MASS-SPECTROMETRY BASED METABOLOMICS TO
DECIPHER STRAIN SPECIFIC *MICROCYSTIS*
CYANOPEPTIDE PROFILES

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in
partial fulfillment of the requirements for the degree of

Master of Science

in

Chemistry with Specialization in Chemical and Environmental
Toxicology

Carleton University

Ottawa, Ontario

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i. Abstract

Over the last one hundred years, ecosystem changes have occurred as a result of human population growth, pollution, increased temperatures, and habitat degradation. A visible change is the increase in frequency and magnitude of toxic cyanobacterial blooms. Cyanobacterial blooms release mixtures of biologically active compounds into freshwater that negatively impact human and ecosystem health as well as having socioeconomic consequences. The factors that influence cyanobacterial growth and toxin production are broadly understood. However, cyanobacteria are a prolific source of structurally diverse and strain specific mixtures of biologically active compounds. Currently, the chemistry, toxicology, environmental concentrations, and risks posed to human and ecosystem health by most cyanobacterial secondary metabolites are unknown. Advances in mass spectrometry and metabolomic techniques can aid in comprehension of complex metabolomes. Here, the use of untargeted and semi-targeted mass spectrometry-based metabolomics is used to decipher the non-ribosomal peptide natural products (cyanopeptides) from five *Microcystis* strains. Cyanopeptides are grouped based on shared structural features, such as the incorporation of non-proteogenic amino acids or partial amino acid sequences that generate diagnostic product ions with the MS/MS of metabolites within the same cyanopeptide group. Global natural product society (GNPS) molecular networking and diagnostic fragmentation filtering (DFF) techniques utilize the similarity in product ion spectra to visualize all variants in the different cyanopeptide groups and the production by *Microcystis* strains. The semi-targeted DFF technique is applied to environmental water and bloom samples for the detection of entire cyanopeptide groups. Finally, growth medium composition effects on *Microcystis* chemical profiles are assessed with the established metabolomic techniques. Optimal growth conditions for cyanopeptide production have been elucidated for the *Microcystis* strains. This will enable the

growth of target strains that produce novel compounds and common environmental contaminants on a large scale for the isolation and purification of new cyanopeptides. With tangible material of novel cyanopeptides, structural and toxicological assessments as well as quantification of environmental concentrations can be performed.

ii. Acknowledgements

I would first like to thank my supervisor, David McMullin, for providing the opportunity to develop and expand the cyanopeptide project with interdisciplinary work. Thank you to Justin Renaud for his expertise with advanced mass-spectrometry and metabolomics as well as Dr Mark Sumarah for the experience in the LRDC lab to better expand our analytical techniques. And thanks to Natasha Desrochers for her metabolomic talents and quick replies. I am grateful for the opportunity to attend the Gordon Research Conference on Phycotoxins and Mycotoxins which expanded my research interests and provided inspiration to my own work.

Thank you to Dr David Miller for many projects and experiences over the course of my time at Carleton University that helped me build up my skills and critical thought processes as a scientist.

Thank you to Francis Pick for the collection and detailed information for the bloom samples collected in the National Capital Region analyzed here.

Thank you to the fellow graduate students for the support and encouragement throughout my time at Carleton University, from brainstorming experimental approaches to trivia night at Mike's Place. I would also like to thank the dedicated undergraduate students that helped perform experiments and did the grunt work.

Finally, thank you to my parents and sisters for the never-ending love and support through this experience.

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i.v. List of abbreviations:

A/Ala – alanine

ACN – acetonitrile

Adda – 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid

Ahda – 3- amino-2-hydroxy decanoic acid

Ahoa – 3-amino-2-hydroxy octanoic acid

Ahp – 3-amino-6-hydroxy-2-piperidone

ANOVA – analysis of variance

ATX – anatoxin

BW – body weight

C/Cys – cysteine

Choi – 2-carboxy-6-hydroxyoctahydroindole

CPCC – Canadian Phycological Culture Collection

DCM – dichloromethane

ddH₂O – distilled deionized water

DFF – diagnostic fragmentation filtering plot

DmA – 1,1-dimethylallyl

E/Glu – glutamic acid

F/Phe – phenylalanine

G/Gly – glycine

GNPS – global natural products society

H/His – histidine

Hph – homo-phenylalanine

Hpla – hydroxy-phenyllactic acid

Hty – homo-tyrosine

HxA – hexanoic acid

I/Ile - isoleucine

K/Lys – lysine

KW – Kruskal Wallis test

L/Leu – leucine
LC – liquid chromatography
M/Met – methionine
 m/z – mass- to- charge ratio
MC – microcystin
Mdha – methyl-dehydroalanine
Mdhb – 2-(methylamino)-2-dibutyric acid
MeOH – methanol
MS – mass spectrometry
MS/MS – tandem mass spectrometry
N/O-*Me* – N-/O-methylated
NOD – nodularin
nRP – non-ribosomal peptide
nRPS – non-ribosomal peptide synthase
OATPs – organic anion transporting polypeptides
P/Pro – proline
PCA – principal component analysis
PKS – polyketide synthase
PP1/2A – protein phosphatase 1/2A
Q/Gln – glutamine
QToF – quadrupole time-of-flight
R/Arg – arginine
RiPP – post-translationally modified peptide
S/Ser – serine
sp. – species
spp. – multiple species
STX – saxitoxin
T/Thr – threonine

Thz – thiazole

Tzc – thiazole carboxylate

Tzl – thiazoline

V/Val – valine

W/Trp – tryptophan

Y/Tyr - tyrosine

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Table S1: LC-HRMS data for cyanotoxins produced by *M. aeruginosa* CPCC 299, 300, 632 and 633, and *M. flos-aquae* CPCC 461.

Table S2: Compiled sequence database for cyanopeptolins previously reported in literature.

Table S3: Compiled sequence database for microginins previously reported in literature.

1. Introduction

1.1 Cyanobacteria

Cyanobacteria or “blue-green algae” are ubiquitous photosynthetic prokaryotes that inhabit aquatic ecosystems including freshwater lakes, marine coastal areas, and drinking water reserves. The long evolutionary history of cyanobacteria has provided them with adaptations to thrive in diverse environmental conditions (Paerl & Millie, 1996). Under normal conditions cyanobacteria will remain at low cell numbers within the environment and not pose a serious threat to ecosystem or human health. The factors that influence cyanobacteria growth are broadly understood and include sunlight, access to macronutrients including nitrogen and phosphorous, and water temperature (Walls et al., 2018). With increased nutrient input from anthropogenic sources as well as an increase in surface water temperatures due to climate change, the excessive proliferation and accumulation of cyanobacterial colonies results in visible surface blooms (cyanobacterial harmful algal blooms, cHABs, Figure 1.1) which can have adverse outcomes. The decomposition of dead cyanobacterial biomass results in decreased dissolved oxygen amounts within the water column. The production of stagnant zones by dense algal mats can generate strong vertical dissolved oxygen gradients and ultimately, anoxic conditions which have been linked with fish mortalities (Watson et al., 2015). Further, the release of cyanobacterial toxins into water during cell senescence pose poorly characterized risks to ecosystem and human health. Further, toxin producing blooms have socioeconomic consequences as a result of contaminated drinking water, irrigation reservoirs, and the diminishment of recreational value in freshwater lakes (Paerl & Otten, 2013).

Figure 1.1: Researchers investigating the *Microcystis* harmful algal bloom in Lake Erie in August 2019 (Johnson, Cleveland.com).

With excess nutrients, the main determinant of cyanobacterial growth and metabolism is temperature because many enzymes that participate in photosynthesis and respiration are temperature dependent (Walls et al., 2018). Cyanobacteria typically dominate the freshwater algae populations at higher temperatures due to their optimum growth rates at such temperatures and their increased affinity for nutrients at higher temperatures in comparison to eukaryotic algae (Figure 1.1). The determinants of surface accumulation and thus bloom formations are highly variable and are dependent on water depth as well as short term changes in weather such as solar radiation and windspeed. Shallow waters paired with low precipitation and high spring temperatures can result in more intense bloom formation (Pick, 2016).

When temperature is constant, the essential nutrients for cyanobacterial growth can be determined. A famous study performed in the 1970s used an entire lake to show the importance of nutrients on cyanobacteria bloom formation and found phosphorous to be the nutrient with the largest impact (Schindler & Fee, 1974). The lake was divided in two with a membrane. One half of the lake was fertilized with phosphorous, nitrogen, and carbon (+P) and the other half fertilized with only nitrogen and carbon (-P, Figure 1.2). The half of the lake fertilized with phosphorous developed a bloom whereas the other side did not.

Figure 1.2: Experimental lake 226 divided with a curtain with half fertilized with phosphorous to show the impact phosphorous has on bloom formation.

Cyanobacteria have several evolutionary adaptations that have provided them with competitive advantages over microalgae species. The competitive advantages include efficiently acquiring

nutrients, buoyancy control allowing an overwintering process, and cyanotoxin production. When water temperatures drop below a level that is optimal for cyanobacterial growth, the cells stock up on their photosynthetically synthesized sugars, sink to benthic layers, slow down primary metabolism and halt secondary metabolism to survive the colder water temperatures (Henao et al., 2020). In the spring, an event known as spring turn over occurs releasing oxygen from the water and results in turn over of the sediment, releasing the cyanobacteria. This is the inoculum for the next years bloom season. This buoyancy control is achieved with the generation of vacuoles which contain photosynthetic oxygen and enable cyanobacteria to access nutrients suspended along the vertical water column (Paerl & Millie, 1996). Another competitive advantage is toxin production (cyanotoxin) that occurs in common bloom forming cyanobacteria genera such as *Microcystis*, *Nodularia*, *Anabaena*, *Aphanizomenon*, and *Dolichospermum*. Cyanotoxins have diverse structures and are grouped at a high level based on their modes of action which can include with hepatotoxins, neurotoxins, dermatotoxins and enzyme inhibitors (Huang & Zimba, 2019). Another competitive advantage that has evolved for some filamentous cyanobacteria genera such as *Anabaena*, *Nostoc*, and *Cylindrospermopsis* are heterocysts, specialized nitrogen-fixing cells that metabolize N₂ gas and nitrates from the atmosphere and water (Paerl & Otten, 2013). Within heterocysts, N₂ gas and nitrates are reduced to amines which are subsequently exported and used by photosynthetic cells and incorporated into complex biomolecules (Zurawell et al., 2005).

Cyanobacteria growth rates vary based on the species, or even strains of the same species, with variable optimal temperatures and nutrient concentrations. Toxic strains of cyanobacteria require larger quantities of nitrogen and phosphorous due to the expense of energy and materials for toxin biosynthesis (Zurawell et al., 2005). Non-heterocystous cyanobacteria such as *Microcystis* and *Planktothrix* require higher nitrogen concentrations for increased toxicity whereas heterocystous

cyanobacteria show increased toxicity when grown in a nitrogen free growth medium. This indicates that toxin production varies in response to ambient nitrogen concentration for these two types of cyanobacteria (Rapala et al., 1997). Further, phosphorous concentrations are linked with toxin production for both heterocystous and non-heterocystous cyanobacteria. There appears to be a negative correlation with toxin production and phosphorous concentration, suggesting phosphorous limitation is a main factor that induces toxin production (Zurawell et al., 2005).

Toxin production increases at warmer temperatures due to increased photosynthetic rates and it is suggested that warmer temperatures impact the proportion of intracellular to extracellular toxin concentrations (Walls et al., 2018). Typically, the toxins remain cell-bound while cyanobacteria cells are alive. Upon cell death and lysis, intracellular cyanopeptides are released into the environment. There are very few defined mechanisms for extracellular toxin release (Walls et al., 2018). Research has shown that the release of secondary metabolites can prevent cellular damage when high photosynthetic output is coupled with unfavourable growth conditions (Smith & Underwood, 2000).

Cyanobacterial strains from the genus *Microcystis* are the focus of this investigation. This genus represents one of the most prolific bloom-forming cyanobacteria with reports of blooms containing *Microcystis* species in over 108 countries with 79 countries reporting association of the hepatotoxins, microcystins, to the bloom (Harke et al., 2016). *Microcystis* occurrence within freshwater phytoplankton communities has increased drastically in the last 15 years, with previous reports of *Microcystis* sp. in less than 30 countries (Zurawell et al., 2005). *Microcystis* species are non-heterocystous, meaning they cannot fix N₂ and thus must have other cellular advantages over heterocystous cyanobacteria. The physiological characteristics of *Microcystis* sp. that favour their ubiquitous dominance over other cyanobacteria species are their optimal growth occurs at 27°C

and ability to produce complex mixtures of toxic secondary metabolites (cyanotoxins) which provides tolerance to zooplankton grazing (Le Manach et al., 2019). In 2014, the Toledo municipal water supply was contaminated by a *Microcystis* bloom that produced large quantities of microcystins, a group of potent hepatotoxins. 400,000 people were left without drinking water for several days while restoration and decontamination efforts were set in place. Large quantities of aluminum and chlorine were added to the water to coagulate the bloom so that biomaterial could be filtered from the water. However, extracellular release of the toxin could not be controlled with this approach. In 2014, there were no US National standards for safe cyanobacteria or cyanobacterial toxin levels within drinking and recreational water which resulted a demand for guidelines to be investigated/released (McKindles et al., 2020).

1.2 Secondary metabolites

Living organisms complete a series of anabolic and catabolic reactions catalyzed by enzymes in a process known as metabolism to synthesize and degrade organic and inorganic materials (A. L. Demain & Fang, 2000). Primary metabolism provides biosynthetic intermediates and energy that are converted into essential macromolecules such a DNA, RNA, proteins, lipids, and polysaccharides. Primary metabolism is a highly regulated and balanced process with the most important resulting molecules (primary metabolites) being nucleic acids, purine nucleotides, vitamins, and organic acids. Primary metabolism is relatively conserved across all living systems as it aids in growth, development, and reproduction (A. L. Demain & Fang, 2000). Secondary metabolism however, primarily occurs in plants and microorganisms, and is usually strain-specific. Secondary metabolites are defined as small biomolecules that are not necessary for growth and development. The compounds have alternative functions that act to improve the survival of the producing organism in competition with other living species. Products of secondary metabolism

are of interest to human health as approximately 25% of all natural products are bioactive and have helped double the human life span during the 20th century through drug development. Roughly 75% of all antibiotics have been obtained from filamentous prokaryotes (Arnold L. Demain, 2014). Secondary metabolites are classified by the route of synthesis into the following categories: alkaloids, polyketide, terpenes, and non-ribosomal peptides. Bioactivities from the reported 23,000 natural products can range from antiviral, cytotoxic, anticancer, and immunosuppressive providing an excellent source for drug candidates (A. L. Demain & Fang, 2000).

Cyanobacteria are known to produce a wide array of bioactive metabolites such as terpenoids, polyketides, depsipeptides, and peptides (Portmann, Blom, Gademann, et al., 2008). The function of these metabolites is proposed as a defensive agent against predators, as many purified cyanotoxins have shown toxicity against grazer crustaceans. Many reports indicate that these metabolites inhibit protease enzymes suggesting an alternative function once within the predator, preventing the degradation of the toxin before reaching its target (Janssen, 2019). Secondary metabolite production may also aid in reducing oxidative stress by diverting energy from the light harvesting complexes. It has been shown that secondary metabolites are often processed, cycled, and disposed of during regular cell growth but may accumulate intra- or extra-cellularly in significant quantities upon environmental stressors. However, most environmental signals that regulate secondary metabolism in cyanobacteria have yet to be deciphered. Overall, it is hypothesized that the production of such large and complex metabolites provides a control mechanism to overcome growth rate disadvantages among microbes when environmental conditions do not provide a competitive growth advantage (Paerl & Millie, 1996). This link to bacterial growth rate further suggests coupling of toxin production to photosynthesis (Walls et al., 2018). The synthesis of these large biomolecules is an energetic process and requires large amounts

of energy, thus when the cells are rapidly dividing and performing photosynthesis at an optimal rate, excess energy is available for secondary metabolite production.

More than 2000 cyanobacteria natural products have been described to date. Oligopeptides (cyanopeptides) derived from non-ribosomal peptide synthetase (NRPS), mixed NRPS-polyketide synthase (NRPS-PKS), and post-translationally modified peptide (RiPP) pathways account for most of this reported chemical diversity (Jones et al., 2020). Other common biosynthetic pathways found in cyanobacteria generate alkaloid or terpene secondary metabolites. Cyanopeptides are the main focus in this investigation and are biosynthesized through via several non-ribosomal and ribosomal pathways such as non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) enzyme systems or they may be synthesized via ribosomally synthesized and post-translationally modified peptide (RiPP) pathways (Tiam et al., 2019). A modular structure of NRPSs is responsible for the sequential amino acid specific extension of peptide chains. The various combinations of modules and their functional domains determines the structure of the final peptide and the associated biological activity (Neilan, Dittmann, Rouhiainen, Bass, Schaub, Sivonen, & Bo, 1999). This mode of synthesis for NRPS can incorporate unusual and non-proteinaceous amino acids into the sequence. As well, some of the modules may be polyketide synthases and result in the addition of polyketide molecular features within a peptide natural product. An example of this combined synthesis is that of congeners in the microginin chemical class (Figure 1.9). These metabolites have proteinaceous amino acids and an N-terminal decanoic acid derivative biosynthesized by a polyketide synthase.

The modular sections within NRPSs have distinct functions responsible for the addition of a defined monomer into one complete natural product (Strieker et al., 2010). This assembly line defines which monomeric units are incorporated, in which particular order, the length and

functionality of the metabolite, as well as the chemical reaction that occurs to link the monomers. There are 4 catalytic steps in NRP synthesis: activation, propagation, condensation, and release (Figure 1.3). The first step, activation, is catalyzed by an adenylation domain (A domain) and must be highly selective and specific toward the incoming monomer. The A domain acts as a gatekeeper enzyme for incoming amino acids and has a 10 amino acid specificity-conferring code which is responsible for substrate binding within the active site and ultimately determines the structure of the final NRP. Sequencing of the A domain and online specificity prediction tools are available and can aid in the isolation of predicted NRPS products (Strieker et al., 2010). Each A domain has a large N-terminal core domain and a small C-terminal subdomain which are connected by a 5-10 residue hinge. The terminal domain amino acid sequences are highly conserved throughout all adenyating enzymes, including acetyl-coA synthase (Strieker et al., 2010). With the help of an ATP molecule, Mg^{2+} as a cofactor, and the specific monomeric amino acid, the first reaction in NRP synthesis occurs and the activated amino acid is transferred to the peptidyl carrier protein (PCP) also known as the thiolation domain, as it contains the prosthetic group 4'-phosphopantetheine (Süssmuth & Mainz, 2017). The now tethered amino acid is moved towards the condensation domain (C domain) where it undergoes a condensation reaction with the nascent peptide. While the activated amino acid is tethered to the PCP, it is possible to be passed to accessory enzymes before reaching the C domain. These accessory enzymes have individual catalytic functions that allow modifications such as methylation, epimerization, heterocyclization, formylation, oxidation and reduction of the amino acid prior to incorporation which aids in increased structural diversity of cyanopeptides (Süssmuth & Mainz, 2017). The finished product is released from the distal end of the assembly line by the termination domain (Te domain) through either hydrolysis, aminolysis, or macrocyclization liberating linear or cyclic products. The Te

domain may cyclize molecules by aiding in folding the linear peptide and catalyzing an intramolecular nucleophilic attack from one end of the metabolite to another (Figure 1.3, Tillett et al., 2000).

Increased structural diversity of cyanopeptides occurs via decreased specificity of the enzymes present within the biosynthetic assembly line for amino acids. Although majority of NRPS have a high specificity for one single amino acid as a substrate, it is possible for NRPS to recognize more than one amino acid as its substrate (Ackerley & Lamont, 2003). If the specificity is high, consistencies between metabolites occurs and results in the formation of a chemical group based on partial amino acid sequences or shared non-proteinaceous amino acids. If the amino acid specificity of the enzyme is low, this generates a highly variable position within the metabolite and thus increases structural diversity.

Figure 1.3: Simplified mechanism of non-ribosomal peptide (NRP) synthesis. (1) The amino acid is activated by the adenylation domain. (2) Transfer of the amino acid onto the PCP domain. (3) Condensation of PCP-bound amino acids. (4) Possibility of amino acid modifications, for example by epimerization domains. (5) Transesterification of the peptide chain from the terminal PCP onto the TE domain. (6) TE domain catalyzed product release by either hydrolysis,

aminolysis, or macrocyclization. The number of modification domains and modules is very variable (Strieker et al., 2010).

The majority of ribosomal peptides from RiPP pathways begin as precursor peptides with an N-terminal leader that guides biosynthetic enzymes responsible for post translational modifications to the core peptide which is eventually cleaved with a protease (Zhang, Chen, et al., 2018). Similar to proteins, these metabolites are synthesized ribosomally, passed along through various enzymatic domains of the synthase system matching an amino acid to a three-letter codon. As these metabolites have a ribosomal origin, their synthesis and structure may be mapped by analyzing the genome of the cyanobacteria, specifically the genes encoding the A domain enzyme. Structural diversity of RiPP synthesized cyanopeptides arises through post-translational modifications catalyzed by methyl-transferases, halogenases, and dehydrogenases.

1.3 Cyanotoxins

Of the many bloom-forming genera, not all populations pose a health risk via the production of toxic metabolites. It is suggested that 25-75% of surface blooms are toxic (Zurawell et al., 2005). Toxic cyanobacteria are defined by the induction of harmful effects on vertebrates and humans and include species within the genera *Microcystis* (studied here), *Dolichospermum* (formerly *Anabaena*), *Lyngbya*, *Cylindrospermopsis*, *Aphanizomenon*, and *Planktothrix*. These genera produce mixtures of cyanotoxins that have neurotoxic, cytotoxic, and/or hepatotoxic effects (Pick, 2016; Zurawell et al., 2005). Toxic species of cyanobacteria produce complex mixtures of cyanotoxins in a strain-specific manner which results in vast structural diversity among cyanobacterial natural products (A. L. Demain & Fang, 2000). Chemotaxonomy is the subclassification of strains within the same genus based on their chemical profiles. Understanding

the metabolites that are generated by strains within the same genus can help determine genetic similarities and evolutionary divergence for classification.

1.3.1 Neurotoxic cyanotoxins

The major cyanotoxins with neurotoxic activity are alkaloid metabolites including anatoxins, also known as the Very Fast Death Factor because of the rapid physiological response, and the toxins associated with paralytic shellfish poisoning (PSP), saxitoxins (Figure 1.3, Yadav et al., 2011). Saxitoxins (STXs) are alkaloid natural products produced by species within the genera *Aphanizomenon*, *Dolichospermum*, *Lyngbya*, and *Cylindrospermopsis* but primarily by dinoflagellates in the genus *Alexandrium* (Testai et al., 2016). These tricyclic alkaloids may be sulfated or non-sulfated and possess a charged guanidine functionality resulting in potent neurotoxicity through binding to voltage-gated sodium ion channels in nerve cells. The resulting biological response causes numbness, paralysis, and death in mammals through respiratory arrest (Yadav et al., 2011). Saxitoxins have been shown to bioaccumulate throughout the marine food web, particularly shellfish, starting by consumption of algal cells by zooplankton and moving into higher organisms. Toxicity analysis of saxitoxins reveals an LD₅₀ with intraperitoneal injection in mice of 10 µg kg⁻¹ BW and environmental concentrations reaching 193 µg L⁻¹ in Washington State with some species reaching a toxin quota per cell of 0.03-1.30 pg cell⁻¹ (Testai et al., 2016).

Anatoxins (ATXs) are produced by both benthic and brackish cyanobacteria species within the following genera *Anabaena*, *Oscillatoria*, *Aphanizomenon*, *Cylindrospermum*, and *Phormidium* (Figure 1.3, (Testai et al., 2016)). ATXs are bicyclic amine alkaloids with acute neurotoxicity through depolarization of neuromuscular blocking agents and provoking fatal asphyxia (Méjean et al., 2014). Prolonged exposure to the toxin results in muscle paralysis with the minimum lethal dose of anatoxin-a residing at 0.25 mg kg⁻¹ BW with intraperitoneal injection in mice. Mortality

events attracted attention when in 2018 several dogs died in New Brunswick, Canada after ingesting large quantities of the toxin near the Saint John River. Depending on the species, as well as the strain of cyanobacteria, ATX is typically found in larger proportions intracellularly but has been detected within the environment extracellularly in concentration up to $1170 \mu\text{g L}^{-1}$ in Washington State (Testai et al., 2016).

β -N-methylamino-L-alanine (BMAA) is a modified non-proteinaceous amino acid produced by many cyanobacteria genera (Figure 1.3). This molecule was first detected in 1967 in the seed of a cycad tree and it has then been theorized that biomagnification of BMAA through the different trophic levels began with a cyanobacterial endosymbiont residing within the roots of the *Cycas* sp. (Botana et al., 2015). Since the initial discovery, the toxin has been reported to be produced by both symbionts and free-living species in all types of habitats, including marine, brackish, and freshwater. Like saxitoxins, this molecule bioaccumulates in fish and shellfish resulting in human exposure through consumption of contaminated seafood. The neurotoxicity of this molecule is complicated with various mechanisms within the cell such as, glutamate excitotoxicity, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, oxidative damage, and toxic protein aggregation leading to motor deficits, neuronal degradation and somatic death (Tan et al., 2018). Long term with low dose exposures of this toxin has been correlated with neurodegenerative diseases such as Parkinson's and Alzheimer's disease with the median LD_{50} with intraperitoneal injection in mice being $3 \mu\text{g kg}^{-1} \text{ BW}$ (Al-Sammak et al., 2015).

Figure 1.3: Chemical structures of neurotoxin cyanotoxins, anatoxin-a (left), saxitoxin (centre), and BMAA (right).

1.3.2 Hepatotoxic cyanotoxins

The alkaloid hepatotoxin cylindrospermopsin (CYL) is produced by several cyanobacteria genera including *Anabaena*, *Aphanizomenon*, *Raphidiopsis*, *Lyngbya*, and *Cylindrospermopsis* (Botana et al., 2015). CYL contains a tricyclic guanidine group with a sulfate group at one end and an uracil at the other end (Figure 1.4). CYL and isomers of this metabolite are biosynthesized through the PKS and NRPS pathways and organisms with these biosynthetic gene clusters are more commonly found in temperate climates (Botana et al., 2015). Bioaccumulation of this toxin occurs in many vertebrates and invertebrates within muscle and hepatopancreatic tissues. Humans are exposed to CYL via consumption of contaminated drinking water or fish. This results in hepatoenteritis as the toxin is distributed to the liver rapidly. *In vitro* studies with intraperitoneal injection of CYL to mice resulted in necrosis or apoptosis depending on the dose and the exposure time, with an LD₅₀ of 2 mg kg⁻¹ BW after 24 hours and 0.2 mg kg⁻¹ BW if administered for 4-6 days (Botana et al., 2015). This data suggests that chronic low dose exposure is more toxic than administration of a larger single dose of CYL.

Figure 1.4: Chemical structure of cylindrospermopsin.

Cyanotoxins synthesized from NRPS synthesis pathways have the highest reports of hepatotoxicity for all previously reported cyanotoxins (Zurawell et al., 2005). The main two groups of cyanobacteria hepatotoxins are microcystins (MCs) and nodularins (NODs) which damage hepatocytes of the liver with death occurring in extreme cases due to intrahepatic hemorrhaging (Yadav et al., 2011). NODs are cyclic pentapeptides produced by species from the genus *Nodularia* and contain the diagnostic non-proteinaceous amino acid moieties 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda, red) and 2-(methylamino)-2-dihydrobutyric acid (Mdhb, blue) (Figure 1.5). The structural similarities between MCs and NODs include the Adda moiety, an *NMe*-Asp, and D-Glu. The primary difference between NODs and MCs is that are pentapeptides and MCs are hexapeptides. Another major difference is that NODs incorporate an Mdhb moiety and MCs incorporate a methyl-dihydroalanine (Mdha, green, Figure 1.5). The Adda moiety present in both these peptide variants results in inhibition of eukaryotic protein phosphatase 1 (PP1) and 2A (PP2A). Although, the substitution of Mdhb to Mdha in MCs results in an increased toxicity as the double bond within Mdha acts as a nucleophile and covalently bonds within the active sites of PPs (Zong et al., 2017). Microcystin congeners and toxicity will be discussed in greater detail in section 1.4.1.

Figure 1.5: Chemical structure of hepatotoxins, nodularin-R (left) and microcystin-LR (right).

1.4 Cyanopeptides from *Microcystis* species

Here metabolites biosynthesized by *Microcystis* sp. with NRPS or combined PKS-NRPS pathways are described. Most cyanopeptides are cyclic or linear non-ribosomal peptides that possess unique non-proteogenic amino acids and are grouped based on common structural features (Welker et al., 2006). Variable NRPS A domain sequences and tailoring enzymes are observed in studied gene clusters, even within strains of the same species suggesting unique metabolomic profiles for cyanobacteria strains. The variation in biosynthetic machinery also provides increased structural diversity in cyanopeptides and the ecotoxicology and toxicokinetics of the vast majority of cyanopeptides are not completely understood.

1.4.1 Microcystins

MCs are a group of more than 300 potent hepatotoxins and possible human carcinogens (IARC Group 2B, (Harke et al., 2016)). MCs are the most studied cyanopeptide group due to their potent biological activities and are known to be produced by several cyanobacterial genera such as *Microcystis*, *Planktothrix*, and *Dolichospermum* (Chernoff et al., 2020). MCs are cyclic

heptapeptides that inhibit eukaryotic protein phosphatases 1 and 2A (PP1 and PP2A, De Figueiredo et al., 2004). The structural features that contribute to MC mode of action are the two non-protein amino acids that characterize this chemical class, Adda (3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid, red) and Mdha (methyl-dehydroalanine, green, Figure 1.6). The long hydrophobic Adda moiety aids in reversible interactions that move the molecule into the pocket of the PP active site while the Mdha moiety interacts irreversibly within the active site by covalently bonding to a nucleophilic site within the pocket (Zong et al., 2017). Both these interactions result in inhibition of the catalytic activity of the phosphatases. Within hepatic cells, this results in hyperphosphorylation of key signal transduction proteins promoting oxidative damage, which can have several deleterious effects on the cell and organ such as cell structure disruption, apoptosis, liver necrosis, and intrahepatic hemorrhage (Zong et al., 2017).

There are two main variable positions within MC congeners, residues 2 and 4 which contribute to the naming convention for the over 300 reported congeners. MC-LR is the most studied congener within this class and has a Leu in position 2 and an Arg in position 4 (Figure 1.6). Environmental concentrations of total MCs can reach $150\ \mu\text{g L}^{-1}$ and are not always the most dominant cyanopeptide group present (Janssen, 2019). MC-LR is the only MC congener that has a provisional guideline set by the World Health Organization (WHO) at $1.0\ \mu\text{g L}^{-1}$ in drinking water and a recreational water guideline of $20\ \mu\text{g L}^{-1}$ or 100,000 cells mL^{-1} set by the Government of Canada. The regulation of only MC-LR poses a problem as MCs are typically produced as mixtures of different MC variants, with some cyanobacteria species producing exclusively other MC congeners. Thus, the total MC levels within drinking and recreational waters may be greatly underestimated.

Figure 1.6: Chemical structure of the microcystin congener MC-LR.

There are few investigations of the toxicokinetics of MC congeners, with the main experimental focus on the major microcystins such as MC-LR. This congener is highly hydrophilic due to the arginine within position 4. Lipophilic congeners with hydrophobic residues within position 4 such as MC-LW and MC-LA, have significantly different toxicokinetics, such as uptake, tissue distribution, and excretion processes (Díez-Quijada et al., 2019). Absorption of MCs is achieved with organic anion transporting polypeptides (OATPs) and distribution to various organs is done rapidly within the blood stream. It has been shown that the higher lipophilicity of the MC congener results in a greater inhibition of PP2A than hydrophilic congeners due to a more efficient transport of the cyanopeptide into the cell (Feurstein et al., 2010). The hydrophobic congeners have also been shown to interact with bilayer membranes suggesting a secondary route into the cell. Experimental models have been investigated to assess the toxicity changes when substitution occurs in position 2 and 4. It was found in mice that the lethal dose was the same for MC-LR and MC-LA with a larger concentration required to achieve a lethal dose for MC-LY and MC-RR (Díez-Quijada et al., 2019). Alternatively, in the protozoan *Tetrahymena pyriformis* there were greater impacts on growth rate when treated with MC-LF and MC-LW in comparison to MC-LY

and MC-LR. This suggests that the experimental model also has a large impact on the toxicity of each MC variant.

1.4.2 Anabaenopeptins

Anabaenopeptins are a class of hexapeptides that share a ureido linkage for cyclization with a Lys residue. The remaining amino acids are highly variable and result in vast structural diversity of the congeners within this chemical class (>100 reported, (Spoof et al., 2016)). These metabolites are synthesized via the NRPS pathway and were first isolated from *Dolichospermum* (Janssen, 2019). There are many subclasses of anabaenopeptins named according to the different genera of cyanobacteria which produce them such as oscillamides isolated from *Planktothrix* (*Oscillatoria*), ferintoic acids from *Microcystis* (Figure 1.7), nodulapeptins from *Nodularia*, and lyngbyaureidamides isolated from *Lyngbya* sp. (Spoof et al., 2016). It should be noted that these metabolites are produced by both marine as well as fresh-water cyanobacteria, further complicating the vast chemical diversity of this class of metabolites.

Figure 1.7: Structure of the anabaenopeptin congener produced by *Microcystis* sp., Ferintoic acid A.

Anabaenopeptin congeners have shown enzyme inhibition against zinc-containing metalloexopeptidases, serine endopeptidases, as well as serine and threonine protein phosphatases (Spoof et al., 2016). The strength and specificity of the enzyme inhibition varies based on classification and amino acid sequence of the cyclic peptide. The concentration of some anabaenopeptins can reach concentrations similar to microcystins in the environment (Janssen, 2019).

1.4.3 Cyanopeptolins

Cyanopeptolins are cyclic depsipeptides containing a 3-amino-6-hydroxy-2-piperidone (Ahp, red, Figure 1.8) moiety at position 4. Position 2 is filled with an *NMe*-aromatic amino acid such as Phe and Tyr, with possible halogenation of the Tyr (blue). The remainder of the molecule is highly variable, with an *exo* position containing two or more substituents typically filled with two amino acids or a fatty acid, such as hexanoic acid. Collectively, cyanopeptolins are potent serine protease inhibitors including trypsin, chymotrypsin, plasmin, thrombin, and elastase (Huang & Zimba, 2019; Janssen, 2019). Structure-activity relationship revealed that the Ahp and residue 5 are important for protease inhibition specificity and potency (Linington et al., 2008). The cyanopeptolin class can be further broken down into subclasses based on the amino acids in position 2 and 3, beside the shared Ahp moiety.

Figure 1.8: Chemical structure of cyanopeptolin A (left) and cyanopeptolin 954 (right). HxA; hexanoic acid, Ac; acetyl.

Fifty-one % of reported cyanopeptolin congeners have the sequence Lxx-Ahp, 32% have the sequence Phe-Ahp, and 17% are substituted with other proteinaceous amino acids, generated the three subclasses of cyanopeptolins (McDonald et al., 2020). Cyanopeptolin A is an example a Lxx-Ahp cyanopeptolin and cyanopeptolin 954 is an example of a Phe-Ahp cyanopeptolin (Figure 1.8). Position 2 is filled with NMePhe in 31% of reported congeners and an NMeTyr in 36%. The remainder of reported congeners have NMeTrp or methylated and/or halogenated NMeTyr. Reported environmental concentrations of these metabolites are similar to anabaenopeptins and microcystins, with production closely related to cell abundance (Janssen, 2019).

1.4.4 Microginins

Microginins are a class of linear N-acyl lipopeptides with four to six amino acids with a 3-amino-2-hydroxy decanoic acid (Ahda, red, Figure 1.9) in the N-terminal position from a PKS pathway. Structural variants arise by modification or substitution of the N-terminal moiety to an octanoic acid derivative (3-amino-2-hydroxy octanoic acid, Ahoa, blue), N-methylation of the amino, halogenation of the acyl chain terminus, and the identity and number of other proteogenic amino

acid building blocks. Other variants within this group have been named oscillaginins and nostoginins, with the naming convention derived by the genus that produces it, *Oscillatoria* and *Nostoc*, respectively (Strangman & Wright, 2016).

Figure 1.9: Chemical structure of microginin 680 (left) and microginin KR 604 (right).

The Ahda moiety is more prevalent in the >90 reported microginin congeners, making up 69% with the Ahoa moiety only 6%. Chlorination of the terminal carbon occurs in the reported congener Microginin 680 and methylation of the amino functionality occurs in the reported congener Microginin KR604 (Figure 1.9). Position 3 contains an *NMe*-amino acid, with no specificity towards the chemistry of the substituted amino acid seen with the substitution from *NMeTyr* to *NMeLeu* (Figure 1.9). This chemical class has shown protease inhibition specifically against ACE and aminopeptidase at low $\mu\text{mol L}^{-1}$ concentrations (Keishi Ishida et al., 2000).

1.4.5 Cyanobactins

Cyanobactins are a large family of ribosomally synthesized cyanopeptides that undergo a range of post-translational modifications described below (Leikoski et al., 2013). Cyanobactin gene clusters are commonly found within the following cyanobacteria genera: *Oscillatoria*, *Arthrospira*, and *Microcystis*. The term cyanobactin encapsulates cyclic peptides within heterocyclized amino acids and further grouped into tenucyclamides, trichamides, lyngbyactins, microcyclamides,

aerucyclamide, aeruginosamides, and anacyclamides. Many cyanobacteria genera produce these complex toxins with over a hundred variants identified (Portmann, Blom, Kaiser, et al., 2008). The heterocycles and the quantity within the molecule dictate which subclass it falls within as well as the number and sequence of proteogenic amino acids. Cyanobactins often show cytotoxicity against different cancer cell lines but have also shown antiviral, antimalarial, and allelopathic activities (Leikoski et al., 2013). The five-membered heterocycles are formed from heterocyclization of Thr, Ser, and Cys into oxazoline and thiazolines. These residues can be further oxidized to oxazoles and thiazoles which occur in majority of the reported cyanobactins, increasing structural diversity of this chemical group (Ge et al., 2019).

Cyclic cyanobactins typically possess six residues, three proteinaceous amino acids and three heterocycles in alternating order. Variation arises with substitution of the proteinaceous amino acids as well as the heteroatoms within the heterocycle and the oxidation state of the heterocycle. For example, *Meoxazolines* are seen within aerucyclamides whereas the oxidized *Meoxazoles* are seen within microcyclamides (Figure 1.10). There are many enzymes attributed to the synthesis and post-translational modification to generate these molecules and results in large cyanobactin diversity.

Figure 1.10: Chemical structure of aerucyclamide A (left) and microcyclamide A (right).

Linear cyanobactins have similar heterocycles as cyclic cyanobactins although post-translational modifications make cyclization impossible (Leikoski et al., 2013). In the case of aeruginosamides B and C, the C-terminal thiazole (Thz) is capped with a methyl ester (thiazole carboxylate, Tzc, blue) preventing C- to N- cyclization (Figure 1.11). These congeners typically have a 4 to 5 amino acid sequence with the unique 1,1-dimethylallyl moiety (DmA, red) at the N-terminus. This particular variety of cyanobactins has prenylated N-terminus and O-methylated C-terminus.

Figure 1.11: Chemical structure of aeruginosamide B (left) and C (right).

1.4.6 Aeruginosins

Aeruginosins are small linear tetrapeptides that contain the unique non-proteinaceous amino acid 2-carboxy-6-hydroxyoctahydroindole (Choi, red, Figure 1.12) and a C-terminal Arg or cyclized Arg, namely Argal. The hydroxyl of the Choi functionality can be functionalized with sulfates, phosphates, and other moieties. Another characteristic features of aeruginosins is an N-terminal hydroxy-phenyllactic acid (Hpla), which may undergo halogenation such as bromination and chlorination (Welker et al., 2006).

Figure 1.12: Chemical structure of aeruginosin 98A.

Aeruginosins are known protease inhibitors like variants of the cyanopeptolin group (Elkobi-Peer & Carmeli, 2015). These metabolites typically appear in structurally related mixtures of compounds with substitutions of the proteinaceous amino acids in position 3 (Figure 1.12). Several cyanobacteria genera produce aeruginosin like compounds such as *Microcystis*, *Oscillatoria*, *Nodularia*, and *Planktothrix* (Yadav et al., 2011).

1.4.7 Microviridins

Microviridins are a complex cyanopeptide group with three peptide chains linked together by ω -ester and ω -amide linkages (Ziemert et al., 2010). These peptides are typically between 1400-2000 Da, with 14 amino acids, and are synthesized through the RiPP synthesis pathway implying that the amino acid building blocks are proteinaceous (Figure 1.13). Without characteristic non-proteinaceous amino acids, classifying this chemical class is solely based on the presence of uncommon proteogenic amino acids such as Trp as well as with a retention time range and identification of doubly and triply charged precursor ions.

Figure 1.13: Amino acid depiction for the chemical structure of microviridin N.

Most microviridin variants show enzyme inhibition activity against serine-type proteases (Ziemert et al., 2010). One variant, microviridin J, is unique due to the substitution of Arg between the Thr and Lys residues which has been suggested to be the key binding site for toxicity as this congener has potent trypsin inhibition (Rohrlack et al., 2003). Other microviridin congeners, such as microviridins B, C, G, H, I have a Leu in the position between the Thr and Lys and show very potent elastase inhibition whereas those with Met, Tyr, or Phe (microviridin D, E, F) in between the residues Thr and Lys show little to no elastase inhibition activity (Rohrlack et al., 2003).

The genetic machinery required for synthesis of these large cage-like proteins include many enzymes such as ligases, acetyl-transferases, and those for lactonization and cyclization (Ziemert et al., 2010). With a large number of amino acids and enzyme complexes involved in microviridin synthesis, there are many opportunities for modification and amino acid substitutions leading to high chemical variation within this class.

1.5 Metabolomics

Metabolomics is the comprehensive study of all metabolites produced by an organism, known as the metabolome (Tiam et al., 2019). Metabolomics employs complex analytical instrumentation

in conjunction with post-data acquisition techniques to monitor and discover metabolic changes in biological systems (Beger, 2013). It has become an important method in recent years to decipher the metabolites that unite and differentiate products of both primary and secondary metabolic pathways based on experimental treatments, cell types, and strains. There are targeted metabolomic methods that require standards for quantification and a predetermined list of known metabolites that are utilized to assess shifts in well-defined metabolic pathways and biological systems. Conversely, there are untargeted metabolomic methods which visualize and interpret an entire chemical profile by gathering information on all metabolites that meet pre-defined intensity parameters (Beger, 2013). With a large untargeted dataset, several metabolite profiling platforms can be used for the rapid identification of previously reported metabolites and potential new metabolites. Untargeted metabolomics is becoming a more common strategy in secondary metabolite investigations and is key for the identification of novel metabolites as potential drug candidates (Tiam et al., 2019). Expansion of chemical libraries with experimental chromatographic and spectral data will aid in rapid dereplication of metabolites within a complex sample and highlight potential new compounds.

1.5.1 High-resolution LC-MS data collection

The analysis of complex samples that contain hundreds of chemicals is possible by combining several chemistry techniques. With advances in chromatography and mass spectrometry (MS) instrumentation, comprehension of complex biological extracts can be achieved. Through the use of sophisticated mass analyzers such as orbitraps or quadrupole time-of-flight (QToF), untargeted data collection provides high-resolution mass data for all ionizable chemicals within a sample (Nagana Gowda & Djukovic, 2014). This first requires chromatographic separation of the sample, gas or liquid, followed by high-resolution mass spectrometry (HR-MS) and tandem mass

spectrometry (MS/MS). This provides chemical information for each ionizable metabolite such as a retention time (relates to metabolite polarity), precursor ion m/z , as well as a number of product ions generated by colliding the molecule with inert gas and detecting the fragments, which are direct clues to the structure of the metabolite. The method used in this investigation is liquid chromatography high-resolution mass spectrometry (LC-HRMS).

Ionization of the metabolites within a sample is one of the most important steps of MS measurements. The most often used methods are electrospray ionization (ESI) and electron impact (EI) ionization (Nagana Gowda & Djukovic, 2014). In LC-HRMS, ESI is the optimal ionization method and used here for multiple reasons. ESI is a soft ionization method and does not readily induce in-source fragments resulting in dominant molecular ions as well as it adequately ionizes both large and small biomolecules within the liquid phase. A drawback of ESI is the generation of salt adducts such as sodiated and ammoniated (Table 2.3) ions with the use of buffered solvent systems. However, in combination with other observed ions they can be confirmatory together.

The mass analyzer is capable of manipulating the ionized compounds and assigning mass to charge (m/z) ratios. Here, an Orbitrap single-configuration mass analyzer is used which provides high-resolution mass spectral (HR-MS) data which is ideal for global profiling and metabolite identification. A data-dependent acquisition (DDA) experiment collects both HRMS and MS/MS spectra for ionized compounds that meet pre-defined criteria. The collection of HRMS/MS data is vital in the dereplication of previously identified compounds and classification of structurally related compounds as compounds with shared structural features will generate common product ions upon fragmentation.

HR-MS and HRMS/MS data provides isotopic analysis and a molecular formula based on the mass defect principle. Every atom has a unique mass defect, meaning their mass is slightly lower than

the sum of the mass of their protons and neutrons. Thus a metabolite will have a characteristic mass defect corresponding to the number and type of atoms it contains (Dettmer et al., 2007). With the molecular formula for a chemical, identification by comparison to literature is simplified. However, isobaric compounds (i.e., compounds with the same molecular formula) cannot be differentiated. For example, the immonium ions generated for the amino acids leucine (m/z 86.0969) and isoleucine (m/z 86.0969), have the same m/z and molecular formula and cannot be differentiated. Structural isomers and stereochemistry cannot be elucidated with HR-MS data. The challenge of discriminating compounds based on molecular formula alone is eased with comprehensive databases and libraries of previously reported common chemicals for rapid dereplication of natural products or establishing a metabolite as potentially novel.

1.5.2 Untargeted mass-spectrometry based metabolomics

The large HRMS and HRMS/MS dataset acquired as described in Section 1.5.1 must be interpreted with sophisticated post-data acquisition strategies to determine similarities and differences within chemical profiles. Multivariate analyses performed with R statistical software and the packages *xcms*, *FactoMineR*, and *MetabolAnalyze* allow for visualization and comprehension of the statistically significant features (compounds) that contribute to diversifying metabolomes and which are common to the investigated metabolomes. Within this investigation, multivariate statistical analyses was performed to assess *Microcystis* chemotypes for five strains, specifically principal component analysis (PCA), factor loading plot analysis, and Kruskal Wallis (KW) test for two or more groups of non-parametric data.

PCA is a means of describing the most pertinent variability within a multivariate dataset. PCA works to condense variation within a dataset into fewer dimensions and is thus desirable when working with massive multivariable datasets (Pearson, 1901). This statistical test is unsupervised

and converts potentially correlated variables into sets of uncorrelated variables called principal components. Therefore, when samples are plotted closely along the principal components the variables that define them are more similar than samples plotted further away along the principal components. Further, to depict which variables distinguish and unite the samples, a factor loading plot may be generated. This type of plot depicts the contribution of each feature to the distribution of the samples within the PCA. Within LC-HRMS based metabolomics, these features are identified compounds detected by LC-HRMS. The KW test is performed on the features within the factor loading plot to provide statistical significance to the variables that define the distribution of the samples within the PCA. A Benjamini-Hochberg correction was utilized here to ensure false positives did not influence the analysis.

Metabolites with similar structural features such as amino acid sequences and/or non-proteinaceous amino acids will generate similar product ions when subjected to LC-HRMS/MS analysis. This peptidyl nature of cyanopeptides enables grouping of the secondary metabolites based on structural similarity and may be used for putative identification and characterization of new and known cyanopeptides. The related nature of these molecules based on their biosynthetic mechanism allows for several post-data acquisition untargeted metabolomic strategies to be applied to sort through the large data set and organize the chemicals that have been detected based on factors, such as structural similarity or which *Microcystis* strain produced them.

Another mode of processing the large untargeted LC-HRMS/MS data sets utilizes the recently developed global natural product social (GNPS) algorithm. This method compares all MS/MS spectra within the data file and generates a network with clusters of structurally related compounds that share product ion profiles (Tiam et al., 2019). Compounds that have similar structural features will generate common product ions within the HRMS/MS spectra and thus generate a cluster

within the molecular network. The nature of the cyanopeptides studied here are an ideal set of compounds for this analysis as the building blocks that make up these metabolites are often shared. Also, as alluded to within previous sections, cyanopeptides are grouped that share unique structural features such as non-proteinaceous amino acids or partial amino acid sequences. With identification of known cyanopeptides within the molecular network, it is possible to identify related metabolites with minor structural modifications such as methylation, halogenation, and/or amino acid substitutions.

1.5.3 Semi-targeted mass-spectrometry based metabolomics

From the information gathered by searching LC-HRMS/MS raw data and the untargeted LC-HRMS/MS data analysis, characteristic features for each chemical group such as retention time ranges, precursor ion m/z ranges, and diagnostic product ions can be used to perform semi-targeted searches of LC-HRMS/MS datasets for any and all metabolites with a particular structural feature relating to a chemical group. A technique known as diagnostic fragmentation filtering (DFF) can be employed to take advantage of the structural similarity. Within the mass spectrometry data analysis software, mzMine, a feature known as diagnostic fragmentation filtering allows for the search of diagnostic product ion pairs or combinations which may be tailored to a specific chemical class (McMullin et al., 2019). The output contains all metabolites within the large data set that contain the specified product ions within their MS/MS.

Cyanobacteria produce diverse and complex mixtures of secondary metabolites that require complex analytical methods for detection and comprehension of the complete chemical profile. There is limited information on the toxicity and chemistry of the many cyanopeptide congeners. Small modifications such as methylation, halogenation, and single amino acid substitutions can have great impacts on the biological role of a molecule and fate within an organism and the

environment. To gain understanding of the total chemical profile, all chemicals within a sample must be detected. Following this, implement a rapid method for the identification of new and dereplication of known chemicals.

1.6 Project aim

Canada contains 20% of the global freshwater resources and in the last one hundred years, its freshwater ecosystems (lakes, rivers, and wetlands) have undergone widespread environmental change. The stress on ecosystems is due to of human population growth, pollution, increased temperatures and habitat degradation. A visible consequence of this environmental change is an increase in frequency and magnitude of cyanobacterial blooms. Positive correlations between toxic cyanobacteria and adverse health effects have been demonstrated in several countries including Canada. Toxic cyanobacteria populations produce diverse and strain-specific mixtures of cyanotoxins with potential hepatotoxic, cytotoxic, and neurotoxic effects. The biological factors that promote cyanobacteria growth and toxin production are broadly understood; however, the chemistry, biological activity, and environmental concentration for the majority of cyanopeptides have not been investigated. The objective of this study was to apply mass-spectrometry based metabolomics to decipher the strain-specific cyanopeptide profiles of five *Microcystis* strains. Multivariate analyses, including PCA and factor loading, are used to assess the metabolites that statistically contribute to *Microcystis* chemotype differences. Cyanopeptide structural diversity is investigated through GNPS molecular networking via groups of structurally related cyanopeptides that are co-produced by *Microcystis* strains. The information gathered regarding the different cyanopeptide groups is applied to environmental surface water and bloom samples collected in Ontario and Quebec for the description of cyanopeptide profiles found within these different ecosystems. Finally, the preliminary cyanopeptide identification will be used to investigate effects

of media composition on *Microcystis* growth and metabolite production. This information will aid in the prioritization of target strains for large scale cultivation for the isolation and purification of novel cyanopeptides and common cyanopeptides contaminating the environment to undergo structural and toxicological characterizations.

2. Materials and Methods

2.1 General experimental procedures.

Cyanobacteria cultures were grown under white light ($30 \mu\text{M m}^{-2} \text{s}^{-1}$) in a Conviron E15 incubator with 40% humidity and a light/dark regime of 12 h. Cyanobacterial growth was monitored via cell number (cells mL^{-1}), counted with a Hausser Scientific Co. counting chamber (catalog #3200) under a VistaVision Microscope. Optical density measurements were recorded with a Molecular Devices SpectraMax 340 PC 384 microplate reader. Cyanobacteria cells were harvested on Whatman glass microfiber filters (GF/A diameter 47 mm, CAT No.1820-047) using a Millipore filtration apparatus. The extraction of intracellular cyanopeptides were performed in VWR disposable culture tubes ($16 \times 100 \text{ mm}$). Vortex and sonication were conducted using a Fisher Scientific Vortex Mixer and Aquasonic VWR Scientific Products Model 50T. Resulting intracellular cyanopeptide extracts were filtered through PTFE syringe filters (Prefilter 30 mm $0.22 \mu\text{m}$, model No. PTFEP030N022O). All solvents were purchased from VWR and chemicals for media were purchased from what was purchased from Eurofins Abraxis and performed according to the manufacturer's instructions. Optical density and absorbance for the PP2A inhibition assay were read with a Molecular Devices SpectraMax spectrophotometer in 96-well tissue culture microplates. Microcystin standards (MC-LR, $[\text{Asp}^3]\text{-MC-LR}$, MC-RR, Nodularin)

were received from the National Research Council in Nova Scotia, Canada and used for identification and quantification of microcystin congeners.

2.2 Cyanobacteria strain cultivation.

Four *Microcystis aeruginosa* strains CPCC 299, CPCC 300, CPCC 632, CPCC 633 and one *Microcystis flos-aquae* strain CPCC 461 were obtained from the Canadian Physiological Culture Centre (CPCC) and selected for metabolomics as pre-screening revealed diverse chemical profiles. Collection data and specific growth rates in three defined cyanobacteria culture media are displayed in Table 2.1 for each studied strain. The three defined culture media (BG-11, MA, BBM) were selected because they are commonly used to grow *Microcystis* species. 25× stock solutions for these media were made according to their recipes (Table 2.2; Watanabe et al., 1996; Anderson 2005). Aliquots of the stock solution were diluted in ddH₂O and autoclaved prior to inoculation with *Microcystis* cells.

Table 2.1: Cyanobacteria strain information from the Canadian Phycological Culture Collection.

Strain	Collection Data	
	Date	Location
<i>M. aeruginosa</i> CPCC 299	08/90	Pretzlaff Pond AB, Canada
<i>M. aeruginosa</i> CPCC 300	08/90	Pretzlaff Pond AB, Canada
<i>M. flos-aquae</i> CPCC 461	08/75	Fox River WI, USA
<i>M. aeruginosa</i> CPCC 632	10/48	Lake Mendota WI, USA
<i>M. aeruginosa</i> CPCC 633	10/54	Little Rideau Lake, ON Canada

Each medium contains a different iron source but all have NaNO₃ as the major nitrogen source. The differences between BBM and BG-11 media are mainly concentration based as majority of the chemicals used are the same with BG-11 containing double the concentration of NaNO₃ (1.5 g L⁻¹) compared to BBM (0.75 g L⁻¹). The main phosphorous source is K₂HPO₄ for BBM (0.175 g L⁻¹) and BG-11 (0.03 g L⁻¹) and BBM has an additional phosphorous source, KH₂PO₄ (0.75 g L⁻¹).

MA is much different than BBM and BG-11 with only a few overlapping chemicals, such as NaNO_3 , Na_2EDTA , and H_3BO_3 , but has an additional nitrogen source being KNO_3 and the major phosphorous source being $\beta\text{-Na}_2\text{-glycerophosphate}$. These fluctuations in the vital nutrients for cyanobacterial growth are believed to impact the growth rate and subsequent toxin production.

All strains were inoculated into sterile media (Table 2.2) at high 10^4 – low 10^5 cells mL^{-1} and grown in 250 mL Erlenmeyer flasks (Pyrex) that contained 50 mL of defined culture media (Table 2.2). Cultures were grown in triplicate and maintained in a growth chamber (Convion E15) under cool white fluorescent light ($30 \mu\text{E m}^{-2} \text{s}^{-1}$) using a light/dark regime of 12 h (Figure 2.1). Cyanobacteria were grown at several different temperatures to assess optimal growth rates, however 25°C was routinely used to grow cyanobacteria to study their cyanopeptide profiles. Flasks were swirled every two days and late-exponential cells were harvested after 15 days and extracted according to the procedure described in Section 2.4.

Figure 2.1: *Microcystis* strains growing in 250 mL Erlenmeyer flasks within the growth chamber.

Each *Microcystis* strain studied was also grown in BG-11 medium supplemented with Na¹⁵NO₃ (Sigma Aldridge) instead of NaNO₃, which represents the major source of nitrogen in the medium (Andersen 2005). These cultures were grown for 20 days and subsequently transferred to fresh BG-11 media containing Na¹⁵NO₃. The process was repeated four additional times and facilitated the biosynthesis of ¹⁵N labelled cyanopeptides by each strain. Strains were also grown as described above in BBM and MA media (Table 2.2), other common media used to *Microcystis* species (Anderson 2005).

Table 2.2: Media components for the defined culture media used to grow *Microcystis* species.

Component	Final concentration (g L ⁻¹)		
	BBM	BG-11	MA
MgSO ₄ • 7H ₂ O	0.075	0.075	-
NaNO ₃	0.75	1.5	0.05
K ₂ HPO ₄	0.175	0.03	-
Na ₂ EDTA	0.001	0.001	0.005
CaCl • 2H ₂ O	0.025	0.036	-
KH ₂ PO ₄	0.75	-	-
NaCl	0.025	-	-
FeSO ₄ • 7H ₂ O	0.00498	-	-
H ₃ BO ₃	0.00011	0.00286	0.02
MnCl ₂ • 4H ₂ O	0.00181	0.00181	0.005
CuSO ₄ • 5H ₂ O	0.000079	0.000079	-
ZnSO ₄ • 7H ₂ O	0.000222	0.000222	-
Co(NO ₃) ₂ • 6H ₂ O	0.0000494	0.0000494	-
Na ₂ MoO ₄ • 2H ₂ O	0.00039	0.00039	0.0008
Biotin	0.000001	0.000001	-
Vitamin B12	0.000001	0.000001	-
Thiamine HCl	0.0002	0.0002	-
Ferric ammonium citrate	-	0.006	-
MnSO ₄ • 7H ₂ O	-	0.075	-
Na ₂ CO ₃	-	0.02	-
FeCl ₃ • 6H ₂ O	-	-	0.0005
Na ₂ SO ₃	-	-	0.04
CoCl ₂ • 6H ₂ O	-	-	0.005
KNO ₃	-	-	0.1
β-Na ₂ -glycerophosphate	-	-	0.1
Bicine	-	-	0.5
ZnCl ₂	-	-	0.0005

2.3 Monitoring the growth of *Microcystis* strains.

To understand how the strain specific physiology and metabolism, as well as nutrient source and concentration impact *Microcystis* growth and toxin production, the strains were grown in the three defined culture media described in section 2.2. Biological growth indicators such as pH, cell number, optical density, and dried biomass were recorded on day 5, 10, 15, 20, and 25 in triplicate. Toxin production was monitored with the same schedule via intracellular extraction and LC-MS/MS analysis (Section 2.4 and 2.6). pH was read with a Fisher brand pH meter calibrated with pH 4, 7, and 10 buffer solutions. Cells mL⁻¹ were counted using 10 µL of culture on a counting chamber and visualized under a microscope. If the cultures were overgrown, the cells were diluted 10× with distilled deionized water in a glass vial. 150 µL of undiluted culture was pipetted into a 96 well plate for an optical density reading at 750 nm for chlorophyll absorbance. Finally, 25 mL of culture was filtered onto dried and pre-weighed glass microfiber filter paper and left overnight at 40°C. The dried filter paper was weighed the following day to obtain dried biomass. Specific growth rates (μ) were calculated using the cell mL⁻² value at day 0 (N_0) and the cell mL⁻¹ value when the cells reached the lag phase. In all experiments, this was the 25 day reading (N_{25}). The following formula was used to provide a comparable specific growth rate with units, days⁻¹ (Anderson, 2005).

$$\mu = \ln(N_t/N_0)/\Delta t$$

2.4 Extraction of cyanopeptides from *Microcystis* cells.

Strain specific differences and media composition effects on *Microcystis* toxin profiles were investigated by intracellular extraction of cyanopeptides from dried biomass and subsequent LC-HRMS/MS (Section 2.6). *Microcystis* cells from 25 mL of culture were harvested on pre-weighed

and dried (overnight at 40 °C) glass microfibre filters (Whatman, GF/A, diameter 47 mm, ~ 1.6 µm) using a filtration apparatus (Millipore). After drying in an oven at 40 °C overnight, the glass microfibre filters with *Microcystis* cells were transferred to disposable culture tubes (16 x 100mm, VWR) and 14 mL of 80% aqueous methanol was added. For cell lysis, tubes were vortexed and sonicated for 30 seconds and stored at -20 °C for 1 hour. Tubes were subsequently thawed to room temperature and the freeze-thaw cycle including agitation and sonication was repeated two additional times. Resulting methanolic extracts containing intracellular cyanopeptides were filtered through 0.22 µm PTFE syringe filters (ChromSpec, Inc.) into amber vials and dried under a gentle stream of nitrogen gas. Extracts of *Microcystis* cells were reconstituted in HPLC grade methanol, transferred to 2 mL amber HPLC vials (Agilent), dried under a gentle stream of nitrogen gas, and stored at -20 °C prior to LC-MS/MS analysis (Section 2.6).

2.5 Cyanopeptide extraction from environmental samples.

Investigation of the cyanopeptides within the environment for analysis of intra- and inter-lake variation was achieved with untargeted LC-HMS/MS and established metabolomic approaches (Section 2.6 and 2.7). Collection data for environmental samples studied are summarized in Table 2.3. To extract cyanopeptides from these samples, 15 mL of surface water was lyophilized and the resulting material was suspended in 3 mL of 80% aqueous methanol. The solution was sonicated and vortexed for 5 minutes each. Surface water samples concentrated on glass microfiber filter papers were processed with the freeze-thaw extraction method used for the cultivated strains. The extracts were filtered through a PTFE syringe filter into an amber vial and dried under a gentle stream of nitrogen gas. Bloom samples were filtered onto glass microfiber filter papers upon collection and processed according to Section 2.4.

Table 2.3: Collection information for bloom and water samples collected in Ontario and Quebec.

Sample Number	Type	Collection Date	Collection Site
(1)	Water	08/19	Wildwood Conservation
(2)	Water	08/19	Scugog Lake
(3)	Bloom	09/19	Lac Breton
(4)	Bloom	10/13	Lake Land
(5)	Bloom	06/13	Lake Land
(6)	Bloom	06/09	Lake Heney
(7)	Bloom	07/13	Lake Land
(8)	Bloom	06/10	Lake Land
(9)	Bloom	06/18	Lake Land

2.6 Untargeted LC-HRMS/MS analysis.

Visualization of the entire cyanopeptide profiles were performed with untargeted LC-HRMS/MS analysis and post-data acquisition metabolomic approaches. LC-HRMS/MS analyses of resulting *Microcystis* extracts and environmental samples was achieved using an Agilent 1290 HPLC (Agilent Technologies) coupled to a Q-Exactive Quadrupole Orbitrap (Thermo Fisher Scientific) mass spectrometer. Chromatography conditions included an Eclipse Plus C18 RRHD (2.1 x 100mm, 1.8 μ m; Agilent Technologies) maintained at 35 °C and mobile phase consisting of 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The 10.5 minute gradient program optimized for cyanobacteria oligopeptide analysis increased from 0 to 35% B over 1.5 min, increased again to 45% B over the following 2.5 min, and finally increased to 100% B over 2 min, where it was held at 100% B for 3 min prior to returning to 0% B over 0.5 min, and maintained at 0% B for 1 min. Five μ L of each intracellular and surface water extract constituted in 90% aqueous methanol was injected and resolved at a flow rate of 0.3 mL/min.

Heated electrospray ionization in positive mode was used with the following settings: capillary 155 voltage, 3.9 kV; capillary temperature, 400°C; sheath gas, 19 units; auxiliary gas, 8 units;

probe 156 heater temperature, 450°C; S-Lens RF level, 45.00. MS data were acquired using untargeted DDA 157 that included a full MS scan at 70 000 resolution, with a scan range of 106.7–1600 m/z ; automatic 158 gain control target, 3×10^6 ; and a maximum injection time of 250 ms. The five highest intensity ions were selected from each full scan for MS/MS analysis using a 1.0 Da isolation window, and were analyzed using the following conditions: resolution, 17 500; automatic gain control target, 1×10^6 ; max IT, 64 ms; normalized collision energy, 37.5; intensity threshold, 1.5×10^5 ; dynamic exclusion, 5 s.

2.7 Metabolite identification.

The metabolomic processing of the large untargeted LC-HRMS/MS datasets acquired utilizes multivariate analysis (PCA, factor loading analysis) in R-software packages (*xcms*, *MetabolAnalyze*, *FactoMineR*), the diagnostic fragmentation filtering (DFF) module within the open-source mass spectrometry platform MZmine2 (<http://mzmine.github.io/>), and the Global Natural Product Social (GNPS) Molecular Networking algorithm (<https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp>, Section 2.9, Figure 2.2).

Figure 2.2: Workflow of the unique LC-MS/MS metabolomic data processing performed.

Untargeted and multivariate techniques such as PCA, factor loading, and GNPS molecular networking enable visualization of metabolome differences between strains, experimental conditions, and environmental samples through comparison and consideration of all compounds present within the sample. The multivariate analysis is an effective data mining strategy for natural product diversity, detection of novel metabolites, and to identify factors that regulate natural product biosynthesis. PCA and factor loading consider only precursor ion m/z data with an additional statistical test within factor loading aiding in understanding of which compounds statistically contributing to sample variation whereas GNPS molecular networking utilizes similar product ion spectra to group compounds that are structurally related. MS/MS spectra contain

characteristic product ions that are unique to the structural features for a metabolite. Structurally related compounds will generate similar product ions within the MS/MS spectra. DFF is a semi-targeted approach which targets specific molecular features via diagnostic product ions to search an untargeted LC-MS/MS dataset for chemicals containing a specific molecular feature. Cyanopeptides are grouped based on the presence of unique non-proteinaceous amino acids or partial amino acid sequences which generate diagnostic product ions that can be used for cyanopeptide group identification. Some identified molecular features of cyanopeptide classes have been identified and the product ions that are generated are summarized in Table 2.4. These structural features were targeted using DFF in this study of cyanopeptide diversity.

Table 2.4: Unique structural features characteristic of different cyanotoxin groups.

Cyanopeptide group	Structural feature(s)	Product ions <i>m/z</i>
Microcystin	Adda	135.0803, 163.1113
Cyanopeptolin	Lxx-Ahp, Phe-Ahp	181.1331, 215.1167
Aeruginosin	Choi	140.1099, 122.0966
Microginin	Di-, mono-, non-Cl- Ahoa	168.0338, 134.0727, 100.1122
	Di-, mono-, non-Cl- NMeAhoa	182.0494, 148.0883, 114.1278

Cyanotoxins are identified here by predicted molecular formula from high-resolution MS data, *de novo* sequencing of MS/MS spectra, and spectral library and metabolite database (in-house or published) searches. This unique metabolomic workflow allows rapid dereplication of previously reported metabolites and identification of potentially novel compounds.

2.8 Untargeted LC-HRMS/MS data processing.

Prior to performing multivariate analyses on the large LC-HRMS/MS data sets, elimination of mass spectral artifacts and deconvolution of peaks is required for confident reporting of compounds identified as unique to each sample. Raw MS data files were converted to centroid .mzML files with MSConvert v3.1.19 (<http://proteowizard.sourceforge.net/>) with peak picking set

at an MS level of 1-2. The mzML files were subsequently processed in this file format in R 3.5. (<https://www.r-project.org/>). Peak areas for metabolites within the data files were extracted using the *xcms* package with the parameters in Table 2.5.

Table 2.5: The peak picking parameters used with the bioinformatics package, *xcms*.

Parameter	Polarity	Scan pre-filter*	<i>m/z</i> deviation (ppm)	s/n ratio cutoff	Peak width range (secs)	Noise level
Set point	+	5 with minimum intensity of 5000	1	5	12-15	500000

*Number of scans a metabolite must be present in with a minimum intensity of 5000.

Deconvolution of the extracted peak list was performed with removal of features outside the oligopeptide retention time range of 2-6.5 minutes, below 400 *m/z*, and if their peak area multiplied by 50 was less than the peak area of the same feature in the blank. Electrospray ionization (ESI) gives rise to adducts, which are charged molecules that can be manipulated by the mass spectrometer and generate multiple precursor ions for the one metabolite. Common adducts generated in positive ESI mode are protonated, dehydrated, sodiated, and aminated ions that correspond to the same molecule (Table 2.6). Multiple adducts for the same metabolite can occur and were removed from the peak list by identification of the characteristic mass differences and alignment of MS/MS patterns. While multiple ESI adducts for the same ionized molecular complicate the analysis of metabolomic data, they can be confirmatory when present in the same MS spectrum. Peak information was gathered into a cyanotoxin database for the metabolites detected from the five intracellular *Microcystis* extracts including retention time, precursor ion *m/z*, adduct *m/z*, *m/z* of product ions, and which strain produced it. The in-house database identified previously characterized metabolites by comparison of high-resolution precursor ion *m/z* and acknowledged cyanotoxins not previously reported (Supplemental database).

Table 2.6: Common adducts generated by electrospray ionization in positive mode.

Adduct Identity	Mass difference (Da)
$[M+H]^+$	+1.0018
$[M+H-H_2O]^+$	-18.0102
$[M+H-SO_3]^+$	-79.9571
$[M+Na]^+$	+21.9879
$[M+NH_4]^+$	+17.0253

Putative structural information was generated by comparison of MS and MS/MS data and proposed fragmentation patterns of the reported structure. Chemicals that did not match literature cyanopeptide reports were designated into a chemical class based on retention time range, precursor ion m/z range, presence of adducts, and diagnostic product ions. Variants within a chemical class were described by comparing the MS/MS to a known metabolite within the same class. Small chemical modifications and amino acid substitutions were characterized with this method as well as comparison of the precursor ion m/z and molecular formula. This MS/MS based approach cannot discriminate residue configurations, nor isobaric residues. For example, Lxx is used to annotate either leucine (Leu) or isoleucine (Ile) except when reporting a known compound.

2.9 Post-Data Acquisition Processing and Visualization.

2.9.1 Principal component analysis and factor loading analysis.

The following multivariate analyses allow for interpretative of the large MS dataset generated and provides significance as to which features (compounds) statistically contribute to the variance between samples. The $\log_{10}$ of the processed peak areas (Section 2.8) were utilized in R for principal component analysis and generation of a loading plot using the packages *MetabolAnalyze* and *FactoMineR*. All dimensions of the PCA were analyzed, with dimensions 1 and 4 showing the best separation and thus variation explained between the 5 studied strains. The triplicate data was

then grouped and a Kruskal-Wallis test with Benjamini-Hochberg correction was performed to investigate the statistical significance of the features and their contribution to the PCA. Features with a p -value < 0.05 were grouped and labelled red within the loading plot. To demonstrate which metabolites contributed to the variation, the loading and PCA data was exported to excel and previously identified metabolites were identified by m/z and retention time and identified by numbers within the loading plot.

2.9.2 Heat map analysis to depict media effects on cyanopeptide production.

Metabolites previously identified from the molecular network were selected for heat map analysis for determination of media effects on cyanopeptide production and production as a function of time. Peak areas were extracted from data files corresponding to day 5, 10, 15, 20, and 25 intracellular extracts of *M. aeruginosa* CPCC 300, CPCC 632 and CPCC 633, and *M. flos-aquae* CPCC 461 when grown in the two defined culture media BG-11 and MA. Peak areas for the selected cyanopeptides were normalized with dried biomass and excluded if it did not exceed $5E^{+04}$ at any time point. Heat maps were generated with $\log_{10}$ normalized peak area per strain, per media, in R using the following packages: *gplots* for heatmap generation and *ColorRamps* for visualization.

2.9.3 GNPS Molecular networking analysis.

The mzML files corresponding to both the intracellular extracts of the five studied *Microcystis* strains and a methanol blank were uploaded to the GNPS network (<https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp>) with FileZilla (<https://filezilla-project.org/>) through the ccms host. A molecular network that groups metabolites by structurally

similarity based on high-resolution MS/MS data was generated with a precursor ion m/z tolerance of 0.02 and a product ion m/z tolerance of 0.03. A minimum of five matching fragment ions and cluster size equal to or greater than two was required for grouping. The graphML file format from the GNPS website was uploaded into cytoscape for visualization (<https://cytoscape.org/>). The blank data file was used for data deconvolution with all metabolites present in the blank deleted from the network. Further deconvolution included the elimination of metabolites outside the retention time range 2-6.5 minutes, below 400 Da, and any remaining single nodes. Nodes were coloured based on which data file they were present in as well as outlined corresponding to their charge (singly, doubly, or triply).

2.9.4 Diagnostic fragmentation filtering (DFF) for cyanopeptide group detection.

The mzML files were uploaded into MZmine v.2.38 (<http://mzmine.github.io/>) and a diagnostic fragmentation filtration plot was generated for the detection of all compounds within the intracellular extract containing diagnostic molecular features characteristic of the studied chemical groups (Table 2.4). The diagnostic product ions m/z 134.0962 and 181.1325 were used for cyanopeptolins containing the sequence NMePhe-Leu-Ahp with a minimum base peak intensity of 10%. For detection of cyanopeptolins containing the sequence NMeTyr-Phe-Ahp, the product ions m/z 150.0917 and 215.1175 were used with a minimum diagnostic ion intensity of 10%. For detection of all cyanopeptolins within environmental samples, the four possible sequences were utilized (NMePhe-Leu-Ahp, NMe-Phe-Phe-Ahp, NMe-Tyr-Leu-Ahp, and NMe-Tyr-Phe-Ahp). All DFF plots generated had an m/z tolerance of 0.02 m/z or 5.0 ppm. Similar analyses were performed for the detection of variants within the microcystin, aeruginosin, and microginin chemical groups.

3. Results

3.1 Untargeted mass-spectrometry based metabolomics: application to cyanopeptide groups

3.1.1 Cyanopeptide identification

Microcystis species are known to biosynthesize structurally diverse mixtures of peptide natural products (cyanopeptides; (Tiam et al., 2019). Untargeted MS metabolomics allows comparison and differentiation of the complete chemical profiles for the five investigated *Microcystis* strains. The strains were grown in BG-11 media at 25°C for 15 days and the intracellular metabolites were extracted with 80% aqueous methanol. The intracellular cyanopeptides that are the focus of this thesis are peptide natural products and are grouped based on the presence of characteristic structural features, non-proteinaceous amino acids, or partial amino acid sequences. These unique structural features generate similar product ions within the MS/MS of structurally related metabolites. We compiled characteristic MS and MS/MS data for each cyanopeptide group including precursor ion ranges, retention time ranges, and product ion spectra patterns to distinguish metabolites in the different cyanopeptide groups (microcystins, cyanopeptolins, anabaenopeptins, microginins, aeruginosins, cyanobactins, and microviridins) within the untargeted MS data processing techniques such as molecular networking, principal component analysis, and factor loading.

3.1.2 Principal component analysis of LC-HRMS data

Untargeted metabolomic strategies such as principal component analysis (PCA), factor loadings plot, and GNPS molecular networking (<https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp>) were utilized to describe the strain specific cyanopeptide diversity from the five *Microcystis* strains studied. With large high-resolution MS data sets, metabolomics approaches are useful for

interpretation, statistical analysis, and visualization of the metabolome. PCA works to describe the variation between complex samples using a collection of variables that defines each sample. To understand the chemical variation of the five *Microcystis* strains, 262 metabolites detected with the *xcms* package are the variables used to assess similarities and differences in the chemical profiles (peak picking parameters described in materials and methods). The $\log_{10}$ peak area for the selected 262 features were analyzed with the packages *MetabolAnalyze* and *FactoMineR* in the R programming environment to generate a PCA (Figure 3.1) and a factor loading plot (Figure 3.2). The factor loading plot pairs with the PCA and provides insight into which chemical variables contribute to the variance and resemblance of the metabolomes for the investigated *Microcystis* cultures.

Figure 3.1: Principal component analysis (PCA) of triplicate *Microcystis aeruginosa* CPCC 299, CPCC 300, CPCC 632, and CPCC 633, and *M. flos-aquae* CPCC 461 cultures grown in BG-11 medium.

In the present study, untargeted MS metabolomic approaches were used to describe the intracellular cyanopeptide diversity from four *Microcystis aeruginosa* (CPCC 299, CPCC 300, CPCC 632, CPCC 633) and one *M. flos-aquae* strains (CPCC 461, Table S1). A total of 262 features were detected using the *xcms* bioinformatics package (search parameters are described in the materials and methods). A PCA plot was constructed with these features to visualize the metabolomic variation between the strains based on cyanopeptide identities and their relative abundances (Figure 3.1). The PCA plot displayed four groups revealing distinct differences between the studied *Microcystis* strains cyanopeptide profiles. The majority of variation is

described with dimension 1 (39.6%) which groups *M. aeruginosa* CPCC 632 (blue) and CPCC 633 (grey) on the positive side of the y-axis and grouping of *M. aeruginosa* CPCC 299 (red), CPCC 300 (yellow), and *M. flos.aquae* CPCC 461 (green) on the negative side of the y-axis. Further description of the variation occurs with the addition of dimension 2 (11.9%), which provides minor separation of the triplicate data points for *M. aeruginosa* CPCC 632 and CPCC 633 but large separation of *M. flos-aquae* CPCC 461 from its grouping in dimension 1 with *M. aeruginosa* CPCC 299 and CPCC 300. *M. aeruginosa* CPCC 299 and CPCC 300 group within the top left quadrant of the PCA plot suggesting similarity in their intracellular metabolomes. These two strains are known microcystin producers (i.e., toxic strains), whereas the other investigated *Microcystis* strains are considered non-toxic (Racine et al., 2019). Differences in the chemical profiles for the two toxic strains are implied because of the slight separation of the set of triplicate data points. The metabolomic profile of *M. flos-aquae* CPCC 461 was vastly different compared to the *M. aeruginosa* strains, appearing alone in the bottom left quadrant of the PCA plot. Grouping of this strain within dimension 1 with the two toxic strains *M. aeruginosa* CPCC 299 and CPCC 300 suggests some metabolome similarity between these strains. *M. aeruginosa* CPCC 632 and CPCC 633 overlap in the PCA plot when considering both dimensions, indicating strong similarity in their cyanopeptide profiles. The specific metabolites and metabolite groups that differentiate the *Microcystis* strains were elucidated through investigation of a factor loadings plot, which visualizes the variables that dictate the spatial separation of the individuals within the PCA.

Further investigation into the features that significantly differentiate the strains was performed with a factor loading plot containing 262 variables. Using the *dplyr* package in R, a Kruskal-Wallis test for non-parametric data with Benjamini-Hochberg correction was performed to determine which metabolites were statistically contributing to the chemical variation of these strains ($p <$

0.05). 140 metabolites were deemed statistically significant when comparing the chemical profiles of the five *Microcystis* strains and are highlighted with red arrows (Figure 3.2). The coordinate location where the arrow lands within this plot is specific and determined by which strain produces it. Cyanopeptides identified based on HR-MS, retention time, fragmentation patterns and database searching have been annotated within the feature loadings plot to highlight the diversity of cyanopeptides and cyanopeptide groups produced by each *Microcystis* strain.

Figure 3.2: The loading plot contains all factor loadings considered in the PCA. Of 787 total variables, 262 are considered in the PCA and visualized in the loading plot. Factors included fall within the precursor ion range m/z 400-1200 (unless present within the molecular network) to target oligopeptide cyanopeptides, 2.0-6.5 min retention time range, and above a peak threshold of $5E^{+06}$. Red arrows indicate statistical significance in contribution to the individual strains in the PCA ($p < 0.05$ Kruskal-Wallis test with Benjamin-Hochberg correction). Specific cyanopeptides identified by their precursor ion m/z , MS/MS data and retention time are numbered.

When analyzing the statistically significant features (red) within the loading plot (Figure 3.2), the identity of MC-LR (m/z 995.5565 $[M+H]^+$; 7 and [Asp³]-MC-LR(m/z 981.5391 $[M+H]^+$; 8) were confirmed by comparison to purified reference materials (NRC Halifax) and agree with the

literature, which states *M. aeruginosa* CPCC 299 and CPCC 300 produce these congeners (Racine et al., 2019). These MC features statistically contribute to the similarity of these strains chemical profiles as well as in differentiating them from the other investigated strains.

Other features within the loading that are significant to the *M. aeruginosa* CPCC 299 and CPCC 300 chemotypes, in a similar manner as the MCs, are aerucyclamide 518, aerucyclamide C, and aerucyclamide D (m/z 519.2382 $[M+H]^+$; 9, m/z 517.2232 $[M+H]^+$; 10, m/z 587.1567 $[M+H]^+$; 6, (Portmann, Blom, Kaiser, et al., 2008)). Features distinguishing *M. aeruginosa* CPCC 300 are four cyanopeptolin congeners, cyanopeptolin A, cyanopeptolin B, cyanopeptolin 963A, and cyanopeptolin 945 (m/z 957.5393 $[M+H]^+$; 1, 929.5072 $[M+H]^+$; 3, 964.5032 $[M+H]^+$; 4, 946.4899 $[M+H]^+$; 2). The first three listed congeners have been previously reported from *M. aeruginosa* and have the partial amino acid sequence *NMe*-Phe-Lxx-Ahp (Bister et al., 2004; Cornel et al., 1993). Distinguishing metabolites for *M. aeruginosa* CPCC 299 are cyanopeptolin 977 and cyanopeptolin 938 (m/z 978.4592 $[M+H]^+$; 11, m/z 939.4628 $[M+H]^+$; 12). These two cyanopeptolins have not been previously reported in the literature. Investigation of their MS/MS patterns revealed they have the partial amino acid sequence *NMe*-Phe-Phe-Ahp by comparison to the MS/MS of the known metabolite Micropeptin 996 with this partial amino acid sequence (Strangman et al., 2018). Other cyanopeptolin features with the partial amino acid sequence *NMe*-Phe-Phe-Ahp identified from the factor loading analysis specific for the *M. aeruginosa* CPCC 299 and CPCC 632 chemotypes cyanopeptolin 954B (m/z 955.4263 $[M+H]^+$; 16), and cyanopeptolin 982 (m/z 983.4639 $[M+H]^+$; 18). Also shared by these two strains are microviridin congeners, microviridin J (m/z 842.8925 $[M+2H]^{2+}$; 19), microviridin 1822 (m/z 912.4276 $[M+2H]^{2+}$; 14), microviridin 1715 (m/z 858.9321 $[M+2H]^{2+}$; 15), and microviridin 1856 (m/z 922.3999 $[M+2H]^{2+}$; 17, (Rohrlack et al., 2003)). The production of similar congeners within both the cyanopeptolin and microviridin

chemical group show overlapping biosynthetic potential by *M. aeruginosa* CPCC 299 and CPCC 632.

The PCA plot (Figure 3.1) shows that *M. aeruginosa* CPCC 632 and CPCC 633 have similar cyanopeptide profiles. Both strains produce the anabaenopeptins, ferintoic acid A and ferintoic acid B (m/z 867.4378 $[M+H]^+$; 23, m/z 881.4552 $[M+H]^+$; 24, (Williams et al., 1996)). The distinct features that distinguish the chemotypes for *M. aeruginosa* CPCC 632 and CPCC 633 are congeners within the groups; cyanopeptolins, microginins, and microviridins, which are known to be produced by *Microcystis* species (Welker et al., 2006). Factor loading analysis showed that *M. aeruginosa* CPCC 632 exclusively produces the previously reported microginin 680 and microginin 646 (m/z 681.2441 $[M+H]^+$; 21, m/z 647.2826 $[M+H]^+$; 22, (Strangman & Wright, 2016)), whereas *M. aeruginosa* CPCC 633 exclusively produces microginin 612 (m/z 613.3225 $[M+H]^+$; 30, (Strangman & Wright, 2016)). These two strains overlap in the production of the not previously documented microginins 660 and 694 (m/z 661.2985 $[M+H]^+$; 25, and m/z 695.2599 $[M+H]^+$; 26). Variation arises in the exclusive production of cyanopeptolin congeners with different partial amino acid sequences. *M. aeruginosa* CPCC 633 produces cyanopeptolin 940 and cyanopeptolin 992 with the partial amino acid sequence NMe-Cl-Tyr-Phe-Ahp and NMeTyr-Phe-Ahp, respectively (m/z 923.4039 $[M+H - H_2O]^+$; 28, m/z 979.4930 $[M+H - H_2O]^+$; 29) and *M. aeruginosa* CPCC 632 produces cyanopeptolin 954B and cyanopeptolin 982 with the partial amino acid sequence (m/z 955.4263 $[M+H]^+$; 16, m/z 983.4639 $[M+H]^+$; 18). Like *M. aeruginosa* CPCC 299 and CPCC 632, CPCC 633 also has the biosynthetic machinery to synthesize microviridin congeners with exclusive production of the congener, microviridin 1630 (m/z 816.4052 $[M+2H]^{2+}$; 27).

M. flos-aquae CPCC 461 produced few metabolites under the growth conditions in comparison to the *M. aeruginosa* strains. There are few NRPS natural products known from *M. flos-aquae* (Urrutia-Cordero et al., 2013). The major metabolite that is unique to this strain and is highlighted within the loading plot is microcyclamide A (m/z 583.1902 $[M+H]^+$; 32, (Portmann, Blom, Kaiser, et al., 2008)) and to a lesser extent, a related microcyclamide metabolite (m/z 585.2058 $[M+H]^+$; 34). Aerucyclamide A is also produced by *M. aeruginosa* CPCC 299 and CPCC 300, as well as *M. flos-aquae* CPCC 461 (m/z 517.2235 $[M+H]^+$; 33, (Portmann, Blom, Gademann, et al., 2008)) supporting the grouping of these strains within dimension 1 of the PCA analysis.

Chemotype information for each *Microcystis* strain has been elucidated via the multivariate analysis of intracellular cyanopeptide extracts. Unique co-production of the different cyanopeptide groups by the *Microcystis* strains proves that each isolate has a strain-specific chemotype. Loading plot analysis identified features (compounds) that statistically contributed to the variance in chemical profiles of the five *Microcystis* strains. Annotation of previously reported cyanopeptides was possible by HR-MS data and product ion spectra, but more in-depth analysis is required to further decipher the complex and individualistic cyanopeptide profiles of the *Microcystis* strains.

3.1.3 Molecular network analysis of LC-HRMS data

GNPS molecular networking is a technique that organizes metabolites based on their MS/MS fragmentation patterns (Tiam et al., 2019). The basis of this tool uses the premise that structurally related metabolites will generate similar product ions and product ion patterns, which can be exploited for the clustering and identification of like compounds. The GNPS algorithm converts each fragmentation spectrum into a vector with normalized product ion intensity for all metabolites to be compared. A cosine function with two vector inputs provide a numerical value of similarity for the two considered metabolites and clustering is visualized within Cytoscape with a node for

each precursor ion and an edge demonstrating a cosine similarity score above the set threshold. The resulting network for the five *Microcystis* strains has 126 nodes representing their cyanopeptides and 232 edges representing the structural similarity between metabolites (Figure 3.3). With nineteen clusters that have more than two nodes, thirteen of the clusters have been categorized by their identified respective chemical group. Fourteen of the twenty-seven clusters (#14-#27) were not able to be identified. Annotation of chemical groups was performed based on identification of individual metabolites within the cluster by HR-MS, retention time, diagnostic product ions, and matches with library or database searches. Of the 126 metabolites, twenty-four were matched with previously reported metabolites. Each annotated group will be described in detail below.

1. Phe-Ahp cyanopeptolins 2. Lxx-Ahp cyanopeptolins

3. microviridins

4. microviridins

5. microviridins

6. microcystins

8. aeruginosamides

9. anabaenopeptins

10. aeruginosins

7. microginins

12. cyanobactins

13. cyanobactins

14.

18.

19.

20.

21.

22.

23.

24.

25.

26.

27.

Figure 3.3: GNPS molecular network generated from metabolite MS/MS spectra produced by the five studied *Microcystis* strains grown in BG-11 medium. Nodes are color coded pi-charts labelled with precursor ion m/z illustrating which strains produced each metabolite (qualitative). The width of lines connecting nodes are proportional to the corresponding cosine score. Precursor and product ion m/z tolerance were set to 0.03 and 0.02 Da, respectively. The minimum base peak intensity was set at $5E^{+06}$. Clustering parameters required a minimum cosine similarity score of 0.6 and five matching product ions. Deconvolution was performed by eliminating all metabolites

present within the blank, outside the retention time range 2-6.5 minutes, and clusters with a size less than 2. Cyanopeptide groups and specific metabolites identified using the GNPS spectral library, interpretation of MS/MS spectra and comparisons to the literature are labelled. Abbreviations used in this figure include Ac; aerucyclamide, Ag; aeruginosamide, Ahp; 3-amino-6-hydroxy-2-piperidone, AP; anabaenopeptin, Cyp; cyanopeptolin, Lxx; leucine or isoleucine, MC; microcystin, Md; microcyclamide, Mg; microginins and Mv; microviridin.

The chemical diversity for the five studied *Microcystis* strains is illustrated in the molecular network (Figure 3.3). Together, the PCA and molecular networking demonstrate extensive strain specific cyanopeptide profiles. No two strains produce the same mixture of chemical groups or congeners within the groups. *M. aeruginosa* CPCC 299 and CPCC 300 are considered “toxic” strains because they both produce MCs. However, they produce many other chemical groups with limited information regarding their toxicity, structures, environmental concentrations and beneficial properties. *M. aeruginosa* CPCC 632 and CPCC 633 also overlap with the production of similar chemical groups including microginins, anabaenopeptins, and microviridins. Again, there is very little to no information in the literature regarding the detected congeners.

Chemical variation of cyanopeptides occurs through amino acid substitutions and post-translational modifications of the molecule, resulting in numerous congeners for each chemical class. The cyanopeptides are grouped based on unique structural features, such as non-proteinaceous amino acids or partial amino acid sequences that result in distinct product ion patterns within the MS/MS spectra of chemicals within the same group. We can exploit this consistency in chemical structures through post acquisition metabolomics techniques for the detection and identification of related structurally related metabolites.

3.1.3a Molecular network analysis: Microcystins

Most cyanopeptide studies focus on microcystins (MCs). Collectively, MCs are group of more than 300 potent hepatotoxins and possible human carcinogens (IARC Group 2B; Bouaïcha et al. 2019). MCs all contain the non-proteinaceous beta amino acid Adda (3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), and in most cases possess Mdha (methyl-dehydroalanine). Within the MS/MS spectra of MCs, diagnostic product ions that for these diagnostic structural features are m/z 135.0808 and m/z 163.1118 of the Adda moiety and m/z 213.0865 for Glu-Mdha (McMullin et al., 2019). Using metabolomic approaches to target these characteristic product ions provides a rapid mechanism to identify all MCs from samples. *M. aeruginosa* CPCC 299 and CPCC 300 are considered “toxic” and produce the same mixtures of MCs according to the molecular network cluster #6, agreeing with previous metabolome reports for these strains (Figure 3.4; Racine et al., 2019). No other studied strains produced MCs. *M. aeruginosa* CPCC 300 and *M. aeruginosa* PCC 7806, the most studied *Microcystis* strain to date, have very similar chemical profiles, with overlapping production of not only MC congeners, MC-LR and [Asp³]-MC-LR but other specific variants within other chemical classes such as cyanopeptolins, cyanobactins, and aeruginosins (Cornel et al., 1993; Portmann, Blom, Gademann, et al., 2008).

Figure 3.4: Representative chemical structure of the MC group, MC-LR (top left). The MC GNPS molecular network cluster # 6 (top right) showing congeners produced by *M. aeruginosa* CPCC 299 (red) and CPCC 300 (yellow) with summary table of MS information for the detected MC congeners. The MS/MS of MC-LR (bottom left) and [Asp³, Dha⁷]-MC-LR (bottom right) with diagnostic product ions and their putative structures, *m/z* 135.0803 and *m/z* 163.1113 of the Adda moiety, and *m/z* 213.0865 of the Mdha.

Three of the four MCs within the molecular network have been previously reported, MC-LR, [Asp³]-MC-LR, and [Asp³, Dha⁷]-MC-LR (Hollingdale et al., 2015). The identity of MC-LR and [Asp³]-MC-LR were confirmed by retention time and HR-MS in comparison to a purified standard (NRC, Halifax). For determining the identity of the MC congener (*m/z* 967.5254 [M+H]⁺), it was considered that it is 14.0131 mass units less than [Asp³]-MC-LR, suggesting a second demethylation. When comparing the MS/MS of MC-LR and [Asp³, Dha⁷]-MC-LR, the loss of the

product ion m/z 213.0865 suggests desmethyl-dehydroalanine in position 7 for this congener (Figure 3.4). The fourth MC within the cluster does not match any congener reported in the MC database suggesting a potential new MC. This congener is 15.9970 mass units greater than MC-LR suggesting hydroxylation of the molecule which could not be elucidated by product ions.

3.1.3b Molecular network analysis: Cyanopeptolins

The cyanopeptolin cyanopeptide group is distinguished by the presence of a 3-amino-6-hydroxy-2-piperidone (Ahp) residue at position 2 of the hexacyclic moiety. The amino acid adjacent to the diagnostic Ahp group aids in further subclassification of cyanopeptolin congeners because the substitution remains relatively consistent between congeners within the subclass. Subclassification is required for cyanopeptolins as there is an immense number of metabolites that contain an Ahp moiety resulting in few common product ions because of the increased structural diversity. Determination of the partial amino acid sequence is done with identification of diagnostic product ions, each of which contain the Ahp moiety and the adjacent amino acid (position 3). For example, cyanopeptolin A is classified as a Lxx-Ahp cyanopeptolin because it contains a Leu in position 3 adjacent to the diagnostic Ahp moiety at position 2 and results in the following diagnostic product ions; m/z 209.1283 $[\text{Leu-Ahp-H}_2\text{O+H}]^+$ and m/z 181.1331 $[\text{Leu-Ahp-H}_2\text{O-CO+H}]^+$ (McDonald et al., 2020)). When identifying cyanopeptide partial sequences by MS/MS approaches, the identity of leucine and isoleucine cannot be determined because they are isobaric, and thus annotated as Lxx. Further exploring this cyanopeptide group, cyanopeptolin 954 is a Phe-Ahp cyanopeptolin as it contains a Phe residue at position 3 neighbouring the Ahp moiety resulting in the following diagnostic product ions; m/z 243.1121 $[\text{Phe-Ahp-H}_2\text{O+H}]^+$ and m/z 215.1174 $[\text{Phe-Ahp-H}_2\text{O-CO+H}]^+$ (Figure 6). The subclassification of cyanopeptolin congeners is illustrated with two clusters annotated as cyanopeptolins within the molecular network, cluster #1 (Phe-Ahp)

and #2 (Lxx-Ahp, Figure 3.3). The substitution of the amino acid at position 3 results in large enough product ion diversity that it generates two separate network clusters representative of the subclasses of cyanopeptolins (Figure 3.5). Increased structural and sequence information arises with the presence of the NMe-aromatic amino acid immonium ion which resides in position 2. NMePhe, NMeTyr, and NMe-Cl-Tyr immonium ions can be seen at m/z 134.0962, m/z 150.0910, and m/z 184.0518, respectively.

The four investigated *M. aeruginosa* strains (CPCC 299, CPCC 300, CPCC 632, CPCC 633) produce diverse and complex mixtures of cyanopeptolins (Table 3.6). *M. aeruginosa* CPCC 300 varies from *M. aeruginosa* CPCC 299, CPCC 632, and CPCC 633 as it produces cyanopeptolins with the partial amino acid sequence, Lxx-Ahp whereas the other three strains produce cyanopeptolins with the partial amino acid sequence, Phe-Ahp (Figure 3.5). These congeners have been differentiated based on product ions patterns and result in large enough structural variation that they cluster separately within the molecular network.

Figure 3.5: The two cyanopeptolin GNPS molecular network clusters, Phe-Ahp cyanopeptolins, cluster #1 (top left) and Lxx-Ahp cyanopeptolins, cluster #2 (bottom left) and representative MS/MS spectra for congeners within these subclasses. The chemical structures of cyanopeptolin 954 (top right) and cyanopeptolin A (bottom right) are examples of each subclass. Key product ions to differentiate the subclasses are highlighted in the MS/MS spectra. For Phe-Ahp cyanopeptolins, m/z 184.0517 (NMe-Cl-Tyr), 215.1167 ([Ahp-Phe-CO-H₂O]⁺) are present and for Lxx-Ahp cyanopeptolins m/z 134.0961 (NMePhe), 181.1331 ([Ahp-Lxx-CO-H₂O]⁺).

M. aeruginosa CPCC 300 produced four previously reported cyanopeptolins: A, B, C, and 963A (Martin et al., Bister et al.). These metabolites share the partial amino acid sequence, NMePhe-Leu-Ahp with variation arising by amino acid substitution in any of the surrounding positions. Within the network cluster, there are four additional variants that do not have literature or database matches to metabolites with the partial amino acid sequence Lxx-Ahp, thus correspond to new potential cyanopeptolin variants (Table 3.1).

M. aeruginosa CPCC 632 produced two previously reported cyanopeptolins, nostopeptin BN920 and its chlorinated form cyanopeptolin 954 (Von Elert et al., 2005). This strain also produces the widest range of variants with other possible congener partial amino acid sequences: *NMePhe-Phe-Ahp*, *NMeTyr-Phe-Ahp*, and *NMe-Cl-Tyr-Phe-Ahp*. *M. aeruginosa* CPCC 299 exclusively produces cyanopeptolins with the partial amino acid sequence *NMePhe-Phe-Ahp* whereas CPCC 633 produces mixtures of cyanopeptolins with either *NMeTyr* or *NMePhe* within position 2. Halogenation of the *NMeTyr* and substitution to *NMePhe* does not merit the generation of a third cyanopeptolin cluster, thus these variants are all grouped together and labeled for the partial sequence they share, *Phe-Ahp* (Table 3.1).

Table 3.1: Summary of all cyanopeptolin variants within the GNPS molecular network.

Cluster #	Name	m/z [M+H-H ₂ O] ⁺	Formula	Error (ppm)	AA1	AA2
1	Cyanopeptolin 904	887.4652	C ₄₆ H ₆₄ N ₈ O ₁₁	-1.08	<i>NMePhe</i>	<i>Phe</i>
1	Cyanopeptolin 920	903.4367	C ₄₃ H ₆₅ N ₈ O ₁₂ Cl	-1.37	<i>NMe-Cl-Tyr</i>	<i>Phe</i>
1	Nosptopeptin BN920	903.4587	C ₄₆ H ₆₄ N ₈ O ₁₂	0.10	<i>NMeTyr</i>	<i>Phe</i>
1	Cyanopeptolin 932	915.4972	C ₄₈ H ₆₈ N ₈ O ₁₁	-0.29	<i>NMeTyr</i>	<i>Phe</i>
1	Cyanopeptolin 940	923.4039	C ₄₅ H ₆₅ N ₈ O ₁₂ Cl	-0.45	<i>NMe-Cl-Tyr</i>	<i>Phe</i>
1	Cyanopeptolin 946	929.5116	C ₄₉ H ₇₀ N ₈ O ₁₁	-1.63	<i>NMeTyr</i>	<i>Phe</i>
1	Cyanopeptolin 954	937.4217	C ₄₆ H ₆₃ N ₈ O ₁₂ Cl	-0.43	<i>NMe-Cl-Tyr</i>	<i>Phe</i>
1	Cyanopeptolin 954B	937.4441	C ₄₉ H ₆₂ N ₈ O ₁₂	-1.41	<i>NMePhe</i>	<i>Phe</i>
1	Cyanopeptolin 966	949.4821	C ₅₁ H ₆₆ N ₈ O ₁₁	0.29	<i>NMePhe</i>	<i>Phe</i>
1	Cyanopeptolin 968	951.4596	C ₅₀ H ₆₄ N ₈ O ₁₂	-1.55	<i>NMeTyr</i>	<i>Phe</i>
1	Cyanopeptolin 980	963.4964	C ₅₂ H ₆₈ N ₈ O ₁₁	-1.04	<i>NMeTyr</i>	<i>Phe</i>
1	Cyanopeptolin 982	965.4525	C ₄₈ H ₆₇ N ₈ O ₁₂ Cl	1.19	<i>NMe-Cl-Tyr</i>	<i>Phe</i>
1	Cyanopeptolin 992	979.4930	C ₅₂ H ₆₈ N ₈ O ₁₂	0.73	<i>NMeTyr</i>	<i>Phe</i>
1	Cyanopeptolin 1010	993.4856	C ₅₀ H ₇₁ N ₈ O ₁₂ Cl	0.08	<i>NMe-Cl-Tyr</i>	<i>Phe</i>
1	Cyanopeptolin 999	1000.5120	C ₅₁ H ₆₉ N ₉ O ₁₂	-1.34	<i>NMePhe</i>	<i>Phe</i>
2	Cyanopeptolin 900	901.5031*	C ₄₄ H ₆₈ N ₈ O ₁₂	0.17	<i>NMePhe</i>	<i>Lxx</i>
2	Cyanopeptolin B	929.5327*	C ₄₆ H ₇₂ N ₈ O ₁₂	-1.66	<i>NMePhe</i>	<i>Leu</i>
2	Cyanopeptolin C	943.5481*	C ₄₇ H ₇₄ N ₈ O ₁₂	-1.90	<i>NMePhe</i>	<i>Leu</i>
2	Cyanopeptolin 945	946.4917*	C ₄₉ H ₆₇ N ₇ O ₁₂	-0.36	<i>NMePhe</i>	<i>Lxx</i>
2	Cyanopeptolin A	957.5391*	C ₄₆ H ₇₂ N ₁₀ O ₁₂	-1.35	<i>NMePhe</i>	<i>Leu</i>
2	Cyanopeptolin 963A	964.5040*	C ₄₉ H ₆₉ N ₇ O ₁₃	1.53	<i>NMePhe</i>	<i>Leu</i>
2	Cyanopeptolin 970	971.5565*	C ₄₇ H ₇₄ N ₁₀ O ₁₂	0.46	<i>NMePhe</i>	<i>Lxx</i>
2	Cyanopeptolin 984	985.5725*	C ₄₈ H ₇₆ N ₁₀ O ₁₂	0.61	<i>NMePhe</i>	<i>Lxx</i>

*[M+H]⁺

3.1.3c Molecular network analysis: Anabaenopeptins

Anabaenopeptins (APs) are a group of hexapeptide cyanopeptides, with a cyclic core composed of five residues and one amino acid in an exo position. This group of cyanopeptides is of interest as its congeners demonstrate a similar mechanism of toxicity as MCs, potent inhibition of protein phosphatases (Janssen et al., 2019). APs share a ureido linkage for cyclization by a lysine residue at position 2. This linkage is common between all congeners within the group and connects the exo residue at position 1 to the residue at position 3. All other positions, aside from the lysine, are variable making this group very structurally diverse (Spoof et al., 2016). The dominant common product ions observed within the MS/MS of anabaenopeptins are m/z 84.0812 and m/z 129.1018, characteristic of the lysine residue. All congeners detected here contain an *NMe*-Ala at position 5 with the diagnostic product ion m/z 58.0658 in all AP MS/MS spectra. Two of the five *Microcystis* strains studied produced similar mixtures of APs, *M. aeruginosa* CPCC 632 and CPCC 633 (Table S1, Figure 3.6).

Figure 3.6: Representative chemical structure of the AP congener, Ferintoic acid A (top left). The AP GNPS molecular network cluster # 9 (top right) showing congeners produced by *M. aeruginosa* CPCC 632 (blue) and CPCC 633 (grey) with summary table of MS information for the detected AP congeners. The MS/MS of Ferintoic acid A (bottom left) and AP 850 (bottom right) with diagnostic product ions and their putative structures, m/z 58.0658 (NMe-Ala), m/z 72.0792 (Val), m/z 84.0812 (Lys), m/z 120.0835 (Phe), m/z 150.0910 (Homo-Tyr; Hty), m/z 134.0964 (Homo-Phe; Hph), and m/z 159.0914 (Trp).

Ferintoic acid A and B (FA A and FA B), AP D, and AP NZ857 are detected congeners that have been previously reported (Spoof et al., 2015). The identity of these previously reported congeners was confirmed based on diagnostic product ions within the MS/MS. For example, immonium ions that confirmed the identity of AP D are m/z 58.0658 (NMeAla), m/z 72.0813 (Val), m/z 84.0812 (Lys), m/z 120.0808 (Phe), and m/z 150.0909 (Hty). Two AP congeners within the network cluster #9 could not be matched to a previously identified metabolites through library or database searches and are considered potential new APs (Figure 3.6). MS analysis shows AP 850 is 15.9925 mass units less than FA A suggesting the loss of an oxygen atom, which is accounted for within the

MS/MS as a substitution of m/z 150.0906 of Hty in FA A to m/z 134.0962 of Hph in AP 850 (Figure 3.6). MS analysis shows AP 882 is 15.9956 mass units larger than FA A suggesting the gain of an oxygen atom. This is explained within the MS/MS as a substitution of m/z 159.0914 of Trp in the exo position of FA A to m/z 175.0863 of 5-hydroxy-Trp of AP 882. These substitutions of one aromatic amino acid to another is common and suggests the synthetic enzyme has low specificity that aids in increased chemical diversity for APs.

3.1.3d Molecular network analysis: Microginins

Microginins (MGs) are a group of linear cyanopeptides containing 4-6 amino acid residues and a characteristic N-terminal 3-amino-2-hydroxydecanoic acid (Ahda) from a polyketide synthesis pathway. Variation in this characteristic residue occurs with a shorter chain length to 3-amino-2-hydroxyoctanoic acid (Ahoa) as well as different chlorination states of the terminal end of the polyketide moiety. Characteristic product ions resulting from the fragmentation of these characteristic microginin N-terminal polyketide moieties include m/z 128.1433, indicative of the Ahda functionality. A mass increase of 33.9606 units to m/z 162.1039 and again to m/z 196.0645 are characteristic of mono- and di-chlorinated Ahda, respectively. The shorter chain congeners generate similar product ions at m/z 100.1122, m/z 134.0727, m/z 168.0338, indicative of the non-, mono-, and di-chlorinated Ahoa residues, respectively (Strangman & Wright, 2016).

Figure 3.7: Representative chemical structure of MG congener, microginin 612 (top left). LC-MS spectrum (top left) of co-eluting MG congeners (MG 612; m/z 613.3225 [M+H]⁺, MG 646; m/z 646.2826 [M+H]⁺, and MG 680; m/z 681.2441 [M+H]⁺) with varying chlorination states seen as a mass difference of 1 chlorine atom (33.98). The MG GNPS molecular network cluster #7 showing congeners produced by *M. aeruginosa* CPCC 632 (grey) and CPCC 633 (blue). The MS/MS of MG 612 (bottom left) and MG 660 (bottom right) with diagnostic product ions labelled, m/z 100.1122 (Ahoa), m/z 148.0833 (Cl-NMe-Ahoa), as well as product ions m/z 70.0658 (Pro), m/z 136.0752 (Tyr) and m/z 150.0910 (NMeTyr) used to confirm the structure.

MGs were produced by two *M. aeruginosa* strains, CPCC 632 and CPCC 633 (Table S1, Figure 3.7). Unlike the APs, the MG mixtures produced by these strains were not the same. The MG

GNPS network cluster (#7) is comprised of twelve MG congeners, eleven of which are produced by *M. aeruginosa* CPCC 633 and six by *M. aeruginosa* CPCC 632. This highlights similar cyanopeptide profiles for *M. aeruginosa* CPCC 633 and CPCC 632, that overlapped in the PCA plot (Figure 3.1). Of the twelve MGs identified, three have previously been identified, MG 612, MG 646, and MG 680 and are exclusively produced by *M. aeruginosa* CPCC 633 (Strangman & Wright, 2016). These congeners possess the uncommon Ahoa residue and vary only by the chlorination state of the N-terminal polyketide chain. An N-methylated series of molecules akin to these previously reported molecules was produced by both strains, MG 626, MG 660, and MG 694 by an increase in mass units of 14.0159 in each case. Upon investigation of the MS/MS of these series of molecules, it is apparent that those produced by *M. aeruginosa* CPCC 632 are structural isomers to the series produced by *M. aeruginosa* CPCC 633. When comparing the MS/MS of the previously reported MGs (MG 612, MG 646, MG 680) to the series of unknown MG metabolites produced by *M. aeruginosa* CPCC 632, the diagnostic product ions associated with the Ahoa and the potential chlorination states are present at m/z 100.1122 (Ahoa), m/z 134.0727 (Cl-Ahoa), and m/z 168.0338 (Cl₂-Ahoa). This suggests the methyl addition is elsewhere. Further investigation reveals that in the MS/MS spectra of MG 626, MG 660, and MG 694 produced by *M. aeruginosa* CPCC 632 there is no immonium ion for Tyr (m/z 136.0762) only NMeTyr (m/z 150.0912), suggesting both residues within the molecule are NMeTyr. For example, the amino acid sequence for MG 626 is Ahoa-NMeTyr-NMeTyr-Pro.

When analyzing the MS/MS spectra for the unknown MG metabolite series produced by *M. aeruginosa* CPCC 633, the diagnostic product ions associated with Ahoa and the potential chlorination states are not present. Instead, they are replaced by a series of product ions with a mass difference of 14.0156 units, m/z 114.1278 (NMe-Ahoa), m/z 148.0883 (NMe-Cl-Ahoa), m/z

182.0494 (*NMe*-Cl₂-Ahoa). This suggest methylation of the amine of the Ahoa as an increase in chain length would not be possible through the polyketide synthase pathway, as well congeners with modification in this manner have been previously reported (Lodin-Friedman & Carmeli, 2018). For example, the amino acid sequence for MG 626 is *NMe*-Ahoa-Tyr-*NMe*Tyr-Pro.

Of the twelve MGs grouped within the molecular network; all are tetra-peptides, nine are chlorinated, ten have the octanoic acid terminal chain, and two have the decanoic acid terminal chain (Figure 3.7). Of the ten congeners with the octanoic acid terminal chain, three have methylated amines (*NMe*-Ahoa), two do not have a hydroxyl on the carbon adjacent to the amine (Amino-octanoic acid, Ahoa), and the remaining five have Ahoa. The isotope pattern as well as retention time gave indication on the level of chlorination of the cyanopeptides. Both strains produced MG variants with Ahda and Ahoa residues with capability for non-, mono-, di-, and tri-chlorination. Nine of the twelve MGs identified are chlorinated, with all possible chain modifications having a mono-chlorinated congener suggesting chlorination of the chain occurs in mono-substitution steps. Not all chain types have a non-chlorinated state or di-chlorinated state.

M. aeruginosa CPCC 633 produced two amino-octanoic derivatives of the known MGs, MG 680 (m/z 681.2441 [M+H]⁺) and MG 646 (m/z 647.2826 [M+H]⁺) with a mass difference of 15.9926 less, MG 660 (m/z 661.2985 [M+H]⁺) and MG 630 (m/z 631.2891 [M+H]⁺), respectively. A mass difference of this size, suggests the loss of an oxygen atom from each of the previously reported metabolites. Further relatedness is confirmed as these sets of compounds share the isotope pattern of di- and mono-chlorinated, respectively. MG 646 has 4 hydroxyl groups that could be removed for the loss of 15.9926 mass units from the precursor ion, 1 on each residue: Pro, Tyr, *NMe*Tyr, and on the Ahoa. When comparing the MS/MS of MG 646 and MG 630, the product ions for both Tyr residues as well as the Pro are present. The Cl-Ahoa immonium ion is also present but the

chain breaks at the carbon with the amine and does not include the hydroxyl, suggesting this is the site without a hydroxyl.

3.1.3e Molecular network analysis: Microviridins

Microviridins (MVs) are tricyclic ribosomally synthesized and post-translationally modified peptides (RiPPs) typically composed of fourteen amino acids with ω -ester and ω -amide bonds between two peptide chains (Rohrlack et al., 2003). These unusual peptides, unlike the other cyanopeptide groups discussed here, are composed of all proteinaceous amino acids with the most reoccurring amino acids between congeners being Tyr, Trp, Phe, Thr, Lys, Asp, Ser, and Glu. The molecular weights of these metabolites are larger (1400-2000 Da range) compared to the other cyanopeptides groups studied. Structural variability is high due to the increased number of positions for amino acid substitution. This structural variability is seen within the molecular network clusters #3, #4, #5 as there are separate clusters to encapsulate all the MV congeners detected (Figure 3.3). All MV nodes within the molecular network are associated with the doubly charged precursor ions. Classification of these molecules as microviridins was done based on retention time range (2-3 minutes), doubly charged precursor ion filtering, and the immonium ions for Trp (m/z 159.0911), Pro (m/z 70.0657), and Lys (m/z 84.0812) within their MS/MS spectra. A large factor that contributes to the dominant immonium ions present within the microviridin MS/MS are attributed to the exo amino acids. MV structures can have up to 4 amino acids that are not cyclized and thus are the first product ions to be generated (Ziemert et al., 2010).

4. microviridins

Figure 3.8: The MV GNPS network cluster #4 (top left) and LC-MS analysis (top right) of congeners produced by *M. aeruginosa* CPCC 299, CPCC 632, and CPCC 633. Full MS scan at 2.76 minutes (bottom left) shows the precursor ion for MV J (m/z 842.8925 $[M+2H]^{2+}$) which was identified by precursor ion m/z and product ions within the MS/MS (bottom right) of m/z 70.0659 (Pro), m/z 84.0808 (Lys), m/z 136.0729 (Tyr), and m/z 159.0935 (Trp). (Zhang, Li, et al., 2018)

Three of the investigated *Microcystis* strains produced microviridins (MVs), *M. aeruginosa* CPCC 299, CPCC 632, and CPCC 633. Each strain produces at least one variant within all three (#3, #4, #5) of the MV clusters annotated (Table S1, Figure 3.3). There are eighteen MV congeners present within the molecular network. Two congeners are identified as previously reported, MV H and MV J, and the remainder are potential new MVs (Rohrlack et al., 2003; Ziemert et al., 2010). The identity of the exo amino acids is hypothesized to generate sufficient structural diversity to separate into three MV clusters. Two of the congeners were identified as MV H and MV J through a match with literature by molecular formula and amino acid immonium ions in the MS/MS spectra. MV H and MV J have similar core amino acids but the identity of the exo amino acids vary. MV H

contains Pro, Asp, and two Tyr residues whereas MV J has Ser, Ile, Gln, and Trp residues. Immonium ions of these exo amino acids are present within the MS/MS of these metabolites. Thus, cluster #4 contains potential MVs with similar exo amino acids as MV H and cluster #5 contains potential MVs with similar exo amino acids as MV J (Figure 3.8). MV cluster #3 did not have any library or database matches but contains MV-like metabolites based on the presence of doubly charged precursor ion m/z , retention time range, and presence of immonium ions. The MS/MS of metabolites within this cluster share many immonium ions with MV H and MV J, but a few outliers exist such as the immonium ion for Phe at m/z 120.0807 and an immonium ion at m/z 115.0866 (NMe-Glu). The exo amino acids for these congeners must vary from those within the congeners of the other network clusters.

3.1.3f Molecular network analysis: Aeruginosins

Aeruginosins (ASs) are small linear tetrapeptides that contain the characteristic 2-carboxy-6-hydroxyoctahydroindole (Choi), a C-terminal Arg or Arg-derivative, and an N-terminal hydroxyphenyl lactic acid (Hpla) with varying substitution of the aromatic ring such as halogenation in the meta positions. Variation increases with O-R modifications of the Choi moiety such as a sulfate group, the cyclization of the Arg to an argal, and finally amino acid substitutions within position 2. Within the MS/MS of AS metabolites, diagnostic product ions are generated from the characteristic Choi moiety, the product ion of Choi at m/z 140.1064 and the dehydrated form 18.0102 mass units less at m/z 122.0962 (Welker et al., 2006).

Two *M. aeruginosa* strains, CPCC 300 and CPCC 632, produced different mixtures of ASs (Table S1, Figure 3.9). None of the AS variants within the molecular network matched library or database searches suggesting five new potential ASs. The clusters for *M. aeruginosa* CPCC 300 and CPCC 632 did not group together indicating structural variability between the congeners produced by

these strains. All congeners were confirmed to be within the AS group based on the presence of product ions characteristic of the Choi moiety.

Figure 3.9: The AS GNPS molecular network clusters, #10 for *M. aeruginosa* CPCC 632 (grey) and #11 for *M. aeruginosa* CPCC 300 (yellow) from the total GNPS network and the congener information. The MS/MS spectrum of AS 688 (bottom left) and AS 650 (bottom right) with diagnostic product ions m/z 140.1069 and m/z 122.0966 of the Choi functionality. Further grouping of this chemical group is done with Phe (m/z 120.0807) or Tyr (m/z 136.0756) substitutions in position 2. Sulfonation of the Choi is possible and visible in the MS as well as the MS/MS as a neutral loss of 79.9571 ([M+H-SO₃]⁺). Partial amino acid sequence was concluded with the product ion corresponding to [Hpla-Residue 2-CO]⁺. For chemicals in cluster 10 contain the sequence [Hpla-Phe – CO]⁺ at m/z 284.1268 and for chemicals in cluster 11 contain the sequence either sequence [Cl-Hpla-Tyr – CO]⁺ at m/z 334.0838 or [Hpla-Tyr – CO]⁺ at m/z 300.1232.

M. aeruginosa CPCC 300 produced three AS variants that contain Tyr within position 2 as well as an Argal (cyclic Arg) in position 4. The Tyr residue is confirmed by the presence of the presence of the Tyr immonium ion in all three fragmentation spectra at m/z 136.0756. The substitution at the C-terminal position was determined as Argal for the ASs produced by *M. aeruginosa* CPCC 300 as the immonium ion for Arg (m/z 114.1025) was not present within the three MS/MS spectra. Upon investigation of the isotope pattern, two of the congeners (AS 684 and AS 650) are singly chlorinated and AS 650 is not chlorinated yet has a mass 33.9606 units less than AS 684 suggesting this metabolite is the non-chlorinated version of AS 684. A b_2 fragment within the MS/MS of AS 650 confirms the partial amino acid sequence as Hpla-Tyr at m/z 300.1232 (Welker et al., 2006). Chlorination within AS 684 and AS 716 on the Hpla is confirmed with the akin fragment 33.9606 mass units greater signifying Cl-Hpla-Tyr at m/z 334.0838.

M. aeruginosa CPCC 632 produces 2 AS variants that contain a Phe within position 2 as well as an Arg in position 4. The Phe residue was confirmed by the presence of the immonium ion for Phe (m/z 120.0807) within the MS/MS for the 2 metabolites and the Arg was confirmed with the product ion for Arg (m/z 114.1025). These metabolites share the characteristic neutral mass loss of 79.9571 m/z from the precursor ion within the mass spectrum as well as in the MS/MS indicating sulfonation of the Choi moiety. The increase in M+2 intensity as well as the mass difference between these two congeners being 33.9606 m/z suggest that AS 688 is not chlorinated and AS 722 is singly chlorinated. The partial sequence Hpla-Phe in AS 688 was confirmed with the product ion m/z 284.1268 for the $[Hpla-Phe-CO+H]^+$. Chlorination of the Hpla in AS 722 is seen with a mass increase of 33.9606 of the aforementioned product ion to m/z 318.0876 of $[Cl-Hpla-Phe-CO+H]^+$.

3.1.3g Molecular network analysis: Cyanobactins

Cyanobactins are a group of highly modified cyanopeptides that contain both proteinaceous amino acids and heterocyclic residues. These residues are a result of a bond between the amino acid side chain heteroatom (oxygen or sulfur atom) from Thr, Ser, and Cys and the preceding amide bond. The enzyme that catalyzes the heterocyclization requires ATP and Mg^{2+} for activity to synthesize the oxazoline and thiazolines, as well as the methyl versions (Ge et al., 2019). These residues can be further oxidized to oxazoles and thiazoles which occur in majority of the reported cyanobactins, increasing structural diversity of this chemical group (Leikoski et al., 2013). Further diversity occurs with substitution of proteinaceous amino acids surrounding the heterocycles. These metabolites may be cyclized or linear peptides and will be discussed in terms of cyclic cyanobactins (cyclamides) and linear cyanobactins (aeruginosamides). The opportunity for oxidation/reduction of the heterocyclized atoms, amino acid substitutions, and linear/cyclic cyanobactins results in increased structural diversity and thus fewer diagnostic product ions generated. Thus, there are 3 clusters (#12, #13, #8) to describe all detected cyanobactins (Figure 3.3).

M. aeruginosa CPCC 299 and CPCC 300, and *M. flos-aquae* CPCC 461 produced different mixtures of cyclamides (Figure 3.3). *M. aeruginosa* CPCC 299 and CPCC 300 produce previously reported aerucyclamide (AC) A and AC C, and *M. aeruginosa* CPCC 300 also produces AC B and AC D and a related congener of AC C, AC 518 as seen in cluster #13 (Figure 3.10, Portmann et al., 2008). With the use of HRMS and the occurrence of sulfur atoms within cyclamides, determining molecular formula for the precursor ion as well as product ions containing sulfur is simplified because of the distinct mass defect of sulfur atoms. Based on the MS/MS of the cyclamides within this network cluster, they each have an *Me*-oxazoline (m/z 84.0448) and Lxx (m/z 86.0969). For those previously reported and structurally characterized (aerucyclamides A-C),

we can say that it possesses an Ile residue but for those that are potentially new cyanobactins, we cannot discriminate between the two isobaric residue, Ile and Leu thus we annotate this residue as Lxx. AC A and AC 518 have the partial sequence Tzln-Lxx which is confirmed with the product ions m/z 86.0064 (Tzln), m/z 154.0683 ([Tzln-Lxx-CONH₂+H]⁺) and m/z 199.0904 (Lxx-Tzln). AC B and AC C have the partial sequence Tzl-Ile which is confirmed with the product ion m/z 197.0741 akin to the oxidized product ion seen in the spectra for AC A and AC 518. The evidence suggests that AC 518 is the oxidized congener of AC C, in a similar manner as AC B is the oxidized congener of AC A. The reduced congener contains the Tzln moiety and the oxidized congener contains the Tzl moiety. Other product ions that confirm the structure of AC 518 are m/z 167.0838 (Val-Ozl) and m/z 235.1438 (Val-Ozl-Lxx). The common proteinaceous amino acids and *Me*-oxazoline present in these metabolites clustered them together within the network. Although, the cyclic nature of these peptides, their uncommon heterocycles, and multiple sites for oxidation/reduction generate a roadblock when searching for variants.

Figure 3.10: Aerucyclamide GNPS network cluster #13 with LC-MS analysis of the congeners produced by *M. aeruginosa* CPCC 299 (red), CPCC 300 (yellow), and *M. flos-aquae* CPCC 461 (green). Representative structure of aerucyclamide A (top right) was confirmed by precursor ion m/z and diagnostic product ions from the MS/MS (bottom left), m/z 86.0969 (Lxx), 154.0683 ([Tzln-Lxx-CONH₂+H]⁺), and 199.0904 (Tzln-Lxx). Potential new aerucyclamide 518 (MS/MS bottom right) was confirmed as reduced aerucyclamide C with diagnostic ions aligning with Tzln functionality m/z 154.0683 and 199.0905, as well as m/z 167.0838 of the Val-Ozl product ion not present in aerucyclamide A or B.

AC D, which did not appear in the molecular network, has a Met in position 4 and a Phe in position 6 in comparison to the AC A structure (Portmann et al., 2008). This metabolite is product by *M. aeruginosa* CPCC 300 and the identity was confirmed with precursor ion m/z and the following product ions m/z 84.0448 (MeOxazoline), m/z 104.0531 (Met), m/z 120.0808 (Phe), and m/z 86.0064 (Tzln). These two substitutions generate enough structural diversity from the other cyclamides that this metabolite did not cluster within the network.

Cluster # 12 illustrates that *M. flos-aquae* CPCC 461 produces microcyclamide A and two related reduced congeners Ac 584 and Ac 586 (Figure 3.11, Portmann et al., 2008). This known congener

has the following hexapeptide sequence: *MeOxl-Ile-Tzl-MeHis-Tzl-Ala*. This molecule is highly oxidized with many sites for reduction to produce the two potential new congeners reported here. The congeners within this network share the product ion for *MeHis* at m/z 96.0685 and *MeOxl-Ala* at m/z 153.0656 confirming the common core structure between these molecules. In the potential new congener, Md 584, the product ion for *Ile-Tzl-MeHis-Tzl* which is at m/z 387.1182 in Md A becomes m/z 389.1213 suggesting oxidation of one of the *Tzl* groups to *Tzln*. To confirm which *Tzl* group is oxidized in Md 584, the product ion for the sequence *MeHis-Tzl* with the m/z 235.0645 is present in both MS/MS spectra for Md A and Md 584 suggesting it is the *Tzl* in position 3 that is oxidized to *Tzln* (Figure 3.11). In the potential new congener Md 586, both *Tzl* residues are oxidized to *Tzln*, thus the mass increase of 4 mass units from the known Md A which is confirmed with the presence of the product ion m/z 391.0996.

Figure 3.11: Microcyclamide GNPS network cluster #12 (top left) with LC-MS analysis of the congeners produced by *M. aeruginosa* CPCC 299 (red) and *M. flos-aquae* CPCC 461 (green). Representative structure of microcyclamide A (m/z 583.1887 [M+H]⁺; top right) was confirmed with product ions m/z 261.0798 (Ala-Tzl-MeHis), 235.0645 (MeHis-Tzl), 153.0656 (MeOxazole-

Ala), and 96.0685 (*MeHis*) within the MS/MS (bottom left). Potential new microcyclamides 584 (MS/MS bottom right) contains the reduced Tzl (Tzln) in position 3 and a Tzl in position 5.

M. aeruginosa CPCC 633 produces linear cyanobactins, known as aeruginosamides (AGs, cluster #8, Figure 3.12). This subgroup contains a C-terminal prenylated Phe, 1,1-dimethylallyl-Phe (Dma-Phe) and an N-terminal Tzl-OMe (Tzc). These characteristic features provide diagnostic product ions within the MS/MS such as m/z 188.1428 for [Dma-Phe-CO+H]⁺ and m/z 144.0109 for Tzc. AG B and AG C are produced by *M. aeruginosa* CPCC 633 and have been previously reported (Leikoski et al., 2013). These metabolites have two Phe residues and a Pro residue which is confirmed by these immonium ions within their MS/MS at m/z 120.0807 (Phe) and m/z 70.0658 (Pro). Based on the precursor ion m/z , both reported congeners (AG B and AG C) have a related congener with a mass difference of 14.0182 less suggesting loss of a methyl. Demethylation of the Tzc to Tzl-OH (which occurs in Aeruginosamide A, (Leikoski et al., 2013)) is seen in the MS/MS of these two congeners as the absence of the product ion m/z 144.0109 and the presence of the product ion m/z 129.9955. The loss of the methyl on the Tzc can also be confirmed with the replacement of the product ion m/z 213.0685 of [Tzc-Pro-CO+H]⁺ to 14.0182 mass units less at m/z 199.0503 [Tzl-OH-Pro-CO+H]⁺. Other congeners within the molecular network cluster #8 have hydroxyl substitutions seen as the gain of 15.9956 mass units from the precursor ion of a previously reported AG, or as an oxidized Tzc (which occurs in cyclamides, (Portmann, Blom, Gademann, et al., 2008)) seen as the gain in 2 mass units from the precursor ion of a previously reported AG.

Figure 3.12: The aeruginosamide GNPS molecular network cluster #8 (top left) with LC-MS analysis of the congeners produced by *M. aeruginosa* CPCC 632 (blue) and CPCC 300 (yellow), and *M. flos-aquae* CPCC 461 (green). Representative structure of aeruginosamide B (top right, MS/MS bottom left) was confirmed with the diagnostic product ions m/z 120.0807 (Phe), 70.0658 (Pro), 144.0109 (Tzc), and m/z 213.0685 (Pro-Tzc). The MS/MS of aeruginosamide 560 (bottom right) shows diagnostic product ions used to elucidate the site of demethylation present at the Tzc to Tzl-OH with the mass difference of 14.0182 at m/z 199.0503.

3.1.3h Molecular network analysis: unknown cluster #14

Interpretation of clusters with no previously reported metabolites or diagnostic product ions were labeled as unknown clusters. Cluster #14 contains a series of metabolites produced by *M. aeruginosa* CPCC 299 and CPCC 633, that are doubly charged ions within the range m/z 720-770 (molecular ions between 1440-1540 Da, Figure 3.13). The doubly charged ion and the calculated molecular ion matched a few metabolites within the database but comparison of the reported structure and the generated MS/MS, it was concluded that the compounds were not a match.

Figure 3.13: The GNPS network cluster #14 (top right) for an unidentified chemical group with congeners produced by *M. aeruginosa* CPCC 299 (red) and CPCC 633 (blue). LC-MS analysis of the congeners (top right) with an MS spectrum (bottom left) at 5.65 minutes to show coelution of two congeners. The MS/MS of the congener Unk 1490 (m/z 746.4204 [M+2H]²⁺), bottom right) with proteogenic immonium ions m/z 70.0659 (Pro), 102.0553 (Gln), 136.0756 (Tyr), and the non-proteogenic and potentially diagnostic product ion m/z 115.0865.

Immonium ions shared between these compounds are m/z 70.0657 (Pro), m/z 84.0451 (Arg), m/z 86.0968 (Lxx), m/z 102.0553 (Glu), m/z 136.0755 (Tyr), and the diagnostic product ion m/z 115.0865 (C₅H₁₁N₂O). This characteristic product ion paired with any of the amino acid immonium ions mentioned could be used to target all congeners with these molecular features.

The LC-MS analysis provides a molecular formula and MS/MS analysis provides information on the building blocks of the metabolite, but connectivity and structure of non-proteogenic amino acids cannot be concluded without other spectroscopic information. Other information that has been derived from this molecular network is the relatedness of the metabolites. For example, in

the MS spectra of Figure 3.13, the two precursor ions Unk 1476 (m/z 739.4133 $[M+2H]^{2+}$) and Unk 1490 (m/z 746.4202 $[M+2H]^{2+}$), have a mass difference of 7.0051 which equates to a mass difference of 14.0185 when factoring that these molecules are doubly charged. This is the difference of a methyl group, suggesting the variation between these metabolites is a methyl modification. Another example is a mass difference of 7.9956 between Unk 1490 (m/z 746.4202 $[M+2H]^{2+}$) and Unk 1474 (m/z 738.4238 $[M+2H]^{2+}$), which equates to a molecular mass difference of 15.9970, or 1 oxygen atom suggesting Unk 1490 has substituted a H for an OH. More information is required to understanding the full structure of these metabolites and their potential toxicity. These will be targets for future chemical characterizations.

3.1.4 Relative abundance of cyanopeptides produced by *Microcystis* strains

The information acquired from untargeted metabolomic techniques such as PCA, loading plot, and molecular networking allows for an understanding of the strain specific metabolites for the investigated *Microcystis* strains. With limited availability for reference materials of the thousands of cyanopeptide congeners reported, interpretation of the proportions of metabolites has been done here with peak area normalized with dried biomass. This normalization provides a proxy for comparison of the relative proportions of different metabolites and metabolite groups within an extract as well as comparison to other strains. Compounds identified within the molecular network are used here to describe the relative amounts of the secondary metabolites produced by the five studied *Microcystis* strains (Figure 3.14).

Figure 3.14: Relative abundance of metabolites on day 15 when grown in BG-11 media at 27°C, generated with normalized peak area of identified secondary metabolites produced by 4 *Microcystis aeruginosa* strains CPCC 299 (top left), CPCC 300 (middle left), CPCC 632 (bottom left), CPCC 633 (middle right) and 1 *Microcystis flos-aquae* strain CPCC 461 (bottom right). Metabolites coloured based on cyanopeptide group.

For all strains that produce cyanobactins (yellow), *M. aeruginosa* CPCC 299, CPCC 300, and CPCC 633 and *M. flos-aquae* CPCC 461, these metabolites have the highest relative abundance when comparing to metabolites within the other chemical classes. The MC congeners produced by *M. aeruginosa* CPCC 299 and CPCC 300 are produced in relatively similar amounts in relation to one another, assuming similar relative responses. For most cyanopeptide groups, there are typically select congeners produced in relatively larger amounts compared to the others. For example, the microginins (grey) MG 646 and MG 680 produced by *M. aeruginosa* CPCC 633 are in higher proportions than the other microginins. Further, for both *M. aeruginosa* CPCC 632 and CPCC 633, the anabaenopeptin ferintoic acid A is produced in larger quantities than ferintoic acid B. This information allows for a better understanding at the production of lower abundance metabolites and the importance of sensitive instrumentation for the detection and subsequent identification of all cyanopeptide variants present within an extract.

3.2 Semi-targeted mass-spectrometry based metabolomics: application to cyanopeptide groups

Advances in mass spectrometry instrumentation and chemometrics together provide opportunities to better study cyanopeptide diversity. Cyanopeptides and other microbial natural products, are typically biosynthesized as mixtures of structurally related compounds, rather than as a single unique entity. The tandem mass spectrometry (MS/MS) spectra of natural products are highly dependent on their chemical structure, meaning groups of structurally related compounds often generate common product ions or neutral losses. This fact was exploited in some of the untargeted analysis described in section 3.1. Post-acquisition data processing strategies can be used to search MS/MS spectra generated with data dependent acquisition (DDA) experiments for sets of characteristic, or diagnostic, product ions that correspond to specific structural features (Renaud et al. 2015; 2017). Previously, the diagnostic fragmentation filtering (DFF) module within the

open-source bioinformatics software platform MZmine2 was developed, which facilitates rapid product ion searching with a user-friendly interface (Walsh et al. 2018). DFF can be used to identify all compounds within a group, including known and new compounds useful for dereplication and identifying new targets for chemical characterizations. The peptidyl nature and common structural features that differentiate cyanopeptide groups generates diagnostic product ions, including immonium ions, in their MS/MS spectra that can be targeted by semi-targeted metabolomics approaches such as DFF. For example, the MC chemical group is unified by the presence of the non-proteinaceous amino acid Adda and cyanopeptolins with Ahp (McDonald et al., 2020; McMullin et al., 2019).

This DFF post-acquisition data processing tool uses MZmine2 (<http://mzmine.github.io/>) to search tandem MS data files for compounds with the diagnostic product ions of interest. The output is a 2-dimensional plot with precursor ion m/z on the x-axis and product ion m/z on the y-axis (for example, Figure 3.16). Each data point within the DFF plot is a product ion, which dictates the y-coordinate, with the exception of intact precursor ions that appear on the diagonal axis. The x coordinate is based on the intact precursor ion that was selected for fragmentation. What appears on the plot are vertical lines, each corresponding to a metabolite that contains the diagnostic product ions of interest. In some cases, if the metabolites are very structurally similar, there is an appearance of a horizontal line, due to many shared product ions. Other parameters that dictate the specificity of these plots, include m/z tolerance, minimum base peak intensity, and precursor ion m/z and retention time ranges. Another feature that can be used to increase selectivity towards a particular group of chemicals, is the input of diagnostic neutral losses. For example, the aeruginosin chemical group has many congeners that are sulfated. The loss of the sulfate group is a neutral loss from the precursor ion, can typically be seen with the full MS scan as well as the

fragmentation spectra. Nonetheless, this can be used as a diagnostic tool for identifying metabolites and thus is useful when considering chemical classes that are sulfated, phosphorylated, etc.

The advantage of having N¹⁵-labelled MS and tandem MS data alongside the unlabelled equivalent is the number of nitrogen atoms within the intact precursor ion and any resultant product ions. With this knowledge, conclusion on a molecular formula and additional evidence of a molecule's putative identity. Further, this can be applied to the tandem MS to aid in identifying the structure of the diagnostic product ion.

The results from the untargeted analysis provide insight into product ions and product ion pairs that can be used in a search for congeners of the chemical group in other untargeted data sets. Furthermore, the parameters used to generate the molecular network potentially exclude metabolites that are structurally diverse and unrelated to other metabolites as individual metabolites are excluded. In this section, DFF plots are used to detect all metabolites with specific structural features from cultured strain extracts and the tool shows its capacity to detect cyanopeptides from surface water and bloom samples (Table 3.2). The chemical classes investigated here are those that are well defined: MCs, cyanopeptolins, aeruginosins, and microginins.

Table 3.2: Collection information for *Microcystis* strains and environmental surface water and bloom samples.

Strain/Sample Name	Sample type	Collection Date	Collection Site
CPCC 299	<i>M. aeruginosa</i> strain	08/90	Pretzlaff Pond AB, Canada
CPCC 300	<i>M. aeruginosa</i> strain	08/90	Pretzlaff Pond AB, Canada
CPCC 461	<i>M. flos-quae</i> strain	08/75	Fox River WI, USA
CPCC 632	<i>M. aeruginosa</i> strain	10/48	Lake Mendota WI, USA
CPCC 633	<i>M. aeruginosa</i> strain	10/54	Little Rideau Lake, ON Canada
(1)	Water	08/19	Wildwood Conservation
(2)	Water	08/19	Scugog Lake
(3)	Bloom	09/19	Lac Breton
(4)	Bloom	10/13	Lake Land
(5)	Bloom	06/13	Lake Land
(6)	Bloom	06/09	Lake Heney
(7)	Bloom	07/13	Lake Land
(8)	Bloom	06/10	Lake Land
(9)	Bloom	06/18	Lake Land

3.2.1 Semi-targeted analysis: Microcystins

The information gathered from the MS/MS of MC standards provided a diagnostic pair of product ions for the Adda moiety (m/z 135.0803 and m/z 163.1113, Figure 3.15) as well as a diagnostic product ion for the non-proteinaceous amino acid Mdha (m/z 213.0865, Figure 3.15, Table 3.3). The product ions selective for the Adda moiety were then used to screen *Microcystis* extracts and environmental water samples for the presence of Adda containing metabolites (Figure 3.16).

Figure 3.15: MS/MS spectrum of MC-LR (m/z 995.5565 $[M+H]^+$, $C_{49}H_{74}N_{10}O_{12}$) produced by *M. aeruginosa* CPCC 300 grown in BG-11 media (left) and $NaN^{15}NO_3$ -supplemented BG-11 (right), with representative structures of product ions generated from the Adda moiety, m/z 135.0803 and m/z 163.1113. The molecular ion increases by 10 mass units indicating the incorporation of 10 nitrogen atoms into the microcystin scaffold.

Table 3.3: Product ions specific to the Adda moiety present within metabolites of the MC chemical group.

Amino acid	Product ion m/z
Adda	135.0803
Adda	163.1113
Mdha	213.0865

Untargeted MS based metabolomics showed that two of the five investigated *Microcystis* strains produce MCs and are considered “toxic”, *M. aeruginosa* CPCC 299 and CPCC 300 (Figure 3.4, (Racine et al., 2019)). Figure 3.16 contains a diagnostic fragmentation filtering (DFF) plot generated with a search for the characteristic product ions from the Adda moiety within the intracellular extract from *M. aeruginosa* CPCC 300. The series of vertical lines between m/z 950-1000 are the three MCs produced by this strain, $[Asp^3, Dha^7]$ -MC-LR, $[Asp^3]$ -MC-LR, and MC-LR (Figure 3.16). The identity of these congeners was determined with a match of high-resolution precursor ion m/z , product ion m/z , and for $[Asp^3]$ -MC-LR and MC-LR comparison of retention time to a purified standard (Racine et al., 2019). The vertical lines between m/z 450-500 represent

the MS/MS spectra generated from the doubly charged precursor ions of [Asp³]-MC-LR and MC-LR.

Figure 3.16: Diagnostic fragmentation filter plot targeting Adda-containing metabolites produced by *M. aeruginosa* CPCC 300 searching for the product ions m/z 135.0803 and m/z 163.1113, characteristic of the Adda moiety.

This tailored method for the detection of MC metabolites was successfully applied to surface water samples and bloom samples. Five MCs were detected within environmental sample #3, with the identity of three confirmed by comparison to a reference standard (Figure 3.17). The two unknown MCs did not match previously reported MC variants.

Figure 3.17: Representative diagnostic fragmentation filtering plot targeting Adda-containing metabolites within the environmental sample #3 searching for the product ions m/z 135.0803 and m/z 163.1113, characteristic of the Adda moiety.

Table 3.4: Microcystins detected by DFF within *Microcystis* strains and environmental water and bloom samples.

Name	[M+H] ⁺ m/z	Molecular formula	Error (ppm)	RT (min)	<i>M. aeruginosa</i> strain		Environmental sample #		
					CPCC 299	CPCC 300	3	4	5
MC-LR	995.5565	C ₄₉ H ₇₄ N ₁₀ O ₁₂	-1.69	3.29	+	+	+	+	+
[Asp ³]-MC-LR	981.5385	C ₄₈ H ₇₂ N ₁₀ O ₁₂	-0.71	3.40	+	+	+		
[Asp ³ , Dha ⁷]- MC-LR	967.5254	C ₄₇ H ₇₀ N ₁₀ O ₁₂	1.76	3.49	+	+			
MC-LA	910.4923	C ₄₆ H ₆₇ N ₇ O ₁₂	0.28	5.22			+		+
MC 998	999.5635			3.25			+		
MC 1008	1009.5732	C ₅₀ H ₇₆ N ₁₀ O ₁₂	1.49	3.41			+		
MC 1012	1013.5663			3.12			+		

3.2.2 Semi-targeted analysis: Cyanopeptolins

A comprehensive cyanopeptolin sequence database (>230 variants as of August 2020) was generated to investigate the distribution and frequency of residues at different positions within the cyanopeptolin scaffold (Supp. Table S2). Rapid identification of common and rare partial cyanopeptolin sequences was enabled as well as identification of new and known cyanopeptolin

congeners. Most variants possess the characteristic Ahp moiety, with approximately 8% containing the methoxy-Ahp (Amp) moiety at residue 4. 14% of all cyanopeptolin congeners within the compiled database contain the partial sequence NMePhe-Lxx-Ahp, 12% contain NMeTyr-Lxx-Ahp, 4% contain NMePhe-Phe-Ahp, and 20% contain NMeTyr-Phe-Ahp. All cyanopeptolins contain an NMe-aromatic amino acid at residue 2, which generates a characteristic immonium within the MS/MS. Another product ion generated within the MS/MS of cyanopeptolins that provides structural information is the [Residue 3-Ahp-H₂O-CO]⁺ product ion. These two product ions provide structural information regarding the parent molecule. The identity of these diagnostic product ions was confirmed by comparison to a purified reference material (Cyanopeptolin A which contains NMePhe-Leu-Ahp), followed by ¹⁵N-labelling experiments (Figure 3.18). The *Microcystis* strains were grown in Na¹⁵NO₃-supplemented BG-11 media which resulted in incorporation of the isotope within the cyanopeptide scaffold. This allows quantification of the number of nitrogen atoms within a product ion, as the mass difference between the product ion within the unlabelled spectra versus the N¹⁵-labelled spectra equates to the number of nitrogen atoms present (Figure 3.18).

Figure 3.18: 37.5 NCE MS/MS spectra of cyanopeptolin A (left) and nostopeptin BN920 (right) produced by *M. aeruginosa* CPCC 300 and CPCC 632, respectively. MS/MS spectra for each cyanopeptolin are displayed when cyanobacteria were grown in BG-11 (top) and Na¹⁵NO₃ supplemented BG-11 (bottom) media. (Top left) Diagnostic product ions for cyanopeptolin A at m/z 134.0963 and 181.1331 correspond to *MePhe* at position 2 and *Leu-Ahp* at positions 3 and 4, respectively. (Bottom left) Incorporation of ¹⁵N into peptide scaffolds supports these diagnostic product ions. Corresponding *MePhe* and *Leu-Ahp* product ions are observed at m/z 135.0935 and 183.1281. (Top right) Diagnostic product ions for nostopeptin BN920 at m/z 150.0912 and 215.1183 correspond to *MeTyr* at position 2, and *Leu-Ahp* at positions 3 and 4, respectively. (Bottom right) Incorporation of ¹⁵N into peptide scaffolds supports these product ions observed at 151.0883 and 217.1119, that contain 1 and 2 nitrogen atoms, respectively.

This diagnostic information regarding the partial amino acid sequence and the frequency within cyanopeptolin congeners, allows for rapid identification and classification of the metabolite. With a semi-targeted approach, all cyanopeptolins within a *Microcystis* extract can be identified by searching MS/MS datasets for pairs of abundant product ions corresponding to specific partial amino acid sequences (Table 3.5).

Table 3.5: Product ions specific to the partial amino acid sequences present within metabolites of the cyanopeptolin chemical class.

Partial sequence	Residue 2 immonium ion	<i>m/z</i>	[Residue 3-Ahp–H ₂ O–CO] ⁺ fragment	<i>m/z</i>
NMePhe-Lxx-Ahp	NMePhe	134.0963	Lxx-Ahp	181.1331
NMeTyr-Lxx-Ahp	NMeTyr	150.0912	Lxx-Ahp	181.1331
NMePhe-Phe-Ahp	NMePhe	134.0963	Phe-Ahp	215.1183
NMeTyr-Phe-Ahp	NMeTyr	150.0912	Phe-Ahp	215.1183
NMe-Cl-Tyr-Lxx-Ahp	NMe-Cl-Tyr	184.0523	Lxx-Ahp	181.1331
NMe-Cl-Tyr-Phe-Ahp	NMe-Cl-Tyr	184.0523	Phe-Ahp	215.1183

In this study, it was determined that four of the five investigated *Microcystis* strains produced cyanopeptolins using the product ion information collected regarding the partial amino acid sequences. The data generated with this semi-targeted approach agrees with the untargeted data generated via the GNPS molecular networking (Figure 3.3). The four *M. aeruginosa* strains (CPCC 299, CPCC 300, CPCC 632, and CPCC 633) produce different mixtures of cyanopeptolin congeners with varying partial amino acid sequences (Table 3.6). *M. aeruginosa* CPCC 300 is the only strain to product congeners with the partial sequence NMePhe-Lxx-Ahp, including previously reported congeners cyanopeptolins A-C and cyanopeptolin 963A (Cornel et al., 1993, Bister et al., 2004). *M. aeruginosa* CPCC 299 and CPCC 633 produced cyanopeptolins with the exclusive partial amino acid NMePhe-Phe-Ahp. *M. aeruginosa* CPCC 632 produces congeners containing the partial amino acid sequence NMeTyr-Phe-Ahp and NMe-Cl-Tyr-Phe-Ahp, including nostopeptin BN920 and the chlorinated counterpart cyanopeptolin 954 (Figure 3.19, Von Elert et al., 2005).

Figure 3.19: Representative DFF plots for cyanopeptolins containing *NMe*-Phe-Lxx produced by *M. aeruginosa* CPCC 300 (top) and *NMe*-Tyr-Phe containing cyanopeptolins produced by *M. aeruginosa* CPCC 632 (bottom). Red horizontal lines are indicative of diagnostic product ions required to be considered cyanopeptolins with designated residues at position 2 and 3. The black solid line represents $[M+H]^+$ or $[M+H - H_2O]^+$ precursor ions as designated in Table 2. Each vertical dot represents product ions detected within the MS/MS spectra of corresponding cyanopeptolin precursor ions.

The tailored semi-targeted approach for identification of cyanopeptolin congeners was applied to surface water and bloom samples collected in the National Capital Region and the Greater Toronto Area (Table 3.2). The samples were screened for cyanopeptolins with a search of all MS/MS spectra for product ion pairs relating to the different partial amino acid sequences that are possible. Fifteen cyanopeptolins, including eight new variants, were detected from the investigated environmental samples and each contained different cyanopeptolin profiles (Figure 3.20, Table 3.7). The cyanopeptolin profiles for the investigated environmental samples are different

suggesting multiple populations of cyanobacteria were residing within the samples water. Previously reported cyanopeptolins that were detected include cyanopeptolin S, cyanopeptolin 972C, micropeptin MZ845, and cyanopeptolin 1013 (McDonald et al., 2020). Cyanopeptolin 954 and the de-chloro version, nostopeptin BN920 were found in both the environmental samples as well as the *Microcystis* extracts (Figure 3.5, Figure 3.20). Of the eight new variants, one variant was detected and is believed to be the smallest cyanopeptolin reported to date with a m/z 733.3880 $[M+H-H_2O]^+$. Many important bloom-forming genera including *Microcystis*, *Planktothrix*, *Dolichospermum*, and *Woronichinia* can all biosynthesize cyanopeptolins resulting in complex cyanopeptolin mixtures with a wide variety of partial amino acid sequences (McDonald et al., 2020).

Figure 3.20: Representative cyanopeptolin DFF plots for water samples 3 (left) and 4 (right). DFF plots for cyanopeptolins containing the partial sequence *NMe*-Tyr-Lxx-Ahp (left) and the partial sequence Cl-*NMe*-Tyr-Phe-Ahp (right).

Table 3.6: Cyanopeptolins detected by DFF from *Microcystis aeruginosa* strains and bloom samples.

Name	[M+H] ⁺ m/z	Molecular formula	Error (ppm)	Rt (min)	Residue 2	Residue 3	<i>M. aeruginosa</i> strain				Environmental sample #				Reference
							CPCC 299	CPCC 300	CPCC 632	CPCC 633	(3)	(4)	(6)	(7)	
Cyp 954B	937.4441 [M+H- H ₂ O] ⁺	C ₄₉ H ₆₂ N ₈ O ₁₂	-1.41	3.92	NMe- Phe	Phe	+								This study
Cyp 954C	955.4556 [M+H] ⁺	C ₄₉ H ₆₂ N ₈ O ₁₂	-0.41	3.31	NMe- Phe	Phe	+								This study
Cyp 938	921.4494 [M+H- H ₂ O] ⁺	C ₄₉ H ₆₂ N ₈ O ₁₁	-1.21	4.35	NMe- Phe	Phe	+								This study
Cyp 922	905.4213 [M+H- H ₂ O] ⁺	C ₄₈ H ₅₈ N ₈ O ₁₁	1.94	3.99	NMe- Phe	Phe	+								This study
Cyp 904	887.4652 [M+H- H ₂ O] ⁺	C ₄₆ H ₆₄ N ₈ O ₁₁	-1.08	4.16	NMe- Phe	Phe	+		+						This study
Cyp 918	901.4815 [M+H- H ₂ O] ⁺	C ₄₇ H ₆₆ N ₈ O ₁₁	-0.35	4.48	NMe- Phe	Phe	+			+					This study
Cyanopeptolin A	957.5391 [M+H] ⁺	C ₄₆ H ₇₂ N ₁₀ O ₁₂	-1.35	3.39	NMe- Phe	Leu		+							Martin et al. 1993
Cyanopeptolin B	929.5327 [M+H] ⁺	C ₄₆ H ₇₂ N ₈ O ₁₂	-1.66	3.32	NMe- Phe	Leu		+							Martin et al. 1993
Cyanopeptolin C	943.5481 [M+H] ⁺	C ₄₇ H ₇₄ N ₈ O ₁₂	-1.90	3.72	NMe- Phe	Leu		+							Martin et al. 1993
Cyp 900	901.5031 [M+H] ⁺	C ₄₄ H ₆₈ N ₈ O ₁₂	0.17	3.16	NMe- Phe	Lxx		+							This study
Cyp 914	915.5178 [M+H] ⁺	C ₄₅ H ₇₀ N ₈ O ₁₂	-0.86	3.18	NMe- Phe	Lxx		+							This study
Cyp 974	975.5527 [M+H] ⁺	C ₄₆ H ₇₂ N ₁₀ O ₁₃	1.75	4.11	NMe- Phe	Lxx		+							This study
Cyo 945	946.4917 [M+H] ⁺	C ₄₉ H ₆₇ N ₇ O ₁₂	-0.36	6.04	NMe- Phe	Lxx		+							This study
Cyp 970	971.5565 [M+H] ⁺	C ₄₇ H ₇₄ N ₁₀ O ₁₂	0.46	4.15	NMe- Phe	Lxx		+							This study
Cyp 1028	1029.5631 [M+H] ⁺	C ₄₉ H ₇₆ N ₁₀ O ₁₄	1.54	4.08	NMe- Phe	Lxx		+							This study
Cyanopeptolin 963A	964.5040 [M+H] ⁺	C ₄₉ H ₆₉ N ₇ O ₁₃	1.53	5.53	NMe- Phe	Leu		+							Bister et al. 2004
Cyp 913	914.4673 [M+H] ⁺	C ₄₈ H ₆₃ N ₇ O ₁₁	1.59	5.53	NMe- Phe	Lxx		+							This study
Cyp 977	960.5074 [M+H- H ₂ O] ⁺	C ₅₀ H ₆₉ N ₇ O ₁₂	-0.45	5.58	NMe- Phe	Lxx		+							This study
Cyp 984	985.5724 [M+H] ⁺	C ₄₈ H ₇₆ N ₁₀ O ₁₂	0.76	4.61	NMe- Phe	Lxx		+							This study
Micropeptin KB928	929.5091 [M+H] ⁺	C ₄₄ H ₆₈ N ₁₀ O ₁₂	0.01	3.28	NMe- Phe	Ile		+							Elkobi- Peer & Carmeli, 2015
Nostopeptin BN920	903.4587 [M+H- H ₂ O] ⁺	C ₄₆ H ₆₄ N ₈ O ₁₂	0.10	3.25	NMe- Tyr	Phe			+			+			von Elert et al. 2005
Cyp 934	917.4752 [M+H- H ₂ O] ⁺	C ₄₇ H ₆₆ N ₈ O ₁₂	-0.91	3.43	NMe- Tyr	Phe			+						This study
Cyp 948	931.4928 [M+H- H ₂ O] ⁺	C ₄₈ H ₆₈ N ₈ O ₁₂	0.51	3.68	NMe- Tyr	Phe			+						This study
Cyp 976	959.5237 [M+H- H ₂ O] ⁺	C ₅₀ H ₇₂ N ₈ O ₁₂	0.01	4.58	NMe- Tyr	Phe			+			+			This study

Cyp 750	733.3880 [M+H- H ₂ O] ⁺	C ₃₄ H ₅₄ N ₈ O ₁₁	0.11	3.44	NMe- Tyr	Phe			+		+				This study
Cyp 990	973.4431 [M+H] ⁺	C ₄₆ H ₆₅ N ₈ O ₁₃ Cl	-0.19	3.48	Cl- NMe- Tyr	Phe			+						This study
Cyanopeptolin 954	937.4201 [M+H- H ₂ O] ⁺	C ₄₆ H ₆₃ N ₈ O ₁₂ Cl	-0.44	3.51	Cl- NMe- Tyr	Phe			+			+			von Elert et al. 2005
Cyp 968	951.4388 [M+H- H ₂ O] ⁺	C ₄₇ H ₆₅ N ₈ O ₁₂ Cl	-1.88	3.90	Cl- NMe- Tyr	Phe			+						This study
Cyp 982	965.4525 [M+H- H ₂ O] ⁺	C ₄₈ H ₆₇ N ₈ O ₁₂ Cl	1.19	4.02	Cl- NMe- Tyr	Phe			+						This study
Cyp 1010	993.4856 [M+H- H ₂ O] ⁺	C ₅₀ H ₇₁ N ₈ O ₁₂ Cl	0.08	4.97	Cl- NMe- Tyr	Phe			+						This study
Cyp 932	915.4972 [M+H- H ₂ O] ⁺	C ₄₈ H ₆₈ N ₈ O ₁₁	-0.29	4.85	NMe- Phe	Phe				+					This study
Cyp 946	929.5119 [M+H- H ₂ O] ⁺	C ₄₉ H ₇₀ N ₈ O ₁₁	-1.39	5.18	NMe- Phe	Phe				+					This study
Cyo 950	933.4516 [M+H- H ₂ O] ⁺	C ₅₀ H ₆₂ N ₈ O ₁₁	1.22	4.47	NMe- Phe	Phe				+					This study
Micropeptin 996	979.4930 [M+H- H ₂ O] ⁺	C ₅₂ H ₆₈ N ₈ O ₁₂	0.73	4.79	NMe- Phe	Phe				+					Strangman et al. 2018
Cyp 980	963.4964 [M+H- H ₂ O] ⁺	C ₅₂ H ₆₈ N ₈ O ₁₁	-1.04	5.31	NMe- Phe	Phe				+					This study
Cyp 1010B	993.5074 [M+H- H ₂ O] ⁺	C ₅₃ H ₇₀ N ₈ O ₁₂	-0.63	4.96	NMe- Phe	Phe				+					This study
Cyp 968B	951.4596 [M+H- H ₂ O] ⁺	C ₅₀ H ₆₄ N ₈ O ₁₂	-1.55	3.96	NMe- Phe	Phe				+					This study
Cyp 982B	965.4755 [M+H- H ₂ O] ⁺	C ₅₁ H ₆₆ N ₈ O ₁₂	-1.27	4.42	NMe- Phe	Phe				+					This study
Cyp 1109	1110.5499 [M+H] ⁺	C ₅₇ H ₇₅ N ₉ O ₁₄	-0.65	3.21	NMe- Tyr	Lxx					+				This study
Cyp 1143	1126.5455 [M+H- H ₂ O] ⁺	C ₅₇ H ₇₇ N ₉ O ₁₆	-0.04	3.44	NMe- Tyr	Phe					+				This study
Cyp 1157	1140.5618 [M+H- H ₂ O] ⁺	C ₅₈ H ₇₉ N ₉ O ₁₆	0.53	3.58	NMe- Tyr	Phe					+				This study
Cyp 1051	1034.4831 [M+H- H ₂ O] ⁺	C ₅₀ H ₆₉ N ₉ O ₁₆	0.15	3.08	NMe- Tyr	Phe					+				This study
Cyanopeptolin 972C	973.5596 [M+H] ⁺	C ₄₈ H ₇₆ N ₈ O ₁₃	-0.85	3.41	NMe- Tyr	Lxx						+		+	Welker et al. 2004
Micropeptin SD944	945.5306 [M+H] ⁺	C ₄₆ H ₇₂ N ₈ O ₁₃	1.69	2.92	NMe- Tyr	Lxx						+			Reshef & Carmeli, 2001
Cyp 1017	1018.5230 [M+H] ⁺	C ₅₀ H ₇₅ N ₉ O ₁₅	1.25	3.37	NMe- Tyr	Phe						+			This study
Cyp 1013	996.4728 [M+H- H ₂ O] ⁺	C ₄₇ H ₆₇ N ₉ O ₁₆	1.40	4.36	NMe- Tyr	Phe						+			This study
Cyp 1017B	1000.5035 [M+H- H ₂ O] ⁺	C ₅₃ H ₆₅ N ₁₁ O ₉	0.26	4.08	NMe- Tyr	Phe						+	+		This study
Cyanopeptolin S	926.4283 [M+H] ⁺	C ₄₀ H ₆₃ N ₉ O ₁₄ S	-0.53	3.34	NMe- Phe	Ile						+	+		Jakobi et al. 1995

Micropeptin MZ845	828.4611 [M+H- H ₂ O] ⁺	C ₄₀ H ₆₃ N ₉ O ₁₁	-0.79	3.23	NMe- Phe	Ile						+	+		Zafrir E & Carmeli, 2010
Cyp 1051	1034.4633 [M+H- H ₂ O] ⁺	C ₅₃ H ₆₆ N ₁₁ O ₁₀ Cl	-1.54	4.48	Cl- NMe- Tyr	Phe						+	+		This study

3.2.3 Semi-targeted analysis: Aeruginosins

The aeruginosin chemical group is characterized by the presence of the Choi functionality.

Diagnostic product ions are generated from the moiety, [Choi+H]⁺ m/z 140.1066 and [Choi – H₂O + H]⁺ at m/z 122.0964 (Table 3.7, Figure 3.21, (Welker et al., 2006)). The structure of these diagnostic product ions was confirmed with N¹⁵-labelling experiments (Figure 3.21).

Figure 3.21: MS/MS spectra of aeruginosin 688 produced by *M. aeruginosa* CPCC 300. MS/MS spectra for the aeruginosin congener are displayed when cyanobacteria were grown in BG-11 (left) and Na¹⁵NO₃ supplemented BG-11 (right) media. (Left) Diagnostic product ions for aeruginosin 688 (m/z 689.2958 [M+H]⁺; C₃₂H₄₄N₆O₉S) at m/z 140.1099 and m/z 122.0964 correspond to the [Choi + H]⁺ and [Choi – H₂O + H]⁺, respectively. Incorporation of ¹⁵N into peptide scaffolds supports these diagnostic product ions. Corresponding [Choi + H]⁺ and [Choi – H₂O + H]⁺ product ions are observed at m/z 141.1029 and m/z 123.0936 that contain 1 nitrogen atom each.

Table 3.7: Product ions specific to the Choi moiety present within metabolites of the aeruginosin chemical class.

Moiety	Product ion m/z
Choi	140.1066
Choi-H ₂ O	122.0964

A second diagnostic feature for aeruginosins is the occurrence of sulfated congeners. Sulfonation is a common chemical modification that occurs on the Choi moiety in many aeruginosins (Welker et al., 2006). If the sulfated and de-sulfated counterpart had the same retention time, it was assumed that this was generated as an in-source fragment and indicates the organism only produces the sulfated metabolite. If the retention times were different, it was assumed that both metabolites were present within the extract. Based on the molecular network, *M. aeruginosa* CPCC 300 and CPCC 632 were screened as aeruginosin producers with the DFF approach for any outstanding metabolites containing the Choi functionality.

Figure 3.22: DFF plot showing Choi containing cyanopeptides produced by *M. aeruginosa* CPCC 632 grown in BBM medium with the product ions m/z 140.1066 and m/z 122.0964.

The DFF of *M. aeruginosa* CPCC 632 shows a search for the pair of diagnostic product ions at m/z 140.1066 and 122.0964 and depicts eight potential aeruginosin metabolites with no metabolite matching literature reports or cyanopeptide databases (Table 3.8). Five of these metabolites display a product ion with a neutral loss of 79.9561 suggesting the loss of a sulfate group, which is depicted by the blue diagonal line (Figure 3.22). The data points directly below the point for the neutral loss

of the sulfate, is the neutral loss of water or dehydration $[M+H - H_2O - SO_3]^+$. The aeruginosins produced by *M. aeruginosa* CPCC 632 are further classified as containing a Tyr or a Phe at residue 2 based on the presence of the immonium ions for Tyr (m/z 136.0756) or Phe (m/z 120.0807) within their MS/MS spectra (Figure 3.21). Chlorination of the Hpla amino acid within aeruginosin metabolites was confirmed via the mass defect of chlorine, visible within the high-resolution mass of the precursor ion as well as by isotopic abundance of the M+2 peaks.

A search for the diagnostic product ions generated for the Choi moiety within the MS/MS data for a *M. aeruginosa* CPCC 633 extract resulted in seven aeruginosin-like metabolites with no previous reports of these compounds within the literature (Table 3.8). This strain shares the production of aeruginosin 688 and aeruginosin 608 with *M. aeruginosa* CPCC 632. The aeruginosin like compounds produced by *M. aeruginosa* CPCC 633 seem to have substitutions of Phe or Lxx within position 2. Interpretation of the MS/MS data for these compounds revealed the characteristic Choi product ions and one of the following immonium ions: Phe (m/z 120.0807) or Lxx (m/z 86.0969). Similar to *M. aeruginosa* CPCC 632, chlorination of the Hpla amino acid was deemed possible with no clear pattern.

Figure 3.23: Diagnostic fragmentation filter plot showing Choi containing metabolites found in bloom samples 5 (left) and 8 (right), with the product ion m/z 140.1066 ($[Choi + H]^+$) and 122.0964 ($[Choi-H_2O+H]^+$).

This semi-targeted approach for the detection of Choi-containing molecules is effective within surface water samples (Figure 3.23). Six potential aeruginosins were detected using a search for the pair of diagnostic product ions specific to the Choi functionality (Table 3.7). A similar pattern of $[M+H]^+$ and $[M+H-SO_3]^+$ was observed. Further, some metabolites precursor ions have isotopic patterns suggesting chlorination. This alongside the presence of the two characteristic product ions of the Choi moiety, suggests the compounds found within this environmental samples are aeruginosins. Majority of the samples, cultured *Microcystis* strains included, share the two potential aeruginosins, As 688 (m/z 689.2958 $[M+H]^+$) and As 722 (m/z 723.2574 $[M+H]^+$). The profiles of aeruginosins are not consistent between the six environmental samples screened (Figure 3.23, Table 3.8). These differences suggest multiple cyanobacterial colonies resided within the water where the samples were collected.

Table 3.8: Chemical information of the Choi-containing metabolites produced by *M. aeruginosa* CPCC 300 and CPCC 632 and found within environmental water and bloom samples detected by DFF.

Name	$[M+H]^+$ m/z	Molecular formula	Error (ppm)	RT (min)	Residue 1	Residue 2	<i>M. aeruginosa</i> strains		Environmental sample #					
							CPCC 300	CPCC 632	1	2	4	5	8	9
As 608	609.3397	C ₃₂ H ₄₄ N ₆ O ₆	-1.68	2.92	Hpla	Phe	+	+	-	-	+	+	-	+
As 650	651.3139	C ₃₃ H ₄₂ N ₆ O ₆	0.70	2.40	Hpla	Tyr	+	-	-	-	-	+	-	-
As 684	685.2758	C ₃₃ H ₄₁ N ₆ O ₆ Cl	0.63	2.32	Cl-Hpla	Tyr	+	-	-	-	-	-	-	-
As 688	689.2958	C ₃₂ H ₄₄ N ₆ O ₉ S	-1.34	2.45	Cl-Hpla	Phe	+	+	+	-	+	+	+	+
As 724	725.2709	C ₃₅ H ₄₁ N ₆ O ₉ Cl	1.31	2.55	Cl-Hpla	Tyr	+	-	-	+	-	-	-	-
As 574	575.3543	C ₂₉ H ₄₆ N ₆ O ₃	-0.61	2.41	Hpla	Lxx	-	+	-	-	-	+	-	-
As 642	643.2984	C ₃₂ H ₄₃ N ₆ O ₆ Cl	-1.38	2.71	Cl-Hpla	Phe	-	+	-	-	-	-	-	-
As 654	655.3111	C ₂₉ H ₄₆ N ₆ O ₉ S	0.22	2.39	Hpla	Lxx	-	+	-	-	-	-	-	-
As 703	704.3313	C ₄₀ H ₄₃ N ₆ O ₆	-0.02	2.31	Hpla	Phe	-	+	-	-	-	-	-	-
As 722	723.2574	C ₃₂ H ₄₃ N ₆ O ₉ SCl	-1.73	2.58	Cl-Hpla	Phe	-	+	+	-	+	+	+	+
As 602	603.3499	C ₃₀ H ₄₆ N ₆ O ₇	0.38	2.31	Hpla	Lxx	-	-	+	-	-	-	-	-
As 634	635.3775	C ₃₁ H ₅₀ N ₆ O ₈	-0.43	2.30	Hpla	Lxx	-	-	+	-	-	-	-	-
As 652	653.3052	C ₃₀ H ₄₅ N ₆ O ₈ Cl	-0.84	2.47	Cl-Hpla	Lxx	-	-	-	+	-	-	-	-
As 624	625.3343	C ₃₂ H ₄₄ N ₆ O ₇	0.05	2.40	Cl-Hpla	Phe	-	-	-	-	-	+	-	-
As 656	657.3256	C ₃₂ H ₄₄ N ₆ O ₉	-0.64	2.34	Hpla	Lxx	-	-	-	-	-	+	+	-
As 642*	643.3016	C ₃₂ H ₄₄ N ₆ O ₆ Cl	0.18	2.54	Cl-Hpla	Phe	-	-	-	-	+	-	+	-

3.2.4 Semi-targeted analysis: Microginins

The structural variability of microginin congeners arises not only with the amino acid sequence but with the chain length, methylation, and chlorination of the N-terminal PKS aliphatic chain (Strangman & Wright, 2016; Welker et al., 2006). The fragmentation of the N-terminus and thus the aliphatic portion of these molecules results in diagnostic product ions that are distinguishable from the immonium ions of proteinaceous amino acids (Table 3.9, Figure 3.24). The formula for the diagnostic product ions is confirmed with inspection of ^{15}N -labelled experiments (Figure 3.24). The N-terminal moiety may be an octanoic acid derivative (Ahoa) or a decanoic acid derivative, which is added on with a PKS synthase. Methylation of the N-terminal polyketide chain on the amino moiety can occur and results in an entirely different set of microginin metabolites. This chain may be halogenated on the terminal carbon, resulting in non-, mono-, di-, and tri-halogenated congeners which can be deduced by investigation of the isotope patterns of the parent ions within the MS/MS (Figure 3.7). Multiple chlorination states were visible within the MS/MS as a mass difference of 1 chlorine atom ($\Delta 33.9606$) when comparing product ions within the non-chlorinated form and the mono-chlorinated form.

Figure 3.24: MS/MS spectra for microginin 646 are displayed when cyanobacteria were grown in BG-11 (left) and $\text{Na}^{15}\text{NO}_3$ supplemented BG-11 (right) media. (Top left) Diagnostic product ions for microginin 646 at m/z 150.0910 and m/z 134.0727 correspond to the NMeTyr and Cl-Ahoa, respectively. Incorporation of ^{15}N into peptide scaffolds supports these diagnostic product ions. Corresponding NMeTyr and Cl-Ahoa product ions are observed at m/z 151.0869 and m/z 135.0684 that contain 1 nitrogen atom each.

Table 3.9: Product ions specific to the variable N-terminal polyketide moiety present within metabolites of the microginin chemical class.

Amino acid sequence	Product ion m/z		
	Non-chlorinated	Mono-chlorinated	Di-chlorinated
Ahoa	100.1122	134.0727	168.0334
NMe-Ahoa	114.1278	148.0883	182.0483
Ahda	128.1433	162.1039	196.0639
NMe-Ahda	142.1589	176.1195	210.0795

To conduct a search for microginins within *Microcystis* extracts, one product ion characteristic of the N-terminal polyketide moiety is used as a pair of diagnostic product ions could not be established. Using all possible N-terminal polyketide moiety product ions described in Table 3.9, a search for all microginins was performed and compiled into one plot (Figure 3.25). This encapsulated the four different chain types (Ahoa, NMeAhoa, Ahda, NMeAhda) and the three different chlorination states (non-, mono-, di-). 2 *M. aeruginosa* strains CPCC 632 and CPCC 633 produce different mixtures of microginins, with the two previously reported congeners microginin

646 and microginin 680 produced by both strains and the congener microginin 613 exclusively produced by *M. aeruginosa* CPCC 633 (Table 3.10, Strangman & Wright, 2016).

Figure 3.25: Diagnostic fragmentation filter plot showing microginins produced by *M. aeruginosa* CPCC 633 when searching for the different product ions that correlate to the N-terminal polyketide moiety. Colours are used to display the variable chain length (Ahoa, NMeAhoa, Ahda, NMeAhda) and shape to designate the chlorination state (non-, mono-, di-).

M. aeruginosa CPCC 632 produced eight microginin congeners, two are non-chlorinated, four are chlorinated, and two are di-chlorinated (Table 3.10). All contain the Ahoa N-terminal chain with two congeners methylated on the amino group. *M. aeruginosa* CPCC 633 produces fifteen microginin congeners, four are non-chlorinated, four are mono-chlorinated, and seven are di-chlorinated (Table 3.10). This strain produces ten congeners with the Ahoa N-terminal polyketide moiety with one being methylated on the amino group and it produces five congeners with the Ahda N-terminal polyketide moiety.

This screening technique for microginin congeners was performed on MS/MS data sets generated from environmental samples collected in the National Capital Region (Figure 3.25, Table 3.10).

Figure 3.25: Representative diagnostic fragmentation filter plots of microginins detected within environmental samples 4 (left) and 8 (right) when searching for the different product ions that correlate to the N-terminal polyketide moiety. Colours are used to display variable chain length and substitution (Ahoa, NMeAhoa, Ahda, NMeAhda) and shape to designate the chlorination state (non-, mono-, di-).

The microginin profile for the six investigated surface water and bloom samples were diverse suggesting multiple cyanobacterial populations growing within the sampled area. 28 microginin congeners were detected with no overlap in the metabolites identified by the two *M. aeruginosa* strains studied (CPCC 632 and CPCC 633). Of the twenty-eight microginins identified within the environmental samples, six have been previously reported, microginin KR 604, microginin 551, microginin 607B, microginin 621B, microginin FR12, and microginin 478 (Stewart et al., 2018). Thirteen of the detected congeners contain an Ahda N-terminal polyketide moiety, nine contain an NMeAhda moiety, and six contain the Ahoa N-terminal polyketide moiety. Five of these detected microginins were mono-chlorinated and only one congener was di-chlorinated.

Table 3.10: Chemical information of the microginins produced by *M. aeruginosa* CPCC 632 and CPCC 633 and found in surface water and bloom samples.

						<i>M. aeruginosa</i> strains		Environmental sample #					
Name	[M+H] <i>m/z</i>	Molecular formula	Error (ppm)	RT (min)	R1	CPCC 632	CPCC 633	1	2	4	5	8	9
Microginin 626	627.3375	C ₃₃ H ₄₆ N ₄ O ₈	-2.11	2.77	Ahoa	+	+	-	-	-	-	-	-
Microginin 640	641.3535	C ₃₄ H ₄₈ N ₄ O ₈	-1.62	2.75	NMeAhoa	+	-	-	-	-	-	-	-
Microginin 644	645.3035	C ₃₃ H ₄₅ ClN ₄ O ₇	-2.25	2.71	Cl Ahoa	+	-	-	-	-	-	-	-
Microginin 646	647.2828	C ₃₂ H ₄₃ ClN ₄ O ₈	-2.22	2.65	Cl Ahoa	+	+	-	-	-	-	-	-
Microginin 660	661.2982	C ₃₃ H ₄₅ ClN ₄ O ₈	-2.49	2.97	Cl Ahoa	+	-	-	-	-	-	-	-
Microginin 674	675.3146	C ₃₄ H ₄₇ N ₄ O ₈ Cl	-1.29	2.73	Cl NMe- Ahoa	+	-	-	-	-	-	-	-
Microginin 680	681.2446	C ₃₂ H ₄₂ Cl ₂ N ₄ O ₈	-0.99	2.72	Cl ₂ Ahoa	+	+	-	-	-	-	-	-
Microginin 694	695.2602	C ₃₃ H ₄₄ Cl ₂ N ₄ O ₈	-1.01	2.72	Cl ₂ Ahoa	+	+	-	-	-	-	-	-
Microginin 584	585.2941	C ₃₀ H ₄₀ O ₈ N ₄	1.95	2.36	Cl ₂ Ahda	-	+	-	-	-	-	-	-
Microginin 598	599.3084	C ₃₁ H ₄₂ O ₈ N ₄	-0.48	2.45	Cl ₂ Ahda	-	+	-	-	-	-	-	-
Microginin 612	613.3225	C ₃₂ H ₄₄ N ₄ O ₈	-1.11	2.53	Ahoa	-	+	-	-	-	-	-	-
Microginin 628	629.3184	C ₃₂ H ₄₄ N ₄ O ₉	0.41	2.47	Ahoa	-	+	-	-	-	-	-	-
Microginin 630	631.288	C ₃₂ H ₄₃ N ₄ O ₇ Cl	-2.03	2.85	Cl Ahoa	-	+	-	-	-	-	-	-
Microginin 640B	641.3536	C ₃₄ H ₄₈ N ₄ O ₈	-1.39	2.89	Ahda	-	+	-	-	-	-	-	-
Microginin 642	643.2974	C ₃₂ H ₄₂ O ₁₀ N ₄	-1.62	2.32	Cl ₂ Ahda	-	+	-	-	-	-	-	-
Microginin 660B	661.2989	C ₃₃ H ₄₅ ClN ₄ O ₈	-1.48	2.65	Cl NMe- Ahoa	-	+	-	-	-	-	-	-
Microginin 664	665.2488	C ₃₂ H ₄₂ N ₄ O ₇ Cl ₂	-2.33	2.92	Cl ₂ Ahoa	-	+	-	-	-	-	-	-
Microginin 666	667.2219	C ₃₁ H ₄₀ N ₄ O ₈ Cl ₂	*	2.65	Cl ₂ Ahoa	-	+	-	-	-	-	-	-
Microginin 674B	675.3149	C ₃₄ H ₄₇ N ₄ O ₈ Cl	-0.94	2.81	Cl Ahda	-	+	-	-	-	-	-	-
Microginin 527	528.2855	C ₂₉ H ₄₀ O ₃ N ₄ Cl	-1.34	2.98	Cl-Ahoa	-	-	+	+	-	-	-	-
Microginin 507	508.3381	C ₂₇ H ₄₆ O ₆ N ₃	-0.08	3.16	NMe-Ahda	-	-	-	+	+	-	-	-
Microginin KR604	605.3907	C ₃₂ H ₅₂ N ₄ O ₇	-0.31	2.85	NMe-Ahda	-	-	-	+	+	+	-	-
Microginin 608	609.3856	C ₃₁ H ₅₂ O ₈ N ₄	-0.28	2.94	NMe-Ahda	-	-	-	+	-	-	-	-
Microginin 654B	655.4064	C ₃₆ H ₅₄ N ₄ O ₇	-0.24	3.1	Ahda	-	-	-	+	-	-	-	-
Microginin 656	657.3853	C ₃₅ H ₅₂ O ₈ N ₄	-0.82	3.05	Ahda	-	-	-	+	-	-	-	-
Microginin 670	671.4014	C ₃₆ H ₅₄ N ₄ O ₈	-0.02	3.12	Ahda	-	-	-	+	-	-	-	-
Microginin 684	685.4189	C ₃₇ H ₅₆ N ₄ O ₈	2.63	3.9	NMe-Ahda	-	-	-	+	-	-	-	-
Microginin 690	691.3467	C ₃₅ H ₅₁ O ₈ N ₄ Cl	-0.05	2.99	Cl-Ahda	-	-	-	+	-	-	-	-
Microginin 700	701.4116	C ₃₇ H ₅₆ N ₄ O ₉	-0.63	3.12	NMe-Ahda	-	-	-	+	-	-	-	-
Microginin 514	515.3442	C ₂₅ H ₄₆ O ₇ N ₄	0.61	2.77	Ahoa	-	-	-	-	-	+	+	+
Microginin 516	517.3157	C ₂₅ H ₄₅ O ₅ N ₄ Cl	1.05	2.81	Cl Ahda	-	-	-	-	-	+	+	-
Microginin 532	533.3101	C ₂₅ H ₄₅ O ₆ N ₄ Cl	0.04	2.73	Cl Ahda	-	-	-	-	+	+	+	+
Microginin 551	551.3438	C ₂₈ H ₄₆ O ₇ N ₄	-0.21	2.73	Ahda	-	-	-	-	+	+	-	-
Microginin 607B	607.4058	C ₃₂ H ₅₄ O ₇ N ₄	-1.16	3.07	Ahda	-	-	-	-	+	+	-	-
Microginin 621B	621.4224	C ₃₃ H ₅₆ O ₇ N ₄	0.29	3.09	NMe-Ahda	-	-	-	-	+	+	+	-
Microginin 661	662.4119	C ₃₄ H ₅₅ O ₈ N ₅	-0.71	2.86	Ahda	-	-	-	-	+	+	-	+
Microginin FR12	784.4857	C ₄₂ H ₆₅ N ₅ O ₉	0.19	3.24	NMe-Ahda	-	-	-	-	+	+	-	-
Microginin 654	655.3699	C ₃₅ H ₅₀ O ₈ N ₄	-0.41	2.65	NMe-Ahda	-	-	-	-	+	-	+	-
Microginin 478	770.4687	C ₄₁ H ₆₃ N ₅ O ₉	-1.51	3.16	Ahda	-	-	-	-	+	-	-	-
Microginin 512	513.3293	C ₂₅ H ₄₄ O ₇ N ₄	2.01	2.37	Ahoa	-	-	-	-	-	-	-	+
Microginin 528	529.3246	C ₂₅ H ₄₄ O ₈ N ₄	2.62	2.35	Ahoa	-	-	-	-	-	-	+	+
Microginin 557	558.3187	C ₃₀ H ₄₃ O ₇ N ₃	2.29	2.73	NMe-Ahda	-	-	-	-	-	-	-	+
Microginin 755	756.3814	C ₃₈ H ₅₃ O ₁₁ N ₅	-0.03	2.92	Ahoa	-	-	-	-	-	-	-	+

Microginin 512B	513.3644	C ₂₆ H ₄₉ O ₆ N ₄	-0.54	2.95	Ahda	-	-	-	-	-	-	+	-
Microginin 548	549.3046	C ₂₅ H ₄₅ O ₇ N ₄ Cl	-0.69	2.69	Cl Ahda	-	-	-	-	-	-	+	-
Microginin 556	557.355	C ₂₇ H ₄₈ O ₈ N ₄	0.87	2.98	Ahoa	-	-	-	-	-	-	+	-
Microginin 566	567.2712	C ₂₅ H ₄₄ O ₆ N ₄ C ₁₂	0.19	2.97	Cl2 Ahda	-	-	-	-	-	-	+	-

3.3 Cyanobacterial growth assessment

Biological indicators (cell count, OD₇₅₀, pH, dried biomass) were monitored to investigate growth and growth rates of four *Microcystis* strains studied in the three culture media BG-11, BBM, and MA. Growth rates were calculated using cell counts after 25 days in all experiments for normalization (Table 3.11). Growth rates remained relatively consistent between BG-11 and BBM culture media for the *M. aeruginosa* strains CPCC 299, CPCC 300, CPCC 632, and CPCC 633, yet increased significantly in BBM for the *M. flos-aquae* strain CPCC 461. In all cases, the growth rate was lowest when grown in MA culture medium (Table 3.11). The major components of BBM and BG-11 media are similar with the largest differences being the concentrations of the essential nutrients, nitrogen and phosphorous. BBM media has a lower concentration of the essential nutrient NaNO₃ (0.75 g L⁻¹) and two sources of phosphorous (KH₂PO₄ and K₂HPO₄), whereas BG-11 has double the concentration of nitrogen (1.5 g L⁻¹) and one source of phosphorous (K₂HPO₄). Another nutrient difference between these two culture media is the iron source which has been shown to impact toxin production and growth rate, BBM has FeSO₄ • 7H₂O and BG-11 has ferric ammonium citrate. MA media has many nutrient differences with BG-11 and BBM, starting with the major nitrogen source being KNO₃ and the major phosphorous source being β-Na₂-glycerophosphate. The source of iron also differs, being FeCl₃ • 6H₂O.

As two of the investigated strains are known MC producers, the phosphatase activity of all *Microcystis* extracts was investigated with a PP2A inhibition assay (Table 3.11). There was no activity from the extracts from *M. aeruginosa* CPCC 632 and CPCC 633. As expected, activity

was seen by the toxic strains *M. aeruginosa* CPCC 299 and CPCC 300, with *M. aeruginosa* CPCC 299 showing a greater activity than *M. aeruginosa* CPCC 300. Unexpectedly, *M. flos-aquae* CPCC 461 showed high inhibition of the phosphatase enzyme with the highest concentration of “MC equivalents” although this strain does not produce MCs nor anabaenapeptins. Investigation into the toxins produced by this *M. flos-aquae* CPCC 461 strain would provide bioactive metabolites against PP2A.

Table 3.11: Strain information and specific growth rates (day^{-1} , μ) at 27°C when grown in BBM, BG-11 and MA.

Strain	Collection data		Specific growth rate, day^{-1} (μ)			PP2A inhibition ([MC] equivalent $\mu\text{g L}^{-1}$)
	Date	Location	BBM	BG-11	MA	
<i>M. aeruginosa</i> CPCC 299	08/90	Pretzlaff Pond AB, Canada	0.240	0.255	0.195	0.71
<i>M. aeruginosa</i> CPCC 300	08/90	Pretzlaff Pond AB, Canada	0.235	0.227	0.316	0.29
<i>M. flos-aquae</i> CPCC 461	08/75	Fox River WI, USA	0.325	0.309	0.103	0.82
<i>M. aeruginosa</i> CPCC 632	10/48	Lake Mendota WI, USA	0.250	0.345	0.139	ND
<i>M. aeruginosa</i> CPCC 633	10/54	Little Rideau Lake, ON Canada	0.260	0.291	0.181	ND

A media composition comparison was performed with four of the five *Microcystis* strains to understand the effects media has on cyanobacterial growth and oligopeptide cyanopeptide production. The two media used in this experiment were BG-11 and MA as they showed major differences in growth rates in preliminary experiments which greatly impact toxin production.

Each strain was inoculated to a cell number on day 0 (high 10^4 -low 10^5 cells mL^{-1}) and biological growth indicators such as cell mL^{-1} and dried biomass, were monitored for triplicate cultures every 5 days until the cells reached mid 10^7 cells mL^{-1} (Figure 3.26).

Figure 3.26: Growth data for *M. aeruginosa* CPCC 300 (top left), *M. flos-aquae* CPCC 461 (top right), *M. aeruginosa* CPCC 632 (bottom left), and *M. aeruginosa* CPCC 633 (bottom right) showing cells mL⁻¹ measurements at each time interval. Orange data points indicate measurements when strains were grown in MA media and blue data points indicate measurements when strains were grown in BG-11 media.

Cell number increased for all *Microcystis* strains studied over the course of 25 days (Figure 3.26). In most cases, cell numbers reached a maximum at day 20 and began to plateau as the cells entered stationary phase. This is well represented with the growth of CPCC 633 (bottom right) as the peak cell number is day 20 in both media and either decreases or stays the same in the final reading on day 25 (Figure 3.26). All strains entered the stationary phase within the range of 3.12×10^7 – 5.24×10^7 cells mL⁻¹ with this end of exponential growth variable based on the strain and the culture media.

Figure 3.27: pH and optical density at 750 nm for *M. aeruginosa* CPCC 300 (top left), *M. flos-aquae* CPCC 461 (top right), *M. aeruginosa* CPCC 632 (bottom left), and *M. aeruginosa* CPCC 633 (bottom right) grown in BG-11 (orange) and MA (blue) growth media. pH data points are represented by circles and optical density data points are represented with squares.

The pH of the culture media BG-11 and MA were adjusted prior to inoculation to 7.5 and 8.6, respectively. There is an increase in the extracellular pH over the 25-day time course for all *Microcystis* cultures (Figure 3.27). The maximum pH for each culture occurred at the end of the exponential phase of growth, with a decrease as the cells progressed in the stationary phase. In all cases, the pH at day 25 was greater when the strains were grown in BG-11 media versus MA. Chlorophyll-a absorbance at 750 nm was utilized as a proxy of bacterial growth. Optical density of the liquid cultures increased in all cases as the cells replicated exponentially.

3.4 Investigation of cyanopeptide production by *Microcystis* strains

With the untargeted MS metabolomics approach, we can view the entire chemical profile of a strain and compare in depth any changes that may occur due to varying growth conditions. The untargeted data presented within the PCA, loading plot, and molecular networking analysis provided identification of cyanopeptides specific to the different *Microcystis* strains. An understanding of their unique metabolomes allows for investigation of compounds changes when growth conditions are varied. With intracellular extracts at time points along the established growth curve, the production of each cyanopeptide is monitored with normalized peak area to compare relative cyanopeptide amounts. For the MC producer, *M. aeruginosa* CPCC 300, intracellular MC concentrations can be quantified with reference material. All values are normalized with dried biomass (mg). Results from these studies will help determine the optimal conditions for the production of understudied cyanopeptide groups including cyanopeptolins and microginins.

3.4.1 Microcystin quantification in toxigenic strains

The identification and quantification of MC congeners within the toxic *Microcystis* extracts was simplified with the use of reference materials. Chemical information such as retention time, precursor ion m/z , product ion m/z and fragmentation patterns, as well as pseudomolecular ions were acquired for nine congeners (MC-LR, [Asp³]-MC-LR, MC-RR, MC-LY, MC-LA, MC-WR, MC-YR, MC-LF, NOD) and is summarized in Table 3.2. A standard curve with a concentration range of a mixture of MC standards, quantification of MC congeners is possible.

Table 3.12: Summary of LC-HRMS data for 8 MC congeners and NOD with reference material.

Name	<i>m/z</i> [M+H] ⁺	RT (min)	Formula	Delta (ppm)
MC-LR	995.5565	3.34	C ₄₉ H ₇₄ N ₁₀ O ₁₂	1.76
[Asp ³]-MC-LR	981.5385	3.34	C ₄₈ H ₇₂ N ₁₀ O ₁₂	-0.71
MC-RR	1038.5726	2.78	C ₄₉ H ₇₅ N ₁₃ O ₁₂	-0.47
MC-LY	1002.5176	5.29	C ₅₂ H ₇₁ N ₇ O ₁₃	-0.66
MC-LA	910.4913	5.15	C ₄₆ H ₆₇ N ₇ O ₁₂	-0.82
MC-WR	1068.5509	3.61	C ₅₄ H ₇₃ N ₁₁ O ₁₂	-0.36
MC-YR	1045.5349	3.26	C ₅₂ H ₇₂ N ₁₀ O ₁₃	-0.39
MC-LF	986.5229	5.78	C ₅₂ H ₇₁ N ₇ O ₁₂	-0.45
NOD	825.4496	3.07	C ₄₁ H ₆₀ N ₈ O ₁₀	-1.11

Production for the congener, MC-LR, was quantified with standard curves for the “toxic” strain *M. aeruginosa* CPCC 300 (Table 3.13, Supplementary info XX).

Table 3.13: Intracellular MC-LR levels for *M. aeruginosa* CPCC 300 grown in BBM media at 27°C.

Media	Maximum production	µg MC-LR mg ⁻¹ biomass
BG-11	Day 20	1.91
MA	Day 10	1.83

The MC-LR production over time by *M. aeruginosa* CPCC 300 was monitored in triplicate culture for comparison of media effects on toxin production. *M. aeruginosa* CPCC 300 was grown in the two defined culture media, MA and BG-11, and the normalized amounts (µg MC mg⁻¹ biomass) produced over time are displayed in Figure 3.28.

Figure 3.28: Quantification of intracellular MC-LR ($\mu\text{g MC-LR mg biomass}^{-1}$) production by *M. aeruginosa* CPCC 300 when grown in the two culture media BG-11 (orange) and MA (blue).

M. aeruginosa CPCC 300 is known as a “toxic” *Microcystis* strain as it produces MC congeners. Quantification of the congener, MC-LR, was performed with a standard curve and production was variable between the two growth media, BG-11 and MA (Figure 3.28). The production of these metabolites is correlated with the growth rate and phase of growth. Intracellular MC-LR concentration spikes quickly at day 10 of growth when CPCC 300 was grown in MA and decreases steadily over the remaining time course. The growth curve for CPCC 300 grown in MA reaches the end of the exponential phase and the beginning of the stationary phase between day 10 and 15, suggesting that the majority of toxin production occurs during the late exponential phase of growth. Max MC production is reported to occur by other strains in the late exponential, or early stationary phase (Watanabe et al., 1996). Our data for *M. aeruginosa* CPCC 300 appear to agree with previous findings. Looking at the BG-11 growth curve, the cells enter the stationary phase at day 20 of growth which correlates to the maximum intracellular concentration of MC-LR.

3.4.2 Effects of media composition on the production of cyanopeptides by *Microcystis* strains

The metabolites presented within the molecular network were targeted for comparison of relative amounts (peak area/biomass) when the producing strain was grown in the two media, BG-11 and MA. Heat maps were generated for each strain and their respective metabolites in R software package with their average normalized peak areas using the packages: *gplots* and *colorRamps*. The overall production profile for all selected compounds can be visualized and compared against the other metabolites produced over time and between the two defined culture media. ANOVA pairwise comparison ($p < 0.005$) and Tukey's LSD test (letters) were used to provide statistical evidence of toxin production over time as well as to compare the production levels of metabolites when grown in different culture media. Very few studies have examined the dynamics of non-MC cyanopeptides. These types of studies are complicated by the very few cyanopeptide reference materials available required for identification and quantitation.

3.4.2a Media effects on cyanopeptide production by *M. aeruginosa* CPCC 300

The “toxic” strain *M. aeruginosa* CPCC 300 produced multiple different chemical groups together with MCs such as cyanopeptolins, aeruginosins, and cyanobactins (Figure 3.3). Figure 3.29 demonstrates the relative amounts of specific metabolites and their production profile over 25 days when grown in two different culture media, BG-11 and MA.

Figure 3.29: Heatmap showing cyanopeptide production by *M. aeruginosa* CPCC 300 over time in two growth media (BG-11; left and MA; right), generated with the average normalized peak area (log₁₀, top). Tukey's HSD test distinguished groups designated with letters to illustrate quantitative differences in the individual cyanopeptide production profile (statistically significant, $p < 0.05$). Data points with the same letter within a cyanopeptide row are not statistically significant from one another. Cell count measurements are depicted to correlate toxin production with the phase of growth (bottom).

The aerucyclamides produced by CPCC 300 appear to be present within the cells at higher amounts compared to other groups at all growth stages. While we could not quantify most cyanopeptides, this observation is in accordance with Natumi and Janssen et al. 2020. Aerucyclamide A and C have the darkest shade of red over the time course when grown in both media. The unknown cyanopeptolins appear to have the lowest concentrations of the represented compounds with blue to green shades over the time course. Of particular interest is the maximum intracellular concentrations, which occur at an earlier time point within the growth curve when the strain is grown in MA media whereas the maximum intracellular concentrations occur at a later time point within the growth curve when the strain is grown in BG-11 (Figure 3.29). A potential explanation for this is the accumulation of the toxin within the cell over time when the strain is grown in BG-

11. The day 25 intracellular concentrations for all compounds when the strain is grown in MA appear to be the lowest suggesting that the metabolites are released from the cell upon lysis within the stationary phase and thus not accumulated intracellularly (Walls et al., 2018).

Table 3.14 compares the relative normalized peak area maximum for major metabolites produced by *M. aeruginosa* CPCC 300 when grown in two separate culture media, BG-11 and MA. Green cells indicate a larger peak area maximum and red indicates a smaller maximum. ANOVA was performed using triplicate peak areas extrapolated from R to assess media effects on intracellular concentrations of identified metabolites. Based on the growth curves generated by CPCC 300 when grown in the two culture media, MA appears to reach the maximum cell number per day at a faster rate than when the culture is grown in BG-11. This difference in growth rate suggests there may be differences in toxin quantities and peak production days. Based on the summary data in Table 3.14, majority of the maximum relative abundances occur on day 15 for both BG-11 and MA media although there are a larger number of day 20 maxima for BG-11 than MA which correlates to the differences in the growth curves. 7 of the 16 investigated compounds show a statistically significant difference in maximum relative abundance suggesting specific chemicals are produced in variable quantities when CPCC 300 is grown with different concentrations of essential nutrients and alternative nitrogen and phosphorous sources. Of the investigated chemical classes, cyanopeptolins are produced in significantly higher concentrations when CPCC 300 was grown in MA whereas cyanobactins and aeruginosins are produced in significantly higher concentrations when the strain is grown in BG-11.

Table 3.14: Maximum relative abundance (green indicates greater peak area) for selected metabolites produced by CPCC 300 when grown in two culture media with an ANOVA pairwise comparison ($p < 0.05$) to add significance to the media effects. The p-values are bolded for statistically significant differences in relative peak area for the metabolites between the two growth media.

Chemical Group	Chemical	Day BG-11	Day MA	p-value (99.5%)
Cyanopeptolin	Cyanopeptolin A	20	15	0
Cyanopeptolin	Cyanopeptolin C	20	20	0
Cyanopeptolin	Cyanopeptolin B	20	15	0.821
Cyanopeptolin	Cyanopeptolin 900	20	15	1
Cyanopeptolin	Cyanopeptolin 945	15	15	0
Cyanopeptolin	Cyanopeptolin 963A	15	15	0.953
Cyanopeptolin	Cyanopeptolin 970	15	15	1
Cyanopeptolin	Cyanopeptolin 984	15	15	1
Cyanobactin	Aerucyclamide A	15	15	0
Cyanobactin	Aerucyclamide C	15	15	0.001
Cyanobactin	Aerucyclamide B	15	15	0
Cyanobactin	Aerucyclamide D	20	15	0.995
Aeruginosin	Aeruginosin 650	15	15	0.749
Aeruginosin	Aeruginosin 684	15	15	0
Microcystin	[Asp ³]-Microcystin-LR	20	15	0.64
Microcystin	Microcystin-LR	20	15	0.113

3.4.2b Media effects on cyanopeptide production by *M. flos-aquae* CPCC 461

This strain did not produce many metabolites aside from a few cyanobactin variants and metabolites from an unknown chemical class. The production profile of these metabolites is shown in Figure 3.30 for comparison of relative amounts.

Figure 3.30: Heatmap depicting cyanopeptide production by *M. flos-aquae* CPCC 461 over time in two growth media (BG-11; left and MA; right), generated with the average normalized peak area (log₁₀, top). Tukey's LSD test distinguished groups designated with letters to illustrate quantitative differences in the individual cyanopeptide production profile (statistically significant, $p < 0.005$). Data points with the same letter within a cyanopeptide row are not statistically significant from one another. Cell count measurements are depicted to correlate toxin production with the phase of growth (bottom).

Microcyclamide is the major metabolite produced by this strain and thus has the highest relative production over the time course. It appears to accumulate over time when the strain is grown in BG-11 yet is always present when grown in MA. The other metabolites are present in much smaller quantities. A similar trend is seen where peak intracellular concentration occurs at an earlier time point when grown in MA versus BG-11. Maximum cyanobactin production occurs at day 20 when *M. flos-aquae* is grown in BG-11 media and at day 10 when grown in MA media. The series of unknown metabolites reach the maximum quantities on day 20 within BG-11 media and on day 15 in MA media. The maximas in metabolite production for both growth media correlate to the late exponential-early stationary phase of growth for *M. flos-aquae* CPCC 461 (Figure 3.30).

Table 3.15 compares the relative peak area maximum for major metabolites produced by CPCC 461 when grown in two separate culture media, BG-11 and MA. The maxima for all compounds was on day 20 when grown in BG-11 and for aeruginosamide B when grown in MA. The remaining

metabolites investigated had maximum relative peak area on day 15 when the strain was grown in MA. The peak day for production correlates with the time that the cells are within the exponential phase and capable of producing large amounts of toxin. The two maxima are different for BG-11 and MA, as CPCC 461 has different growth profiles in these media. The outlier, aeruginosamide B, is in statistically higher relative proportions when the strain is grown in MA.

Table 3.15: Maximum relative abundance (green indicates greater peak area) for selected metabolites produced by CPCC 461 when grown in two culture media with an ANOVA pairwise comparison ($p < 0.05$) to add significance to the media effects. The p-values are bolded for statistically significant differences in relative peak area for the metabolites between the two growth media.

Chemical Group	Chemical	Day BG	Day MA	p-value (99.5%)
Cyanbactin	Microcyclamide A	20	15	1
Cyanbactin	Microcyclamide 584	20	15	1
Cyanbactin	Microcyclamide 586	20	15	1
Cyanbactin	Aeruginosamide B	20	20	0
Unknown	Unknown 612	20	15	1
Unknown	Unknown 692	20	15	0.992
Unknown	Unknown 794	20	15	1
Unknown	Unknown 810	20	15	1

3.4.2c Media effects on cyanopeptide production by *M. aeruginosa* CPCC 632

M. aeruginosa CPCC 632 produced a wide range of cyanopeptide groups including anabaenopeptins, microviridins, cyanopeptolins, microginins, and aeruginosins (Figure 3.3). The production profile of a subset of the produced metabolites over a 25-day time course and when grown in two different culture media is shown in Figure 3.31.

Figure 3.31: Heatmap depicting cyanopeptide production by *M. aeruginosa* CPCC 632 over time in two growth media (BG-11; left and MA; right), generated with the average normalized peak area (log₁₀, top). Tukey's LSD test distinguished groups designated with letters to illustrate quantitative differences in the individual cyanopeptide production profile (statistically significant, $p < 0.05$). Data points with the same letter within a cyanopeptide row are not statistically significant from one another. Cell count measurements are depicted to correlate toxin production with the phase of growth (bottom).

Cyanoepetolin 954B and aeruginosin 688 have the highest relative intracellular concentrations compared to the subset of metabolites depicted with this analysis method. These metabolites are present within the cell for each of the 5 measurements when the strain is grown in MA. The abundance of dark blue data points for the metabolites when the strain is grown in BG-11 suggest that there is very little to no metabolite present within the cell at the first time point. For *M. aeruginosa* CPCC 632, it is apparent that maximum toxin production for each chemical group investigated occurred at the end of the exponential growth phase (Figure 3.31).

Table 3.16 compares the relative peak area maximum for major metabolites produced by CPCC 632 when grown in two separate culture media, BG-11 and MA. Majority of the relative peak area

maxima are on day 25 when this strain is grown in BG-11 and on day 15 when grown in MA. This correlates with the phase of growth the strain is in when in BG-11 versus MA. The strain has a faster growth rate in MA as it reaches the stationary phase of growth at an earlier time point than when grown in BG-11. This is further seen with peak area maximums for the investigated metabolites in Table 3.16 showing highest relative intracellular concentrations at day 15 for MA and at a later time point for BG-11, day 25. Ferintoic acid A was produced in statistically higher intracellular concentrations when CPCC 632 was grown in MA whereas microginin 626 was present in statistically higher levels when the strain was grown in BG-11.

Table 3.16: Maximum relative abundance (green indicates greater peak area) for selected metabolites produced by CPCC 632 when grown in two culture media with an ANOVA pairwise comparison ($p < 0.05$) to add significance to the media effects. The p-values are bolded for statistically significant differences in relative peak area for the metabolites between the two growth media.

Chemical Class	Chemical	Day BG	Day MA	p-value (99.5%)
Anabanopeptin	Ferintoic acid A	25	15	0
Anabanopeptin	Ferintoic acid B	25	15	1
Anabanopeptin	Anabaenopeptin D	25	15	1
Anabanopeptin	Anabaenopeptin 850	25	15	1
Anabanopeptin	Anabaenopeptin 882	25	15	1
Cyanopeptolin	Cyanopeptolin 904	25	15	1
Cyanopeptolin	Nostopeptin BN 920	25	15	1
Cyanopeptolin	Cyanopeptolin 941	15	15	1
Cyanopeptolin	Cyanopeptolin 954B	15	15	0.314
Cyanopeptolin	Cyanopeptolin 954	25	15	1
Microviridin	Microviridin H	15	20	0.559
Microviridin	Microviridin J	15	15	1
Microviridin	Microviridin 1822	15	15	0.601
Microginin	Microginin 626	20	15	0
Microginin	Microginin 640	25	15	1
Microginin	Microginin 660	20	15	1
Microginin	Microginin 714	25	20	1
Aeruginosin	Aeruginosin 722	25	15	1
Aeruginosin	Aeruginosin 688	15	15	0.418

3.4.2d Media effects on cyanopeptide production by *M. aeruginosa* CPCC 633

This strain produces congeners within the following chemical classes: cyanopeptolin, microviridin, aeruginosamide, microginin, and anabaenopeptin. A few congeners were selected for heatmap analysis for rapid comparison of their production profiles when CPCC 633 is grown in the two culture media, BG-11 and MA (Figure 3.32).

Figure 3.32: Heatmap depicting cyanopeptide production by *M. aeruginosa* CPCC 633 over time in two growth media (BG-11; left and MA; right), generated with the average normalized peak area (log₁₀, top). Tukey's LSD test distinguished groups designated with letters to illustrate quantitative differences in the individual cyanopeptide production profile (statistically significant, $p < 0.05$). Data points with the same letter within a cyanopeptide row are not statistically significant from one another. Cell count measurements are depicted to correlate toxin production with the phase of growth (bottom).

The linear cyanobactins, aeruginosamides, have the highest relative intracellular concentration based on heatmap analysis. Ferintoic acid A and microginin 680 are also present in high abundance within the cell in comparison to the other metabolites investigated here. It appears as one metabolite dominates the production for the group and the other congeners within said group are produced at lower levels. Maximum production of the metabolites produced by *M. aeruginosa*

CPCC 633 vary based on the growth media (Figure 3.32). Peak production of secondary metabolites when the strain was grown in BG-11 media occur when the cells exit exponential phase of growth and enter stationary phase. When the strain was grown in MA media, peak metabolite production occurs mid-exponential growth phase.

Table 3.17 compares the relative peak area maximum for major metabolites produced by CPCC 633 when grown in two separate culture media, BG-11 and MA. Peak area maxima vary between the compounds investigated when the strain was grown in BG-11 however day 15 appears to be the maximum day for all but one metabolite investigated. Ferintoic acid A and two microginins, 646 and 680 have statistically higher intracellular levels when CPCC 633 was grown in BG-11 media.

Table 3.17: Maximum relative abundance for selected metabolites produced by CPCC 633 when grown in two culture media with an ANOVA pairwise comparison ($p < 0.05$) to add significance to the media effects. The p-values are bolded for statistically significant differences in relative peak area for the metabolites between the two growth media.

Chemical Class	Chemical	Day BG	Day MA	p-value (99.5%)
Anabaenopeptin	Ferintoic acid A	25	15	0.001
Anabaenopeptin	Ferintoic acid B	25	15	1
Anabaenopeptin	Anabaenopeptin D	15	15	1
Cyanopectolin	Cyanopectolin 946	15	15	1
Cyanobactin	Cyanopectolin 966	15	15	1
Cyanobactin	Cyanopectolin 992	15	15	1
Cyanobactin	Aeruginosamide B	20	15	1
Cyanobactin	Aeruginosamide C	20	15	0.917
Microviridin	Microviridin H	15	15	1
Microginin	Microginin 612	25	15	1
Microginin	Microginin 646	25	15	0.049
Microginin	Microginin 660	15	15	1
Microginin	Microginin 674	20	15	1
Microginin	Microginin 680	20	15	0
Microginin	Microginin 714	20	20	1

4. Discussion

Over the last one hundred years, cyanobacterial blooms have been increasing in both frequency and magnitude. The biological factors that promote cyanobacterial growth and toxin production are broadly understood. The generation and release of cyanotoxins into the environment pose a threat to human and ecosystem health. The majority of research efforts have been invested in MCs due to their potent hepatotoxicity and classification as a possible human carcinogen (IARC group 2B). The chemistry, toxicology, and environmental concentrations of the vast majority of other cyanotoxins are unknown. Untargeted and semi-targeted mass-spectrometry based metabolomics was used to study the strain specific variation in chemical profiles for five *Microcystis* strains. Target strains have been prioritized for large scale cultivation and isolation of novel cyanotoxins for structural and toxicological characterizations.

4.1 Untargeted mass-spectrometry based metabolomics to understand strain specific variation between *Microcystis* species.

4.1.1 Discussion of multivariate analyses: PCA and loading plot.

Microcystis species are known to produce diverse and complex mixtures of cyanopeptides (Janssen, 2019). The PCA analysis visualized which *Microcystis* strains have similar or different cyanopeptide profiles (Figure 3.1). Supporting the PCA, the factor loading plot distinguished specific features (compounds) characteristic of chemical profiles for the four *Microcystis aeruginosa* strains (CPCC 299, CPCC 300, CPCC 632, CPCC 633) and one *M. flos-aquae* (CPCC 461, Figure 3.2). Additionally, a Kruskal Wallis test with Benjamini-Hochberg correction provided statistical significance to the features that were strain specific.

As these strains are all within the genus *Microcystis*, it is assumed that there are similarities within the primary metabolism (growth, development, reproduction, (Neilan, Dittmann, Rouhiainen, Bass, Schaub, Sivonen, & Börner, 1999)). We are viewing the entire intracellular metabolome, which is likely to contain primary metabolites. The loading plot contains many features that did not match databases or spectral libraries, but are central within the loading plot meaning that they are shared metabolites produced by the *Microcystis* strains. A hypothesis is that these unidentified features are from primary metabolism pathways. To determine which features unite and differentiate the chemotypes, features furthest away from the center of the loading plot were investigated.

The two most similar metabolic profiles are those of *M. aeruginosa* CPCC 632 and CPCC 633, which share the production of cyanopeptides within the microviridin, microginin, cyanopeptolin, and anaebaenopeptin groups. The triplicate data points for these strains overlap within the PCA plot suggesting a strong agreement in their metabolic profiles (Figure 3.1). Further investigation reveals that similar mixtures of congeners within the microginin and anabaenopeptin cyanopeptide groups are produced by these strains. Variation between *M. aeruginosa* CPCC 632 and CPCC 633 arise as *M. aeruginosa* CPCC 632 is capable of producing aeruginosins and *M. aeruginosa* CPCC 633 cannot produce these metabolites but synthesizes linear cyanobactins. More variation arises with cyanopeptolins as *M. aeruginosa* CPCC 632 produces congeners with the partial amino acid sequences NMe-Cl-Tyr-Ahp and NMeTyr-Phe-Ahp whereas *M. aeruginosa* CPCC 633 produces cyanopeptolins with the partial amino acid sequence NMePhe-Phe-Ahp (Table 3.6). Therefore, although there is strong genetic overlap in the NRPS synthase gene clusters for these two strains, differences manifest in the less abundant metabolites produced which distinguish their chemical profiles (Figure 3.3)

M. aeruginosa CPCC 299 and CPCC 300 have the next closely related metabolome according to the PCA (Figure 3.1, Figure 3.2). These two strains are known “toxic” *Microcystis* strains as they both produce congeners of the hepatotoxic cyanopeptide group, Microcystins (Racine et al., 2019). Factor loading analysis provides agreement with previous reports that the two MC congeners they produce as well as the aerucyclamide congeners are the same. Yet the congeners of cyanopeptolins produced by *M. aeruginosa* CPCC 299 and CPCC 300 varied. *M. aeruginosa* CPCC 299 produced cyanopeptolins with the partial amino sequence NMePhe-Phe-Ahp whereas *M. aeruginosa* CPCC 300 produced congeners with the partial amino acid sequence NMePhe-Lxx-Ahp. As with *M. aeruginosa* CPCC 632 and CPCC 633, the formerly mentioned strains do not share the production of specific metabolite groups such as aeruginosins by *M. aeruginosa* CPCC 300 and further the congeners within shared cyanopeptide groups such as cyanopeptolins distinguish their chemical profiles.

The triplicate data points for *M. flos-aquae* CPCC 461 are isolated within the PCA (Figure 3.1) suggesting the largest difference in total chemical profile when comparing the *Microcystis* strains. This strain produces few metabolites with the major metabolites being microcyclamides, which are not produced by the *M. aeruginosa* strains (K. Ishida et al., 2000). A few features that statistically contribute to the variance of the chemical profile for *M. flos-aquae* CPCC 461 are not previously reported and do not contain any diagnostic features that relate to the other cyanopeptide groups. This depicts the limitations of untargeted LC-MS/MS, as here we can distinguish this strain as significantly different from the *M. aeruginosa* strains and have the *m/z* and retention time for these compounds, but we are unable to characterize explicitly which chemicals they are. Further analysis, such as genomics or compound isolation, purification, and characterization is required to answer this question.

4.1.2 Discussion of the molecular network data for five *Microcystis* strains.

Multivariate methods such as PCA and factor loading analysis aid in visually depicting the similarities and differences in chemical profiles between samples. The variables assessed are all the compounds detected that meet the given parameters and can be thousands of compounds, identified only by m/z and retention time. Pairing this method with GNPS molecular networking provides a starting point for identifying compound classes and dereplication of natural products. GNPS molecular networking is a method that utilizes the concept that structurally related chemicals will fragment in similar manners and generate common product ions within the MS/MS. The resulting molecular network has clusters of chemicals that contain common structural features. This method is useful for *Microcystis* extracts as the majority of their secondary metabolites are peptides that arise from NRPS, NRPS-PKS and RiPP pathways. Further, most cyanopeptide groups share diagnostic structural features including partial amino acid sequences or non-proteinaceous amino acids that result in unique product ions (Table 2.3).

The chemical profiles of the five studied *Microcystis* strains are significantly different and strain specific metabolites have been identified here (Figure 3.2, Figure 3.3). The untargeted metabolomic strategies applied to their extracts show the metabolic variation with the chemical groups of cyanopeptides that are produced as well as the congeners produced within each class. Furthermore, the molecular network suggests that these strains have different metabolomes but contain various overlapping biosynthetic mechanisms. Of interest is the generation of multiple network clusters for the same chemical group such as microviridins, cyanopeptolins, and cyclic cyanobactins. Multiple network clusters for the same chemical group suggests that there are large enough structural differences between the congeners that results in them clustering separately. This is important as the structural diversity of cyanopeptides is vast and annotation of all congeners

proves difficult when inspecting a peak list of m/z and retention times alone. Molecular networking provides a place to start with unknown congener annotation, as it gives a gauge of structural similarity of the metabolites to previously identified compounds and allows for in-depth subclassification of cyanopeptides.

Alongside compound identification and dereplication, molecular networking illustrates metabolomic similarities and differences. For example, *M. aeruginosa* CPCC 299 produces metabolites within the following chemical classes: microviridin, microcystin, cyanobactin, and cyanopeptolins whereas *M. aeruginosa* CPCC 300 produces metabolites within the following classes: microcystins, cyanobactins, and cyanopeptolins (Figure 3.3). These two strains have an overlap in their biosynthetic potential as they both can make microcystins, cyanobactins, and cyanopeptolins, although *M. aeruginosa* CPCC 299 has additional biosynthetic genes for the synthesis of microviridins. Microviridins are synthesized by the RiPP pathway (Ziemert et al., 2010), a pathway different than the nRPS pathway the other chemical classes are synthesized by, suggesting *M. aeruginosa* CPCC 299 has the capacity to utilize another biosynthetic pathway for cyanopeptide production. Dissecting these two chemical profiles further, it is now known that the congeners within these shared chemical classes are different. *M. aeruginosa* CPCC 299 produces strictly Phe-Ahp containing cyanopeptolins (molecular network cluster #1) whereas *M. aeruginosa* CPCC 300 produced strictly Lxx-Ahp containing cyanopeptolins (molecular network cluster #2, Figure 3.3, (Cornel et al., 1993; Von Elert et al., 2005)). The small variation within the peptide sequence has been differentiated by the diagnostic product ions generated within the MS/MS of known cyanopeptolins within these two subclasses (Figure 3.5). *M. aeruginosa* CPCC 299 has an NRPS enzyme cluster for cyanopeptolins with specificity for a Phe residue in position 2 with *M. aeruginosa* CPCC 300 having specificity for a Lxx residue in position 2. This minor change in

enzyme-substrate selectivity generates a whole new subclassification of cyanopeptolins. This variability in enzyme structure and thus amino acid specificity results in the production of hundreds of congeners for each metabolite produced by the NRPS and RiPP pathway (Strieker et al., 2010). RiPP synthesized peptides may have increased structural variation as the identity, number, and location of the post-translational modification is independent of the core peptide synthesis.

An interesting chemotypic difference between these strains visualized within the molecular network is that four of the five *Microcystis* strains produce metabolites within the cyanobactin group. *M. aeruginosa* CPCC 299 and CPCC 300 produce cyclic cyanobactins (aerucyclamides, molecular network cluster #13) whereas *M. aeruginosa* CPCC 633 produces linear cyanobactins (aeruginosamides, molecular network cluster #8) and *M. flos-aquae* CPCC 461 produces both cyclic cyanobactins (microcyclamides, molecular network cluster #12) and the linear aeruginosamides (Figure 3.3, (K. Ishida et al., 2000; Leikoski et al., 2013; Portmann, Blom, Gademann, et al., 2008; Portmann, Blom, Kaiser, et al., 2008)). Cyanobactins are generated by the nRPS pathway and are cyclized through an enzymatically catalyzed reaction with a carboxy terminal and a terminal amine. *M. aeruginosa* CPCC 633 and *M. flos-aquae* CPCC 461 both produce aeruginosamides, which contain a methyl group on the carboxy terminal making N to C macrocyclization impossible (Leikoski et al., 2013). An alternative explanation for this is that the enzyme complex responsible for synthesizing these metabolites does not contain a macrocyclase module. The cyclic cyanobactins produced by *M. aeruginosa* CPCC 299 and CPCC 300, and *M. flos-aquae* CPCC 461 are termed cyclamides and are broken into two subclasses: microcyclamides and aerucyclamides. *M. aeruginosa* CPCC 299 and CPCC 300 produce aerucyclamides and *M. flos-aquae* CPCC 461 produces microcyclamides. The difference between these classes are the

heterocyclized amino acid moieties and their number within the molecule. The mixture of aerucyclamide congeners produced by *M. aeruginosa* CPCC 299 and CPCC 300 are the same suggesting conserved biosynthetic genes between these strains. The microcyclamide congeners produced by *M. flos-aquae* CPCC 461 differ from the aerucyclamides with substitution of the proteinaceous amino acids. Both microcyclamides and aerucyclamides contain three heterocycles, two of which are generated by cyclization of cysteine (thiazole or thiazoline) and one is formed by cyclization of either serine or threonine (oxazole or oxazoline, (Ge et al., 2019)). This supports the hypothesis that 10-30% of all cyanobacteria have the genetic material for a cyanobactin pathway (Leikoski et al., 2013).

4.1.3 Discussion of relative cyanopeptide abundance within *Microcystis* extracts.

In this study, the relative abundance of metabolites identified within the molecular network and utilized as features within the multivariate analysis are compared by normalizing peak area with biomass. This is an accepted proxy for comparison of relative amounts of metabolites that cannot be quantified with a purified standard (Tiam et al., 2019). Utilizing normalized peak area has advantages as it enables comparison of metabolite “quantities” in reference to other chemicals as well as relative production over time (Figures 3.29-3.32). For MC-LR, absolute quantities in units of $\mu\text{g mg biomass}^{-1}$ were calculated as we have a purified reference standard for this cyanopeptide (Figure 3.28, Table 3.13). Quantification of all the other detected cyanopeptides could not be performed due to lack of available reference material.

Within the relative abundance figure (Figure 3.14), a snapshot of the intracellular profile of cyanopeptides on day 15 of growth is visualized for the five *Microcystis* strains. Varying amounts of cyanopeptides are seen with some metabolites appearing in slightly greater intracellular concentrations over the other congeners. A recent investigation of cyanopeptide co-production

dynamics elucidated that when growth conditions are varied the proportions of cyanopeptide groups remains constant (Natumi & Janssen, 2020). This suggests that the mechanisms for cyanopeptide co-production are intertwined and are not related to nutrient requirement changes during the growth cycle.

Within the heatmap analysis, the time point for maximum intracellular metabolite concentrations are in the late exponential phase of growth (Figure 3.29-3.32). Although in some cases, the maximum production varied based on the compound of interest. For example, when *M. aeruginosa* CPCC 300 was grown in BG-11 maximum aeruginosin 650 concentration occurs at day 10 whereas maximum intracellular concentration of microcystin-LR occurs at day 20 (Figure 3.26). Variables that must be considered are the potential for the metabolite to be exported from the cell, and the total intracellular metabolite concentration would not include excreted metabolites.

There are complications with using peak area as a proxy as these molecules are structurally different, varying in size, cyclic vs linear, as well as amino acid sequences, the extent of ionization varies resulting in differences in peak shape and size. For example, some metabolites may ionize very well and give a large peak area as each molecule has been ionized providing a larger signal. In contrast, a poor ionizing molecule will produce a smaller signal in comparison to how much compound is actually present. Therefore, comparing relative abundance of compounds within the same chemical group makes more sense than comparing between cyanopeptide groups as the structural similarity resulting from the shared chemical features would provide similar ionization capacities.

4.2 Semi-targeted mass-spectrometry based metabolomics for detection of cyanopeptide groups in Microcystis extracts and environmental samples.

4.2.1 Discussion of semi-targeted approach for microcystin detection.

Microcystins are known hepatotoxins with more than 300 congener reports and copious information regarding their toxicity and environmental concentrations. With the number of congener reports steadily increasing, rapid detection and putative identification of microcystin congeners must evolve. The established semi-targeted approach for the detection of Adda containing cyanopeptides was tested with *Microcystis* extracts and validated with detection of congeners within environmental samples. Interestingly, similar microcystin congener profiles were detected within the *M. aeruginosa* CPCC 299 and CPCC 300 extracts as well as the environmental samples (Table 3.16-3.17). The identity of the MC-LR and [Asp³]-MC-LR congeners was confirmed with purified reference material with putative identification of [Asp³, Dha⁷]-MC-LR and MC-LA based on HR-MS and product ion spectra. Previous reports of MC-LA being a persistent microcystin congener within the water column and within tissues of bivalves and fish suggest that production of this congener is advantageous to the producing species since it does not bind to sediment but remains active within the water column and can bioaccumulate within predators (Díez-Quijada et al., 2019).

4.2.2 Discussion of semi-targeted approach for cyanopeptolin detection.

To further understand cyanopeptolin diversity produced by the *Microcystis* strains of interest, DFF plots were generated searching for different possible partial amino acid sequences (Table 3.5). The four *M. aeruginosa* strains investigated produce different mixtures of cyanopeptolins (CPCC 299, CPCC 300, CPCC 632, and CPCC 633). The subclassification of cyanopeptolins based on partial amino acid sequence derived from the molecular networking aided in the semi-targeted search for cyanopeptolins in both *Microcystis* cultures and environmental samples. Variation in the partial

amino acid sequence was great enough to generate two cyanopeptolin molecular network clusters and arose from the residue directly adjacent to the characteristic non-proteinaceous amino acid present in all cyanopeptolins, Ahp. *M. aeruginosa* CPCC 300 exclusively produced Lxx-Ahp cyanopeptolins as described in the molecular network, with 14 cyanopeptolins detected via the DFF approach which is 6 more congeners than detected within the molecular network. *M. aeruginosa* CPCC 299, CPCC 632, and CPCC 633 produce cyanopeptolin congeners with the partial amino acid sequence Phe-Ahp. Further subclassification occurs between these cyanopeptolin profiles as *M. aeruginosa* CPCC 299 and CPCC 633 produce exclusively cyanopeptolins with a NMePhe in position 3 whereas *M. aeruginosa* CPCC 632 produces cyanopeptolins with either NMeTyr or NMe-Cl-Tyr in position 3. Some cyanopeptolin biosynthesis gene clusters encode a gene for a halogenase enzyme which catalyzes halogenation of specific amino acid residues (Nishizawa et al., 2011). This property explains the coproduction of both chlorinated and non-chlorinated cyanopeptolins by *M. aeruginosa* CPCC 632 and non-chlorinated cyanopeptolins by the remainder of *M. aeruginosa* strains. *M. flos-aquae* CPCC 461 did not produce any detectable cyanopeptolins and none of the studied strains produced cyanopeptolins with the partial amino acid sequence NMeTyr-Lxx-Ahp which accounts for 12% of all cyanopeptolin congeners according to the generated cyanopeptolin sequence database (Supp Table S2, McDonald et al., 2020).

4.2.3 Discussion of semi-targeted approach for aeruginosin detection.

Five aeruginosin congeners were detected and annotated within the molecular network produced by *M. aeruginosa* CPCC 300 and CPCC 632 (Figure 3.3). The congeners detected within the molecular network generated two subclassifications of aeruginosins based on partial amino acid sequences, Phe-Choi aeruginosins (*M. aeruginosa* CPCC 300) and Tyr-Choi aeruginosins (*M.*

aeruginosa CPCC 632). Diagnostic product ions were identified for the Choi moiety and subsequently used to search *Microcystis* strains and environmental samples for potential aeruginosins (Table 3.7). The result of the semi-targeted search returned more aeruginosin congeners than shown within the molecular network, five congeners produced by *M. aeruginosa* CPCC 300 and seven congeners produced by *M. aeruginosa* CPCC 632 (Table 3.8). Both *M. aeruginosa* CPCC 300 and CPCC 632 display the capability of halogenating the Hpla moiety of aeruginosin congeners, as seen within the structure of aeruginosin 89 reported from *Microcystis* species (Welker et al., 2006). This is visible within the full scan MS by the isotopic abundance of the M+2 peak. Investigating the MS/MS data for the additional aeruginosin congeners detected by DFF revealed the missing link between the molecular network data and the DFF data. Two congeners containing the partial amino acid sequence Lxx-Choi and three containing Phe-Choi did not meet the molecular networking requirements for product ion spectra similarity and thus were not present or clustered within the network. Also not depicted within the molecular network is the overlap in production of As 609 and the sulfated version As 689 by *M. aeruginosa* CPCC 300 and *M. aeruginosa* CPCC 632. Instead, it is visualized that *M. aeruginosa* CPCC 632 produces these compounds and *M. aeruginosa* CPCC 300 does not. The levels of the aeruginosin congeners within the *M. aeruginosa* CPCC 300 extract may be low and resulted in poor fragmentation or non-inclusion within the molecular network.

4.2.4 Discussion of semi-targeted approach for microginin detection.

Cyanopeptides within the microginin group have few common and diagnostic product ions as the N-terminal acyl chain derived from a PKS synthase pathway can have many modifications such as chain length, halogenation, and/or methylation. The remainder of the molecule is highly variable based on a structural database of all previously reported microginin congeners (Supp. Table S3).

For detection of these congeners via a semi-targeted approach, a filter within the DFF search must be used. This encapsulates the different possible N-terminal acyl chain variations that are biosynthesized (Table 3.9).

Two of the five investigated strains produced different mixtures of microginin congeners, *M. aeruginosa* CPCC 632 and CPCC 633. Based on DFF analysis for microginins, *M. aeruginosa* CPCC 632 produces congeners with exclusively an eight carbon chain N-terminal chain variants (R-Ahoa) whereas *M. aeruginosa* CPCC 633 produces both eight carbon chain and ten carbon chain N-terminal groups (R-Ahoa, R-Ahda, Table 3.10). Methylation of the amino group on the N-terminal chain was more commonly seen within the microginin congeners produced by *M. aeruginosa* CPCC 632.

The majority of previously reported microginin congeners contain the Ahda N-terminal moiety with variable chlorination and methylation, with incorporation of Ahoa and its variants as the N-terminal moiety being extremely rare (Strangman & Wright, 2016). This is proven with the detection of thirteen R-Ahda containing microginins within the environmental samples and only six R-Ahoa containing microginins (Table 3.8). Possible underreporting of R-Ahoa containing microginins may have occurred in previous reports due to lack of purified standards and few characterized congeners. These seem like rare new compounds worth pursuing.

4.2.5 Optimizing cyanopeptide detection and identification.

It has been shown here that the *Microcystis* genus is a prolific source of peptide natural products with vast structural diversity. Most research efforts are towards the hepatotoxic microcystins, with little information surrounding the toxicity, fate, and environmental concentrations of the other cyanopeptide classes. As cyanobacterial blooms escalate in their occurrence and severity, it is

believed the complexity of the cyanopeptides that are produced will escalate as well (Tiam et al., 2019). Rapid detection, identification, and toxicological assessment of the cyanopeptides is required to ensure the safety of the general public and the environment.

There are advantages and disadvantages of untargeted metabolomics when attempting to understand and describe the chemical diversity within and between samples. A large advantage is gathering chemical information for thousands of chemicals within a sample. A factor that must be fine-tuned is the chromatography prior to mass analysis as the data dependent acquisition method utilizes the abundance of ions to select which molecules will be fragmented. If you have a large number of chemicals eluting within a small period of time, the most abundant compounds will be selected for fragmentation and the lower abundance compounds will not be acknowledged. A major challenge of operating with large data sets is the processing and interpretation of the data. Without large and comprehensive databases/libraries to describe the chemicals that may be present within the sample, the chemicals that are detected cannot be annotated or assigned an identity. As this approach becomes more widely used, such databases are being compiled and readily accessible on public websites (Strangman et al., 2018; Tiam et al., 2019). With high resolution MS data, concluding on a molecular formula is possible and aids in matching a molecule to literature. As well, matching fragmentation patterns to key structural features within a molecule will help confirm an identity. But when identifying new congeners of a chemical, one cannot conclude on the chemical structure without isolation, purification, and NMR characterization of the compound. But this technique can supply a place to start for said isolation.

Here we have combined an untargeted LC-MS/MS approach with multivariate analysis and semi-targeted searches for rapid identification and annotation of different cyanopeptide groups. It was seen here that molecular networking is not a perfect technique with underreporting of the number

of congeners present within a sample based on follow-up DFF analysis. But the development of the DFF approach was reliant on the data gathered from the molecular networking as a starting point for compound classification. Molecular networking provides vital information for compound identification and classification in a dataset comprised of thousands of compounds. Understanding the molecular features of the cyanopeptides present within a sample can help with potential new compound identification as well as target cyanobacterial strains for large scale cultivation and cyanopeptide purification, for structural and toxicological characterizations.

Confirmation of a metabolites identity and quantification cannot be done without a purified standard. MC-LR and [Asp³]-MC-LR are the only metabolites identified within this study that have purified standards accessible and thus intracellular concentrations could be quantified (Figure 3.28). These metabolites have undergone extensive toxicological assessment and are high priority cyanopeptides due to their hepatotoxicity. It is seen here that these two metabolites are not the only compounds produced by the two “toxic” *Microcystis* strains, CPCC 299 and CPCC 300. More studies are required for the isolation and structural characterization of congeners within the other identified chemical groups such as anabaenopeptins which are known (and less studied) PP2A inhibitors (Spoof et al., 2016). Hypotheses can be made regarding the toxicity of a metabolite based on structural motifs and structural similarities to compounds that have been used in pharmacokinetic and pharmacodynamic studies. But with tangible material, unambiguous characterization of toxicokinetics can be done as well as quantification of intra/extra-cellular and environmental concentrations.

4.3 Media composition effects on cyanopeptide production by *Microcystis* strains.

To assess media composition effects on cyanopeptide production, biological parameters must be considered as toxin production is closely correlated with photosynthetic rates (Walls et al., 2018).

Prior to media composition changes, it was evident that growth rates and physiology of the five *Microcystis* strains varied (Table 3.11). The phenotypic changes, such as the extent of their green colour suggesting changes in pigment ratios as well as the formation of biofilms resulting in thick mats suggest that the chemical profile differences of the *Microcystis* strains is a reflection of physiological differences.

Bacterial growth rates were calculated with cell mL⁻¹ for the five *Microcystis* strains when grown in media containing different concentrations and sources of essential nutrients. Table 3.11 clearly depicts that MA media resulted in the lowest growth rate for all strains, ranging from 0.103-0.216 days⁻¹. It has been previously reported that reduction of nutrients (N, P, micronutrients (Fe, Zn, etc.)) results in a significant reduction of growth but only reductions in phosphorous or micronutrients resulted in a significant reduction in total cyanopeptide quota (Natumi & Janssen, 2020). Thus, the changes in growth seen within the *Microcystis* strains when grown in MA versus BG-11 media could be due to the complex phosphorous source, β -Na₂-glycerophosphate, and a smaller total phosphorous availability.

In our analyses, we compared maximum relative peak area for select metabolites to gage shifts in metabolite production based on media composition over a 25-day time course. Based on the heatmap analysis, the metabolites that were produced in the largest quantities remained the same suggesting that total intracellular cyanopeptide quota did not shift significantly when the strains were grown in the two pre-defined culture media. Interestingly, when the *Microcystis* strains were grown in MA medium, the maximum relative intracellular toxin levels occurred within the exponential phase of growth suggesting that when the cells enter the stationary phase of growth cell lysis occurs and the cell-bound toxin levels are released suggesting unfavourable growth conditions (Figures 3.29-3.32, Walls et al., 2018). Whereas when the *Microcystis* strains were

grown in BG-11, it appears that the toxin levels continually accumulate with maximum relative peak area occurring when the cells enter the stationary phase of growth.

Contrary to previous reports, a profound media effect is observed on the production of metabolites by *M. flos-aquae* CPCC 461. When the strain is grown in BG-11 media, the dominant cyanobactin is the cyclic microcyclamide A whereas when the strain is grown in MA media, the dominant cyanobactin is the linear aeruginosamide B (Figure 3.30). There is a large growth rate shift for *M. flos-aquae* CPCC 461 when grown in BG-11 (0.309) and MA (0.103, Table 3.11) suggesting that the shift from cyclic cyanobactins to linear cyanobactins is more energetically favourable when the cells are not growing efficiently. A hypothesis for this is the capacity for the strain to utilize the complex phosphorous source (β -Na₂-glycerophosphate).

4.4 Discussion of the toxicity of the five Microcystis strains.

Growth of *Microcystis* species in waterbodies used for recreational purposes or as drinking water resources are of public health concern because of the ability to produce hepatotoxins (Pick, 2016). Hepatotoxin-producing strains of *Microcystis* are classified as toxigenic strains due to protein phosphatase inhibition whereas those without hepatotoxic capabilities are classified as non-toxigenic strains. The cyanopeptide groups with protein phosphatase activity are microcystins and nodularins, and anabaenopeptins (Urrutia-Cordero et al., 2013; Zurawell et al., 2005). In this study, two of the five *Microcystis* strains are considered toxigenic (*M. aeruginosa* CPCC 299 and CPCC 300) as they can biosynthesize microcystin congeners. The toxicity of all five strains was assessed with a PP2A inhibition assay (Table 3.11). The strains classified as toxigenic displayed PP2A activity with calculated microcystin equivalents at 0.71 $\mu\text{g L}^{-1}$ for *M. aeruginosa* CPCC 299 and 0.29 $\mu\text{g L}^{-1}$ for *M. aeruginosa* CPCC 300. Unexpectedly, *M. flos-aquae* CPCC 461 displayed PP2A inhibition with microcystin equivalents calculated at 0.82 $\mu\text{g L}^{-1}$, larger than both the

toxigenic strains. Upon screening *M. flos-aquae* CPCC 461 for Adda-containing metabolites with our semi-targeted approach, no potential microcystins, nor nodularins were detected. The metabolites annotated here produced by the *M. flos-aquae* CPCC 461 strain are cyanobactins (microcyclamides) which have cytotoxic and antimicrobial activity that cannot be attributed to the protein phosphatase inhibition (K. Ishida et al., 2000). Previous reports of a *M. flos-aquae* strain show strong inhibition of freshwater amoeba as a result of anabaenopeptin variants (Urrutia-Cordero et al., 2013). Potentially, low abundant anabaenopeptins are produced by this strain and were not annotated within the molecular network due to little structural similarity with other metabolites. A screening method for anabaenopeptins via determination of diagnostic product ions for this chemical class would aid in the rapid identification of anabaenopeptin variants.

The non-toxigenic strains (*M. aeruginosa* CPCC 632 and CPCC 633) produce anabaenopeptin congeners (Figure 3.3, Table S1). Without any displayed PP2A toxicity (Table 3.11), it is assumed that the produced variants are either produced in low abundance or have very little activity against PP2A specifically. Other cyanopeptide groups produced by these non-toxigenic strains are cyanopeptolins, microviridins, microginins, aeruginosins, and cyanobactins. Variants within these cyanopeptide groups have bioactive properties such as protease inhibition and present therapeutic potential with anticancer, antifungal, antibacterial, antiviral, and potent enzyme-inhibiting properties. Large scale cultivation of target *Microcystis* strains for cyanopeptide isolation for structural and toxicological assessment would reveal the changes in toxicokinetics from amino acid substitutions, halogenation, methylation, and sulfonation of the general cyanopeptide group core structures. *Microcystis* strains provide vast chemical diversity of natural products with various biological activities that are attractive potential drug candidates and therapeutics.

5. Conclusion

The use of untargeted and semi-targeted mass-spectrometry based metabolomics to describe the strain specific cyanopeptide profiles was described here. The metabolome differences of four *Microcystis aeruginosa* strains (CPCC 299, CPCC 300, CPCC 632, CPCC 633) and one *M. flos-aquae* strain (CPCC 461) were deciphered with the use of untargeted and semi-targeted mass-spectrometry based metabolomic techniques. Strain specific profiles were elucidated as the co-production of multiple cyanopeptide groups and characteristic variants. These profiles were compared and visualized through GNPS molecular networking and diagnostic fragmentation filtering analyses. Information gathered from the untargeted techniques was used for the rapid identification of microcystins, cyanopeptolins, aeruginosins, and microginins within environmental samples. Changes in total chemical profile were assessed after alterations to culture media composition. The largest effect was seen in the growth rate which altered the production profiles of the cyanopeptides over time monitored with relative abundances of the metabolites. The production profile analysis provides insight for large scale cultivation of *Microcystis* strains for the optimal production and isolation of cyanopeptides. Toxicity of the *Microcystis* extracts was assessed with PP2A inhibition assays which provided agreement with the classification of the toxigenic strains *M. aeruginosa* CPCC 299 and CPCC 300, and surprising toxicity of the non-toxigenic strain *M. flos-aquae* CPCC 461. Further investigation for the structural and toxicological characterization of the less studied metabolites produced by *Microcystis* could reveal potential new bioactive metabolites.

6. References

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7. Supplementary Information

Table S1: LC-HRMS data for cyanotoxins produced by *M. aeruginosa* CPCC 299, 300, 632 and 633, and *M. flos-aquae* CPCC 461

Cluster #	Cyanotoxin group	Name	[M+H] ⁺ m/z	Formula	Error (ppm)	RT (min)	Microcystis strain (CPCC)				
							299	300	461	632	633

1	Cyanopeptolin	Uk Cyp 904	887.4652 ¹	C ₄₆ H ₆₄ N ₈ O ₁₁	-1.08	4.2	+			+	
1	Cyanopeptolin	Uk Cyp 920	903.4367 ¹	C ₄₃ H ₆₅ N ₈ O ₁₂ Cl	-1.37	3.40				+	
1	Cyanopeptolin	NP BN 920	903.4587 ¹	C ₄₆ H ₆₄ N ₈ O ₁₂	0.10	3.25				+	
1	Cyanopeptolin	Uk Cyp 932	915.4972 ¹	C ₄₈ H ₆₈ N ₈ O ₁₁	-0.29	4.85					+
1	Cyanopeptolin	Uk Cyp 941	923.4039 ¹	C ₄₅ H ₆₅ N ₈ O ₁₂ Cl	-0.45	3.38				+	
1	Cyanopeptolin	Uk Cyp 946	929.5116 ¹	C ₄₉ H ₇₀ N ₈ O ₁₁	-1.63	5.19					+
1	Cyanopeptolin	Cyp 954	937.4217 ¹	C ₄₆ H ₆₃ N ₈ O ₁₂ Cl	-0.43	3.51				+	
1	Cyanopeptolin	Uk Cyp 954	937.4441 ¹	C ₄₉ H ₆₂ N ₈ O ₁₂	-1.41	4.16	+			+	
1	Cyanopeptolin	Uk Cyp 945	946.4917	C ₄₉ H ₆₇ N ₇ O ₁₂	-0.36	5.35		+			
1	Cyanopeptolin	Uk Cyp 966	949.4821 ¹	C ₅₁ H ₆₆ N ₈ O ₁₁	0.29	5.00	+				+
1	Cyanopeptolin	Uk Cyp 968	951.4596 ¹	C ₅₀ H ₆₄ N ₈ O ₁₂	-1.55	3.96				+	+
1	Cyanopeptolin	Uk Cyp 980	963.4964 ¹	C ₅₂ H ₆₈ N ₈ O ₁₁	-1.04	5.31					+
1	Cyanopeptolin	Uk Cyp 982	965.4525 ¹	C ₄₈ H ₆₇ N ₈ O ₁₂ Cl	1.19	4.02				+	
1	Cyanopeptolin	Uk Cyp 992	979.4930 ¹	C ₅₂ H ₆₈ N ₈ O ₁₂	0.73	4.79					+
1	Cyanopeptolin	Uk Cyp 1010	993.4856 ¹	C ₅₀ H ₇₁ N ₈ O ₁₂ Cl	0.08	4.97				+	+
1	Cyanopeptolin	Uk Cyp 999	1000.5120	C ₅₁ H ₆₉ N ₉ O ₁₂	-1.34	3.90	+				
2	Cyanopeptolin	Uk Cyp 900	901.5031	C ₄₄ H ₆₈ N ₈ O ₁₂	0.17	3.17		+			
2	Cyanopeptolin	Cyp B	929.5327	C ₄₆ H ₇₂ N ₈ O ₁₂	-1.66	3.69		+			
2	Cyanopeptolin	Cyp C	943.5481	C ₄₇ H ₇₄ N ₈ O ₁₂	-1.90	3.70		+			
2	Cyanopeptolin	Cyp A	957.5391	C ₄₆ H ₇₂ N ₁₀ O ₁₂	-1.35	3.75		+			
2	Cyanopeptolin	Cyp 963A	964.5040	C ₄₉ H ₆₉ N ₇ O ₁₃	1.53	5.31		+			
2	Cyanopeptolin	Uk Cyp 970	971.5565	C ₄₇ H ₇₄ N ₁₀ O ₁₂	0.46	4.14		+			
2	Cyanopeptolin	Uk Cyp 984	985.5725	C ₄₈ H ₇₆ N ₁₀ O ₁₂	0.61	4.72		+			
3	Microviridin	Uk Mv 1630	816.4052 ²	C ₇₂ H ₁₁₂ N ₁₇ O ₂₆	-0.35	2.81				+	+
3	Microviridin	Uk Mv 1651	826.9047 ²	C ₈₆ H ₁₀₇ N ₁₆ O ₁₈	-0.02	2.70	+			+	
3	Microviridin	Uk Mv 1669	835.9076 ²	C ₈₀ H ₁₁₃ N ₁₄ O ₂₅	0.36	2.79	+				
3	Microviridin	Uk Mv 1804	903.4258 ²	C ₈₈ H ₁₁₄ N ₁₉ O ₂₃	2.00	2.80	+			+	
3	Microviridin	Uk Mv 1856	922.3999 ²	C ₈₇ H ₁₁₂ N ₁₇ O ₂₈	-0.55	2.81	+			+	
3	Microviridin	Uk Mv 1850	926.4323 ²	C ₉₃ H ₁₁₆ N ₁₉ O ₂₂	-2.18	2.86					+
3	Microviridin	Uk Mv 1858	930.4302 ²	C ₉₁ H ₁₁₆ N ₁₉ O ₂₄	1.13	2.87	+			+	
3	Microviridin	Uk Mv 1874	938.4132 ²	C ₈₈ H ₁₁₆ N ₁₇ O ₂₉	-1.75	2.89				+	
4	Microviridin	Uk Mv 1567	784.8342 ²	C ₈₀ H ₉₁ N ₁₄ O ₂₀	0.31	3.21	+				+
4	Microviridin	Uk Mv 1589	800.3928 ²	C ₇₁ H ₁₀₉ N ₁₇ O ₂₅	0.61	2.64				+	+
4	Microviridin	Mv J	842.8925 ²	C ₈₀ H ₁₀₅ N ₁₉ O ₂₂	1.31	2.75	+			+	

4	Microviridin	Uk Mv 1715	858.9321 ²	C ₈₈ H ₁₁₅ N ₁₆ O ₂₀	1.36	2.90	+			+	
4	Microviridin	Uk Mv 1822	912.4276 ²	C ₈₈ H ₁₁₆ N ₁₉ O ₂₄	-1.87	2.80	+			+	
4	Microviridin	Mv H	919.4114 ²	C ₈₉ H ₁₁₄ N ₁₇ O ₂₆	-2.11	2.87	+			+	+
5	Microviridin	Uk Mv 1459	730.8624 ²	C ₈₂ H ₉₁ N ₁₆ O ₁₀	-0.15	2.44	+				+
5	Microviridin	Uk Mv 1612	807.4003 ²	C ₇₂ H ₁₁₁ N ₁₇ O ₂₅	0.06	2.80					+
5	Microviridin	Uk Mv 1790	896.4191 ²	C ₈₀ H ₁₁₆ N ₁₉ O ₂₈	0.06	2.64	+			+	
5	Microviridin	Uk Mv 1826	914.4168 ²	C ₉₀ H ₁₁₂ N ₁₉ O ₂₃	0.66	2.81	+			+	
6	Microcystin	[Asp ³ , Dha ⁷]-MC-LR	967.5254	C ₄₇ H ₇₀ N ₁₀ O ₁₂	-1.69	3.29	+	+			
6	Microcystin	[Asp ³]-MC-LR	981.5385	C ₄₈ H ₇₂ N ₁₀ O ₁₂	-0.71	3.40	+	+			
6	Microcystin	MC-LR	995.5565	C ₄₉ H ₇₄ N ₁₀ O ₁₂	1.76	3.49	+	+			
6	Microcystin	Uk MC 1010	1011.5535	C ₄₉ H ₇₄ N ₁₀ O ₁₃	-0.41	3.37	+	+			
7	Microginin	Mg 612	613.3225	C ₃₂ H ₄₄ N ₄ O ₈	-1.11	2.53					+
7	Microginin	Uk Mg 626	627.3374	C ₃₃ H ₄₆ N ₄ O ₈	0.49	2.70				+	+
7	Microginin	Uk Mg 628	629.3184	C ₃₂ H ₄₄ N ₄ O ₉	0.11	2.31					+
7	Microginin	Uk Mg 630	631.2891	C ₃₂ H ₄₃ N ₄ O ₇ Cl	-0.32	2.60					+
7	Microginin	Uk Mg 640	641.3535	C ₃₄ H ₄₈ N ₄ O ₈	-1.54	2.75				+	+
7	Microginin	Mg 646	647.2826	C ₃₂ H ₄₃ N ₄ O ₈ Cl	-2.22	2.56					+
7	Microginin	Uk Mg 660	661.2985	C ₃₃ H ₄₅ N ₄ O ₈ Cl	-1.66	2.70				+	+
7	Microginin	Uk Mg 664	665.2503	C ₃₂ H ₄₂ N ₄ O ₇ Cl ₂	0.41	3.04					+
7	Microginin	Uk Mg 674	675.3148	C ₃₄ H ₄₇ N ₄ O ₈ Cl	-1.06	2.72				+	+
7	Microginin	Mg 680	681.2441	C ₃₂ H ₄₂ N ₄ O ₈ Cl ₂	-1.44	2.65					+
7	Microginin	Uk Mg 694	695.2596	C ₃₃ H ₄₄ N ₄ O ₈ Cl ₂	-1.97	2.75					+
7	Microginin	Uk Mg 694	695.2599	C ₃₃ H ₄₄ N ₄ O ₈ Cl ₂	-0.39	2.89					+
7	Microginin	Uk Mg 714	715.2053	C ₃₂ H ₄₁ N ₄ O ₈ Cl ₃	-1.36	2.86				+	+
8	Aeruginosamide	Uk Ag 560	561.2522	C ₃₁ H ₃₆ N ₄ O ₄ S	-0.68	2.97					+
8	Aeruginosamide	Uk Ag 562	563.2695	C ₃₁ H ₃₈ N ₄ O ₄ S	-1.86	3.09					+
8	Aeruginosamide	Ag B	575.2678	C ₃₂ H ₃₉ N ₄ O ₄ S	0.43	3.55		+	+		+
8	Aeruginosamide	Uk Ag 590	591.2635	C ₃₂ H ₃₈ N ₄ O ₅ S	-0.28	3.08					+
8	Aeruginosamide	Uk Ag 661	660.3217	C ₃₆ H ₄₆ N ₅ O ₅ S	0.08	3.16					+
8	Aeruginosamide	Ag C	674.3357	C ₃₇ H ₄₇ N ₅ O ₅ S	0.79	3.85					+
8	Aeruginosamide	Uk Ag 659	690.3328	C ₃₇ H ₄₇ N ₅ O ₆ S	1.18	3.56					+
9	Anabaenopectin	Ap D	828.4298	C ₄₄ H ₅₇ N ₇ O ₉	-0.06	3.91				+	+
9	Anabaenopectin	Uk Ap 850	851.4453	C ₄₆ H ₅₈ N ₈ O ₈	-0.10	4.97				+	+
9	Anabaenopectin	Uk Ap 857	858.438	C ₄₅ H ₅₉ N ₇ O ₁₀	-1.82	3.44				+	+

9	Anabaenopeptin	FA A	867.4378	C ₄₆ H ₅₈ N ₈ O ₉	-1.44	3.93				+	+
9	Anabaenopeptin	FA B	881.4552	C ₄₇ H ₆₀ N ₈ O ₉	1.13	4.18				+	+
9	Anabaenopeptin	Uk Ap 882	883.4334	C ₄₆ H ₅₈ N ₈ O ₁₀	-1.66	3.46				+	+
10	Aeruginosin	Uk Ag 722	643.2986 ⁴	C ₃₂ H ₄₃ N ₆ O ₉ SCl	-1.38	2.60				+	
10	Aeruginosin	Uk Ag 650	651.3141	C ₃₃ H ₄₂ N ₆ O ₆	0.70	2.24		+			
10	Aeruginosin	Uk Ag 684	685.2750	C ₃₃ H ₄₁ N ₆ O ₆ Cl	0.63	2.34		+			
10	Aeruginosin	Uk Ag 688	689.2947	C ₃₂ H ₄₄ N ₆ O ₉ S	-1.34	2.50				+	
10	Aeruginosin	Uk Ag 734	717.3002 ¹	C ₂₉ H ₄₆ N ₆ O ₇ Cl ₂	0.98	2.44		+			
11	Cyanobactin	Md A	583.1901	C ₂₆ H ₃₀ N ₈ O ₄ S ₂	0.85	3.15			+		
11	Cyanobactin	Uk Md 584	585.2058	C ₂₆ H ₃₂ N ₈ O ₄ S ₂	-0.03	2.96	+		+		
11	Cyanobactin	Uk Md 586	587.2216	C ₂₆ H ₃₄ N ₈ O ₄ S ₂	-0.20	3.84	+		+		
12	Cyanobactin	Ac A	535.2161	C ₂₄ H ₃₄ N ₆ O ₄ S ₂	0.98	5.78	+	+			
12	Cyanobactin	Ac B	533.2006	C ₂₄ H ₃₂ N ₆ O ₄ S ₂	1.27	5.82		+			
12	Cyanobactin	Ac C	517.2235	C ₂₄ H ₃₂ N ₆ O ₅ S	1.42	5.85	+	+			
12	Cyanobactin	Uk Ac 518	519.2385	C ₂₄ H ₃₄ N ₆ O ₅ S	-0.89	5.77		+			
13	Unknown	Uk 1448	725.3962 ²	C ₇₃ H ₁₀₆ N ₁₅ O ₁₄ S	1.02	5.38	+				+
13	Unknown	Uk 1461	731.4159 ²	C ₆₈ H ₁₁₄ N ₁₅ O ₁₈ S	-0.92	5.81	+				+
13	Unknown	Uk 1462	732.4059 ²	C ₇₀ H ₁₁₀ N ₁₆ O ₁₆ S	-2.16	5.50	+				+
13	Unknown	Uk 1474	738.4238 ²	C ₆₉ H ₁₁₆ N ₁₅ O ₁₈ S	-0.75	5.81	+				+
13	Unknown	Uk 1476	739.4133 ²	C ₇₁ H ₁₁₂ N ₁₆ O ₁₆ S	-1.42	5.61	+				+
13	Unknown	Uk 1488	745.4307 ²	C ₇₀ H ₁₁₈ N ₁₅ O ₁₈ S	-2.07	5.91	+				+
13	Unknown	Uk 1490	746.4202 ²	C ₇₂ H ₁₁₄ N ₁₆ O ₁₆ S	-1.69	5.56	+				+
13	Unknown	Uk 1504	753.4296 ²	C ₇₃ H ₁₁₆ N ₁₆ O ₁₆ S	-1.95	5.69	+				+
13	Unknown	Uk 1508	755.4265 ²	C ₇₃ H ₁₁₈ N ₁₅ O ₁₅ S ₂	0.67	5.41	+				+
13	Unknown	Uk 1522	762.4341 ²	C ₇₄ H ₁₂₀ N ₁₅ O ₁₅ S ₂	0.74	5.57	+				+
14	Unknown	Uk 676	659.3628 ¹	C ₃₀ H ₄₈ N ₁₀ O ₈	-0.11	5.75		+		+	+
14	Unknown	Uk 678	661.3791 ¹	C ₃₀ H ₅₀ N ₁₀ O ₈	0.12	5.94		+		+	+
14	Unknown	Uk 816	799.4315 ¹	C ₃₅ H ₅₆ N ₁₄ O ₉	-0.82	6.12				+	+
15	Unknown	Uk 3314	1105.9709 ³	C ₁₈₈ H ₂₄₇ N ₂₇ O ₂₇	2.34	6.05	+			+	+
15	Unknown	Uk 3316	1106.6386 ³	C ₁₈₈ H ₂₄₉ N ₂₇ O ₂₇	-0.05	5.84				+	+
15	Unknown	Uk 3953	1318.7494 ³	C ₁₉₈ H ₂₉₇ N ₄₁ O ₄₄	0.01	6.05				+	+
16	Unknown	Uk 612	613.3250	C ₃₂ H ₄₄ N ₄ O ₈	2.96	2.95			+		
16	Unknown	Uk 692	693.2809	C ₃₂ H ₄₄ N ₄ O ₁₁ S	1.34	3.03			+		
16	Unknown	Uk 794	795.2213	C ₃₄ H ₄₂ N ₄ O ₁₅ S ₂	-0.05	3.00			+		

16	Unknown	Uk 810	811.1867	C ₄₀ H ₃₅ N ₄ O ₁₃ S	-2.61	3.02			+		
17	Unknown	Uk 1013	1014.6271	C ₅₆ H ₈₃ N ₇ O ₁₀	-0.27	6.04				+	+
17	Unknown	Uk 1029	1030.6211	C ₅₆ H ₈₃ N ₇ O ₁₁	0.22	5.91				+	+
18	Unknown	Uk 359	360.1929	C ₁₉ H ₂₅ N ₃ O ₄	2.82	2.33	+				
18	Unknown	Uk 559	560.2925	C ₂₄ H ₄₁ N ₅ O ₁₀	-0.21	2.20	+				
19	Unknown	Uk 608	609.4340	C ₃₁ H ₅₆ N ₆ O ₆	1.00	6.22		+			
19	Unknown	Uk 622	623.4494	C ₃₂ H ₅₈ N ₆ O ₆	0.64	6.24		+			
20	Unknown	Uk 495	513.3036 ⁵	C ₂₃ H ₄₀ N ₆ O ₇	1.02	6.25				+	+
20	Unknown	Uk 674	692.3845 ⁵	C ₂₅ H ₄₆ N ₁₂ O ₁₀	-0.80	5.49	+	+	+	+	+
21	Unknown	Uk 669	670.4092	C ₃₈ H ₅₁ N ₇ O ₄	2.28	5.62	+		+		
21	Unknown	Uk 831	832.4614	C ₄₄ H ₆₁ N ₇ O ₉	1.29	5.63	+		+		+
22	Unknown	Uk 758	759.3113	C ₃₇ H ₄₂ N ₈ O ₁₀	0.03	5.45				+	+
22	Unknown	Uk 760	761.3274	C ₃₇ H ₄₄ N ₈ O ₁₀	2.72	5.66		+		+	+
23	Unknown	Uk 410	393.2088 ¹	C ₁₉ H ₃₀ N ₄ O ₆	-2.36	2.32				+	+
23	Unknown	Uk 477	478.2753	C ₂₂ H ₃₅ N ₇ O ₅	-1.70	2.17				+	+
24	Unknown	Uk 334	335.1596	C ₁₇ H ₂₂ N ₂ O ₅	-1.63	2.89		+		+	+
24	Unknown	Uk 366	349.1753 ¹	C ₁₇ H ₂₆ N ₄ O ₅	-2.71	3.55				+	+
25	Unknown	Uk 516	534.3126 ⁵	C ₂₃ H ₄₀ N ₄ O ₉	-0.82	2.28			+		+
25	Unknown	Uk 560	578.3389 ⁵	C ₂₅ H ₄₄ N ₄ O ₁₀	-0.16	2.30			+		+
25	Unknown	Uk 604	622.3652 ⁵	C ₂₇ H ₄₈ N ₄ O ₁₁	-0.70	2.32			+		+
26	Unknown	Uk 946	947.5921	C ₄₆ H ₇₈ N ₁₀ O ₁₁	-0.34	6.20	+	+			
26	Unknown	Uk 960	961.6081	C ₄₇ H ₈₀ N ₁₀ O ₁₁	0.07	6.29	+	+			
26	Unknown	Uk 974	975.6237	C ₄₈ H ₈₂ N ₁₀ O ₁₁	0.05	6.32		+			

¹[M+H-H₂O]⁺; ²[M+2H]²⁺; ³[M+3H]³⁺; ⁴[M+H-SO₃]⁺; ⁵[M+NH₄]⁺

Abbreviations used in the table: Ac; aerucyclamide, Ag; aeruginosamide, Ahp; 3-amino-6-hydroxy-2-piperidone, Ap; anabaenopeptin, Cyp; cyanopeptolin, FA; ferintoic acid, Lxx; leucine or isoleucine, MC; microcystin, Md; microcyclamide, Mg; microginin, Mv; microviridin, NP; nostopeptin, Uk; unknown

Table S2: Compiled sequence database for cyanopeptolins previously reported in literature.

Compound name	Formula	Theoretical [M+H] ⁺	Theoretical [M+H-H ₂ O] ⁺	Residue 1	Residue 2	Residue 3	Residue 4	Residue 5	Residue 6	Residue 7	Residue 8
Tasipeptin B	C40 H62 N6 O9	771.4651	753.4545	L-Val	NMe-L-Phe	L-Leu	Ahp	L-Leu	L-Thr		
Micropept in MZ771	C38 H61 N9 O8	772.4716	754.4610	Ile	NMe-Phe	Ile	OMe-Ahp	Arg	Thr		
Micropept in MM836	C43 H60 N6 O11	837.4393	819.4287	Ile	NMe-Phe	Phe	Ahp	Leu	Thr		

planktopeptin BL843	C41 H61 N7 O12	844.4451	826.4345	L-Ile	NMe-OMe-L-Tyr	L-Thr	Ahp	L-Leu	L-Thr	L-Gln	
Micropeptin in MZ845	C40 H62 N9 O11	846.4720	828.4614	Ile	NMe-Phe	Ile	Ahp	Arg	Thr		
Micropeptin in MM850	C44 H62 N6 O11	851.4549	833.4444	Ile	NMe-Phe	Phe	OMe-Ahp	Leu	Thr		
Micropeptin in MZ859	C41 H65 N9 O11	860.4876	842.4771	Ile	NMe-Phe	Ile	OMe-Ahp	Arg	Thr		
Tasipeptin A	C45 H71 N7 O10	870.5335	852.5230	L-Val	NMe-L-Phe	L-Leu	Ahp	L-Leu	L-Thr	L-Val	
Cyanopeptolin 880	C45 H64 N6 O12	881.4655	863.4549	Ile	NMe-Phe	Ile	Ahp	Htyr	Thr		
micropeptin in 88A	C44 H65 N7 O12	884.4764	866.4658	L-Ile	NMe-L-Phe	L-Val	Ahp	HcAla	L-Thr	L-Glu	
Micropeptin in HU895A	C40 H62 Cl N9 O12	896.4279	878.4174	Val	NMe-OMe-Cl-L-Tyr	Ile	Ahp	Arg	Thr		
Micropeptin in HU895B	C40 H62 Cl N9 O12	896.4279	878.4174	Ile	L-NMe-Cl-Tyr	Ile	Ahp	Arg	Thr		
Micropeptin in HU909	C41 H64 Cl N9 O12	910.4436	892.4330	Val	NMe-OMe-Cl-L-Tyr	Ile	OMe-Ahp	Arg	Thr		
micropeptin in SF909	C45 H63 N7 O13	910.4557	892.4451	L-Ile	L-NMe-Tyr	L-Leu	Ahp	L-Gln	L-Thr	L-Hpla	
Micropeptin in LH911A	C39 H61 N9 O14 S	912.4131	894.4026	Val	NMe-Phe	Ile	Ahp	Arg	Thr		
Micropeptin in LH911B	C39 H61 N9 O14 S	912.4131	894.4026	Ile	NMe-Phe	Val	Ahp	Arg	Thr		
Micropeptin in LH911C	C41 H65 N7 O14 S	912.4383	894.4277	Ile	NMe-Phe	Ile	Ahp	NMe-Lys	Thr		
Micropeptin in MM916	C43 H60 N6 O14 S	917.3961	899.3855	Ile	NMe-Phe	Phe	Ahp	Leu	Thr		
Somamide B	C46 H62 N8 O12	919.4560	901.4454	Val	NMe-Tyr	Phe	Ahp	Abu	Thr	Gln	
Nostopeptin BN9	C46 H64 N8 O12	921.4716	903.4611	L-Val	NMe-L-Tyr	L-Phe	Ahp	L-Leu	L-Thr	L-Gln	
Micropeptin in LH920	C46 H64 N8 O12	921.4716	903.4611	Val	NMe-Tyr	Phe	Ahp	Gln	Thr	Gly	
Cyanopeptolin 920	C47 H68 N8 O11	921.5080	903.4975	Val	NMe-Phe	Phe	Ahp	Lys	Thr	Leu	
cyanopeptolin S	C40 H63 N9 O14 S	926.4288	908.4182	L-Ile	NMe-L-Phe	L-Ile	Ahp	L-Arg	L-Thr		
Micropeptin in MZ925	C40 H63 N9 O14 S	926.4288	908.4182	Ile	NMe-Phe	Ile	Ahp	Arg	Thr		
Micropeptin in LH925	C42 H67 N7 O14 S	926.4539	908.4434	Ile	NMe-Phe	Ile	Ahp	N,NdiMe-Lys	Thr		
nostopeptin B	C46 H70 N8 O12	927.5186	909.5080	L-Ile	NMe-L-Tyr	L-Ile	Ahp	L-Leu	Hmp	L-Gln	
nostopeptin C	C46 H70 N8 O12	927.5186	909.5080	Ile	NMe-L-Tyr	Ile	Ahp	Leu	Hmp	Gln	
Micropeptin in KB928	C44 H68 N10 O12	929.5091	911.4985	Ile	NMe-Phe	Val	Ahp	Arg	Thr	Asp	
Cyanopeptolin B	C46 H72 N8 O12	929.5342	911.5237	Val	NMe-Phe	Leu	Ahp	Lys	Thr	Asp	
Cyanopeptolin 930	C45 H70 N8 O13	931.5135	913.5029	Val	NMe-Tyr	Leu	Ahp	Lys	Thr	Glu	
Micropeptin in MM932	C43 H60 N6 O15 S	933.3910	915.3804	Ile	NMe-Tyr	Phe	Ahp	Leu	Thr		
Micropeptin in MZ939A	C41 H65 N9 O14 S	940.4444	922.4339	Ile	NMe-Phe	Ile	OMe-Ahp	Arg	Thr		

Micropept in MZ939B	C41 H65 N9 O14 S	940.4444	922.4339	Ile	NMe-Phe	Ile	OMe-Ahp	Arg	Thr		
Cyanopep tolin C	C47 H74 N8 O12	943.5499	925.5393	Val	NMe-Phe	Leu	Ahp	NMe-Lys	Thr	Asp	
micropept in SD944	C46 H72 N8 O13	945.5292	927.5186	L-Val	NMe-L- Tyr	L-Ile	Ahp	L-Lys	L-Thr	L-Asp	
Scryptolin A	C45 H69 Cl N8 O14	946.5006	928.4900	Val	L-NMe- Cl-Tyr	Thr	Ahp	Leu	Thr	Thr	Ala
Micropept in KT 946	C44 H62 N6 O15 S	947.4067	929.3961	Ile	NMe-Phe	Ile	Ahp	Htyr	Thr		
Anabaeno peptilide 90A	C46 H64 N8 O14	953.4615	935.4509	Ile	NMe- OMe-Tyr	Thr	Ahp	Htyr	Thr	Gln	
Cyanopep tolin 954	C46 H63 Cl N8 O12	955.4327	937.4221	L-Val	NMe-Cl- L-Tyr	L-Phe	Ahp	L-Leu	L-Thr	L-Gln	
nostopepti n A	C48 H74 N8 O12	955.5499	937.5393	L-Ile	NMe-L- Tyr	L-Ile	Ahp	L-Leu	Hmp	L-Gln	
nostopepti n D	C48 H74 N8 O12	955.5499	937.5393	L-Ile	NMe-L- Tyr	L-Ile	Ahp	L-Leu	Hmp	L-Gln	
nostopepti n E	C47 H73 N9 O12	956.5451	938.5346	L-Ile	NMe-L- Tyr	L-Leu	Ahp	L-Leu	Hmp	L-Gln	
Cyanopep tolin A	C46 H72 N10 O12	957.5404	939.5298	Val	NMe-Phe	Leu	Ahp	Arg	Thr	Asp	
Micropept in KB956	C46 H72 N10 O12	957.5404	939.5298	Ile	NMe-Phe	Val	OMe-Ahp	Arg	Thr	OMe-Asp	
Cyanopep tolin D	C48 H76 N8 O12	957.5655	939.5550	Val	NMe-Phe	Leu	Ahp	N,NdiMe- Lys	Thr	Asp	
Cyanopep tolin 958	C45 H70 N10 O13	959.5197	941.5091	Val	NMe-Tyr	Leu	Ahp	Arg	Thr	Glu	
micropept in B	C47 H74 N8 O13	959.5448	941.5342	L-Val	NMe-L- Tyr	L-Leu	Ahp	L-Arg	L-Thr	L-Glu	
Cyanopep tolin 960	C45 H64 N6 O15 S	961.4223	943.4117	Ile	NMe-Phe	Ile	Ahp	Htyr	Thr		
Micropept in HH 960	C49 H68 N8 O12	961.5029	943.4924	Val	NMe-Phe	Leu	Apo	Leu	Thr	Asn	
micropept in 90	C42 H59 N9 O15 S	962.3924	944.3818	Val	NMe-Tyr	L-Phe	Ahp	Arg	L-Thr		
cyanopept olin 963A	C49 H69 N7 O13	964.5026	946.4920	L-Val	NMe-L- Phe	L-Leu	Ahp	L-Tyr	L-Thr	L-Asp	
micropept in E1964	C46 H64 N10 O13	965.4727	947.4621	L-Ile	NMe-L- Tyr	L-Phe	Ahp	L-Arg	L-Thr	L-Asp	
Somamide A	C48 H67 N7 O12 S	966.4641	948.4536	Val	NMe-Tyr	Phe	Ahp	Abu	Thr	Met(O)	
Micropept in T20	C46 H59 N6 O15 P	967.3849	949.3743	L-Ile	N-Me-L- Tyr	L-Phe	L-Ahp	L-Phe	L-Thr	D-Gac- Phos	
nostopepti n I	C48 H75 N9 O12	970.5608	952.5502	L-Ile	NMe- OMe-L- Tyr	L-Leu	Ahp	L-Leu	Hmp	L-Gln	
nostopepti n F	C48 H75 N9 O12	970.5608	952.5502	L-Ile	NMe-L- Tyr	L-Leu	OMe-Ahp	L-Leu	Hmp	L-Gln	
Micropept in KB970A	C47 H74 N10 O12	971.5560	953.5455	Ile	NMe-Phe	Val	Ahp	Arg	Thr	OMe-Asp	
Micropept in KB970B	C47 H74 N10 O12	971.5560	953.5455	Ile	NMe-Phe	Val	OMe-Ahp	Arg	Thr	Asp	
Micropept in KB970C	C47 H74 N10 O12	971.5560	953.5455	Val	NMe-Phe	Val	OMe-Ahp	Arg	Thr	OMe-Asp	
Anabaeno peptilide 90B	C45 H61 Cl N8 O14	973.4069	955.3963	Ile	L-NMe- Cl-Tyr	Thr	Ahp	Htyr	Thr	Gln	
Cyanopep tolin 972	C46 H72 N10 O13	973.5353	955.5247	Val	NMe-Tyr	Leu	Ahp	Arg	Thr	Asp	

Micropept in 478-A	C40 H62 Cl N9 O15 S	976.3847	958.3742	Ile	L-NMe- Cl-Tyr	Ile	Ahp	Arg	Thr		
Micropept in HU975	C40 H62 Cl N9 O15 S	976.3847	958.3742	Val	NMe- OMe-Cl- L-Tyr	Ile	Ahp	Arg	Thr		
micropept in EI992	C48 H68 N10 O13	977.5091	959.4985	L-Ile	NMe-L- Tyr	L-Phe	Ahp	L-Arg	L-Thr	L-Asp	
Micropept in HM 978	C49 H70 N8 O13	979.5135	961.5029	Val	NMe-Phe	Ile	Ahp	Leu	Thr	Asn	
Micropept in MM978	C49 H70 N8 O13	979.5135	961.5029	Ile	NMe-Tyr	Phe	Ahp	Gln	Thr	Thr	
Micropept in HH 978	C49 H70 N8 O13	979.5135	961.5029	Val	NMe-Phe	Leu	Ahp	Leu	Thr	Asn	
micropept in SD 979	C49 H69 N7 O14	980.4975	962.4870	L-Val	NMe-L- Tyr	L-Ile	Ahp	L-Tyr	L-Thr	L-Asp	
Micropept in GH 979	C49 H69 N7 O14	980.4975	962.4870	Val	NMe-Phe	Ile	Ahp	Leu	Thr	Asp	
Micropept in HA 983	C46 H62 Cl N9 O13	984.4228	966.4123	Ile	L-NMe- Cl-Tyr	Phe	Ahp	Gln	Thr	Gln	
nostopepti n J	C49 H77 N9 O12	984.5764	966.5659	L-Ile	NMe- OMe-L- Tyr	L-Leu	OMe-Ahp	L-Leu	Hmp	L-Gln	
Micropept in KB984	C48 H76 N10 O12	985.5717	967.5611	Ile	NMe-Phe	Val	OMe-Ahp	Arg	Thr	OMe-Asp	
Micropept in K139	C47 H74 N10 O13	987.5510	969.5404	Ile	NMe-Tyr	Ile	Ahp	Arg	Thr	Asp	
Cyanopep tolin 986A	C47 H74 N10 O13	987.5510	969.5404	Val	NMe-Tyr	Leu	Ahp	Arg	Thr	Glu	
micropept in A	C49 H78 N8 O13	987.5761	969.5655	L-Val	NMe-L- Tyr	L-Leu	Ahp	L-Arg	L-Thr	L-Glu	
Micropept in HU989	C41 H64 Cl N9 O15 S	990.4004	972.3898	Ile	NMe- OMe-Cl- L-Tyr	Ile	Ahp	Arg	Thr		
Micropept in HH 992	C50 H72 N8 O13	993.5292	975.5186	Val	NMe-Phe	Leu	Mpc	Leu	Thr	Asn	
Cyanopep tolin 992	C50 H72 N8 O13	993.5292	975.5186	Val	NMe-Tyr	Phe	Ahp	Lys	Thr	Glu	
Micropept in KB992	C50 H72 N8 O13	993.5292	975.5186	Val	NMe-Phe	Ile	OMe-Ahp	Leu	Thr	Asn	
Micropept in E (or cyanopept olin 993)	C50 H71 N7 O14	994.5132	976.5026	Val	NMe-Tyr	Leu	Ahp	Tyr	Thr	Glu	
micropept in SF995	C48 H73 N11 O12	996.5513	978.5407	L-Val	L-NMe- Trp	L-Ile	Ahp	L-Arg	L-Thr	L-Asp	
Micropept in KR998	C51 H66 N8 O13	999.4822	981.4716	Val	NMe-Tyr	Phe	Ahp	Tyr	Thr	Gln	
micropept in SD999	C47 H73 N11 O13	1000.5462	982.5356	L-Val	NMe-L- Kyn	L-Ile	Ahp	L-Arg	L-Thr	L-Asp	
micropept in SD1002	C51 H70 N8 O13	1003.5135	985.5029	L-Val	NMe-L- Trp	L-Ile	Ahp	L-Tyr	L-Thr	L-Asp	
Micropept in KR1002	C51 H70 N8 O13	1003.5135	985.5029	Val	NMe-Tyr	Phe	Ahp	HcAla	Thr	Gln	
Cyanopep tolin SS	C40 H63 N9 O17 S2	1006.3856	988.3750	Ile	NMe-Phe	Ile	Ahp	Arg	Thr		
Cyanopep tolin 1006A	C49 H70 N10 O13	1007.5197	989.5091	Val	NMe-Tyr	Phe	Ahp	Arg	Thr	Asp	
Micropept in DR 1006	C51 H74 N8 O13	1007.5448	989.5342	Ile	NMe-Phe	Leu	Ahp	Leu	Thr	Gln	
Micropept in T2	C52 H73 N9 O12	1016.5451	998.5346	Val	NMe-Trp	Phe	Ahp	Lys	Thr	Glu	

Micropept in MZ1019	C41 H65 N9 O17 S2	1020.4013	1002.3907	Ile	NMe-Phe	Ile	OMe-Ahp	Arg	Thr		
Cyanopep tolin 1020	C50 H72 N10 O13	1021.5353	1003.5247	Val	NMe-Tyr	Phe	Ahp	Arg	Thr	Glu	
Cyanopep tolin 1020B	C52 H76 N8 O13	1021.5605	1003.5499	Val	NMe-Tyr	Phe	Ahp	Lys	Thr	Glu	
Micropept in HU1021	C40 H63 N9 O18 S 2	1022.3805	1004.3700	Ile	NMe-L- Tyr	Ile	Ahp	Arg	Thr		
Micropept in LH1021	C50 H71 N9 O14	1022.5193	1004.5088	Val	NMe-Tyr	Phe	Ahp	Gln	Thr	Thr	Gly
Micropept in F (or cyanopept olin 1021)	C52 H75 N7 O14	1022.5445	1004.5339	Val	NMe-Tyr	Leu	Ahp	Tyr	Thr	Glu	
Aeruginop eptin 917S-C	C51 H74 N8 O14	1023.5397	1005.5292	Ile	NMe-Tyr	Leu	Ahp	Leu	Thr	Gln	
Micropept in C	C53 H69 N7 O14	1028.4975	1010.4870	Val	NMe-Tyr	Phe	Ahp	Tyr	Thr	Glu	
Micropept in KR1030	C53 H74 N8 O13	1031.5448	1013.5342	Val	NMe-Tyr	Phe	Ahp	HcAla	Thr	Gln	
micropept in 88N	C54 H78 N8 O14	1031.5812	1013.5706	L-Ile	NMe-L- Phe	L-Val	Ahp	L-Tyr	L-Thr	L-Glu	L-Leu
Oscillapep tilide 97-B	C52 H73 N9 O13	1032.5401	1014.5295	Ile	NMe-Tyr	Phe	Ahp	Leu	Thr	Gln	Pro
Micropept in HU1041	C39 H60 Cl N9 O18 S 2	1042.3259	1024.3153	Val	L-NMe- Cl-Tyr	Ile	Ahp	Arg	Thr		
Micropept in KT1042	C53 H70 N8 O14	1043.5084	1025.4979	Val	NMe-Phe	Ile	Ahp	Tyr	Thr	Gln	
Aeruginop eptin 228A	C52 H68 N8 O15	1045.4877	1027.4771	Ile	NMe-Phe	Thr	Ahp	Tyr	Thr	Gln	
micropept in 103	C52 H72 N10 O13	1045.5353	1027.5247	L-Val	NMe-L- Trp	L-Phe	Ahp	L-Gln	L-Thr	L-Thr	Gly
Oscillapep tilide 97- A	C53 H75 N9 O13	1046.5557	1028.5451	Ile	NMe- OMe-Tyr	Phe	Ahp	Leu	Thr	Gln	Pro
Micropept in KB1046	C53 H74 N8 O14	1047.5397	1029.5292	Ile	NMe-Phe	Val	Ahp	HcAla	Thr	Gln	
Aeruginop eptin 228B	C52 H72 N8 O15	1049.5190	1031.5084	Ile	NMe-Phe	Thr	Ahp	HcAla	Thr	Gln	
Micropept in KB1048	C49 H77 Cl N10 O13	1049.5433	1031.5327	Ile	L-NMe- Cl-Tyr	Ile	OMe-Ahp	Arg	Thr	OMe-Asp	
Micropept in LH1048	C52 H76 N10 O13	1049.5666	1031.5560	Val	NMe-Tyr	Phe	Ahp	Arg	Thr	Glu	
Cyanopep tolin 1048	C52 H76 N10 O13	1049.5666	1031.5560	Val	NMe-Tyr	Phe	Ahp	Arg	Thr	Glu	
Anabaeno peptilide 202A	C51 H71 N9 O15	1050.5142	1032.5037	Ile	NMe- OMe-Tyr	Thr	Ahp	Htyr	Thr	Gln	Pro
Micropept in T1	C55 H70 N8 O13	1051.5135	1033.5029	Val	NMe-Trp	Phe	Ahp	Tyr	Thr	Glu	
Symplostas tin 2	C52 H74 N8 O13 S	1051.5169	1033.5063	Val	NMe-Tyr	Phe	Ahp	Abu	Thr	Met(O)	Ile
Symploca mide A	C46 H71 Br N10 O13	1053.4438	1035.4332	L-Val	NMe,OM e-L-Br- Tyr	L-Ile	Ahp	L-Cit	L-Thr	L-Gln	But

Micropept in 478-B	C40 H62 Cl N9 O18 S 2	1056.3416	1038.3310	Ile	L-NMe- Cl-Tyr	Ile	Ahp	Arg	Thr		
Micropept in D	C55 H73 N7 O14	1056.5288	1038.5183	Val	NMe-Tyr	Phe	Ahp	Tyr	Thr	Glu	
Micropept in DR 1056	C54 H72 N8 O14	1057.5241	1039.5135	Ile	NMe-Phe	Leu	Ahp	Tyr	Thr	Gln	
Microcyst ilide A	C54 H72 N8 O15	1057.5241	1039.5135	Ile	NMe-Tyr	Leu	Ahp	Tyr	Thr	Gln	
Micropept in DR 1060	C54 H76 N8 O14	1061.5554	1043.5448	Ile	NMe-Phe	Leu	Ahp	HcAla	Thr	Gln	
planktope ptin BL1061	C50 H79 N9 O16	1062.5718	1044.5612	L-Ile	NMe- OMe-L- Tyr	L-Thr	Ahp	L-Leu	L-Thr	L-Gln	L-Leu
oscillapep tin C	C55 H79 N7 O14	1062.5758	1044.5652	L-allo-Ile	NMe-L- Phe	L-Ile	OMe-Ahp	HcAla	L-Thr	D-Htyr	
micropept in 88E	C54 H78 N8 O14	1063.5710	1045.5605	L-Ile	NMe-L- Phe	L-Val	Ahp	L-Leu	L-Thr	L-Glu	L-Phe
Micropept in LH1062	C53 H78 N10 O13	1063.5823	1045.5717	Val	NMe-Tyr	Phe	Ahp	Arg	Thr	OMe-Glu	
Micropept in HU1069	C41 H64 Cl N9 O18 S 2	1070.3572	1052.3466	Ile	NMe- OMe-Cl- L-Tyr	Ile	Ahp	Arg	Thr		
Anabaeno peptilide 202B	C50 H68 Cl N9 O15	1070.4596	1052.4491	Ile	L-NMe- Cl-Tyr	Thr	Ahp	Htyr	Thr	Gln	Pro
Aeruginop eptin 917S-A	C54 H72 N8 O15	1073.5190	1055.5084	Ile	NMe-Tyr	Leu	Ahp	Tyr	Thr	Gln	
Hofmanno lin	C54 H71 N7 O16	1074.5030	1056.4924	Val	NMe-Tyr	OMe-Tyr	Ahp	Tyr	Thr	Glu	
Aeruginop eptin 917S-B	C54 H76 N8 O15	1077.5503	1059.5397	Ile	NMe-Tyr	Leu	Ahp	HcAla	Thr	Gln	
Micropept in 88B	C53 H74 N8 O16	1079.5296	1061.5190	L-Ile	NMe-L- Phe	L-Val	Ahp	L-Glu	L-Thr	L-Glu	L-Phe
micropept in 88Y	C55 H72 N8 O15	1085.5190	1067.5084	L-Ile	NMe-L- Phe	L-Val	Ahp	L-Tyr	L-Thr	L-Glu	L-Tyr
oscillapep tin F	C51 H76 N8 O16 S	1089.5173	1071.5067	L-allo-Ile	NMe-L- Phe	L-Ile	Ahp	L-Lys	L-Thr	D-Htyr	
Cyanopep tolin 975	C47 H66 N10 O18 S	1091.4350	1073.4244	Leu	NMe-Tyr	Phe	Ahp	Arg	Thr	Asp	
Micropept in 1106	C54 H79 N11 O14	1106.5881	1088.5775	Ile	NMe-Phe	Val	Ahp	Arg	Thr	Glu	Tyr
Oscillapep tin G	C53 H77 N9 O17	1112.5510	1094.5405	Ile	NMe-Tyr	Thr	Ahp	Leu	Thr	Gln	Hty
micropept in 88C	C57 H76 N8 O15	1113.5503	1095.5397	L-Ile	NMe-L- Phe	L-Val	Ahp	L-Tyr	L-Thr	L-Glu	L-Phe
Nostocycl in	C56 H76 N8 O16	1117.5452	1099.5346	Val	NMe-Tyr	Phe	Ahp	Hse	Thr	Hse	Ile
micropept in 88D	C57 H80 N8 O15	1117.5816	1099.5710	L-Ile	NMe-L- Phe	L-Val	Ahp	HcAla	L-Thr	L-Glu	L-Phe
Micropept in 1120	C55 H81 N11 O14	1120.6037	1102.5932	Ile	NMe-Phe	Val	Ahp	Arg	Thr	OMe-Glu	Tyr
Scryptolin B	C52 H80 Cl N9 O16	1122.5484	1104.5379	Val	L-NMe- Cl-Tyr	Thr	Ahp	Leu	Thr	Thr	Ala (x2)
planktope ptin BL1125	C54 H79 N9 O17	1126.5667	1108.5561	L-Ile	NMe,OM e-L-Tyr	L-Thr	Ahp	L-Leu	L-Thr	L-Gln	L-Htyr
micropept in 88F	C58 H78 N8 O15	1127.5659	1109.5554	L-Ile	NMe-L- Phe	L-Val	Ahp	L-Tyr	L-Thr	OMe-L- Glu	L-Phe

oscillapeptin D	C54 H77 N7 O17 S	1128.5169	1110.5064	L-allo-Ile	NMe-L-Phe	L-Ile	Ahp	HcAla	L-Thr	D-Htyr	
oscillapeptin E	C55 H75 N7 O17 S	1138.5013	1120.4907	L-allo-Ile	NMe-L-Phe	L-Ile	Ahp	L-Htyr	L-Thr	D-Htyr	
Lyngbyastatin 4	C53 H68 N8 O18 S	1139.4403	1121.4297	L-Val	NMe-Tyr	Phe	L-Ahp	Abu	L-Thr	L-Htyr	L-Ala
Aeruginopeptin 95A	C56 H75 N9 O17	1146.5354	1128.5248	Ile	NMe-Phe	Thr	Ahp	Tyr	Thr	Gln	Thr
Oscillapeptin J	C47 H68 N10 O18 S	1149.4557	1131.4452	Ile	NMe-Tyr	Thr	Ahp	Arg	Thr	Tyr	
Aeruginopeptin 95B	C56 H79 N9 O17	1150.5667	1132.5561	Ile	NMe-Phe	Thr	Ahp	HcAla	Thr	Gln	Thr
oscillapeptin A	C56 H77 N7 O18 S	1168.5119	1150.5013	L-allo-Ile	NMe-OMe-L-Tyr	L-Ile	Ahp	L-Htyr	L-Thr	L-Htyr	
Oscillapeptin	C56 H77 N7 O18 S	1168.5119	1150.5013	Ile	NMe-OMe-Tyr	Ile	Ahp	Tyr	Thr	Tyr	
oscillapeptin B	C57 H79 N7 O18 S	1182.5275	1164.5169	L-allo-Ile	NMe-OMe-L-Tyr	L-Ile	Ahp	L-7-Me-Htyr	L-Thr	L-Htyr	
largamide E	C56 H82 Cl N9 O17	1188.5590	1170.5484	L-Val	L-NMe-Cl-Tyr	L-Thr	L-Ahp	L-Leu	L-Thr	L-Val	L-Ala
largamide D	C56 H82 Br N9 O17	1232.5085	1214.4979	L-Val	L-NMe-Br-Tyr	L-Thr	L-Ahp	L-Leu	L-Thr	L-Val	L-Ala
largamide F	C59 H80 Br N9 O18	1282.4878	1264.4772	L-Val	L-NMe-Br-Tyr	L-Thr	L-Ahp	L-Tyr	L-Thr	L-Val	L-Ala
largamide G	C60 H82 Br N9 O18	1296.5034	1278.4928	L-Val	L-NMe-Br-Tyr	L-Thr	L-Ahp	L-Htyr	L-Thr	L-Val	L-Ala

Table S3: Compiled sequence database for microginins previously reported in literature.

Compound name	Molecular formula	Theoretical m/z [M+H] ⁺	Residue 1	Residue 2	Residue 3	Residue 4	Residue 5	Residue 6
MG FR1	C36 H57 N5 O9	728.4229	Ahda	Ala	NMe-Leu	Tyr	Tyr	

MG FR2	C39 H59 N5 O9	742.4386	<i>NMe-Ahda</i>	Ala	MLeu	Tyr	Tyr	
MG FR3	C37 H53 N5 O10	728.3865	Ahda	Thr	Pro	Tyr	Tyr	
MG FR4	C38 H55 N5 O10	742.4022	<i>NMe-Ahda</i>	Thr	Pro	Tyr	Tyr	
MG FR5	C33 H55 N5 O9	666.4073	Ahda	Val	Pro	Tyr	Tyr	
MGFR6	C39 H57 N5 O9	740.4229	Me Ahda	Val	Pro	Tyr	Tyr	
MG FR7	C38 H57 N5 O10	744.4178	Ahda	Ser	Me Leu	Tyr	Tyr	
MG FR8	C41 H61 N5 O9	768.4542	Me Ahda	Pro	Me Leu	Tyr	Tyr	
MG FR9	C39 H54 N6 O9	751.4025	Me Ahda	Thr	Pro	Tyr	Trp	
MG FR10	C40 H56 N6 O8	749.4232	Ahda	Val	Pro	Tyr	Trp	
MG FR12	C42 H65 N5 O9	784.4855	Me Ahda	Val	Me Leu	Me Tyr	Tyr	
MG FR13	C41 H63 N5 O9	770.4699	Me Ahda	Val	Ile/Leu	Me Tyr	Tyr	
MG	C37 H55 N5 O9	714.4073	Ahda	Ala	Val	Me Tyr	Tyr	
MG T1	C36 H50 Cl N5 O9	732.3370	Cl-Ahda	Ala	Pro	Tyr	Tyr	
MG T2	C36 H51 N5 O9	698.3760	Ahda	Ala	Pro	Tyr	Tyr	
MG 51-A	C49 H68 N6 O11	917.5019	Ahda	Tyr	Me Val	Me Tyr	Pro	
MG 51-B	C50 H70 N6 O11	931.5175	Me Ahda	Tyr	Me Val	Me Tyr	Pro	
MG 91-A	C28 H51 Cl N4 O6	575.3570	Cl Ahda	Ile	Me Leu	Pro	N/A	
MG 91-B	C28 H50 Cl2 N4 O6	609.3180	Cl2 Ahda	Ile	Me Leu	Pro	N/A	
MG 91-C	C37 H61 N5 O8	704.4593	Ahda	Ile	Me Leu	Pro	Tyr	
MG 91-D	C37 H60 Cl N5 O8	738.4203	Cl Ahda	Ile	Me Leu	Pro	Tyr	
MG 91-E	C37 H60 Cl2 N5 O8	773.3892	Cl2 Ahda	Ile	Me Leu	Pro	Tyr	
MG 99-A	C40 H58 Cl N5 O8	772.4047	Cl Ahda	Tyr	Leu	Me Tyr	Pro	
MG 99-B	C40 H57 Cl2 N5 O8	806.3657	Cl2 Ahda	Tyr	Leu	Me Tyr	Pro	

MG 299-A	C45 H67 Cl N6 O10	887.4680	Cl Ahda	Val	Me Val	Me Tyr	Pro	Tyr
MG 299-B	C45 H66 Cl2 N6 O10	921.4290	Cl2 Ahda	Val	Me Val	Me Tyr	Pro	Tyr
MG 299-C	C45 H68 N6 O10	853.5070	Ahda	Val	Me Val	Me Tyr	Pro	Tyr
MG 299 D	C36 H57 Cl2 N5 O8	758.3657	Cl2 Ahda	Val	Me Val	Me Tyr	Pro	
MG 478	C41 H63 N5 O9	770.4699	<i>NMe-Ahda</i>	Val	Me Val	Me Tyr	Tyr	
MG 511	C25 H41 N3 O6 S	512.2789	Ahda	Ala	Met	Tyr	N/A	
MG 527	C25 H41 N3 O7 S	528.2738	Ahda	Met	Tyr	Tyr	N/A	
MG 527 Methyl Ester	C26 H43 N3 O7 S	542.2894	Me Ahda	Met	Tyr	Tyr	N/A	
MG 535	C27H42N4O7	535.3000	Ahda	Ala	Pro	Tyr	N/A	
MG 551	C27H42N4O8	551.1000	Ahda	Ser	Pro	Tyr	N/A	
MG 565A	C29H48N4O7	565.0000	Ahda	Ala	Me Leu	Tyr	N/A	
MG 565B	C29H48N4O7	565.2000	Ahda	Thr	Pro	Tyr	N/A	
MG 568	C27H41N4O7Cl	569.1000	Cl Ahda	Ala	Pro	Tyr	N/A	
MG 576	C26H53N4O6Cl	577.2000	Cl Ahda	Ile	Me Lxx	Val	N/A	
MG 577	C26H53N4O6Cl	638.2300	Cl Ahda	Ile	Me Lxx	Val	N/A	
MG 579A	C30H50N4O7	578.9000	Me Ahda	Ala	Me Lxx	Tyr	N/A	
MG 579B	C29H46N4O8	579.2000	Me Ahda	Thr	Pro	Tyr	N/A	
MG 579C	C29H46N4O8	579.2000	Ahda	Ala	Me Tyr/Me Htyr	Tyr	N/A	
MG 581	C29H48N4O8	581.2000	Ahda	Ser	Me Lxx	Tyr	N/A	
M 591B	C31H50N4O7	591.2000	Ahda	Tyr	Me Lxx	Pro	N/A	
MG 598	C28H43N4O8Cl	599.2000	Cl Ahda	Thr	Pro	Tyr	N/A	
MG 607A	C32H54N4O7	607.4000	Me Ahda	Val	Leu/Ile	MeTyr/Htyr	N/A	
MG 607B	C32H54N4O7	607.4000	Ahda	Val	MeLeu/Me Ile	MeTyr/Htyr	N/A	
MG 612	C32 H44 N4 O8	613.3232	Ahoa	Gly	Tyr	Tyr	Pro	
MG 621A	C33H56N4O7	621.3000	Me Ahda	Val	Me Leu/Me Ile	MeTyr/Htyr	N/A	

MG 621B	C33H56N4O7	621.4000	Me Ahda	Val	Me Leu/Me Ile	Tyr	N/A	
MG 646	C32 H43 Cl N4 O8	647.2842	Cl Ahoa	Gly	Tyr	Tyr	Pro	
MG 650	C33H55N5O8	650.0000	Ahda	Ala	Val	Leu/Ile	Tyr	
MG 674	C34 H50 N4 O8 S	675.3422	Me Ahda	Met	Tyr	Tyr	N/A	
MG 680	C32 H42 Cl2 N4 O8	681.2452	Cl2 Ahoa	Gly	Tyr	Tyr	Pro	
MG 688	C35 H52 N4 O8 S	689.3579	Ahoa	Met	Tyr	Tyr	N/A	
MG 690	C34 H50 N4 O9 S	691.3371	Me Ahoa	Met	Tyr	Tyr	N/A	
MG 704	C35 H52 N4 O9 S	704.3450	Ahda	Val	Me Met	Tyr	Me Tyr	
MG 711	C39 H59 N5 O6	694.4538	An	Val	Me Leu	Tyr	Tyr	
MG 713	C37 H55 N5 O9	714.4073						
MG 715	C38 H50 Cl O7 N5	724.3472	ClAn	Val	Pro	Tyr	Tyr	
MG 756	C38 H58 N5 O9	729.4307	Ahda	Val	Leu	HTy	Tyr	
MG 757	C39 H59 N5 O10	746.4335	Ahda	Thr	Val	Tyr	Tyr	
MG 764	C40 H56 N6 O9	765.4182	Me Ahda	Thr	Pro	Tyr	Trp	
MG 767	C41 H61 N5 O9	768.4542						
MG 767B	C41 H61 N5 O9	768.4542						
MG 770	C39 H60 N5 O9	743.4464	Me Ahda	Val	Leu	HTy	Tyr	
MG AL584	C28 H45 Cl N4 O7	585.3050	Cl Ahda	Gly	Val	Tyr	N/A	
MG KR604	C32 H52 N4 O7	605.3909	Me Ahda	Tyr	<i>NMeLeu</i>	Pro	N/A	
MG KR638	C32 H51 Cl N4 O7	639.3519	Cl-NMe Ahda	Tyr	<i>NMeLeu</i>	Pro	N/A	
MG KR767	C41 H61 N5 O9	768.4542	Me Ahda	Tyr	<i>NMeLeu</i>	Pro	Tyr	
MG KR781	C42 H62 N5 O9	781.4520	<i>NMe Ahda</i>					
MG KR787	C40 H58 Cl N5 O9	788.3996	Cl Ahda					
MG KR801	C41 H61 Cl N5 O9	803.4231	Cl-NMe-Ahda	Tyr	Me Leu	Pro	Tyr	

MG KR815	C42 H62 N5 O9	781.4620	Cl-NMe-Ahda					
MG KR835	C41 H59 Cl2 N5 O9	836.3763	Cl2-NMe-Ahda	Tyr	Me Leu	Pro	Tyr	
MG SD755	C40 H61 N5 O9	755.4464	MAhda	Val	Me Ile	Me Tyr	Tyr	
Microginin neu								
Microginine microginine	C37 H55 N5 O9	714.4073	Ahda	Ala	Val	Me Tyr	Tyr	
Microginine 711 711	C37 H53 N5 O9	712.3916	An	Val	Me Leu/Me Ile	Tyr	Tyr	
Microginine 757	C38 H59 N5 O10	745.4256	Ahda	Thr	Me Leu	Tyr	Tyr	
Microginine 764 764	C40 H56 N6 O9	765.4182	Me Ahda	Thr	Pro	Tyr	Trp	
Microginine 767 767	C41 H61 N5 O9	768.4542	Me Ahda	Tyr	Me Ile	Pro	Tyr	
Microginine GH787 GH787	C40 H58 Cl N5 O9	788.3996	Cl Ahda	Tyr	Me Ile	Pro	Tyr	
Microguanidine KT636	C19 H32 N4 O14 S	572.1630						
Cyanostatin A	C38 H57 N5 O9	728.4229	Ahda	Ala	Val	Me Tyr	Hty	
Cyanostatin B	C40 H59 N5 O9	754.4386	Ahda	Tyr	Me Ile	Me Pro	Tyr	
Nostoginin BN578	C30 H51 N4 O7	580.3831						
Nostoginin BN741	C39 H59 N5 O9	742.4286						
Oscallaginin A	C29 H47 Cl N4 O8	615.3155	Cl Ahda	Ser	Me Val	HTyr	N/A	
Oscallaginin B	C29 H48 N4 O8	581.3545	Ahda	Ser	Me Val	HTyr	N/A	

Figure S1: Standard curve for quantitative analysis of MC-LR in toxigenic *Microcystis* strain extracts.