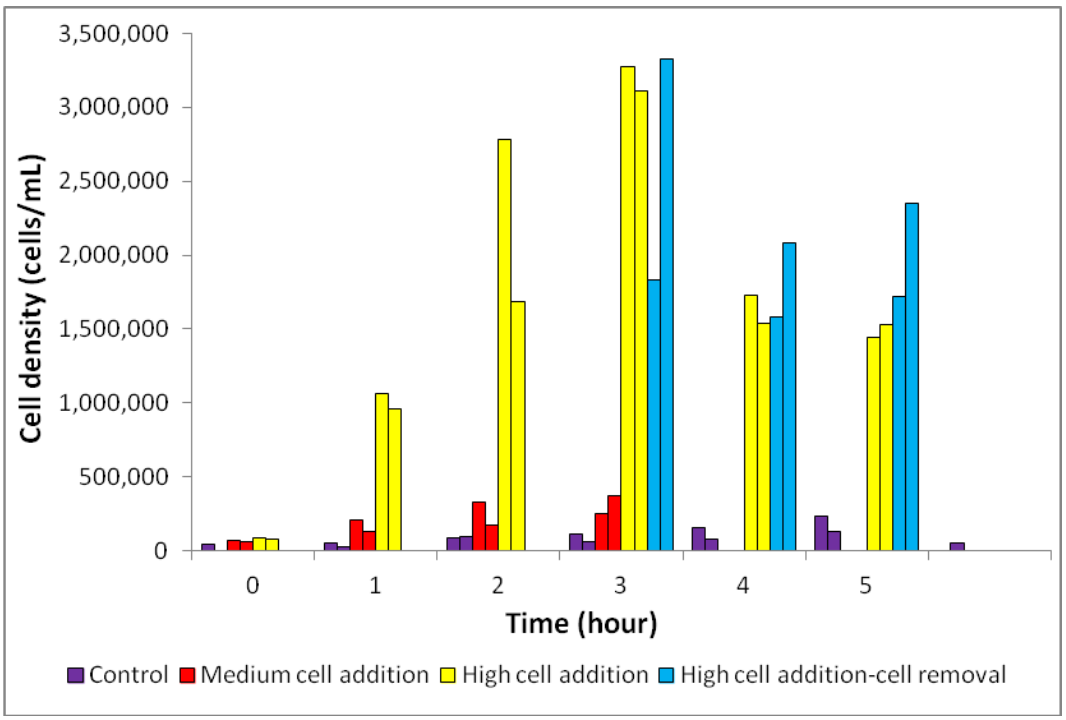
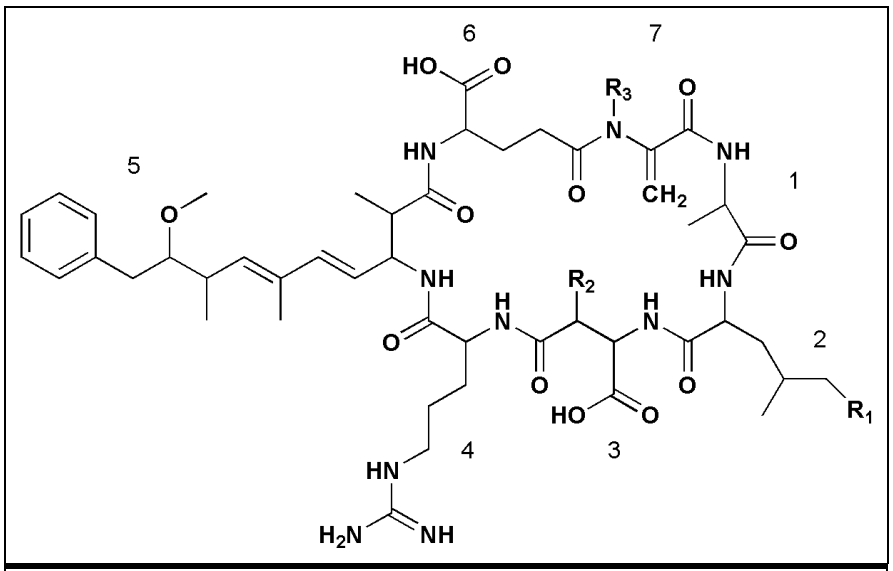


Figure 4.11: Cell density in mesocosms over the sampling period.

4.2.3 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of Microcystin Quota

Liquid chromatography coupled to mass spectrometry (LC-MS) analysis of the concentrated extract of lake water showed that the predominant peak present in the liquid chromatogram corresponded to a mass-to-charge ratio (*m/z*) of 981.5 in the positive ion mode. Peaks corresponding to *m/z* of 967.5 and 995.5 were also detected in the sample. A thiol derivatisation technique using β-mercaptoethanol indicated that *m/z* 995.5, 981.5 and 967.5 ions were highly likely to be microcystins (MCs) as a 78 Da mass increase was observed following incubation.[131] The *m/z* 979.5, 981.5 and 995.5 ions were detected at the expected retention time of ca. 5.8 minutes. Tandem mass spectrometry of *m/z* 981.5, 967.5 and 995.5 ions indicated the presence of several characteristic daughter ions for [Dha^{7}] MC-LR and [D-Asp^{3}, Dha^{7}] MC-LR (Figure 4.12 and Table 4.1).



Microcystin	M_r (Da)	R_1	R_2	R_3
[Asp^3, Dha^7] MC-LR	966.5	-H	-H	-H
[Dha^7] MC-LR	980.5	-H	-CH_3	-H

Figure 4.12: Structures of microcystin congeners identified in Lake Rotorua

Table 4.1: Fragment ions detected from the *m/z* 981.5 and 967.5 microcystin (MC) ions from the concentrated Lake Rotorua sample.

Fragment Assignment	[Dha^7] MC-LR	[D-Asp^3, Dha^7] MC-LR
M + H	981	967
M – $CH_2NHCN_2H_3$ + H	909	895
M – (Me)Asp + H	852	
M – Glu + H	852	838
(Me)Asp-Arg-Adda-Glu + H	728	714
Arg-Adda-Glu + H	599	599
(Me)Asp-Arg-Adda + H	599	
Dha-Ala-Leu-(Me)Asp-Arg + NH_4	556	542
Dha-Ala-Leu-(Me)Asp-Arg + H	539	525
Ala-Leu-(Me)Asp-Arg + H	470	
Adda'-Glu-Dha-Ala + H	432	432
Adda'-Glu-Dha + H	361	361

Two LC-MS injections were required to quantify MC-LR, [D-Asp^3, Dha^7] MC-LR and [D-Asp^3] MC-LR. Therefore, the multiple reaction monitoring (MRM) method and LC gradient described in Section 6.1.2 was used as it included these three congeners and a number of other microcystin congeners could also be detected at the same time if present.

The microcystin quota determined from the number of toxic cells given by QPCR (Appendix D) was not congruent with the microcystin quota determined using the cell density given by microscopy. No consistent pattern in differences between cell enumeration from microscopy and from QPCR was observed. The cell density determined from microscopy was used to determine microcystin quota as this was a more direct measure and was closer to the expected values given from previous studies.[57]

The microcystin quota remained relatively constant for each mesocosm over the time-course of the experiment and was at similar levels between mesocosms of different cell density, apart from the 'control' mesocosms which appeared to have a slightly higher microcystin quota (Figure 4.13). Extracellular microcystins were not detected.

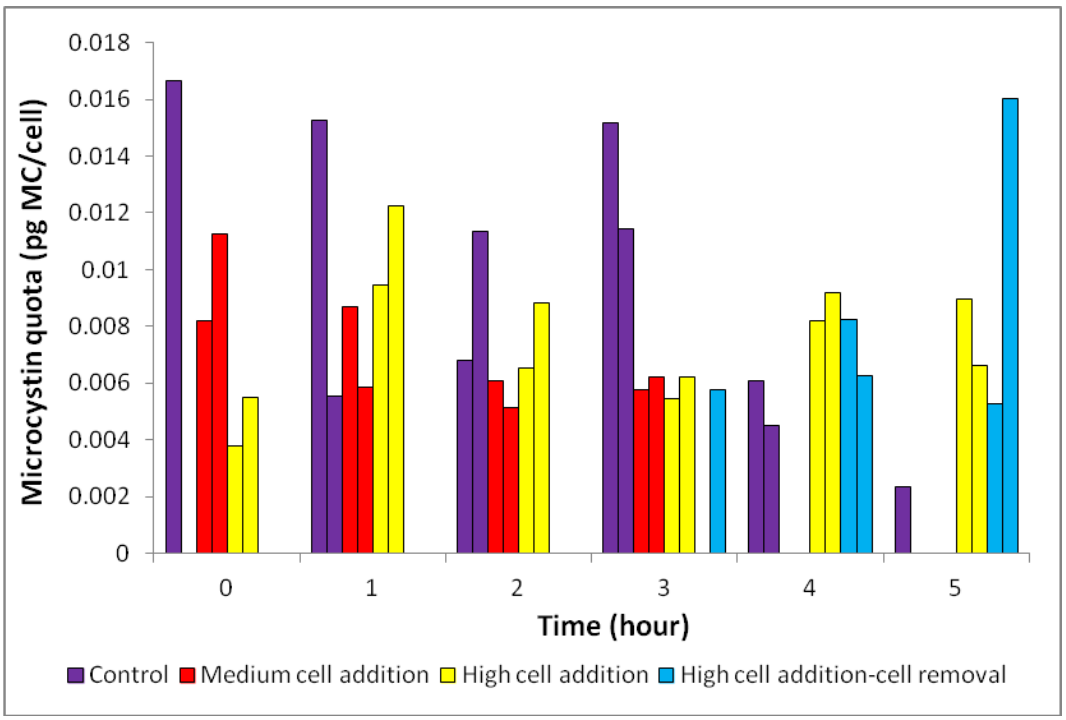


Figure 4.13: Microcystin quota in mesocosms over the sampling period.

4.3 Discussion

4.3.1 Abiotic Parameters

The observed changes in surface temperature and light intensity were most likely influenced by the position of the sun in the sky and time of day.[57] The decrease in temperature observed in the 'high cell addition' and 'high cell addition-cell removal' mesocosms after three hours is likely to be attributed to the mixing of the mesocosms which occurred at three hours. The lower temperatures observed in the mesocosms at 20 cm depth indicates a temperature gradient, thus mixing would have redistributed heat from the surface of the water (where the sensors were placed) throughout the mesocosm. A temperature gradient indicates stratification within the mesocosms which provides conditions that are likely to induce more pronounced buoyancy control and increase potential for scum formation at the surface.

The temperature-adjusted pH and dissolved oxygen appeared to be positively correlated with cell density. A higher rate of photosynthesis in the high cell density treatments would result in increased consumption of carbon dioxide and the subsequent formation of oxygen by Cyanobacteria.[132] Therefore, the depletion of carbon dioxide is likely to have caused the observed increase in pH, while the production of oxygen increased the dissolved oxygen concentration observed with higher cell densities.[1,4] A study carried out on Steilacoom Lake, Washington observed a similar effect in that the pH was positively correlated with *M. aeruginosa* biovolume.[132] The reduction in pH and dissolved oxygen observed in the 'high cell addition' and 'high cell addition-cell removal' mesocosms was likely due to mixing, allowing the dissolved oxygen and carbon dioxide on the surface to be redistributed throughout the mesocosm.

The decrease in NH_4-N concentration with increased cell density could be a result of the consumption of NH_4-N by *Microcystis* for cell metabolism and growth. Non-nitrogen fixing Cyanobacteria such as *M. aeruginosa* have been shown to preferentially use NH_4-N as a source of nitrogen over NO_3-N.[133] This would explain the rapid consumption of NH_4-N in the 'high cell density' mesocosms while NO_3-N concentrations remained relatively stable. The initial increase in dissolved NH_4-N concentration after cell removal in the 'high cell addition' and 'high cell addition-cell removal' mesocosms was likely a result of mixing and diffusion of the NH_4-N throughout the mesocosms. This initial increase was followed by consumption of dissolved NH_4-N as per before cell removal.

The consumption of NH_4-N was mirrored by the increase in total nitrogen and total phosphorus with increased cell density. Several studies have shown that

Cyanobacteria require phosphorus and nitrogen for protein synthesis and growth,[2,132,134] substantiating the expected positive relationship between total nutrients and cell density. In line with this, an expected decline in total phosphorus and nitrogen after cell removal in the 'high cell density-cell removal' mesocosms was expected. The failure to reduce cell density could be an explanation as to why this was not observed. Upon assessment, the cell density in the 'high cell addition-cell removal' mesocosms remained at similar levels to the 'high cell addition' mesocosms after cell removal.

4.3.2 Cell Density and Microcystin Quota

The changes in microcystin quotas with cell density demonstrated in the previous Lake Rotorua mesocosm study[57] were not observed in this study. It is possible that the threshold cell concentration and associated environmental stressors required to induce microcystin production were not reached. In the previous study, there were three cell addition steps at 30 minutes, one hour and two hours, allowing a higher maximum cell density to be reached of ca. 7,000,000 cells/mL compared with ca. 3,000,000 cells/mL achieved in this study. However, a notable increase in microcystin quota at ca. 3,000,000 cells/mL was still observed in the previous study.

The lower levels of extracellular microcystins could also explain why there was no increase in microcystin quota in this study. The process of concentrating cells using a plankton net excludes any extracellular microcystins associated with those cells. This is in stark contrast to a natural aggregation event caused by wind or wave action which is postulated to cause an increase in extracellular microcystins by increased cell death or export of the dense cell population.[62,74] Therefore, a

longer period of time would be required for microcystins in the extracellular environment to build up and be sensed by surrounding cells which would then upregulate microcystin production. The same can be said for any other quorum sensing molecule or environmental stressors associated with cell density such as pH or dissolved oxygen. It is possible that the period over which the experiment took place was not long enough to observe any effects from increased cell density.

However, the previous mesocosm study found that while microcystin quota increased with increased cell density, there was no relationship between extracellular microcystins and cell density.[57] These results indicate that microcystins do not play a role outside the cell in triggering microcystin production and that the low level of extracellular microcystins cannot be used to explain why no increase in microcystin quota was observed in this study. It is possible that some other factor or a complex interplay of factors synergistic with cell density is actually the trigger of microcystin production. For example, microcystins have been suggested to play a role in response to oxidative stress caused by high light exposure[76] and a recent study found that microcystins can bind to cysteine residues in proteins to protect them from degradation under high UV exposure.[64]

4.4 Conclusions

Microcystin quota remained relatively constant throughout the study, despite induced changes in cell density and associated environmental parameters. Therefore, it is difficult to speculate on the triggers of microcystin production. Drawing from the results of this study and previous *in situ* studies, it is likely that cell density is not the only trigger of microcystin production, but instead, multiple stressors in congruence with cell density are required to enhance microcystin synthesis.

CHAPTER 5: FUTURE DIRECTIONS

The sample processing experiment carried out in this study demonstrated that, apart from filtration, the different processing methods tested resulted in similar amounts of microcystins being extracted for CYN60, CYN11 and CYN06. Further investigation as to why processing by filtration yielded significantly lower microcystin quotas could also prove useful, as the separation of cells for collection of the intracellular fraction of microcystins using GF/C filters in the field is more feasible than the use of a portable centrifuge or collecting two subsamples for total microcystins and extracellular microcystins. The use of a sonic probe to extract microcystins from the cells on the filter paper may yield higher extraction efficiencies compared with the sonic bath currently used. Investigation of whether the different sample processing methods influence microcystin quotas from environmental samples could reveal different results from this laboratory-based study.

Recent experiments performed in the field have shown larger changes in microcystin quota than in other laboratory-based studies,[57,74] suggesting greater sensitivity to change than cultured cyanobacterial strains. The assessment of a wider range of extraction methods could also be advantageous in determining the optimal processing method. For example, one study demonstrated that microcystin extraction using a microwave oven yielded similar results to lyophilisation with methanol extraction.[102] If extraction using a microwave oven yielded similar microcystin concentrations to the other methods for extracting

microcystins, it would save time and reduce the requirement for expensive equipment.

This study also showed that incubation of cells after concentration by centrifugation for up to five hours had no influence on the microcystin quota, however, the experiment was only carried out once on a single strain (CYN11). More repetitions of the same experiment and using other cyanobacterial strains (for example, CYN06 and CYN60) and environmental samples would increase confidence in the validity of these results.

The comparative microcystin export (CME) assay developed during this study has the potential to be a useful tool for determining the active export of microcystins and could thus help solve the mystery of the ecophysiological role of microcystins. The CME assay was designed to reveal the proportion of extracellular microcystins present as a result of cell lysis, and thus if any active export of microcystins had occurred. This would be accomplished by comparing the proportion of extracellular microcystins with the proportion of extracellular aeruginosin (auxiliary peptide), which would only be present due to cell lysis. More experiments optimising the CytoTox 96 Non-Radioactive Cytotoxicity Assay and/or another assay which can be used to measure the level of induced cell lysis would be beneficial for validation of the CME assay. This would allow the proportion of extracellular aeruginosin as a result of cell lysis to be validated and confirm that aeruginosin itself does not get actively exported. Determination of whether any degradation of microcystins or aeruginosin occurs during normal sample storage times is also required to validate this assay. Once the assay has been validated, expansion of its utility to more cyanobacterial species/strains and

environmental samples would allow for culture-based and field-based experiments to determine whether active export of microcystins occurs under an array of conditions. For example, culturing experiments which test whether active export occurs after cell addition, rapid chilling or heating, nutrient depletion or the addition of non-toxic cyanobacterial strains could be carried out.

One of the main limitations of the cell density mesocosm experiment carried out during this study was the inability to pre-determine the abundance of the cells used for the cell additions. The use of an on-site method for cell enumeration would indicate if there were major differences in cell density between replicates so they can be pooled together for data analysis and whether a high enough cell density had been reached in the 'high cell addition' mesocosms to induce microcystin production.

The drop in temperature, pH and dissolved oxygen observed after mixing of the 'high cell addition' and 'high cell addition-cell removal' mesocosms was most likely a result of their redistribution and diffusion from the surface (where the sensors were placed) throughout the mesocosms. In addition, at 20 cm depth there was a consistently lower temperature and light intensity compared with the surface of the mesocosms, causing cell accumulation and scum formation at the surface and prompting the development of a gradient of abiotic parameters such as pH and dissolved oxygen within the mesocosms. Samples analysed for microcystin content collected at different depths, under different mixing regimes, would provide additional data for comparison in future mesocosm experiments.

No increase in microcystin quota with cell density was observed in this cell addition mesocosm study. It is possible that a longer period of time was required for microcystins in the extracellular environment to build up and be sensed by surrounding cells which would then upregulate microcystin production. However, the previous mesocosm study found that while microcystin quota increased with increased cell density, there was no relationship between extracellular microcystins and cell density.[57] A mesocosm study investigating the effect of microcystin addition (as opposed to *Microcystis* cells) would determine whether microcystins act outside the cell as autoinducers or whether another factor associated with cell density causes upregulation of microcystin production.

CHAPTER 6: MATERIALS AND METHODS

6.1 General Materials and Methods

6.1.1 Commonly Used Materials

Deionised water (>18 MΩ·cm resistivity; purified on an E-pure still; Barnstead) was used for the preparation of all solutions in this study. Table 6.1 lists the chemicals used to prepare the solutions and the suppliers of the solutions.

Table 6.1: Chemicals used and their suppliers.

Chemical	Source
Anhydrous magnesium sulphate; Boric acid; Calcium chloride dehydrate; Copper (II) sulphate pentahydrate; Formic acid (88%); HPLC-grade methanol; Hydrochloric acid (HCl); Magnesium sulphate heptahydrate; Manganese (II) chloride tetrahydrate; Potassium carbonate; Sodium bicarbonate; Sodium hydroxide	Ajax Chemicals
Ammonium chloride; Iodine; Iron (III) chloride hexahydrate; Potassium iodide; Sodium nitrate	BDH Chemicals
MC-LR standard; MC-RR standard; MC-LA standard	DHI Lab Products
HPLC-grade acetonitrile (ACN)	Honeywell International
Cobalt (II) chloride hexahydrate; Sodium nitrite	Hopkins and Williams
Dipotassium phosphate; Sodium molybdate dehydrate	May and Baker
Selenous acid	Riedel-de Haën
HPLC-grade ethanol; Sodium ethylenediaminetetraacetic acid (EDTA); Zinc sulphate heptahydrate	Scharlau
Angiotensin I; β-Mercaptoethanol; Biotin; Thiamine·HCl; Vitamin B12	Sigma-Aldrich

The composition of the commonly used solutions in this study are listed in Table 6.2.

Table 6.2: The composition of commonly used solutions.

Solution	Composition
10% Formic acid	11.4 mL of 88% formic acid (10%; v/v) - Make up to 100 mL with H_2O and filter (0.45 μm)
LC-MS Solvent A	10 mL of 10% formic acid (0.1%; v/v) 20 mL of HPLC-grade acetonitrile (2%; v/v) - Make up to 1 L with H_2O
LC-MS Solvent B	10 mL of 10% formic acid (0.1%; v/v) 10 mL of H_2O (2%; v/v) - Make up to 1 L with HPLC-grade acetonitrile
MLA vitamin stock	10 mg of thiamine·HCl (332 μM) 50 μL of 409 μM biotin (205 nM) 50 μL of 73 μM vitamin B_{12} (37 nM) - Make up to 100 mL with H_2O and store at 4 °C
MLA micronutrient stock	4.36 g of $Na_2EDTA·2H_2O$ (11.7 mM) - Add 800 mL H_2O and stir over a low heat to dissolve 1.58 g of $FeCl_3·6H_2O$ (5.8 mM) 0.60 g of $NaHCO_3$ (3.5 mM) 0.36 g of $MnCl_2·4H_2O$ (1.8 mM) 10 mL of 4 mM $CuSO_4·5H_2O$ (40 μM) 10 mL of 7.7 mM $ZnSO_4·7H_2O$ (77 μM) 10 mL of 4.2 mM $CoCl_2·6H_2O$ (42 μM) 10 mL of 2.5 mM $Na_2MoO_4·2H_2O$ (25 μM) - Make up to 1 L with H_2O and store at 4 °C
40× MLA stock	10 mL of 200 mM $MgSO_4·7H_2O$ (8 mM) 20 mL of 1 M $NaNO_3$ (80 mM) 50 mL of 40 mM K_2HPO_4 (8 mM) 10 mL of 40 mM H_3BO_3 (1.6 mM) 10 mL of 10 μM H_2SeO_3 (400 nM) 10 mL of vitamin stock 10 mL of micronutrient stock - Make up to 250 mL with H_2O, filter sterilise (0.2 μm) and store at 4 °C
MLA sodium bicarbonate stock	4.23 g of $NaHCO_3$ (200 mM) - Make up to 250 mL with H_2O, autoclave and store at 4 °C
MLA calcium chloride stock	0.29 g of $CaCl_2·2H_2O$ (200 mM) - Make up to 100 mL with H_2O, autoclave and store at 4 °C
MLA culturing medium	25 mL of 40× MLA Stock 10 mL of sodium bicarbonate stock (2 mM) 1 mL of calcium chloride stock (200 μM) - Make up to 1 L with autoclaved H_2O and store at 4 °C
Lugol's solution	10 g of potassium iodide (602 mM) - Dissolve in 70 mL H_2O 5 g of iodine (394 mM) 10 mL of acetic acid (10%; v/v) - Make up to 100 mL with H_2O and store in the dark

6.1.2 Commonly Used Laboratory Methods

Freeze-Drying

Samples were frozen in liquid nitrogen prior to lyophilisation using a FreeZone6 freeze-drier (Labconco).

Centrifugation

Centrifugation was undertaken on one of three centrifuges; a Jouan C4i centrifuge (Thermo Scientific) for general samples greater than 1.5 mL, an Eppendorf MiniSpin Plus centrifuge (Global Science) for general samples less than 1.5 mL or an Eppendorf 5414 R centrifuge (Global Science) during DNA extraction.

Sonication

Samples and solvents were sonicated at ambient temperature in a Transonic T 700/H ultrasonic bath (Elma).

Cell Enumeration

Cell enumeration was carried out using an inverted microscope (IX71; Olympus). When colonial strains were assessed, samples were mechanically ground (Tissue Grinder; Wheaton, USA) for 30 s to separate *Microcystis* colonies and diluted. Subsamples (1 mL) were settled in chambers (Plankton Chamber; Hydrobios Kiel, Germany) and cells from ten random fields of view (FOV) were counted at 400× magnification.[135]

Cell concentrations were calculated as:

$N = C \cdot f \cdot (A/b \cdot a \cdot V)$

where:

N = number of cells per mL in original water sample

C = total number of cells counted in all FOVs

A = total area of bottom of the settling chamber (mm^2)

a = total area of FOV= (mm^2)

b = number of FOVs counted

f = dilution factor

V = volume of lake water that was settled (mL)

Cell enumeration on filamentous strains of Cyanobacteria was carried out by measuring the length of all filaments in a single transect using an inverted microscope (IX71, Olympus) at 100× magnification. A light microscope (BX51; Olympus) attached to a digital microscope camera (DP70; Olympus) was used to measure the length of a maximum of ten cells in up to six filaments at 1,000× magnification.[135] The number of cells per filament was calculated by dividing the average length of a filament by the average length of a cell. The total number of cells was calculated by multiplying the number of cells per filament by the number of filaments in a transect.

Cell concentrations were calculated as:

$N = C \cdot f \cdot (A/a \cdot V)$

where:

N = number of cells per mL in original water sample

C = total number of cells counted in transect

A = total area of bottom of the settling chamber (mm^2)

a = total area of transect (mm^2)

f = dilution factor

V = volume of sample settled (mL)

Cyanobacterial Culturing

All cyanobacterial strains were cultured in screw capped plastic containers (60 mL; Biolab) with MLA medium[136] (ca. 30 mL) or in glass Erlenmeyer flasks with MLA medium (500 mL) in a Contherm Biosyn 6000 CP Incubator (photon flux of 100 µmol m^{-2} s^{-1}, 20 ºC) under a 12:12 hour light/dark cycle.

General Microcystin Extraction and Concentration

Extraction of microcystins was generally carried out by four freeze-thaw cycles where freezing was one hour at -20 ºC and thawing was carried out by sonication (30 min). The resulting extract was centrifuged and the supernatant concentrated via solid phase extraction (C_{18} StrataX cartridge, 50 mg; Phenomenex) using a vacuum manifold. The cartridge was primed with MeOH/ACN (4 mL; 1:1) and equilibrated with H_2O (4 mL) before the extract was loaded onto the cartridge and slowly drawn though the packing material. The cartridge was washed with 10% MeOH (8 mL) followed by elution of the microcystins in 80% MeOH (8 mL).

β-Mercaptoethanol Derivatisation

Concentrated lake water extract (800 µL) was added to a septum-capped LC vial with $NaHCO_3$ (200 µL; 200 mM), left to equilibrate at 30 °C and an injection analysed by liquid chromatography couple to mass spectrometry (LC-MS). Following this injection, β-mercaptoethanol (5 µL) was added to the vial and the sample was left to incubate for approximately two hours before an injection of the derivatised sample was analysed by LC-MS.

Liquid Chromatography-Mass Spectrometry Analysis

Microcystin concentrations were determined using a high performance liquid chromatography (HPLC) system (UltiMate 3000, Dionex) directly coupled to an electrospray ionisation (ESI)-ion trap mass spectrometer (MS; AmaZon X; Bruker Daltonics). HyStar (Bruker Daltonics) was used as an interface to control the two systems. Samples were separated on a C_{18} column (Aeris PEPTIDE XB-C18, 100×2.1 mm, 3.6 µm; Phenomenex) using the water to acetonitrile (ACN) gradient in Table 6.3.

Table 6.3: HPLC gradient for microcystin quantitation by LC-MS/MS.[a]

Time (min)	**%A (98:2 H_2O/ACN + 0.1% FA[b])**	**%B (2:98 H_2O/ACN + 0.1% FA[b])**
0	80	20
1	80	20
2	70	30
12	40	60
13	0	100
15	0	100
16	80	20
20	80	20

[a] Flow rate = 250 µL/min; Column temperature = 40 °C.
[b] FA = Formic Acid

Microcystins were detected using a multiple reaction monitoring (MRM) method to detect and quantify ions belonging to the microcystins produced at moderate to high levels in each strain. The concentration of microcystins present was determined by comparing the abundance of the $[M-H_2O-H]^-$ ions using two separate injections (Table 6.4 and Table 6.5), with standard curves constructed using a mixture of two microcystin standards (MC-LR and MC-RR) or three microcystin (MC) standards (MC-LR, MC-RR and MC-LA), which were dispersed throughout the sample run in order to account for response drift. The MC-LR standard curve was used for quantification of MC-LR, desmethyl (dm)MC-LR, didmMC-LR, MC-RA, MC-Raba, MC-YR, MC-WR, MC-FR, MC-LA, dmMC-LA, MC-YA, MC-WA, MC-FA, MC-Laba, MC-Faba, MC-Waba, MC-FL and MC-WL microcystin congeners. The MC-RR standard curve was used for quantification of MC-RR, dmMC-RR and didmMC-RR microcystin congeners. When the MC-LA standard was available, the standard curve was used for quantification of MC-YA, MC-WA, MC-LA, MC-FA, dmMC-LA, MC-Laba, MC-Faba, MC-Waba, MC-FL and MC-WL and the MC-LR standard curve was not utilised for these congeners. The limits of quantitation (LOQ) for MC-RR and MC-LR for the MRM methods used were determined by converting the integrated peak value to pg/mL, multiplying this value by three, and then averaging this for all calibration curves acquired for each standard.

Table 6.4: MRM-A segments.

Retention time (min)	Microcystin	$[M-H]^-$	$[M-H_2O-H]^-$
0.0-4.7	MC-RR	1036.5	1018.5
	dmMC-RR	1022.5	1004.5
4.7-6.3	dmMC-LR	979.5	961.5
	MC-LR	993.5	975.5
6.3-9.0	MC-RA	951.5	933.5
	MC-RAba	965.5	947.5
9.0-10.7	MC-LA	908.5	890.5
	MC-FA	942.5	924.5
10.7-20.0	MC-FAba	956.5	938.5
	MC-WAba	995.5	977.5

Table 6.5: MRM-B segments.

Retention time (min)	Microcystin	$[M-H]^-$	$[M-H_2O-H]^-$
0.0-4.7	didmMC-RR	1008.5	990.5
	MC-YR	1043.5	1025.5
4.7-5.9	MC-YR	1043.5	1025.5
	didmMC-LR	965.5	947.5
5.9-8.0	MC-FR	1027.5	1009.5
	MC-WR	1066.5	1048.5
8.0-9.68	MC-YA	958.5	940.5
	dmMC-LA	894.4	876.5
9.68-12.0	MC-WA	981.5	963.5
	MC-LAba	921.5	903.5
12.0-20.0	MC-FL	984.5	966.5
	MC-WL	1023.5	1005.5

QuantAnalysis (Bruker Daltonics) software was used to create extracted ion chromatograms according to the mass-to-charge ratio (*m/z*) of each microcystin and to integrate peak areas for each microcystin congener a sample.

6.2 Work Described in Chapter Two

6.2.1 Experimental Design

A colonial *Microcystis* strain (CYN06-CAWBG11; code of isolator-Cawthron code), a single celled *Microcystis* strain (CYN11-CAWBG16) and a filamentous *Planktothrix* strain (CYN60-CAWBG59) were obtained from the Cawthron Institute Microalgae Culture Collection (www.cultures.cawthron.org.nz; Nelson, New Zealand) and maintained in culture as described in Section 6.1.2.

Effect of cell processing methods on microcystin quota

Three replicate experiments were carried out on different days for each strain. Three treatments were compared: (1) direct freezing (no prior cell concentration), (2) cell concentration by centrifugation, and (3) cell concentration by filtration. Triplicate subsamples (1 mL) for each treatment were taken from the same batch of culture after swirling cultures to homogenise them. Cell enumeration of the culture used for each experimental replicate was carried out as specified in Section 6.1.2.

Method one- Direct freezing

Subsamples of culture were frozen at -20 °C, -80 °C or in liquid nitrogen (-196 °C). Microcystin extraction was carried out by three freeze-thaw cycles (Appendix E) interspersed with a sonication step (30 min). After extraction,

formic acid (final concentration 0.1% v/v) was added to samples before centrifuging (5 min, 6,000 × *g*) and transferring the supernatant (800 µL) to a septum-capped vial (1.8 mL) containing MeOH (800 µL).

Method two - Centrifugation

Subsamples of culture were centrifuged (5 min, 6,000 × *g*) to separate the extracellular medium (supernatant) from the cell pellet. The supernatant was removed, syringe filtered (0.2 µm) and diluted with MeOH (1:1) in a septum-capped vial (1.8 mL). The cell pellets were frozen in liquid nitrogen and freeze-dried overnight. The lyophilised sample was extracted in MeOH (1 mL; Appendix F) by sonication (30 min). After extraction, samples were centrifuged (5 min, 6,000 × *g*) and 800 µL of supernatant was placed in a septum-capped vial (1.8 mL) containing deionised water (800 µL).

Method three - Filtration

Subsamples of culture were syringe filtered onto glass fibre filter paper (GF/C 25 mm diameter; MicroScience). The extracellular medium (filtrate) was syringe filtered (0.2 µm) and diluted in MeOH (1:1) in a septum-capped vial (1.8 mL). The filter was placed in an Eppendorf tube (1.5 mL), frozen in liquid nitrogen and freeze-dried overnight. The lyophilised sample was extracted in MeOH (1 mL) by sonication (30 min). After extraction, samples were centrifuged (5 min, 6,000 × *g*) and 800 µL of supernatant was placed in a septum-capped vial (1.8 mL) containing de-ionised water (800 µL). The microcystin concentration detected in the filtrate was subtracted from the microcystin concentration detected for samples that were directly frozen to adjust for extracellular microcystins present in the culturing medium and allow microcystin quota to be determined.

Control

An additional three subsamples of culture were frozen in liquid nitrogen, freeze-dried overnight and extracted in MeOH (1 mL) with sonication (30 min). Samples were centrifuged (5 min, 6,000 × *g*) and the supernatant (800 µL) was placed in a 1.8 mL septum-capped vial containing deionised water (800 µL). This treatment was used as a control to ensure that the two extraction procedures utilised (aqueous and organic) yielded similar efficiencies.

Temporal effect of centrifugation on microcystin quota

Two experiments were carried out on different days for CYN11. Triplicate subsamples (1 mL) were taken from the same batch culture after swirling cultures to homogenise them. Subsamples were centrifuged (5 min, 6,000 × *g*) to concentrate cells. The supernatant was (1) removed immediately and discarded whilst the pellet was left at room temperature for zero, one, two, three, four and five hours, before being frozen in liquid nitrogen. The sample was then freeze-dried and the lyophilised sample was extracted in MeOH (1 mL) by sonication (30 min). After extraction, samples were centrifuged (5 min, 6,000 × *g*) and the supernatant (800 µL) was placed in a septum-capped vial (1.8 mL) for intracellular microcystin analysis by LC-MS. In the second experiment, the supernatant was left in the tube with the pellet at room temperature for zero, one, two, three, four and five hours after which the supernatant (800 µL) was placed a septum-capped vial (1. 8 mL) for extracellular microcystin analysis by LC-MS and the pellet was frozen in liquid nitrogen. The pellet was then freeze-dried and the lyophilised sample was extracted in MeOH (1 mL) by sonication (30 min). After extraction, samples were centrifuged (5 min, 6,000 × *g*) and the supernatant

(800 µL) was placed in a septum-capped vial (1.8 mL) for intracellular microcystin analysis by LC-MS.

6.2.2 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

LC-MS analysis was carried out using the instrumentation and protocols outlined in Section 6.1.2. The MRM method in Section 6.1.2 was used to detect and quantify ions belonging to the microcystins produced at moderate to high levels in CYN06 (Table 6.6),[37] CYN11 ([Asp^3] MC-LR and MC-LR; Appendix G.1)[137] and CYN60 ([Asp^3] MC-LR and MC-LR; Appendix G.2).[107]

Table 6.6: Microcystin congeners in CYN06 detected by the MRM method.

Microcystin	
MC-RR	MC-YR
[Asp^3] MC-RR	[Asp^3, Dha^7] MC-LR
[Asp^3] MC-LR	MC-FR
MC-LR	MC-WR
MC-RA	MC-YA
MC-RAba	MC-WA
MC-LA	MC-LAba
MC-FA	MC-FL
MC-FAba	MC-WL
MC-WAba	

6.2.3 Statistical Treatments

Statistical analyses were undertaken using R (http://www.r-project.org). To account for variation between experimental days and to enable pooling of all data points across all strains, microcystin concentrations for each strain were standardised by dividing each value by the corresponding control (liquid nitrogen,

freeze-dried and methanol extracted) samples value. Analyses of variance (ANOVAs) were then used to compare median concentrations for each extraction treatment. Post-hoc pairwise comparisons were undertaken using Tukey honest significant difference (HSD).

6.3 Work Described in Chapter Three

Four different *Microcystis* strains (Rotorua A, B, C and D) collected from the shoreline of Lake Rotorua (Kaikoura, South Island) were isolated by Dr. Susie Wood (Cawthron Institute, Nelson). These strains were transported to the University of Waikato and cultured as described in Section 6.1.2.

6.3.1 Morphological Characterisation

Morphological characterisation of the four strains was carried out using a light microscope (BX51, Olympus) attached to a digital microscope camera (DP70, Olympus) at 1,000× magnification. Features such as colour, cell shape, and whether cells were undergoing division were noted and Image J was used to measure the diameter of cells (n=30).

6.3.2 Molecular Characterisation

Subsamples (1 mL) from each strain were centrifuged (1 min, 10,000 × g) and the supernatant removed. DNA was extracted from the cell pellets using a PureLink™ Genomic DNA Kit (Invitrogen, USA) according to the protocol supplied by the manufacturer for Gram-negative bacteria, with the alteration that incubation (at 55 °C) was overnight to increase the DNA yield. The extracted DNA from each strain was quantified by using the Beer-Lambert equation to calculate the

concentration based on the UV/Vis absorbance at 260 nm (NanoDrop 1000, Thermo Fisher Scientific). Polymerase chain reaction (PCR) amplification of a region of the 16S ribosomal RNA (rRNA) gene was undertaken in two segments using two different sets of cyanobacterial-specific primers, 27F/809R[138] and 740F/1494R.[139] PCR was undertaken in two separate 50 μL reaction mixes containing i-Taq 2× PCR master mix (25 μL; Intron, Korea), 500 nM of each primer set (1 μL; 27F/809R or 740F/1494R at 10 μM) and template DNA (10-30 ng). A positive control (cyanobacterial DNA from the Cawthron culture collection *Anabaena spiroides* CYN101/CAWBG547), a negative control (deionised water) and the DNA extraction control were PCR amplified along with the samples (DNA Engine Peltier Thermocycle, BIO-RAD). Conditions for the PCR reaction were an initial denaturation at 94 °C for 2 min, followed by twenty cycles of denaturation at 94 °C for 20 s, annealing at 54 °C for 20 s and extension at 72 °C for 50 s, with a final extension at 72 °C for 5 min. The PCR products were separated by agarose gel electrophoresis (1%) and visualised with SYBR® Safe (Invitrogen, New Zealand) stain and UV illumination. The amplified DNA was purified using Quickclean II PCR Extraction Kit (GenScript, USA) and unidirectional Sanger sequencing carried out using the same primers as above at the University of Waikato DNA Sequencing Facility. All sequences were edited using Finch TV and contigs assembled using MEGA4 to create one sequence for each strain covering almost the entire region of the 16S rRNA gene sequence. A ClustalW alignment of the four sequences was undertaken in MEGA4 to determine the similarity between sequences. Each sequence was compared with 16S rRNA sequences from the NCBI GenBank database by performing a nucleotide blast search using the Blastn algorithm.[140]

6.3.3 Chemical Characterisation

Subsamples (15-50 mL) of each cultured strain were extracted and concentrated as described in Section 6.1.2. The microcystins present in each strain were identified by an LC-MS screen in both positive and negative ion mode. The ions which had *m/z* corresponding with the molecular mass of known microcystin congeners and that were at the correct retention time were fragmented by tandem mass spectrometry (MS/MS).

An additional diagnostic test, β-mercaptoethanol derivatisation,[131] was carried out on the concentrated extract of each strain as outlined in Section 6.1.2.

The Rotorua A strain was selected for determination of the presence of other auxiliary peptides. Significant peaks in the liquid chromatogram were scanned for abundant ions of *m/z* that corresponded to any known non-ribosomal peptides which met these criteria. Any suspected auxiliary peptides were further characterised by MS/MS analysis.

6.3.4 Comparative Microcystin Export Assay Method Development

The Rotorua A strain was selected for the comparative microcystin export assay development. A method fragmenting the parent ion of the selected peptide (aeruginosin) and microcystins over a variety of collision amplitudes, starting at 0.45 and ending at 1.10 and increasing by 0.05 increments, was used to determine the optimal collision amplitude at which the detection of the daughter ions selected for quantification was most abundant. A variety of liquid chromatography gradients was tested to give the shortest run time with the peptide and microcystins still eluting separately and within the gradient window. An

MRM method analysing for [Dha^7] MC-LR ([M−H]$^-$ =979.5), [D-Asp^3, Dha^7] MC-LR ([M−H]$^-$ = 965.5) and aeruginosin ([M−H]$^-$ = 601) was developed using the optimal collision amplitude and the chosen LC-MS gradient.

The use of Angiotensin I as an internal standard was tested by incorporating it into the MRM method. A method fragmenting the parent ion of the selected peptide (Angiotensin I) and microcystins over a variety of collision amplitudes, starting at 0.5 and ending at 1.20 and increasing by 0.1 increments, was used to determine the optimal collision amplitude at which the detection of the daughter ions selected for quantification was most abundant.

The reproducibility of this method was tested by running six injections of an intracellular and extracellular microcystin extract, each spiked with 250 nM of angiotensin I, and analysing the relative standard deviation of the area of the integrated peaks.

6.3.5 Comparative Microcystin Export Assay Method Validation

A lactate dehydrogenase (LDH) assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega) was tested for its efficacy in measuring the degree of cell lysis occurring in cyanobacterial samples (Rotorua Strain A). The assay was carried out according to the protocol supplied by the manufacturer and absorbance at 490 nm was measured (Microplate Reader 680, BIO-RAD).

The LDH assay was carried on a serial dilution series (1/10 and 1/2) starting with 7,675,700 cells/mL of Rotorua A that had undergone four freeze-thaw cycles to induce cell lysis.

Subsamples of Rotorua A at 6,515,400 cells/mL were subjected to CAPB (0-150 mg/L, increasing in 25 mg/L increments) and left to incubate for one, two, three and four hours in order to find the optimal incubation time.

Subsamples of Rotorua A at 8,884,700 cells/mL were treated with CAPB as the cytotoxic agent (0-250 mg/L, increasing in 25 mg/L increments). The variability in triplicate samples was used to assess the reproducibility of the assay using CAPB.

6.3.6 Experimental Utility of the Comparative Microcystin Export (CME) Assay

Culturing Experiment and Sample Collection

Rotorua strain A was cultured as described in Section 6.1.2 in an Erlenmeyer flask (500 mL). Triplicate subsamples (1 mL) were collected from the same batch culture at zero, two, four, seven, eight, ten and eleven days after swirling cultures to improve cell homogeneity. Subsamples were preserved in Lugol's iodine solution for cell enumeration, additional subsamples were frozen at -80 ºC for analysis of total microcystins and subsample filtrate (GF/C, Whatman) was collected and frozen at -80 ºC for extracellular microcystin analysis.

Sample Analysis

Cell enumeration was carried out as specified in Section 6.1.2. Subsamples collected for total microcystin analysis were thawed and sonicated (30 min) followed by three additional freeze-thaw cycles to rupture cells and release microcystins. The samples were centrifuged and the supernatant (ca. 1 mL) was placed in a septum-capped LC vial (1.8 mL). Subsamples collected for

extracellular microcystin analysis were thawed at room temperature and placed in septum-capped LC vials (1.8 mL).

The concentration of microcystins and aeruginosin was determined as described in Section 6.1.2, but using the MRM method (Table 6.7) and HPLC gradient developed in Section 6.3.4 with standard curves constructed using only MC-LR as the microcystin standard (1-1000 nM).

Table 6.7: MRM method for the comparative microcystin export assay.

Retention time (min)	**Peptide**	**$[M-H]^-$**	**Quantitation Fragment**
0-4.10	Aeruginosin	601.3	541.3
4.10-14	[Asp^3] MC-LR	979.5	961.5
	[Asp^3, Dha^7] MC-LR	965.5	947.5

6.4 Work Described in Chapter Four

6.4.1 Study Site and Sample Collection

Samples were collected at Lake Rotorua (South Island, New Zealand; 42°24′05 S, 173°34′57 E) on 23 April 2013. Lake Rotorua is a small (0.55 km²), shallow (max. 3 m), highly eutrophic lake.[57] It is surrounded by farmland, regenerating scrub and native bush and has one small outflow. An 8 m floating pontoon was constructed and positioned on the south east side of Lake Rotorua (Figure 6.1).

Figure 6.1: Satellite map indicating the position of the pontoon in Lake Rotorua (not to scale).

Five mesocosms were attached to the outer end of each side of the pontoon (10 total; Figure 6.2). Each mesocosm was made from a low-density clear polyethylene bag of 60 μm thickness, attached to a float formed from a 40 m coil of high-density polyethylene pipe (15 mm external diameter). Each polyethylene bag was 570 mm deep with an internal diameter of 380 mm to give a total volume of approximately 55 L.

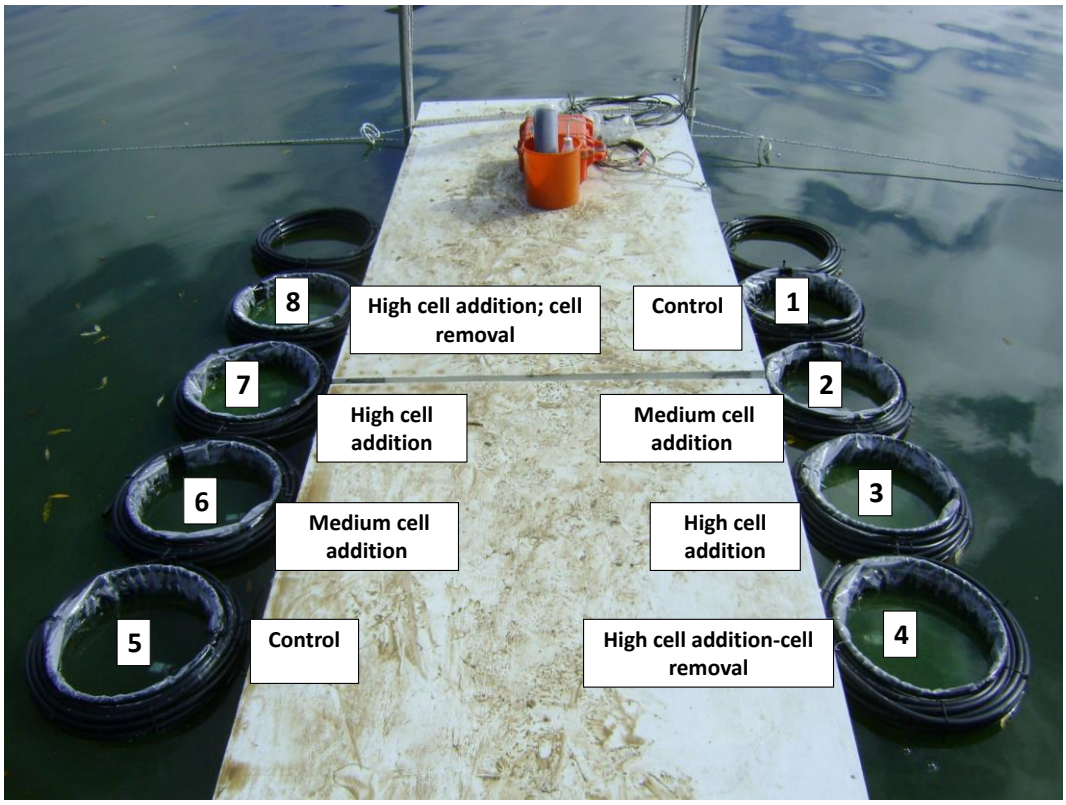


Figure 6.2: Position and treatment of mesocosms attached to pontoon.

The mesocosm experiment commenced at 10:40. Prior to starting, lake water at an average concentration of 56,800 *Microcystis* cells per mL was added to 'control' mesocosms (one and five). Cyanobacterial cells in the lake water adjacent to the mesocosms were concentrated using a 20 μm plankton net and 5 L of the concentrated cells were added to 'medium cell addition' mesocosms (two and six) and topped up with regular lake water to bring the average concentration up to 63,600 *Microcystis* cells/mL. Concentrated cells (10 L) were added to 'high cell addition' and 'high cell addition-cell removal' mesocosms (three, four, seven and eight) to bring the average concentration up to 81,100 *Microcystis* cells/mL.

Over the following 45 min, additional lake water was concentrated and added to 'high cell addition' and 'high cell addition-cell removal' mesocosms until a final average concentration of 1,011,000 *Microcystis* cells/mL was achieved. The

additional cells were added to simulate the formation of a dense cyanobacterial scum. After three hours, cells were removed from 'high cell addition-cell removal' mesocosms by filtering the water from the mesocosms through a 20 μm plankton net and adding the filtered water back into the mesocosms. In order to maintain consistency, 'high cell addition' mesocosms were mixed at intervals during this process to replicate the mixing occurring in 'high cell addition-cell removal' mesocosms.

Sensors recording temperature and light intensity every 5 min (UA-002-08 HOBO; Onset Computer Corporation) were attached to each mesocosm just below the surface of the water and in one replicate of each mesocosm (one, three and four) at a depth of 20 cm. A weather station (Weather Transmitter WXT520; Vaisala) was set up at the end of the pontoon which recorded wind speed, wind direction and air temperature every 1 min for the duration of the study. Dissolved oxygen (Fibox 3 LCD trace v7 and PSt3 oxygen sensor with temperature control, Presens) and pH (pHTestr 20 Waterproof Tester; Eutech Instruments) were measured at the surface of each mesocosm at specific time points (Table 6.8 and Table 6.9).

Table 6.8: Time points for surface pH measurements in each mesocosm (X).

	Control		Medium cell addition		High cell addition		High cell addition-cell removal	
Time	**1**	**5**	**2**	**6**	**3**	**7**	**4**	**8**
11:00	X	X						
11:52	X	X	X	X	X	X	X	X
12:28	X	X	X	X	X	X	X	X
13:03	X	X	X	X	X	X	X	X
14:05	X	X			X	X	X	X
15:14	X	X			X	X	X	X
15:48	X	X			X	X	X	X

Table 6.9: Time points for dissolved oxygen measurements in each mesocosm (X).

	Control		Medium cell addition		High cell addition		High cell addition-cell removal	
Time	**1**	**5**	**2**	**6**	**3**	**7**	**4**	**8**
10:32	X	X	X	X	X	X	X	X
11:41	X	X	X	X	X	X	X	X
12:15	X	X	X	X	X	X	X	X
12:49	X	X	X	X	X	X	X	X
13:23	X	X	X	X	X	X	X	X
13:54	X	X	X	X	X	X	X	X
14:23	X	X			X	X	X	X
14:53	X	X			X	X	X	X
15:28	X	X			X	X	X	X
16:02	X	X			X	X	X	X

Samples (180 mL) were collected from the water surface of ‘controls’ and ‘high cell addition’ mesocosms at zero, one, two, three, four and five hours, ‘medium cell addition’ mesocosms at zero, one, two and three hours and ‘high cell addition-cell removal’ mesocosms at three, four and five hours. Subsamples

(15 mL) were preserved in Lugol's iodine solution for cyanobacterial identification and cell enumeration. Aliquots (5 mL) were placed in cryovials and snap frozen in liquid nitrogen for analysis of total microcystins while filtrate (5 mL; GF/C, Whatman) was collected for extracellular microcystin analysis in cryovials which were initially stored on ice. Subsamples (10-50 mL) were filtered (GF/C, Whatman) and filters were stored in Eppendorf tubes on ice for DNA extraction. Subsamples for total nutrients (30 mL unfiltered) and dissolved nutrients (30 mL, filtered; GF/C, Whatman) were placed in Falcon tubes and stored on ice. Apart from cell enumeration samples (stored at room temperature away from light), all samples were frozen (-20 °C) within ten hours for long term storage.

6.4.2 Sample Analysis

Concentrations of dissolved nutrients (NH_4, NOx, NO_2, NO_3 and PO_4) were determined using a discreet photometric analyser (AQUAKEM 200 Cd, Thermo Scientific). Total oxidised nitrogen (NOx-N) was analysed using SMWW/APHA Standard Method 4500 - NOx - E and EPA Method 353.4. NO_2-N concentrations were determined by SMWW/APHA Standard Method 4500 NO_2. NO_3-N was determined by subtracting NO_2-N from the NOx-N value. PO_4-P was analysed using Methods for the Examination of Water and Associated Materials: Phosphorus in Waters, Sewage and Effluents 1981, ISBN 011751582.5 and EPA Method 365.1. NH_4-N was analysed using Methods for the Examination of Water and Associated Materials: Ammonia in Waters 1981, ISBN 0117516139. Concentrations of total nitrogen and total phosphorus were determined by simultaneous persulphate digestion based on EPA methods 353.1 and 365.3 respectively.[141]

Cyanobacterial cell identification and enumeration was carried out as described in Section 6.1.2.

DNA was extracted from the GF/C filters (Power Soil® DNA Isolation Kit; MO BIO, USA) according to the protocol supplied by the manufacturer. Quantitative PCR was undertaken in 12.5 µL reaction mixes containing 2× Rotor Gene Probe PCR master mix (6.25 µL, Qiagen), primers targeting a region within the *McyE* ORF of the microcystin synthase gene (0.4 µM mcyE-F2 and MicmcyE-R8),[127] McyE probe[129] (0.2 µM) and template DNA (1 µL). Quantitation was based on a calibration curve produced from a dilution series of DNA extracted from a pure culture of Rotorua Strain A with a known cell concentration. An internal-control assay to indicate any PCR inhibition was run in a separate 12.5 µL reaction mix containing 2× Rotor Gene Probe PCR master mix (6.25 µL, Qiagen), primers targeting the internal transcribed spacer region 2 of the rRNA gene operon of chum salmon, *Oncorhynchus keta* (0.4 µM Sketa F2 and Sketa R3), TaqMan probe synthesised with a CAL fluor red 610 reporter dye at the 5´-end and a Black Hole Quencher 2 at the 3´-end (0.2 µM; Biosearch Technologies, United States), salmon sperm DNA (15 ng; Sigma, United States) and sample DNA (1 µL).[128] The cycling profile was 95 °C for 3 min, followed by 50 cycles of 95 °C for 3 s and 58 °C for 10 s. If any samples had a higher than expected cycle threshold (Ct) for the salmon sperm assay, the samples were diluted and re-analysed.

Subsamples collected for total microcystin analysis were thawed and sonicated (30 min) followed by three additional freeze-thaw cycles to rupture cells and release microcystins. Formic acid (5 µL, 0.1% v/v) was added to samples which were left for 15 min before centrifugation (5 min, 6,000 × *g*). A sub-sample of the

supernatant (ca. 1 mL) was placed in a septum-capped LC vial (1.8 mL). Subsamples collected for extracellular microcystin analysis were thawed at room temperature, formic acid (5 µL, 0.1% v/v) added and sample left for 15 min before centrifugation (5 min, 6,000 × *g*) and a subsample (ca. 1 mL) of the supernatant placed in a septum-capped LC vial (1.8 mL).

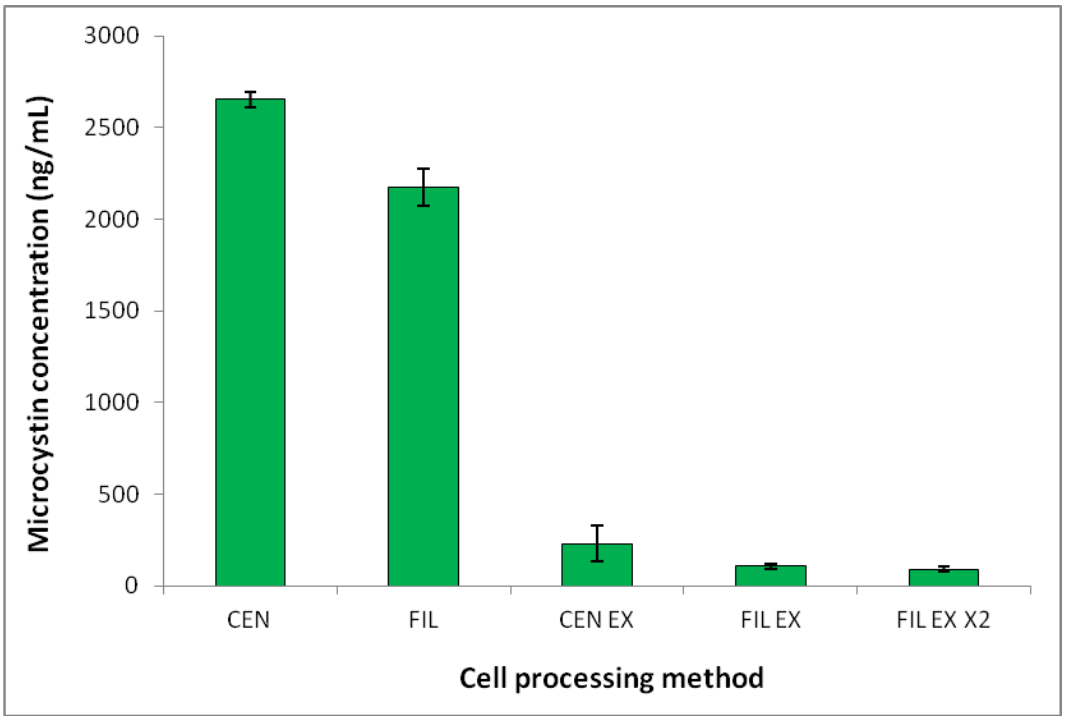
Microcystins present in the lake water were identified by an LC-MS screen in both positive and negative ion mode of an extract of concentrated lake water (Section 6.1.2). The ions that had *m/z* corresponding with the molecular mass of known microcystin congeners and which were at the correct retention time were then fragmented by MS/MS to confirm the microcystin congeners present based on the expected fragment ions produced. β-Mercaptoethanol derivatisation[131] was also carried out on the lake water extract as outlined in Section 6.1.2 to further verify the identity of the microcystin congeners.

The concentration of microcystins present was determined using the MRM method and LC gradient described in Section 6.1.2 with multiple standard curves constructed using a mixture of three microcystin standards (MC-LR, MC-RR and MC-LA).

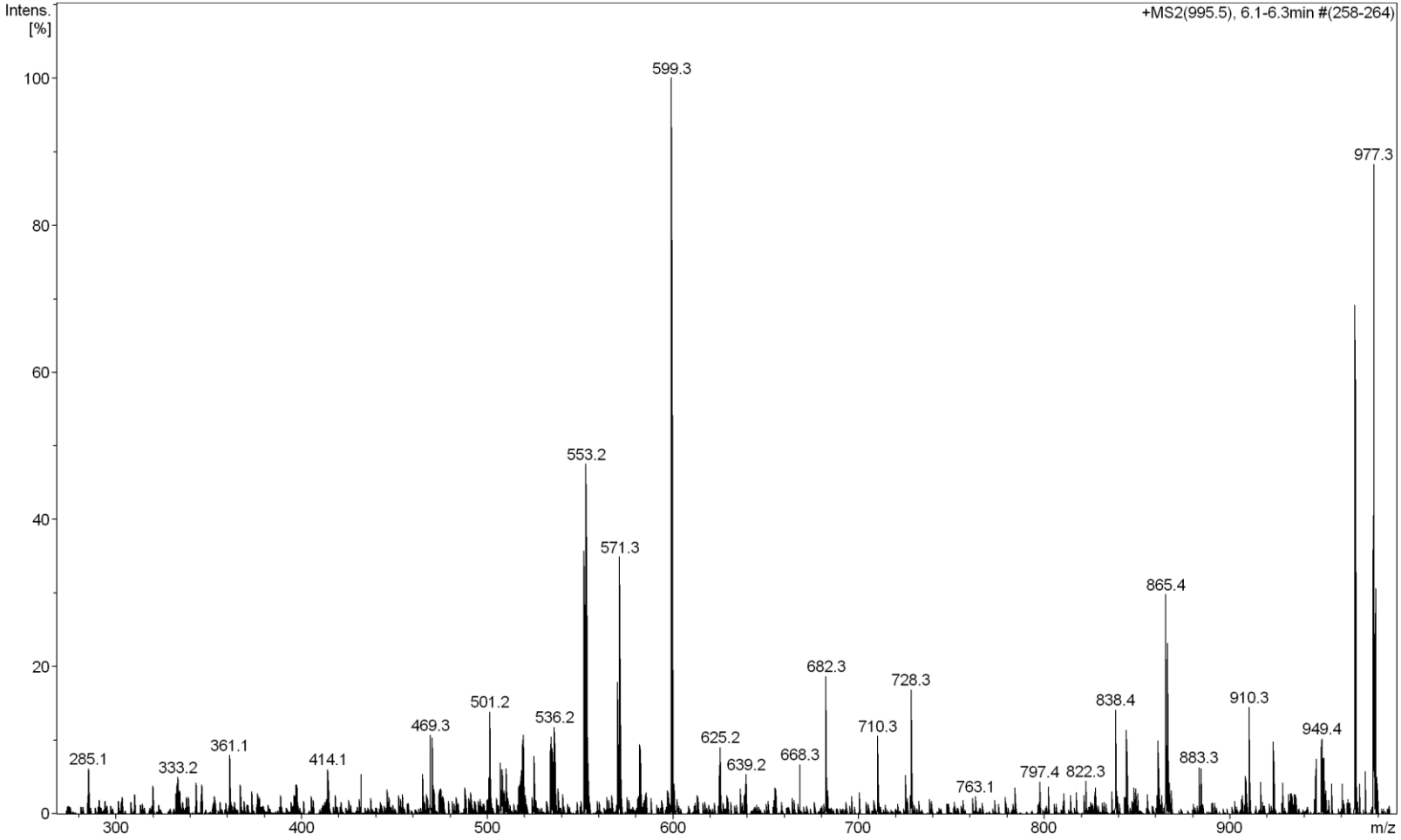
APPENDICES

Appendix A: Determination of loss of cells through filtration.

Triplicate subsamples (1 mL) were taken from the same batch culture of CYN06 *Microcystis* strain. Subsamples for intracellular and extracellular microcystins processed by centrifugation (CEN and CEN EX) and filtration (FIL and FIL EX) were assessed as described in Chapter 6.2.1 with the exception that after filtration, half of the extracellular medium (filtrate) was syringe filtered (0.20 µm; FIL EX X2) and both fractions were analysed. All extracellular microcystin samples also underwent four freeze-thaw cycles in order to lyse any cells that may have passed through the filters. Undiluted extracts were placed in a septum-capped vial (1.8 mL) and LC-MS analysis was carried as described in Chapter 6.1.2.



Appendix B: Tandem mass spectrum of the 995.5 ion in Rotorua A strain (putatively [Dha^7] MC-HilR).



Appendix C: The use of angiotensin I as an internal standard.

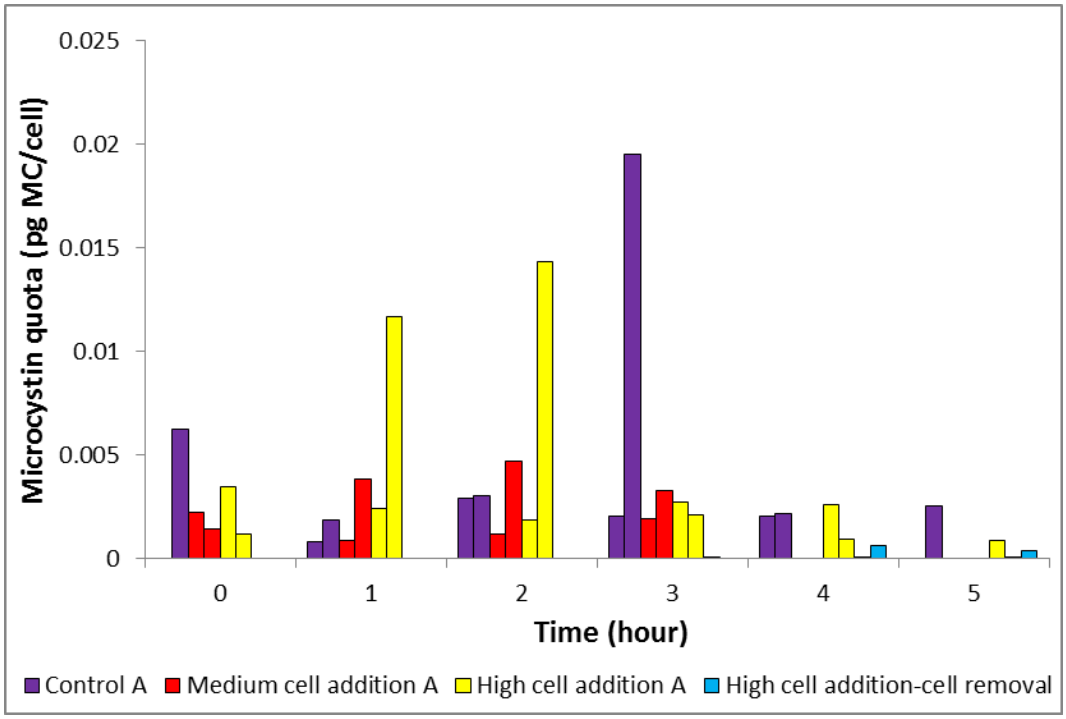
The repeatability of the MRM method created to quantify angiotensin I, aeruginosin, [Dha] MC-LR and [D-Asp^3, Dha^7] MC-LR was poor for extracellular samples. The relative standard deviation for angiotensin I was 104.6% for extracellular samples despite all samples (both intracellular and extracellular) containing the same concentration of this internal standard.

Peptide	**Relative Standard Deviation Intracellular (%)**	**Relative Standard Deviation Extracellular (%)**
Aeruginosin	19.85	67.69
Angiotensin I	9.50	104.6
[Dha^7] MC-LR	5.37	13.86
[Asp^3, Dha^7] MC-LR	3.85	4.04

In order to eliminate the possibility that the transition between two different collision amplitudes in the same window was influencing the ionisation of angiotensin I, the repeatability of the same method, but at the collision amplitude of 1.2 for both aeruginosin and angiotensin I was analysed. A high relative standard deviation (49 %) was still observed for angiotensin I.

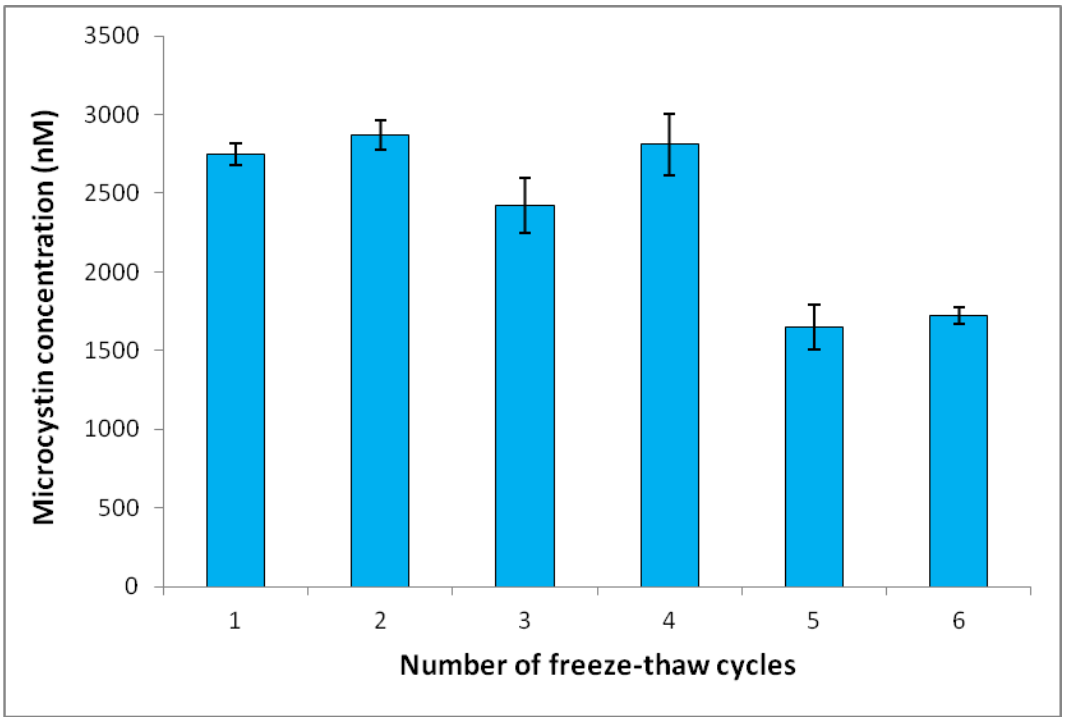
The ratio of $[M-H]^-$ ion (*m/z* = 1294.5) and $[M-H_2O-H]^-$ ion (*m/z* = 1276.5) abundance remained the same for all intracellular and extracellular samples indicating that full fragmentation had occurred. The integrated peak area for the extracted ion chromatograms of both ions reduced over the sample time indicating the possible degradation of angiotensin I in the cell extract or media. Therefore, the use of angiotensin I as an internal standard was rejected.

Appendix D: Microcystin quota based on QPCR toxic cell enumeration.



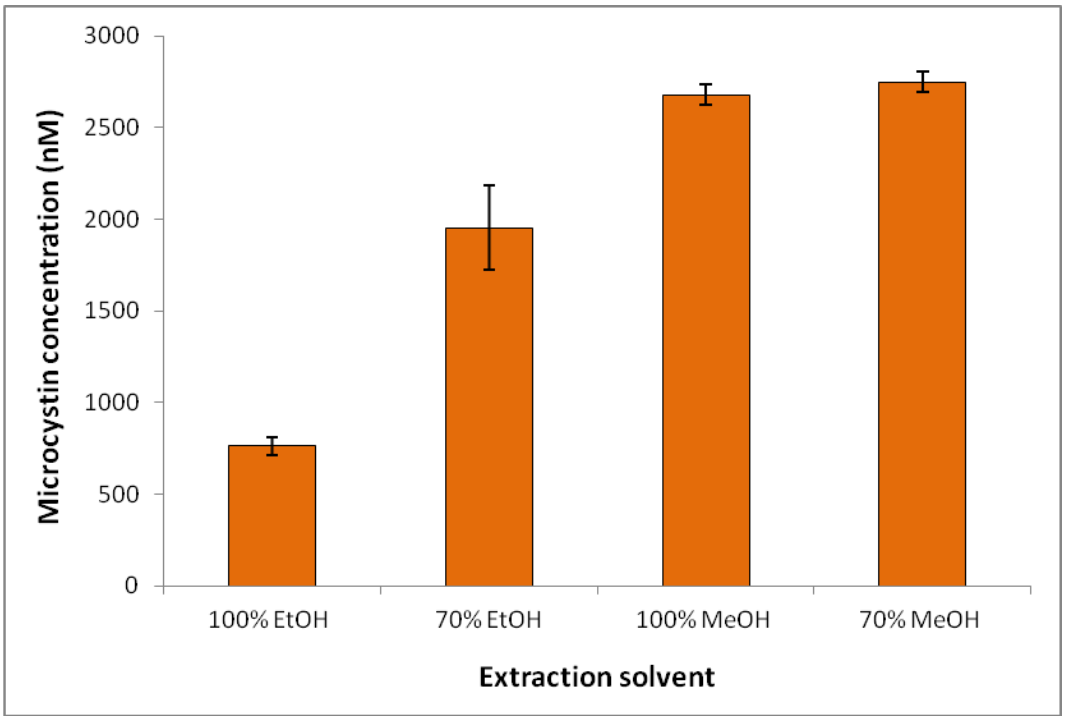
Appendix E: Determination of number of freeze-thaw cycles for most efficient microcystin extraction.

Duplicate subsamples (1 mL) were taken from the same batch culture of CYN06 *Microcystis* strain and subjected to one, two, three, four, five or six freeze-thaw cycle/s. In one freeze-thaw cycle, samples were frozen at -20 ºC (one hour) followed by sonication (30 min) and centrifugation. The undiluted supernatant was placed in a septum-capped vial (1.8 mL) and LC-MS analysis was carried as described in Chapter 6.1.2. There was no significant difference in the microcystin yield after one, two, three and four freeze-thaw cycles. The microcystin yield decreased significantly after five and six freeze-thaw cycles, most likely due to degradation. Four freeze-thaw cycles gave the highest microcystin yield and was thus chosen as the number of freeze-thaw cycles to be used in the sample processing experiment.



Appendix F: Determination of most efficient extraction solvent.

Duplicate subsamples (1 mL) were taken from the same batch culture of CYN06 *Microcystis* strain. Subsamples were centrifuged and cell pellet resuspended in 70% ethanol (EtOH), 100% EtOH, 70% methanol (MeOH) or 100% MeOH before sonication (30 min) and centrifugation. The undiluted supernatant was placed in a septum-capped vial (1.8 mL) and LC-MS analysis was carried out as described in Chapter 6.1.2. Results showed a significant difference in microcystin concentration following extraction in 100% EtOH and 70% MeOH (P = 0.0006) and 100% EtOH and 100% MeOH (P = 0.0007). Extraction in 70% EtOH, 100% MeOH or 70% MeOH all gave similar results in terms of extraction efficiency. Extraction in 100% MeOH was chosen to be used in the sample processing experiment as it gave one of the highest microcystin yields and was most convenient.



Appendix G.1: Fragment ions detected for *m/z* 995.5, 981.5 and 967.5 ions in CYN11.

Fragment Assignment	**MC-LR**	**[Asp^3] MC-LR**	**[D-Asp^3, Dha^7] MC-LR**
M+H	995	981	967
M – Ala + H			896
M – $CH_2NHCN_2H_3$ + H	923	909	
M – (Me)Asp + H			
M – Glu + H		852	838
M – Adda sidechain + H	861	847	833
(Me)Asp-Arg-Adda-Glu + H	728		714
(Me)Asp-Arg-Adda + H	599		
Arg-Adda-Glu + H	599	599	599
Arg-Adda + H			
(M)dha-Ala-X-(Me)Asp-Arg + NH_4	570	556	
Ala-X-(Me)Asp-Arg + NH_4			
(M)dha-Ala-X-(Me)Asp-Arg + H	553	539	525
Ala-X-(Me)Asp-Arg + H			
Mdha-Ala-X-(Me)Asp + H	397		369
Adda'-Glu-(M)dha-Ala + H			432
Adda'-Glu-(M)dha + H	375	375	361

Appendix G.2: Fragment ions detected for *m/z* 995.5, 981.5 and 967.5 ions in CYN60.

Fragment Assignment	**MC-LR**	**[Asp^3] MC-LR**	**[D-Asp^3, Dha^7] MC-LR**
M+H	995	981	967
M – Ala + H	924		
M – $CH_2NHCN_2H_3$ + H	923	909	
M – (Me)Asp + H		866	
M – Glu + H			838
M – Adda sidechain + H	861	847	
(Me)Asp-Arg-Adda-Glu + H			714
(Me)Asp-Arg-Adda + H	599		
Arg-Adda-Glu + H	599	599	599
Arg-Adda + H	470		
(M)dha-Ala-X-(Me)Asp-Arg + NH_4		556	542
Ala-X-(Me)Asp-Arg + NH_4	487		
(M)dha-Ala-X-(Me)Asp-Arg + H	553	539	525
Ala-X-(Me)Asp-Arg + H	470		
Mdha-Ala-X-(Me)Asp + H	397		
Adda'-Glu-(M)dha-Ala + H	446		
Adda'-Glu-(M)dha + H	375	375	361

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