AUTHOR: ARIF ABDUL RAHMAN, T.

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An investigation on the effects of cyanopeptides on the growth and secondary metabolite production of *Microcystis aeruginosa* PCC7806

By

Thaslim Arif A.R.

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy to Robert Gordon University, Aberdeen, U.K.

April 2016

DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise acknowledged, and has not been submitted in any form for another degree or qualification at any other academic institution. Information derived from published or unpublished work of others has been acknowledged in the text and a list of references is given.

Thaslim Arif A.R.

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Abstract:

Cyanobacteria are one of the oldest forms of photosynthetic life and may have contributed significantly to the evolution of oxygen into the then anoxic environment. Cyanobacteria are also one of the best sources of natural secondary metabolites (cyanopeptides) some of which have harmful effects on the ecosystem, while others may be beneficial. It is known that these secondary metabolites are continuously produced during growth, however, it is not known whether the producing cyanobacteria actually benefit from these metabolites. The overarching aim of this study was to answer the question 'Why do cyanobacteria produce secondary metabolites?'.

With this aim in mind, preliminary work focused on understanding the growth and secondary metabolite production characteristics of *Microcystis aeruginosa* PCC7806. The technique of labelling secondary metabolites with ^{15}N was successfully employed in differentiation and quantification of *ex-novo* and *de-novo* metabolites. The effect of exogenous cyanopeptides such as microcystins, aerucyclamides, anabaenopeptins, aeruginosamide, cyanopeptolin and aeruginosin on *M. aeruginosa* PCC7806 was evaluated using a rapid bioassay approach along with an automated cell enumeration technique. The results indicate that at least some cyanopeptides (microcystins-LR, microcystin-LF, aeruginosamide, anabaenopeptin B and aerucyclamide A) induce significant changes to cell division and metabolite production rate. In an ecological scenario, the release of such secondary metabolites by lysing cells (such as when blooms collapse), may be perceived as an alarm signal by surrounding live

cells, which may in turn slow cell division and prepare for re-invasion. This may be a strategy for species survival and dominance.

While the results from this study do not confirm a role for cyanopeptides, it is thought that the results are clearly indicative of the role played by cyanopeptides for the producing organism. In order to confirm a role, it is recommended that monitoring ribosomally synthesised metabolites (e.g. aerucyclamides) along with chlorophyll-a gene expression, with sophisticated techniques such as qPCR are used.

Keywords – Cyanobacteria, Cyanopeptides, Secondary metabolites, *Microcystis aeruginosa* PCC7806, microcystin, demethyl microcystin-LR, cyanopeptolin, aerucyclamide, anabaenopeptin, aeruginosamide, flowcytometry, de-novo production, ex-novo, UPLC, LC-MS/MS

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Chapter I

Introduction

1.1 Cyanobacteria – background

Cyanobacteria are a group of photosynthetic organisms which are spread across varied ecosystems, including extreme ones [1]. Their range of habitats includes freshwater, brackish and marine environments. Some strains are known to grow in terrestrial ecosystems; however, moisture is an absolute requirement for their survival. This group of bacteria can be clearly differentiated from other forms owing to the presence of chlorophyll-a and phycocyanin [2]. As photosynthetic organisms, their nutrient requirement is relatively simple and consists of inorganic carbon, nitrogen, phosphates, traces of inorganic minerals, sunlight and water. This simple requirement and their ability to adapt to changing environments has enabled them to thrive in a wide range of ecosystems [3].

Cyanobacterial fossils have been dated back to 3.5 billion years confirming their presence even before the oxygenation of the earth's atmosphere [4]. In those days, the atmosphere could not support oxygen enabled metabolism as its primary elements were mainly CH_4 and CO_2 and H_2O. It is thought that cyanobacteria predominated in this atmosphere evolving oxygen, ultimately leading to the oxygenation of the atmosphere [5]. In fact, it has been proven that the present day higher photosynthetic organisms evolved as a result of endosymbiosis of primitive cyanobacterial cells [6].

Cyanobacteria are sometimes referred to as blue-green algae owing to the presence of the blue phycocyanin and the green

chlorophyll-a pigments. However, unlike algae which are eukaryotes, cyanobacteria are truly prokaryotes as they lack a well-defined nucleus, organised intracellular structures and reproduce asexually [7]. Presently, cyanobacteria are classified under prokaryotes as cyanophycaen members, which form a large monophyletic group (Figure 1.1).

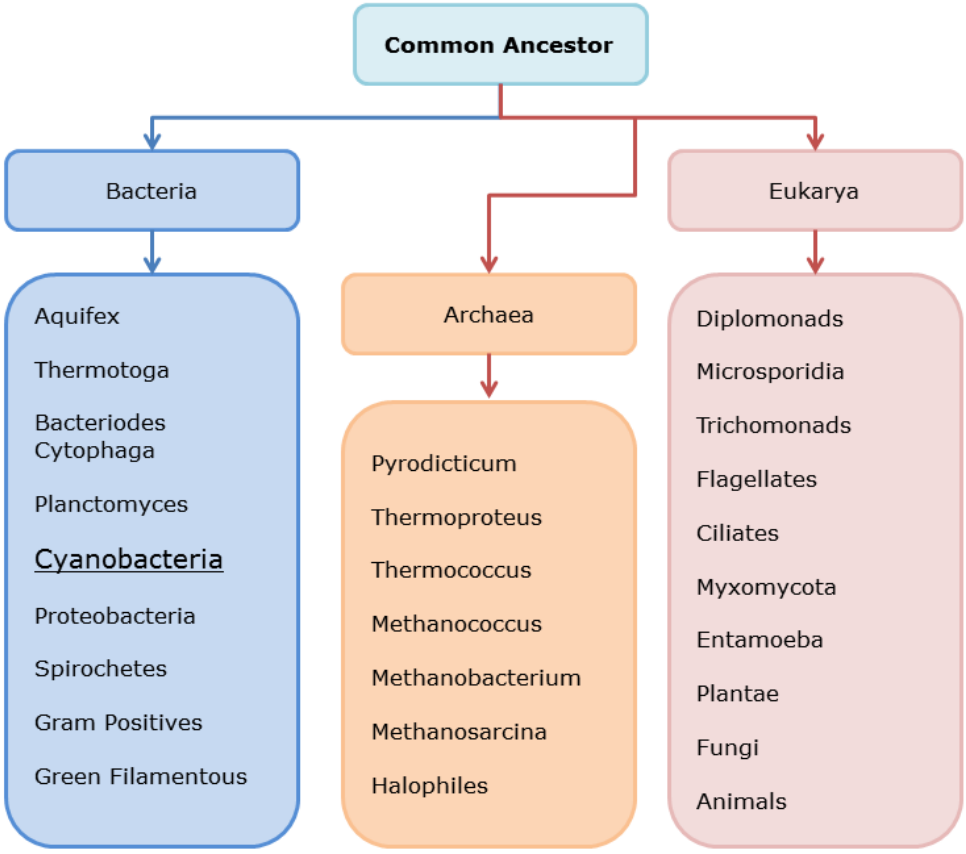


Figure 1.1: Phylogenetic tree of life depicting classification of cyanobacteria [8].

1.2 Blooms

Cyanobacteria are capable of prolific growth under ambient atmospheric conditions. Factors such as low turbulence, warm temperature, high pH, optimal sunlight, low N:P ratio and low grazing

rates encourage bloom formation. Recently, it was reported that bloom frequencies have increased as a net result of global warming. Blooms can occur under wide ranging environmental conditions, although the tropical and subtropical climates seem to favour bloom formation [9]. Blooms may be widely classified as non-toxic and toxic based on the toxicity of the predominant organism that has formed the bloom. Overall, it has been estimated that up to 75% of blooms may be toxic i.e. the compounds secreted by the predominant organism may be toxic to humans or animals [10]. In 1996, 76 people were reported to have died due to Caruaru syndrome with symptoms such as hepatomegaly, jaundice, necrosis and apoptosis of hepatocytes. Later, it was confirmed that the symptoms were due to poisoning from microcystin contaminated water used for renal dialysis [11]. In 2003, a report suggested that the death of a large number of Lesser Flamingos in Kenya may be attributed to the presence of microcystin-LR and anatoxin-a in the Bogoria lake [12]. Recently, between 2007 and 2011, the US department of health reported that 12 canines have succumbed to anatoxin poisoning, 3 to microcystin and 1 to brevetoxin [13].

In UK, a number of dog deaths have been attributed to neurotoxin poisoning caused by consumption of cyanobacterial bloom contaminated water. It was confirmed that the cause of these deaths were due to anatoxin-a produced by *Oscillatoria* sp [46]. Bloom frequency have been reported to increase during warm dry summers in UK and Europe, causing the death of sheep, dogs and some cases of human illness [18]. Considering the implications of cyanotoxins, WHO

has set a provisional guideline value of 1 µg / L of total (free and cell bound) microcystin-LR for drinking water [14]. Even when the bloom type is non-toxic, it may still result in an adverse effect on the water body by depleting nutrient and oxygen levels and blocking sunlight penetration to the underwater photosynthetic organisms thereby inhibiting them. Also, when blooms collapse, they release excess ammonia into the ecosystem causing disease to fish [10].

Recent research suggests that cyanobacterial blooms are promoted by higher temperatures as well as increased anthropogenic nutrient loads. It is also suggested that changing weather conditions will alter rainfall which may in turn contribute to further nutrient inflow. Further, climate change is predicted to be inevitable with increased temperatures, enhanced vertical stratification of aquatic ecosystems and alteration in annual weather patterns, all of which favour cyanobacterial growth [15 - 17]. Also, as toxic blooms have been reported in Australia, USA, UK, India, Africa and Europe it is imperative that toxic cyanobacteria are studied in depth to understand their growth and toxin production dynamics [18 - 20].

The predominant bloom forming cyanobacteria are the *Microcystis, Anabaena, Nodularia, Cylindrospermopsis, Oscillatoria* and *Planktothrix.* Blooms formed by toxic cyanobacteria such as *Microcystis, Cylindrospermopsis, Nodularia* release an array of their secondary metabolites into the aquatic ecosystem. Many of these secondary metabolites have been shown to exert a toxic effect against mice hepatocytes (hepatotoxin) or affect the nerve junctions

(neurotoxins) [10]. Along with the toxic effect, these blooms also have the additional negative effects of non-toxic blooms e.g. O_2 depletion. These combined effects drastically affect water quality; kill fish and other aquatic life forms. Hence, cyanobacterial secondary metabolites have become a concern for healthcare as well as environmental sectors. Though cyanobacteria have been studied for several decades and several hundreds of secondary metabolites have been identified, their ecological significance and the benefit to the producing organism are yet to be fully understood.

1.3 Secondary metabolites

Cyanobacterial secondary metabolites have become a subject of intense research due to two main reasons. Firstly, some of the secondary metabolites have been reported as toxic to humans, animals as well as fish [10 - 13]. This has prompted the establishment of guideline values for some cyanotoxins. Secondly, cyanobacteria have become one of the best sources of new bioactive compounds. Terrestrial cyanobacteria have been reported to produce antiviral (cyanovirin N from *Nostoc ellipsosporum* and scytovirin from *Scytonema varium*) and anticancer (e.g. cryptophycins) compounds whereas marine cyanobacteria have been reported to produce compounds with activities ranging from antibacterial, (serine) protease inhibitors, cytotoxic and antineoplastic properties [21]. Cyanobacteria are known to produce nearly 800 different low molecular weight secondary metabolites some of which have been identified as toxins.

The toxins of cyanobacteria can still further be classified into hepatotoxins, neurotoxins and endotoxins based on their site of action.

1.3.1 Hepatotoxins

Hepatotoxins are compounds that affect liver cells (hepatocytes) in mammals. Cyanobacterial hepatotoxins are widely distributed among aquatic environments and have been attributed to some cases of livestock deaths [10]. Generally, these hepatotoxins are small cyclic peptide molecules ranging in size between 800 to 1100 Daltons, water soluble and released along with cell lysis [11]. Microcystins (MC) are one of the well-known hepatotoxins produced by species such as *Microcystis, Anabaena, Planktothrix and Nostoc*. Microcystins show considerable structural variance and to date more than 80 different structural variants have been reported. Microcystin-LR (microcystin-LR) is the most commonly found variant (Figure 1.2).

MCs are monocyclic heptapeptides with a conserved basic structure which consists of three D-amino acids at positions 1, 3, 6, the amino acid N-methyldehydroalanine (Mdha) at position 7, an unusual amino acid 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid (adda) at position 5. The two amino acids at position 2 and 4 have always been reported as L-forms and are highly variable and MCs are named according to these amino acids (Table 1.1) [22].

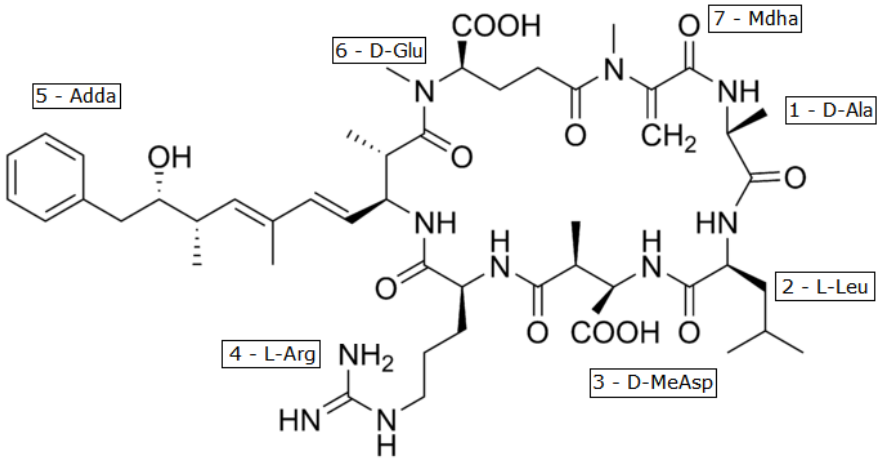


Figure 1.2 – General structure of Microcystins. The amino acids at position 2 and 4 are variable and always L-forms and gives rise to numerous variants (Table 1.1).

Table 1.1 – Some common MC variants showing the amino acids at position 2, 4

Variant	**Mol Wt**	**Position 2**	**Position 4**
Microcystin-LA	910.1	Leu	Ala
Microcystin-LF	986.2	Leu	Phe
Microcystin-LR	995.2	Leu	Arg
Microcystin-LW	1025.2	Leu	Trp
Microcystin-LY	1002.2	Leu	Tyr
Microcystin-RR	1038.2	Arg	Arg
Microcystin-YR	1045.2	Tyr	Arg
Microcystin-WR	1068.3	Trp	Arg

Once ingested, microcystins are transported to the liver where they inhibit protein phosphatases Type 1 and type 2A enzymes. Protein phosphatases perform regulatory activities such as glycogen metabolism, cell division, apoptosis, and protein synthesis. When the

enzymes are inhibited, the cells become more vulnerable towards tumor formation and in higher concentrations the cells die [22].

Another potent hepatotoxin is nodularin (NOD) (Figure 1.3). Though structurally similar to microcystin, nodularin is a pentapeptide, primarily produced by and identified in *Nodularia spumigena.* The general structure of NOD is cyclo-(D-MeAsp-L-arginine-Adda-D-glutamate-Mdhb). *Nodularia* has been repeatedly isolated from blooms across the Baltic sea, coastal lagoons in Australia, German North Sea coast, New Zealand, North America and in River Murray, South Australia [23 – 27]. Like microcystin, NOD also inhibits protein phosphatase enzymes leading to functional disturbance within cells. However, nodularin is even more potent when compared to microcystin as nodularin penetrates cells better. It has been found that microcystin is a tumor promoter (a substance that has no carcinogenic potential but amplifies the cancer inducing effect of other carcinogens) whereas nodularin is a carcinogen [28, 29].

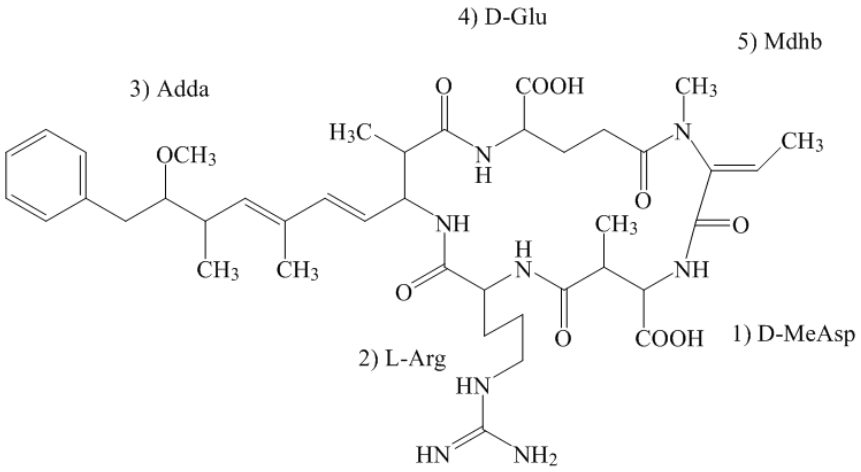


Figure 1.3 – Chemical structure of nodularin ($C_{41}H_{60}N_8O_{10,}$ Mol wt 824.98). NOD is structurally different from MC in that two amino acids at position 1 and 2 of MC are missing and Mdha (N-methyldehydroalanine) is replaced by Mdhb (N-methyldehydrobutyrine) at position 5.

NOD is of particular concern to the sea food industry as it is known to accumulate in mussels, fish, mysid shrimp and prawns at concentrations sufficient to induce hepatotoxicity to the consumers [30]. Though chemically similar to MC, NOD has fewer variants and thus far only 9 variants have been reported (Table 1.2) [31].

Table 1.2: A list of reported variants of NOD

Variant	$[M+H]^+$	Ref
NOD-R	825	[31]
Linear NOD	843	
[D-Asp]NOD	811	
[DMAdda]NOD	811	
[dhb]NOD	811	
[MeAdda]NOD	839	
Glu(OMe)]NOD	839	
[L-Har]NOD	839	[32]
[L-Val]NOD	768	[33]

Where, $[M+H]^+$ stands for protonated molecule in positive ion mode of LC-MS

Cylindrospermopsin (CYN) is yet another potent hepatotoxin, but varies in its mode of action. CYN is produced by freshwater cyanobacteria such as *Cylindrospermopsis, Anabaena*, *Aphanizomenon, Lyngbya* and *Planktothrix* [34] Chemically, CYN is a tricyclic sulphated uracil (Figure 1.4) derived alkaloid and is highly polar in nature. The toxicity of CYN is primarily attributed to its pyrimidine (Uracil) ring which binds to cytochrome P450 [35]. CYN inhibits protein synthesis, inhibits glutathione and cytochrome P450 which in severe cases leads to liver and kidney malfunctions. It has been reported that CYN inhibits protein synthesis irreversibly, induces membrane proliferation, detaches ribosomes from rough endoplasmic reticulum and promotes accumulation of lipids within cells. There are also reports that CYN may covalently bind and modify DNA [36 – 39].

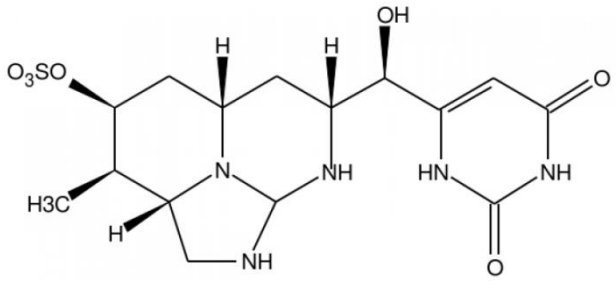


Figure 1.4 – Chemical structure of Cylindrospermopsin (CYN).
Chemical formula $C_{15}H_{21}N_5O_7S$, Mol wt 416.12

1.3.2 Neurotoxins

Neurotoxins are a class of compound that acts on neurons, and either inhibit nerve impulse transmission or stimulate neurotransmitter release. Cyanobacteria produce a number of cyanotoxins such as anatoxin, antillatoxins, saxitoxin and L-β-N-methylamino-L-alanine (L-BMAA). Based on their chemical components, cyanobacterial neurotoxins can be classified as neurotoxic alkaloids, lipopeptides and neurotoxic amino acids.

The alkaloid anatoxin-a (MW = 165 Da) and its closely related methylene homolog homoanatoxin-a (MW = 179 Da) are low molecular weight bicyclic cyanotoxins. Some of the organisms known to produce anatoxin-a are *Oscillatoria*, *Anabaena* [40] and *Aphanizomenon* [41] while homoanatoxin-a is produced by *Oscillatoria*, *Anabaena* and *Phormidium* [42, 43]. The organism *Raphidiopsis mediterranea* produces both the toxins [44]. In the past, there have been several incidences of animal poisoning where these toxins have been fatal for cows [45], dogs [46] and flamingos [12]. Anatoxin-a (Fig 5) was first

described as 'very fast death factor' as it kills mice within a short period at an intraperitoneal dose of LD_{50} of 250 µg/kg [47]. It is reported that the death of the animal is due to neuromuscular blockage followed by membrane depolarization which leads to respiratory arrest, hypoxia, acidosis and death [48]. The homolog homoanatoxin-a (Figure 1.5) was shown to be a potent agonist of muscular and neuronal nicotinic acetylcholine receptors [49].

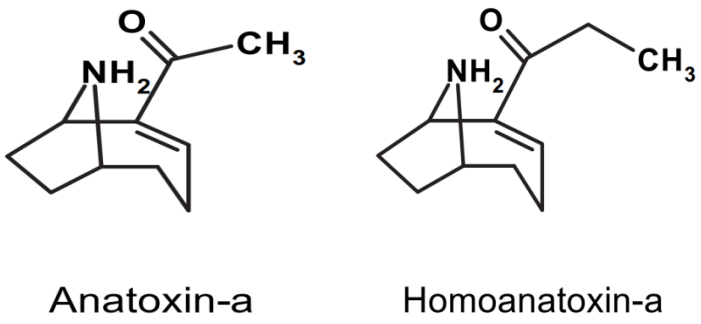


Figure 1.5 – Anatoxin-a (165 Da) and Homoanatoxin-a (179 Da)

The neurotoxin produced by *Anabaena flos-aquae strain* NRC 525–17, anatoxin-a(s) (Figure 1.6) has been shown to have exceptional anticholinesterase activity (LD_{50} (i.p) of 20 to 50 µg/kg mice). The letter 's' in anatoxin-a(s) represents the characteristic symptom salivation induced by the toxin in vertebrates [50, 51]. Chemically, it is a guanidine methyl phosphate ester and irreversibly binds to acetylcholinesterase. Intoxication with anatoxin-a(s) is characterised by cholinergic symptoms such as reduced pulse and arterial blood pressure, salivation, convulsion and respiratory arrest. Anatoxin-a(s) acts as an irreversible but potent inhibitor of acetylcholine esterase, preventing it from hydrolysing the neurotransmitter acetylcholine thus preventing termination of synaptic

transmission. This results in continuous muscle stimulation and when respiratory muscles are involved, death occurs due to respiratory failure and brain hypoxia. It is reported that death due to anatoxin-a(s) poisoning occurs typically within 30 mins of intoxication [52].

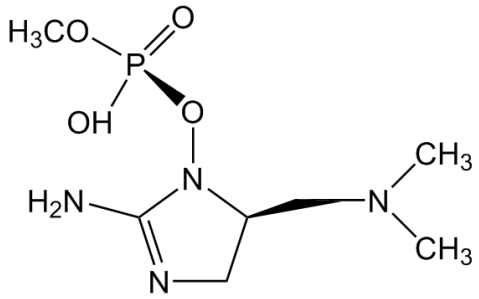


Figure 1.6: Chemical structure of Anatoxin-a(s). Mol Wt 252 Da.

Another group of alkaloids, called saxitoxins (STX) is best known for their potent lethality in shellfish. Originally identified from dinoflagellates such as *Alexandrium*, *Gymnodinium* and *Pyrodinium*, it was later found that cyanobacterium such as *Anabaena circinalis, Planktothrix*, *Aphanizomenon gracile, Cylindrospermopsis raciborskii* and *Lyngbya wollei* also produce the toxin [53 – 57]. The term saxitoxins refers to a group of related analogs including saxitoxin, neosaxitoxin, gonyautoxins, C-toxins and decarbamylosaxitoxin. STX are trialkyl tetrahydropurine precursor molecules giving rise to about 30 naturally different derivatives. The variable positions may be hydroxylated, sulphated or carbamoylated and most of the variants have been detected in both cyanobacteria and dinoflagellates [58]. However, one analog of STX, the zetekitoxin, isolated from the frog *Atelopus zeteki* has been found to be 580-fold more potent than STX [59]. Intoxication with STX results in symptoms such as vomiting,

diarrhoea, loss of motor control and paralysis. STX acts as a potent blocker of voltage gated sodium channel. Recently, it has also been reported as a calcium channel blocker and to prolong the gating of potassium channels [60, 61]. STX are responsible for a lethal human illness called paralytic shellfish poisoning (PSP). PSP poisoning occurs mainly due to the consumption of bivalve molluscs which are filter feeders and tend to accumulate STX producing dinoflagellates or marine eukaryotic alga. The LD_{50} for humans has been determined to be 5.7 µg/kg and hence this toxin is considered as highly toxic to humans. In fact, it was observed that these toxins were about 1000 times more potent that the nerve gas sarin [62, 63].

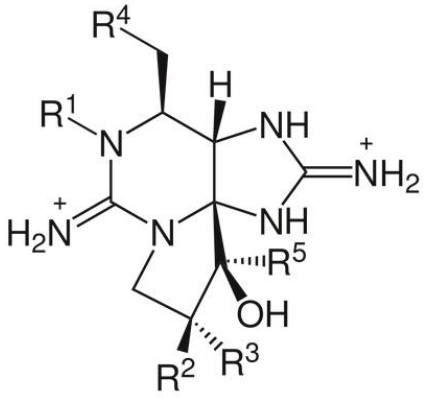


Figure 1.7: General structure of Saxitoxin.

Cyanobacteria produce neurotoxic lipopeptides such as antillatoxins, kalkitoxins and Jamaicamides. Antillatoxin a (Figure 1.8) is a cyclic tripeptide that forms linkages with methylated lipid sections thus forming a lipopeptide. A N-methyl homophenylalanine homolog has also been reported (antillatoxin B) [64, 65]. Both antillatoxin A and B have been reported to cause neuronal death by inducing Na^+

influx. Hence these groups of toxins are also named as sodium channel activators [66, 67].

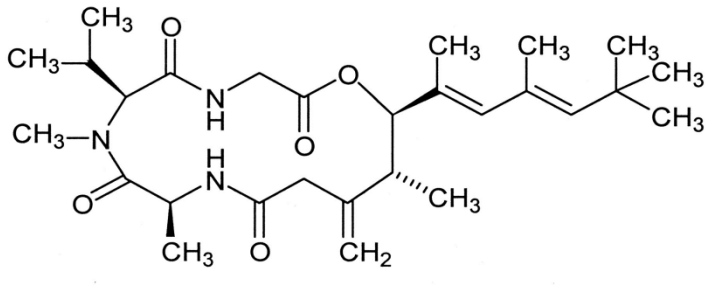


Figure 1.8 – General Structure of Antillatoxin

1.3.3 Other bioactive compounds from cyanobacteria

Anticancer compounds

Cancer may be described as uncontrolled cell division leading to the formation of tumors, which may migrate to distant sites and establish new growth centres. Marine cyanobacteria, especially benthic strains have become an immense source of interesting bioactive compounds. Cyanobacterial strains *Lyngbya, LeptoLyngbya, Symploca, Prochloron, Nostoc and Oscillatoria* have been reported to produce compounds with antineoplastic properties. In 2001, a survey of 424 cyanobacterial secondary metabolites revealed that about 15 % of the compounds could have anticancer properties (Figure 1.9) [67].

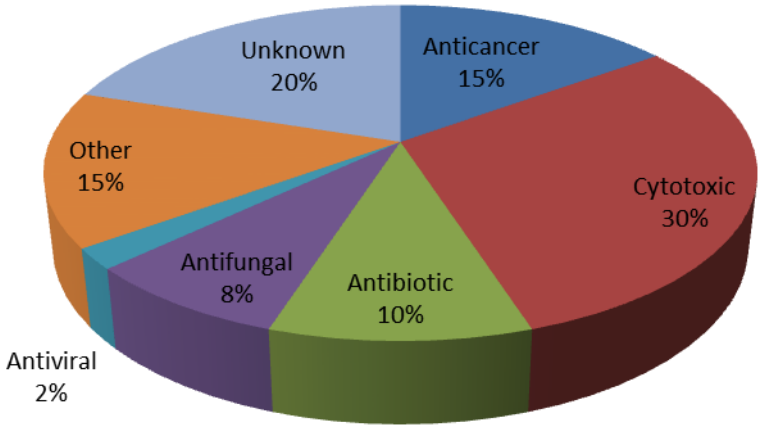


Figure 1.9: The reported range of activity of marine cyanobacterial secondary metabolites

One of the ways by which some cyanopeptides arrest tumor growth is by inhibiting microtubules and actin proteins. The cyanopeptides cryptophycin 52, calothrixin A, dolastatin 10, 15 and symplostatin 1, hectochlorin and Lyngbyabellins are reported to inhibit tumor growth by arresting cell cycle at the G_2/M phase. Certain cyanopeptides such as aurilides A & B, cryptophycin 1, swinholide A are known to induce tumor cytotoxicity by inducing damage to mitochondrial DNA. An important pathway to induce tumor cell apoptosis is by the induction of caspase pathway. It should be noted that, unlike necrosis, apoptosis does not give rise to inflammation and hence induction of this pathway is considered an ideal pathway to use for cancer treatments. The cyanotoxins symplostatin 1, cryptophycin 1, 52, somocystinamide A are some of the examples of caspase pathway stimulators (Table 1.3).

Table 1.3: Anticancer compounds produced by cyanobacteria

Organism	Compound	Type	Potency	Ref
Scytonema sp	Scytonemin	Alkaloid	IC50 = 7.8 μM	[67]
Lyngbya majuscula	Apratoxin A	Lipopeptide	IC50 < 0.5 nM	[68]
L. majuscula	Hectochlorin	Lipopeptide	GI50 < 5.2 μM	[69]
L. majuscula	Lyngbyabellin E - I	Lipopeptide	LC50 < 5 μM	[70]
L. majuscula	Curacin A	Lipopeptide	IC50 < ng/ml	[71]
Nostoc sp	Cryptophycin	Lipopeptide	IC50 = 5 pg/ml	[72]
Symploca hydnoides	Dolastatin 10	Lipopeptide	IC50 < ng/ml	[73]
Symploca sp	Symplostatin 1	Lipopeptide	IC50 = 3.9 - 10.3 nM	[74]
Symploca sp	Belamide A	Lipopeptide	IC50 = 0.74 μM	[75]
Symploca sp	Largazole	Lipopeptide	IC50 = 7.6 nM	[76]
L. majuscula	Microcolin A	Lipopeptide	TC50 = 22.6 nM	[77]
Prochloron sp	Patellamide D	Octapeptide	IC50 = 50 μM	[78]
Lyngbya confervoides	Obyanamide	Lipopeptide	IC 50 = 0.58 lg/ml	[79]
L. majuscula	Jamaicamides A	Lipopeptide	IC 50 = 15 μmol/L	[80]
L. majuscula	Aurilides B	Lipopeptide	LC50 = 0.01 to 0.13 μM	[81]

Organism	Compound	Type	Potency	Ref
Lyngbya semiplena	Wewakpeptins A - D	Lipopeptide	LC50 0.4 μM	[82]
L. majuscula	Somocystinamide A	Lipopeptide	IC 50 = 1.4 μg/ml	[82, 83]
Lyngbya sp	Palauimide	Lipopeptide	IC 50 = 13 nM	[84]
Lyngbya sp	Ulongapeptin	Lipopeptide	IC 50 = 0.63 μM	[85]
Oscillatoria sp	Largamide A, G, H	Lipopeptide	IC 50 = 4 - 25 μM	[85]

1.4 Anti-Infective Compounds

Cyanobacteria produce a good range of antibacterial as well as antiviral compounds. The cyanobacterium *Nostoc* sp. ATCC 53789 produces cryptophycins, antifungal agents. Cryptophycins (Figure 1.10a) were also later found to have broad spectrum of activities against drug sensitive and drug resistant murine and human tumors. However, owing to their toxicity to non-tumor cells, clinical trials have been discontinued [86]. The compound cyanovirin-N (from *Nostoc*) has received much attention due to its HIV inactivation properties. Cyanovirin-N irreversibly inactivates diverse strains of HIV, including the sexually transmitted variant. As cyanovirin-N is stable under denaturating conditions, detergents, organic solvents and multiple heat thaw cycles, it is being considered for development as an anti-HIV agent [87]. The organism *Oscillatoria nigroviridis* has been reported to produce compounds that are antibiotic against *Plasmodium falciparum, Leishmania donovani* and *Trypanosoma cruzi.* The compound was

termed as viridamide (Figure 1.10b) [88]. In addition, the alkaloid Nostocarboline (Figure 1.10c) from *Nostoc* sp. 78-12A was also found to be active against *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum* with IC_{50} values ranging from 0.5 to 0.194mM [89]. Aerucyclamide C isolated from *Microcystis aeruginosa* PCC7806 was also found to be active against *T. brucei*, while aerucyclamide B (Figure 1.10d) against *P. falciparum* with submicromolar IC_{50} values [90].

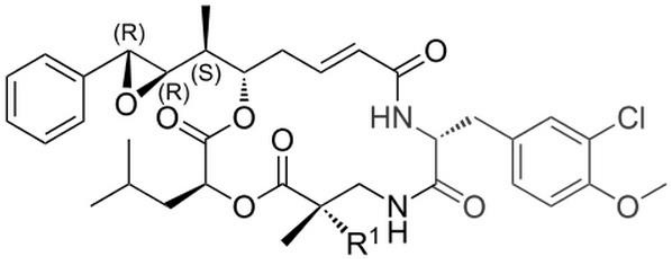


Figure 1.10a – Cryptophycin 1 and 52

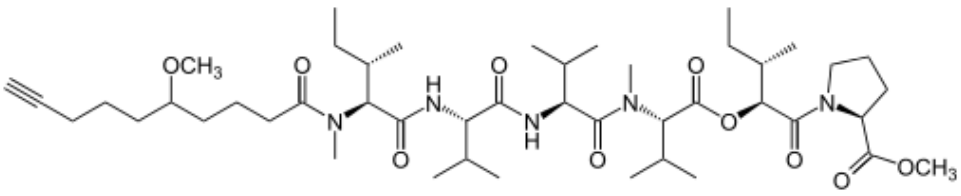


Figure 1.10b – Viridamide A

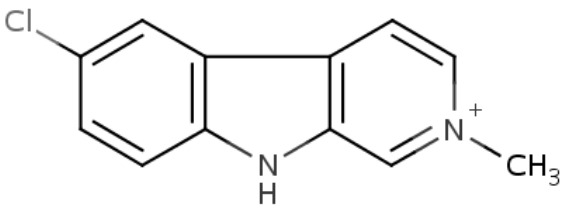


Figure 1.10c – Nostocarboline

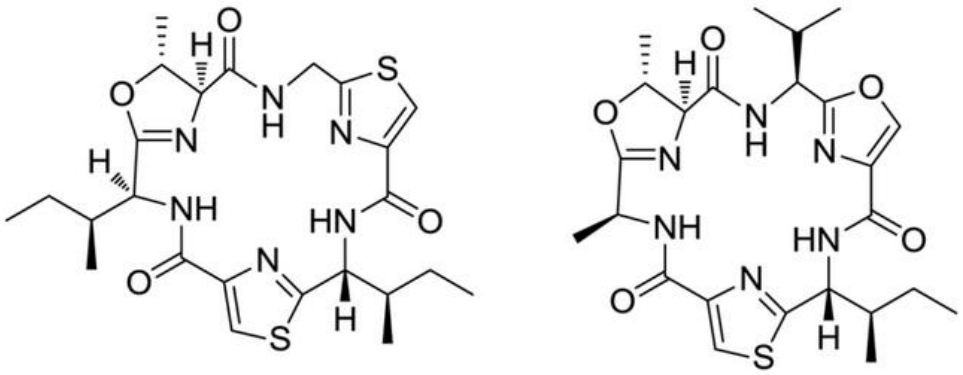


Figure 1.10d – Aerucyclamide B and Aerucyclamide C

1.5 Environmental conditions that affect secondary metabolite production

Cyanobacteria are prolific producers of secondary metabolites. In recent reports, it was mentioned that eutrophication and global warming could be one of the reasons for the increase in bloom frequencies thus leading to an increased secretion of toxins into the ecosystem [91]. Cyanobacterial growth requires optimal physical and environmental conditions like temperature, light, pH, phosphates, nitrates and trace metals [92]. In general, it is accepted that factors that enable enhanced growth enhances toxin synthesis [93].

In the case of *Microcystis*, factors such as light [94], temperature [95], pH [96], high nitrogen [97], low phosphorous [98] and low iron [99] invariably affect cell growth and indirectly influence microcystin production. Further, it has also been suggested that growth rate directly influences microcystin production rate [95, 100]. However, some recent reports suggest that toxin production is a

complicated process and involves many more extrinsic and intrinsic factors and hence needs to be evaluated further [101].

In the case of *Nodularia*, low nitrogen to phosphorous ratio was observed to increase cell division which led to increased toxin levels [102]. In contrast to this finding, some researchers also report that chemostat cultures of *N. spumigena* grown in phosphate starved condition did not significantly decrease toxin levels [103]. These findings suggest that there may be more than one factor responsible for enhanced toxin synthesis and that a combination of optimal growth conditions such as light, nutrients, CO_2 content play a complex role in enhancing the growth rate of an organism. This enhanced growth might lead to increased toxin levels in the environment. This is further confirmed in the case of *Cylindrospermopsis* where low levels of fixed nitrogen were observed to enhance toxin production [104]. In the case of *C. raciborskii* T3 which produces saxitoxin, the presence of extracellular NaCl played a significant role in the toxin production and a linear concentration dependent response was observed [105].

Most cyanobacterial blooms are comprised of toxic as well as non-toxic strains of one or more species. Thus, toxic and non-toxic strains coexist in the field [106, 107]. Surprisingly, a study on bloom succession concluded that bloom dynamics in lakes mainly result from the succession of a large number of genotypes and that non-toxic strains dominated at the end of bloom while in other lakes the toxic strains dominated. The authors conclude that while the growth parameters were similar, succession by certain genotypes followed a

seasonal pattern or it may be that other intrinsic factors may be influencing bloom types [94].

Although cyanobacteria produce a wide range of secondary metabolites, it is still a matter of debate as to why they produce them. To date, no clear evidence has been reported regarding the usefulness of these secondary metabolites to the parent organism. Apart from reasons such as heavy metal chelation [109], anti-grazing [110], nitrogen storage [111], Photo/UV protection [112], quorum sensing [113], one possible reason could be that cyanobacteria communicate with each other using these metabolites (Allelopathy). During bloom formation, numerous biotic and abiotic factors play a role [114] and allelopathy may be one of the reasons [115]. Allelopathy has also been known to shape microbial communities in aquatic ecosystems [114, 116, 117].

1.6 Allelopathy

The term allelopathy is derived from the Greek word 'allelos' meaning mutual and 'pathos' meaning suffering. Allelopathy was first used by Prof Molisch H, 1937, while reporting the effect of some plants on others [118]. Though this concept was initially thought of as a negative feedback phenomenon within higher plants, later it was broadened to define the direct or indirect and harmful or beneficial effect of organisms on one another by the release of chemical compounds. The compounds that induce this effect are called allelochemicals.

1.6.1 Allelopathy in bacteria

Initially, allelopathic interactions were thought to be present only among higher plants, however, in recent times, allelopathy has been reported among insects, animals and microbes [119]. The actinomycetes group of bacteria has been reported to produce compounds with interesting qualities. The soil bacterium *Streptomyces saganonensis* has been reported to produce herbicidin which inhibits the growth of several monocot and dicot plants [120]. The compound blasticidin from this genus was specifically shown to inhibit only dicots without any effect on monocots [121]. It should also be noted that compounds like cycloheximide, nystatin, streptomycin, bleomycin are all produced by this genus and are well known antibiotics (negative allelopathy to other bacteria).

Within the cyanobacterial community, the first report of allelopathy was in the organism *Scytonema hofmanii* which produced the compound cyanobacterin (a natural herbicide) [121 - 123] and a later report on hapalindole A (antimicrobial and antimycotic) from *Hapalosiphon fontinalis* [124]. Allelopathy has been documented in many species such as *Microcystis, Nodularia, Aphanizomenon, Nostoc, Oscillatoria, Phormidium* and *Scytonema* [125].

1.6.2 Cyanobacterial allelopathy

Cyanobacteria form massive outbreaks of growth (blooms) when optimal conditions for growth occur. Adding to this, it has also been

thought that allelochemicals secreted by the growing cells may signal bloom formation [115]. In a study with *C. raciborskii,* it was observed that an allelochemical inhibited the surrounding phytoplankton hence reducing competition for nutrients [126]. In a recent study, it was noticed that the presence of exogenous microcystin toxin increased the intracellular toxin levels of the host organism. Hence, it was thought that the toxin may act as an infochemical (i.e., radar) for the host organism [127]. Further, it was reported that a compound (yet to be fully characterised) secreted by *Planktothrix agardhii* doubled the toxin production of microcystin and nodularin [128].

Though allelopathy has been studied for some time, there is still no convincing evidence why cyanobacteria secrete allelochemicals into the ecosystem. It is thought that allelochemicals are used to increase cellular fitness [129], to compete for resources or to signal blooming [130]. Several biotic and abiotic factors have been thought to trigger allelopathic secretions. Some of the biotic factors include competition among species [131 – 133] and allelochemical degrading heterotrophs [134]. The abiotic factors include light, temperature, pH and nutrient levels [135].

Cyanobacteria are known to produce a large variety of secondary metabolites (Table 1.4). Unlike other organisms, their mode of synthesis is mainly by using the non-ribosomal peptide synthetase (NRPS) and the polyketide synthase (PKS) biosynthetic pathways. This process enables them to produce diverse peptides with unique

bioactive functions, although, very few have shown to have a growth inhibitory or stimulatory effect.

Cyanobacterin

The first report of cyanobacterin (Figure 1.11) was in 1982 when it was isolated from *Scytonema hofmanii*. It was initially thought of as an antibiotic, however, an allelopathic role was later identified. Cyanobacterin specifically inhibited the growth of photosynthetic organisms, but had no effect on bacteria or protozoans. Cyanobacterin has been shown to inhibit the growth of *Bracteacoccus* sp. and *Anabaena sp.* at a concentration of 300 µg/ml [122]. However, a purified form of the toxin has been shown to have some inhibition even at a dose of about 2 µg/ml [136].

The compound is hydrophobic with a molecular weight of 430 Da and empirical formula $C_{23}H_{23}O_6Cl$. Cyanobacterin inhibits the growth of photosynthetic bacteria by disrupting the thylakoids structure. It also is known to inhibit the oxygen evolution pathway and hence this compound is only active against photosynthetic organisms, mostly cyanobacteria [136]. It was also discovered that this compound could have a negative effect on angiosperms as well. In these plants, the compound specifically inhibits Hill reaction centres and hence confirms that cyanobacterin inhibits oxygen evolving photosynthetic electron transport. Since cyanobacterin does not have any effect on photosystem I, the most probable site of action is supposed to be photosystem II [143].

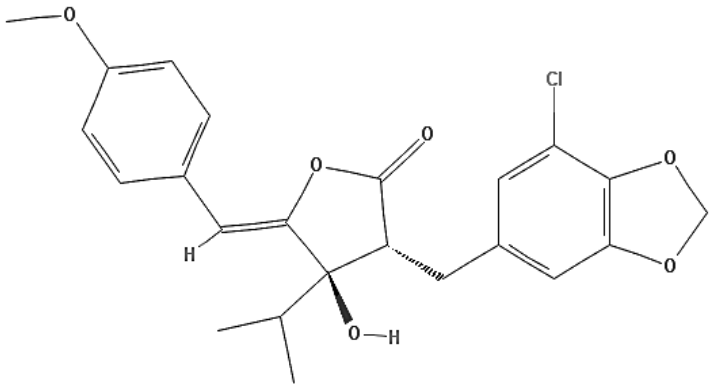


Figure 1.11 - Structure of the allelochemical Cyanobacterin

Table 1.4 – Summary of some known allelochemicals and their mode of action

Organism	**Allelochemical**	**Target organism**	**Mode of Action**	**Ref**
Fischerella sp	Hapalindoles , Fischerellins	Other Cyanobacteria, bacteria, fungi, protozoa, algae	RNA synthesis/ polymerase inhibition	[137] [138] [139]
Microcystis sp	Microcin, microcystin-LR, microcarborin	*Versicularia dubyana*, *Peridinium gatunense*	Photosynthesis inhibition, growth inhibition	[142] [140] [141]
Nodularia sp	Norharmane	Other Cyanobacteria	Cytotoxicity, growth inhibition	[123]
Nostoc insulare	Norharmane, 4,4-dihydroxybiphenyl	Other Cyanobacteria	Cytotoxicity	[117]
Calothrix sp	Calothrixin	Other cyanobacteria, bacteria and mammalian cells	RNA synthesis/ polymerase inhibition, growth inhibition	[137]

Fischerellin

Fischerellin was identified in 1991 from the organism *Fischerella muscicola.* It is a hydrophobic compound which exists in nature as two isoforms. Fischerellin A (Figure 1.12a) is known to be a potent cytotoxin, whereas the less cytotoxic form is named as Fischerellin B (Figure 1.12b). It has been reported that this compound may also be produced by other species such as *Fischerella ambigua* and *Fischerella tisserantii*.

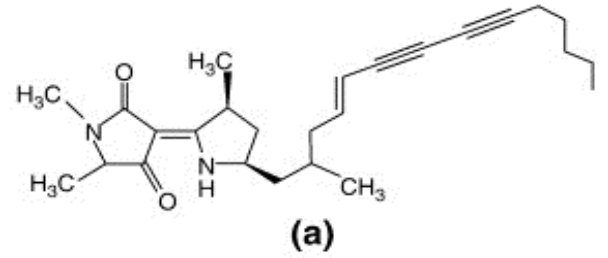


Figure 1.12a: Structure of fischerellin a

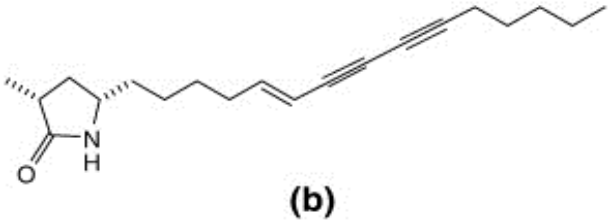


Figure 1.12b: Structure of fischerellin b

This compound showed growth inhibitory activity against *Anabaena sp, Phormidium sp. Synechococcus sp. and Synechocystis sp.* However, this compound did not have any effect on non-photosynthetic bacteria such as *Bacillus sp.* or *Pseudomonas sp.* Comparatively, this compound is more potent than cyanobacterin and is capable of inhibitory activity at the nanogram level [144]. Fischerellin inhibits cyanobacterial growth by

inactivation of PSII reaction centres and disturbing oxygen evolution [145].

Calothrixin

The first report of Calothrixin (Figure 1.13) was in 1999 [146]. It is a pentacyclic bioactive secondary metabolite produced by *Calothrix* sp. In nature, Calothrix exists in two forms A ($C_{19}H_{10}N_2O_3$) and B ($C_{19}H_{10}N_2O_2$).

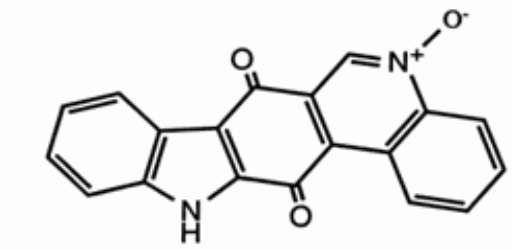


Figure 1.13 – Structure of Calothrixin A

Calothrixin is one of the compounds that show inhibitory activity against *Hela* cell lines and *Jurkat* cancer cell lines by inducing intracellular DNA damage [147]. Calothrixin A functions by inhibiting RNA synthesis and DNA replication of other cyanobacteria and algae and RNA polymerase of *E. coli* [148, 137]. This compound has received much attention from the medical community, as the compound is capable of inhibiting the chloroquine resistant malarial parasite *Plasmodium falciparum* at a concentration of 58 nanomoles [146].

Microcin SF608

Microcin, as the name indicates, is produced by *Microcystis aeruginosa*. The empirical formula of Microcin is $C_{32}H_{45}N_6O_6$ (Figure 1.14). When tested against the water moss *Versicularia dubyana*, it was found that Microcin significantly inhibited microsomal glutathione-S-transferase. In *Daphnia magna*, the peroxidase was inhibited. In the photosynthetic organisms *V. dubyana*, the oxygen production rate as well as the ration of chlorophyll a / b showed stress. In general, microcin is regarded as a serine protease inhibitor. Although, microcin has not been studied extensively, its inhibitory activity against unrelated aquatic organisms suggests that it might play an allelopathic role in combination with microcystin-LR [142].

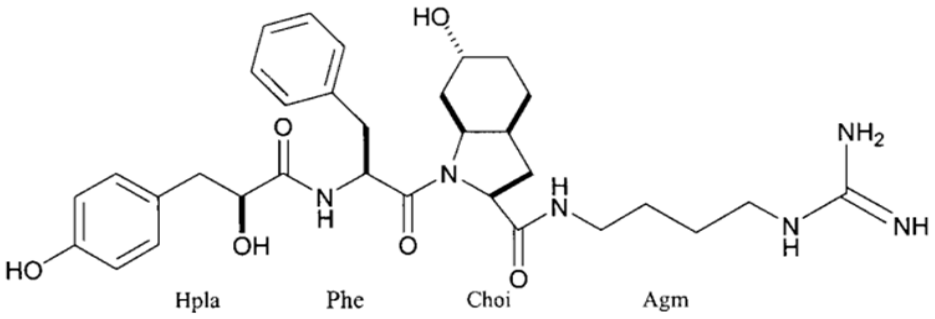


Figure 1.14 – Structure of Microcin (SF608)

It could be concluded that at least some cyanobacterial secondary metabolites do play a part in allelopathy. Even the hepatotoxin microcystin has been thought of as an allelochemical. Initially,

microcystins were thought of as a defense chemical [149, 150]. However, a phylogenetic study concluded that microcystins were being synthesised even before the occurrence of grazers. This strongly suggests that microcystins could not be a defence chemical [151]. A recent study provided some evidence that the presence of extracellular microcystin increased their intracellular concentration, indicating that the cells detected the death of other cells and increased their cellular fitness [127].

Another study indicated that microcystins could have metal chelation properties [152**,** 99]. These types of allelochemicals may bind to metals such as iron, copper, cadmium, zinc and act as siderophores to the parent organism. Indirectly, this could negatively affect the survival of competitors [130]. Also, the concentrations of key nutrients play an important role in allelochemical secretion. It is thought that low nutrient conditions stimulate allelochemical production, thereby decreasing competitor concentrations and increasing the chances of survival of a species [131]. Though conditions like light, temperature, nutrient shortage and the presence of competitors are thought to be conditions that induce allelopathic interactions, it is still not very clear as to how important allelochemicals are to the parent organism.

It could be generalized that of the hundreds of metabolites produced by cyanobacteria, only some have been characterised and even less have been fully studied. Of these few compounds, some of the above mentioned compounds have been suggested to have allelopathic

ecological roles and this may justify their production. However, within cyanobacterial ecology, there are many more unique compounds which are still poorly defined and their ecological role is still unclear. Hence a study of these compounds may enhance our understanding about the biological role of these secondary metabolites. Further, an understanding of these compounds may lead to the discovery of unique compounds with potential applications such as bloom mitigation, biomedical research tools and commercial benefits. Since only a fraction of the diverse pool of these compounds have been characterised, the chance of identifying a new role for a compound is greater. Therefore, a study into this field guarantees novelty will be challenging and may be commercially beneficial. In order to study allelopathy in cyanobacteria, a model organism needs to be chosen. The organism *Microcystis aeruginosa* PCC7806 seemed to be an ideal candidate as it has several features required for this type of study.

1.7 Introduction to *Microcystis aeruginosa* PCC7806

M. aeruginosa (order Chroococcales) is a freshwater cyanobacteria distributed worldwide. The cells are spherical and about 3 to 6 μm in diameter (Figure 1.15). Due to the presence of chlorophyll-a, the cells appear green. In ambient conditions, the cells aggregate to form large colonies of indefinite shapes. However, under laboratory conditions, the cells occur in singles, doubles or tetrads which are easily disassociated. It is one of the most common bloom forming organisms and is well known

as a predominant toxin producer. Its annual life cycle consists of 4 stages, which are pelagic (rising to the surface and bloom formation) during summer, sedimentation during autumn, benthic during winter and reinvasion of the water body during spring [153]. *Microcystis* sp. achieve buoyancy through the use of their gas vesicles. During autumn through winter, the water temperature reduces slowing their metabolism. This leads to decreased respiration and increased accumulation of carbohydrates intracellularly which increases cellular density causing sedimentation [154].

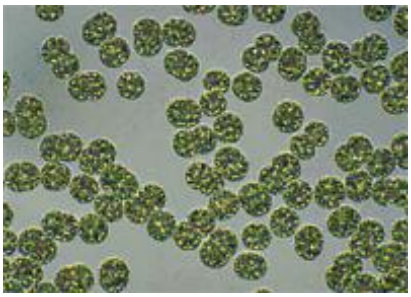


Figure 1.15 – Light micrograph of *M. aeruginosa* at 1000x [180].

One of the main nutrient requirements for the growth of *Microcystis sp.* is Nitrogen (N). Sources of N in a water system may be from lake eutrophication, N fixers and ammonia released from anaerobic metabolism. When N is not the limiting factor, the doubling time of *Microcystis* sp. is between 1.24 to 1.39 days [155]. Some recent studies suggest that excess N can promote abundant growth as well as increase intracellular microcystin content [156] and N limitation reduces microcystin quota disproportionately [157].

M. aeruginosa is well adapted to fluctuating phosphate (P) concentrations. Although phosphate is an essential nutrient for its growth,

it has evolved mechanisms for P uptake and storage enabling it to thrive in P depleted ecosystems [158]. In a recent study it was reported that the production of microcystin was directly proportional to the concentration of P up to 5 mg/L, above which the microcystin production decreased significantly. However, the authors also note that P along with N play a role in growth and microcystin synthesis and that an N:P ratio of 50:1 gave maximum microcystin concentration per cell [159]. It is also thought that Iron (Fe^2+) may play some role in inducing toxin production [160]. Iron is known to induce oxidative stress and hence may decrease cell growth indirectly. Hence a decreased biomass could give a higher calculated value for microcystin content. Also, a relationship between the inorganic carbon availability and the total microcystin content (intra and extracellular) have also been reported. It was reported that under depleted inorganic carbon concentrations, the intracellular and extracellular microcystin concentration increased [161]. However, as carbon is one of the primary nutrients, it could also be that the cells may have been starved resulting in reduced growth rate or increased death rate thereby releasing the intracellular microcystins.

1.7.1 Secondary Metabolites of *M. aeruginosa* PCC7806

The organism *M. aeruginosa* PCC7806 has been studied extensively for over 40 years. The strain was originally isolated from Braakman Reservoir, The Netherlands in 1972. It is one of the most common bloom

forming cyanobacteria occurring widely in fresh water ecosystems [8]. One of the key characteristics of *M. aeruginosa* PCC7806 is its ability to produce an array of secondary metabolites such as aerucyclamides, cyanopeptolins and microcystin-LR (Figure 1.16). Among its secondary metabolites, microcystins are the most frequently observed and well-studied.

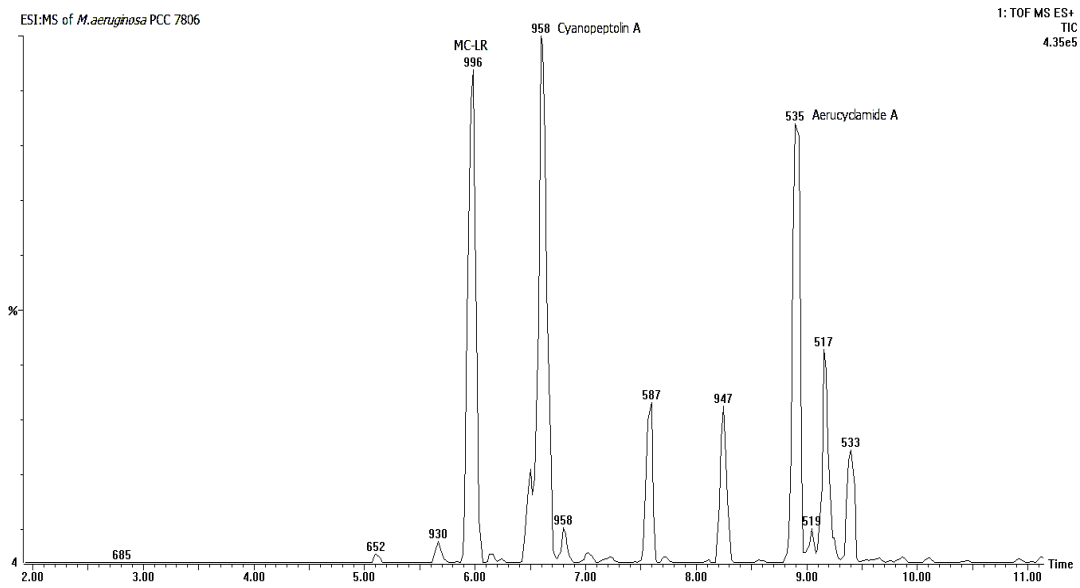


Figure 1.16: HPLC chromatogram of *M. aeruginosa* PCC7806 extracted with 80% Methanol (MeOH). The peaks: 996 Dalton is microcystin-LR, 958 Da is cyanopeptolin A and 535 Da is aerucyclamide. The organism also produces other secondary metabolites such as aerucyclamide D (587 Da) and aerucyclamide C (517 Da).

Aerucyclamides

Cyanobacteria are prolific producers of secondary metabolites which include polyketides, peptides and cyclamides. In 2008, Portmann *et al*., [162] reported that *M. aeruginosa* PCC7806 produces aerucyclamides A, B, C and D. Aerucyclamides are a rare example of cyclamides that feature oxazoline, thiazoline and thiazole moieties in one compound. The ecological relevance of aerucyclamides is under investigation; however, a study revealed that aerucyclamides are about 1 order less toxic than microcystins [160, 90] which could be attributed to their hydrophobic nature (Figure 1.17). A later study has revealed that Aerucyclamide B showed potent activity against the chloroquine resistant strain of *Plasmodium falciparum* K1 but showed little or no activity against Rat Myoblast L6 cell lines. The compound Aerucyclamide C showed moderate activity against *Trypanosoma brucei rhodesiense* STIB 900 [90]. It may be that in the future some of these compounds could be evaluated for biomedical purposes.

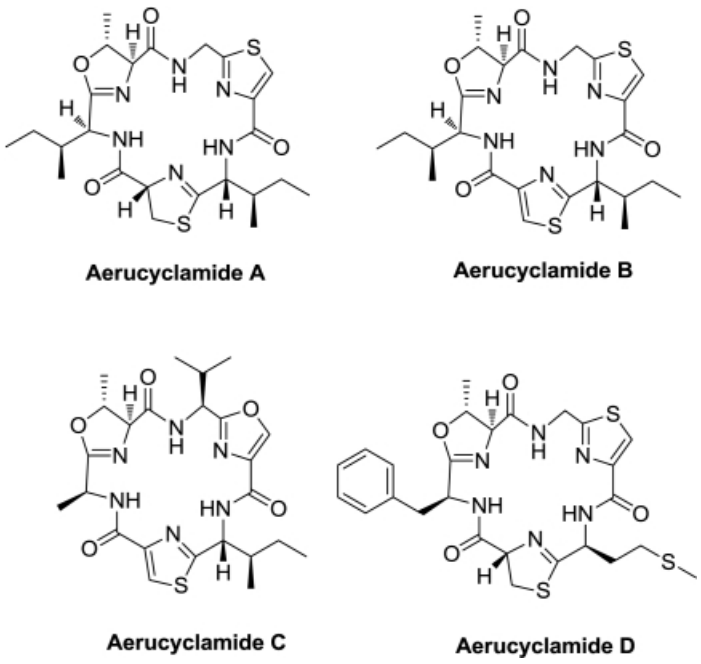


Figure 1.17: Chemical structures of aerucyclamides from *M. aeruginosa* PCC7806.

Cyanopeptolins

In 1993, Martin *et al*., [163] reported that the organism *M. aeruginosa* PCC7806 produces 4 depsipeptides i.e., Cyanopeptolins A, B, C and D. These are peptide lactones which possess a ring structure built up by condensation of the aldehyde of reduced L-glutamic acid with the amino group of L-leucine (Figure 1.18). They possess identical structures consisting of cyclic L-glutamic acid-y-aldehyde, L-leucine, 7-methyl-phenylalanine, L-valine, L-threonine, L-aspartic acid, hexanoic acid and a variable basic amino acid. This variable amino acid can be L-arginine (cyanopeptolin A), L-lysine (cyanopeptolin B), N-methyl-L-lysine

(cyanopeptolin C) and N-N-dimethyl-L-lysine (cyanopeptolin D), respectively [176]. In 1995, a sulphur containing cyanopeptolin-S was reported by Jakobi *et al*, [164].

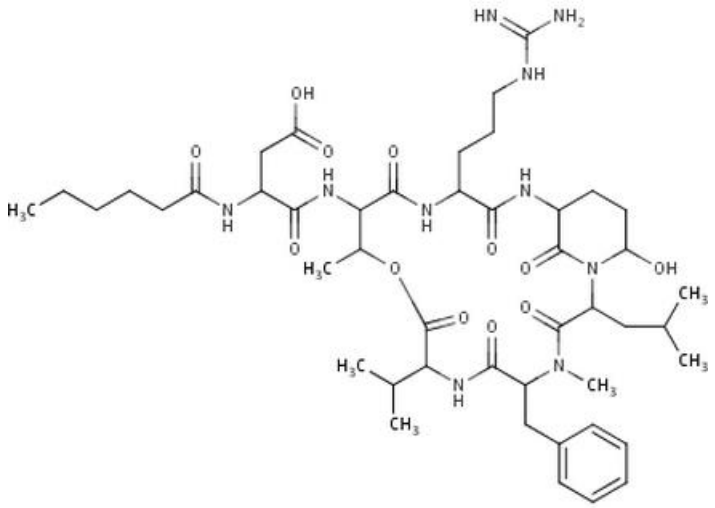


Figure 1.18: Chemical structure of cyanopeptolin A, Mol Wt 958 Da

Biologically, cyanopeptolins are known to be protease inhibitors. Although the function of cyanopeptolin A is as yet unknown, the congeners cyanopeptolin S inhibits plasmin and thrombin where as cyanopeptolin 963A (reported in 2004 [164]) inhibits chymotrypsin. Apart from these functions, other cyanopeptolins have been found to be toxic to zebra fish embryos affecting their DNA damage recognition and repair, circadian rhythm, response to light, and to some extent metabolic activities [165]. They report that cyanopeptolins should be considered as neurotoxins which may have human health consequences.

Microcystin-LR (microcystin-LR)

Microcystins are produced by several genera such as *Anabaena* sp, *Aphanizomenon* sp., *Planktothrix* sp. and *Microcystis* sp. and different variants of microcystins have been reported. *M. aeruginosa* PCC7806 produces only the leucine-arginine variant Microcystin-LR in abundance and its demethylated congener in relatively small quantities. Microcystin-LR is a small (996 Da) monocyclic heptapeptide with a conserved structure of D-Ala-L-leu-D-MeAsp-L-Arg-Adda-D-Glu-Mdha (Figure 1.19). The unique amino acid Adda (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid) is present ubiquitously within cyanobacterial species only.

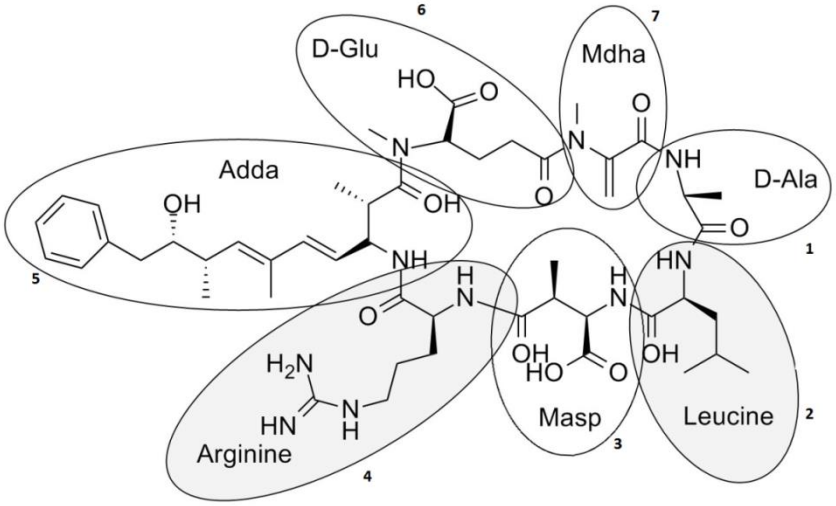


Figure 1.19 - Chemical structure of Microcystin-LR. In position 2 is leucine (L) and position 4 is arginine (R) both of which are variable regions

Within microcystin-LR, about 22 variants occur and the variation could be due to demethylation, variations at the 6th or 7th amino acid or

modification of the amino acid Adda. The majority of the microcystin variants are produced by *Microcystis* sp, however, some strains of *Anabaena* sp and *Planktothrix* sp have been reported to produce microcystins. One of the characteristics of microcystin-LR is its ability to bind irreversibly to protein phosphatase 1 (PP1). The binding is brought about by the adda fitting into the hydrophobic grove of PP1, the carboxylate group and the carbonyl oxygen bond with the metal binding site and L-Leu binds to a tyrosine at the C terminal of PP1 [166]. PP1 (and PP2) perform regulatory activities such as glycogen metabolism, cell division, apoptosis, and protein synthesis. When the enzymes are inhibited, the cells become more vulnerable towards tumor formation and in higher concentrations the cells die. It is thought that the presence of the adda amino acid along with hydrophobic amino acids is required for microcystin-LR toxicity and a substitution with hydrophilic amino acid such as arginine (at sites 2 or 4) significantly reduces its binding properties and hence reduces toxicity [167].

The microcystin-LR toxin, by its property of binding to PP1 and PP2 are predominantly localised within hepatocytes. Hence, microcystin-LR can be classified as a hepatotoxin. However, microcystin-LR by its nature cannot penetrate cell membranes, but require the multispecific bile acid transport systems for its uptake into hepatocytes [168]. Within the hepatocytes, microcystin-LR inhibits PP1 and PP2 and disrupts the critical phosphorylation / dephosphorylation balance disrupting cellular functions such as carbohydrate and lipid metabolism, gene expression, protein

synthesis, cell division [169]. At the cell membrane level, microcystins alter cytoskeleton components resulting in bleb formation, invagination and loss of microvilli [170] and ultimately resulting in formation of tumor cells [171, 172].

The property of microcystin-LR as a hepatotoxin and its widespread occurrence has interested many researchers to study their biosynthetic pathway. The knowledge of such a biosynthetic pathway may lead to the development of technologies to minimize toxin secretions into the ecosystem. The biosynthesis of microcystin-LR (and other microcystins) is unique as it does not follow the central dogma of protein synthesis but uses enzyme complexes to add amino acids without the employment of ribosomes. Such a pathway was later designated as non-ribosomal peptide synthesis and the enzyme complex was named as NRP synthetase [173]. In addition to NRPS a polyketide synthetase system has also been reported to be partially involved in microcystin-LR biosynthesis [174]. In 1997, Dittmann *et al*., [175] reported the presence of three genes i.e., *mcy* A, B and D to be involved in microcystin-LR synthesis. In 2000, Tillet *et al*., [176] reported the complete structure of the microcystin-LR biosynthetic pathway (Figure 1.20). They report the presence of two operons (mcy A to C in forward and mcy D to J in reverse orientation) to a common promoter [176]. Although, 10 genes comprise the microcystin synthetase cluster, the genes *mcy* A, B and D are thought to be the key genes as insertional mutation of any of these genes results in complete cessation of microcystin synthesis [173 – 176].

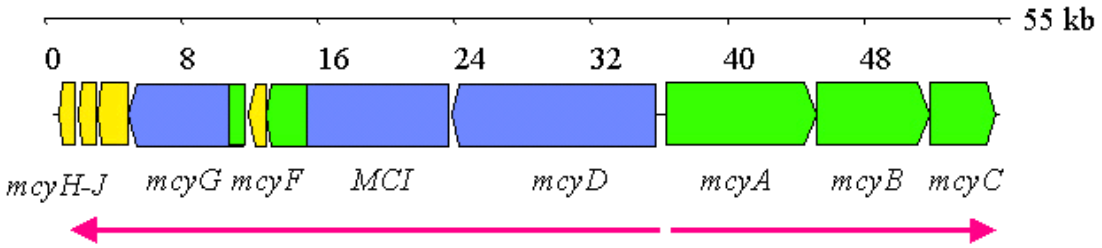


Figure 1.20. Structural organization of the microcystin synthetase gene complex (*mcy* A-J). Green – genes encoding NRPS modules. Blue – genes encoding PKS modules and Yellow – genes encoding modifying enzymes [176].

Apart from the genes described, several factors have been known to influence toxin biosynthesis. As a general rule, it is thought that microcystin is synthesised and accumulated continuously during growth phase and released during the stationary or decline phase. It is reported that during stationary phase, *Microcystis* sp. may accumulate up to 0.53 ± 0.06 pg/cell of microcystins. Factors such as temperature as well as pH are important for microcystin synthesis, however, these factors influence the optimal growth of the organism rather than microcystin induction. Light intensity may be one important factor for toxin biosynthesis and subsequent release in the ecosystem. In 1992, it was reported that as light intensity increased from 20 to 40 μE/m^2/s the intracellular microcystin-LR increased from 2 to 5 ng/μg total cellular protein and beyond 40 μE/m^2/s light, the toxin content remained same [177]. However, the reported values are in toxin content per unit total cellular protein and may indicate only the intracellular toxin content. As the biosynthesis of microcystins are carried out by enzyme complexes, the

effect of heavy metals (as cofactors) have been thought to be important. Only the metals iron and zinc are known to affect toxin production. In the organism *M. aeruginosa* PCC7806, it has been reported that Zn concentration of 0.25 μM increased growth by 1.5 times and toxin yield by 30 percent; however, higher concentrations were lethal to the cells. Similarly, Iron has also been reported as an important cofactor and the concentration of microcystin was inversely proportional to the available iron (Fe^{2+}) concentration. In this view, it was hypothesised that microcystins are heavy metal chelators required for optimal growth [160].

Several functions have been reported for microcystin-LR. Initially it was thought that microcystins were produced to dissuade phytoplankton grazers, but in 2003 a phylogenetic study concluded that the genes for microcystin synthesis evolved much earlier to the occurrence of grazers and hence could not be assembled in response to grazer stress [151]. However, it is not clear if the oldest known cyanobacteria harboured the microcystin genes (in its functional form) even then. In a recent study, it was reported that microcystin-LR may function to remove competitors from the immediate surroundings and ensure bloom formation [178]. Some of the other functions attributed to microcystins are signalling and gene regulation. It was reported that microcystin-LR may be produced in response to high light signal and regulate essential gene. However this hypothesis has not been conclusively proved and the authors suggest that additional mechanisms may be needed to sense high light intensities along with microcystin-LR [135].

Although microcystins have been studied for over 30 years, their primary function has remained unclear. One of the aims of this study is to investigate the role of microcystin-LR on the producing organism i.e., *M. aeruginosa* PCC7806. It should be noted that *M. aeruginosa* PCC7806 produces several other secondary metabolites (aerucyclamides and cyanopeptolins) along with microcystin-LR and it would be interesting to see the effect of microcystin-LR on the production of *de-novo* metabolites. Further, in order to evaluate possible allelopathic interactions, the secondary metabolites produced by other cyanobacteria (cyanopeptides) will also be tested on *M. aeruginosa* PCC7806 and its parameters such as growth rate and secondary metabolite production rate will be monitored.

In order to understand the role of secondary metabolites, several basic tools such as analytical methods to differentiate between *de-novo* and *ex-novo* metabolites and a small scale rapid bioassay protocol needs to be developed. To enhance the accuracy of the data, a methodology to enumerate cyanobacteria using flow cytometry will need to be developed. Also, most previous studies in this field lack information in areas such as secondary metabolite production rate, an accurate quantification of the metabolite within a single cell, accurate count of the cell numbers, the *de-novo* production of these metabolites and the fate of the secondary metabolites already present in the surroundings. In most studies, microcystin is quantified as a measure of the change in total microcystin concentration over time. This type of quantification yields only an overall picture of the quantity of microcystins, but does not reveal any

information regarding microcystins metabolism (synthesis vs catabolism). Further, while calculating the rate of production, the total biomass is represented only in terms of total protein or the total chlorophyll content [100]. This leads to errors like the total biomass may be a composition of live and dead cells, which do not contribute to microcystin synthesis. Even studies that report a linear correlation between mcy D (one of the genes in microcystin synthesis operon) expression and microcystin concentration [179] fails to quantify the microcystin production against live cell numbers. This study aims to bridge this knowledge gap by developing tools that accurately quantify the *de-novo* production rate of secondary metabolites by providing ^{15}N enriched media, hence allowing the study of the turnover rates of secondary metabolites.

1.8 Research Aims

Cyanobacteria produce an array of secondary metabolites employing complex NRPS/PKS enzyme systems at the expense of energy. Although, cyanobacterial secondary metabolites (cyanopeptides) have been studied for several decades, a functional role for them or a distinct advantage to the host cells have not been convincingly proven. The aim of this study was to evaluate the role of cyanopeptides on *M. aeruginosa* PCC7806 and possibly determine if allelopathy is a reason for the production of secondary metabolites.

Objectives

1. **To understand the chromatographic profile of the various secondary metabolites of *M. aeruginosa* PCC7806**

   In order to quantify the *de-novo* secondary metabolites produced by *M. aeruginosa* PCC7806, the methodology of replacing the ^{14}N in the growth media with a stable isotopic ^{15}N would be used. Further, robust analytical methods to differentiate *de-novo* metabolites will be used.

2. **To evaluate the role of exogenous microcystin-LR on *M. aeruginosa* PCC7806**

   Microcystin-LR is one of the well-studied secondary metabolites and its thought that it may function as an allelochemical. In order to evaluate this hypothesis, *M. aeruginosa* PCC7806 would be exposed to 3 different concentrations of exogenous microcystin-LR and its effects on the growth and secondary metabolite production will be evaluated. This study may give some insights into the role of microcystin-LR and since *M. aeruginosa* PCC7806 is also a producer of microcystin-LR, this might shed some light on the advantages conferred by microcystin-LR on its hosts

3. **To evaluate the effect of cyanopeptides on *M. aeruginosa* PCC7806**

   A range of cyanopeptides would be chosen and their effect on the growth and secondary metabolite production of *M. aeruginosa* PCC7806 would be studied. The growth rate and secondary metabolites production rate would be compared to that of a control which would reflect the effect of cyanopeptides on *M. aeruginosa* PCC7806. Additionally, the expression of genes *mcyA* and *mcyE* (responsible for microcystin-LR biosynthesis) would also be investigated and compared against the control.

Chapter II

Materials and Methods

2.1 Cyanobacterial Culture

The cyanobacterium *Microcystis aeruginosa* PCC7806 (Pasteur Culture Collection, Paris) was selected as it had several features that made it an ideal candidate for this study. The strain was originally isolated from the Braakman Reservoir, The Netherlands, in 1972. One of the main features of this organism is that it produces several well characterised secondary metabolites such as microcystins (microcystin-LR & DM-LR), cyanopeptolins (A, B, C & D) and aerucyclamide (A, B, C & D). It is a unicellular organism; hence enumeration would be straight forward using haemocytometry or flow cytometry. Furthermore, *M. aeruginosa* PCC7806 has been extensively studied for its general physiology as well as its molecular characteristics.

2.2 Media Preparation

M. aeruginosa PCC7806 is a freshwater strain, hence the growth medium of choice was BG11 [181,182]. The preparation of BG11 was done as prescribed by Stanier *et al*., 1971 [183] without any modification. The components listed in stock I (Table 2.1) were prepared as individual stock solutions in Milli-RO and the required volume representing the concentration mentioned in stock 1 were taken. The media was prepared in the order listed in Table 2.1 and finally 1 ml from the trace element stock solution was added. The media was appropriately distributed into conical flasks and autoclaved at 121 °C at 15 psi for 15 mins.

Table 2.1. Composition of BG11 media for the culture of *M. aeruginosa* [183]

Stock I				**Final**
	Components	**Formula**	**g/L**	**mM**
1	Sodium Nitrate	$NaNO_3$	1.5	17.65
2	Dipotassium Hydrogen Phosphate	K_2HPO_4	0.04	0.23
3	Magnesium Sulphate Heptahydrate	$MgSO_4.7H_2O$	0.075	0.30
4	Calcium Chloride Dihydrate	$CaCl_2.2H_2O$	0.036	0.24
5	Citric Acid	$C_6H_8O_7$	0.006	0.03
6	Ammonium Ferric Citrate – Green	$FeSO_4.7H_2O$	0.006	0.02
7	EDTA - Disodium Salt	$C_{10}H_{16}N_2O_{8.}2Na$	0.001	0.003
8	Sodium Carbonate	Na_2CO_3	0.02	0.19
	Trace elements stock	**Formula**	**g/L**	**µM**
9	Boric Acid	H_3BO_3	2.86	46.26
10	Manganese Chloride Tetrahydrate	$MnCl_2.4H_2O$	1.81	9.15
11	Zinc Sulphate Heptahydrate	$ZnSO_4.7H_2O$	0.22	0.77
12	Sodium Molybdate Dihydrate	$Na_2MoO_4.2H_2O$	0.39	1.61
13	Copper Sulphate Pentahydrate	$CuSO_4.5H_2O$	0.08	0.32
14	Cobalt Nitrate Hexahydrate	$Co(NO_3)_2.6H_2O$	0.05	0.17

2.2.1 Determination of *de-novo* secondary metabolite production

As the production of secondary metabolites by *M. aeruginosa* PCC7806 is a continuous process, the need to quantify/differentiate between *de-novo* and *ex-novo* secondary metabolites arises. Also, most publications report only the quantity of intracellular or extracellular secondary metabolites at a particular sampling time point rather than actual *de-novo* production (fg/cell/day). To determine actual production, the methodology of growing *M. aeruginosa* PCC7806 in a media containing ^{15}N nitrogen was employed [184, 185]. The BG11 media prepared with sodium nitrate ($Na^{15}NO_3$) would be termed as BG11-^{15}N. In the case of preparation of BG11–^{15}N media, all other preparation procedures remained the same as that of preparation of BG11-^{14}N. Sodium nitrate ($Na^{15}NO_3$) was purchased from Cambridge Isotope Laboratories, Inc., USA. *M. aeruginosa* PCC7806 grown in ($Na^{15}NO_3$) media will have isotopic nitrogen as the only source of nitrogen and synthesis its metabolites incorporating only ^{15}N. The atomic composition of each of the secondary metabolites of *M. aeruginosa* PCC7806 is well known and hence this knowledge could be used to differentiate between *de-novo* and *ex-novo* metabolites. For example, microcystin-LR (Chemical formula $C_{49}H_{74}N_{10}O_{12}$) is a heptapeptide containing 10 nitrogen atoms in a molecule (Figure 2.1). The atomic mass difference between ^{14}N and ^{15}N is 1 and hence the total mass of the *de-novo* microcystin-LR would differ by 10 i.e., microcystin-LR which has an average mass of 996, will have an overall increase of 10 mass units when all the nitrogen is ^{15}N, thus 1006

mass units (Figure 2.2). This mass difference can be differentiated by LC-MS allowing an accurate measure of the *de-novo* synthesised microcystin-LR.

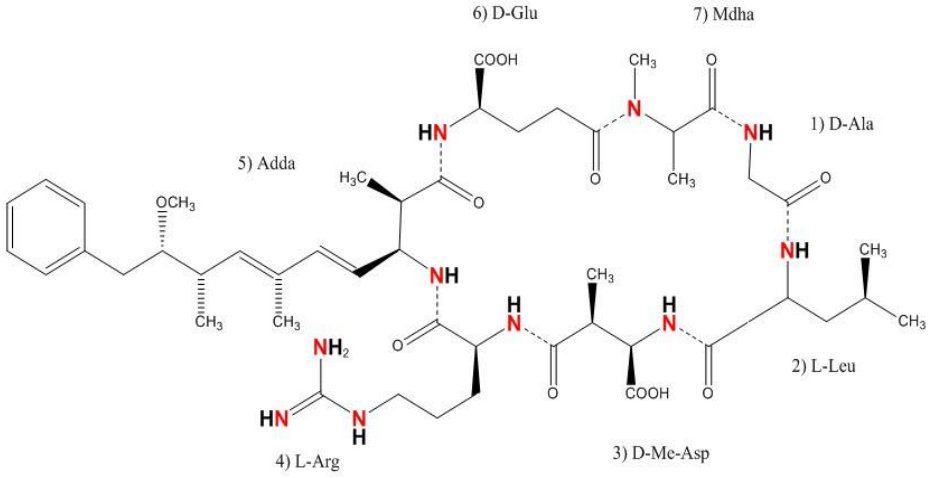


Figure 2.1: Structure of microcystin-LR highlighting the position of nitrogen atoms

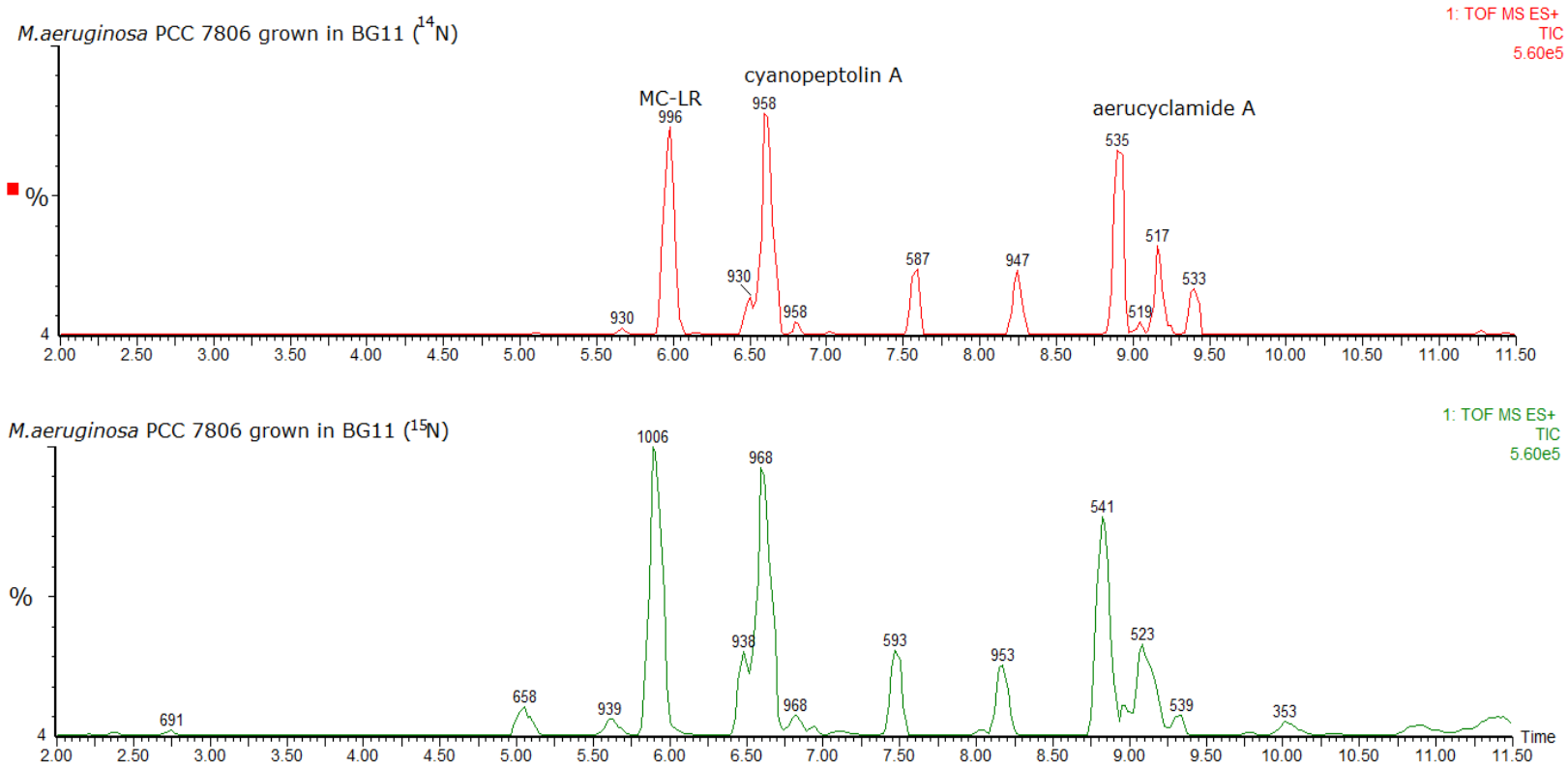


Figure 2.2: HPLC-MS chromatogram of the methanol extracts of *M. aeruginosa* PCC7806 showing the mass shifts of its secondary metabolites when grown in BG11-^{14}N or BG11-^{15}N media.

2.3 Growth conditions

For routine culture maintenance, sterile BG11-^{14}N was used. *M. aeruginosa* PCC7806 was grown under continuous light of about 10 µmols/m^2/s and temperature 21 ± 1 °C and sub-cultured once in 15 days so that an actively growing inoculum would be readily available. However, for experimental assays, the light intensity was maintained at ~20 µmols/m^2/s (continuous) and temperature was maintained at 21 ± 1 °C. Lighting was provided by Philips cool white fluorescent lamps (58 watts). Light intensity was monitored daily using LI-COR LI-250A light meter by moving the flasks aside and placing the probe in its place. The flasks would be mixed once a day and the sampling was timed roughly at the same time each day. The flasks were incubated for the duration required by the experiment and samples were withdrawn after mixing the flasks thoroughly.

2.4 Experimental setup

2.4.1 Evaluation of microcystin as a signalling molecule

To determine if microcystins function as a signalling molecule, *M. aeruginosa* PCC7806 cells were grown in media containing exogenously added microcystin. The presence of exogenous microcystin may alter the growth and/or metabolism of PCC7806 which can be detected as differences in cell counts or the differences in production of secondary

metabolites. The growth media was BG11-^{15}N i.e., BG11 media prepared with $Na^{15}NO_3$. Three treatment conditions were used i.e., 0.1, 1 and 10 µg/ml microcystin-LR (m/z 996) was added exogenously. A control was maintained to which no exogenous microcystin was added. For each treatment, 4 replicates were maintained.

A 10% inoculum of *M. aeruginosa* PCC7806 was added to sterile BG11 – ^{14}N and allowed to grow for 15 days at 21 ± 1 °C at continuous illumination of 10 µmol/m^2/s to reach active growth phase. This was used as the starting inoculum for the experiment. To remove traces of unlabelled nitrogen, the cells were centrifuged at 2000 *g* for 20 mins and the supernatant was discarded. The pelleted cells were washed once with nitrate free BG11 and centrifuged again. The pellet was then resuspended in sterile BG11-^{15}N. This procedure allowed the removal of any unlabelled nitrogen present in the media and provided only labelled nitrogen as the sole nitrogen source. An aliquot from this suspension was taken for cell enumeration. Sterile BG11 – ^{15}N (100 ml) was placed in conical flasks (250 ml) and *M. aeruginosa* PCC7806 was inoculated at a final cell density of 2.5 x 10^6 cells/ml of culture. Purified microcystin-LR (m/z 996), 1 mg was dissolved in Milli-Q by vortexing vigorously for several minutes. This preparation was filter sterilized (0.22 µm) using a syringe filter unit. Sterile microcystin-LR (m/z 996) was added to the treatments to a final concentration of 0.1, 1 and 10 µg/ml. The flasks were incubated under continuous light of 20 µmol/m^2/s and temperature of 22 ± 1 °C for a period of 35 days. Sampling was carried out in 7 day intervals starting

from day 0 until day 35 and samples processed as described in Figure 2.3.

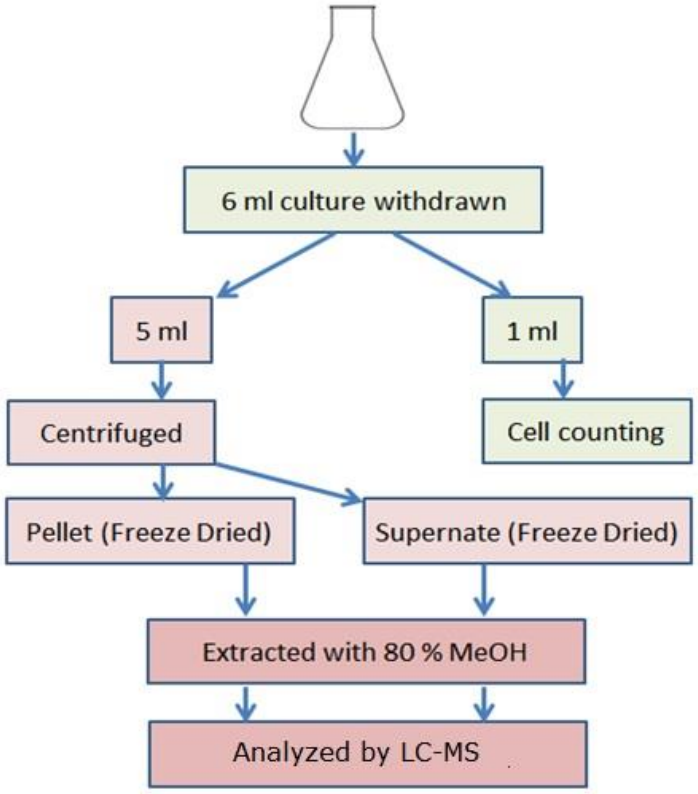


Figure 2.3: Overall sample processing flowchart for evaluating the role of exogenously added microcystin-LR (m/z 996).

2.4.2 Evaluating the effect of exogenously added cyanopeptides

The culture and growth conditions were as described in section 2.1, 2.2 and 2.3. As a general laboratory practice, an actively growing inoculum was readily made available by routine subculture of the

organism every 15 days. Where an older inoculum was required, a few culture flasks were left to incubate for 50 days.

In the previous assay, the cultures were grown in 250 ml conical flasks as they required multiple sample withdrawal. However, in order to evaluate the effect of several cyanopeptides, a faster approach was required. A number of publications have reported that samplings between 5 - 10 days were sufficient to determine the effect of exogenously added compounds [185 - 190] on *Microcystis* sp. Hence, it was decided that a single sample would be withdrawn at day 10 and that a 20 ml culture be taken in 50 ml conical flasks would provide similar culture conditions and an appropriate volume of culture for analysis. The choice of lower culture volume is advantageous when considering the use of expensive resources such as purified compound (e.g. cyanopeptides), labelled nitrate and the relatively smaller space occupied by the culture vessels and hence more test conditions could be carried out at one time.

The inoculum (cells grown in BG11-^{14}N) was centrifuged at 2000 *g* for 20 min and washed with sterile nitrate free BG11. The pellet was resuspended in 10 ml of BG11-^{15}N and the volume required for 2.5 million cells per ml was added in to 3 L of sterile BG11-^{15}N. From this, aliquots of 60 ml were distributed into sterile 100 ml conical flasks and labelled appropriately. To these aliquots, sterile peptides solution was added. The flasks were mixed thoroughly and 20 ml aliquots were withdrawn and distributed into sterile 3 x 50 ml conical flasks. The preparation of the

peptide solutions is described in section 2.5. A control was maintained with equal quantity of Milli-Q water added instead of a peptide solution.

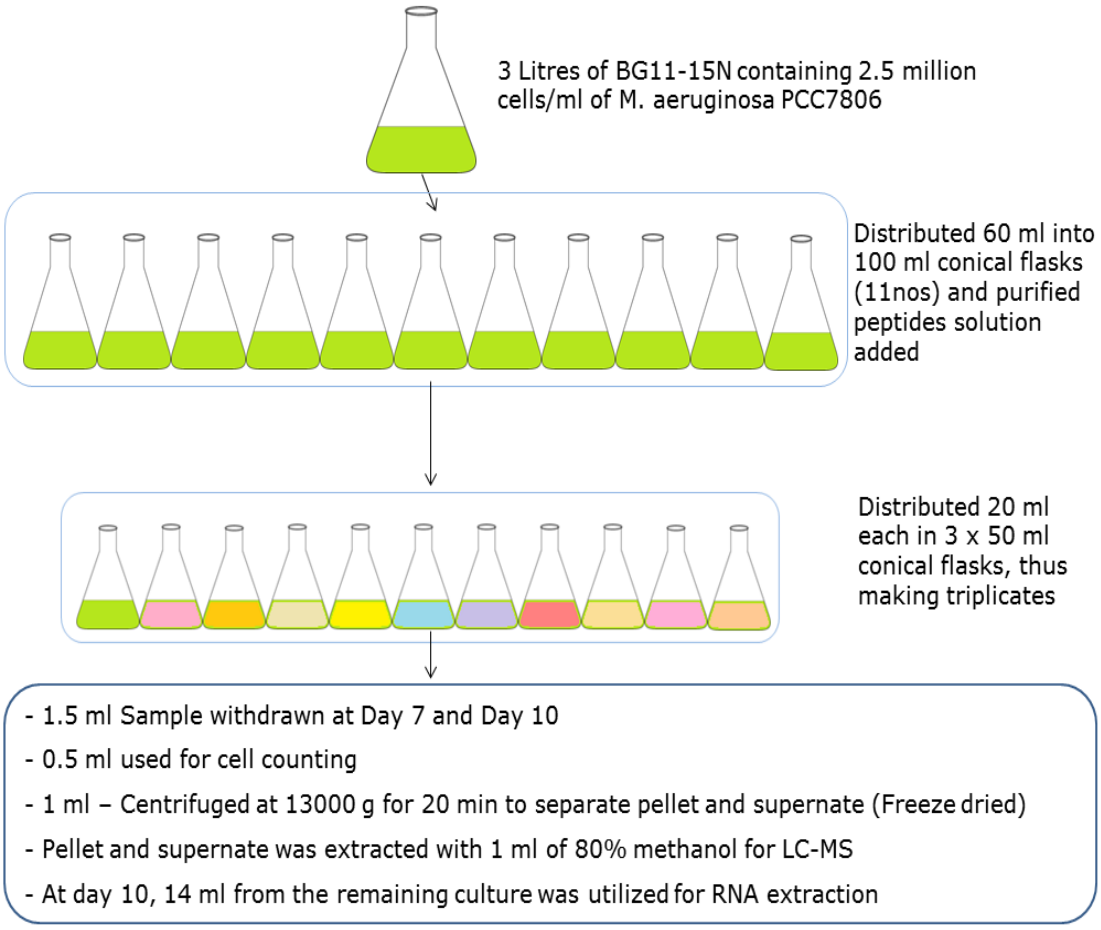


Figure 2.4: Overview of culture preparation and sample processing flowchart to evaluate the effect of different cyanopeptides on *M. aeruginosa* PCC7806.

2.5 Preparation of peptides solutions

The cyanopeptides microcystin-LR, microcystin-LF, MC-RR, cyanopeptolin A, aerucyclamide A and D, aeruginosin, aeruginosamide, anabaenopeptin A and B were chosen. The intended peptide dosage was 10 µg/ml, however due to difference in solubility, the concentration of the peptides in the final peptides solution varied. The purified peptides were kindly provided by Dr Christine Edwards, School of Pharmacy and Life Sciences, Robert Gordon University, Aberdeen, UK.

To 700 µg (100 µg x 7 vials) of each of the dried peptide, 1 ml of Milli-Q water was added to the first vial. This was vortexed vigorously and its contents transferred to the next vial and vortexed. This was repeated until the 7^{th} vial. A further 1 ml of Milli-Q water was added to the first vial and vortexed and the steps repeated until the 7^{th} vial had 2 ml of the peptide in solution. The concentration of this peptide solution was quantified against a purified peptide standard on the LC-MS. It was noticed that except for aeruginosamide, all the other peptides had dissolved reasonably well in Milli-Q water. Hence, only for aeruginosamide, the vials were sonicated (amplitude 50%, for 30 seconds, pulse mode) using a probe sonicator. Finally, the 2 ml peptide solution of each of the peptides was filter sterilized (0.22µm) using a Millex® 13 mm dia PVDF syringe filter assembly and added to the 60 ml culture taken in 100 ml conical flask. From this 20 ml x 3 aliquots were

transferred to 50 ml conical flasks, which were then incubated as described in section 2.4.2

2.6 Cell counting

The organism chosen for this study was unicellular in nature, moreover it also has been reported to contain intracellular pigments such as chlorophyll-a and phycocyanin [181, 191]. Hence, the choice of different cell counting methods such as Neubauer haemocytometer, Beckmann Coulter® EPIC XL-MCL flow cytometer and the Beckman's Multisizer™-3 were available. In order to make the best use of time, a protocol was optimized for all the cell counting instruments such that the counts obtained from any automated instrument are comparable to that of the basic haemocytometric cell counts. The Sedgewick rafter or the Utermohl counting chamber were not considered as the culture being investigated is a pure culture, relatively small in size (~ 3 µm in dia) and that the cell numbers were in the order of million cells per ml.

2.6.1 Cell counting using Neubauer haemocytometer

In order to accurately measure cyanobacterial growth, *M. aeruginosa* PCC7806 was counted using improved Neubauer haemocytometer. This method of cell counting was preferred to Sedgewick rafter or an Utermohl counting chamber as the cell size of *M.*

aeruginosa PCC7806 is between 3 to 4 µm and would take a longer time to settle (1 to 2 hours). The Sedgewick rafter or an Utermohl counting chamber is the preferred counting method for larger and low concentrations of phytoplanktons whereas the Neubauer haemocytometer has been used extensively for counting *Microcystis* cells [97, 156]. Moreover, the cell numbers used for our experiments would in the range of million cells per ml and hence getting sufficient numbers of cells to count would not be a problem. The cell culture was diluted appropriately using sterile nitrate free BG11 and no additional staining methods were employed as the cells naturally appear green in colour. The slide and cover slip was cleaned with ethanol using a lens paper prior to cell counting. The coverslip was pressed on the slide until the coverslip is firmly held by the haemocytometer. A small volume of the sample was taken in a Pasteur pipette and allowed to be drawn by the capillary action into the haemocytometer. The slide was placed under a microscope and observed at 400x magnification. One of the four corner squares was focused and cells were counted starting from the top left corner and moving towards the bottom right corner square. If cells fall on the triple grid lines, only the cells that appear on the top and left grid lines were counted and the cells on the bottom and right gridlines are ignored. Dilution of the culture was adjusted so that around 20 to 250 cells can be counted within one corner square. For every sample, four of the corner squares were counted and the average value taken. Each corner square is 1 mm^2 and a depth of 0.1 mm and hence the volume occupied would be

0.1 mm^3, which would contain a liquid volume of 0.1 µl. Hence, in order to convert to per ml, the cell numbers are multiplied by 10^4.

Cell number per millilitre was calculated using the below formula.

Cells per ml = Number of cells per corner square x 10000 x Dilution factor

Table 2.2: Example of cell counts of *M. aeruginosa* PCC7806 on haemocytometer

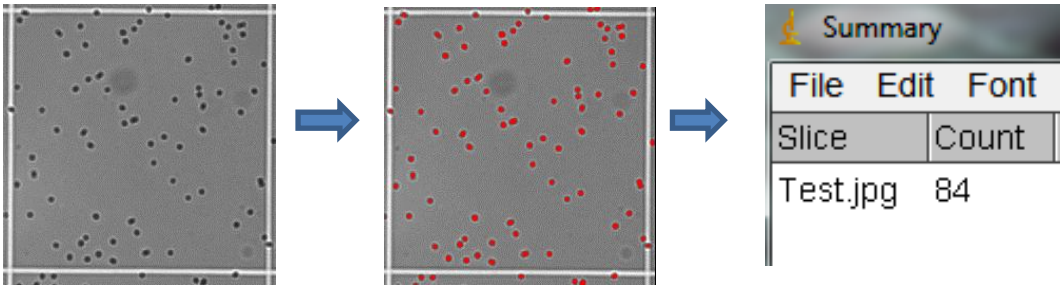
	Cell per square						x 10000		
Conc	A	B	C	D	Dilution	Average	Cells/ml in Million	STDEV	%RSD
100	111	115	99	124	1:10	1122	11.2	1037224	9.2
80	91	85	83	106	1:10	912	9.1	1040433	11.4
60	75	88	72	69	1:10	760	7.6	838744	11.0
40	57	35	43	39	1:10	433	4.3	960152	22.1
20	250	219	230	222	0	230	2.3	139613	6.1
10	130	180	127	138	0	143	1.4	246086	17.1
5	40	70	49	55	0	53	0.5	126095	23.6

2.6.1.1 Cell counting using ImageJ

The cells were counted by haemocytometer as per the protocol as described previously (section 2.6.1). Cell counting using microscopy is preferred when the sample numbers are low and time is not a constraint. However, where a large number of samples need to be quantified, it becomes difficult to process all the samples in a suitable time; hence a semi-automated approach was explored. Most modern microscopes are equipped with image capture options and have software that can assist in counting cells. The ImageJ software is a freeware developed and maintained by 'The National Institute of Health, USA' and is capable of automated cell counting. The overall process of using the haemocytometer remained the same, except that the manual counting part was replaced by taking a monochrome image of the microscopic view of cells (Figure 2.5). The image thus taken was opened in the ImageJ application and processed as follows

1) File>Open
2) Image>Type>8-bit
3) Image>Adjust>Threshold (move slider to highlight cells)
4) Analyze>Analyze Particles. On the pop-up window select summarize and click Ok. Cell counts will be displayed on a new pop-up window.

A sample demonstration is shown.



Raw image from light microscope | cells identified by ImageJ | Cell count given by ImageJ

Figure 2.5: Screenshots of cell counting using the Image-J software.

2.6.2 Cell counting using flow cytometry

Flow cytometry is a rapid particle counting technique and has been suitably adapted for the enumeration of phytoplankton including cyanobacteria. The technique is very effective in particle detections hence some researchers are using it for the identification and enumeration of cyanobacteria, new species of bacteria and sometimes even viruses [192, 193]. The basic principle behind flow cytometry is that cells are aligned into single file by pressurized sheath fluid and passed through a narrow tube. An argon laser (488 nm) beam passes perpendicular to the stream of cells. Each time a cell passes through the beam, the light is deflected and this is counted as an event (Figure 2.6). The deflection is dependent on the density, size and the presence of pigments within the cell. In the case of cyanobacteria, the fluorescence emitted by pigments such as

chlorophyll-a can be measured as an event (Figure 2.7). The cell counts were obtained from the flow cytometer using the Expo32 ADC© software and using MS-Excel software the final cell numbers were calculated considering the dilution factor.

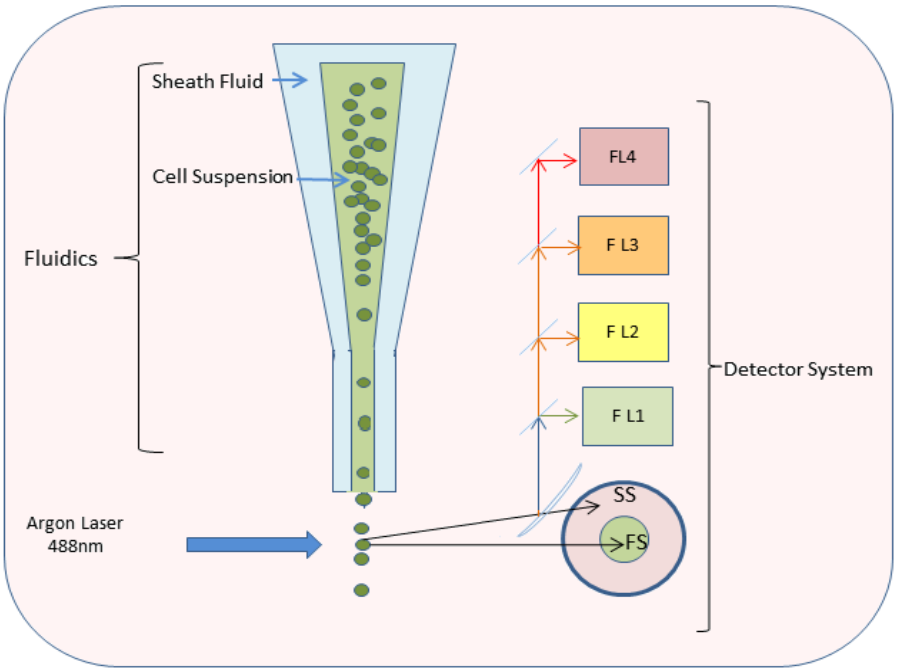


Figure 2.6 – Flow cytometer schematics. Detectors range FL1 – 525 nm, FL2 – 575 nm, FL3 – 620 nm and FL4 – 675 nm.

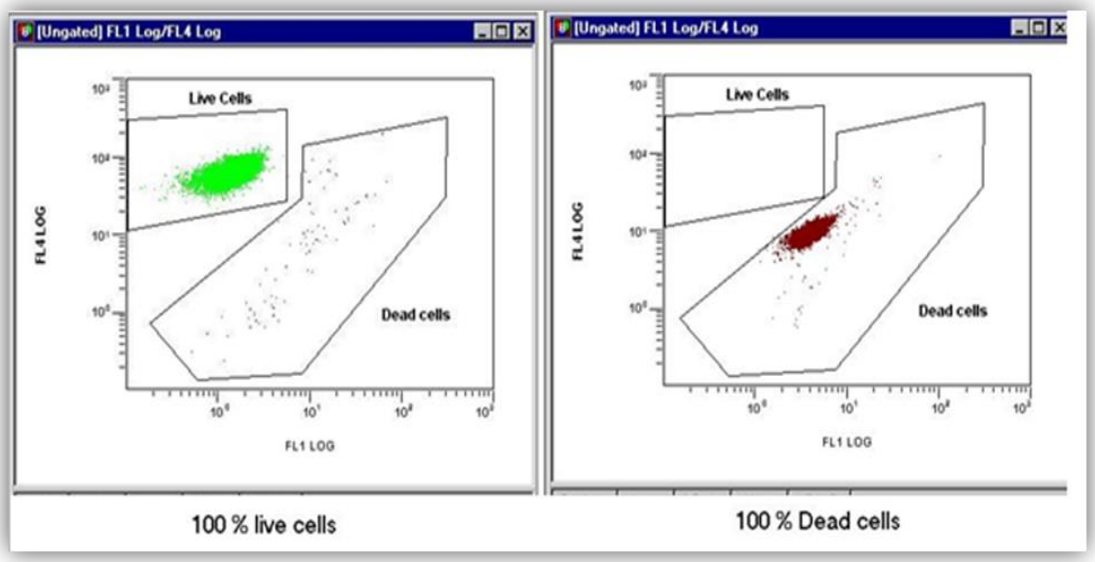


Figure 2.7: Screen shots of flow cytometer showing dot plots. The gates were manually applied to distinguish between live and dead cells. Live cells were directly sampled from a culture of *M. aeruginosa* PCC7806 and dead cells were prepared by heat treating (45 °C for 10 min) an aliquot of the same culture.

The organism *M. aeruginosa* PCC7806 is reported to possess chlorophyll–a (chl-a) and phycocyanin, which have an excitation wavelength of 430 nm and 625 nm respectively. The Coulter® EPICS-XL-MCL™ flow cytometer is equipped with the 488 nm argon laser which makes it an ideal instrument owing to its capability to excite the chlorophyll-a pigment and detect its autofluorescence. The excited chl-a fluoresce at a higher wavelength of 669 nm which is detected by the FL4 detector. Due to their single celled nature of *M. aeruginosa* PCC7806, cells can be counted and differentiated based on their fluorescence wavelength or lack of emission and classified as live or dead cells.

Table 2.3: Flow cytometer settings for enumeration of *M. aeruginosa* PCC7806. The dot plot was chosen taking FL4 on Y axis and FL1 on X axis.

Excitation	488 nm	
Detectors	Voltage (V)	Gain
FL1 (Green) - 525 nm	836	1.0
FL4 (Red) - 675 nm	800	1.0
Flow rate	15 µl/min (Low)	
Time/sample	60 Sec	

The flow cytometer was switched on and allowed to warm up for 15 minutes. *M. aeruginosa* PCC7806 culture was diluted in 0.2 µm filtered BG11 and analysed in setup mode at a fixed flow rate of 15 µl/min. On the x axis FL1 (Log) was selected and on the y axis FL4 (log) was selected. The parameters such as voltage and gain were adjusted so that the event oriented towards the central region of the plot (Table 2.3). Once the cells (detected as events) appear within central region, gates were applied to enumerate and differentiate live and dead cells.

In order to differentiate between live and dead cells, an aliquot of the mid-log culture was taken in a thin walled vial and immersed for 10 min in a water bath heated to 45 °C. The resultant cell suspension was considered as 'dead cells' of *M. aeruginosa* PCC7806 and used only for the purpose of differentiating the live cells from the population of a culture. It should be noted that the cells (events) occurring within the dead cell gates may be whole cells with little or no fluorescence, cellular debris or

empty cell walls. Also, the gate applied to differentiate live and dead cells serves only the function of differentiating live actively growing cells and does not serve to quantify the dead cell population from the suspension.

A mixture of 50% live cells and 50% dead cells was prepared and diluted in filtered (0.2 µm filtered) BG11. This preparation was analysed in the flow cytometer in setup mode (which continually analyzes the sample allowing parameters to be optimized) and the required plots were chosen. The dot plot was chosen as it provides the number of events occurring within a gate. The logic behind this setup is that when the cyanobacterial pigments (chl-a) are excited by the argon laser, fully functional and intact chl-a would fluoresce red with a higher wavelength which would be detected by the FL4 detector. However, when the cyanobacterial cells are unhealthy, one of the characteristics is that the cultures appear pale green, an indication that the pigments are bleached, and hence the cells are compromised. It may also be that chl-a are unable to absorb any light and let the light pass through with minimal interference. This light would be detected by the FL1 detector and would give an indication of the count of unhealthy cells. The mixture of 50 % live and dead cells gave two distinct events population clearly separated from each other. By analyzing a mid-log cell suspension, one of the dense plots were identified as indicative of live cells and the same procedure was repeated with the heat treated cell suspension and two gates were drawn to differentiate live and dead cells. The setting was saved and a sample of known cell density (previously determined by haemocytometer)

was analysed after making an appropriate dilution. The voltage and gain was again adjusted such that the number of events, correspond to the known cells per ml (after appropriate calculation).

Cell numbers were calculated using the below formula.

$$Cells\,per\,ml = Number\,of\,events\,within\,a\,gate\;x\frac{1000}{F}x\;Dilution\,factor$$

Where F represent the flow rate (µl/min), and it was set at 15 µl/min

While preparing dilutions, it should be noted that the maximum number of events the flow cytometer can count in one second is approximately 200. Higher number of events may lead to poor detection resulting in underestimation of cell counts. Any suspension with a higher cell density was further diluted so as to stay within the detection limits for the instrument.

In order to accurately measure cyanobacterial growth using a flow cytometer, the cells of *M. aeruginosa* PCC7806 were first counted in the haemocytometer and then the flow cytometer was calibrated against the haemocytometer cell counts. In this way the cell counts would be comparable to traditional cell counting methods. The count of live and dead cells was obtained as described previously. Appropriate dilutions were made in filtered BG11 prior to analysis on flow cytometer (Table 2.4).

Table 2.4: Cell counts of *M. aeruginosa* PCC7806 on flow cytometer

	Events per gate					=(x/15) * 1000		
Conc	A	B	C	Dilution	Average	Cells/ml	STDEV	%RSD
100	854	846	743	200	162833	10.9	829045	7.6
80	654	541	666	200	124083	8.27	917928	11.1
60	530	431	530	200	99417	6.63	760178	11.5
40	276	265	273	200	54250	3.62	76376	2.1
20	155	160	153	200	31167	2.08	50918	2.5
10	6282	6208	6268	1	6253	0.41	2621	0.6
5	2908	3022	3063	1	2998	0.20	5354	2.7

The values of cell numbers obtained through the flow cytometer were comparable to that of those observed by the haemocytometer (Table 2.2) at higher concentrations. However, at lower concentrations the difference was significant. The relative standard deviation while using haemocytometer was between 6 to 24% while in flow cytometer it was between 0.6 to 12% and the error seemed to be higher in higher cell densities. The counts obtained from flow cytometer analysis represent only the live cell count. Since the culture used was mid-log, a small number of cells (events) appeared in the dead cell gate (5 %) and were excluded from the counting. Although some researchers have used flow cytometer to quantify *Microcystis* sp. their methods were complex and required additional dyes. The described method here is straightforward and gave consistent cell counts and could be modified further when

additional analysis is required. Further, the methodology could be adopted for other unicellular cyanobacteria by way of utilizing the emission properties of their intracellular pigments. However, for routine cell counting, the method described above is sufficiently accurate and robust.

There are several advantages of using flow cytometry for counting unicellular cyanobacterial cells. The technique is straight forward and does not require sample fixing procedure. When accurate growth rates and production rates are to be calculated, the need to differentiate between live or dead cells arises. It could be concluded that flow cytometry can be used as effectively as a haemocytometer to enumerate unicellular cyanobacteria, but with a high degree of accuracy.

2.7 Growth Rate and doubling time determination

The specific growth rate per day (μ) and doubling time (G) of *M. aeruginosa* PCC7806 was calculated using the formula given below [220].

$$\text{Growth rate } (\mu) \text{ per day} = [\log(X) - \log(X_0)] / [\log(2) * \Delta t]$$

Where, X_0 is the initial cell count, X is the obtained cell count and Δt is the time interval taken between initial and obtained cell counts.

The doubling time in days (G) was calculated as:

$$G = 1/\mu$$

where μ = growth rate.

2.7.1 – Calculation of production rate (per cell per day) of secondary metabolites

In order to calculate the production rate of secondary metabolites, the cell numbers and the concentration of *de-novo* intracellular secondary metabolite (e.g. cyanopeptolin A) per ml was obtained. From this data, the production rate (per cell per day) was calculated as shown below.

Steps

W – The 'new cell numbers' were obtained by subtracting latest weeks cell numbers from the corresponding flasks previous weeks cell number.

X – The concentration of 'new' secondary metabolite (e.g Cyano A) was obtained by subtracting latest weeks *de-novo* intracellular secondary metabolites concentration (per ml) from the corresponding flasks previous weeks *de-novo* secondary metabolite concentration (per ml).

Y – The ‘new’ concentration of secondary metabolites (X) was divided by ‘new cell numbers’ (W) and the answer multiplied by 1×10^9 to convert µg/ml to femtograms per ml.

Z – In order to obtain the concentration per cell per week, Y was divided by duration between two sampling points i.e. 7 days.

Sample	Cyanopeptolin A (µg/ml)	Cells/ml	New Cells (W)	New Cyano A (X)	Cyanopeptolin A per cell per week(Y)	Cyanopeptolin A per cell per day (Z)	Average	STDEV
C1	0.0000	2597867						
C2	0.0000	2604000						
C3	0.0000	2626667						
C4	0.0000	2635333						
C1	0.2079	8994667	6396800	0.208	32.502	4.643		
C2	0.3787	8877333	6273333	0.379	60.368	8.624		
C3	0.4517	9177333	6550666	0.452	68.955	9.851		
C4	0.2801	8828000	6192667	0.280	45.229	6.461	7.395	2.308
C1	1.0626	42652000	33657333	0.855	25.394	3.628		
C2	1.0247	48592000	39714667	0.646	16.266	2.324		
C3	0.9911	43112000	33934667	0.539	15.895	2.271		
C4	1.0242	47652000	38824000	0.744	19.165	2.738	2.740	0.628
C1	1.5353	76334400	33682400	0.473	14.035	2.005		
C2	1.4541	84855600	36263600	0.429	11.842	1.692		
C3	1.4004	76042800	32930800	0.409	12.430	1.776		
C4	1.3737	73364400	25712400	0.350	13.595	1.942	1.854	0.145

2.8 Analytical methods (LC-MS analysis)

The analysis of the secondary metabolites of *M. aeruginosa* PCC7806 was performed using Waters ACQUITY UPLC® system equipped with a QTOF/MS (Quadrupole Time of flight Mass spectrometer) and a PDA detector (Photodiode array) [185, 198, 199]. All solvents such as methanol, acetonitrile, formic acid used were of HPLC grade. Samples for HPLC analysis were prepared as described in section 2.3 or 2.4 as per the experimental design. Briefly, the cell culture was centrifuged and the pellet and the supernatant separated. The pellet and supernatant were freeze dried and extracted with 80% methanol. The methanolic extract was centrifuged to remove debris and the supernatant was taken in HPLC vials for analysis. The BEH (Bridged Ethylene hybrid) C18 column (2.1 mm diameter, 130Å pore size, 100 mm length, 1.7 µm particle size, Waters®, Elstree, UK) was used to achieve separation of the metabolites. The mobile phase used was Milli-Q water and acetonitrile containing 0.1% formic acid. The flow rate was maintained at 0.2 ml per minute. The solvent gradient of acetonitrile was initially 20% and was gradually increased to 80% over a period of 10 minutes. This was followed by a washing step with 100% acetonitrile and column equilibration. The capillary voltage was 3.0 kV and cone voltage was set to 25 V. The source and desolvation temperatures were 80 °C and 300 °C respectively. Mass spectrometry analysis was performed in positive ion electrospray mode, scanning from m/z 50 to 2000 Da with a scan time of 1 second.

Table 2.5: The MS and UV values used for extraction of peak areas using MassLynx™ software for the different secondary metabolites

	Ex-novo		*De-novo*	
	MS	UV	MS	UV
microcystin-LR	996	238	1006	238
Cyanopeptolin A	958	N/A	968	N/A
Cyanopeptolin B	930	N/A	939	N/A
Aerucyclamide A	535	N/A	541	N/A
Aerucyclamide C	517	N/A	523	N/A
Aerucyclamide D	587	N/A	593	N/A

Peak areas for the corresponding *ex-novo* and *de-novo* secondary metabolites were obtained using the MassLynx™™™ application (Table 2.5). Quantification of microcystin-LR was performed by comparing the peak area of the unknown sample against the peak area of a known standard. Previously purified and quantified microcystin-LR (m/z 996 of >99% purity by HPLC) standard was used to prepare a dilution series. The limit of detection of this method is reported to be about 0.5 pg/µl. The standard was prepared in 80% methanol to final concentration of 50, 10, 5 and 1 µg/ml in triplicate and analysed in HPLC-MS. The MS peak areas were obtained from the data using the proprietary MassLynx™™™ data analysis software. Plotting peak area on the Y-axis and the concentration on the X-axis a simple linear regression curve was plotted and correlation co-efficient was obtained. Also, a line equation was obtained for the linear regression curve ($y = mx + c$) and concentration of the unknown

sample was obtained by solving for x (Figure 2.11, 2.12 and Table 2.6, 2.7).

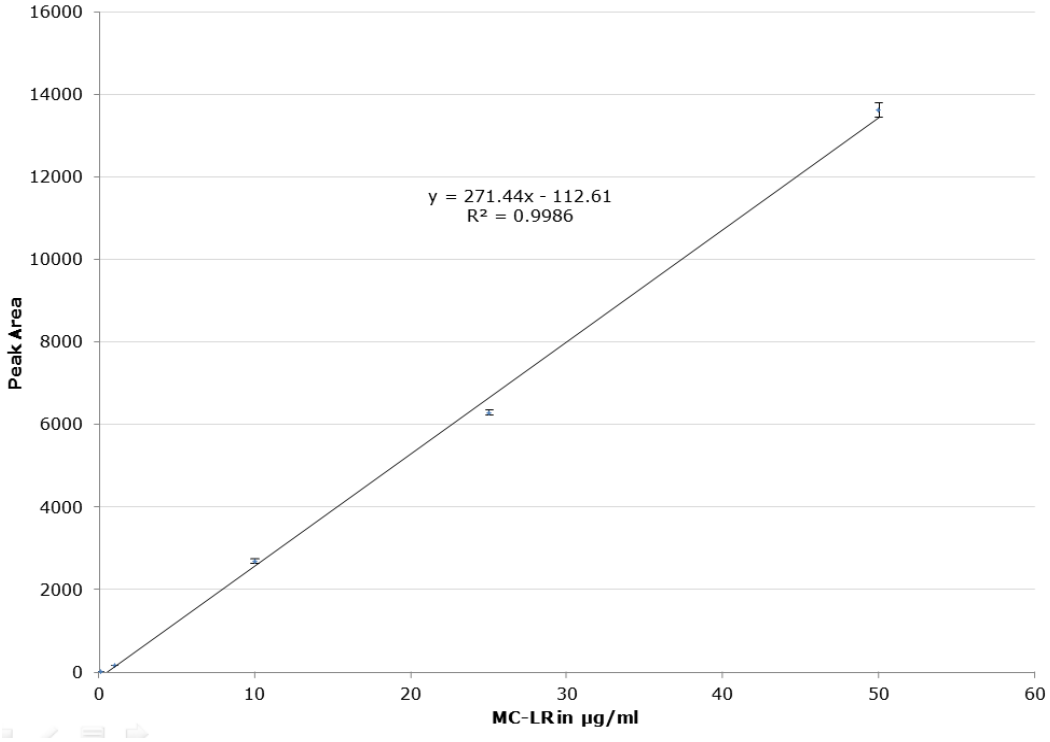


Figure 2.11 – Standard curve of microcystin-LR (m/z 996) by the ESI-MS, quantified using peak area at m/z 996. Error bars = 1 SD, n=3.

Table 2.6: An example showing calculations for finding the unknown concentration of microcystin-LR considering ESI peak area at m/z 996

Peak area of Sample	y = 271.44x - 112.61	microcystin-LR in µg/ml
249	=(249+112.61)/271.44	1.33
228	=(228+112.61)/271.44	1.25
261	=(261+112.61)/271.44	1.38
	Average	1.32
	STDEV	0.06

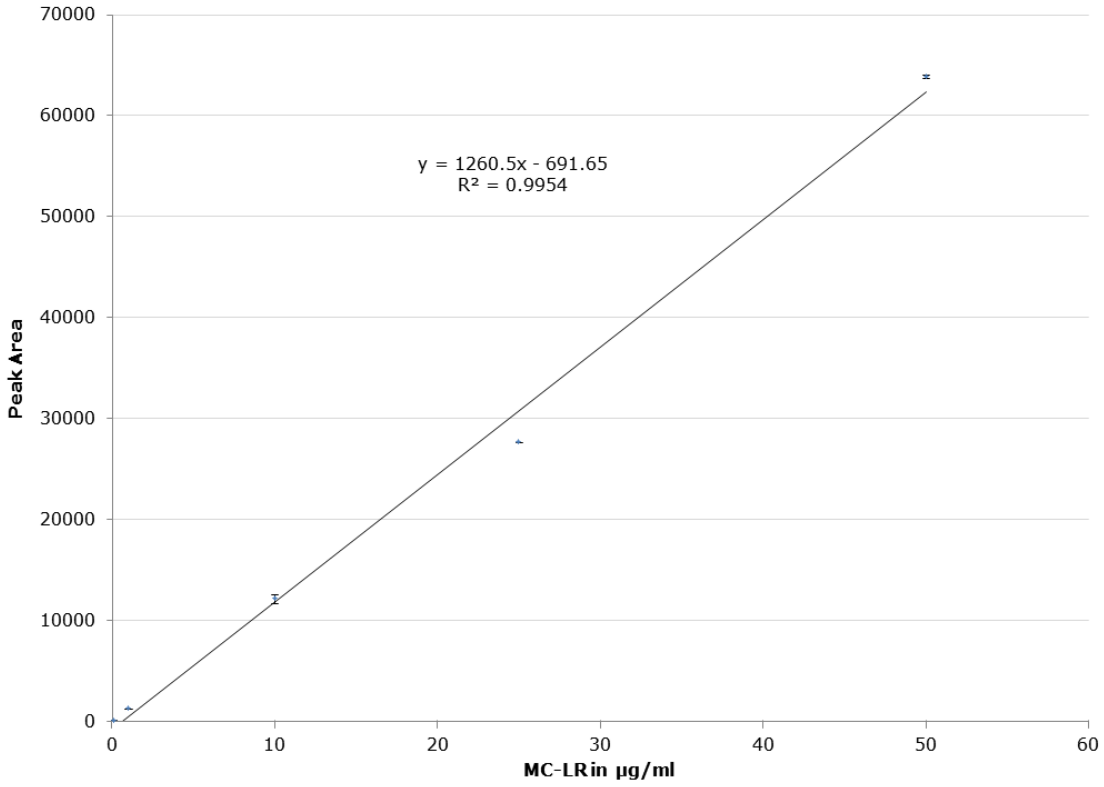


Figure 2.12 – Standard curve of microcystin-LR by the UV quantified at 238 nm. Error bar = 1 SD, n=3.

Table 2.7: An example showing calculations for finding the unknown concentration of microcystin-LR considering UV peak area at 238 nm.

Peak area of Sample	y = 1260.5x - 691.65	microcystin-LR in µg/ml
1219	=(1968+691.55)/1260.5	1.52
976	=(1850+691.55)/1260.5	1.32
1309	=(2117+691.55)/1260.5	1.59
	Average	1.48
	STDEV	0.14

2.9 RNA extraction and purification

In order to study the gene expression profile of *M. aeruginosa* PCC7806, mRNA was extracted and the mRNA of 16s rRNA, the microcystin synthetase genes *mcyA* and *mcyE* were amplified. At day 0 of the experiment, prior to the addition of peptides, 40 ml of cells in triplicate (2.5 million cell/ml) was taken in ice cold Falcon tubes. This would be considered as day 0 (baseline) and serve to compare with gene expression. At day 10, the culture was centrifuged at 4000 *g* for 20 min at 4 °C and the supernatant discarded. The RNA extraction procedure was performed using the TRIZOL™ Max™ Bacterial RNA Isolation Kit (Invitrogen, UK) with some modifications.

To the pellet, 200 µL of Max™ Bacterial Solution was added and mixed by pipetting. The samples were incubated in water bath maintained at 95°C for 4 min. To this, 1 mL of TRIzol® Reagent was added and mixed, 200 µl of cold chloroform was added and mixed

thoroughly. The tubes were centrifuged at 4000 *g* for 45 min at 4 °C. The upper aqueous layer was transferred to fresh tubes and 300 µl of cold isopropyl alcohol was added to precipitate RNA. The tubes were mixed and allowed to incubate at room temperature for 10 mins. The tubes were centrifuged at 4,000 *g* for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in 500 µL of chilled 75% aqueous ethanol. The samples were vortexed thoroughly and then centrifuged at 4,000 *g* for 15 min at 4°C. This step was repeated twice. The pellets were air dried and to this 100 µL of RNase-free water was added. The samples were stored at -80°C in a freezer until purification.

2.9.1 RNA purification

RNA purification was performed using RNeasy Mini Kit (Qiagen, UK) and DNase treatment using RNase-free DNase set (Qiagen, UK). To 100 µL of the extracted RNA, 350 µL of RLT buffer and 200 µL of chilled absolute alcohol was added. The samples were mixed and immediately transferred to Qiagen RNeasy columns. The columns were centrifuged at 12,000 *g* for 1 min at room temperature. The flow through was discarded and 350 µL of buffer RW1 was added and centrifuged for 12,000 *g* for 1 min. Then, 80 µl of DNase I mix (10 µl of DNase I stock with 70 µl of RDD buffer) was added to the column and allowed to incubate at room temperature for 15 mins. After incubation, 350 µl Buffer RW1 was added and centrifuged for 12,000 *g* for 1 min. Subsequently, 500 µl Buffer RPE was added to the column and centrifuged at 12,000 *g* for 2 min. The column was placed in new

1.5 ml collection tube and 30 µl RNase-free water was directly added to the column membrane. The columns were centrifuged for 1 min at 12,000 *g* to elute the RNA. The concentration and purity of RNA samples were determined by 260/280 nm ratio using Genova NanoSpectrometer (Jenway). It is generally accepted that a 260/280 ratio of ~ 2 is acceptable for RNA. The RNA samples were stored at -80°C in a freezer until use.

$$\text{Purity of RNA} = \frac{\text{Absorbance at 260 nm}}{\text{Absorbance at 280 nm}}$$

$$\text{Concentration of RNA} = \frac{OD_{260}}{\text{Path length}} \times \text{Standard coefficient x dilution}$$

Where, standard coefficient for RNA is 40 µg/ml.

2.10 Semi-quantitative RT-PCR

The Qiagen® OneStep RT PCR kit was used for amplification of specific mRNA sequences. The primers were designed and purchased from Eurofins MWG, UK.

16s rRNA Gene - Accession no - U03402		Base pairs	Tm (°C)	% GC	Product size
Forward	5′-CTAAAGGCGGTGGAAACTGGC-3′	21	61.8	57.1	214 bp
Reverse	5′-ACAAGCCACGCCTAGTATCC-3′	20	59.4	55.0	214 bp

mcyE Gene					
Forward	5′-CGAGAAATCTTGGGGCTACTTGAAC-3′	25	63	48.0	535 bp
Reverse	5′-CAATGGGAGCATAACGAGTC-3′	20	57.3	50.0	535 bp

mcyA Gene					
Forward	5′-GCTGCCCAGATGCGAGAATG-3′	20	61.0	60	334 bp
Reverse	5′-CCTCTAAAACCCGCAGTAAGTAATC-3′	25	61.0	44	334 bp

The reaction mixture for RT-PCR was as follows:

Qiagen RT-PCR Buffer, 5x	2.50 µL
dNTP mix (10 mM each)	0.50 µL
Primer (Forward) (0.6 µM)	0.75 µL
Primer (Reverse) (0.6 µM)	0.75 µL
RT Enzyme	0.50 µL
RNase-free Water	5.00 µL
RNA Template (100 ng)	2.50 µL
Total	12.5 µL

The PCR conditions were as follows:

No of cycles	Step	Temp (°C)	Time (min)
1	Reverse Transcription	50	30.0
1	Activates Taq DNA polymerase	95	15.0
23	Denaturation	94	0.5
	Annealing	55	0.5
	Extension	72	0.5
1	Final extension	72	10.0

The PCR products were run in 1% Agarose gel prepared in 1x Tris-Boric Acid-EDTA (TBE) Buffer containing GelRed™ nucleic acid stain. The gels were viewed using Gel Documentation System: UVITEC, Cambridge and images were captured using UVIDoc HDS software. The gel bands were quantified using Quantity One® 1-D analysis software (BioRad).

2.11 Statistical analysis

The one-way ANOVA statistical method was used to determine statistical significance. The mean of two groups e.g. the average cells numbers of 'control' at Day 10 to that of average cell numbers of

'treatment' was statistically compared and the resultant P-value gives an indication of the statistical significance between the groups. A P-value of less than 0.05 was considered statistically significant while a P-value greater than 0.05 is statistically insignificant. A web based version of the Anova statistical software was used for the calculations [30]. Post data analysis, the Tukey's HSD post-hoc test was performed with a confidence interval set to 95 %.

Chapter III

Incorporation of ^{15}N into secondary metabolites of *M. aeruginosa* PCC7806

3.1 Introduction

The production of secondary metabolites by cyanobacteria has recently received much attention as many of these metabolites are either toxic to higher organism or have interesting biochemical characteristics [200]. The organism *Microcystis aeruginosa* PCC7806 produces several interesting molecules such as the hepatotoxin microcystin-LR, the protease inhibiting cyanopeptolins and the antimalarial aerucyclamides [90, 175, 201]. Although this organism has been studied extensively for decades, questions such as production dynamics, accurate quantification, production per cell per day and excretion rate of these compounds remain largely unanswered.

The production of cyanobacterial secondary metabolites may be governed by extrinsic factors such as light, temperature, nutrient availability and intrinsic factors like growth rate, metabolite concentrations and gene regulations [97, 202 - 206]. While earlier reports indicated that growth limiting factors such as nitrogen depletion indirectly controlled microcystin production, by limiting growth rate [100], later reports add that microcystin production is a complicated process and factors such as nitrogen as well as light play a combined role [94]. In fact, it was reported that photon irradiance acts directly on the transcription and/or transcript stability of microcystin synthetase genes [207]. Further, the production of different microcystin variants may respond to environmental factors in different ways [208]. It has been reported that in *Planktothrix agardhii,* the

production of microcystin-RR decreased two fold, while the production of microcystin-LR increased three fold when photon intensity increased from 60 to 100 μmols/m^2/s [101].

The reason for the complexity of peptide production could be explained by the fact that most of the secondary metabolites produced by cyanobacteria are a product of multifunctional enzyme complexes formed from non-ribosomal peptide synthetase and polyketide synthase (NRPS/PKS) [207, 183]. The NRPS/PKS system in *M. aeruginosa* PCC7806 was elucidated In 2000 and the authors suggest that microcystin-LR synthesis is achieved by a bidirectional operonic cluster consisting of 10 genes (*mcyA - J*) in which the genes *mcyA - C* code for NRPS while the genes *mcyD – J* code for PKS [176]. Further, In 2008 the gene sequence for aerucyclamides was identified and designated as *mca (A - G)* and in 2009 the gene sequence for cyanopeptolin was identified and designated as *mcn (A - E)* [209, 210]. Although the genes for most of the secondary metabolites of *M. aeruginosa* PCC7806 have been identified, their inter gene relationships are largely unknown and it is thought that they operate independent of each other [211]. This was demonstrated when certain genes necessary for microcystin biosynthesis were inactivated which in turn abrogated microcystin production but the production of other metabolites were unaffected [175, 181]. Hence, if it were argued that environmental factors affected gene expression, then it is possible that the production of different cyanopeptides will be affected differently by the same culture conditions.

Under typical laboratory batch growth conditions, cyanobacteria grow exponentially, then transition to a light-limited stage of linear growth before finally reaching a non-growth stationary phase. In cyanobacteria, cultures can be limited in their growth by self-shading that prevents light penetration into deep culture layers. The macro and microelement limitations and limitation by high content of dissolved O2 and/or by low content of dissolved CO_2 or of HCO_3 are often superimposed on the light limitation. At the gene level, the transcription of approximately 10% of the genes in the wild type was differently expressed between the exponential and linear phase. It has been reported that the expression of photosynthetic and regulatory genes are under expressed while genes for DNA photolyase (DNA repair enzyme) and Chl-binding protein are strongly induced [108]. In a study on the unicellular cyanobacterium *Cyanothece* sp, it was reported that the specific growth rate in exponential growth phase was less dependent on the incident irradiance than the photosynthetic activity. They also comment that the transition from exponential to linear phase is caused by a light limitation and the transition from linear to stationary phase by nitrogen limitation [102].

In terms of cell growth and microcystin biosynthesis, a consistent culture system or a set of growth condition have not been reported for the study of *M. aeruginosa* PCC 7806. While some researchers have monitored the growth for up to 60 days [94], some have monitored growth for 20 days [161, 214] and others report monitoring growth for 6 days or less [127, 215]. Nitrate seems to be an important requirement and in terms of NO_3 concentration used,

authors have reported using between 2 mM to 24 mM [217, 218]. In an earlier work on *M. aeruginosa* PCC7806, it has been reported that the when light intensity is 5 ± 1 µmol/m^2/s and nitrogen concentration is 9 mM of $NaNO_3$ and starting cell density is 1 x 10^5 cells /ml, the lag phase growth is up to 5 days while the exponential phase could last up to 40 days. However, at the same $NaNO_3$ concentration and cell densities, light intensity of 39 ± 4 µmol/m^2/s the exponential growth phase lasted only up to 10 days [216]. Another report has added that light intensities above 32 µmols/m^2/s inhibits *mcyB* transcription, meaning microcystin production is inhibited [220] while others report that high light intensities induce passive release of intracellular metabolites [94, 127]. Enumeration of cells was also widely different and several cell counting methods such as microscopy [108], flowcytometry, measuring optical density, Chl fluorescence [102] and dry weight have been used. Hence, a direct comparison between growth parameters and the response of the organism to these parameters was not possible. In the case of *Microcystis* sp, a statistical report concludes that $NaNO_3$ > 1.27 mM, K_2HPO_4 > 0.1 mM, iron > 0.01 mM and temperature > 18.8 °C stimulates growth and microcystin production [219]. As the synthesis of secondary metabolites is achieved through NRPS/PKS complex, it could be argued that these secondary metabolites would be synthesised continuously. In fact, it has been reported that about 67 % variability in toxin production (as determined my *mcyB* transcripts) may be attributable to growth phase and temperature [220]. Combining published data and drawing on the experience of previous lab members [185], it was

decided that a culture grown for 15 days would provide the cells sufficient time to acclimatise and start multiplying. Such a culture would be an ideal starting inoculum for experimental purposes. Also, the starting inoculum cell density was decided to be kept consistent at around 2.5 million cells per ml. While light intensity is an important factor, in the case of *M. aeruginosa* PCC7806, light intensity above 32 μmol/m^2/s is detrimental to microcystin production [220] while light intensity between 10 to 20 μmols/m^2/s has been reported to have no effect on cell productivity (as determined by protein content, 4.93 ± 0.66 pg/cell) [94]. Hence it was decided that the inoculum would be grown at 10 μmols/m^2/s but the experiments would be conducted at 20 μmols/m^2/s so as to provide ample lighting to the culture, when cell shading occurs as a net result of increased cell numbers. Such an approach adds consistency to experimental procedures while providing optimal growth conditions for the culture.

In this study, the production of *de-novo* cyanopeptides was monitored by growing the culture in BG11 media containing a stable isotopic nitrate (^{15}N) salt i.e. ($Na^{15}NO_3$). As the exact number of nitrogen in each of the cyanopeptide is known, mass differences between *de-novo* and *ex-novo* cyanopeptides could be differentiated by LC-MS, and hence accurate measure of day to day production could be achieved. The combination of ^{15}N labelling and TOF (time of flight) technology has been used previously and has been proven to be a robust, accurate and straight forward tool in the study of cyanopeptides [212]. The technique could be invaluable when studying the turnover rates of multiple metabolites of a single strain, its

intracellular and extracellular fate, how production rate and growth rate correlates. Also, owing to the slow growth, the cyanopeptide's intermediates and their order of synthesis may also be deduced. In future, this study might be useful to compare the effect of environmental factors, allelopathic interactions and when coupled with ^{15}N labelling of primary metabolites could aid in understanding complex interactions between growth and intracellular metabolism [213].

3.2 Aims

To study the incorporation of ^{15}N into the secondary metabolites of *M. aeruginosa* PCC7806

Objectives

- To determine the chromatographic and ion profile of the secondary metabolites of *M. aeruginosa* PCC7806
- To observe the fragmentation pattern of the secondary metabolites viz microcystin-LR, cyanopeptolin and aerucyclamides and to study the partial incorporation of ^{15}N into secondary metabolites of *M. aeruginosa* PCC7806
- To establish a protocol for the use of ^{15}N in BG11 media to study the *de-novo* synthesis of secondary metabolites of *M. aeruginosa* PCC7806.

3.3 Material and Methods

3.3.1 Culture, media and growth conditions

The culture, media preparation and growth conditions were as described in section 2.1, 2.2 and 2.3. Cell counting was performed as described in section 2.6.1 and samples were analysed as described in section 2.8.

3.3.2 Experimental setup

In order to evaluate the growth characteristics of *M. aeruginosa* PCC7806, a 15 day old inoculum of the culture was aseptically inoculated into 200 ml x 3 BG11-^{15}N media taken in 500 ml conical flasks. The cultures were incubated for 21 days at conditions described in section 2.3 and samples were withdrawn every day, roughly at the same time of the day. Samples were only withdrawn after thorough mixing of the flasks to ensure homogenous cell densities. Once samples were withdrawn, they were processed as shown in Figure 2.3.

3.4 Results

3.4.1 Growth curve of *M. aeruginosa* PCC7806

The aim of this study was to monitor the growth of the organism for a period of 21 days, however due to unforeseen circumstances the growth of *M. aeruginosa* PCC7806 could only be monitored for a period of 19 days (Figure 3.1). To maintain consistency between experiments, the starting inoculum cell density was maintained around 2.5 to 3 million cells per ml [185].

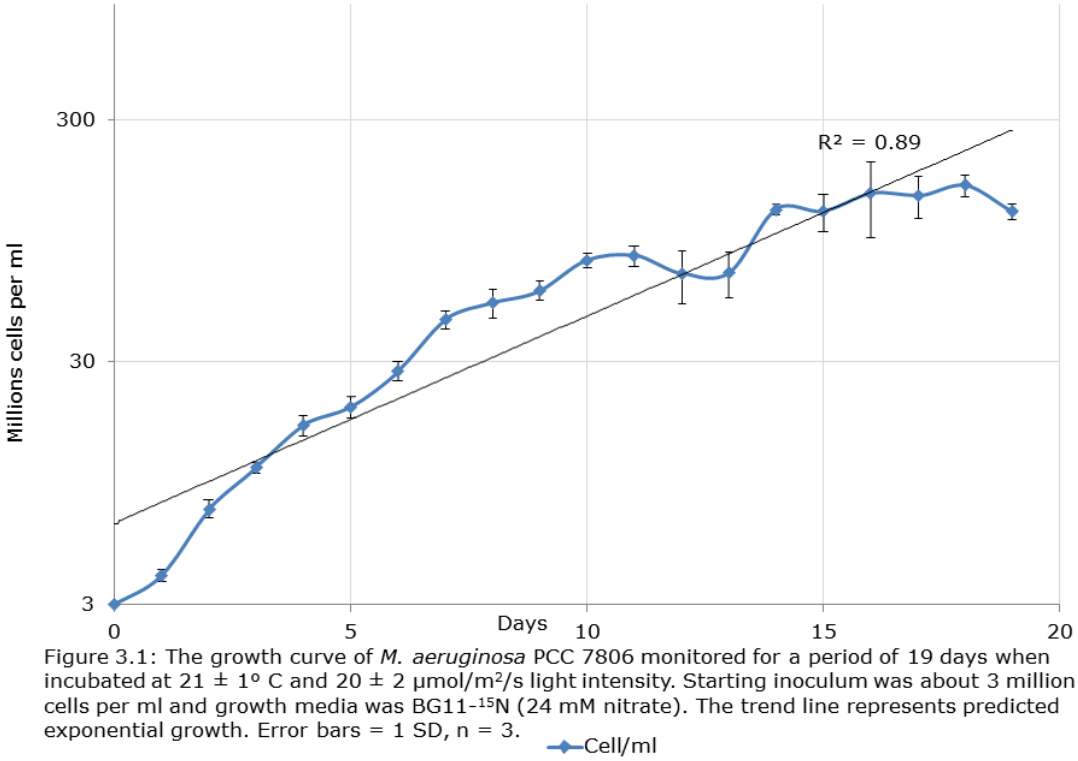


Figure 3.1: The growth curve of *M. aeruginosa* PCC 7806 monitored for a period of 19 days when incubated at 21 ± 1° C and 20 ± 2 µmol/m²/s light intensity. Starting inoculum was about 3 million cells per ml and growth media was BG11-^{15}N (24 mM nitrate). The trend line represents predicted exponential growth. Error bars = 1 SD, n = 3.

From the cell numbers monitored for a period of 19 days, the specific growth rate (per day) was calculated using the formula described in section 2.7. Statistically, the growth rate between day 1 to 5 & 5 to 15 was insignificant ($p = 0.21$), indicating that the cells are

growing at a similar rate between day 1 to 15, beyond which stationary phase begins (Table 3.0). The doubling time (G) i.e. time taken for a cell to divide also correlated with the growth rate i.e. as growth rate decreased, the time taken for cells to divide increased. In literature, it has been reported that the growth rate of *M. aeruginosa* PCC7806 ranges from 0.2 to 0.5 per day [160, 200 - 221] under various growth conditions. Hence, it could be stated that the observed growth rates correlates well within literature reported values. Further, the doubling time observed here is also well correlated with that of reported values of 1.8 to 4.8 days [220-221].

Table 3.0 – The growth rate and doubling time of *M. aeruginosa* PCC7806 monitored for a period of 19 days.

Days	Growth rate /day (μ)	Doubling Time in Days (G)
1 to 5	0.39 ± 0.07	1.84 ± 0.45
5 to 15	0.32 ± 0.05	2.27 ± 0.40
15 to 19	0.23 ± 0.02	3.08 ± 0.29

The organism *M. aeruginosa* PCC7806 cannot fix atmospheric nitrogen and hence an exogenous nitrogen source needs to be supplied for its growth. In BG11 media, if the nitrate (in the form of Na $^{14}NO_3$) is replaced with a stable isotopic nitrate (in the form of Na $^{15}NO_3$) salt, the cells would utilize this nitrogen to form its secondary metabolites. The mass of the *de-novo* secondary metabolite thus produced will be heavier by the number of nitrogen atoms making up the metabolite.

For example, microcystin-LR contains 10 ^{14}N nitrogens and has an average mass of 996, but when all the nitrogen is incorporated as ^{15}N, the average mass of the molecule raises to 1006 Da. The intracellular secondary metabolites of *M. aeruginosa* PCC7806 was also monitored for the period of 19 days. With the use of ^{15}N stable nitrogen as the sole source of nitrogen, the *de-novo* (newly synthesised) as well as the *ex-novo* (previously synthesised when growing in ^{14}N inoculum) intracellular metabolites could be distinguished. The organism produces several secondary metabolites such as microcystins (MC's), aerucyclamides and cyanopeptolins (Figure 3.2 – 3.6).

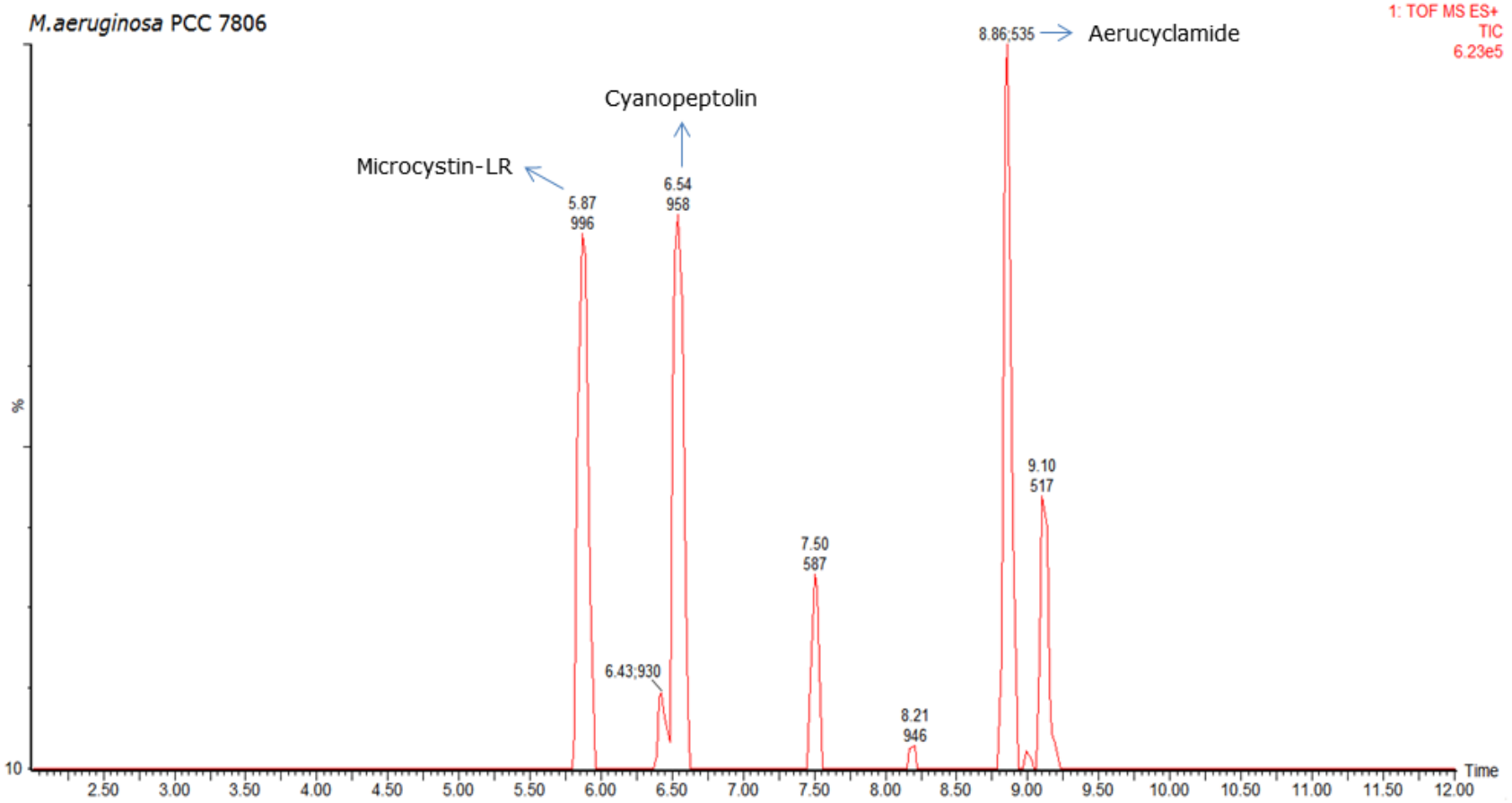


Figure 3.2: The HPLC-MS chromatogram of *M. aeruginosa* PCC7806 grown in BG11(^{14}N) extracted with 80% methanol and separated on BEH-C18 column (Waters®). The peak at time 5.8 min represents unlabelled (*ex-novo*) microcystin-LR (*m/z* 996), at 6.5 min represents cyanopeptolin A (*m/z* 958), at 8.8 mins represents aerucyclamide A (*m/z* 535). The smaller peak at time 6.4 mins is cyanopeptolin B (*m/z* 930), at 7.5 mins is aerucyclamide D (m/z 587) and at 9.1 mins is aerucyclamide c (*m/z* 517). This organism also produces demethyl microcystin-LR (*m/z* 982), but elutes a few seconds after microcystin-LR and could not be separated as a single peak.

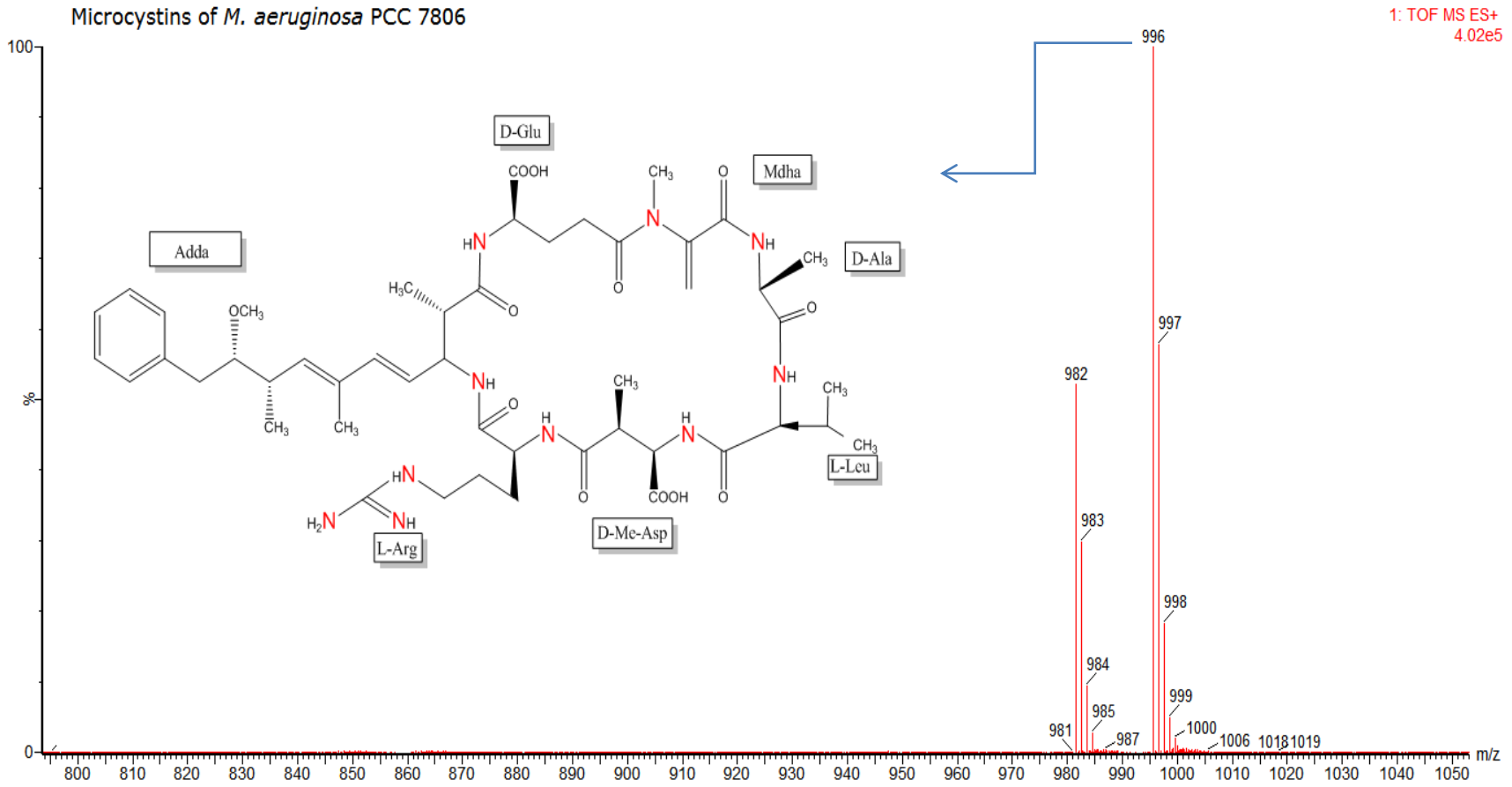


Figure 3.3: The mass spectrum of microcystins of *M. aeruginosa* PCC7806 as seen in the positive ion mode. The peak at *m/z* 996 is microcystin-LR (microcystin-LR) and the peak at *m/z* 982 is demethylated microcystin-LR. Microcystin-LR chemical formula $C_{49}H_{74}N_{10}O_{12}$, average mass = 995.18 Da. The position of nitrogen atoms are highlighted in red.

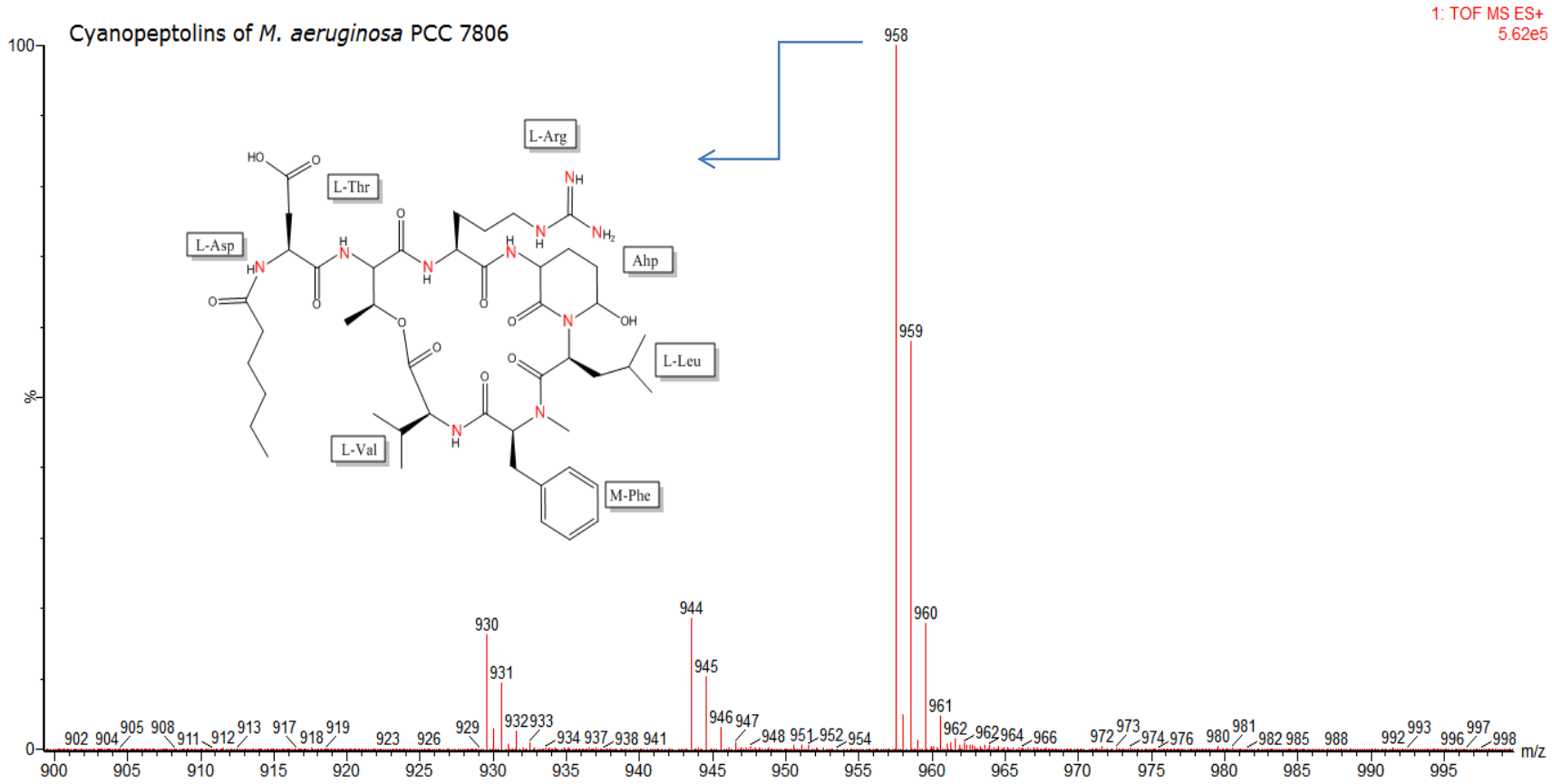


Figure 3.4: The mass spectrum of cyanopeptolins of *M. aeruginosa* PCC7806 as seen in the positive ion mode. The peak at *m/z* 958 is cyanopeptolin A, the peak at *m/z* 930 is cyanopeptolin B and the peak *m/z* 944 is cyanopeptolin C. Cyanopeptolin A chemical formula $C_{46}H_{72}N_{10}O_{12}$, average mass = 957.13 Da. The position of nitrogen atoms are highlighted in red.

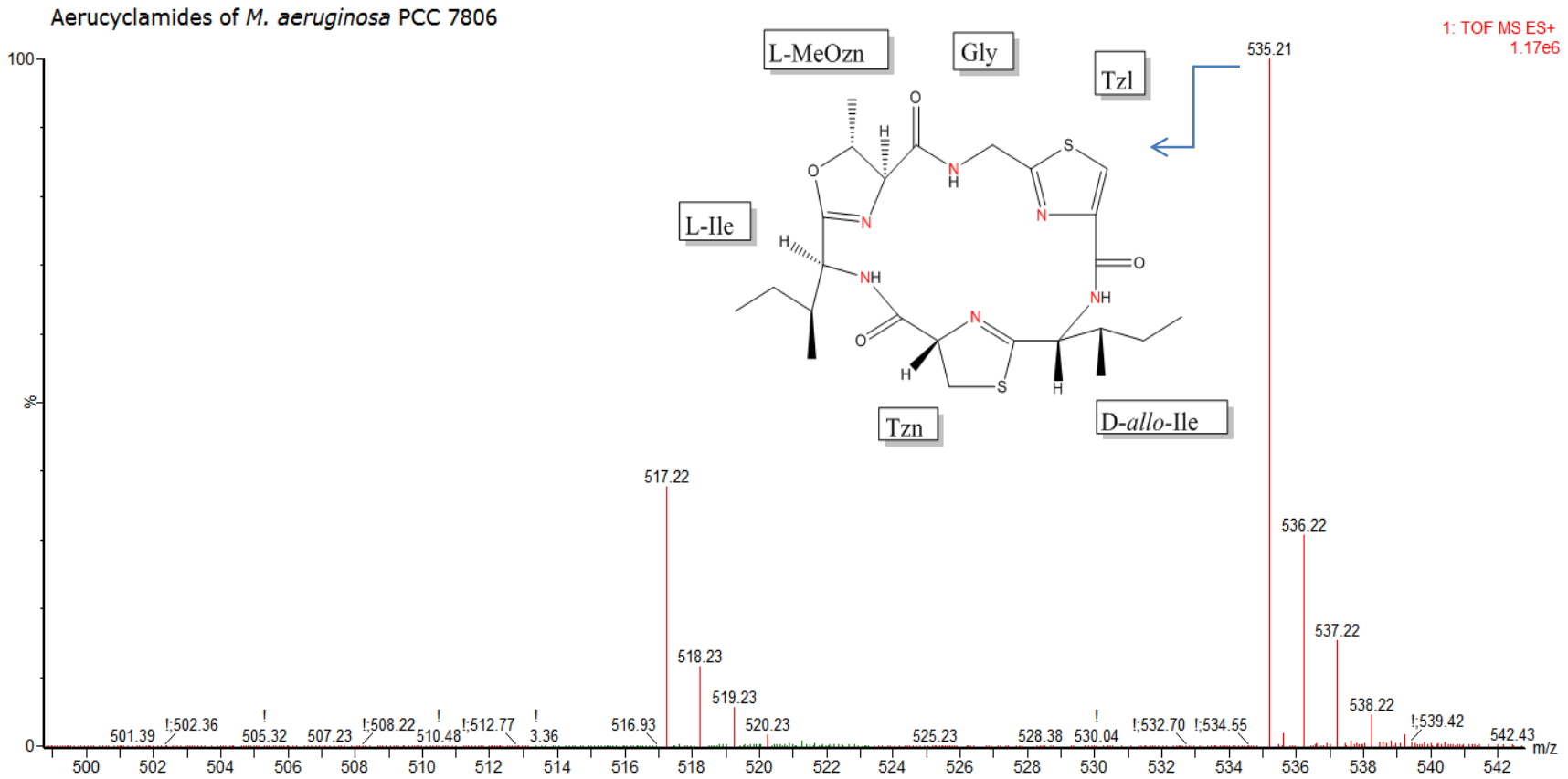


Figure 3.5: The mass spectrum of aerucyclamides of *M. aeruginosa* PCC7806 as seen in the positive ion mode. The peak at *m/z* 535 is aerucyclamide A, the peak at *m/z* 517 is aerucyclamide C. Aerucyclamide A chemical formula $C_{24}H_{34}N_6O_4S_2$, average mass = 535.21 Da. The position of nitrogen atoms are highlighted in red.

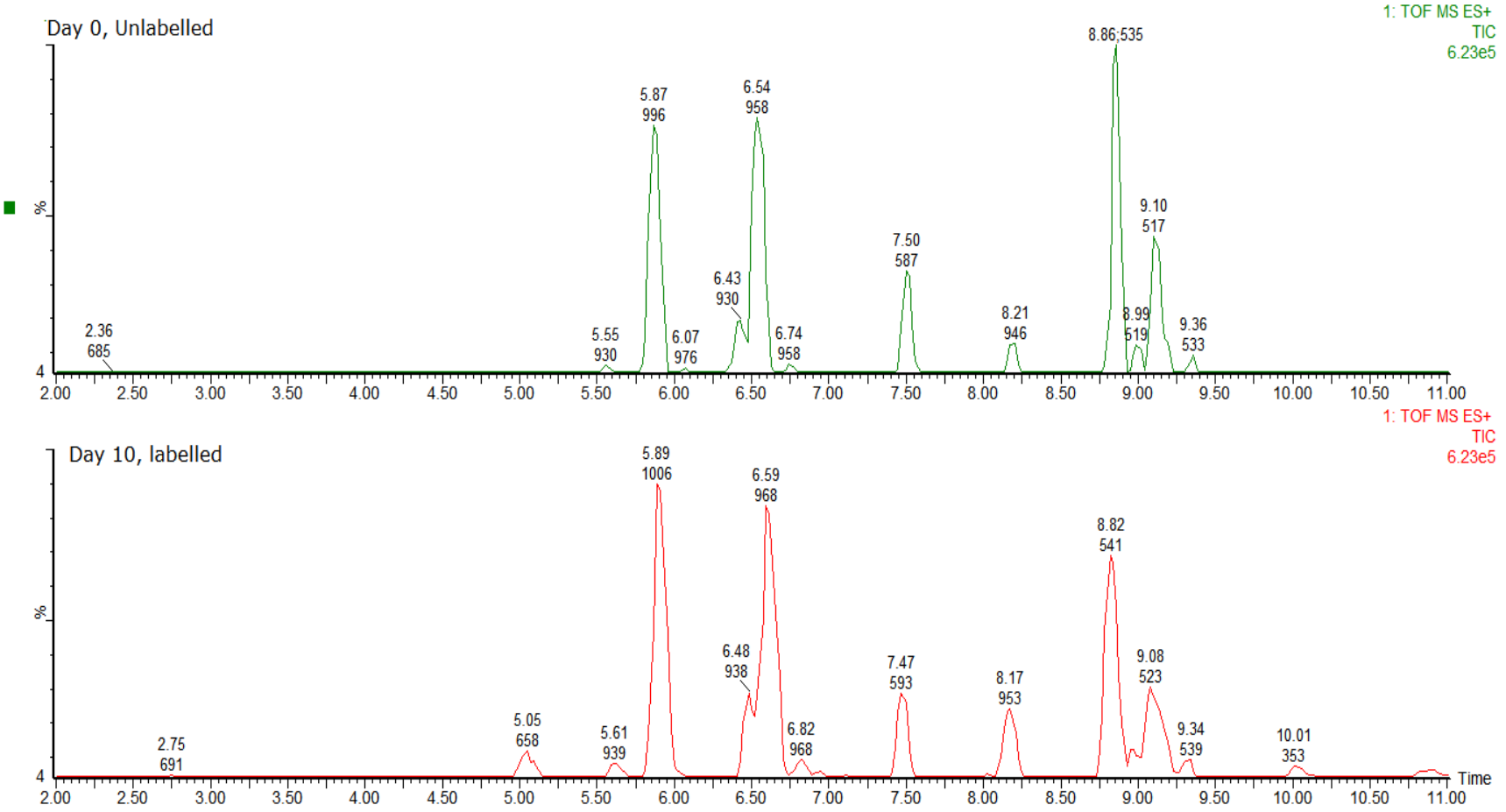


Figure 3.6: Comparison of HPLC-MS chromatogram of *M. aeruginosa* PCC7806 grown in ^{14}N and ^{15}N and extracted with 80% methanol. The peak at time 5.8 min represents microcystin-LR (*m/z* 996, unlabelled and m/z 1006, labelled), at 6.5 min epresents cyanopeptolin A (*m/z* 958, unlabelled and m/z 968 labelled), at 8.8 mins represents aerucyclamide A (*m/z* 535, unlabelled and m/z 541, labelled).

3.4.2 ESI-MS fragments of the secondary metabolites of *M. aeruginosa* PCC7806

In recent years, the combination of liquid chromatography (LC) with electron spray ionization mass spectrometry (ESI-MS) has become a robust tool for the detection of natural compounds [222]. ESI can either be positive or negative, but in this case the positive ion mode was used i.e. the spraying nozzle was kept at a positive potential. In positive ion mode, ion generation is achieved via transfer of protons and hence the ions thus generated are protonated molecules [223] and is represented by the general notation $[M + H]^+$. As a result, the molecule of interest is detected with 1 mass unit increase e.g. microcystin-LR of average mass 995.18 Da is detected on the mass detector as [995.18 + 1] = 996.18. An interesting feature of ESI-MS is the generation of multiple charged ions of large molecules, which yields additional information aiding in structure determination of an unknown molecule. In the case of microcystins, the formation of $[M + H]^+$ and $[M + 2H]^{2+}$ are widely reported [224].

Next, the generated ions collide against an inert gaseous target resulting in fragmentation of the molecule into product ions [225]. ESI-MS of microcystins results in the formation of multiple charged ions and the ratio of $[M + H]^+$: $[M + 2H]^{2+}$ is reported to be dependent on the presence of arginine residues within the molecule. In the case of microcystin-LR, which contains 1 arginine, the ratio is reported to be

around 100:11, whereas, in the case of microcystin-RR (containing 2 arginine) the ratio is 21:100 and in the case of microcystin-LA (containing 0 arginine) the ratio is 31:0 [224]. The ionic spectrum of a compound is thought to vary according to the CID-ESI-MS/MS conditions, yet certain characteristic ions present within a sample is indicative of the presence of a compound of interest. For example, the presence of the fragment ion *m/z* 135 indicates the presence of adda ($[PhCH_2CHOMe]^+$) which indicates that the sample may contain microcystins or nodularin while the presence of the ion *m/z* 498 indicates the presence of microcystin-LR [226, 227]. A list of the published fragment ions of microcystin-LR and demethyl-microcystin-LR is provided in Table 3.1, however, it should be noted that the *m/z* values shown for ^{15}N containing MC's are calculated values derived from the published data.

Table 3.1: Some of the published ion fragments of microcystin-LR and demethyl-microcystin-LR

	m/z-^{14}N	m/z-^{15}N	m/z-^{14}N	m/z-^{15}N	Ref
Ion Structure	microcystin-LR		demethyl-microcystin-LR		
$[M+H]^+$	996	1006	982	992	224, 228
$[PhCH_2CHOMe]^+$	135	135	-	-	224, 228, 229
[Glu-Mdha+H]$^+$	213	215	198	200	229, 230, 231
[Arg-MeAsp+H]$^+$	286	291	271	276	230, 231
[C11H15O-Glu-Mdha+H]$^+$ or Adda + (Glu or MeAsp) + Mdha + H+ ($-NH_3-134$ Adda)]	375	377	360	362	226, 229, 230
$[M + 2H]^{2+}$	498	503	491	496	232, 229, 231
[Mdha-Ala-Leu-MeAsp-Arg+H]$^+$ or Ala + Arg + (Glu or MeAsp) + Leu + Mdha + H]	553	561	538	546	226, 232, 231
[Mdha + Ala + Leu + MeAsp - Arg - NH2]	570	578	555	563	232, 230, 231
[MeAsp-Arg-Adda+H]+ or Adda + Arg + (Glu or MeAsp) + H^+]	599	605	584	590	226, 230
[M+2H-135]$^+$	861	871	847	857	228, 226, 231

The chromatographic peak representing microcystin-LR of *M. aeruginosa* PCC7806 (Figure 3.3, 3.7) actually consists of two major ions i.e. microcystin-LR (*m/z* 996) and its demethylated (Asp-3) congener demethyl-microcystin-LR (*m/z* 982). As a congener, both the microcystins showed similar fragmentation patterns. The mass spectrum also reveals that demethyl-microcystin-LR amounts to > 50 % of microcystin-LR, which is unusually high for *M. aeruginosa* PCC7806. It is thought that this might be an acquired characteristic of a culture being maintained for prolonged duration under light limiting conditions, however, this remains to be confirmed.

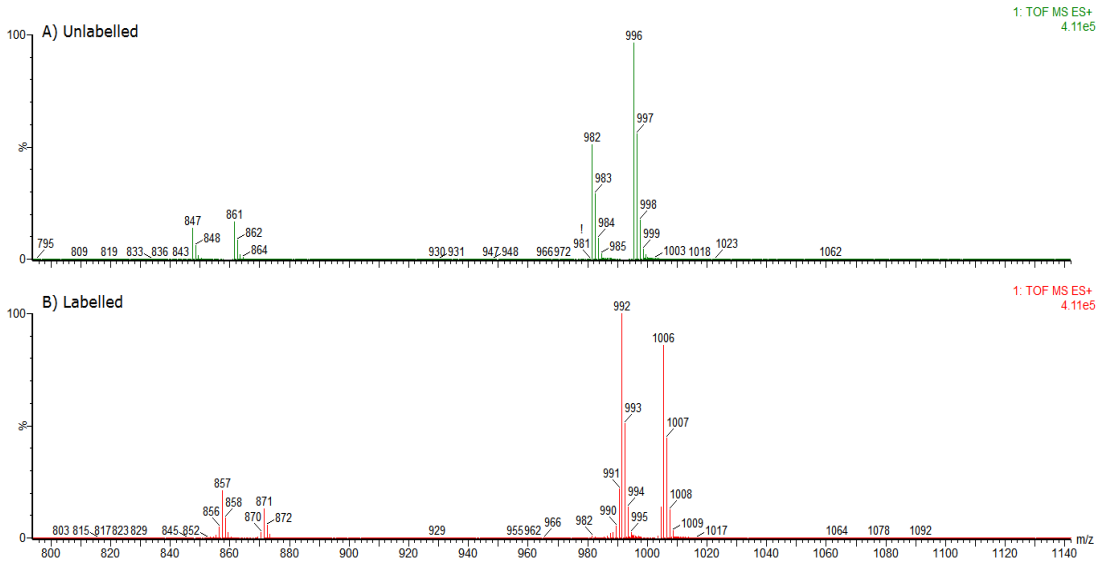


Figure 3.7: The mass spectra of the MC peak of *M. aeruginosa* PCC7806. A) Day 0, mass spectra when the organism was grown in ^{14}N replete media and B) Day 10, when grown in ^{15}N replete media. The ions at *m/z* 996 is unlabelled microcystin-LR and *m/z* 982 is unlabelled demethyl-microcystin-LR. The ion at m/z 1006 is fully labelled microcystin-LR and *m/z* 992 is labelled demethyl-microcystin-LR. The ions *m/z* 847, 861 and

857, 871 represent the adda cleaved products of their respective major ions.

Apart from the base peak at *m/z* 995.6, the peaks *m/z* 996.6, 997.6 and 998.6 represent microcystin-LR formed from the naturally occurring isotopes of C, H, N and O. Such isotopomers were observed for all the metabolites studied. Table 3.2 shows the relative abundance of fully ^{15}N labelled microcystin-LR isotopomers.

Table 3.2: Relative abundance of labelled microcystin-LR isotopomers arising from naturally occurring isotopes

m/z	Peak Area	Relative %
1005	1655	12.77
1006	12962	Base Peak
1007	5724	44.16
1008	1986	15.32
1009	608	4.69

Within the microcystin peak, the ion characteristic for microcystins i.e. *m/z* 135 could be detected. Relative to the major ions, the *m/z* 135 ion was present only at about 1%. It should be noted that the adda fragment does not contain any nitrogen atoms and hence could not be differentiated between *ex-novo* and *de-novo*. This characteristic ion was present in all of the samples.

The next major ion within the ESI-MS ion spectra was the ion with *m/z* 498 which is reported to be representative of $[M + 2H]^{2+}$, the doubly charged ion of microcystin-LR [224]. The relative abundance of $[M + 2H]^{2+}$ of each of the ions were calculated with regards to the protonated

ion $[M + H]^+$ and it revealed that for the *de-novo* produced microcystins it amounted to 7 to 20 %, which correlated with literature values of 11 to 45% (Table 3.3) [224, 226, 231].

Table 3.3: The relative abundance of $[M + H]^+$ and $[M + 2H]^{2+}$ ions in the ESI mass spectra of microcystin. The ion abundance (%) was normalized to the base peak (100%) ion. Sample = Day 10, intracellular extracted with 80% MeOH

Ion	^{14}N microcystin-LR	^{14}N demethyl-microcystin-LR	^{15}N microcystin-LR	^{15}N demethyl-microcystin-LR
$[M + H]^+$	342 (100%)	383 (100%)	5437 (100%)	3763 (100%)
$[M + 2H]^{2+}$	92 (27%)	52 (14%)	1141 (20%)	278 (7%)
Values outside the brackets represent peak area and values within the brackets are relative abundance in percentage				

Along with the doubly charged ions, three other ions could be seen in relative high abundance to that of the doubly charged ions. Figure 3.8 shows the respective ions comparison between day 0 and day 10. It should be noted that at day 0, no *de-novo* MC's would be present whereas at day 10, the intracellular pool would predominantly be *de-novo* MC's and this would reflect in the *m/z* ratio of the respective ions. The ions with *m/z* 491 are reported to be the doubly charged $[M + 2H]^{2+}$ ion of demethyl-microcystin-LR ion while the ion *m/z* 498 corresponds to its microcystin-LR congener (Figure 3.8, 3.9). The ions with *m/z* 475 are reported to be the doubly charged demethyl-microcystin-LR ion of [M +

2H – MeOH]$^{2+}$ while the ion *m/z* 482 corresponds to its microcystin-LR congener [231].

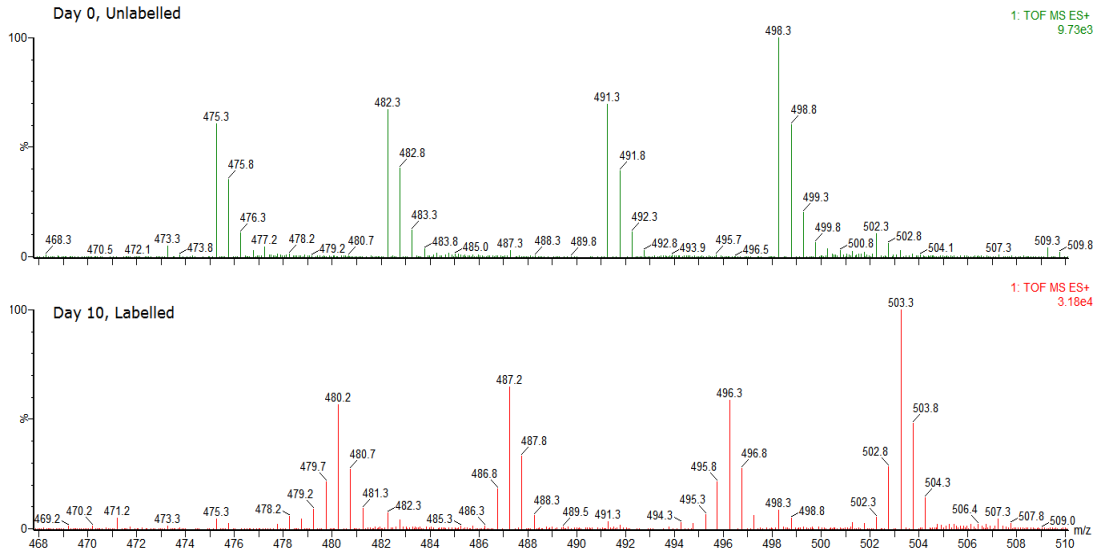


Figure 3.8: ESI-MS ion spectrum comparison between double charged ions between day 0 (unlabelled) vs day 10 (labelled). Ions *m/z* 498 and 491 represent [M + 2H]$^{2+}$ *ex-novo* microcystin-LR and demethyl-microcystin-LR, *m/z* 503 and 496 are *de-novo* microcystin-LR and demethyl-microcystin-LR. The ion *m/z* 482 is *ex-novo* [M + 2H - MeOH]$^{2+}$ of microcystin-LR and 475 is of demethyl-microcystin-LR. Its *de-novo* counterparts are *m/z* 487 and 480 respectively.

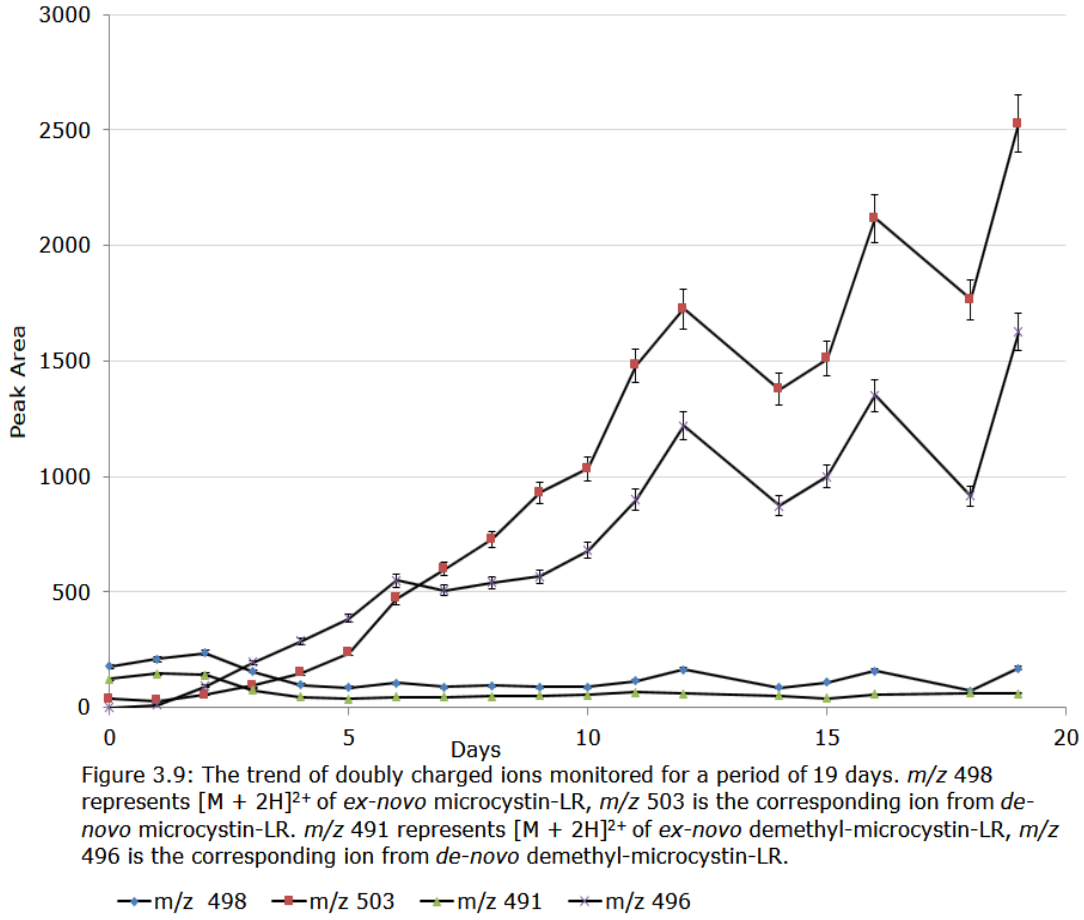


Figure 3.9: The trend of doubly charged ions monitored for a period of 19 days. *m/z* 498 represents $[M + 2H]^{2+}$ of *ex-novo* microcystin-LR, *m/z* 503 is the corresponding ion from *de-novo* microcystin-LR. *m/z* 491 represents $[M + 2H]^{2+}$ of *ex-novo* demethyl-microcystin-LR, *m/z* 496 is the corresponding ion from *de-novo* demethyl-microcystin-LR.

The next sets of major ions were the *m/z* 861 and 847, which are proposed to be the cleavage of adda fragment (*m/z* 135) from the respective doubly charged MC congeners [228]. The relative abundance of these cleavage products with respect to the parent ions are given in Table 3.4. It should be noted that the cleavage products are resultant of the CID of ESI-MS/MS process and do not occur naturally and hence, quantification of *de-novo* microcystins should accommodate these ions as well (Figure 3.10, 3.11).

Table 3.4: The relative abundance of $[M + H]^+$ and $[M + 2H -135]^{2+}$ ions in the ESI mass spectra of microcystin LR. The ion abundance (%) was normalized to the base peak (100%) ion. Sample = Day 10, intracellular extracted with 80% MeOH

Ion	^{14}N microcystin -LR	^{14}N demethyl-microcystin -LR	^{15}N microcystin -LR	^{15}N demethyl-microcystin -LR
$[M + H]^+$	342 (100%)	383 (100%)	5437 (100%)	3763 (100%)
$[M + 2H - 135]^{2+}$	68 (20%)	94 (25%)	871 (16%)	956 (25%)
Values outside the brackets represent peak area and values within the brackets are relative abundance in percentage				

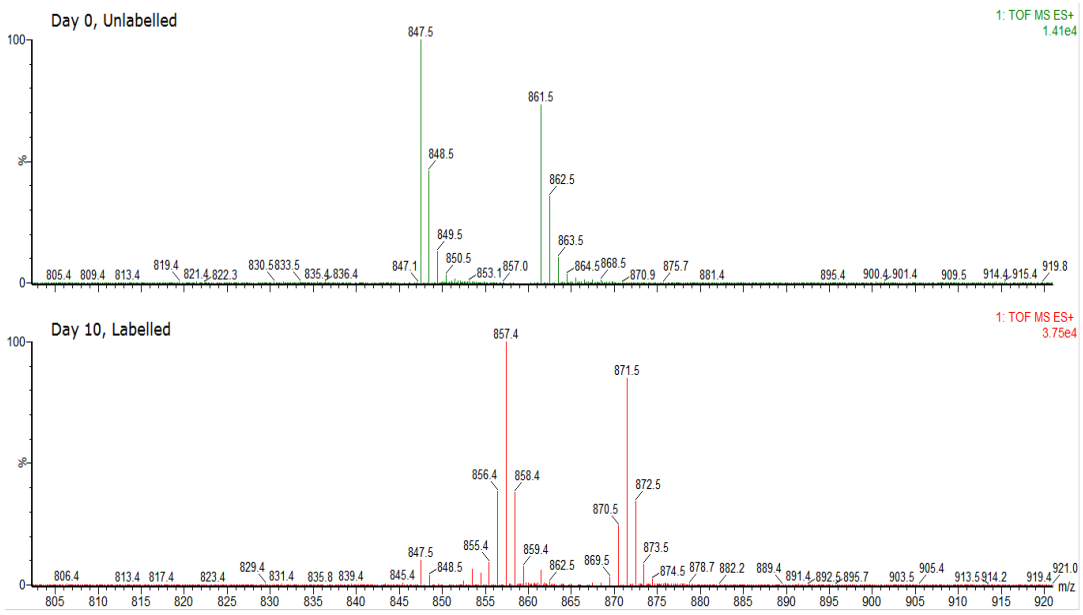


Figure 3.10: ESI-MS ion spectrum comparison between the adda fragment $[M + H - 135]^+$ cleaved ions of MC's between day 0 (unlabelled) vs day 10 (labelled).

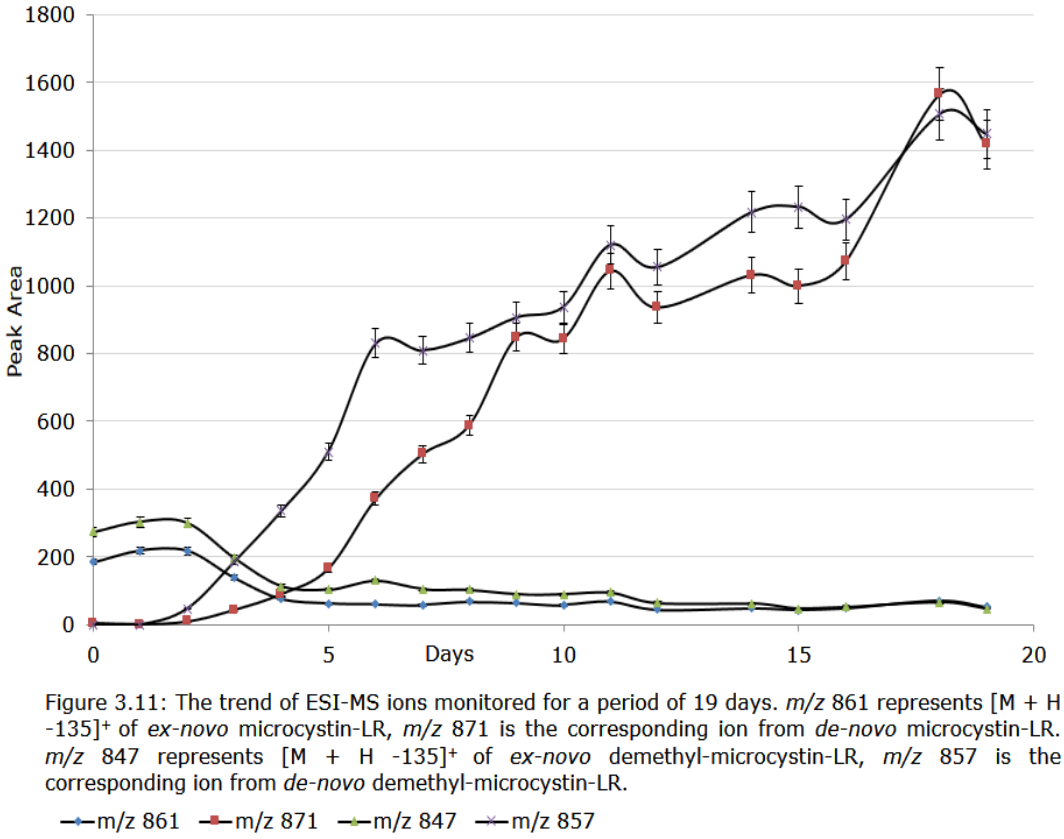


Figure 3.11: The trend of ESI-MS ions monitored for a period of 19 days. *m/z* 861 represents [M + H -135]$^+$ of *ex-novo* microcystin-LR, *m/z* 871 is the corresponding ion from *de-novo* microcystin-LR. *m/z* 847 represents [M + H -135]$^+$ of *ex-novo* demethyl-microcystin-LR, *m/z* 857 is the corresponding ion from *de-novo* demethyl-microcystin-LR.

3.4.3 Partial Incorporation of ^{15}N into microcystins

The aim of this study was to evaluate the rate at which ^{15}N is incorporated into *de-novo* microcystins. The experimental design was such that *M. aeruginosa* PCC7806 would be grown in BG11 media containing ^{14}N until mid-log following which the cells would be centrifuged, washed with nitrate free BG11 to remove traces of ^{14}N and resuspended in BG11 media containing ^{15}N. As microcystin-LR contains 10 nitrogen atoms, the number of ^{15}N that can go into the synthesis of

de-novo microcystin-LR can range between 0 and 10. At day 0, owing to the fact that the inoculum was grown in ^{14}N, only unlabelled microcystin-LR (m/z 995) could be detected with high relative abundance. The intracellular concentration of the unlabelled microcystin-LR remained mostly unchanged for the duration of the experiment (Figure 3.13). It could be stated that, microcystin-LR being an intracellular molecule is neither utilized nor is exported. In terms of daily biosynthesis, until day 2, no *de-novo* MC's could be detected, however, at day 3 the completely formed demethyl-microcystin-LR could be detected and in comparison 60 % less of microcystin-LR could also be detected. It is unclear if demethyl-microcystin-LR is synthesised from microcystin-LR by a demethylation process or vice-versa.

Since the cells contain some amount of ^{14}N (or partially synthesised ^{14}N containing aminoacids) and the media contains large quantities of ^{15}N, the microcystin-LR thus synthesised may contain nitrogen from either of the isotopes i.e. partially ^{15}N incorporated microcystin-LR. In order to study the abundance of partially ^{15}N incorporated microcystin-LR to that of fully ^{15}N incorporated microcystin-LR, the relative abundance of each of the possible $^{14}N/^{15}N$ combinations was quantified. As microcystin-LR contains 10 nitrogen, the peak areas between m/z 994 to m/z 1006 were considered (Figure 3.12), where m/z 996 would represent microcystin-LR formed with 10 ^{14}N and m/z 1006 would be all ^{15}N. As the natural abundance of the ^{15}N isotope is ~0.36%, the probability of synthesising a

microcystin-LR molecule containing one or more ^{15}N atoms in cells growing in the unlabelled media is very low.

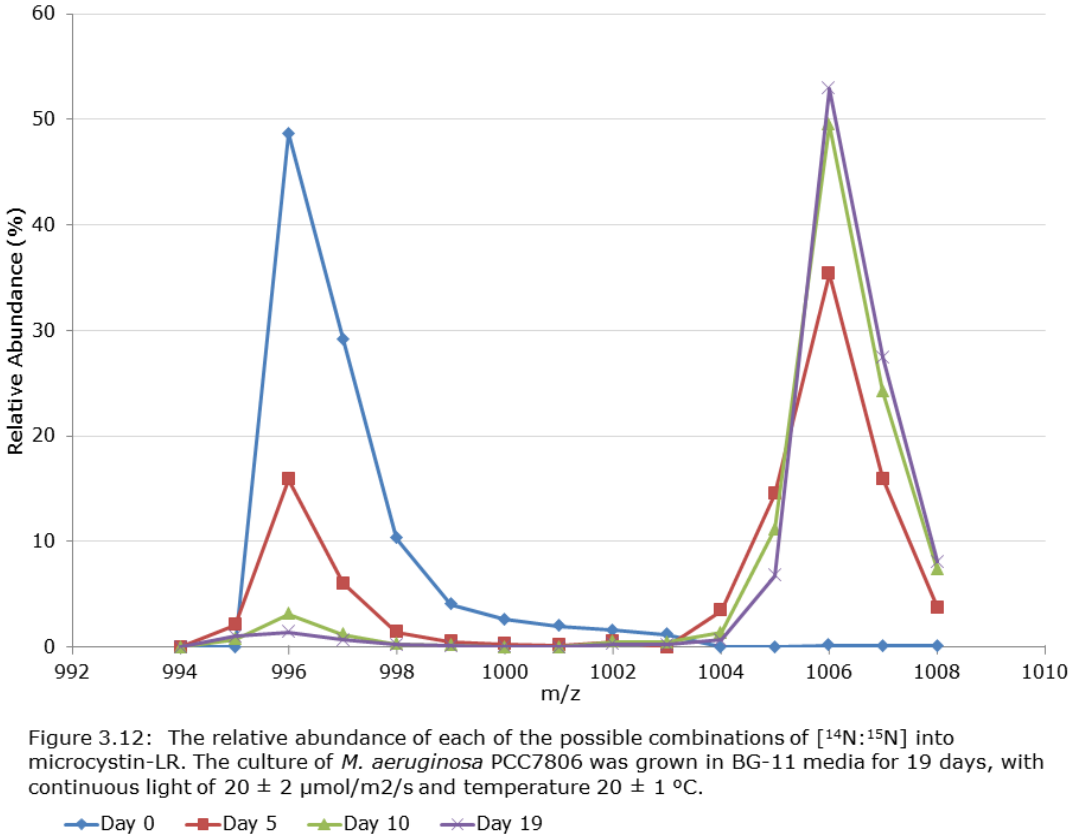


Figure 3.12: The relative abundance of each of the possible combinations of [^{14}N:^{15}N] into microcystin-LR. The culture of *M. aeruginosa* PCC7806 was grown in BG-11 media for 19 days, with continuous light of 20 ± 2 µmol/m2/s and temperature 20 ± 1 ºC.

The relative abundance of m/z 996 at day 0 was the highest, which reduced to 14 % by day 5 and continued to decline further on (Figure 3.12). At the same time, the relative abundance of m/z 1005 (containing 1 ^{14}N nitrogen and 9 ^{15}N nitrogen) was 9 % by day 3 and reached 13 % by day 5. This amount of m/z 1005 was stable until day 8 after which its concentration decreased to 9 % and was stable from there on. The concentration of m/z 1006 (10 ^{15}N) was negligible at day 0, but increased to 32 % by day 5 and reached 50 % by day 9 and remained stable henceforth. A previous work reported that the major ion at day 0 was m/z

995, but by day 18 the major ions had transformed to m/z 1004 [230]. In comparison, this data suggests that the major ions at day 0 m/z 996 while at day 19 it is m/z 1006. Although, the experimental and detection methodologies are very different, it may be stated that this data correlates well with published findings. The evidence presented here suggests that it takes at least 2 days for the formation of fully ^{15}N labelled microcystin-LR and that nitrogen is assimilated into microcystin-LR in order of its abundance in the media (Figure 3.13).

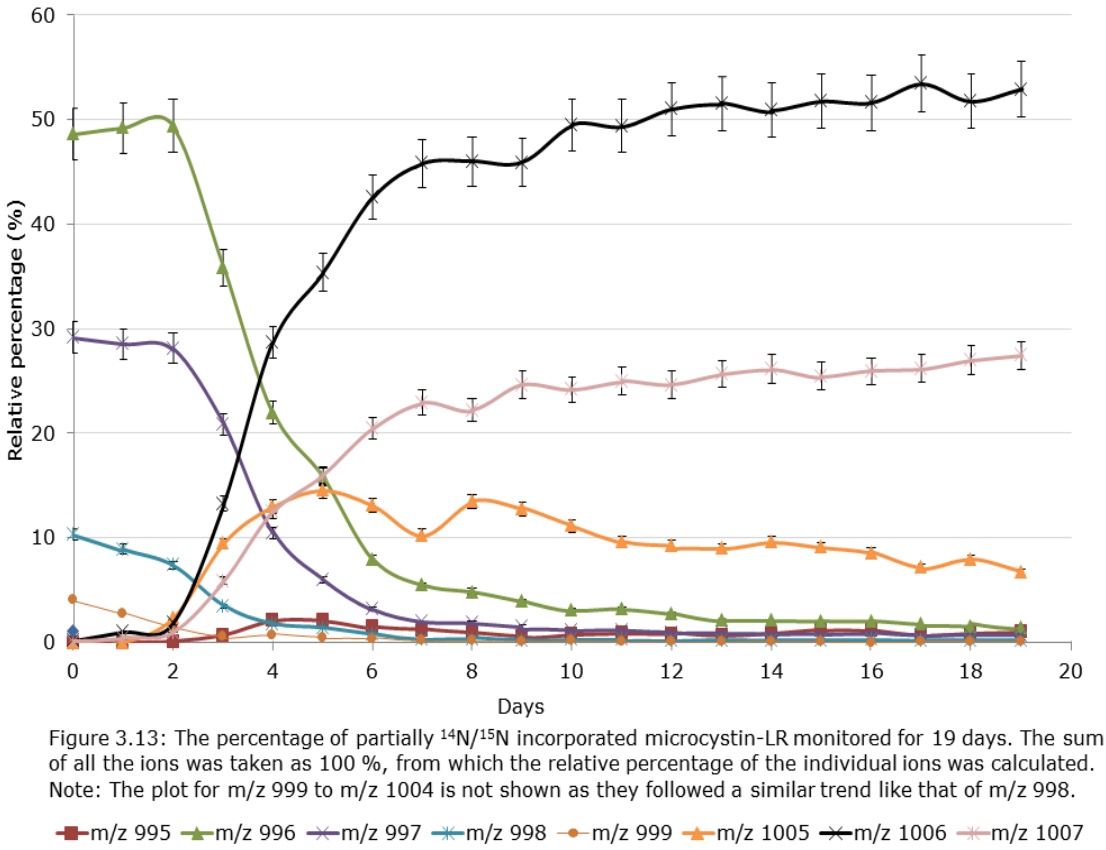


Figure 3.13: The percentage of partially $^{14}N/^{15}N$ incorporated microcystin-LR monitored for 19 days. The sum of all the ions was taken as 100 %, from which the relative percentage of the individual ions was calculated. Note: The plot for m/z 999 to m/z 1004 is not shown as they followed a similar trend like that of m/z 998.

The pool of all the ions between m/z 995 to m/z 1007 was analysed for the relative percentage of each of the individual ions. It revealed that

m/z 996 and m/z 997 were the predominant ions in the unlabelled pool whereas m/z 1006 and m/z 1007 were the predominant ions in the labelled pool. All of the other ions were present in low concentrations only i.e. less than 10 % only. It was also observed that it takes 2 days for complete ^{15}N labelling of microcystin-LR (Figure 3.13). Although partial incorporation studies on microcystin-LR is rare, some studies have investigated incorporation of ^{15}N into metabolites such as jamaicamides, curazole, carmabin A, apratoxin A and hectochlorin have reported that it takes 10 days for complete incorporation to occur [212]. Interestingly, a study on partial incorporation of ^{15}N into chlorophyll determined that complete incorporation occurs in about 2.08 days [213]. It should be noted that unlike secondary metabolites, chlorophyll is innately related to the metabolism of the organism and hence may explain its faster turnover rate. Overall, it may be stated that complete labelling of secondary metabolites takes a longer time period than labelling of primary metabolites and in the case of microcystin-LR, it takes 2 days for complete labelling.

3.4.4 Partial incorporation of ^{15}N into Cyanopeptolin

M. aeruginosa PCC7806 is reported to produce several cyanopeptolins such as cyanopeptolin A, B, C and D and 963A [201, 163]. Compared to microcystins, literature on cyanopeptolins is rather limited, but their anti-protease activity may lead to extensive studies in the

future. Cyanopeptolins are cyclic peptides containing 7 to 9 amino acids, one of which is variable. The L-glutamic acid aldehyde and the amino group of L-leucine form an unusual 3-amino-6-hydroxy-2-oxo-l-piperidine system (Aph) (Table 3.5).

Table 3.5: Cyanopeptolins from *M. aeruginosa* PCC7806

Amino acid	Cyanopeptolin variants				
	A	B	C	D	963A
L-Arginine	+				
L-Lysine		+			
N-Methyl-L-lysine			+		
N,N-Dimethyl-L-lysine				+	
Tyrosine					+
L- Leucine	+	+	+	+	+
N-Methyl-L-phenylalanine	+	+	+	+	+
L-Valine	+	+	+	+	+
L-Threonine	+	+	+	+	+
L-Aspartic acid	+	+	+	+	+
Hexanoic acid	+	+	+	+	+
Chemical Formula	$C_{46}H_{72}N_{10}O_{12}$	$C_{46}H_{72}N_8O_{12}$	$C_{47}H_{74}N_8O_{12}$	$C_{48}H_{76}N_8O_{12}$	$C_{49}H_{69}N_7O_{13}$
m/z [M + H]+ (^{14}N)	958.14	930.13	944.15	958.18	965.13
m/z [M + H]+ (^{15}N)	968.14	938.13	952.15	966.18	972.13

Under the conditions of the UPLC-MS used, the chromatogram of *M. aeruginosa* PCC7806 revealed that cyanopeptolins eluted after microcystin-LR at about 6.3 mins, which may indicate that cyanopeptolins are comparatively less polar than microcystin-LR. Of the 5 cyanopeptolins reported, only cyanopeptolin A peak was predominant, however, the ion spectrum revealed that cyanopeptolin A and D co-eluted (Figure 3.14, 3.15).

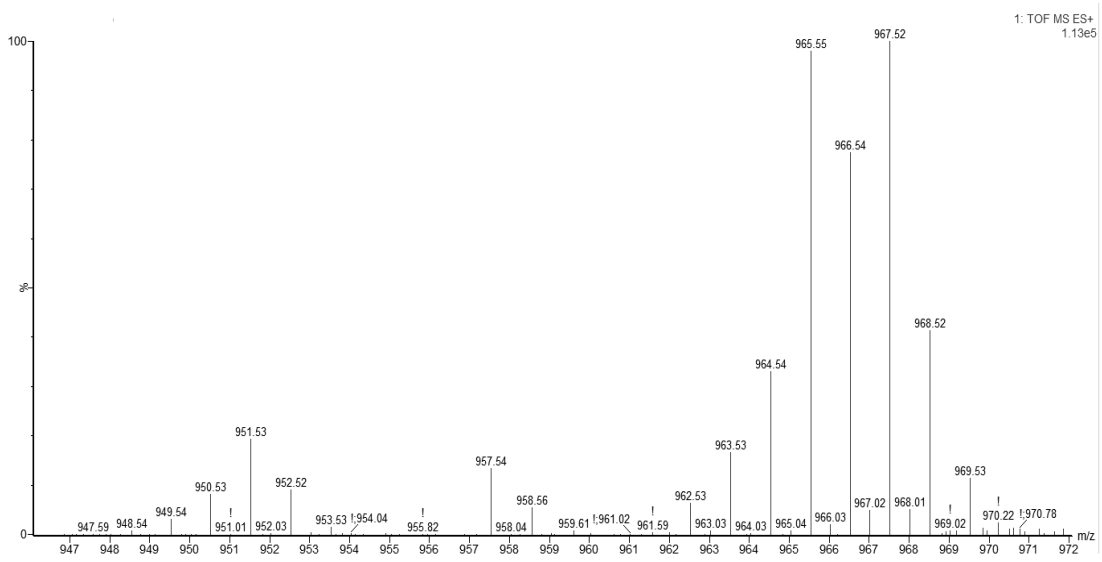


Figure 3.14: The positive ESI ion spectra of the ions of cyanopeptolin peak at time 6.3 mins. The ion m/z 965.55 represents ^{15}N labelled cyanopeptolin D (Ret time 6.33 min) while the ion m/z 967.52 represents cyanopeptolin A (Ret Time 6.31 mins).

As the mass spectra of both cyanopeptolin A and D overlapped, it was impossible to differentiate their corresponding peaks. Also, a literature search for the fragmentation pattern of cyanopeptolin A or D

was unfruitful. The low energy ion spectra of cyanopeptolin A and D revealed several major peaks at m/z 474, m/z 475, probably representing doubly charged ions $[M - CH_3 + H]^{2+}$ of both the molecules respectively (Figure 3.14). A more detailed analysis of the fragmentation pattern of cyanopeptolins may need to be undertaken in the future to predict the composition of the observed fragments.

For the above stated reasons, cyanopeptolin A and D were excluded for the ^{15}N partial incorporation analysis, while cyanopeptolin B (m/z 929) seemed to be an ideal candidate. Cyanopeptolin B contains 8 nitrogen atoms and hence the possibility of ^{15}N incorporating within it range from 0 to 8. Hence, the peak areas between m/z 929 to 937 were obtained and the relative ions percentage was calculated (Figure 3.15). The peak at m/z 930 represented $[M + H]^+$ of unlabelled molecule whereas the peak at m/z 938 represented $[M + H]^+$ of fully labelled (^{15}N) molecule. At day 0, the unlabelled peak showed the highest intensity, but by day 3 had reduced to less than 20 % of the ion pool, which reduced to below 10 % by day 4 and continued to decline. At the same time, the relative abundance of m/z 937 (containing 1 ^{14}N nitrogen and 7 ^{15}N nitrogen) was 18 % by day 3 and remained steady henceforth. This concentration of m/z 938 ($^{15}N_8$) was 6% at day 2, rapidly increasing to 25% by day 3 and reached 40% by day 5. Interestingly, the percentage of m/z 939, synthesised out of the naturally occurring stable isotopes of C or O, followed a similar trend to that of m/z 937. Overall, it may be stated that the partial ^{15}N incorporation of cyanopeptolins and microcystin-LR are

similar, but like microcystin-LR it takes about 2 days for the complete ^{15}N labelling of cyanopeptolin A.

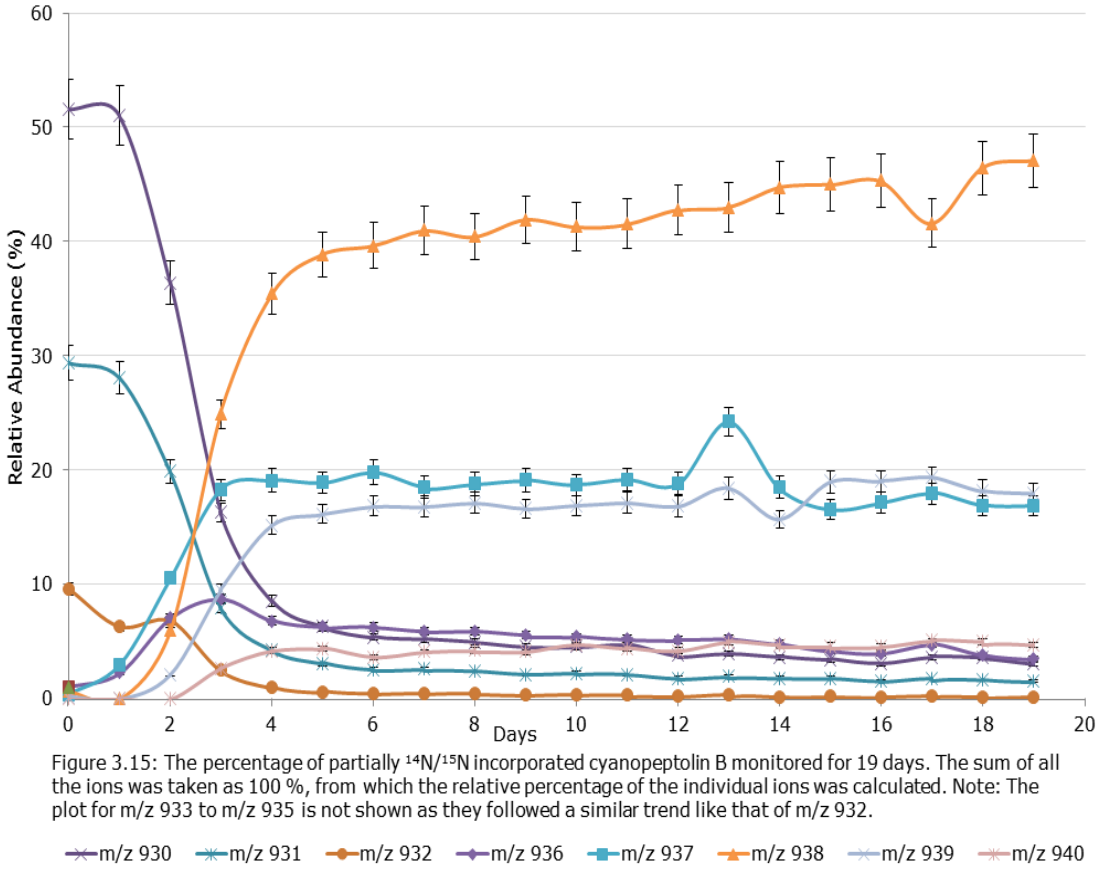


Figure 3.15: The percentage of partially $^{14}N/^{15}N$ incorporated cyanopeptolin B monitored for 19 days. The sum of all the ions was taken as 100 %, from which the relative percentage of the individual ions was calculated. Note: The plot for m/z 933 to m/z 935 is not shown as they followed a similar trend like that of m/z 932.

3.4.5 Partial incorporation of ^{15}N into aerucyclamides

M. aeruginosa PCC7806 also produces aerucyclamides A to D, but unlike other secondary metabolites aerucyclamides are ribosomally synthesised [90, 162]. Here again, a literature search for the fragmentation pattern of aerucyclamides was unfruitful, however, the structure and amino acid composition of all the aerucyclamides of *M. aeruginosa* PCC7806 has been predicted (Table 3.6). Aerucyclamides

differ from the other secondary metabolites studied here, as they are sulphur containing secondary metabolites.

Table 3.6: Aerucyclamides from *M. aeruginosa* PCC7806

Amino acid	Aerucyclamide variants			
	A	B	C	D
D-allo-Isoleucine	+	+		
Glycine	+	+		+
L-Threonine	O-acyl	O-acyl	+	+
L-Methyloxazoline		+	+	+
Thiazoline	+			+
Thiazole	+	+	+	+
L-Isoleucine	+	+	+	
Oxazole			+	
L-Alanine			+	
L-Valine			+	
Phenylalanine				+
Chemical Formula	$C_{24}H_{34}N_6O_4S_2$	$C_{24}H_{33}N_6O_4S_2$	$C_{24}H_{32}N_6O_5S$	$C_{26}H_{31}N_6O_4S_3$
m/z [M + H]+ (^{14}N)	535.71	533.70	517.62	588.77
m/z [M + H]+ (^{15}N)	541.71	539.70	523.62	594.77

The UPLC chromatogram of the methanolic extract of *M. aeruginosa* PCC7806 revealed that this organism produces about 50% more of aerucyclamide A when compared to microcystin-LR (Figure 3.16) and for this reason aerucyclamide A was chosen for partial incorporation of $^{14}N/^{15}N$ analysis. The positive ion spectra of aerucyclamide A did not reveal any significant fragments except for the base peak (Figure 3.17).

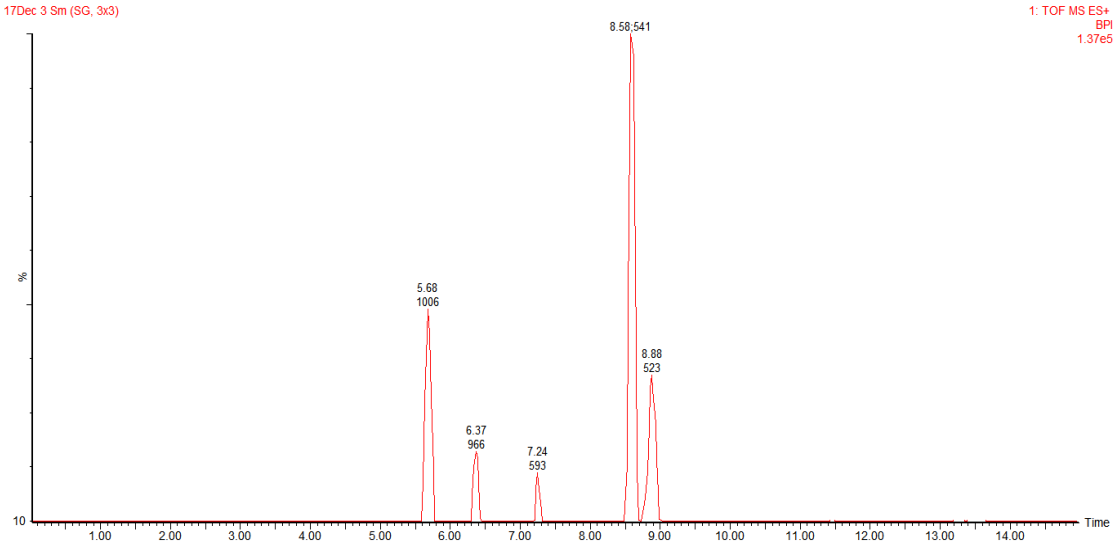


Figure 3.16: The BPI (base peak intensity) chromatogram of *M. aeruginosa* PCC7806 methanolic extracts. The aerucyclamides elute after microcystin-LR and hence can be regarded as more hydrophobic than microcystin-LR. The peak m/z 593 represents ^{15}N labelled aerucyclamide D, m/z 541 is aerucyclamide A and m/z 523 is aerucyclamide C.

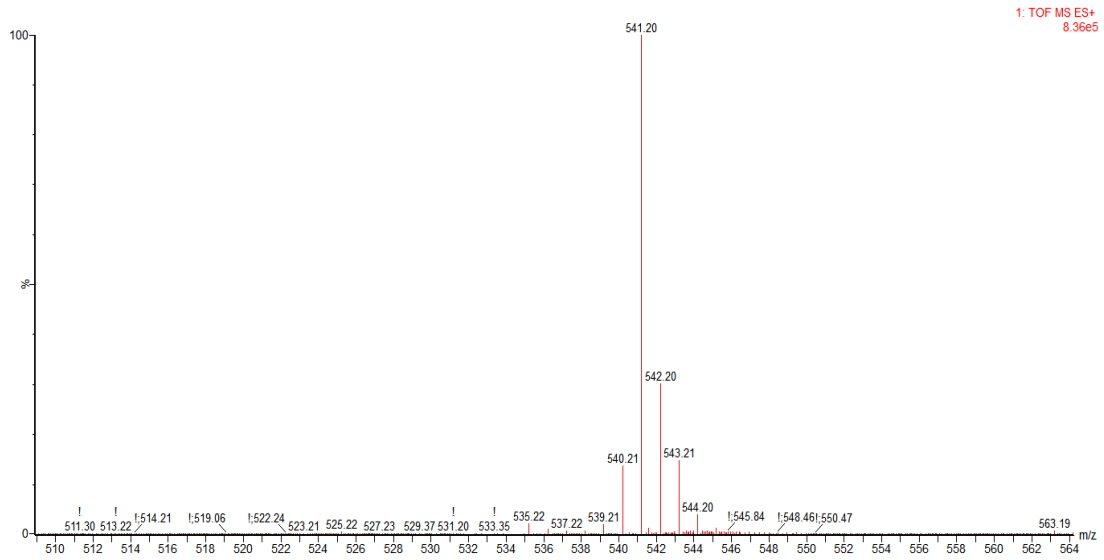


Figure 3.17: The positive ESI-MS of labelled aerucyclamide A m/z 541.

Aerucyclamide A contains 6 nitrogen, and during growth, the probability of ^{15}N incorporating within may range from 0 to 6. Hence, the peak areas between m/z 535 to 541 were obtained and the relative ions percentage was calculated (Figure 3.18). The peak at m/z 535 represented $[M + H]^+$ of unlabelled molecule whereas the peak at m/z 541 represented $[M + H]^+$ of fully labelled (^{15}N) molecule. At day 0, the unlabelled peak showed the highest intensity, but by day 3 had reduced to 30% of the ion pool, which further reduced to below 20% by day 4 and continued to decline further. At the same time, the relative abundance of m/z 536 (containing 1 ^{14}N nitrogen and 5 ^{15}N nitrogen) was 12% by day 3 and steadily declined henceforth. This concentration of m/z 541 ($^{15}N_6$) was 9% at day 2, rapidly increasing to 30% by day 4 and reached 40% by day 5. By day 10, the fully labelled molecule concentration had

reached 50% of the ion pool and remained steady henceforth. Interestingly, the percentage of m/z 542, synthesised out of the naturally occurring stable isotopes of C or O, followed a similar trend to that of cyanopeptolin B, but only reached a maximum of 15%. Overall, it may be stated that the partial ^{15}N incorporation of aerucyclamides and microcystin-LR are similar and that both the molecules take 2 days for complete ^{15}N labelling.

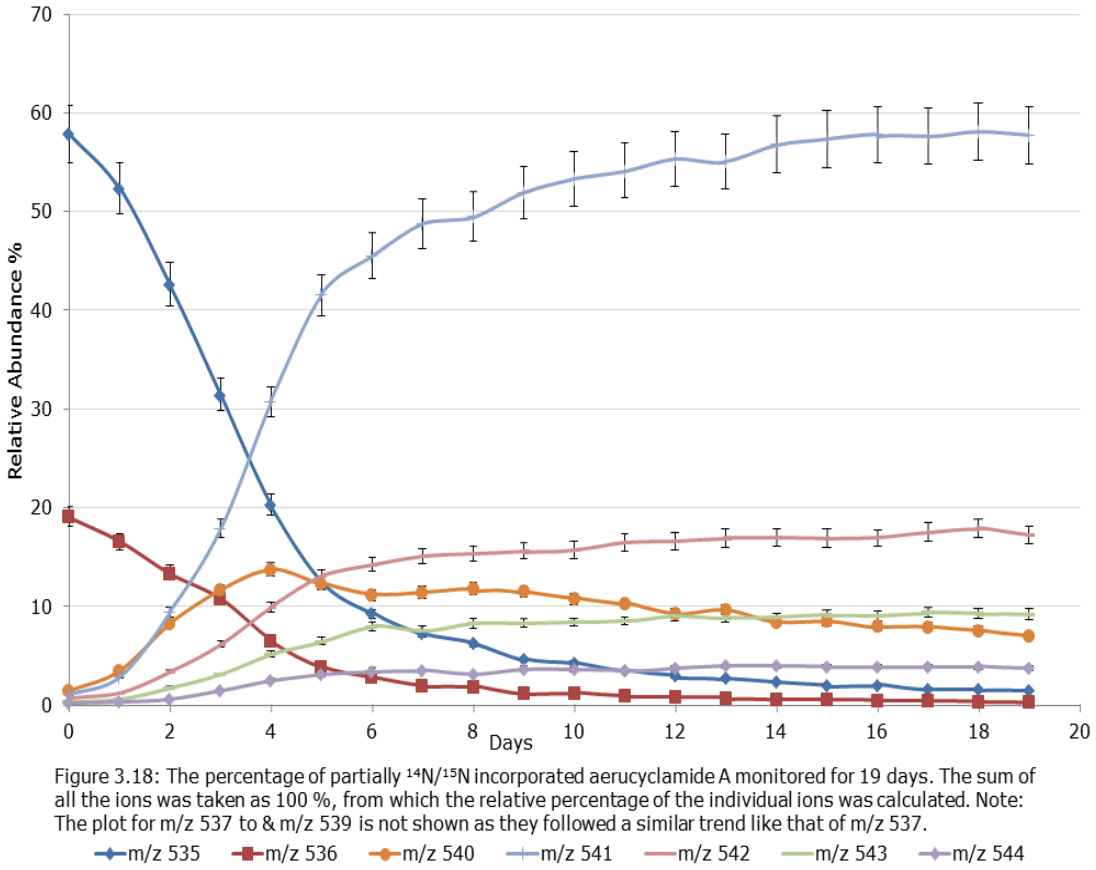


Figure 3.18: The percentage of partially $^{14}N/^{15}N$ incorporated aerucyclamide A monitored for 19 days. The sum of all the ions was taken as 100 %, from which the relative percentage of the individual ions was calculated. Note: The plot for m/z 537 to & m/z 539 is not shown as they followed a similar trend like that of m/z 537.

3.5 Discussion

This study tried to understand the growth and secondary metabolite production characteristics of the freshwater toxic organism *M. aeruginosa* PCC7806. In the environment, elevated water temperature, low photon irradiance, high water-column stability, intense zooplankton grazing on competing species, high pH, and low nitrogen/phosphorus ratio are generally the most important factors stimulating the growth of harmful cyanobacteria [234]. It has been reported that nitrate (NO_3) concentrations may vary between negligible amounts to 142.8 μM and total phosphorous may range between 1.61 to 2.91 μM. Light intensities typically would be in the range of 200 to 500 μmols/m^2/s, however it is known that *Microcystis* sp may avoid high light intensities by decreasing its buoyancy. Temperatures are also largely variable, but it is reported that temperatures of about 21 ± 2 °C are favourable for bloom formation [235 - 240]. Compared to real life conditions, the parameters used in laboratory experiments are designed to provide ample nutrients and growth conditions for the growth of *M. aeruginosa* PCC7806. In the conditions used here, it appears that the organism multiplies at a rate comparable to literature reported values. The conditions used in this study would be considered as mild when compared to environmental conditions. However, it is well known that conditions such as water turbulence, UV exposure, grazer population, cyanophages, nutrients are largely variable in the environment and *M. aeruginosa* PCC7806, by virtue of its gas vesicles is well capable of adapting to changing environments

[241]. In terms of growth characteristics, a direct comparison could not be made with other published studies, firstly because a consistent culture method (inoculum age, duration, light cycles, nutrient concentration) were largely variable and secondly this is the first study where *M. aeruginosa* PCC 7806 has been grown with ^{15}N as its sole source of nitrogen. Moreover, the aim of this study was only to elucidate partial incorporation patterns of ^{15}N into its secondary metabolites.

This study tried to elucidate the secondary metabolites produced by *M. aeruginosa* PCC7806 using LS-MS/MS. According to literature, this organism produces several secondary metabolites such as microcystin-LR, demethyl-microcystin-LR, cyanopeptolins A to D, 963A and aerucyclamides A to D. In the HPLC protocol used here, it was observed that microcystin-LR, cyanopeptolin D, aerucyclamides A, C and D were the predominant peaks. The BPI of the chromatogram reveals that aerucyclamide A was present in the highest concentration followed by microcystin-LR at about 50% of the BPI. Cyanopeptolin D, aerucyclamides C and D were present in less than 20% concentration in the BPI picture. It was also observed that microcystin-LR and demethyl-microcystin-LR peaks could not be resolved, as were cyanopeptolin A and D. The peaks for cyanopeptolins B, C and 963A were not observable in the BPI picture and neither was aerucyclamide B (Figure 3.16).

In terms of ion fragmentation, microcystin-LR and demethyl-microcystin-LR showed several well documented ions such as $[M + 2H]^{2+}$

and $[M + 2H - 135]^{2+}$. Also, the adda fragment m/z 135 could be observed. As for cyanopeptolins, the ion fragments m/z 474 and m/z 475 were observed, however, their composition could not be readily confirmed. In contrast none of the aerucyclamides showed any fragmentations.

In terms of partial incorporation, it seemed that irrespective of the isotopicity, *M. aeruginosa* PCC7806 seem to assimilate the available nitrogen into its molecules for the biosynthesis of its secondary metabolites. In fact, it was observed that the relative abundance of the unlabelled molecules decreased owing only to the lack of ^{14}N in the media. In terms of the time taken for *M. aeruginosa* PCC7806 to assemble fully ^{15}N incorporated metabolites, this study indicates that it takes about two days for complete biosynthesis of microcystin-LR (probably demethyl-microcystin-LR as well), aerucyclamides A and cyanopeptolin B.

It was observed that the growth rate of *M. aeruginosa* PCC7806 ranges between 0.3 to 0.7 per day and the doubling time ranged between 1.5 to 3.5 days even within the logarithmic growth phase. When the growth rate was compared to the secondary metabolite production rate, a linear correlation for all of the major secondary metabolites such as microcystin-LR, cyanopeptolin and aerucyclamide was observed. It was also noted that even when the growth rate was at its lowest, a small amount of all of the mentioned secondary metabolites was continuously produced.

Overall, it could be stated that this study has highlighted some interesting features of secondary metabolite production of *M. aeruginosa* PCC7806. While studying cyanobacteria, it is also imperative to closely monitor cellular metabolic activity. In this view, it is also suggested that future studies monitor the secondary metabolites production as well as one other molecule that closely correlates cellular activity. Chlorophyll-a (or its breakdown product pheophytin-a, $C_{55}H_{74}N_4O_5$, m/z 871.2) seems to be an ideal candidate and has been successfully studied using the same ^{15}N (and HPLC-MS) methodology. As chlorophyll-a is a molecule that is actively synthesised and degraded continuously, it may serve as an indicator of cellular metabolic activity when coupled with labelling studies [213, 233].

Chapter IV

Evaluation of microcystin-LR as a signalling molecule

4.1 Introduction

Cyanobacteria produce a diverse range of secondary metabolites which include small peptides, polyketides and alkaloids [242]. When optimal conditions of sunlight, temperature, nutrients (nitrates, phosphates) and low turbulence occur, cyanobacteria rapidly multiply and form blooms [114]. Although factors such as nutrient concentrations and low grazing populations of zooplanktons are advantageous, no single factor has been directly attributed to bloom formation. It is thought that increased anthropogenic eutrophication of water bodies in recent years enhanced by global warming may be one of the leading causes to the increased occurrences of blooms [243 - 245]. During late exponential growth phase and bloom collapse, the intracellularly produced secondary metabolites are released into the ecosystem. It has been reported that up to 75% of the blooms could be secreting secondary metabolites that may be toxic to the environment [122].

The range of secondary metabolites produced by cyanobacteria has drawn much scientific interest in recent years. It is reported that cyanobacteria produce about 800 different secondary metabolites, whose activities range from hepatotoxic to neurotoxic to antibacterial, antifungal and enzyme inhibitory activities [247]. Although cyanobacterial secondary metabolites have been widely studied for their potential applications in commercial as well as pharmaceutical applications, the key question as to why cyanobacteria produce such a wide array of metabolites remains

unanswered. Only in recent years, it is thought that interspecies communication i.e., allelopathy may be one of the reasons.

Allelopathy in cyanobacteria has been reported as early as 1948, however intensive research in this field began only in the 1980s [123]. Several cyanobacterial secondary metabolites have been classed as allelochemicals based on the properties these compounds exhibit on other cohabiting organisms. One of the first allelochemicals to be studied was cyanobacterin from *Scytonema hofmanii* which was reported to be specifically active against algae but did not have any significant effect on non-photosynthetic organisms [122]. Later, Moore *et al.,* [124] reported the isolation of 18 hapalindoles from *Hapalosiphon fontinalis* which were either antibacterial or antimycotic in nature. Following this discovery, the search for allelochemicals continues and the compounds calothrixin A and fisherellin are some well known allelochemicals [137, 138].

The organism *Microcystis* has been studied for several decades as it is one of the most common bloom forming cyanobacteria [248] as well as its characteristic hepatotoxin microcystins (MC's) produced by some species. MC's are a group of heptapeptides containing a unique amino acid Adda along with a conserved structure of D-Ala-L-X-D-MeAsp-L-Y-Adda-D-Glu-Mdha. Most of the variants of MC's arise from the variable L-amino acids at position X and Y. MC's are unusual in their biosynthetic pathway involving a mixed Non Ribosomal Peptide Synthesis (NRPS) /Polyketide Synthetase (PKS) enzyme complex [176]. In spite of the

complex nature of MC's and its synthesis, MC's do not seem to play a vital role in the host organism's survival or play a significant role in the immediate habitat of the host. Some functions, such as heavy metal chelation [99] or dissuasion of grazers have been attributed to microcystins although none of these functions have been conclusively proven [151].

As MC's are secondary metabolites (which are not directly involved in the host organism's metabolism), it is thought that MC's may have an allelochemical role. Recently, Schatz *et al.,* [127] reported that the exposure of *Microcystis* cells to MC, micropeptin or microginin (from lysates of *Microcystis* cells) resulted in significant increase in the accumulation of McyB (gene product of *mcyB* of the microcystin operon) as well as MC. With this evidence, the authors claim that these metabolites may have a signalling role. However, the authors fail to convincingly explain how the overproduction of microcystin by the host organism benefits its fitness or its survival.

In 2005, Hu *et al.,* [249] reported that the presence of exogenous microcystin inhibited the growth of *Synechococcus elongates* while significantly increasing its glutathione as well as glutathione peroxidase activities. They concluded that exogenous microcystin induced significant oxidative stress on target organisms which in turn responded by up regulating antioxidative measures. However, the concentration of 100 ng/ml of exogenous microcystin used in the study would be considered

high when compared to ecological scenarios and hence allelopathic interactions between the organisms may not be relevant in nature. Also, from the study it could not be concluded if the induction of oxidative stress is a direct effect of exogenous microcystin on the organism or a nonspecific effect of MC's localising within the thylakoids of the target organism [249].

A further study by Babica *et al.,* on the effect of MC's on five photoautotrophs namely *Chlamydomonas reinhardtii, Chlorella kesslerii, Pediastrum duplex, Pseudokirchneriella subcapitata* and *Scenedesmus quadricauda* found no allelopathic effect within ecologically relevant concentrations of MC. However, the researchers had considered only growth as a parameter and the maximum exposure duration was only 11 days [250]. In aquatic ecosystems, it is unlikely that cyanobacteria and their cohabitors are only exposed for a short duration. Also, it should be noted that the authors have not reported any significant reduction in the growth of the chlorophytes.

Some molecular studies may suggest that there is indeed a role for MC's or at least they might have been useful in the past. The presence of a transport protein McyH encoded by a sequence within the microcystin biosynthetic cluster, as well as the complete loss of microcystin biosynthesis with the deletion of the *mcyH* gene suggest that microcystin is produced with strong intention of extracellular export [181]. Further, the discovery of a transcription factor (NtcA) that up regulates

microcystin synthesis during nitrogen stress adds benefit to the hypothesis that microcystin serves some function to the host organism, at least under stress conditions such as nitrogen limitation [182]. Hence it may be inferred that in order to fully understand the allelopathic role of MC's, further studies are required and factors such as longer exposure durations, growth parameters such as growth rate, photosynthesis rate, effect of exogenous MC's on the secondary metabolites and other parameters which reflect the molecular state of the organism need to be evaluated.

The organism *M. aeruginosa* PCC7806, originally isolated from Braakman Reservoir (in 1972, Netherlands) has been studied extensively for several decades. It originates from one of the most common bloom forming cyanobacteria occurring widely in fresh water ecosystems [251]. One of the key characteristics of *M. aeruginosa* PCC7806 is its ability to produce an array of secondary metabolites such as aerucyclamides, cyanopeptolins and microcystins (Figure 4.1). Among its secondary metabolites, microcystins are the most frequently observed and well-studied.

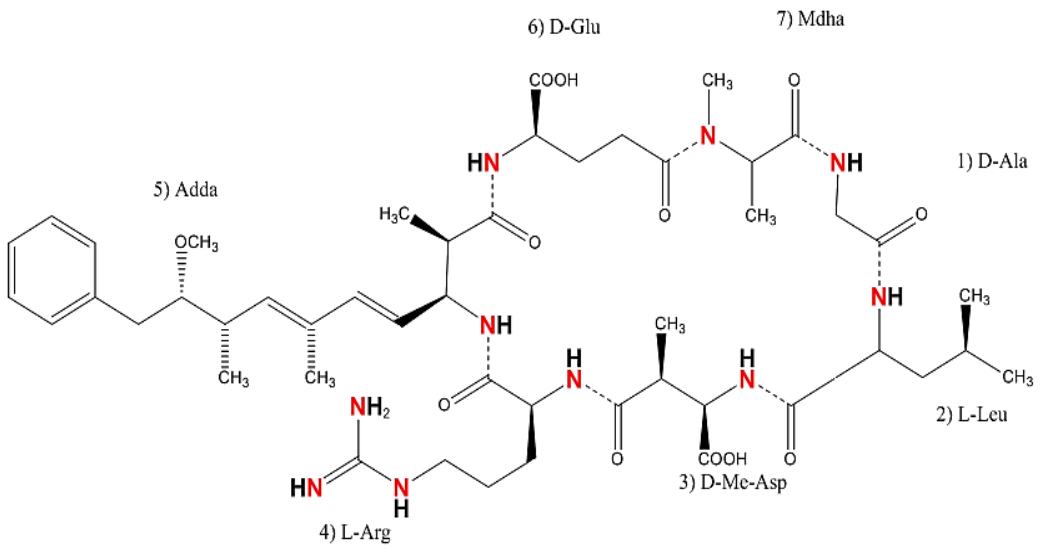


Figure 4.2 - Chemical structure of microcystin-LR. In position 2 is leucine and position 4 is arginine both of which are L forms and variable.

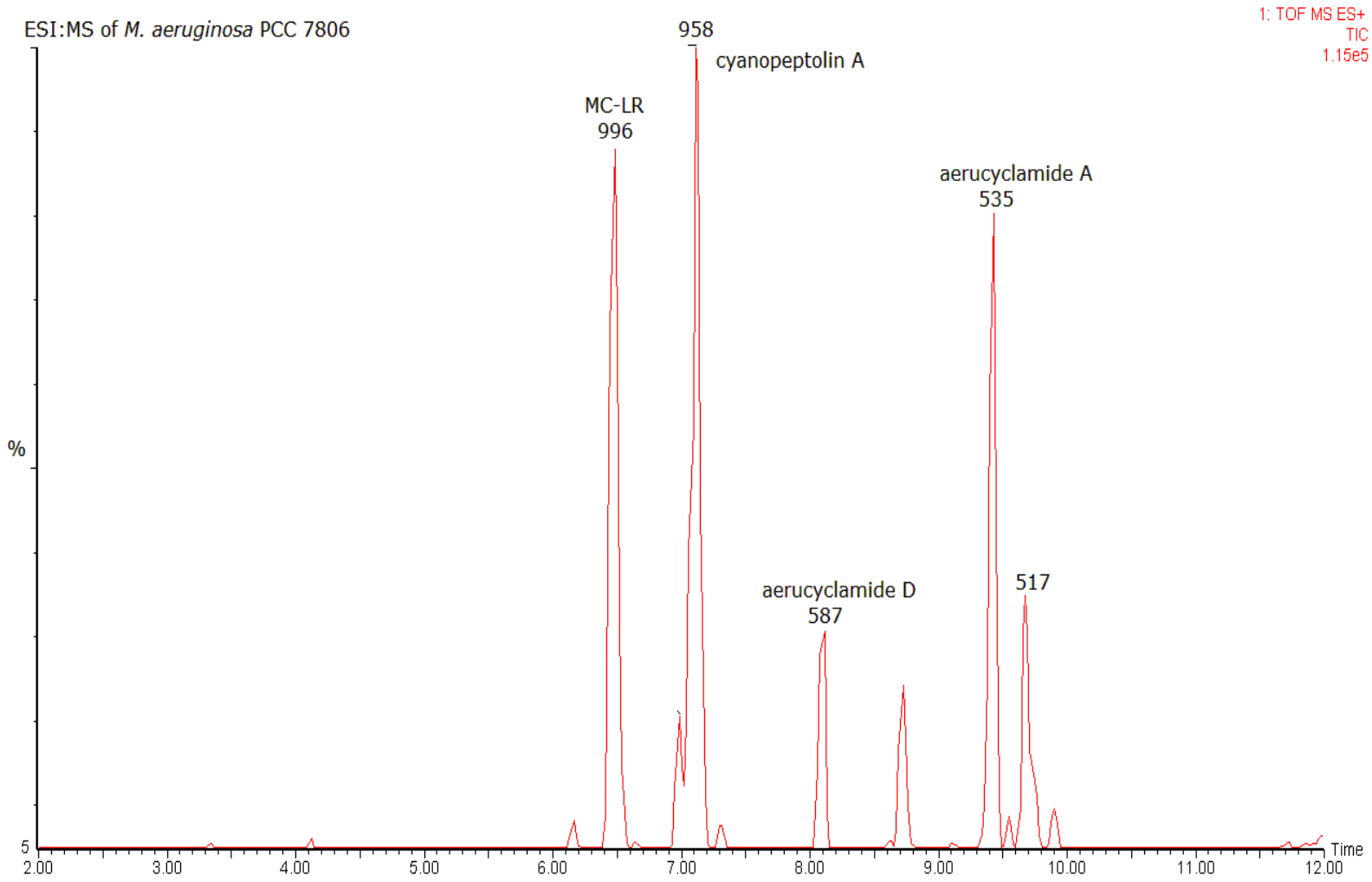


Figure 4.1: HPLC-MS chromatogram of *M. aeruginosa* PCC7806 extracted with 80% methanol (MeOH). The peaks 996 Dalton is microcystin-LR, 958 Da is cyanopeptolin A and 535 Da is aerucyclamide. The organism also produces other secondary metabolites such as aerucyclamide D (587 Da) and aerucyclamide C (517 Da).

Microcystins are monocyclic heptapeptides with a conserved structure of D-Ala-L-leu-D-MeAsp-L-Arg-Adda-D-Glu-Mdha (Figure 4.2). The unique amino acid adda (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid) is present ubiquitously within cyanobacterial species only [252]. One of the characteristics of microcystin-LR is its strong inhibition of protein phosphatases (type PP1 and PP2A of plant and mammals), but 1000 fold less affinity to PP2B. The binding of microcystin-LR to PP1 is through the carboxyl group of MeAsp with Arg96 and Tyr134, followed by the linkage of Mdha side chain to Cys-273. The binding of microcystin-LR to PP2A is through the adda side chain binding with the amino acids (Trp200, His 191, Gln 122 and Ile123) of the binding pocket, followed by van der Waals interactions between the adda with the residues of the binding pocket and later the covalent linkage of Cys269 of the enzyme with Mdha side chain [253, 254]. Protein phosphatases perform regulatory activities such as glycogen metabolism, cell division, apoptosis, and protein synthesis. When the enzymes are inhibited, the organism becomes vulnerable towards tumor formation and in higher concentrations the cells die.

As stated, although microcystins have been studied for several decades, their primary function to the host organism has remained unclear. Besides ecological concerns, the effect of MC’s on human health has drawn significant attention towards the study of microcystins. One approach to study this molecule is to determine how microcystin affect the growth and secondary metabolite production of a known microcystin

producing organism such as *M. aeruginosa* PCC7806. An understanding of how the molecule interacts with the host organism may provide an explanation of why the organism is producing the molecule. The study by Schatz *et al.,* provided some evidence that the presence of extracellular microcystin increased their intracellular concentration, indicating that the cells detected the death of other cells and increased their cellular microcystin content [127]. It is also worthwhile to note that very few studies have reported the effect of MC's on its host organism. Since *M. aeruginosa* PCC7806 produces several other secondary metabolites along with microcystin, it would be interesting to see the effect of microcystin-LR on its own production as well as on the other metabolites produced by the organism. To further enhance the accuracy of the data, a methodology to enumerate cyanobacteria using flow cytometry will be employed as this method enables one to differentiate between live cells and lysing cells.

Also, previous studies in this field often lack information in areas such as production rate, an accurate quantification of the metabolite within a single cell, accurate count of the cell numbers, the *de-novo* production of these metabolites and what is the fate of the secondary metabolites already present in the surroundings. In most studies, microcystin is quantified at time points i.e., measure of the change in total microcystin over time. This type of quantification yields only an overall picture of the quantity of microcystins, but does not reveal any information regarding microcystins metabolism (synthesis vs

degradation). Further, while calculating the rate of production, the total cell population is often only represented in terms of total protein or the total chlorophyll content [100]. This leads to errors such as over or underestimation of the cell density, which in turn leads to errors in microcystin quantification. Even studies that attempt to report a linear correlation between *mcy D* (one of the genes in microcystin synthesis operon) expression and microcystin concentration [255] do not quantify the microcystin production against live cell numbers. This study aims to bridge this knowledge gap by developing tools that accurately quantify cell numbers and differentiate between live and dead cyanobacterial cells, quantify the production of *de-novo* (newly synthesised) secondary metabolites and evaluate how the exogenous presence microcystin-LR affects the growth and the production of other peptides of the host organism. In order to accurately measure the daily production of new microcystin (*de-novo*), yet another novel methodology of replacing the nitrogen source in the growth media with a stable isotopic nitrogen (^{15}N) containing sodium nitrate would be employed. While the organism grows in such a media, the metabolites thus synthesised will incorporate the isotopic nitrogen whereby increasing its mass equivalent to the number of nitrogen atoms in a molecule of MC. This mass difference is detectable on the HPLC-MS and serves to differentiate between the *de-novo* and native MC.

4.2 Aim

To investigate the role of microcystins as signalling molecules in a microcystin producing cyanobacteria

Objective

1) To determine the effect of exogenously added microcystin-LR on the growth of *M. aeruginosa* PCC7806
2) To determine if exogenously added microcystin-LR affects the production of secondary metabolites such as microcystin-LR, aerucyclamides and cyanopeptolins produced by *M. aeruginosa* PCC7806

4.3 Material and Methods

4.3.1 Cultures

The cyanobacterium *M. aeruginosa* PCC7806 (Pasteur Culture Collection, Paris) was selected as it had several features that made it an ideal candidate for this study.

4.3.2 Media and growth conditions

Media preparation and growth conditions were followed as described in section 2.2 and 2.3.

4.3.3 Flow cytometry

Cell counting in this study was performed using flowcytometer. The protocol used for cell counting was as described in section 2.6.2.

4.3.4 Analytical methods (LC-MS analysis)

Analysis of secondary metabolites of *M. aeruginosa* PCC7806 was performed as described in section 2.8

4.3.5 Monitoring *de-novo* microcystin-LR production

Monitoring *de-novo* secondary metabolite production was carried out by providing ^{15}N as the sole source of nitrogen. The principle behind this technique is described in section 2.2.1

4.3.6 Experimental setup

The details of experimental setup were as described in section 2.4.1. Briefly, *M. aeruginosa* PCC7806 was exposed to exogenously added purified microcystin-LR at concentrations of 0.1 µg/ml, 1 µg/ml and 10 µg/ml. A control was also maintained without the addition of any exogenous microcystin-LR. The reason behind choosing high concentrations of microcystin was that during blooms collapse, high localised concentrations of microcystin-LR of up to 1500 µg/g dry weight of biomass has been reported [285]. It is thought that high concentrations of microcystins may trigger cellular survival mechanisms. Also, previous work in this field has used similar concentration of microcystins, to study its effects on other strains of *Microcystis* sp [185].

M. aeruginosa PCC7806 was grown in the presence of exogenously added microcystin-LR for a period of 35 days and samples were withdrawn once a week and analysed for cell numbers and *de-novo* secondary metabolites. As the doubling time for this organism is reported to be about 3 days, samples were withdrawn once per week in order to allow the cells to sufficiently multiply. Besides, many publications in this

field report samplings between 7 to 10 days [220, 221]. Also, withdrawing samples frequently would increase significant changes in the culture system while adding the risk of contamination.

4.3.7 Data extraction and analysis of cell growth

The cells counts were obtained from the flow cytometer using the Expo32 ADC© software and MS-Excel software the final cell numbers were calculated considering the dilution factor. The protocol followed was as described in section 2.6.2

4.3.8 UPLC Data extraction

Peak areas for the corresponding secondary metabolites were extracted using the MassLynx™™ application (Table 4.1) and the data entered into MS-Excel application. In MS-Excel, unknown concentration of the secondary metabolite was calculated by obtaining its peak area and comparing it to that of a known concentration of microcystin-LR in UV and MS mode. Statistical significance (P-values) was obtained using a one-way Analysis of Variance (Anova) analysis. The Anova was chosen as it compares the mean of two groups and the resultant P-value gives an indication of the statistical significance between the groups. A P-value of less than 0.05 is considered statistically significant while a P-value greater

than 0.05 is statistically insignificant. A web based version of the Anova statistical software was used for the calculations [257].

Table 4.1: The MS and UV values used for extraction of peak areas using MassLynx™ software for the different secondary metabolites

	Native		*De-novo*	
	MS	UV	MS	UV
microcystin-LR	996	238	1006	238
Cyanopeptolin A	958	-	968	-
Cyanopeptolin B	930	-	939	-
Aerucyclamide A	535	-	541	-
Aerucyclamide C	517	-	523	-
Aerucyclamide D	587	-	593	-

4.4 Result

The hypothesis that microcystins may perform the role of a signalling molecule was proposed by Schatz *et al.* in 2007 [127]. Their research suggested that the presence of exogenous microcystin (and other secondary metabolites such as microginin or micropeptin) increased the intracellular concentrations of microcystin, indicating that the producing organism is also able to detect the presence of these metabolites and subsequently upregulate the production of intracellular microcystin. It has also been reported that the expression of NRPS/PKS genes involved in microcystin production occurred throughout the day-night cycle [206] indicating that MC's are produced continuously and may have an important purpose. A growth study between MC-producing and non-MC-producing strains revealed that the non-MC producing strain dominated the microcystin culture and it was thought that due to the high energy requirement of toxin production, the MC-producing strain could not compete with its mutant counterpart [258]. Hence it could be understood that a toxin producing organism diverts considerable amount of energy towards microcystin production. However, it also important to note that a biological or an allelochemical role for microcystin has not been convincingly proved [149].

An experiment was designed in such a way that an organism that produces microcystin-LR would be exposed to 3 concentrations of microcystin-LR added exogenously. The growth of the organism would be

monitored for a period of 35 days with sampling once a week. Along with growth, the production of microcystin-LR would be monitored. The organism *M. aeruginosa* PCC7806 produces several other secondary metabolites (such as cyanopeptolins and aerucyclamides). Therefore, the production of these metabolites was also monitored. Hence a comprehensive picture of the effect of exogenous microcystin-LR on the growth and the secondary metabolite production of an organism would be explored against a control i.e., without addition of any exogenous microcystin-LR. All other experimental parameters such as temperature, light and nutrient concentration were comparable to that of the control. Exogenous microcystin-LR was added to the flasks at a final concentration of 0.1, 1 and 10 µg/ml

The fate of exogenously added microcystin-LR was also monitored throughout the duration of the experiment to determine if added microcystin-LR remained unaltered or if *M. aeruginosa* PCC7806 is able to assimilate it. The use of BG11-^{15}N as the sole source of nitrogen enables us to differentiate the added microcystin-LR from the *de-novo* synthesised microcystin-LR. The mass spectrometric analysis of the exogenously added microcystin-LR revealed that the sample contained roughly 25% of demethylated microcystin-LR (Demethy-microcystin-LR, MW 982 Da) and 75% methylated microcystin-LR (MW 996 Da). This would not affect the experimental setup but while determining the production of *de-novo* microcystin-LR, the sum of Demethy-microcystin-LR and microcystin was determined. Also, since a known concentration (50 µg/ml) of the same

microcystin-LR preparation was used to prepare standard dilution series, the peak area representing the concentration of the standard should also be obtained as *m/z* (982+996). The UV detector of the HPLC-MS detects microcystin-LR by its absorbance property at 238 nm and hence no adjustment is needed in order to quantify microcystin-LR by UV method.

4.4.1 Analysis of the exogenously added microcystin-LR

It is generally accepted that microcystin-LR is a stable molecule and could persist in the environment for several days. It has been reported that microcystin-LR could withstand pH extremes and is stable at temperatures up to 300 °C [259]. In the environment, the half-life of microcystin-LR is thought to be about 10 weeks. However, in the absence of sunlight, microcystin-LR may remain stable for several months to years [260]. The use of labelled nitrogen (^{15}N) in the media serves to accurately track the fate of exogenous added microcystin-LR as it is unlabelled while the *de-novo* produced microcystin-LR is labelled. Moreover, the intracellular as well as extracellular unlabelled microcystin-LR can be quantified independently to determine if there is any change in the distribution or location.

The exogenous unlabelled microcystin-LR data (Figure 4.4) indicates that at lower concentrations, the concentration remained mostly unaltered for the duration of the assay (5 weeks). Although the intended dosage was 0.1, 1 and 10 µg/ml the actual average concentration the

flasks had received were determined to be 0.1, 0.94 and 9.14 µg/ml. This could be due to the fact that purified microcystin-LR is poorly soluble in water and some loss may have occurred during the process of dissolving and filtration. The treatment that received 9.14 µg/ml microcystin-LR showed a 9.6% increase by week 2 and thereafter declined steadily with 6.5 µg/ml (29% decrease) at week 5. Interestingly, this trend was not observed in the lower concentrations but the concentration seemed to be maintained although some variability could be seen. If it was assumed that the organism *M. aeruginosa* PCC7806 assimilated microcystin-LR or that the microcystin-LR has been degraded, then it would be expected the same trend would have been observed even in the lower concentration treatments as well. Further, with the help of the *ex-novo* i.e., unlabelled microcystin-LR data (Figure 4.5) it could be seen that the intracellular concentration of unlabelled microcystin-LR remains steady throughout the duration of the experiment. Hence it could be interpreted that the exogenously added microcystin-LR is not assimilated by *M. aeruginosa* PCC7806. The loss of microcystin-LR seen at higher exogenous concentration of microcystin-LR may be due to non-biological reasons such as aggregation or microcystin-LR binding to cell components and this going undetected. An interesting point to note here is that the quantity of intracellular *ex-novo* microcystin-LR remaining constant, even though the cells are actively dividing through week 1 to 3 then division is declining. It would be expected that the concentration of intracellular *ex-novo*

microcystin-LR decreases over time as it is known that microcystin-LR is produced during active growth and released during senescence.

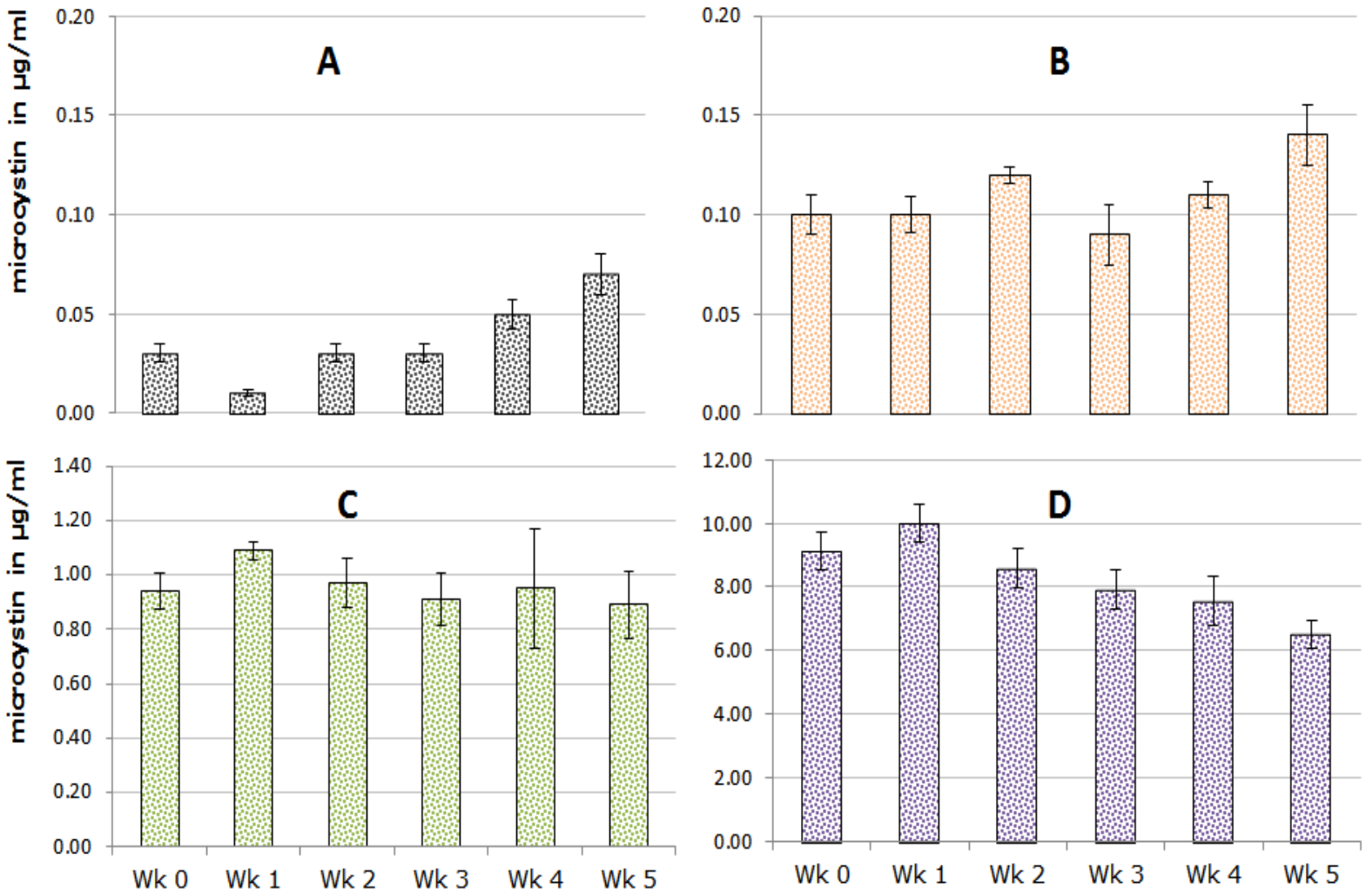


Figure 4.4: The trend of exogenously added microcystin-LR (m/z 982+996) tracked for 5 weeks. Plot A) Control. B) 0.1 µg/ml exogenous microcystin-LR. C) 1 µg/ml exogenous microcystin-LR & D) 10 µg/ml exogenous microcystin-LR. Error bars=1SD, n=4.

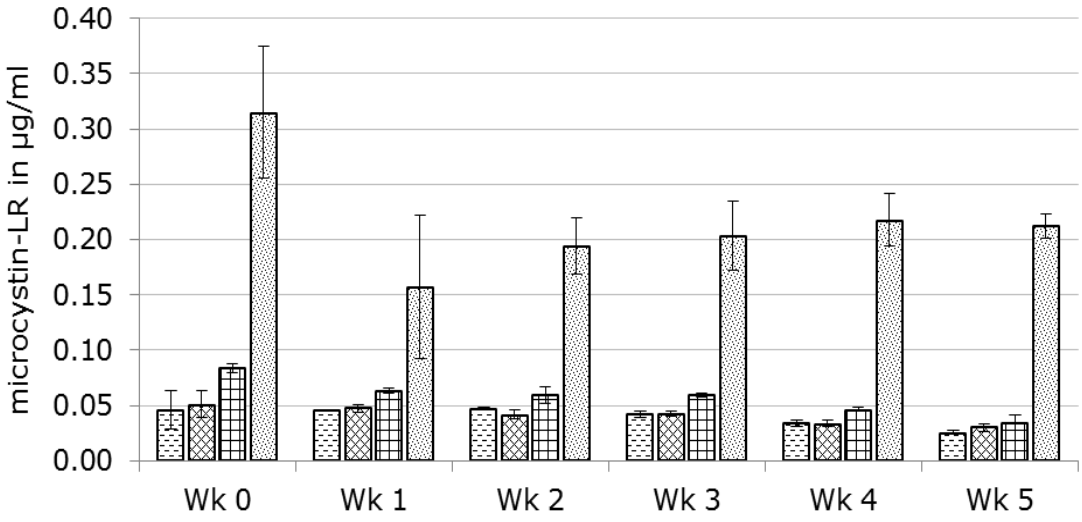


Figure 4.5 - The intracellular unlabelled microcystin-LR (m/z 996+982) over 5 week period. Samples were withdrawn once a week and analyzed in HPLC-MS/MS. Error bars - 1 SD, n-4. Control 0.1 µg/ml 1 µg/ml 10 µg/ml

4.4.2 Effect of exogenous microcystin on growth of *M. aeruginosa* PCC7806

The growth of *M. aeruginosa* PCC7806 was monitored using flow cytometry and in particular the cells appearing within the 'live cells' gate were considered for analysis purposes. As the experiment design had a control group that did not receive any exogenous microcystin-LR, the treatments were individually compared to the control at any given week, to determine the effect of exogenous microcystin-LR on the cell numbers.

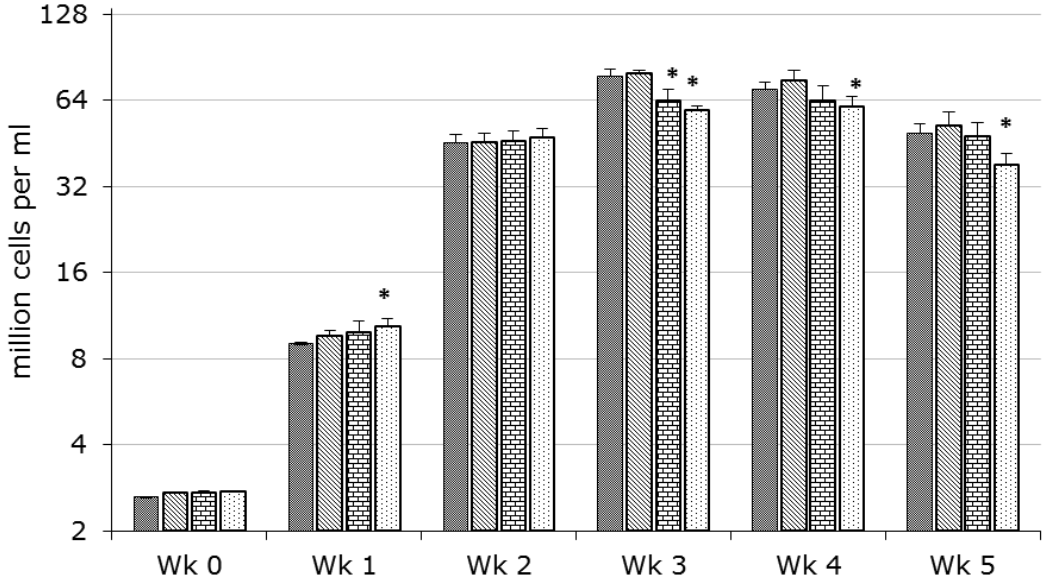


Figure 4.6 - The effect of exogenous microcystin-LR on the growth of *M. aeruginosa* PCC7806. The cells were grown in BG11-^{15}N media at 20 ± 1 °C and light 20 ± 2 µmols/m^2/s. Samples were withdrawn once a week and cell counts estimated by flowcytometry. Error bars - 1SD, n - 4. Statistical analysis was performed using Anova and * - indicates $p \leq 0.05$. Control 0.1 µg/ml 1 µg/ml 10 µg/ml

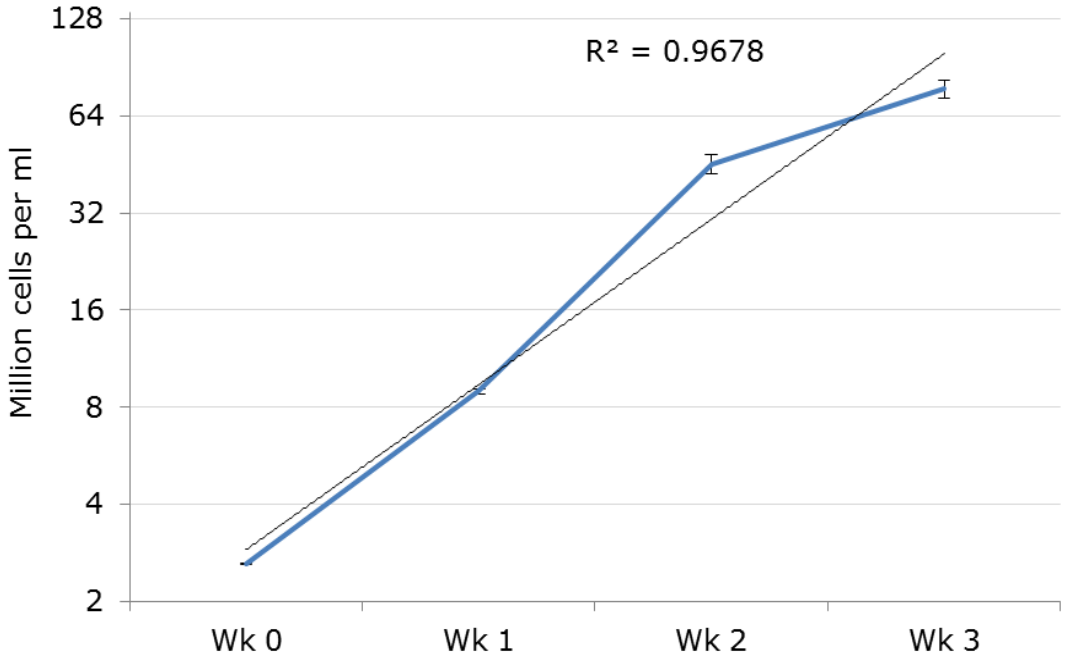


Figure 4.6.1: The three week cell numbers of the control was taken and an exponential trend line plotted to determine the linearity of logarithmic growth curve. linearity was good as 96.78 %. Error bars = 1 SD and n = 4

From the plot of live cells, it was evident that the log phase growth of *M. aeruginosa* PCC7806 was up to week 3 (Figure 4.6 and 4.6.1) and the stationary phase was between week 3 and week 4 after which cell numbers declined. In a recently published study, it has been reported that when a toxin producing *Microcystis sp.* was grown in BG11 containing 1.5 g/L of sodium nitrate, the cell grow exponentially until day 9 and then remain stable until day 21 [261]. In a previous publication, it has been reported that the growth curve of *M. aeruginosa* PCC7806 showed logarithmic growth until day 10 followed by a stationary phase [262]. However, this data shows slower growth which could be a result of inoculum differences. An important observation here is that most publications denote the growth of the cells as either a quantification of

Chl-a content, biomass (g/L) or haemocytometric method, while this method used flow cytometry and differentiated the live cells from the population. Estimation of chl-a requires multiple extraction steps and the measured optical density provides only a rough indication of the cell density and the health of the cells, while haemocytometry is a tedious process and cell counts may not be very accurate. Compared to the chl-a and haemocytometry, the protocol developed in the flow cytometer is rapid and cells are counted based on the principle of autoflourescence of chl-a. Hence only cells that emit a strong fluorescent signal will fall within the live cell gate and are enumerated as a live cell.

Although the cells were exposed to exogenous microcystin-LR from day 0, an indication of the effect could only be observed at week 3. It is likely that during logarithmic growth, *M. aeruginosa* PCC7806 tolerates the presence of exogenous microcystin-LR. Between week 2 and week 3 the treatments that received 1 and 10 µg/ml microcystin showed reduced growth (20% less) when compared to the control and the treatment that received 0.1 µg/ml microcystin. From week 4 onwards no active growth was seen and the cell numbers started to decline. In order to determine if the difference in cell numbers observed between the control and treatments was statistically significant, analysis was performed for individual weeks comparing the control to the treatments. The statistical analysis was performed excluding week 0 as all the flasks in this week received the same cell numbers during inoculation.

At week 1, the P-value of the treatment 10 µg/ml indicated that their difference in cell numbers were significantly different from that of the control. However, an observation of the percentage variability of the cell number within the treatment showed that the difference was a mere 6.8%. It should also be noted that the anova test compares the variability within the replicates of a sample set and compares that with the other. At week 1, the variability within the control was 1.7% only while the variability within treatments 0.1 µg/ml was 4.2%, 1 µg/ml was 9.7% and 10 µg/ml was 6.8%. The treatment 10 µg/ml, although different from the control showed a small variability within the replicates and hence the statistical analysis indicates that the values are statistically significant from the control by virtue of its variability within the replicates. In the case of the treatment 1 µg/ml, the variability within the replicates was comparatively larger and hence the difference observed in respect to the control may not be significant. This fact is further confirmed by the P-values seen at week 2 where none of the treatments were statistically different from the control. Hence it could be inferred that *M. aeruginosa* PCC7806 tolerates exogenous microcystin-LR concentration of up to 10 µg/ml while actively growing. In an earlier report, it was found that a *Microcystis* bloom might secrete up to 5 ng/ml of microcystins into the water system [214] while other reports suggest that microcystin concentrations can vary between 5 to 43 ng/ml [39]. Higher concentrations reported were between 1.3 to 1.8 µg/ml [264] where the cells had been exposed to algicide treatment. In a broad sense, it could

be stated that MC-producing *Microcystis* cells are always exposed to some exogenous microcystins but in varying concentrations and seemed to survive and reinoculate the waters system when conditions turn favourable. From this study, it could be stated that *M. aeruginosa* PCC7806 are able to tolerate exogenous microcystin-LR up to 0.1 µg/ml (i.e., 100 µg/L) and higher concentrations may negatively affect cell division. It should also be noted that the cells need not be in an actively growing state in order to tolerate this concentration of microcystin-LR.

In an incubation period of 35 days, week 3 seems to be the peak of cell density following which the cell numbers begin to decline. However it should be noted that this observation may be applicable only when the *M. aeruginosa* PCC7806 is grown in BG11 media containing 1.5 g/L of sodium nitrate and a static culture system. From the growth curve plot, it could be seen that week 3 was also the end of log phase growth for all the treatments. Further, it is also observed that the presence of 0.1 µg/ml microcystin-LR is in fact mildly favourable to the growth of *M. aeruginosa* PCC7806 although not found to be statistically significant. This has been observed by a previous work which reported an 18% increase in cell numbers [265] in the presence of exogenous microcystin-LR. However, in the present study, only a 2 to 8% increase in cell numbers could be observed between the control and the 0.1 µg/ml treatment. The difference may be due to cell counting procedures which in this case used flow cytometer and live and dead cells could be differentiated. Taken together, it is clearly evident that presence of low concentrations of

exogenous microcystin-LR is mildly beneficial for the host and in ecological scenarios, this may confer an advantage towards species survival irrespective of the growth phase of the host organism [266].

Treatment with 1 µg/ml showed significant decrease in cell numbers when compared to the control at week 3. As this difference was not observed in week 2, it could be concluded that under certain conditions (such as nutrient limitation, accumulation of metabolic by-products), microcystin-LR concentration of higher than 0.1 µg/ml negatively affects the growth of *M. aeruginosa* PCC7806. This trend was also observed at week 4 and 5, although not statistically significant. As evident from the growth curve plot, at week 4 and 5 there are factors which affect the growth of cells even in the control. This may also have introduced large variability within the control and hence when this is compared to the treatment, the difference is not statistically significant.

The treatment 10 µg/ml showed significant decrease in cell numbers from week 3 to week 5 when compared to the control. Unlike the 1 µg/ml treatment, the difference was not transient and hence it could be confirmed that under static culture conditions, microcystin-LR concentrations of above 1 µg/ml may affect cell division while a microcystin-LR concentration of above 10 µg/ml strongly inhibits cell division and or survival. It remains to be determined as to how microcystin-LR brings about this affect and if the same effects could be observed in a continuous culture system.

The use of flow cytometer was primarily to enumerate cells and to some extent differentiate between live and dead cells (events that fall out of the live cells gate in the flow cytometer). When the growth curve of the live cells was interpreted along with the dead cells, it was observed that there was no significant difference between the control and the treatments in any given week (figure 4.6.2).

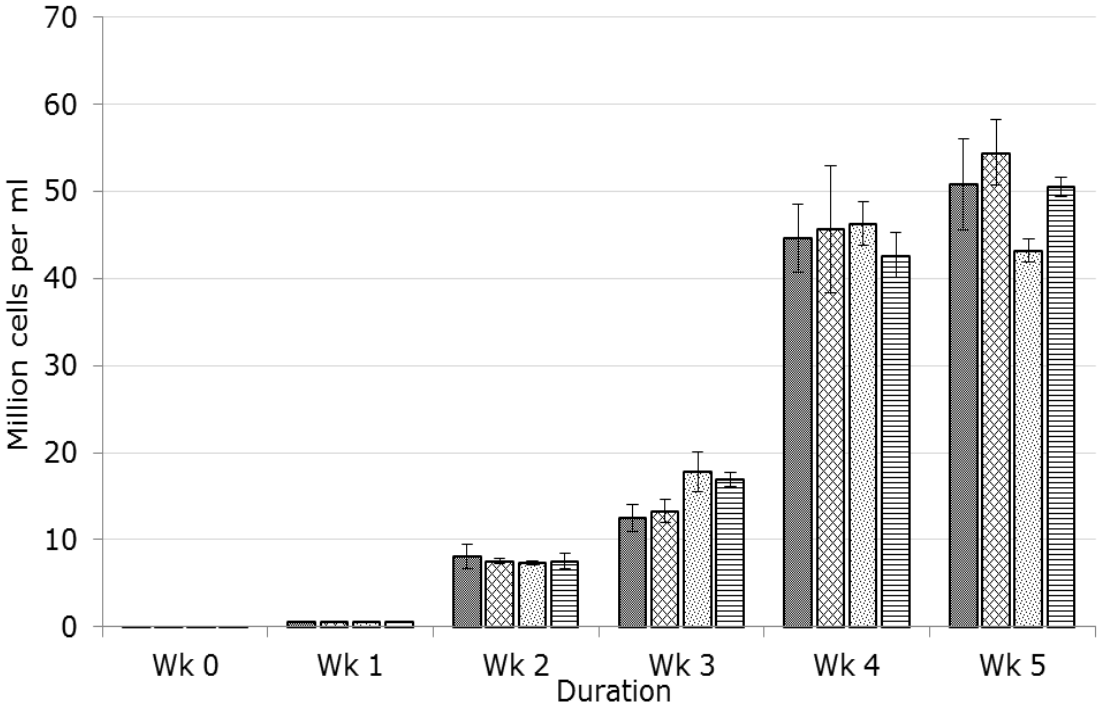


Figure 4.6.2: A plot showing the population of cells appearing in the 'Dead cells' gate of the flowcytometric dot plot. In this study actively growing cells of *M. aeruginosa* PCC7806 was grown in the presence of exogenously added microcystin-LR and samples were withdrawn once a week and cells enumerated using flowcytometer. Error

A statistical analysis of the dead cells would be erroneous as the events appearing within the dead cell gate may represent anything between an empty cell wall, disintegrating cell debris or a whole cell with

little Chl-a fluorescence. It may be worthwhile to adjust the dead cell gate to capture only events with a higher signal strength and observe differences. However, with the present data, it could be concluded that the presence of even a high concentration of microcystin-LR (10 mg/L) does not significantly increase cells death of the host organism. Taken together, it appears that microcystin-LR does not induce cell death but inhibits cell division. Ecologically such a scenario might occur during bloom senescence; however the cells may never be exposed to such concentrations. The rapidly growing cells utilize the available nutrients and may reach a stationary phase of growth. The presence of the produced microcystin-LR in their immediate surrounding (assuming low turbulence) may signal the organism to further decrease cell division and prolong generation time. In this way, species survival might be guaranteed for long periods of stress.

In order to confirm if exogenous microcystin-LR increases the generation time the live cell population alone was considered and generation time calculated. It revealed that during mid-log growth, the generation time of *M. aeruginosa* PCC7806 was about 3 days (Table 4.3). In some studies, it was reported that the generation time of *Microcystis* sp. is between 1.24 to 1.7 days [155, 267, 268]. However, other studies report a doubling time of 3.25 days [269]. Hence it could be stated that the generation time of *M. aeruginosa* may vary between 1.24 to 3.25 days depending on conditions of light, temperature, aeration, irradiation and nutrients.

During late exponential growth, the generation time of *M. aeruginosa* PCC7806 had extended to 9 days (3 folds) and it is understood that nutrients limitation could have been one of the factors. This increase of generation time was further exacerbated with the presence of microcystin-LR where the extension of generation time was proportional to the microcystin-LR concentration. Interestingly, the presence of 0.1 µg/ml microcystin-LR seemed to mildly decrease the generation time, however, as the difference was a mere 3%, it's not considered a significant effect. For the treatment that received 1 µg/ml microcystin-LR, the generation time had extended to 15.3 days while the highest concentration tested i.e., 10 µg/ml, the generation time was extended to 22.48 days (2.5 fold compared to the Wk 3 control). The generation time was extrapolated beyond 3 weeks and it was surprising to note that the generation time for the treatments that received 1 µg/ml and above microcystin-LR was beyond and considerably longer (around 120 days). However, as the formula used to calculate the generation time requires that the cells be in their logarithmic growth phase, and hence the value obtained beyond week 3 may be an artefact.

Table 4.3: The calculated doubling time (in days) of *M. aeruginosa* PCC7806

	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5
Control	3.94	2.99	9.08	-	-
0.1 µg/ml microcystin-LR	3.83	3.12	8.80	-	-
1 µg/ml microcystin-LR	3.76	3.14	15.33	-	-
10 µg/ml microcystin-LR	3.63	3.20	22.48	-	-

The fact that the presence of microcystin-LR does not significantly induce cell death is interesting to note as it confirms that microcystin-LR is not toxic to the producing organism even at very high concentrations. In that case, the question remains as to how microcystin-LR extends generation time significantly. One possibility may be that microcystin-LR may bind to metal ions and make them unavailable for the host. Another possibility is that microcystin-LR is known to bind to intracellular proteins and localised within thylakoids. As thylakoids are centres of respiration, the overabundance of microcystin-LR might interfere with the cells respiration and hence slow cell growth. A recent report found that microcystin-LR is localised within the thylakoid membrane causing a decrease in photosystem II activity without any negative effects on the cell survival [270].

4.4.3 Effect of exogenous microcystin-LR on production of Microcystin

The effect of exogenous microcystin-LR on *M. aeruginosa* PCC7806 was studied with a view to understanding if there are any effect seen at the cellular as well as molecular level. The production of microcystin can be considered as a reflection of the metabolic state of the organism and if any changes are seen, it would indicate that exogenous microcystin has an effect on the producing organism at a molecular level. Also, as this organism produces several other secondary metabolites, a comprehensive analysis of the other metabolites such as aerucyclamides and cyanopeptolins would indicate if the effect occurs at multiple levels.

The *de-novo* microcystin was quantified by obtaining the peak areas for the mass *m/z* 1006+992 i.e., the methylated microcystin (microcystin-LR) as well as the demethylated-MC (Demethy-microcystin-LR). It was thought that *M. aeruginosa* produced about 90% methylated microcystin-LR and 10% Demethy-microcystin-LR [271]. However, the PCC7806 culture used in this work was seen to produce significantly higher quantities of Demethy-microcystin-LR i.e., of the total amount of *de-novo* microcystin produced, Demethy-microcystin-LR ranged between 45 to 55% (Figure 4.7, 4.8). It was also observed that the control and 0.1 µg/ml treatment produced equal or slightly lower Demethy-microcystin-LR (10% less) than microcystin-LR, whereas the treatments that received 1 and 10 µg/ml microcystin-LR produced about 15% higher Demethy-

microcystin-LR (Figure 4.8). It is unusual that PCC7806 produces more than 10% of demethylated microcystin and it may be explained by the culture being maintained under low light for several years. However, confirmatory evidence is not available at this time.

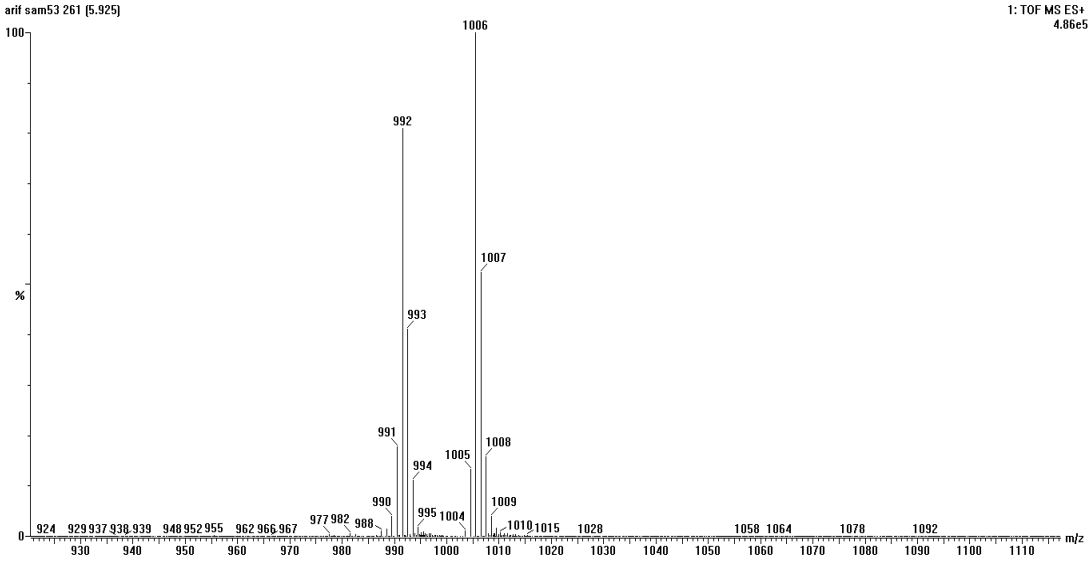


Figure 4.7: Intracellular ESI-MS ions of *m/z* 992 (Demethy-microcystin-LR) and 1006 (microcystin-LR) as seen in the control at week 3

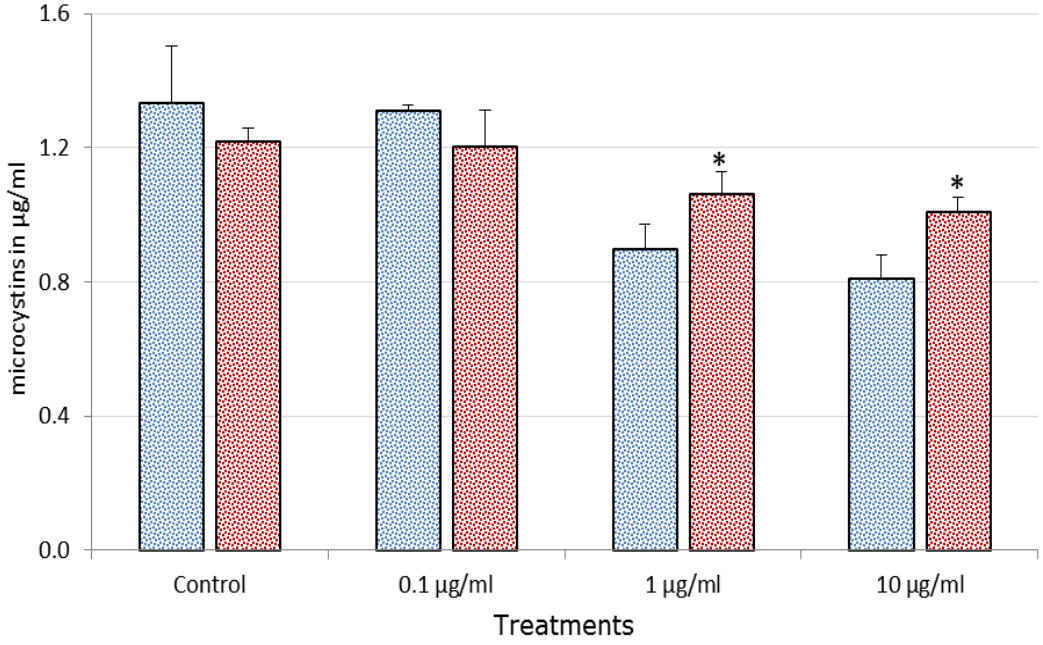


Figure 4.8: The quantities of microcystin-LR and Demethyl-Microcystin-LR produced intracellularly at week 3 by *M. aeruginosa* PCC7806. Error bars - 1SD, n=4. Statistical analysis was performed using Anova and * - indicates $p \leq 0.05$. Microcystin-LR Demethyl-Microcystin-LR

Statistical analysis was performed between the two microcystin variants at week 3 and it appeared that the presence of exogenous microcystin-LR of 1 µg/ml or higher, the ratio of Demethy-microcystin-LR to microcystin-LR was significantly higher. It remains to be explored if the effect seen was indeed from the exogenous microcystin-LR. As the *M. aeruginosa* PCC7806 produces methylated as well demethylated microcystin in abundance, the quantification of microcystin was performed by adding the quantities of both the isomers to give an account of the total *de-novo* microcystin produced. At week 0, as one would expect no traces of *de-novo* microcystin were seen whereas by week 1, the intracellular microcystin content had reached to 0.33 ± 0.07 µg/ml. Even

at week 1, the effect of exogenous microcystin-LR was detectable and at concentrations higher than 0.1 µg/ml seemed to inhibit the synthesis of *de-novo* microcystin (Figure 4.9).

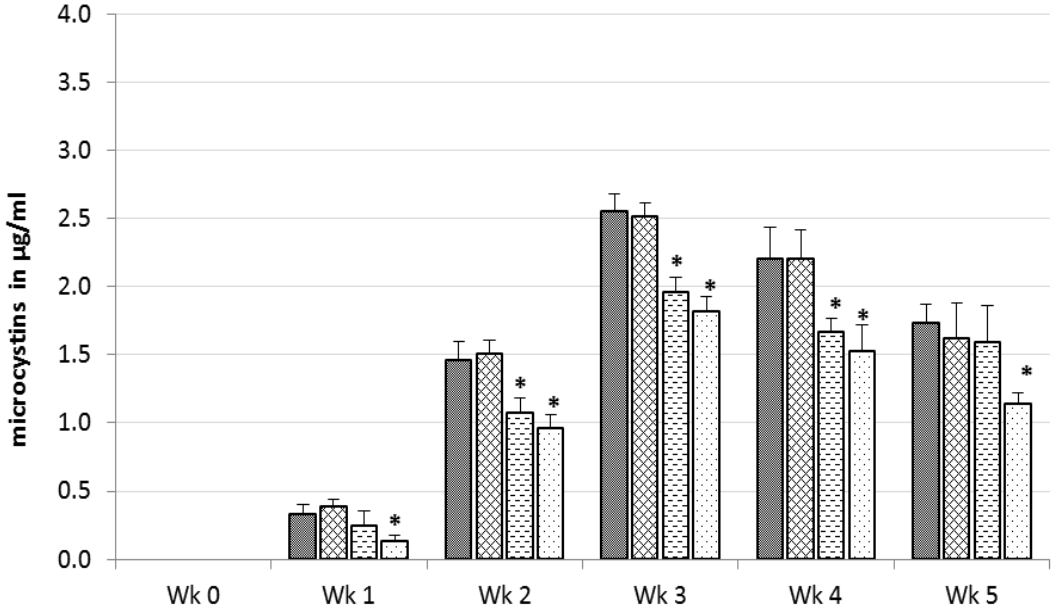


Figure 4.9: The effect of exogenous microcystin-LR on the intracellular de-novo microcystin biosynthesis by *M. aeruginosa* PCC7806. Samples were withdrawn once a week and analysed in HPLC-MS/MS for de-novo microcystin. Total de-novo microcystin was the sum of m/z 992+1006. Error bars = SD and n = 4. * indicates statistically significant difference from the respecitve weeks control ($p \leq 0.05$) as determined by Anova.

Statistical analysis of the intracellular *de-novo* microcystin content revealed a very interesting picture. The treatment with exogenous microcystin-LR at 0.1 µg/ml showed similar intracellular concentrations to that of the control throughout the experiment. The treatment with exogenous microcystin-LR at 1 µg/ml showed significant difference beginning week 2 and continuing until week 4. The treatment with exogenous microcystin-LR at 10 µg/ml showed significant difference with

respect to the control for the entire 5 weeks. Collectively, it appears that the presence of exogenous microcystin-LR beyond 0.1 µg/ml may inhibit microcystin biosynthesis. It should be noted that the cell numbers did not mirror this effect and until week 3, no significant differences could be noticed (Figure 4.9). This data contradicts the results of Schatz *et al.* (127) wherein they report that the presence of exogenous microcystin-LR enhances the intracellular microcystin production. The differences may be due to the concentrations of microcystin-LR they had used (10 - 50 ng/ml), while in this study concentrations ranged from 100 ng/ml to 10 µg/ml. Also, the method of quantification used here is very sensitive compared to using antimicrocystin-LR antibodies. Although the results presented here contradicts the published data, it should be noted that the treatment that received 0.1 µg/ml did show some positive effect on the microcystin content. They also state that exposing cells to greater than 10 ng/ml of microcystin has no additional effect on microcystin production, however, the present data shows that at higher concentrations, cell numbers as well as intracellular microcystin content are reduced. However, a dose dependent correlation could not be established but overall, the treatment 1 µg/ml showed a 25% reduction (average for 5 weeks) while the 10 µg/ml treatment showed a 37.7% reduction.

It has also been reported that presence of exogenous microcystin-LR is known to induce morphological and physiological changes such as cell aggregation, cell volume increase as well as increase in cell pigmentation [272]. However, these parameters were not measured in

this assay, but it could be stated with some confidence that the flasks that received exogenous microcystin-LR did not show any changes in aggregation compared to the control. It may also have been because the flasks were mixed once a day to enable homogenous cell distribution and even light penetration.

In order to confirm that the intracellular microcystin synthesis had indeed reduced, the *de-novo* microcystin content was divided by the cell number (live) such that an indication of the production per cell could be obtained. Here, the calculations are done with the assumption that the live cells retain most of the microcystin produced whereas the aged cells would have leaked their intracellular content [273]. It was calculated that the average microcystin quota (for control) was 33.8 (± 2.2) femtograms (fg) of microcystin per cell and this rate remained constant for the duration of 5 weeks (Figure 4.10). In literature, it has been reported that the intracellular microcystin-LR quota for *M. aeruginosa* PCC7806 ranges between 35 to 51 fg per cell [221, 94]. It was encouraging to note that the values observed correlated well within the range of reported values.

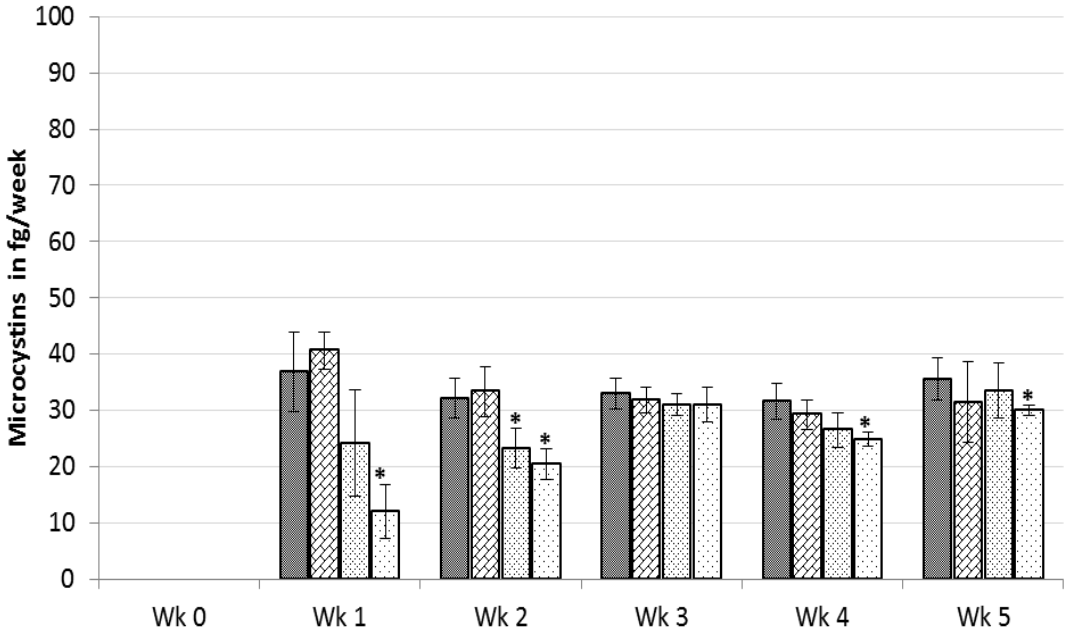


Figure 4.10: The intracellular *de-novo* microcystin (m/z 992+1006) content at a particular week. Errors bars - 1SD, n-4. * statistically significant difference, p value ≤ 0.05 as determined by Anova.

At week 1, the effect of exogenous microcystin-LR was most pronounced compared to any other week followed by week 2. Comparing week 1, 2 and 3 it could be that the cells exposed to 1 and 10 µg/ml microcystin-LR were actively adapting themselves to the new environment and by week 3 their intracellular toxin level was comparable to that of the control. It should be noted that the cultures were exposed to the exogenous microcystin-LR throughout the assay and the question of how the cells are able to almost fully recover microcystin production at a later stage arises. It is well known that microcystin-LR biosynthesis is an enzymatic process involving a system of NRPS/PKS enzymes [176]. Hence, if even one of the enzymes in the biosynthetic pathway is inhibited

(or its production reduced), the production of microcystin-LR would suffer initially but should be able to recover with time, as characteristic of an enzymatic reaction. As the experimental design did not accommodate to monitor this fact, at this stage it could only be hypothesised that an enzyme is being inhibited by the exogenous microcystin-LR and this is reflected in the production rate.

Beyond week 3, the intracellular toxin production was affected, however, only the 10 µg/ml treatment showed a significant reduction compared to its control. The average intracellular toxin production was calculated in order to determine if there was any net reduction on the intracellular toxin production [Figure 4.11]. Comparing to the control, the treatment 0.1 µg/ml did not show much significant difference but it also revealed that the treatments 1 µg/ml (P=0.02) and 10 µg/ml (P=0.02) were significantly different from the control. As the cell numbers have also been accounted for in this calculation, it appears that the exogenous microcystin-LR has had some effect on the toxins production. This confirms that the presence of exogenous microcystin-LR inhibits the production of *de-novo* MC, however the effect is not concentration dependent i.e., lower concentrations (0.1 µg/ml) may mildly stimulate microcystin-LR synthesis whereas higher concentrations inhibit synthesis in a dose independent manner. Such a finding has also been reported previously [127] where they claim that exogenous microcystin-LR concentrations above 50 ng/ml had little effect on the toxin biosynthesis and as the culture aged, the response was diminished.

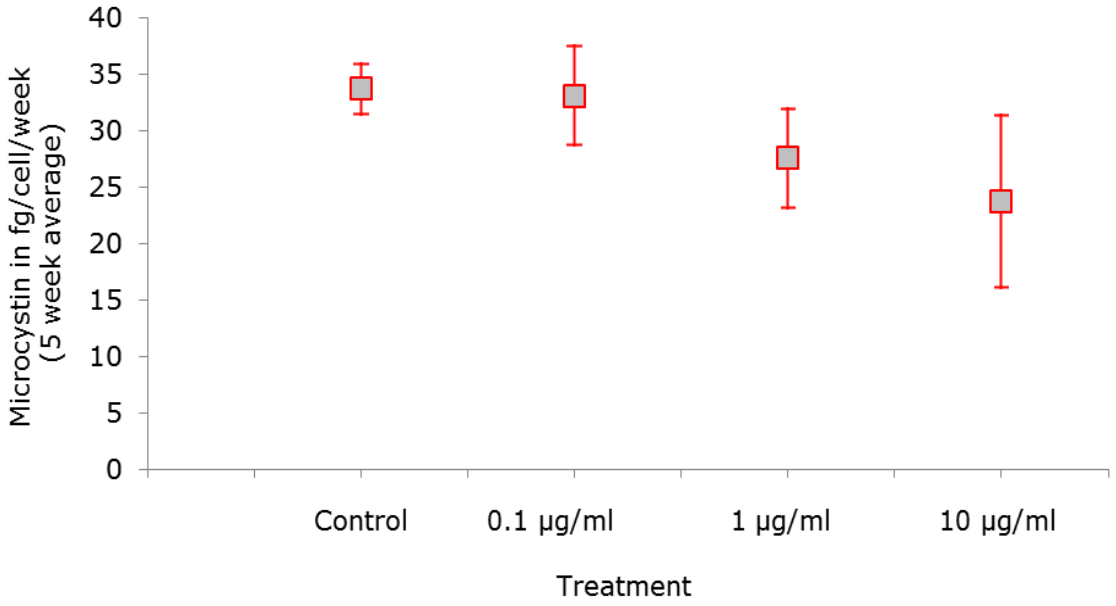


Figure 4.11: The average microcystin production per week (fg/cell/week) for *M. aeruginosa* PCC7806 under different exogenous microcystin-LR concentrations. Values were calculated by taking the mean de-novo microcystin (m/z 992+1006) production per week.

An attempt was made to calculate the microcystin production rate i.e., intracellular production of *de-novo* microcystin-LR per cell per day (Figure 4.12). The weeks 0, 4 and 5 were not considered in the calculation as at week 0 no *de-novo* microcystin-LR was detected and at weeks 4 and 5, the net production was negative compared to the previous week. The reason that no net increase can be seen in the intracellular microcystin may be due to culture aging and causing the cells to leak their intracellular contents.

Between week 0 and 1, the control seemed to be producing microcystin at a rate of 7.4 ± 1.4 fg/cell/day. Compared to the control, the treatment 0.1 µg/ml showed about 8% increase whereas the

treatments 1 and 10 µg/ml showed a 36% and 68% decrease in production respectively. By week 2, the control and the 0.1 µg/ml treatment seemed to have reduced its production rate by about 40% when compared to week 1, and is maintained at the same rate by week 3. It is interesting to note during mid-log growth, the microcystin production rate has actually reduced while in literature it is reported that the production rate linearly correlates with specific growth rate [100, 97]. However, there are major differences in cell enumeration and microcystin-LR quantification between the published work and methodology applied here. In this work, cell numeration was using flow cytometry and microcystin quantification was highly sophisticated (considering only *de-novo* MC) whereas in the published work, cell counting was either by microscopy or by dry weight and microcystin detection was by the PDA detection system monitoring only the total microcystin pool at a given time point. Also, in terms of culture conditions, their work was in nitrogen deplete conditions (0.2 mM $NaNO_3$) whereas this work was in nitrogen replete (17.65 mM $NaNO_3$) conditions.

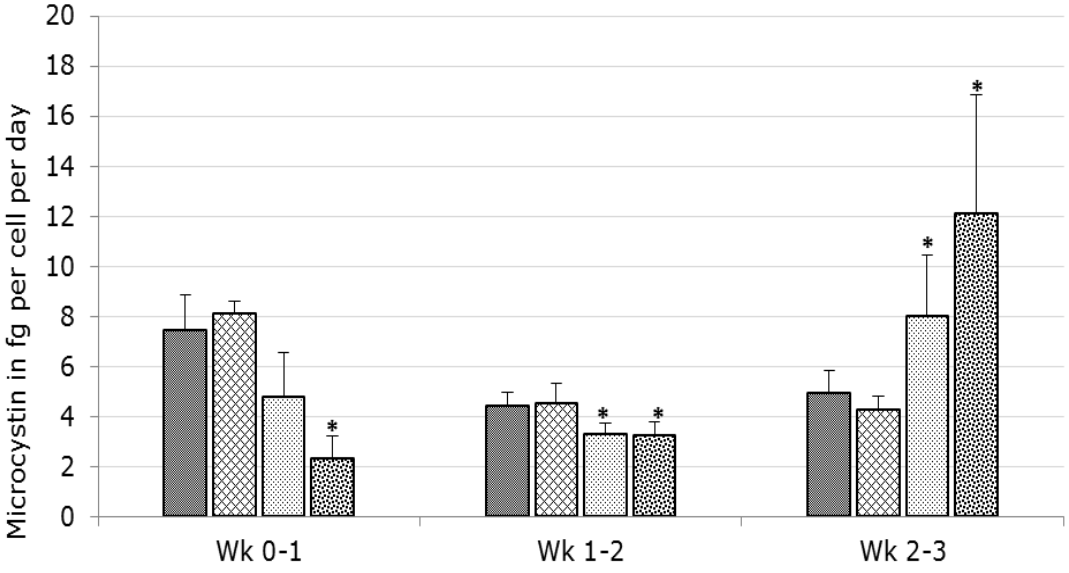


Figure 4.12 - The production rate (per cell per day) of *de-novo* microcystin (m/z 992+1006) by M. aeruginosa PCC7806 in response to exogenously added microcystin-LR. Error bars = 1 SD, n = 4. *** -** Statistically significant ($p \leq 0.05$) compared to control, as determined by Anova.

Between week 1 and 2, the treatments 1 and 10 µg/ml were again seen to be reduced compared to the control while at week 3, their intracellular production was significantly higher than the control. However, when the sum of the *de-novo* production for the three weeks was calculated it revealed that the net production was about 16.8 fg with about 5% difference among the control and treatments. Hence, it may only be concluded that although the presence of exogenous microcystin-LR may seem to have an influence on the microcystin-LR production initially, in the long run, the net production reaches levels comparable to the control. It may be speculated that as microcystin-LR biosynthesis is an enzymatic process, the observations made herein are only transient reflections of the intracellular molecular events and in order to

conclusively determine the effect of exogenous microcystin-LR (on intracellular microcystin-LR), further experiments which precisely monitor growth parameters are required.

The organism *M. aeruginosa* PCC7806 is known to secrete microcystin into the ecosystem during late log and stationary phase of its growth cycle [274]. A graph of the extracellular microcystin (m/z 992+1006) excluding the exogenously added microcystin-LR (m/z 996+982) was plotted and it was evident that there was an unusually high amount of microcystin-LR present (Figure 4.13) compared to reported values. It has been reported that for PCC7806, the amount of extracellular microcystin-LR would range between 10 to 16% [221]. In another report, it has been reported that the extracellular microcystin-LR would amount to 7% of the total microcystin-LR produced at day 20 [214]. However, in this case at day 21, the extracellular microcystin-LR amounted to 26% of the total microcystin-LR at that time point. The reason for unusually high amounts of extracellular microcystin-LR could not be explained, however, it is speculated that changes in light intensity from 5 to 20 μmol/m^2/s as well as replete nitrogen conditions might have caused the cells to release higher microcystin into the extracellular. This could also serve as an indication that replete nitrogen conditions (such as in eutrophic lakes) may cause pronounced microcystin release into water bodies. Such a phenomenon has been previously reported by Weidner *et al.* [94] where they report a 20 fold increase in extracellular microcystin-LR when the light intensity changed from 10 to 40 μmol/m^2/s. However

they still report that at any time point the extracellular microcystin-LR was only about 2.47% of the total microcystin-LR.

The percentage of extracellular microcystin to that of the total microcystin (Intra + Extra (m/z 992+1006)) was calculated and it was noticed that the extracellular microcystin content increased progressively with time. If it were suspected that the presence of exogenous microcystin-LR may have induced the release, it could be seen that the extracellular microcystin data for the control as well as the treatment did not show any pattern in relation to the concentration of exogenous microcystin-LR. As the methodology employed specifically differentiates between exogenously added and secreted *de-novo* microcystin-LR, it can be confirmed that exogenous microcystin-LR did not have a pronounced effect on the secretion of intracellular MC.

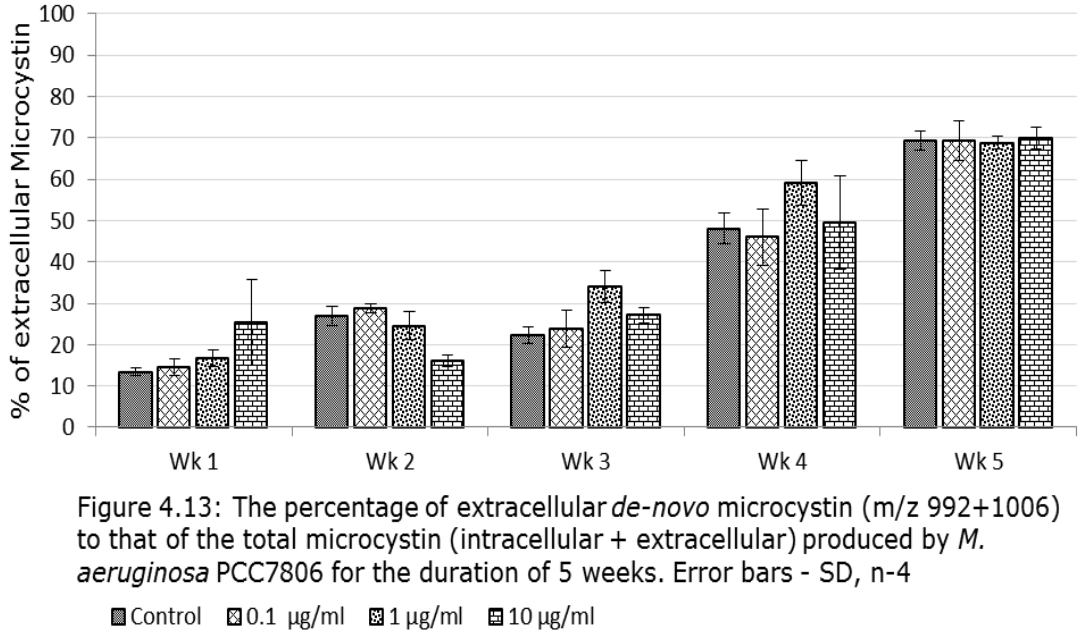


Figure 4.13: The percentage of extracellular *de-novo* microcystin (m/z 992+1006) to that of the total microcystin (intracellular + extracellular) produced by *M. aeruginosa* PCC7806 for the duration of 5 weeks. Error bars - SD, n-4

4.4.4 The effect of exogenous microcystin-LR on cyanopeptolin and aerucyclamides

The organism *M. aeruginosa* PCC7806 produces several variants of cyanopeptolins such as A, B, C and D (Table 4.4), however, not all of them have been chemically characterised (275, 201). Cyanopeptolins are depsipeptides, possessing structures consisting of cyclic L-glutamic acid- γ -aldehyde, L-leucine, N-methyl-phenylalanine, L-valine, L-threonine, L-aspartic acid, hexanoic acid and a variable basic amino acid. This variable amino acid can be L-arginine (cyanopeptolin A), L-lysine (cyanopeptolin B), *N*-methyl-L-lysine (cyanopeptolin C) and *N/N* dimethyl-L-lysine (cyanopeptolin D), respectively. The L-glutamic acid- γ -aldehyde and the amino group of L-leucine form an unusual 3-amino-6-hydroxy-2-oxo-l-piperidine system. With the use of HPLC-MS, these compounds were separated and their respective peak areas were obtained using the MassLynx™™ software. However, as only a cyanopeptolin A standard was available, the quantification of only cyanopeptolin A was performed while the peak area of cyanopeptolin B is shown as an example.

Table 4.4: The mass of compounds of *M. aeruginosa* PCC7806 when grown in BG11 (^{14}N) and BG11 (^{15}N)

Compound	*m/z* in BG11 (^{14}N)	*m/z* in BG11 (^{15}N)
cyanopeptolin A	958	968
cyanopeptolin B	930	938
cyanopeptolin C	944	952
cyanopeptolin D	958	966
cyanopeptolin 970	972	982
cyanopeptolin 963 A	963	971

Note – Cyanopeptolin A and D have the same molar mass but differ by one amino acid i.e, cyanopeptolin A has L-arginine while cyanopeptolin D has N_{ε},N_{ε}-Dimethyl-L-lysine.

The effect of exogenous microcystin-LR on the production of cyanopeptolin A was evaluated. In order to quantify cyanopeptolin A, a standard curve of the respective peptide was plotted taking known concentration of the purified peptide (Figure 4.14). From this standard curve, a line equation was obtained and using this equation, the unknown concentration of the sample was determined (Figure 4.15).

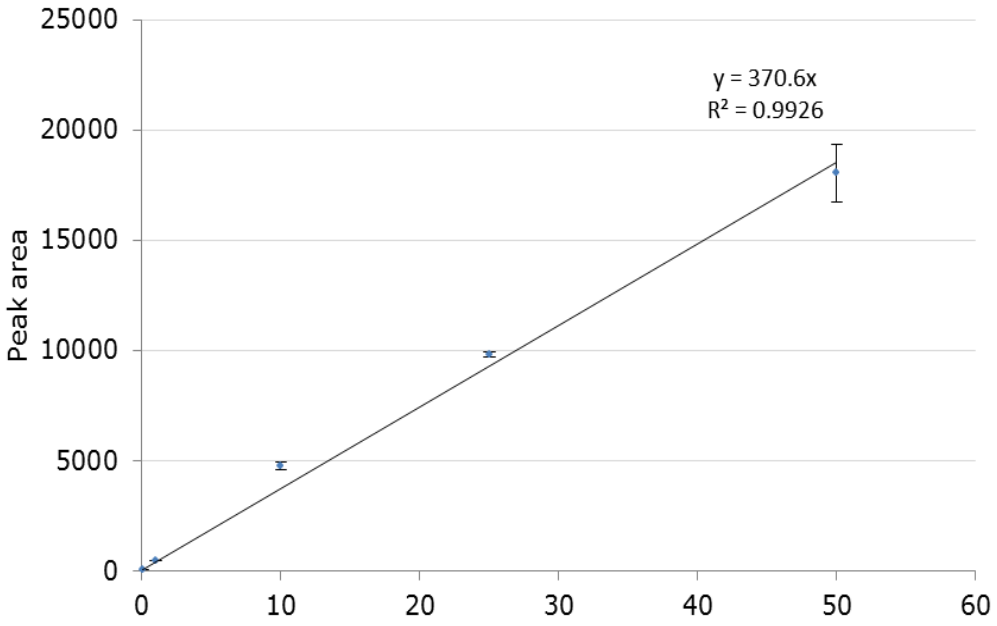


Figure 4.14 - Standard curve for cyanopeptolin A by ESI-MS, quantified using peak area at m/z 958. The trend line was set to intercept at 0 and a line equation obtained. Error bars=1SD and n=3

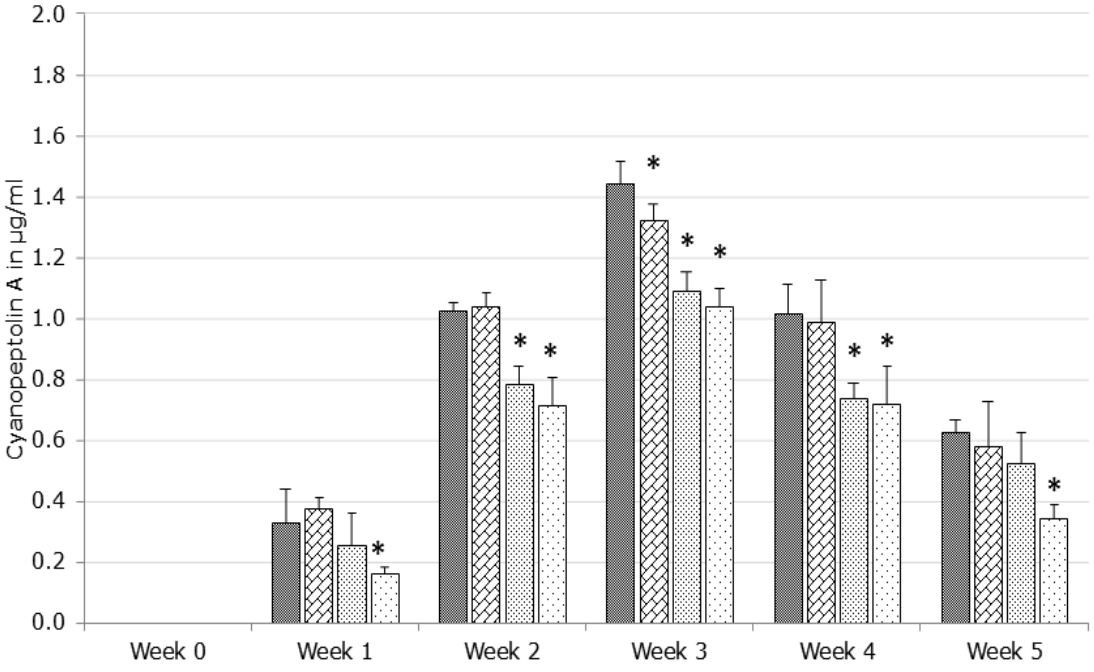


Figure 4.15 : Effect of exogenous microcystin on intracellular *de-novo* cyanopeptolin A synthesis (*m/z* – 968). *M. aeruginosa* PCC 7806 was grown in ^{15}N BG11 media and allowed to grow for 5 weeks. Error bars = 1SD, n = 4. ***** - statistically significant difference ($p<0.05$) compared to control as determined by Anova. Control 0.1 µg/ml 1 µg/ml 10 µg/ml

The intracellular *de-novo* cyanopeptolin A concentration increased until week 3, following which there was a decline (Figure 4.15). This superimposed with the cell number data seen during this period, could indicate that cyanopeptolin biosynthesis follows the growth curve. An exogenous microcystin-LR concentration of 0.1 µg/ml did not show any significant inhibition for the duration of the experiment except on week 3. However, an exogenous microcystin-LR concentration of 10 µg/ml showed significantly lower concentration of intracellular cyanopeptolin A throughout the duration of the experiment. The treatment that received 1 µg/ml exogenous microcystin-LR showed significant differences at week 2, 3 and 4. It was clearly evident that the presence of a high concentration of exogenous microcystin-LR negatively affected the intracellular concentration of the metabolite, however, it could not be concluded as to how this inhibition was brought about. It is also interesting to note that even though the cells continue to divide after three weeks, the intracellular cyanopeptolin content decreased similar to the 'live cell' numbers. This could indicate that the cells were becoming aged and leaking their intracellular contents.

Statistical analysis revealed that the presence of exogenous microcystin-LR significantly reduces the cyanopeptolin-A content and this effect was non-linearly proportional to the concentration of exogenous microcystin-LR of above 0.1 µg/ml. The 0.1 µg/ml treatment showed comparable or slightly higher concentration of intracellular cyanopeptolin, however, not significant. However, it should be noted that, as in the

earlier case, this reduction or increase seen could be due to the cell numbers and may not indicate actual reduction in total cyanopeptolin per cell. The higher treatments 1 and 10 µg/ml showed significant reductions and in particular the 10 µg/ml treatment showed significant reduction all through the five weeks of the assay. When compared to the intracellular *de-novo* microcystin-LR production, it was observed that both these metabolites followed a similar trend. This could be taken as an indication that exogenous microcystin-LR has no specific influence on any of these metabolites and may be affecting this change at a higher point in their biosynthetic pathway. It remains to be explored if the reduction seen is a result of microcystin-LR binding to a specific substrate or a particular enzyme or if it is a net non-specific effect of the high concentration of exogenous microcystin-LR.

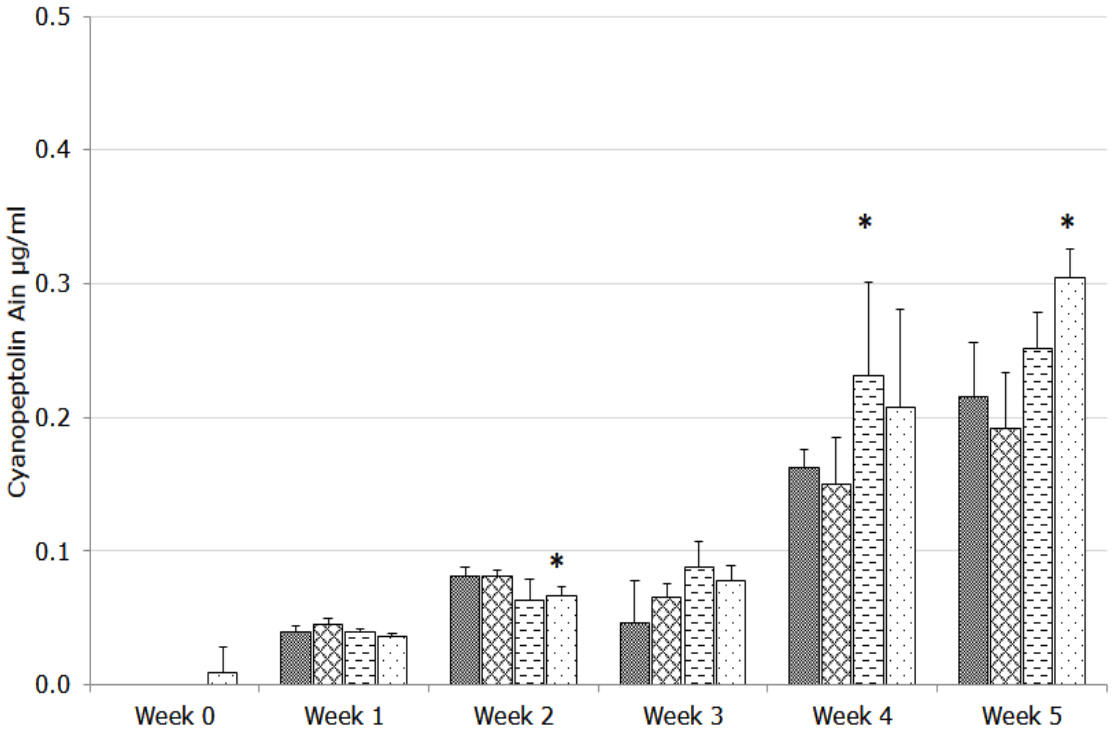


Figure 4.16: The concentrations of extracellular *de-novo* cyanopeptolin A (m/z – 968) of *M. aeruginosa* PCC7806 when exposed to exogenous microcystin. Error bars = 1SD, n = 4. * statistically significant difference compared ($p \leq 0.05$) to the control, as determined by Anova

The extracellular *de-novo* cyanopeptolin A was also tracked for 5 weeks and it was evident that the extracellular concentration did not tally with the intracellular production/release which had occurred between week 3 to 5 (Figure 4.16). This may well indicate that cyanopeptolins are unstable molecules exogenously or it may well be that *M. aeruginosa* PCC7806 may be causing the degradation. This needs to be further investigated. Another observation was that the presence of exogenous microcystin-LR does not seem to trigger the release of intracellular cyanopeptolins when the cells are in log phase, but may have contributed to its release as the cells aged.

The extracellular cyanopeptolin concentration (Figure 4.16) was also analysed for statistical significance and it appeared that the treatment 0.1 µg/ml had no effect on the release of cyanopeptolin from within the cells. The 1 µg/ml treatment had significant effect on the release of intracellular cyanopeptolin starting from week 3 onwards, however at week 5 the variability of the replicates within the control was high and hence nothing significant could be seen. From the growth curve of *M. aeruginosa* PCC7806 (Figure 4.6), it could be deduced that week 3 indicates the end of log phase growth followed by stationary growth until week 4 and cells death occurring thereafter. In treatment 10 µg/ml, the effect is seen to start as early as week 2 and continues throughout the duration of the experiment. The contradiction at week 4 could be due to high variability within the replicates of the 10 µg/ml treatment.

A comparison between the intracellular and the extracellular cyanopeptolin levels revealed that, unlike microcystin-LR, the percentage of the extracellular cyanopeptolin to the total content was only about 6% until week three after which the cells seemed to release higher amounts. Compared to microcystin-LR, it may be the differences in their relative structure or their hydrophobicity coupled with the activity of their respective transport proteins causing the differences in release (276).

Considering the intracellular *de-novo* cyanopeptolin concentration and the live cell numbers, the production rate was calculated i.e., the production per cell per day was calculated (Figure 4.17). It was calculated

that the *M. aeruginosa* PCC7806 produces cyanopeptolin A at the rate of 4 ± 2.98 fg/cell/day during its logarithmic growth phase. It is thought that this is the first report where production rate of cyanopeptolin A has been reported. One way Anova statistical analysis was performed on the production rate data and it revealed that although some significant differences were observed sporadically, a consistent pattern could not be observed. Hence it may be concluded that the presence of exogenous microcystin-LR inhibits the growth of *M. aeruginosa* PCC7806 which in turn inhibits intracellular metabolite synthesis. However, it is beyond the scope of this experiment to determine how microcystin-LR inhibits cell growth or any of its secondary metabolites. This should be investigated further and it may even reveal an intracellular binding site for microcystin-LR.

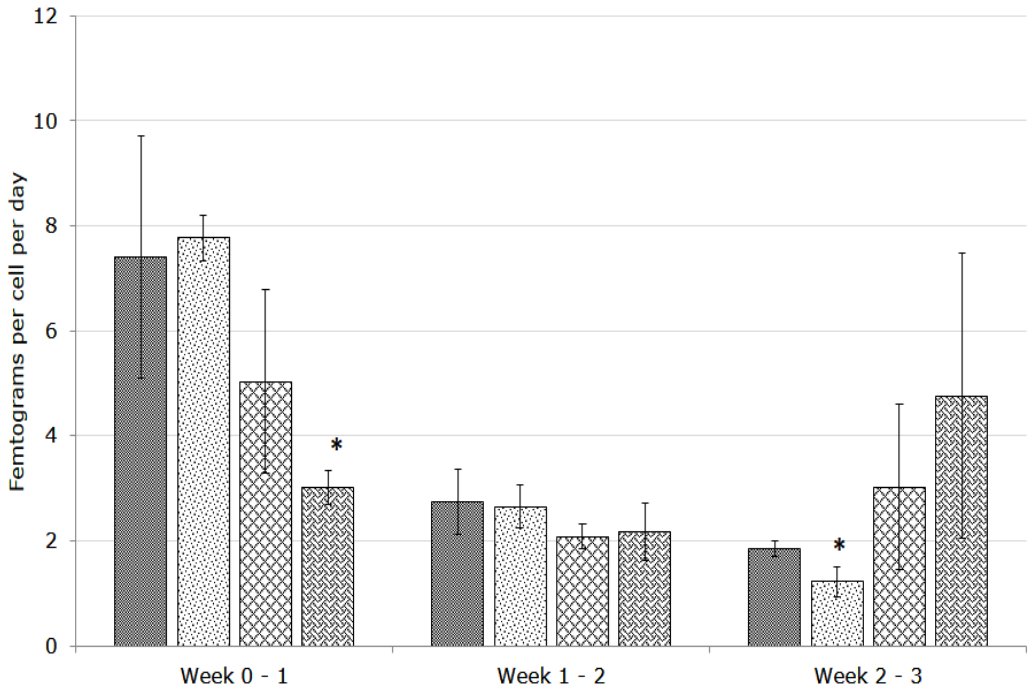


Figure 4.17: The production rate (per cell per day) of *de-novo* cyanopeptolin A (m/z 968) by *M. aeruginosa* PCC7806 in response to different concentrations of exogenously added microcystin. * - Statistically significant difference ($p \leq 0.05$) compared to that weeks control (determined by Anova).
Control 0.1 µg/ml 1 µg/ml 10 µg/ml

M. aeruginosa PCC7806 also produces cyanopeptolin B, about 25% to that of cyanopeptolin A. Its intracellular *de-novo* cyanopeptolin B concentration could be detected using HPLC-MS, however, extracellular concentration was seen to be too low for detection using this system. A quantification of cyanopeptolin B could not be performed due to non-availability of purified standards and hence only the peak areas have been reported so as to give an indication of its intracellular concentrations (Figure 4.18).

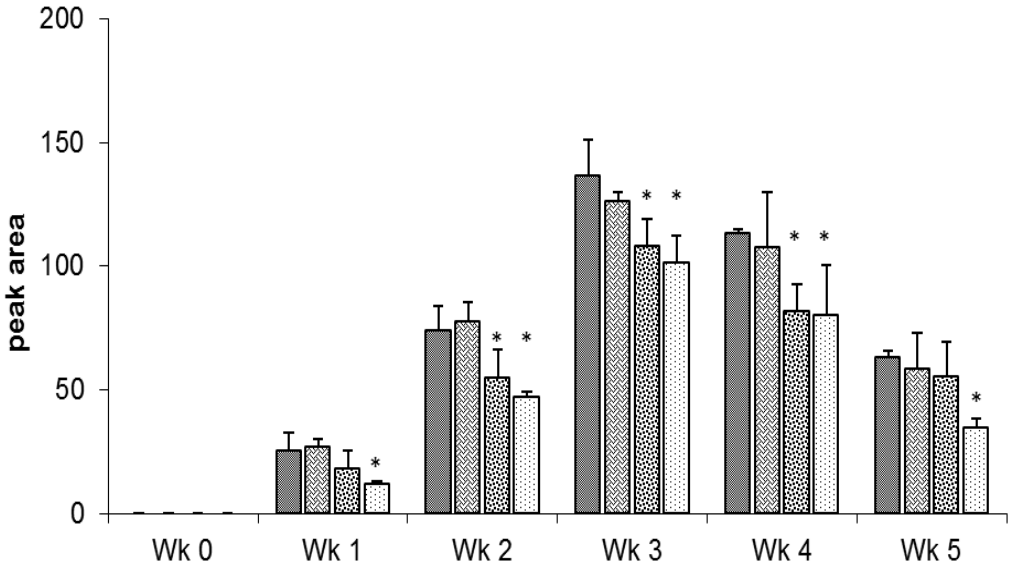


Figure 4.18: The effect of exogenous microcystin on intracellular de-novo cyanopeptolin B (*m/z 938*) synthesis. Quantification of cyanopeptolin B could not be performed due to lack of a purified standard. * - indicates statistically significant difference ($p \leq 0.05$) compared to that weeks control (determined by Anova). Error bars = 1SD, n = 4. Control 0.1 - µg/ml 1 - µg/ml 10 - µg/ml

In comparison to cyanopeptolin A, cyanopeptolin B also showed similar intracellular production ratios and hence its behaviour in response to exogenous microcystin-LR also closely resembled that of cyanopeptolin A. In a report by Tonk *et al.* [277], it is shown that at 20 °C, *M. aeruginosa* PCC7806 produced about 10% cyanopeptolin A when compared to microcystin-LR. However, in this study, it is seen that cyanopeptolin A is about 50% compared to that of microcystin-LR. This was also the case in the treatments 0.1, 1 and 10 µg/ml exogenous microcystin-LR and hence it is concluded that the presence of exogenous microcystin-LR does not significantly affect the production ratios of cyanopeptolins to microcystin-LR. It is thought that differences between the literature reported and observed values may be due to the different

media used. Although several publications have reported the isolation and characterization of cyanopeptolins [201, 164, 283], no literature on the production dynamics of cyanopeptolins by *Microcystis* sp. (or any other cyanobacteria) could be found. A report by Uwe *et al.,* estimates that cyanopeptolins may amount to roughly 0.1% of the dry weight of a bloom. However, the authors declare that since a purified standard was not available, their estimates may be inaccurate. In fact, they also add that their estimation was based on comparison with a microcystin standard in the UV mode at 225 nm absorption [284].

The organism *M. aeruginosa* PCC7806 also produces aerucyclamides A – D [162, 90] which were first identified in 2008 (Table 4.5). Unlike microcystins, aerucyclamides are thought to be synthesised ribosomally and usually contain at least one sulphur atom. Furthermore, all the aerucyclamides contains just 6 nitrogen atoms. The function of aerucyclamides is still being investigated, however, potential commercial uses for aerucyclamides as antiplasmodial as well as antitrypanosomal agents have been demonstrated [278].

Table 4.5: The mass of aerucyclamides of *M. aeruginosa* PCC7806 when grown in BG11 (^{14}N) and BG11 (^{15}N)

Compound	*m/z* in BG11 (^{14}N)	*m/z* in BG11 (^{15}N)
aerucyclamide A	535	541
aerucyclamide B	533	539
aerucyclamide C	517	523
aerucyclamide D	587	593

In order to quantify aerucyclamides, a standard curve was drawn using known concentrations of purified aerucyclamide A taking the concentration on x axis and peak area on y axis (Figure 4.19).

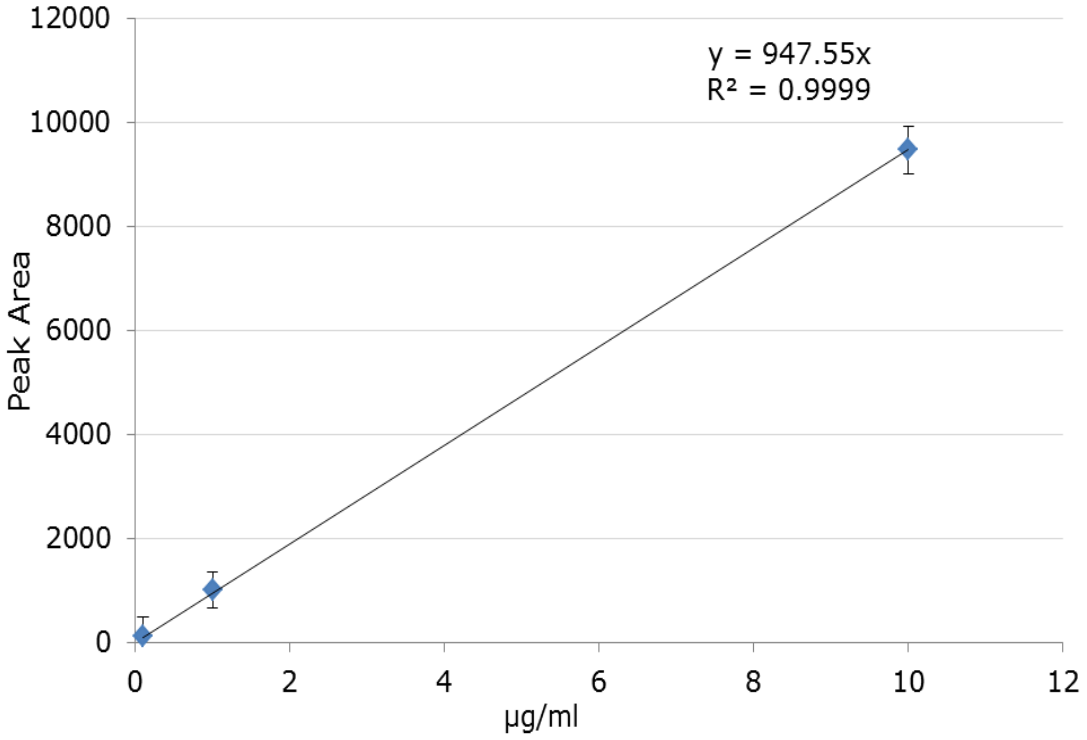


Figure 4.19: Standard curve of aerucyclamide A (m/z 535) as measured using HPLC-MS/MS. Purified aerucyclamide A was taken in 80 % methanol in concentrations of 0.1, 1 and 10 µg/ml. Error bars = 1 SD and n = 4

The concentration of the intracellular and extracellular *de-novo* aerucyclamide-A was deduced using the line equation derived from the standard curve. The graph revealed that the intracellular aerucyclamide concentration was inhibited by exogenous microcystin-LR concentrations higher than 1 µg/ml while concentrations of up to 0.1 µg/ml had no observable effect (Figure 4.20). Here again, the pattern of synthesis closely followed the growth curve i.e., increased production until week 3, following which the net production reduced. Statistical analysis was performed on each week comparing the 3 treatments individually to the control. The analysis revealed that the presence of up to 0.1 µg/ml microcystin-LR did not have any significant effect on the intracellular aerucyclamide concentration while concentrations higher than 1 µg/ml demonstrated significant effect. A key observation here is that, although aerucyclamide biosynthesis is different i.e., ribosomal instead of non-ribosomal, its pattern of biosynthesis as well as response to exogenous microcystin-LR remains similar to the other metabolites.

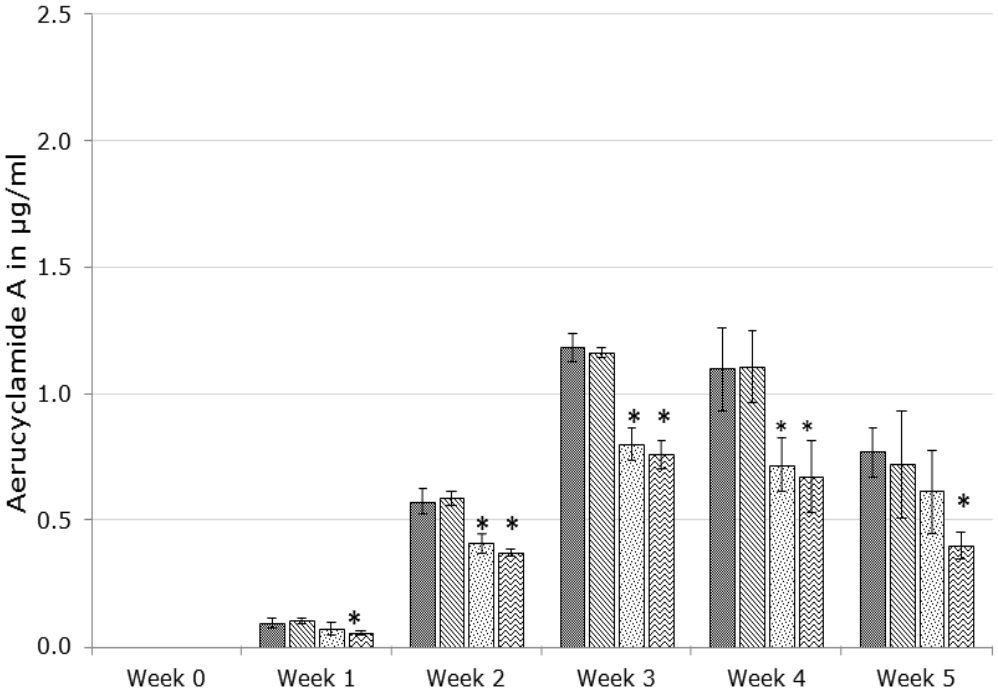


Figure 4.20 : Effect of exogenous microcystin on intracellular *de-novo* aerucyclamide A synthesis (m/z - 541). *M. aeruginosa* PCC 7806 was grown in ^{15}N BG11 media and allowed to grow for 5 weeks. Error bars - 1SD, n-4. * statistically significant difference ($p \leq 0.05$) compared to the control, as determined by Anova.

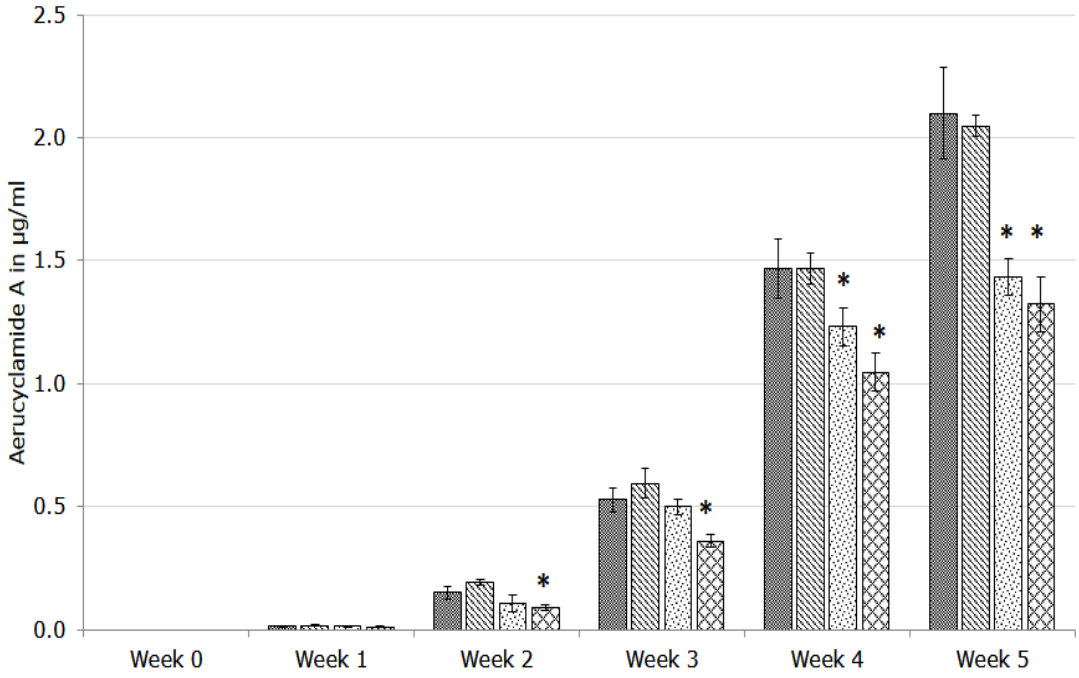


Figure 4.21: The concentrations of extracellular *de-novo* aerucyclamide A (m/z – 968) of *M. aeruginosa* PCC7806 when exposed to exogenous microcystin. Error bars = 1SD, n = 4. [* statistically significant difference compared ($p \leq 0.05$) to the control, as determined by Anova]

Statistical analysis was also performed on the quantities of extracellular aerucyclamide-A and it appeared that the presence of up to 1 µg/ml exogenous microcystin-LR did not have any significant effect on the release of intracellular aerucyclamide. Whereas, an exogenous microcystin-LR concentration of above 1 µg/ml may have a significant effect on the release of intracellular aerucyclamides and this effect could be seen as early as from week 2 onwards. However, when comparing the percentage extracellular to the total aerucyclamide, it revealed that the presence of exogenous microcystin-LR had no net effect on the release or intracellular aerucyclamides (Table 4.6).

Table 4.6: The percentage of extracellular aerucyclamide A to the total pool. Paired t-test was performed between the control and the three treatments for 5 weeks

% Extra	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	P-value
Control	12.89	21.21	31.21	57.58	73.04	ND
0.1 µg/ml	14.19	25.10	34.62	57.24	72.29	0.188
1 µg/ml	15.07	21.52	39.08	63.29	72.34	0.1314
10 µg/ml	16.33	20.01	33.03	60.98	76.72	0.0723

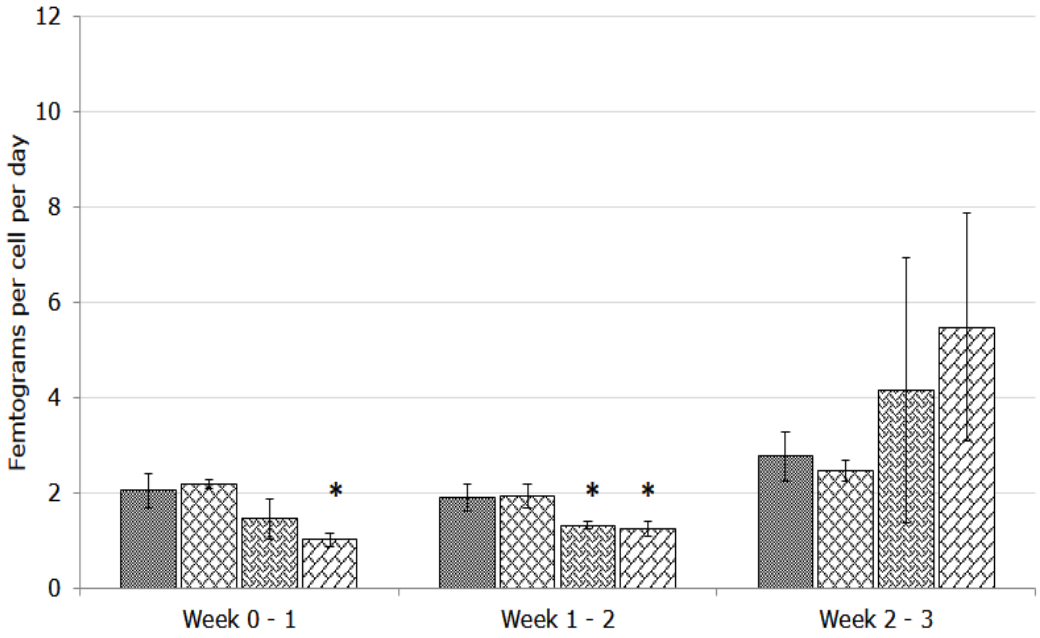


Figure 4.22: The production rate of *de-novo* aerucyclamide A in response to exogenously added microcystin. * - indicates statistically significant ($p \leq 0.05$) compared to the control, as determined by Anova. Error bars = 1SD, n = 4. Control 0.1 µg/ml 1 µg/ml 10 µg/ml

The production rate of intracellular aerucyclamide A was calculated (Figure 4.22) and the overall rate of aerucyclamide A was 2.24 ± 0.46 fg/cell/day. It also revealed that the presence of exogenous microcystin-LR may inhibit the intracellular aerucyclamide A production at least when the microcystin-LR concentration is as high as 10 µg/ml. The discrepancy seen at week 3 may be explained by the decrease in cell numbers at that week when compared to the control.

Aerucyclamide B is an oxidative derivative of aerucyclamide A with a difference of just 2 hydrogen atoms [162] and amounting to just 8% of aerucyclamide A. However, aerucyclamide C and D were only 35% and 28% to that of aerucyclamide A. Even in the presence of exogenous microcystin-LR, the production ratios remained unchanged i.e., exogenous microcystin-LR had no specific effect on the production of any of these metabolites. It should be noted that the quantification of aerucyclamide B and C could not be performed, however, a purified standard for aerucyclamide D was available and this peptide was quantified. The intracellular *de-novo* aerucyclamide D profile revealed that exogenous microcystin-LR concentrations of above 1 µg/ml were inhibitory to the synthesis of intracellular aerucyclamide D (Figure 4.24). Again, the overall intracellular aerucyclamide D profile followed the growth curve of the organism.

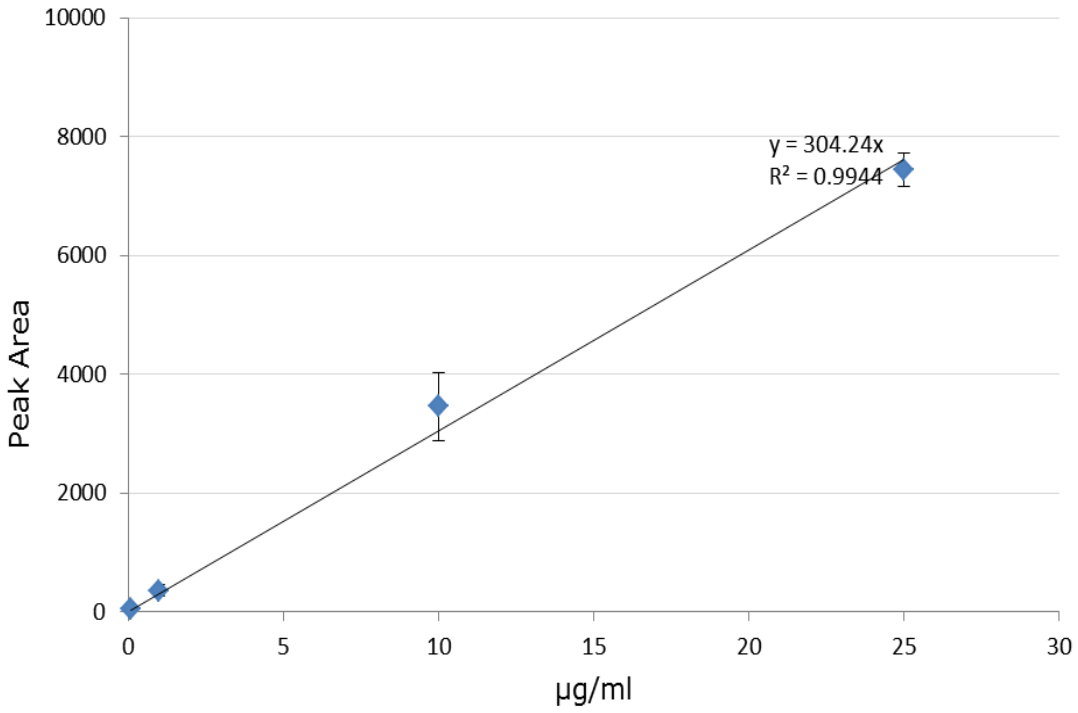


Figure 4.23: Standard curve of aerucyclamide D (m/z 587) measured using HPLC-MS/MS. Purified aerucyclamide D was taken in 80 % methanol in concentrations of 0.1, 1 10 and 25 µg/ml. Error bars = 1 SD and n = 4

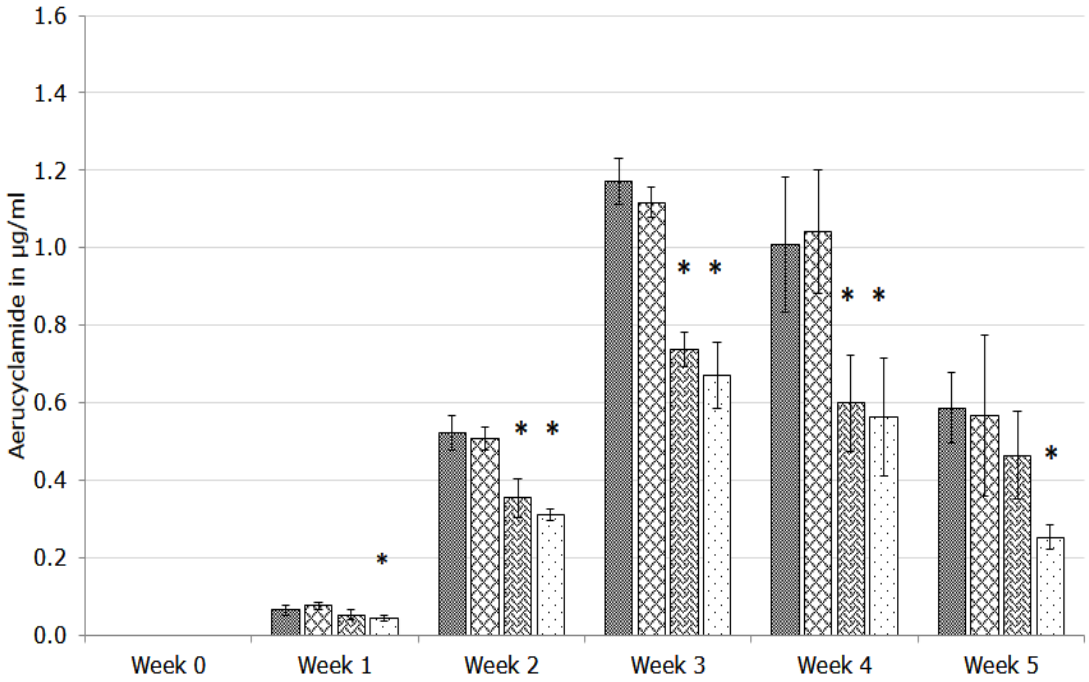


Figure 4.24 : Effect of exogenous microcystin on intracellular aerucyclamide D synthesis (m/z - 593). *M. aeruginosa* PCC 7806 was grown in ^{15}N BG11 media and allowed to grow for 5 weeks. Error bars - 1SD, n-4. * statistically significant difference ($p \leq 0.05$) compared to the control, as determined by Anova.

It was also noted that the concentrations of aerucyclamide D and A were similar to each other i.e 1.1 µg/ml at week 3 and hence it may be concluded that *M. aeruginosa* PCC7806 produces aerucyclamides A & D in similar concentrations. However, the extracellular aerucyclamide D profile was unlike that of aerucyclamide A but closely resembled the pattern of cyanopeptolins i.e it seemed that aerucyclamide D was stable intracellularly whereas unstable exogenously (Figure 4.25).

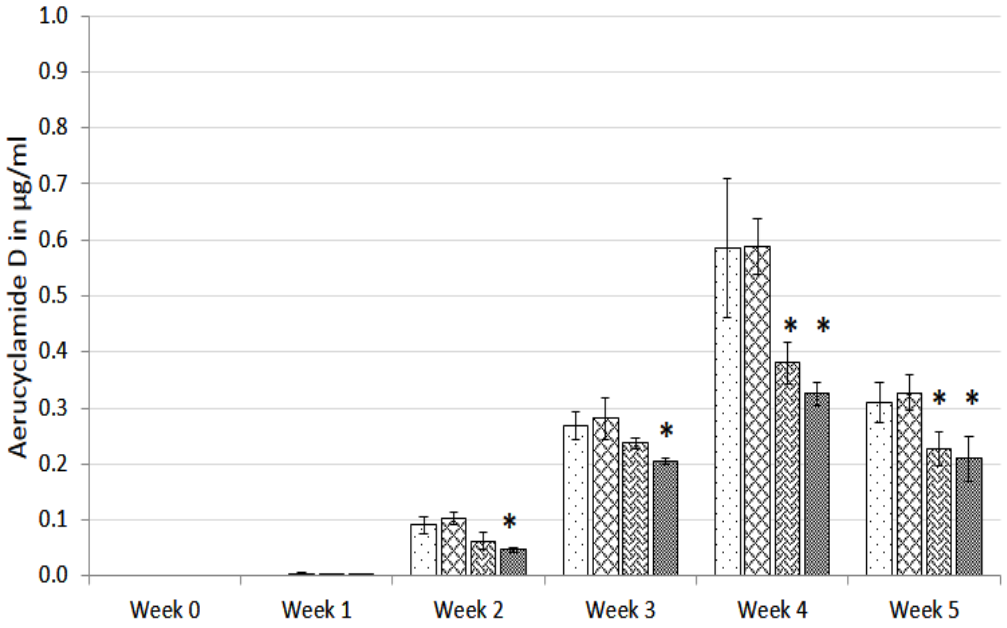


Figure 4.25 : The concentrations of extracellular *de-novo* aerucyclamide D (m/z - 593) of *M. aeruginosa* PCC7806 when exposed to exogenous microcystin. Error bars - 1SD, n-4. * statistically significant difference ($p \leq 0.05$) compared to the control, as determined by
□Control ▨0.1 µg/ml ▨1 µg/ml ▩10 µg/ml

Considering the intracellular *de-novo* aerucyclamide D and the live cell numbers, the production rate of aerucyclamide D was calculated. It was estimated that *M. aeruginosa* PCC7806 produces aerucyclamide D at the rate of 2.03 ± 0.77 fg/cell/day. The production also revealed that presence of exogenous microcystin-LR may significantly inhibit the biosynthesis of aerucyclamide D (Figure 4.26). The ambiguity observed at week 3 may merely be due to the effect of the decline in cell numbers of the treatments when compared to the control.

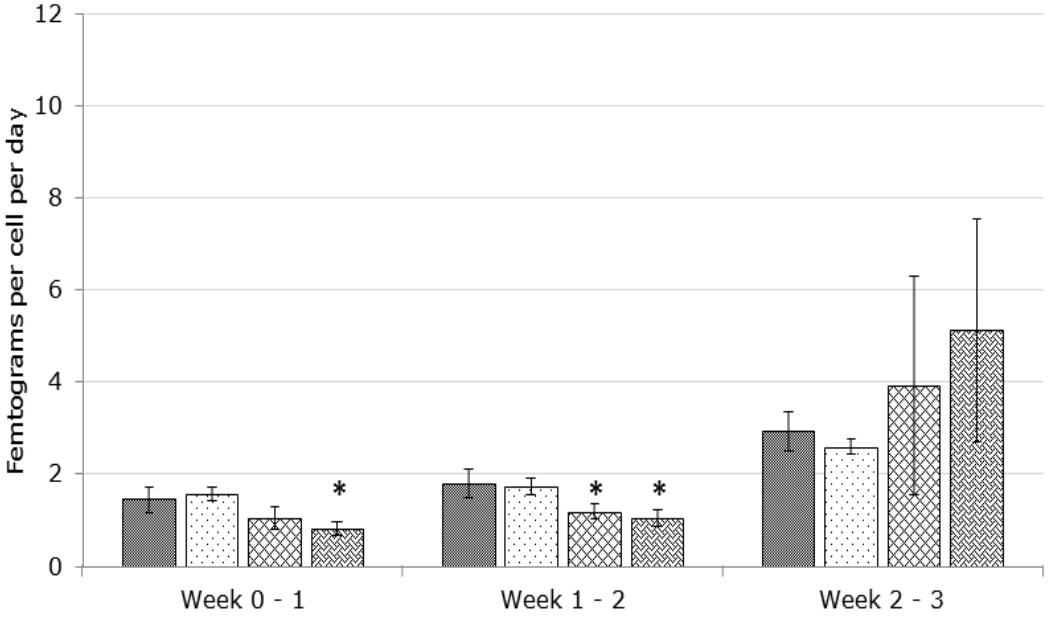


Figure 4.26 - The production rate (per cell per day) of aerucyclamide D was calculated for 5 weeks. Beyond week 3 no net increase in production was observed. Error bars=1SD, n=4. Statistical analysis was performed using Anova and * - indicates $p < 0.05$.

An interesting observation was made when the percentage of extracellular metabolite to that of the intracellular metabolite was calculated. At week 5, cyanopeptolin A and aerucyclamide D were the two metabolites whose extracellular concentrations were about 39% and 53% of their respective intracellular concentrations. The other metabolites such as microcystin-LR, aerucyclamide A, B and C were higher than their respective intracellular concentrations i.e., 176%, 272%, 158% and 142% respectively. From this, it could be concluded that microcystin-LR, aerucyclamides A, B and C are not assimilated or degraded by *M. aeruginosa* PCC7806, however, contrary to the belief that these are (at

least microcystin-LR) predominately intracellularly stored molecules a significant quantity of these metabolites are released to the extracellular environment. The cyanopeptolins A, B and aerucyclamide D, although their extracellular concentration is about 50% that of their intracellular content at week 5, the question arises as to what would have happened to the quantities produced during the previous weeks. It may be that these metabolites are stable when inside the cell, but once secreted are unstable or may have been degraded.

Aerucyclamides are relatively newly characterised compounds and very little data is available on their production dynamics. The interest in aerucyclamides is increasing as they have potential application (or at least their derivatives) as antimalarial compounds [278]. Also, as aerucyclamides are ribosomally synthesised compounds and their gene sequence recently elucidated, they may be useful in studying cyanobacteria at the transcriptional level [209]. In this view, the findings of this study may serve as a measure of aerucyclamide A and D production rate under optimal conditions, from which efficiency of genetically modified clones could be ascertained.

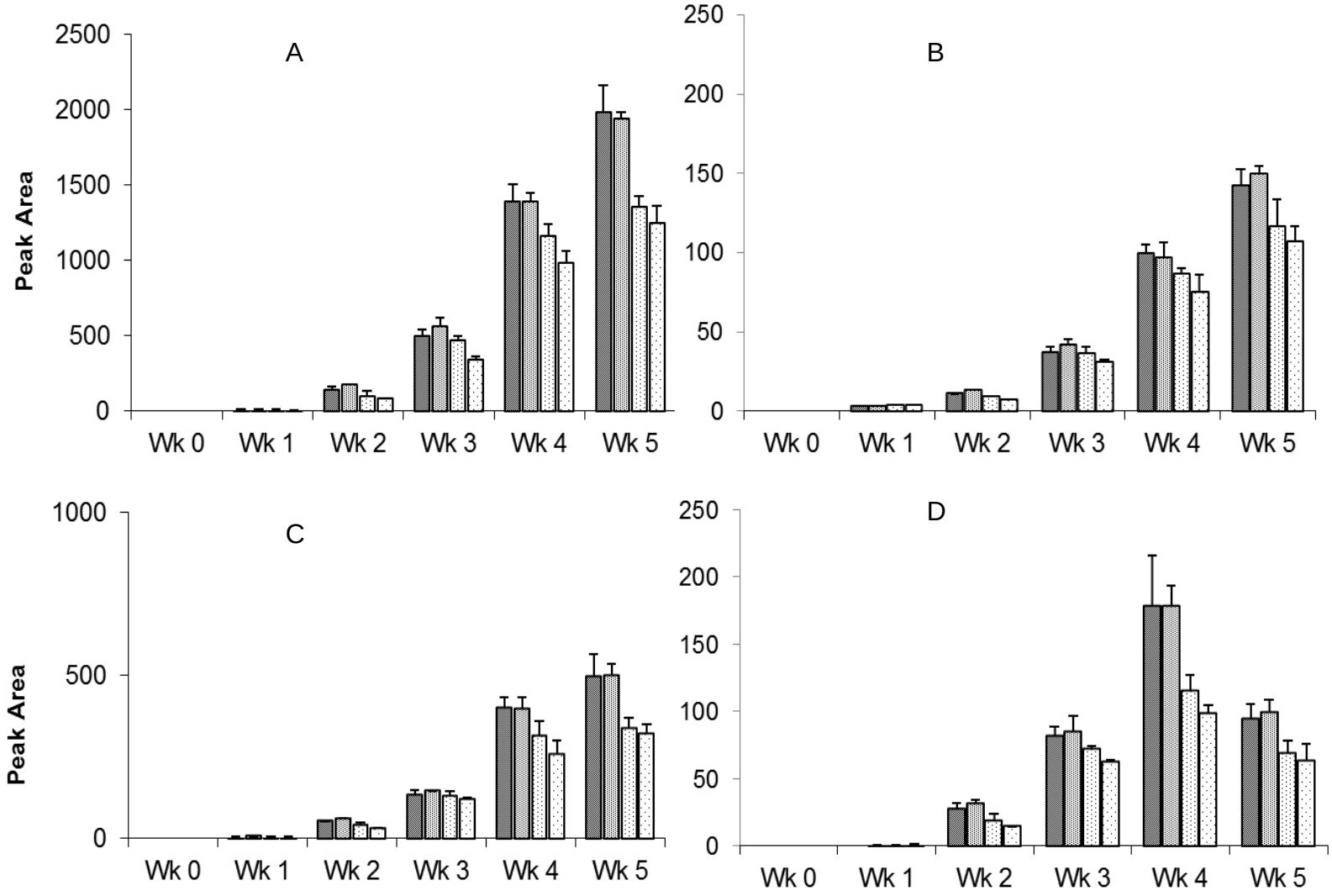


Figure 4.27: The concentrations of extracellular *de-novo* aerucyclamides A (m/z 541), B (m/z 539), C (m/z 523) and D (m/z 593) of *M. aeruginosa* PCC7806 when exposed to exogenous microcystin. Error bars = 1 SD and n=4. Only the peak areas are shown as a means of comparison.

4.5 Discussion

The aim of this study was to evaluate if microcystin-LR functions as a signalling molecule to its host organism *M. aeruginosa* PCC7806. The effect of exogenous microcystin-LR on the growth of *M. aeruginosa* PCC7806 and its secondary metabolite production was studied. The growth curve of *M. aeruginosa* PCC7806 showed similar trends compared to literature values i.e., log phase growth was until day 21 in a static culture system, however, between reported results and the data observed here, there were differences such as media used, light intensity, temperature and aeration and hence data could not be readily compared [258, 94, 161].

The use of flow cytometry on *M. aeruginosa* PCC7806 has proved to be a reliable tool to enumerate and differentiate between healthy and unhealthy cells. Hence, it is recommended that this protocol be adapted for further studies on this organism. This protocol may also be extrapolated for the study of other pigmented unicellular cyanobacteria. With the aid of the flow cytometric technique, the effect of exogenous microcystin-LR on the growth of *M. aeruginosa* PCC7806 was studied with special interest on the live cell population. The results indicated that exogenous microcystin-LR concentrations of above 1 µg/ml may have a significant effect on the growth of *M. aeruginosa* PCC7806. It could also be stated that exogenous microcystin-LR does not cause cell death, but may serve to prolong the generation time significantly. Such an effect

may be brought by the interference of localised microcystin-LR within thylakoid membranes and around polyphosphate bodies [270], however this needs to be confirmed with further studies. It is recommended that, similar future studies be coupled with the analysis of one other parameter reflective of the cells metabolism i.e., photosynthetic activity, in order to precisely evaluate the effect of exogenous microcystin-LR.

In respect to the intracellular microcystin content, literature values report a concentration of 1.5 µg/ml microcystin-LR at day 20 whereas about 2.6 µg/ml at day 21 was observed here. Further, the extracellular microcystin content is 10% compared with the intracellular content [214], whereas here it is 23% at day 21 and 176 % at day 35. These differences could again be attributed to the difference in culture conditions, inoculum age and nitrate content of the media, however, as the organism in comparison is *M. aeruginosa* PCC7806 it could be argued that the only comparable value could be the microcystin production rate which is 5.6 ± 1.6 femtograms/cell/day which is broadly corroborated in several publications [221, 94, 279]. Another interesting feature of this organism was highlighted i.e. the organism seemed to produce 81% of Demethy-microcystin-LR when compared to microcystin-LR, however, in literature it has been reported that Demethy-microcystin-LR is mostly about 6% of microcystin-LR produced [279]. Although some reports suggest that certain *Microcystis aeruginosa* blooms may contain Demethyl-microcystin-LR ranging from 16 to 220% of the microcystin-LR in lower pH conditions [280], such a finding for *M. aeruginosa* PCC7806 has not been previously

reported. It remains to be explored if any of the culture conditions used, such as light, nitrate or continuous illumination may have caused the high production of Demethyl-microcystin-LR. It also is unknown if Demethyl-microcystin-LR is an intermediate in the biosynthesis of microcystin-LR or vice versa. However, such investigations are beyond the scope of this experimental setup.

The use of stable labelled nitrogen as the only source of nitrogen enabled accurate quantification of *de-novo* production of all the secondary metabolites of *M. aeruginosa* PCC7806. This comes as an invaluable technique to differentiate between exogenously added microcystin-LR and the newly produced microcystin-LR i.e. the production dynamics of microcystin-LR could be monitored. By the use of this technique, it was clearly evident that the concentration exogenously added microcystin-LR remained almost unchanged throughout the duration of the experiment i.e. 35 days. This confirmed that *M. aeruginosa* PCC7806 does not catabolise the produced microcystin and that it was stable about at least one month in the conditions tested here. An intriguing question arises as to why an organism should produce a compound that it does not utilize. It also remains to be evaluated if this is the fate of the other secondary metabolites of *M. aeruginosa* PCC7806.

The production of *de-novo* microcystin-LR followed the growth and hence it could be stated that the presence of exogenous microcystin-LR does not significantly inhibit the biosynthesis of microcystin-LR, although

initial suppression of production could be seen. A recent metabolomics analysis has reported that wild type *M. aeruginosa* PCC7806 is able to quickly adapt to saturated light conditions owing to its ability to synthesis microcystin. Further they also conclude that a microcystin deficient mutant accumulates stress markers in a similar situation, indicating that microcystin may function as a stabilizing agent under stressful conditions [281]. This conclusion fits well within these findings where it is not possible to find a specific role for microcystin as the cultures were not stressed. Alternatively, it is not understood why high concentrations of exogenous microcystin-LR should significantly suppress growth. If this is argued as a direct effect of stress, it would mean that the high exogenous microcystin-LR had overwhelmed the cells and ultimately suppressed growth. The 0.1 µg/ml exogenous microcystin-LR consistently showed mildly beneficial effects on growth which may be an indication of microcystin-LR acting as a stabilizing molecule, however, as the effect was not significant, it may only be stated that this hypothesis needs to be evaluated further. It is thought that due to diminished intracellular activity, the production is delayed rather than inhibited. It is noted that microcystin-LR and the other secondary metabolites of *M. aeruginosa* PCC7806 are produced by and NRPS/PKS enzyme complex and hence quantification of these may not strictly reflect the intracellular metabolic state of the organism. Hence it would be a wise choice to include other parameters which are tightly bound to the metabolic process to evaluate the effect of microcystin-LR on the metabolic state of the organism.

The quantification of intracellular secondary metabolites may reveal the effect of exogenous microcystin-LR on their production dynamics while, the quantification of the extracellular *de-novo* secondary metabolites may shed some light on the release of the secretion of these metabolites. This is thought to be the first study to report the production rates of several of the metabolites of *M. aeruginosa* PCC7806 (Table 4.7). As seen from this study, the presence of exogenous microcystin-LR does not have any net effect on the release of intracellular metabolites. It is also seen that the release of metabolites is directly related to its intracellular concentration.

Table 4.7: The daily production rates of few of the metabolites of *M. aeruginosa* PCC7806

Metabolite	fg/cell/day
microcystin-LR	5.6 ± 1.6
Cyanopeptolin A	4.0 ± 2.9
Aerucyclamide A	2.2 ± 0.5
Aerucyclamide D	2.0 ± 0.7

All the secondary metabolites of *M. aeruginosa* PCC7806 i.e., microcystin-LR, cyanopeptolins and aerucyclamides behaved with similar production and release dynamics. Hence, it is concluded that the presence of only high exogenous microcystin-LR has a significant effect on the production of any of *M. aeruginosa* PCC7806 secondary metabolites. Interestingly, it was noted that while microcystin-LR, aerucyclamides A, B

and C are not assimilated or degraded by *M. aeruginosa* PCC7806, the cyanopeptolins A, B and aerucyclamide D, although appearing stable when inside the cell, are unstable, once secreted.

In conclusion, although this study was robust in it design to expose *M. aeruginosa* PCC7806 to very high concentrations of one of its secondary metabolites (microcystin-LR), the response of the organism was only mild and temporary. This observation does not confirm or negate the hypothesis that microcystins may have a signalling function. The fact that, unlike some secondary metabolites that are unstable once outside the cell, microcystin-LR is stable for prolonged period of time indicates that this molecule was synthesised with a specific function. Whether this molecule serves a functional role to M. aeruginosa PCC7806 or another organism in its vicinity, needs to be investigated in future studies.

Chapter V

The effect of cyanopeptides on *M. aeruginosa* PCC7806

5.1 Introduction

Cyanobacteria are prolific producers of secondary metabolites [286]. Due to the varied ecosystems these cyanobacteria inhabit, it is thought that challenging environmental conditions as well as competition pressures have influenced cyanobacteria to produce a wide variety of secondary metabolites [287, 288] ranging from small peptides to polyketides to alkaloids [289]. However, there is considerable debate as to the primary function of any of these metabolites. While several hypotheses such as colony survival, cellular fitness and dissuasion of predators exist, none of these have been convincingly proven.

One way of evaluating the functional role of cyanopeptides is by exposing these peptides to a cyanobacterium, which has been well characterised, and studying the effects of the peptides on its growth/metabolic characteristics [6, 290]. The organism *M. aeruginosa* PCC7806 fits the purpose as this is one of the most studied cyanobacterium owing to its capability to synthesis a wide variety of secondary metabolite synthesis capability. In this study, *M. aeruginosa* PCC7806 was exposed to an array of cyanopeptides and its growth, secondary metabolite production rate and gene expression patterns were studied. The range of peptides chosen for this study was microcystins (microcystin-LR, microcystin-LF and microcystin-RR), anabaenopeptins (A & B), aerucyclamides (A & D), aeruginosin, aeruginosamide and cyanopeptolin A. It should be noted that *M. aeruginosa* PCC7806 in itself

produces cyanopeptides such as microcystin-LR, cyanopeptolins, aeruginosin and aerucyclamides.

One of the most frequently encountered and widely studied cyanopeptides are the microcystins. These hepatotoxic heptapeptides are produced by several genera such as *Microcystis*, *Nostoc, Oscilatoria, Planktothrix and Anabaena sp* [291, 292]. To date, nearly 100 different variants have been reported, mostly arising from the substitution of different amino acids at position two and four of the molecule [293]. Recently, it was reported that a single species (*Microcystis viridis* NIES 102) could produce up to 47 microcystin variants [294]. Although highly variable, all microcystins have a common unique β-amino acid Adda (3*S*-amino-9*S*-methoxy-2*S*,6,8*S*-trimethyl-10-phenyldeca-4*E*,6*E*-dienoicacid), which is known to impart the molecules characteristic hepatotoxicity [295].

The toxin microcystin-LR is one of the most commonly encountered cyanopeptides and along with microcystin-RR (microcystin-RR) accounts for nearly 45 to 99% of total microcystin concentration in certain natural blooms [296, 297]. Another isomer of microcystin-LR, Demethylated-microcystin-LR (D-MeAsp at position 3 is demethylated to Asp), is also commonly encountered within strains that produce microcystin-LR [298]. Microcystin-RR (Figure5.1a), a variant of microcystin-LR, wherein the L-amino acid at position 2 is replaced by arginine (R) is also sometimes encountered along with microcystin-LR [299].

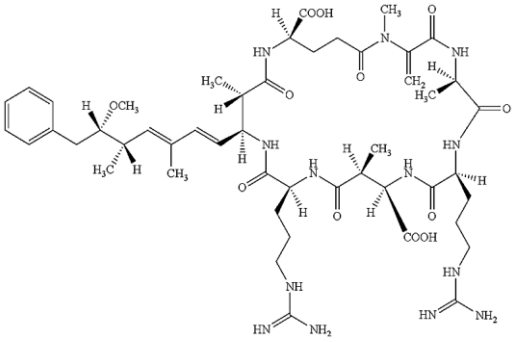


Figure 5.1a: Microcystin-RR

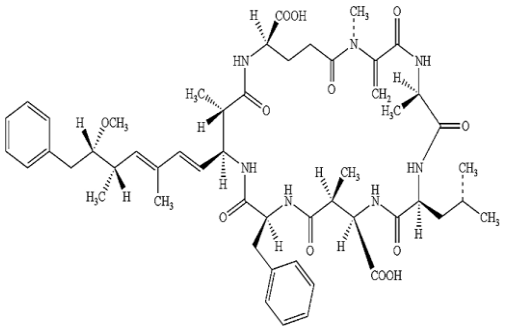


Figure 5.1b: Microcystin-LF

In terms of toxicity, microcystin-LR is one of the most toxic microcystin with an LD_{50} of 50 nmol/kg while that of microcystin-RR is 580 – 630 nmol/kg when tested intraperitoneally on mice [300, 301]. Another microcystin variant, microcystin-LF (phenylalanine at position 4, Figure 5.1b) is one of the least studied and no *in-vivo* data is available to date, however, a recent *in-vitro* study reports its toxicity to be 7 times higher than microcystin-LR to primary human hepatocytes [302].

Like microcystins, anabaenopeptins are also protein phosphatase inhibitors and were first reported by Harada *et al* In 1995 [303, 304]. In recent years, anabaenopeptins have also been reported in *Planktothrix, Aphanizomenon, Nodularia* and *Microcystis*. Anabaenopeptins are cyclic peptides with a conserved lysine at position 5 and a unique ureido bond between the amino acid and the ring (Figure 5.2) [305]. It has also been reported that certain anabaenopeptins have potent carboxypeptidase A inhibitory activities [306].

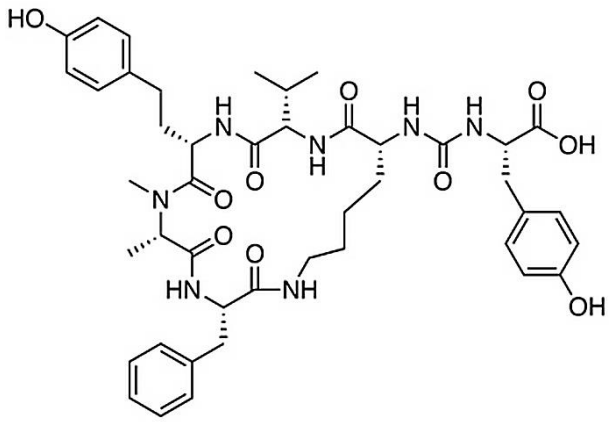


Figure 5.2: Structure of Anabaenopeptin A, chemical formula $C_{44}H_{57}N_7O_{10}$. Mol wt – 843.96

Another class of compounds, the aerucyclamides (Figure 5.3) were only very recently isolated and characterised from *M. aeruginosa* PCC7806 [162]. Compared to microcystins, these were an order less toxic, but interestingly, they showed promising and selective activity against malarial parasite (aerucyclamide B, IC_{50} = 0.7 μM) and Human African Trypanosomiasis (HAT) (aerucyclamide C, IC_{50} = 9.2 μM) [307]. Unlike most cyanopeptides, aerucyclamides are thought to synthesised ribosomally and hence may be used as tools to study the metabolic profile of certain cyanobacteria [90].

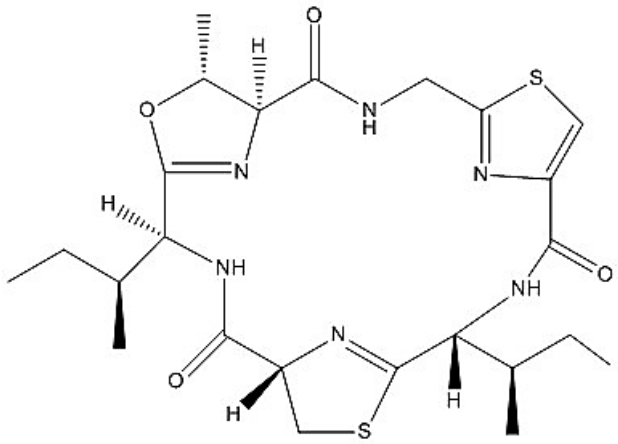


Figure 5.3: Structure of Aerucyclamide A, $C_{24}H_{34}N_6O_4S_2$, Mol wt – 534.69 Da

The organism *M. aeruginosa* PCC7806 is also known to produce the linear peptide, aeruginosin (Figure 5.4), which has been shown to inhibit serine protease [217]. Although, aeruginosins were initially thought to be restricted to *Microcystis*, recent reports suggest that species such as *Planktothrix* and *Nodularia*, may also synthesize its congeners [308]. Chemically, aeruginosins are unique in that they contain a central Choi moiety (2-carboxy-6-hydroxyoctahydroindole) and Hpla ((4-hydroxy) phenyl lactic acid) moieties [309]. In terms of toxicity, aeruginosins are considered to be only slightly less toxic than microcystins but additionally show potent inhibitory activities against thrombin (IC_{50} = 21.8 nM) and trypsin (IC_{50} = 112 nM). Currently aeruginosins are being investigated for their potential applications in life threatening conditions such as venous thrombosis.

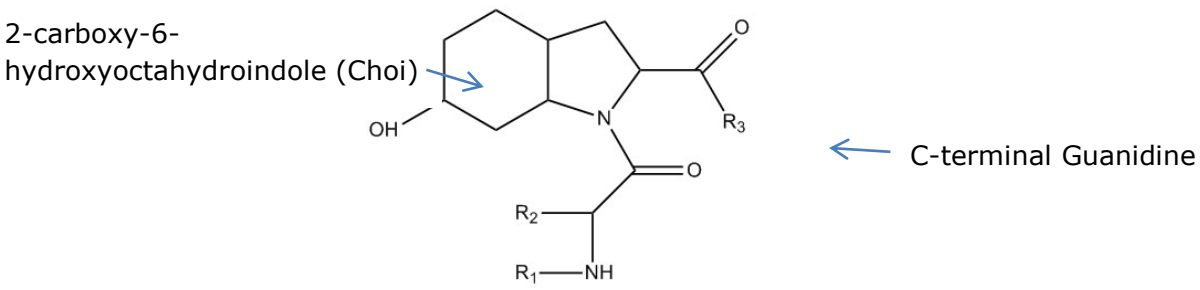


Figure 5.4: General structure of aeruginosins. R1 – N-terminus, R2 – hydrophobic amino acid

Another unique linear peptide, aeruginosamide (Figure 5.5), has also been reported to be produced by *Microcystis,* although very little literature is available on the molecules functional properties. Aeruginosamides are characterised by the presence of diisoprenylamine and the carboxylated thiazole moieties which are not reported in other cyanobacterial metabolites [310]. However, like aerucyclamides, aeruginosamides are also ribosomally synthesised and hence may prove potentially useful in studying cyanobacterial metabolic phases [311].

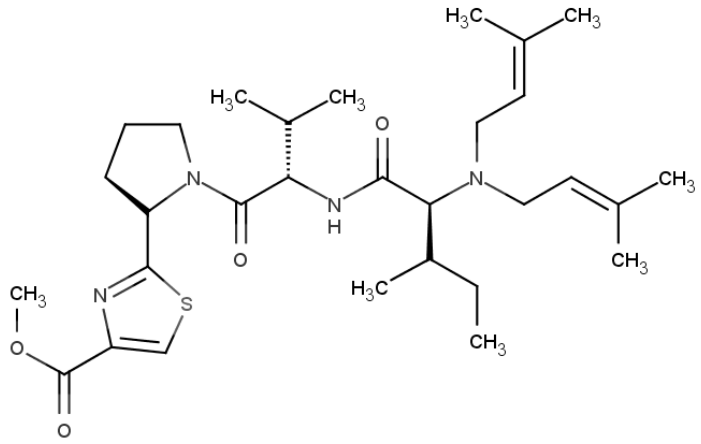


Figure 5.5: Structure of Aeruginosamide, $C_{30}H_{48}N_4O_4S$, Mol wt – 560.79 Da

The organism *M. aeruginosa* PCC7806 also produces several cyclic depsipeptides called cyanopeptolins (Figure 5.6) [163]. These are molecules that contain the structural element AHP (3-amino-6-hydroxy-2-piperidone), and show potent serine protease activity at micromolar concentrations [201]. Apart from *Microcystis*, cyanopeptolins are also known to be produced by *Planktothrix* and *Scytonema hofmanii* [312]. Although initially cyanopeptolins were thought be to non-toxic, recent evidence suggest that cyanopeptolins are potent inhibitors of trypsin (IC_{50} = 670 picomoles), human kallikerin (4.5nM) and factor Xia (3.9nM), while micromolar concentrations were required for inhibition of plasmin (0.49 μM) and chymotrypsin (0.9 μM) [313].

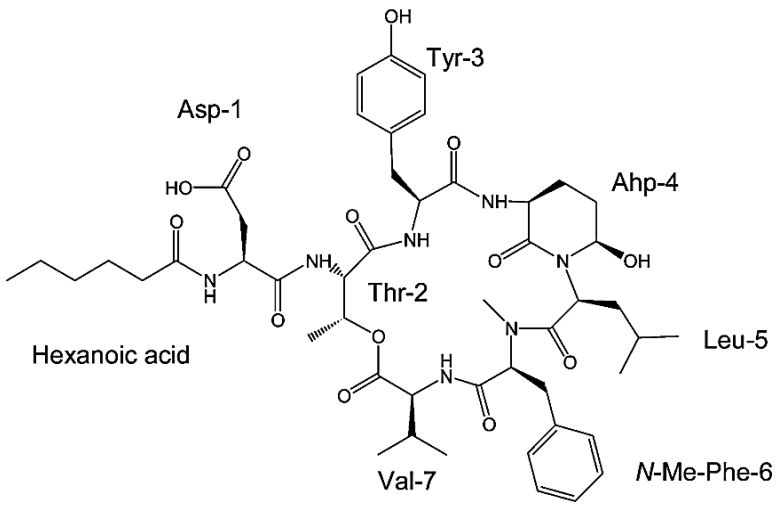


Figure 5.6: Cyanopeptolin 963A, $C_{49}H_{69}N_7O_{13}$, Mol wt – 963.48

Although cyanobacteria are a rich source of unique metabolites, very little is known about their production dynamics, and even less about why they produce such unique compounds. A single organism such as *M. aeruginosa* PCC7806 is known to produce 4 classes of unique peptides i.e., microcystins (microcystin-LR, demethyl-microcystin-LR), aeruginosins, aeruginosamides (A-D) and cyanopeptolins (A-D, 963A) [6]. Considering the conserved nature of the corresponding genes as well as the energy expenditure diverted towards the production of these peptides, it is highly unlikely that these peptides are remnants of ancient genes. This study was aimed to evaluate the effect of several of these cyanopeptides on the growth and peptide production of dynamics of *M. aeruginosa* PCC7806. It is hoped that this study may reveal the subtle changes induced by these peptides on its host and may shed some light

on the principle reason of what cyanobacteria stand to benefit from these peptides.

5.2 Aim

To evaluate the effect of cyanopeptides on *M. aeruginosa* PCC7806

5.2.1 Objectives

1) To determine the effect of exogenously added cyanopeptides namely, microcystin-LR, microcystin-RR, microcystin-LF, aeruginosin, aerucyclamide A and D, cyanopeptolin A, aeruginosamide, anabaenopeptin A and B, on the growth of *M. aeruginosa* PCC7806.
2) To study the effect of these cyanopeptides on the secondary metabolites production of *M. aeruginosa* PCC7806.
3) To evaluate the effect of these cyanopeptides on expression of genes *mcy A* and *mcy E* of microcystin-LR biosynthesis cassette.

5.3 Materials and Methods

5.3.1 Culture and growth conditions

The culture and growth conditions were as described in section 2.1 and 2.3. The experimental setup was as described in section 2.4.2. The preparation of various peptides solution was as described in section 2.5

5.3.2 Sampling and sample processing

Samples were withdrawn on day 0 and 10. On the sampling day, 1.5 ml of the culture was aseptically withdrawn from each flask and 0.5 ml used for cell counting and the remaining 1 ml was used for LC-MS analysis.

5.3.3 Cell counting

Cell counting was performed using haemocytometer using the protocol described in section 2.6.1

5.3.4 Sample preparation for HPLC-MS/MS

From 1 ml sample taken on day 0 and 10, the samples were processed for LC-MS. The cells were centrifuged at 13000 *g* for 20 minutes and the supernatant and the pellet separated. The supernatant

was freeze dried. To the pellet and freeze dried supernatant, 1 ml of aqueous 80% methanol was added and allowed to extract for 1 hr. This was centrifuged again at 13000 *g* for 20 minutes and the supernatant was taken in HPLC vials for analysis. The analysis of the metabolites was performed as described previously [2.8] except that the column used was CORTECS™ UPLC® C18+, 90 Å pore size, 1.6 µm particle dia, 2.1 x 100 mm solid core. This column was chosen as it enabled separation of demethyl-microcystin-LR from microcystin-LR and hence quantification of the two microcystins could be performed independently.

5.3.5 RNA extraction, purification and semi-quantitative PCR.

RNA extraction was carried out as described in section 2.9 and semi-quantitative PCR was performed as described in section 2.10

5.4 Results

In order to evaluate the effect of cyanopeptides on the growth of *M. aeruginosa* PCC7806, it was essential to determine the amount of cyanopeptide added exogenously at day 0 and their concentrations at Day 10 (Figure 5.7).

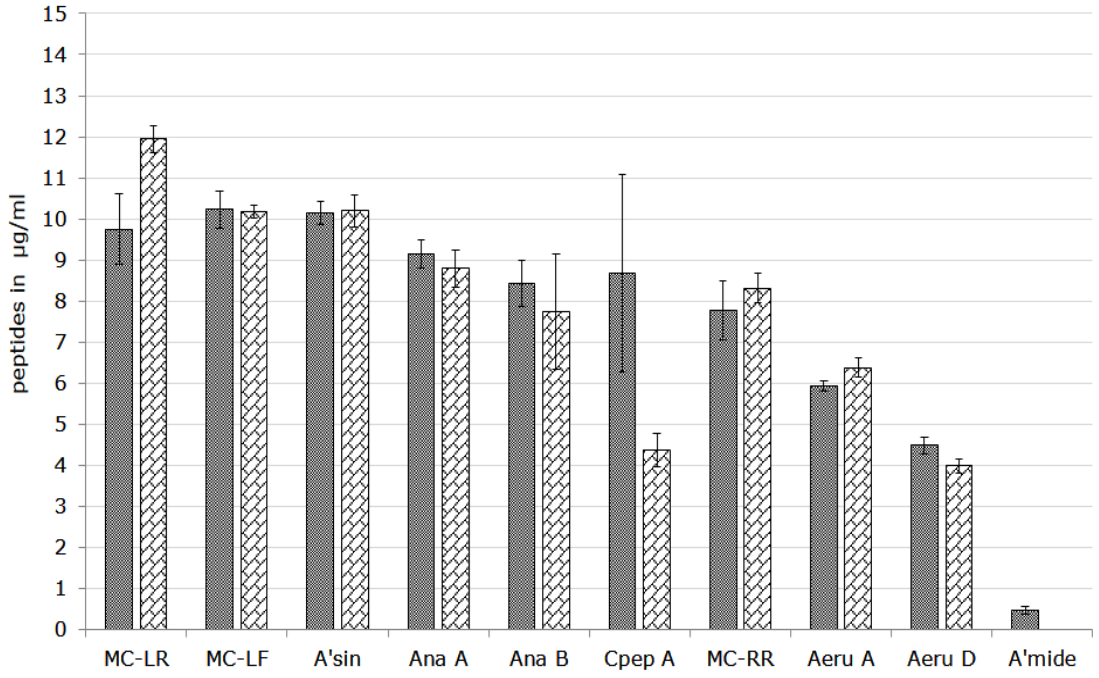


Figure 5.7: The concentration of exogenously added cyanopeptides at days 0, 7 and 10. Note that the quantities represent only the *ex-novo* concentrations. Error bars = 1SD and n = 4

The intended concentration was 10 µg/ml, however due to differences in solubility of the peptides, this could not always be achieved. The peptides aeruginosin (A'sin) and cyanopeptolin A (Cpep A) were the only peptides found to be readily soluble in water, however, all the other peptides required additional vortexing steps to dissolve. The peptide aeruginosamide (A'mide) was the least soluble in water and required

sonication. Even after sonication, it was observed that the maximum concentration that was added exogenously to the final culture was about 0.47 ± 0.1 µg/ml. It could be either that the peptide was poorly soluble in water or that the additional sonication step may have destroyed the peptide. It would be advisable, in future, to initially dissolve aeruginosamide in DMSO.

In order to quantify the different peptides, a standard curve of each of the peptides was performed taking several dilutions of the purified peptide in 80% aqueous methanol. These were analysed in HPLC-MS and a linear standard curve was plotted. The peptides microcystin-LR, microcystin-LF, aeruginosin, anabaenopeptin A and cyanopeptolin A were present at approximately 10 µg/ml at day 0. Except cyanopeptolin A, the concentrations of all the peptides remained unchanged for the duration of the study i.e., until day 10. In fact, even the other peptides that showed poor solubility also remained stable over the duration of 10 days. It may be concluded that *M. aeruginosa* PCC7806 is unable to degrade these peptides except cyanopeptolin A, which may either have been degraded by the activity of the culture or is naturally unstable. The unstable nature of cyanopeptolin A has been previously noted in Chap IV, Figure 4.16, where it was seen that the release of intracellular *de-novo* cyanopeptolin A did not correlate with the amount present extracellularly.

5.4.1 Effect of cyanopeptides on the growth of *M. aeruginosa* PCC7806

The cell density measured at day 0 was 2.5 million cells and by day 10 they had increased 50 fold (Figure 5.8). At day 10, the cells density had reached 124 million cells/ml which was 1.5 fold higher than that observed previously (~80 million (Figure 3.1)). The reason for this may be due the difference in the inoculum cells preparation which were grown in BG11 containing 0.75 g/L of sodium nitrate. This may serve as an indication that, although this organism does not fix its own nitrogen, excessive amounts of nitrogen may have a growth suppressing effect.

At day 10, the cells grown in the presence of microcystin-LF, microcystin-RR, A'mide, Ana A & B, and Aeru A & D showed significant growth inhibition compared to the control. At this point, it was not known if this was due to growth inhibition or toxicity of these cyanopeptides to the cells of *M. aeruginosa* PCC7806. In the case of aeruginosin, it may be stated that this cyanopeptide does not have an effect on the growth of *M. aeruginosa* PCC7806. The fact that *M. aeruginosa* PCC7806's genome contains a gene cluster for aeruginosin suggests that it should be producing its own aeruginosin, however, analysis of HPLC data did not provide any evidence for this. It is thought that *M. aeruginosa* PCC7806 may be producing a variant of aeruginosin [6] thus far not characterised. From this study, it is also known that aeruginosin is readily soluble in water (easily diffusible into the surroundings), which is an ideal

characteristic for an allelochemical. A study on the effect of this cyanopeptide on other cyanobacteria/diatoms may provide some evidence of an ecological role.

Likewise, the cells exposed to cyanopeptolin A also showed no effect on the growth of *M. aeruginosa* PCC7806. It is thought that the decrease in concentration of cyanopeptolin A (by 50% compared to day 0) may be the reason. At the moment, it is not known if cyanopeptolin A has been degraded or has been modified in some way. It is suggested that the stability of cyanopeptolin A be further studied in order to establish its suitability in bioassays. It is interesting to note that aerucyclamide D and aeruginosamide, even though being in low concentrations showed significant growth inhibitory effects. It should be noted here that *M. aeruginosa* PCC7806 produces microcystin-LR and aerucyclamides (A & D) and hence the inhibition seen may be a combined effect of the respective *ex-novo* and *de-novo* cyanopeptides.

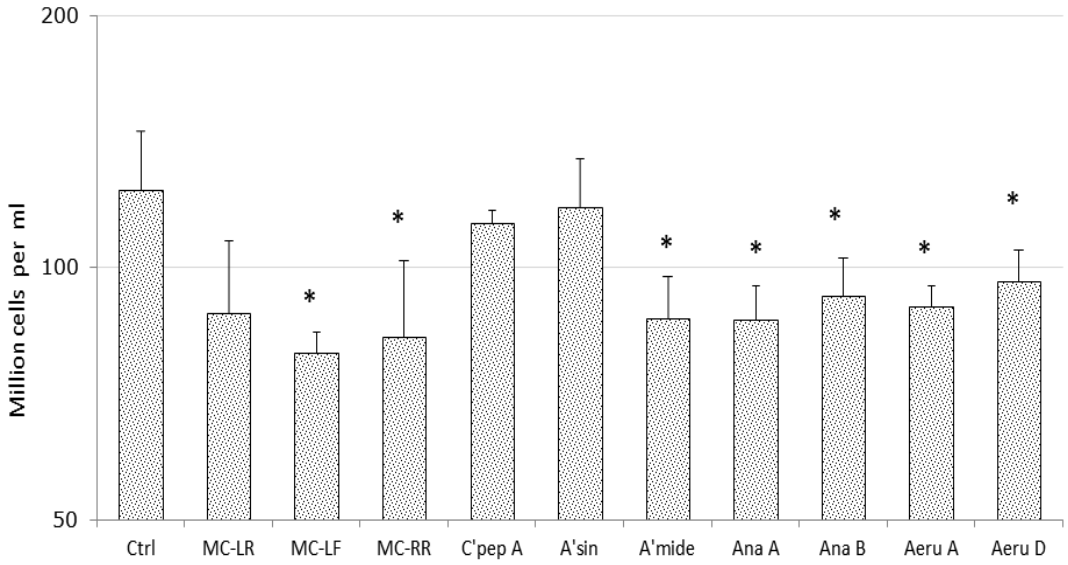


Figure 5.8: The effect of cyanopeptides on the growth of *M.aeruginosa* PCC 7806 at day 10. The starting inoculum was 2.5 million cells/ml, culture was incubated at 21 ± 1º C for 10 days and 20 ± 2 µmol/m^2/s of light continuously. Purified peptides were aseptically added. An appropriate control was also maintained without any exogenous cyanopeptide. Statistical significance was determined by comparing each treatment to the control. * indicates $p \leq 0.05$. Error bars = 1SD, n=3.

From the cell numbers, the doubling time of the different treatments was calculated and compared to the control for statistical significance. The formula used to calculate doubling time is described in section 2.7. It was observed that the average doubling time of *M. aeruginosa* PCC7806 was 1.77 ± 0.08 by day 10 (Table 5.1). The doubling time of *M. aeruginosa* PCC7806 has been reported to be between 2 to 4 days in some studies [269, 221] while others have reported it to be between 1.8 to 2.9 [220], although growth conditions were different in each study. In this study, the doubling time reported can be stated as the highest for *M. aeruginosa* PCC7806. The fast doubling time seen here may be attributed to the cumulative effect of growing the inoculum in BG11 media containing 12 mM nitrate, optimal growth conditions provided by

growing the culture in 20 ml volume taken in 50 ml conical flask and approximately 20 µmols/m^2/s light and 20 °C temperature.

From the doubling times data, it appears that certain cyanopeptides such as microcystin-LR, microcystin-LF, aeruginosamide, aerucyclamide A and anabaenopeptin B seem to be significantly extending the doubling time, when compared to the control. The fact the exogenous microcystin-LR extends the doubling time has been reported in a previous assay (Chapter IV, Table 4.3), which confirms that high concentrations of cyanopeptides in the surroundings significantly affect cell division rates. It would be an interesting exercise to evaluate the mechanism by which cyanopeptides bring about this change. Table 5.1: The mean doubling time (in days) of *M. aeruginosa* PCC7806 under the effect of cyanopeptides

Treatment	Day 0 – 10
Control	1.77 ± 0.08
Aeruginosin	1.79 ± 0.06
Cyanopeptolin A	1.81 ± 0.02
Aerucyclamide A	1.99 ± 0.03*
Aerucyclamide D	1.89 ± 0.05
microcystin-LR	1.98 ± 0.10*
microcystin-RR	1.91 ± 0.06
microcystin-LF	1.94 ± 0.05*
Aeruginosamide	1.95 ± 0.07*
Anabaenopeptin A	1.95 ± 0.12
Anabaenopeptin B	1.92 ± 0.03*

* - indicates statistically significant from that week's control. $p \leq 0.05$

5.4.2 The effect of cyanopeptides on microcystin

The use of stable labelled nitrogen (^{15}N) enabled differentiation between *de-novo* (newly synthesised) and *ex-novo* (already present) microcystins within cells. Further, the use of the CORTECS™ UPLC® C18+ column for HPLC-MS was useful in differentiating between the two forms of microcystins viz; demethyl-microcystin-LR and microcystin-LR produced by *M. aeruginosa* PCC7806. The amount of intracellular *de-novo* demethyl-microcystin-LR (m/z 992) was quantified against a standard of purified microcystin-LR, at day 0 and 10. The one-way Anova analysis was performed so as to determine statistical significance (Figure 5.9).

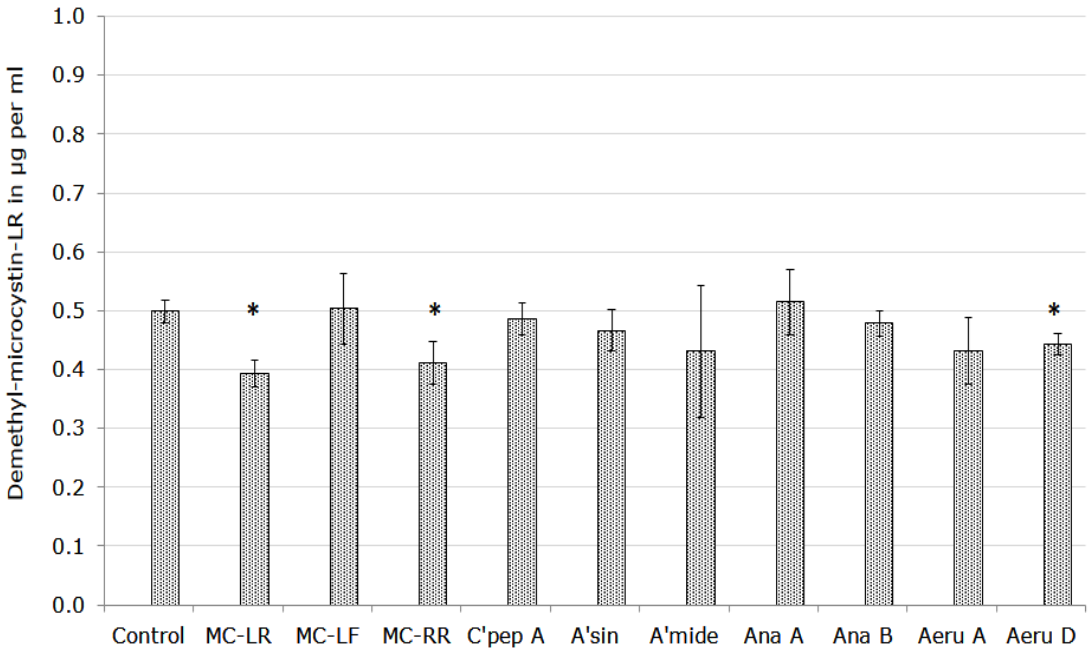


Figure 5.9: The effect of exogenously added cyanopeptides on intracellular *de-novo* Demethyl-microcystin-LR (m/z 992) biosynthesis. *M. aeruginosa* PCC7806 cells were exposed to purified cyanopeptides and grown for 10 days in BG11-^{15}N media. Using Anova, statistical significance was determined by comparing each treatment to its week's control. * indicates $p \leq 0.05$.Error bars = 1SD, n=3.

As expected, at day 0, no *de-novo* demethyl-microcystin-LR could be detected. However, by day 10 the detectable quantities of demethyl-microcystin-LR had accumulated within cells. The cells that were exposed to microcystin-LR, microcystin-RR and aerucyclamide D (Aeru D) showed significantly reduced quantities of intracellular demethyl-microcystin-LR while none of the other treatments showed any significant change. Considering the age of the inoculum used (33 days old) and the doubling time, it could be suggested that the day 10 observations could represent the actual effect of the exogenous peptides on *M. aeruginosa* PCC7806. Although previous reports have quantified demethyl-microcystin-LR independently, most publications report the microcystin concentration as the sum of microcystin-LR and demethyl-microcystin-LR [314 - 316] and methodologies published [317], this is the first report where demethyl-microcystin-LR separation has been adequately achieved and the *de-novo* production reported (Figure 5.10).

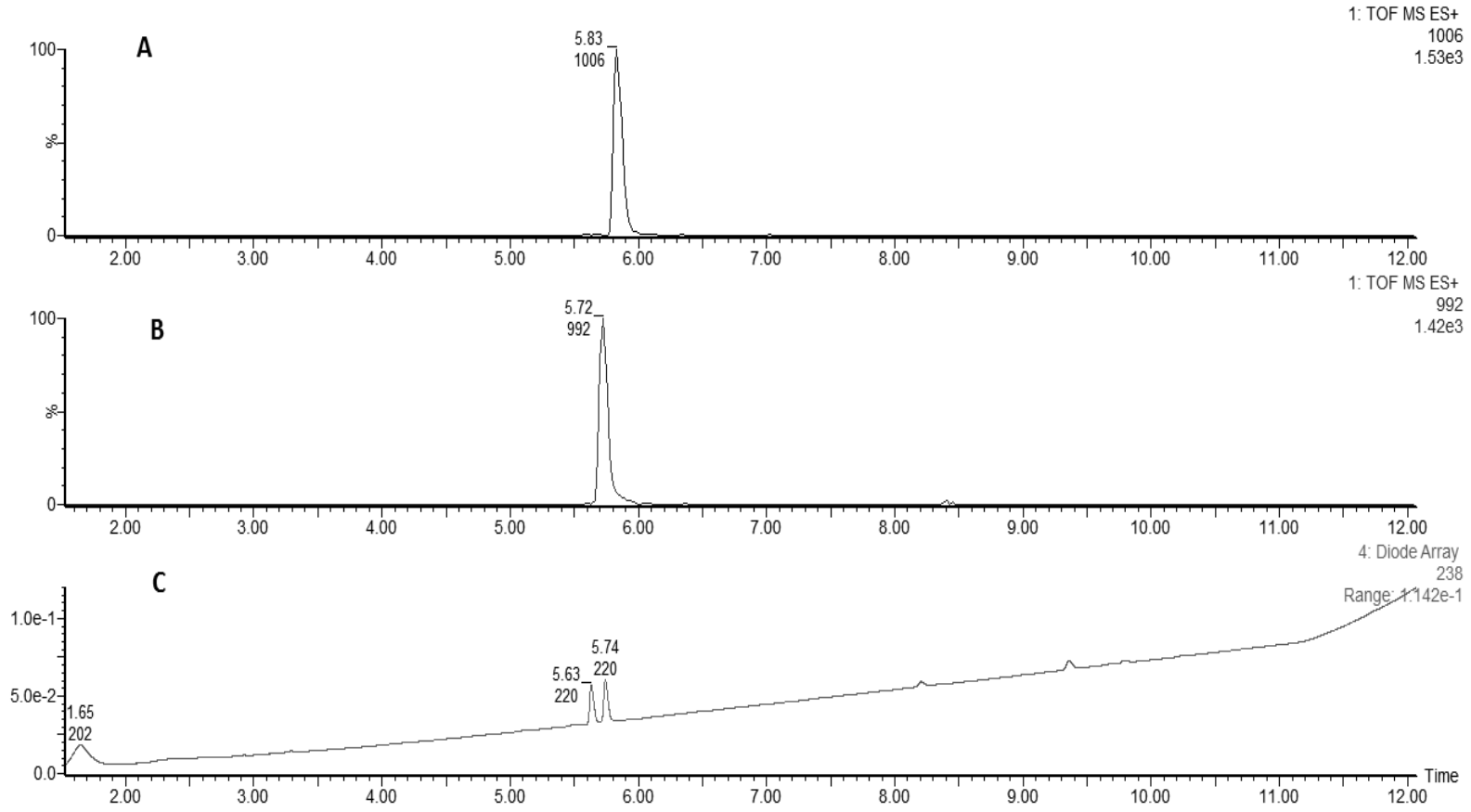


Figure 5.10: The chromatogram of microcystins of *M. aeruginosa* PCC7806 using the CORTECS® column. A is ESI-MS of 15-N labelled microcystin-LR (m/z 1006), B is ESI-MS of 15-N labelled demethyl-microcystin-LR (m/z 992) and C is UV (238 nm) spectra of demethyl-microcystin-LR and microcystin-LR.

In order to obtain a complete picture of the effect of cyanopeptides on the microcystin production of *M. aeruginosa* PCC7806, the quantification of *de-novo* microcystin-LR (m/z 1006) was also performed and it could be observed that at day 10, only aerucyclamide A and D showed significant sustained inhibition (Figure 5.11). From the growth curve of *M. aeruginosa* PCC7806 reported earlier (Chap IV, Figure 4.6) the logarithmic growth phase is between day 7 to day 14 and hence it could be stated that during active growth, the presence of certain exogenous peptides such as aerucyclamide A and D may have a significant effect on the biosynthesis of microcystin-LR, at a dose of 4 to 6 µg/ml. It remains to be explored if cyanopeptolin A or aeruginosamide would also have a similar effect; however, in this study we observed that cyanopeptolin concentrations decreased with time while poor solubility of aeruginosamide limited its availability in the assay.

The intracellular *de-novo* microcystin-LR concentrations seen here was compared to values reported elsewhere and at day 10 the intracellular microcystin-LR concentrations were comparable i.e., 0.6 ± 0.08 µg/ml. This is an interesting observation as the cell numbers were 1.5 fold higher, yet the intracellular content remains almost similar. This could serve as an indication that microcystin biosynthesis and cell growth rate are independent processes, in fact a negative correlation between growth rate and microcystin biosynthesis can be observed.

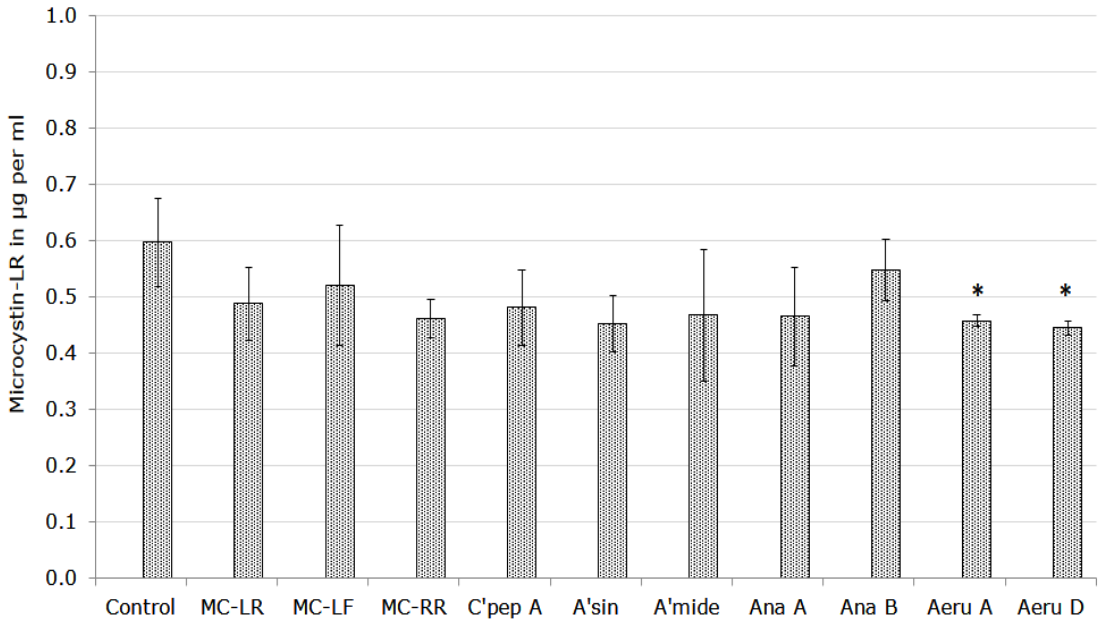


Figure 5.11: The effect of cyanopeptides on intracellular *de-novo* microcystin-LR (m/z 1006) biosynthesis. *M. aeruginosa* PCC7806 cells were exposed to purified cyanopeptides and grown for 10 days in BG11-^{15}N media. Using Anova, statistical significance was determined by comparing each treatment to its week's control. * indicates $p \leq 0.05$.Error bars = 1SD, n=3.

Although the use of the Cortecs® columns enabled the separation of microcystin-LR from demethyl-microcystin-LR, studies report that both these congeners are equally potent toxins [318] and in the ecosystem these congeners are often co-produced [319 - 322]. The ratio of demethyl-microcystin-LR to microcystin-LR might vary between 9 to 50%, however, in most studies with *Microcystis* the microcystins are reported as the sum of the congeners [271]. In order to further understand the effect of cyanopeptides on total microcystin biosynthesis, the net *de-novo* microcystin content i.e. microcystin-LR + demethyl-microcystin-LR was calculated [Figure 5.12]. It should be noted that in this study, we observed that demethyl-microcystin-LR was present in equal concentrations to that of microcystin-LR.

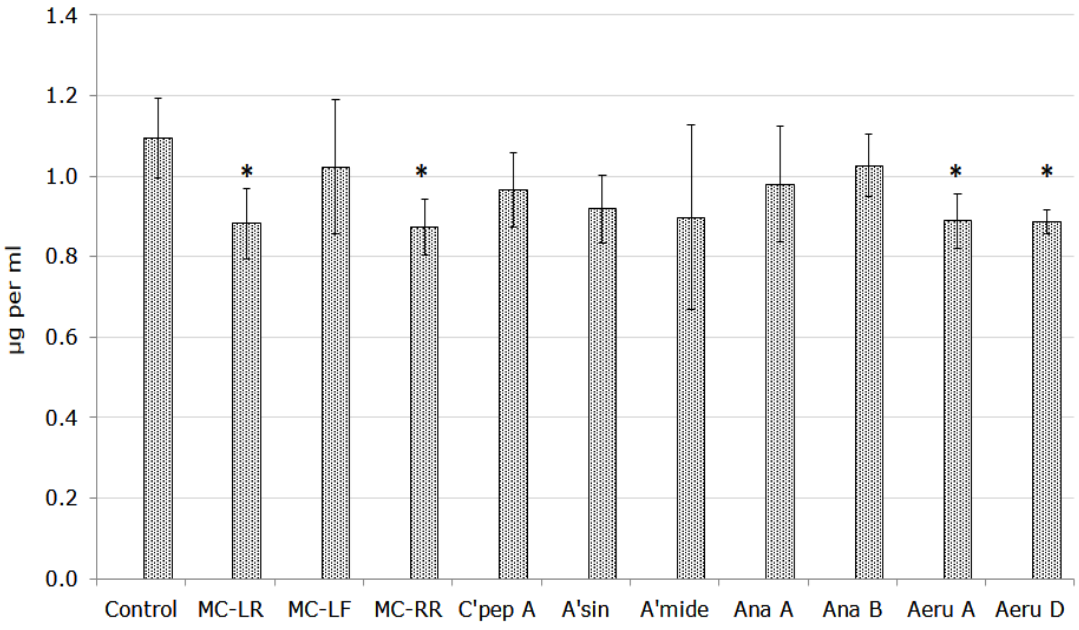


Figure 5.12: The effect of cyanopeptides on intracellular *de-novo* microcystin (m/z 992+1006) biosynthesis. *M. aeruginosa* PCC7806 cells were exposed to purified cyanopeptides and grown for 10 days in BG11-^{15}N media. Using Anova, Statistical significance was determined by comparing each treatment to its week's control. * indicates $p \leq 0.05$. Error bars = 1SD, n=3.

The total *de-novo* intracellular microcystin concentration at day 10 was about 1.09 ± 0.09 µg/ml which correlated well with previous studies (Chap IV, Figure 4.9). In a typical ecosystem microcystin concentration are known to range widely (4 to 1500 µg/g dry weight of biomass) and several factors such as temperature and light availability have been known to affect this [323 - 324]. Hence it could only be interpreted that even in controlled laboratory studies, some microcystin variability should be expected and it is recommended that appropriate controls are maintained when a study such as this is performed.

In terms of the effect of cyanopeptides, only microcystin-LR, microcystin-RR, aerucyclamide A and D sustained the inhibition by day 10. This data corroborates a previous observation (chap IV, Figure 4.9),

but contradicts the findings of Schatz *et al*. [127], wherein they report that the presence of exogenous microcystin-LR (and microginin and micropeptin) upregulated microcystin production. However, it should be noted that the concentration of microcystin-LR used in this study was much higher than may be found ecologically, except under localised conditions. Considering the fact that several other peptides used in this study were of equally high concentrations, yet *M. aeruginosa* PCC7806 did not show any significant inhibition, it may be concluded that even high concentrations of exogenous cyanopeptides is non-toxic to the host. Additionally, the inhibition shown by microcystin-RR may also be due to the same mechanism as microcystin-LR, however, this needs to be confirmed by further studies.

M. aeruginosa PCC7806 produces microcystins and aerucyclamides and it is an interesting observation that on exposure to aerucyclamides (or microcystin-LR), an inhibition in microcystin biosynthesis is seen although the organism seems to be insensitive to other peptides such as anabaenopeptins or aeruginosin. If it is argued that the seeming reduction in microcystin biosynthesis could be due to inhibited cell numbers, observation of the cell numbers (Figure 5.8) reveal that apart from microcystins and aerucyclamides, the anabaenopeptins and aeruginosamide also showed significantly inhibited cell numbers but this inhibition did not translate into microcystin biosynthesis (Figure 5.12). In order to further investigate this question, the production per cell per day

(production rate per day) of *de-novo* microcystin (m/z 992+1006) was calculated (Figure 5.13).

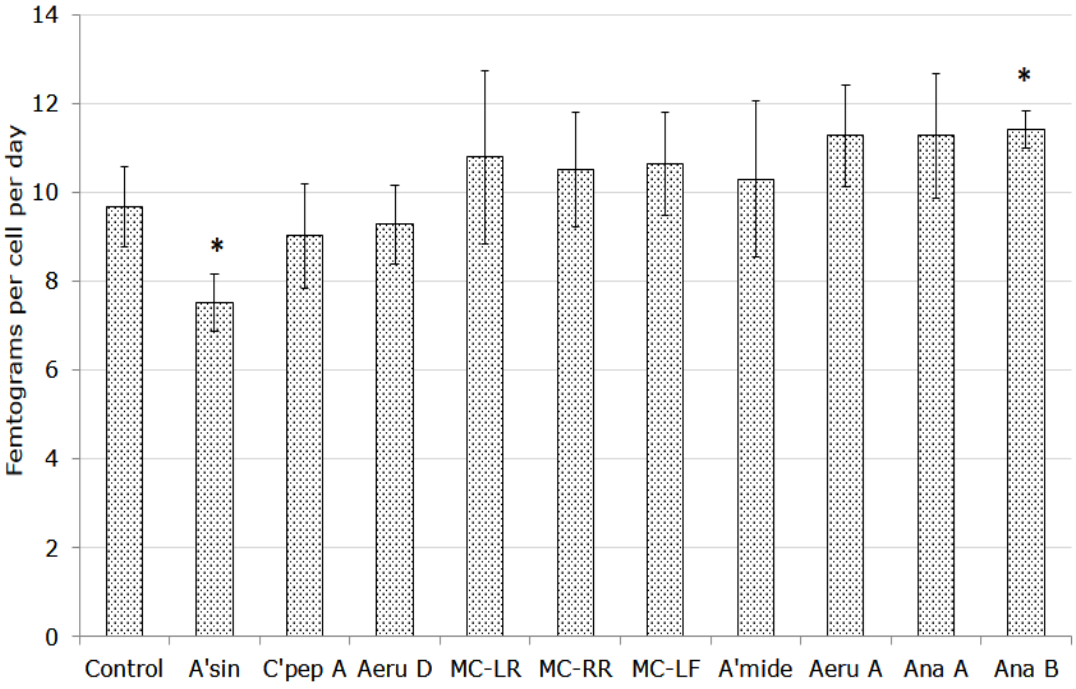


Figure 5.13: The production rate of microcystin (m/z 992+1006) when exposed to exogenous cyanopeptides. *M. aeruginosa* PCC7806 cells were exposed to purified cyanopeptides and grown for 10 days in BG11-^{15}N media. Using Anova, statistical significance was determined by comparing each treatment to its week's control. * indicates $p \leq 0.05$. Error bars = 1SD, n=3.

Analysis of production rate revealed that between day 0 to 10, the net *de-novo* production rate was 9.67 ± 0.9 fg/cell/day. Previously, we have observed that the production rate between day 0 to 7 was 7.4 ± 1.5 fg/cell/day and 4.4 ± 0.5 fg/cell/day between day 7 to 14 (Chap IV, Figure 4.12). The difference observed could be explained by the fact that the nitrate concentration was 50% less in this study but growth was at least 1.5 fold higher. Also, the inoculum used in this study was one month

old compared to the mid-log inoculum (15 days) used previously. This suggests that although *M. aeruginosa* PCC7806 requires nitrogen for its growth, nitrate at a final concentration of 12 mM (0.75 g/L of $NaNO_3$) is sufficient for optimal growth when compared to 24 mM (or 1.5 g/L of $NaNO_3$). Another observation is that, although reduced nitrate enhances growth, the microcystin production seems to be disconnected and seems to follow a rate that is independent of growth.

As for the effect of cyanopeptides on the production rate of microcystin (Figure 5.13), a contradicting picture emerged as none of the peptides that had shown an inhibitory activity on the total *de-novo* microcystin (Figure 5.12) showed a significant inhibition on the production rate at day 10. However, exogenously added aeruginosin showed significant inhibition in the production rate while anabaenopeptin B showed an enhancing effect. The contradicting results observed may be explained by the fact that microcystin production is a complex biosynthetic process involving several enzyme complexes [176], and the suppression or upregulation of a few enzymes in the pathway may not translate into the quantities of the final product at a particular time point. It may also be that the reduced doubling time (table 5.1) exhibited by these peptides may have caused the intracellular MC's to accumulate and ultimately give a high intracellular production rate.

The primary aim of this study was to evaluate the role of these cyanopeptides on *M. aeruginosa* PCC7806 and if possible extrapolate this

information into the general role of these metabolites in the ecosystem. Although microcystins have been studied for the past 30 years, conclusive evidence for their role is still being debated. It is clear the cyanopeptides are secondary metabolites i.e. these are not involved in the host's primary metabolism and hence it should be expected that their absence/presence in the host organism may cause only subtle changes and it would be a challenge to detect/measure these changes.

Although several cyanopeptides have been isolated and characterised, only microcystins have been extensively studied. Hence, most literature available focuses on determining the role of microcystins in its host or the ecosystem. In an earlier study, it was reported that microcystins were produced to dissuade grazers such as daphnids [325, 326], however, a contradicting conclusion that the daphnids showed no selectivity towards toxic and non-toxic strains and a recent study reporting that the microcystin synthetase genes evolved prior to the origin of metazoans [151] disprove this hypothesis.

One key observation of many of these cyanopeptides is that they are predominately intracellular molecules and hence intracellular functions such as iron chelation and signalling/gene regulation have been suggested. Microcystins have been thought of as iron chelators and it had been reported that at low concentrations of Fe (≤2.5 μM) the cell growth rate decreased, but their intracellular toxin content increased [160]. However, later studies contradicted this finding and reported that

microcystin content decreased with iron deplete conditions [329]. The authors also report that toxic strains of *Microcystis* maintain their levels of intracellular Fe^{2+} while non-toxic strains have low levels of Fe^{2+} [99]. The differences in findings may be due to differences in the strains and culture conditions, but mostly due to insensitive microcystin quantification methods. Moreover, microcystins have been found to be localised in the thylakoids and around phosphate bodies, which do not support the hypothesis of siderophores [270].

5.4.3 The effect of cyanopeptides on the genes of microcystin biosynthesis

In 2000, the complete structure of the microcystin synthetase genes were published [176], which paved way for the investigation of the role of microcystins at the molecular level. Structurally, *mcy* (55 kb gene cluster for microcystin) is composed of 10 genes arranged in the form of two operons *mcyABC* and *mcyD-J* arranged in opposite directions [Figure 5.14].

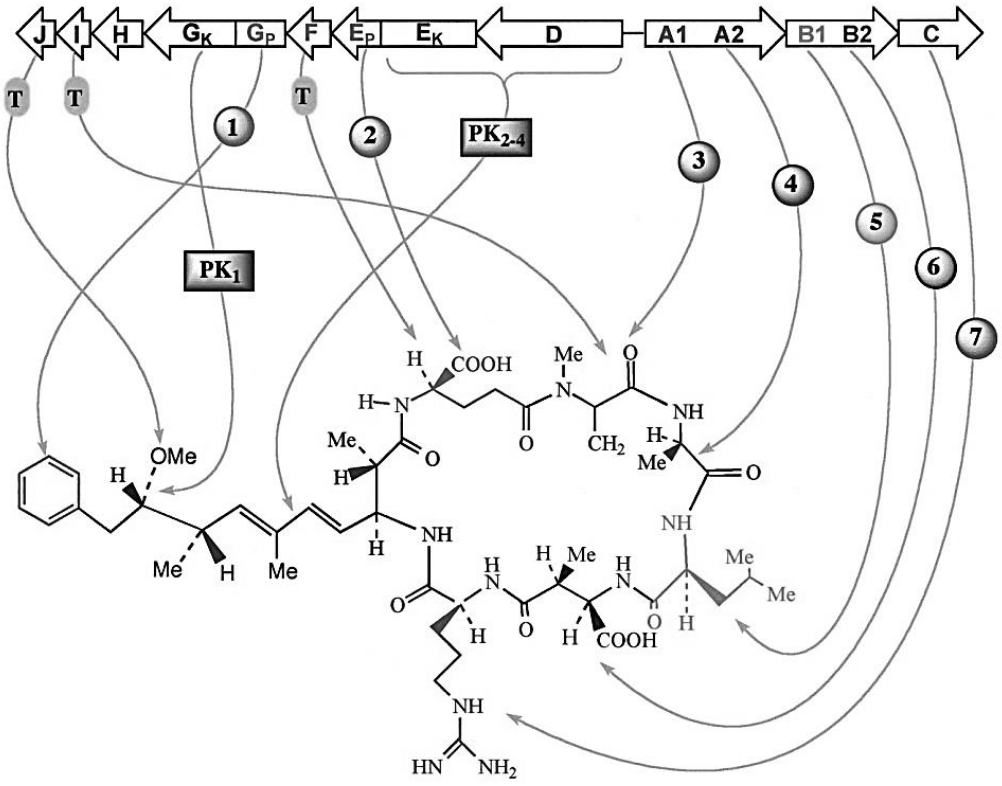


Figure 5.14: Structural organisation of microcystin genes showing the proposed sequence of microcystin assembly. 'T' indicates genes with a putative tailoring function. (Image: Kaebernick *et al*., FEMS Microbiology Ecology, 2001 [327])

One early study, investigating the effect of exogenous microcystin on the gene expression of *mcyB* gene concluded that *Microcystis* cells are able to sense the lysis of surrounding cells and increase their intracellular microcystin production, thereby indicating that microcystin may function as an allelochemical (signalling molecules) [127]. Further, a study on the partial deletion of the putative transporter *mcyH* gene resulted in the complete absence of intracellular MC. The authors claim that MC's may be

produced with an intention to export and hence may provide an extracellular role for microcystin [181]. However, it is generally accepted that MC's are predominately intracellular molecules and the mutation of a transport gene should not affect their intracellular production.

A recently published study hypothesised that as MC's are intracellular molecules, they may play a significant protective role, by binding to several essential proteins and protecting the cells against proteases and oxidative stress [266]. Another study, which exposed *Microcystis* to high light intensities of 250 µmol/m^2/s found that wild type cells adapted faster to the increased light intensity and accumulated glycolate, with a view of decreasing their buoyancy. The authors claim that this may cause the cells to sink to lower water columns thereby avoiding high light intensities [281]. It has also been reported that microcystin deficiency upregulates a number of genes involved in secondary metabolism and could be complemented by the addition of exogenous MC. Further, they also report that exogenous microcystin is never imported by neighbouring cells but MC's may pass on the signal through a network of sensors located on the cell surface [328] and hence MC's may play an intra as well as extracellular role. Although the evidence presented indicates a multilevel role for microcystins, in the ecosystem, both microcystin-producing strains and non-MC producing wild type strains co-exist and dominate each other in water systems. Further, it has also not been explained why non-toxic mutant strains spontaneously generate from toxic strains [151].

The results of the present study thus far indicate that the presence of exogenous peptides reduce cell numbers, which may explain the differences in intracellular microcystin quantified. In order to investigate the effect of exogenous peptides at the molecular level, it was decided to study the expression profile of some of the genes involved in microcystin. The study design was such that day 10 samples would be used for gene expression analysis as this would represent active cell growth period. The *mcyA* gene, which encodes for N-methyltransferase and *mcyE,* which encodes a polyketide synthase module at the N-terminus and a peptide synthetase module at the C-terminus were chosen as they have been reported to be important for microcystin synthesis and insertional mutagenesis of these genes have reported to abolish microcystin production [330, 174]. Further, *mcyA* has been documented only in microcystin producing strains while *mcyE* is known to play an important role in the incorporation of Adda and D-Glutamic acid moieties in the microcystin biosynthesis [330, 331]. Total mRNA from the cells was extracted and purified as described in section 2.9. Following this, the mRNA specific for *mcyA* and *mcyE* and 16s rRNA was amplified by reverse transcription-PCR (RT-PCR) thus making DNA copies. This DNA was then separated on 1.2% agarose gel. The band intensities was quantified (using Gel-doc, UVITEC) and the relative band intensities of the mcyA and mcyE genes were determined in comparison to the band intensity of 16s rRNA. The relative band intensities, thus obtained, were reported as fold change in comparison to the control. The rationale behind the use of 16s

rRNA gene is that it is considered as a good housekeeping gene, meaning it is stably expressed [255]. Besides, several previous works involving the use of semi-quantitative RT-PCR have used the band density of this gene to comment on the efficiency of the RNA extraction procedure and as an internal control to normalize the band intensity of the gene in question [255, 332 - 333].

A visual observation of the 16s cDNA (from rRNA sequence) bands confirmed sufficient extraction of RNA and that comparable quantities of the sample has been loaded onto agarose gels. However, analysis of the band intensity using image analysis software (Gel-doc, UVITEC) showed that the average band intensity varied by ± 6.45%. In order to reduce errors in interpretation of results, the band intensities of *mcyA* and *mcyE* were normalized against their respective 16s rRNA bands. Between day 0 and 10, the change in *mcyA* expression was 2 fold while that of *mcyE* was a 3 fold increase. This may be explained by the fact that the inoculum was obtained from a stationary phase culture while the day 10 sample would represent a mid-log culture.

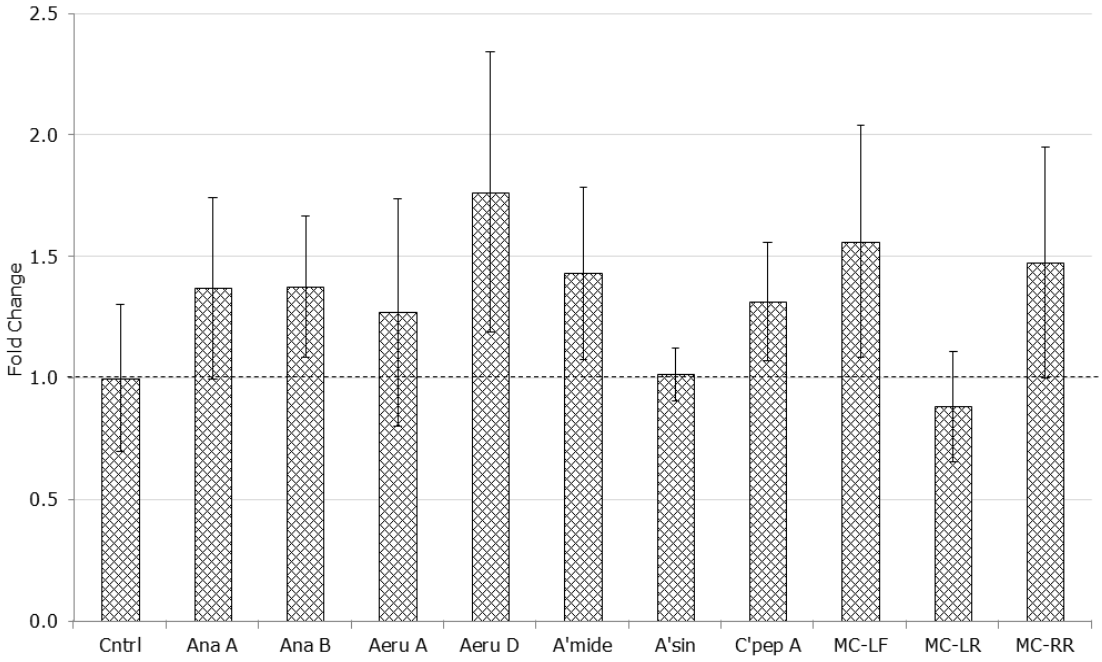


Figure 5.15: The effect of cyanopeptides on the expression of *mcyA* gene of *M.aeruginosa* PCC 7806. *M. aeruginosa* PCC7806 was exposed to different cyanopeptides and allowed to grow for a period of 10 days. The cells were harvested and mRNA extracted and analyzed for expression of *mcyA* gene. Statistical analysis revealed no statistical significance when compared to control. Error bars = 1SD, n = 3

The *mcyA* gene expression profile revealed that except for the cells exposed to aeruginosin and microcystin-LR, all the other treatments showed some overexpression (Figure 5.15). Statistical analysis revealed that none of the expressions were significantly different from that of the control, but, it should also be noted that the semi-quantitative PCR used here is an insensitive technique. It may only be interpreted that the cells exposed to cyanopeptides (except aeruginosin) show some differences in the expression of the *mcyA* gene. It is interesting to note that, the cells exposed to aerucyclamide D showed the highest relative overexpression of *mcyA*, even when its exogenous concentration was only about 4 µg/ml and in spite of the insensitive quantification technique used.

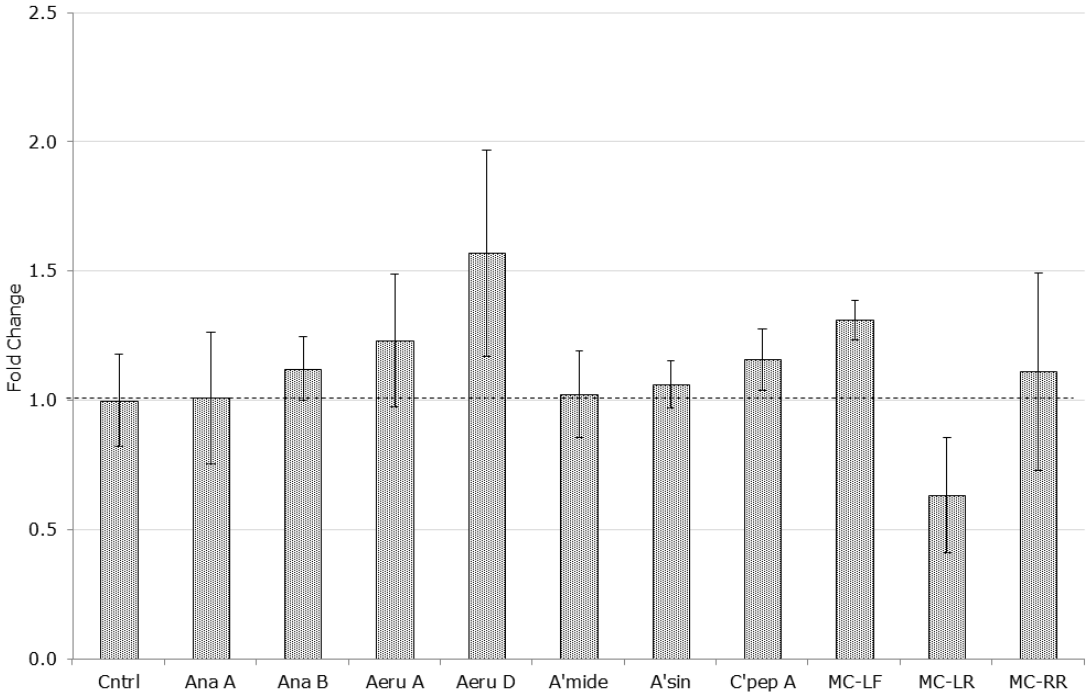


Figure 5.16: The effect of cyanopeptides on the expression of *mcyA* gene of *M.aeruginosa* PCC 7806. *M. aeruginosa* PCC7806 was exposed to different cyanopeptides and allowed to grow for a period of 10 days. The cells were harvested and mRNA extracted and analyzed for expression of *mcyA* gene. Statistical analysis revealed no statistical significance when compared to control. Error bars = 1SD, n = 3

The expression of profile of *mcyE* gene revealed that many cyanopeptides, i.e. anabaenopeptin A & B, aeruginosamide, aeruginosin, cyanopeptolin A and microcystin-RR showed little or no difference in expression compared to the control (Figure 5.16). Only, the cells exposed to aerucyclamide D showed relatively high overexpression of *mcyE.* In fact, between *mcyA* and *mcyE* expression profiles, it was seen that only aerucyclamide D exposed cells consistently overexpressed these genes.

Overall, it could be stated that the presence of at least some of the cyanopeptides such as aerucyclamide D and microcystin-LF causes an overexpression of *mcyA* and *mcyE* genes, whereas only microcystin-LR inhibits the expression of its own biosynthetic genes. The presence of aeruginosin and to some extent cyanopeptolin A seemed to have no effect

at all, although it should be noted that cyanopeptolins are known to degrade in the presence of *M. aeruginosa* PCC7806. The lack of statistical significance in Figure 5.15 (or 5.16) should not be construed as invalid results, as it is well known that semi-quantitative RT PCR produces highly variable results even when ample care has gone into sample handling and analysis procedures. Besides, quantification of bands using band intensity as a parameter is also highly variable. In such cases, qualitative interpretation of bands would be more accurate and at least give a relative indication of a positive or negative effect on gene expression levels (Figure 5.17).

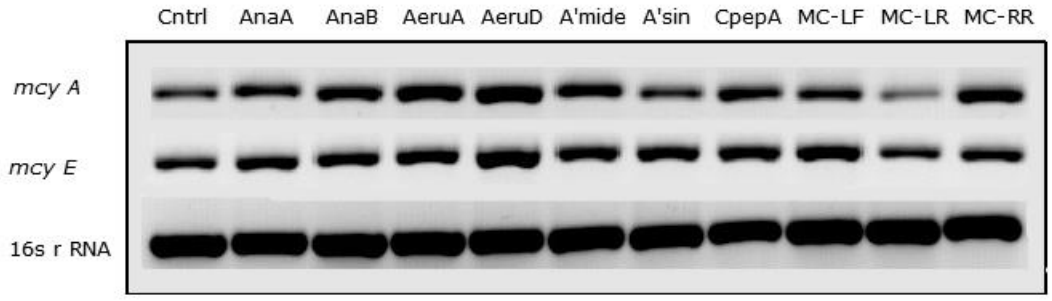


Figure 5.17: Agarose gel (1.2 %) bands of genes *mcyA*, *mcyE* and 16s rRNA of *M. aeruginosa* PCC7806 exposed to cyanopeptides for a period of 10 days.

Comparing the gene expression to the net microcystin production (Figure 5.12), it was observed that the consistent overexpression of *mcyA* and *mcyE* does not translate into net microcystin production. It was also observed that exogenous microcystin-LR inhibits the expression of *mcyA* and *mcyE* which also does not translate into decreased production rate. The evidence presented here contradicts the findings of *Schatz et al*.

[127], where they report that the presence of exogenous microcystin induces the overexpression of mcyB (another gene in the microcystin biosynthesis operon) which translated into increased microcystin production. However, differences such as the strain of *Microcystis* used and microcystin quantification methods may explain the contradicting results. Moreover, they report that the gene *mcyB* is overexpressed whereas we report that gene *mcyA/mcyE* is underexpressed. Overall, from this study it could not be decisively concluded if exogenous cyanopeptides exhibit any significant effect on *M. aeruginosa* PCC7806, but it is obvious that the presence of at least some of the cyanopeptides causes a change in the expression profile of these genes.

The correlation between *mcy* gene expression levels and microcystin concentrations are controversial. One of earliest work on the effect of light on *mcyB* and *mcyD* genes concluded that total intracellular microcystin content and the expression of these genes are not correlated. They argued that the high light intensities studied may have induced the release of intracellular toxins and hence given lower values for intracellular toxin [255]. A study on iron availability and *mcyD* expression levels concluded that at day 10, although gene expression was only mildly enhanced, a significant increase in microcystin-LR was noticed [179]. Later, a study on the effect of nitrate on *mcyD* expression concluded that even though the gene was underexpressed, the net microcystin-LR per cell was unchanged [332]. In another study using *mcyE*, the authors conclude that only *mcyE* gene copies correlated strongly with microcystin

concentration whereas McyE transcript levels did not correlate [331]. Overall, it may only be stated that microcystin biosynthesis is a complex process and it is difficult to establish a correlation between gene expression and microcystin concentrations. Moreover, as the gene products of most of the *mcy* genes is an enzyme, the overexpression or underexpression of a few genes may not translate into actual variations in microcystin concentrations. From this study, it is concluded that studying the expression of just two genes (although important for microcystin biosynthesis) is insufficient to determine the effect of cyanopeptides on *M. aeruginosa* PCC7806. In order to obtain a comprehensive view, it is recommended that all the genes involved in microcystin biosynthesis be monitored. Moreover, the genes for cyanopeptolins and aerucyclamides have recently been identified [328] and when studied along with microcystin-LR genes, would give a broad picture on the effects of cyanopeptides on *M. aeruginosa* PCC7806. Further, monitoring the metabolic state of cells, in addition to monitoring growth of cells, by measuring chlorophyll-a genes or pheophytin (breakdown product of chlorophyll-a) may provide additional information on the intracellular activities of a cell when exposed to cyanopeptides. Finally, it is recommended that gene expressions are monitored using robust techniques such as qPCR as they have the advantage of reduced variability and contamination, online monitoring and do not require post reaction analyses. Further, some of these systems are capable of multiplexing and high-throughput analysis.

5.4.4 Effect of exogenous cyanopeptides on the release of intracellular metabolites

The presence of high concentrations of cyanopeptides may cause the cells to behave differently and leak their intracellular contents faster. The effect of cyanopeptides on the release of intracellular microcystin was also monitored. Analysis of the extracellular microcystin (m/z 992+1006) revealed that the total *de-novo* microcystin present in the extracellular was 0.32 ± 0.06 µg/ml which is about 30% of the intracellular synthesised (Figure 5.18). Further, the extracellular total microcystin data (m/z 992+1006) showed that none of the exogenously added peptides significantly affected the release of intracellular microcystin at day 10. This has also been corroborated with the findings reported in chap IV, Figure 4.13. While in the intracellular region, the ratio of microcystin-LR to demethyl-microcystin-LR was higher, in the extracellular region it seems that demethyl-microcystin-LR was higher than microcystin-LR. Taken together, it may be interpreted that while some exogenous peptides may cause inhibition in the release of intracellular microcystin, none of these peptides seemed to sustain this inhibition by day 10. Considering the age of the culture, the picture seen at day 10 may be considered to be the direct effect of exogenous peptides. The question of whether demethyl-microcystin-LR is more easily released from the

intracellular region compared to microcystin-LR remains to be further investigated.

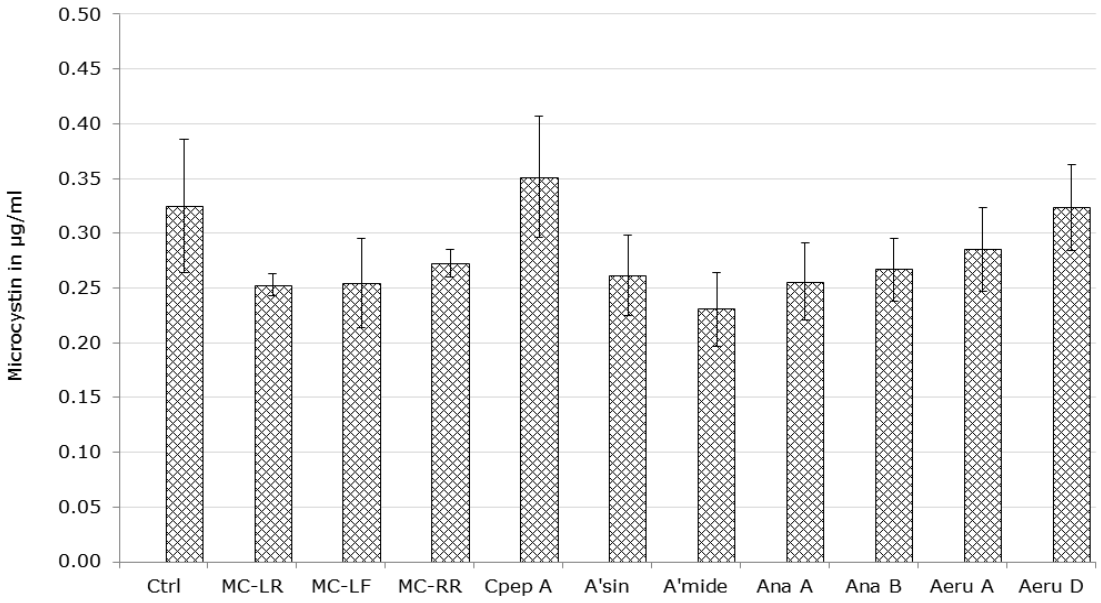


Figure 5.18: The concentration of extracellular de-novo microcystin (m/z 992+1006) when the cells of M. aeruginosa PCC7806 was exposed to different cyanopeptides. *M. aeruginosa* PCC7806 was exposed to different cyanopeptides and allowed to grow for 10 days. At day 10, the supernatant was analyzed in HPLC-MS/MS for de-novo microcystin. Statistical analysis was performed, however no statistically significant differences were found. ■ Day 0 ⊠ Day 10

5.4.5 Effect of cyanopeptides on Cyanopeptolins

The organism *M. aeruginosa* PCC7806 produces several variants of cyanopeptolins namely A, B, C, D and 963A (Table 5.2) [163, 201]. The peak areas of the respective cyanopeptolins were obtained and the intracellular as well as the extracellular quantities were determined. Cyanopeptolin A (m/z 968 (*de-novo*)) was present in abundance and was quantifiable whereas *de-novo* cyanopeptolin B (m/z 939), C (m/z 951) and 963A (m/z 970) were present in negligible quantities and hence could not be quantified. *De-novo* cyanopeptolin D (m/z 966) was also present in

equal concentrations to cyanopeptolin A, but seemed to co-elute and in the absence of a suitable standard could not be quantified. Since chemically cyanopeptolin D is dissimilar to cyanopeptolin A in that its *N*ε,*N*ε-Dimethyl-L-lysine is replaced with L-arginine, quantification of one may not be related to the other (Figure 5.19).

Table 5.2: Table of cyanopeptolins produced by *M. aeruginosa* PCC7806

Name	Formula	^{14}N Mass	^{15}N Mass
Cyanopeptolin A	$C_{46}H_{72}N_{10}O_{12}$	957	968
Cyanopeptolin B	$C_{46}H_{72}N_8O_{12}$	929	938
Cyanopeptolin C	$C_{47}H_{74}N_8O_{12}$	943	951
Cyanopeptolin D	$C_{48}H_{76}N_8O_{12}$	957	966
Cyanopeptolin 963A	$C_{49}H_{69}N_7O_{13}$	963	970

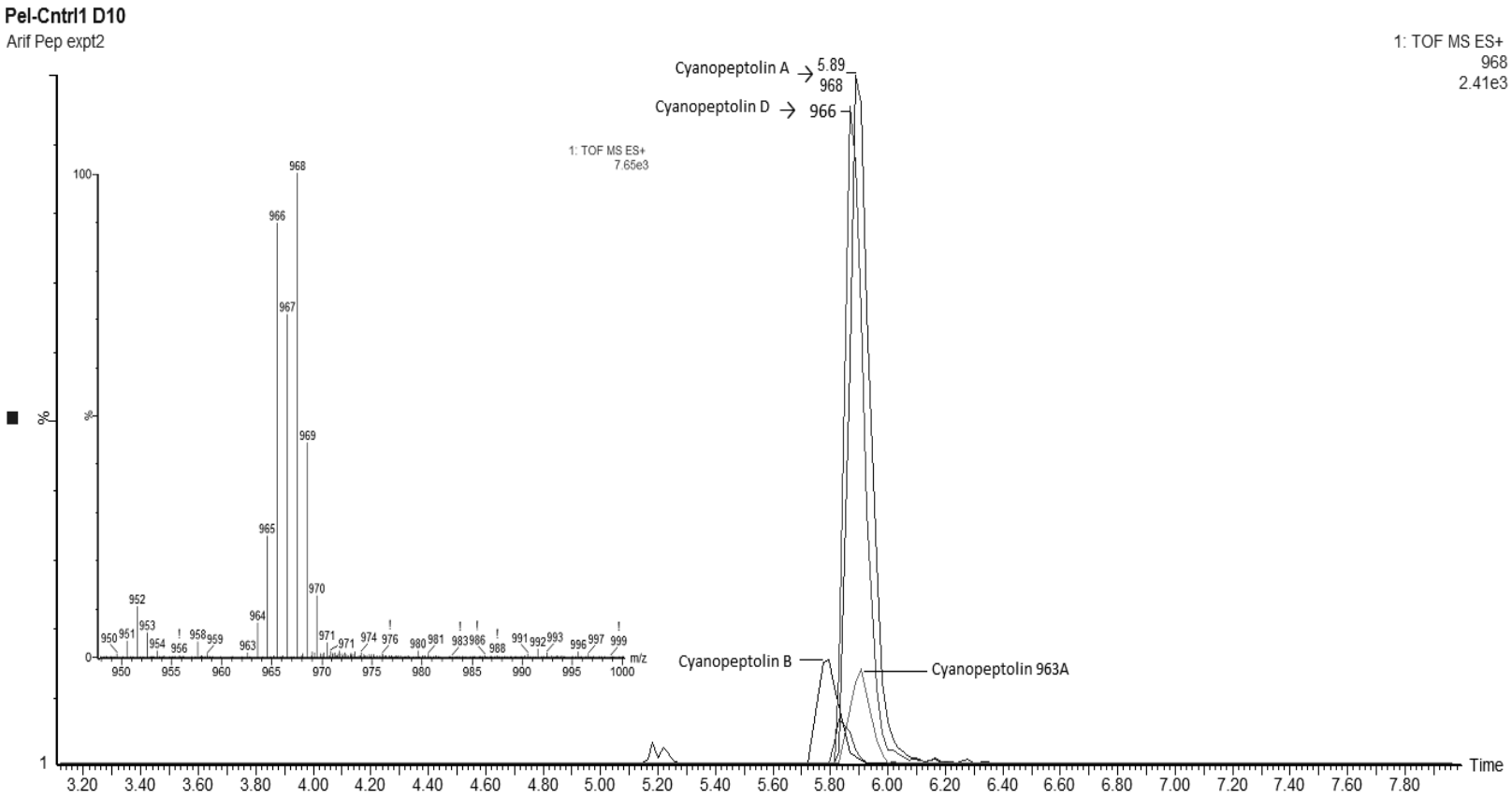


Figure 5.19: The ESI-MS chromatograms of the cyanopeptolins A, B, C, D and 963A showing their relative abundance and elution time. *M. aeruginosa* PCC7806 was grown for 10 days in BG11 (^{15}N) and the cell pellet was extracted with 80% methanol and analysed in HPLC-MS/MS.

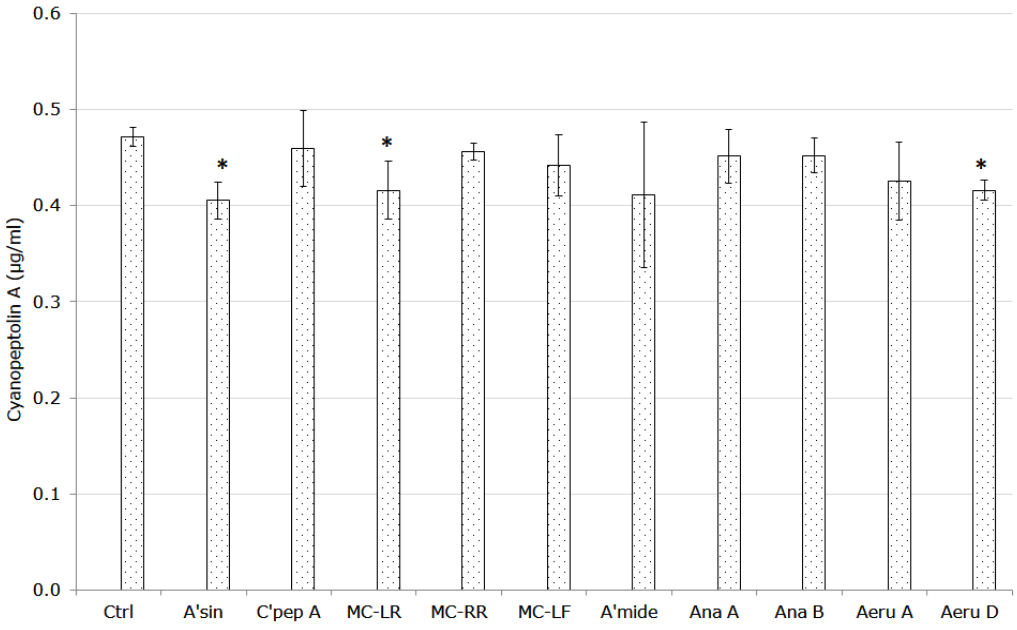


Figure 5.20: The effect of cyanopeptides on intracellular *de-novo* cyanopeptolin A (m/z 968) biosynthesis. *M. aeruginosa* PCC7806 was grown in the presence of exogenously added cyanopeptides in BG11-^{15}N media for 10 days. Using Anova, statistical significance was determined by comparing each treatment to its week's control. * indicates $p \leq 0.05$.Error bars = 1SD, n=3. ■Day 0 □Day 10

At day 10, although differences were seen with many of the peptides, only the cultures that were exposed to aeruginosin (A'sin), microcystin-LR, and aerucyclamide D (Aeru D) showed sustained inhibition in their net quantities (Figure 5.20). Using the intracellular *de-novo* cyanopeptolin A content and the respective weeks cell number, the production rate i.e., the production per cell per day was determined (Figure 5.21).

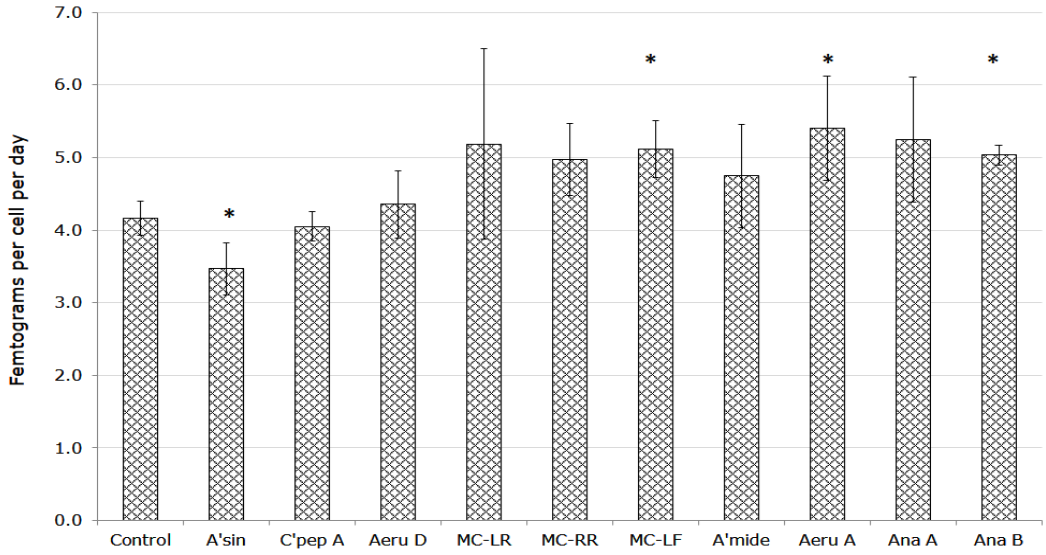


Figure 5.21: The production rate of cyanopeptolin A (m/z 968) by *M. aeruginosa* PCC7806 when exposed to exogenous cyanopeptides. The cells were grown in the presence of cyanopeptides in ^{15}N BG11 media for a period of 10 days. Statistical analysis was performed between the control and treatment using Anova. Error bars = 1SD, n=3.

In terms of production rate, at day 10, the cultures that were exposed to aeruginosin showed significantly lower production rate compared to the control, whereas the cultures that were exposed to microcystin-LF, aerucyclamide A and anabaenopeptin B showed a significantly higher production rate. As stated previously, what seems to be enhanced peptide production may well be a direct effect of lowered cell growth rate, leading to the accumulation of more metabolite within the cells. Within cyanobacteria, it is generally accepted that secondary metabolite production follows growth rate [297], but later studies also noted that higher growth rates resulted in reduced peptide production [277]. This study's observations support the latter, at least in the case of microcystin and cyanopeptolin A production. Some previous work on cyanopeptolins have been published, however, the authors declare that due to absence of purified standards, their calculations would be inaccurate. Production details of cyanopeptolins have so far never been reported in literature

and hence to our knowledge, this is the first report where *de-novo* cyanopeptolin production has been deduced.

The effect of cyanopeptides on the release of intracellular cyanopeptolin A was studied and it revealed that none of the exogenously added peptides contributed to the significant release of intracellularly produced cyanopeptolin A (Figure 5.22). It has been previously reported that cyanopeptolins are unstable once outside the cell and hence this may have been a contributing factor here as well.

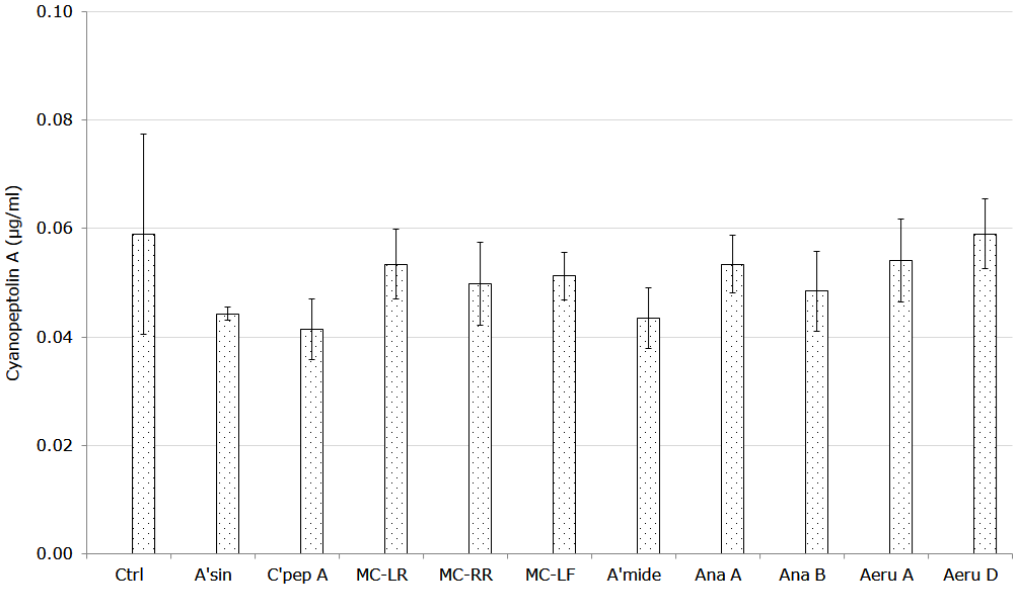


Figure 5.22: The concentration of extracellular *de-novo* cyanopeptolin A (m/z 968) when the cells of *M. aeruginosa* PCC7806 was exposed to different cyanopeptides. *M. aeruginosa* PCC7806 was exposed to different cyanopeptides and allowed to grow for 10 days. At day 10, the supernatant was analyzed in HPLC-MS/MS for *de-novo* microcystin. Statistical analysis was performed, however no statistically significant differences were found. Error bars = 1SD and n = 3 ■Day 0 □Day 10

5.4.6 Effect of cyanopeptides on Aerucyclamides

M. aeruginosa PCC7806 produces several aerucyclamides such as A (m/z 535), B (m/z 533), D (m/z 587) and C (m/z 517). Aerucyclamides are chemically different in that they contain sulphur

and unlike other secondary metabolites of PCC7806, are thought to be synthesised ribosomally (Table 5.3) [162, 90].

Table 5.3: Table of aerucyclamides produced by *M. aeruginosa* PCC7806

Name	Formula	^{14}N Mass	^{15}N Mass
Aerucyclamide A	$C_{24}H_{34}N_6O_4S_2$	534	540
Aerucyclamide B	$C_{24}H_{33}N_6O_4S_2$	532	538
Aerucyclamide C	$C_{24}H_{33}N_6O_5S$	516	522
Aerucyclamide D	$C_{26}H_{31}N_6O_4S_3$	586	592

Purified standards of aerucyclamide A and D were available and hence only these metabolites were quantified from the samples. The intracellular *de-novo* aerucyclamide A profile revealed that most of the treatments showed no significant effect in their intracellular quantities except exogenously added aerucyclamide A & D showed a significant inhibition in its intracellular content (Figure 5.23). In order to further investigate the effect of cyanopeptides, the production rate of aerucyclamide A was calculated (Figure 5.24).

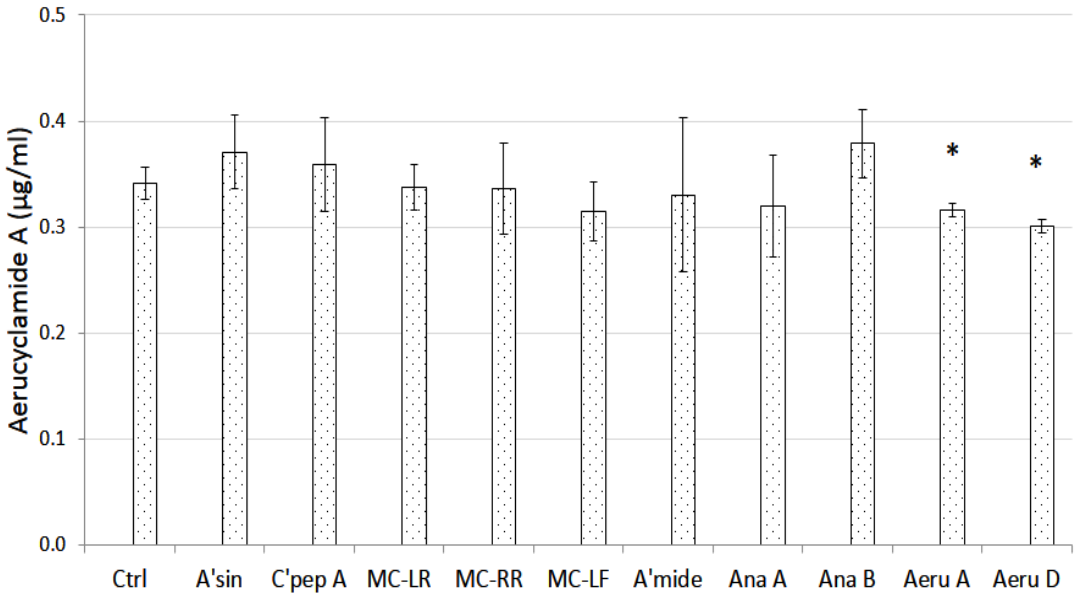


Figure 5.23: The effect of cyanopeptides on intracellular *de-novo* aerucyclamide A (m/z 541) biosynthesis at day 10. *M. aeruginosa* PCC7806 cells were grown in ^{15}N containing BG-11 media in the presence of purified cyanopeptides. Using Anova, statistical significance was determined by comparing each treatment to its week's control. * indicates $p \leq 0.05$. Error bars = 1SD, n=3.

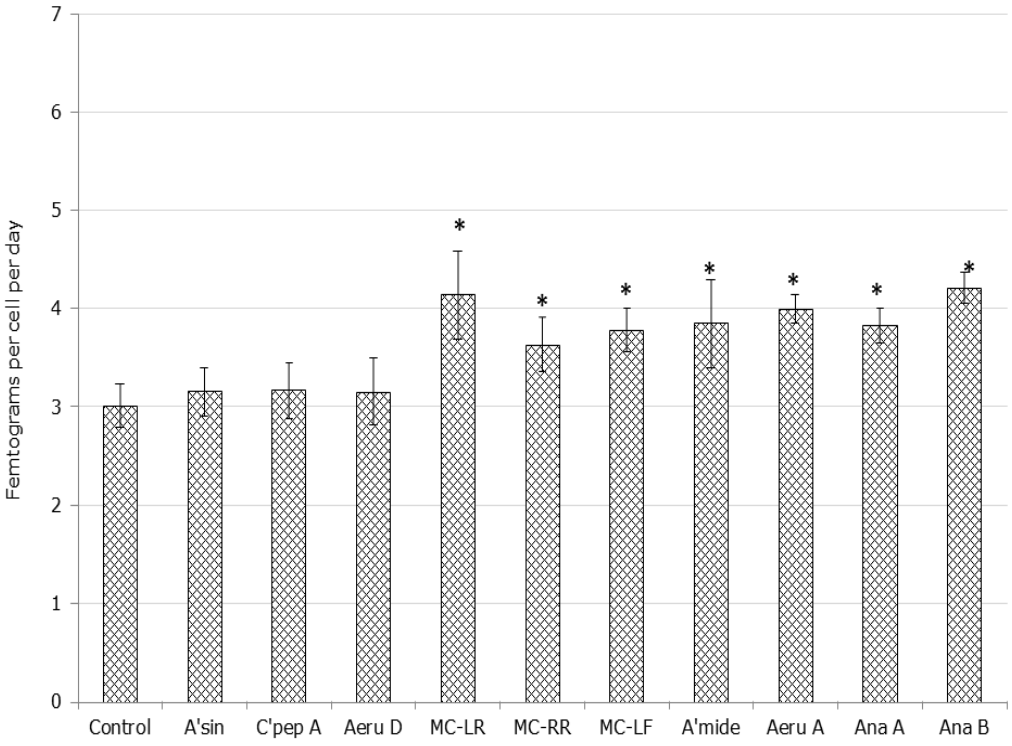


Figure 5.24: The production rate of Aerucyclamide A (m/z 541) by *M. aeruginosa* PCC7806 when exposed to exogenous cyanopeptides. The cells were grown in the presence of cyanopeptides in ^{15}N BG11 media for a period of 10 days. Statistical analysis was performed between the control and treatment using Anova. Error bars = 1SD, n=3. ⊠Aeru A(Day 0 to 10)

The production rate of aerucyclamide A, between day 1 to 10 revealed that *M. aeruginosa* PCC7806, aerucyclamide A was produced at 3.01 ± 0.21 fg/cell/day. In terms of the effect of cyanopeptides on aerucyclamide A, at day 10, the treatments microcystin-LR, microcystin-RR, microcystin-LF, aeruginosamide, anabaenopeptin A & B, and aerucyclamide A showed significant increase in their production rate compared to the control. It seems that microcystins, anabaenopeptins and some aerucyclamides may have a positive effect on the production of aerucyclamides. This observation may well be considered pivotal, since aerucyclamides are ribosomal peptides (unlike microcystin and cyanopeptolins which are NRPS/PKS enabled) and may reflect cellular dynamics better than non-ribosomally synthesised peptides. In this view, it appears that cells exposed to certain cyanopeptides are metabolically more active than their unexposed counterparts, even though this activity does not translate to increased cell numbers (Table 5.1). It should also be pointed out here that, as *M. aeruginosa* PCC7806 produces many of the metabolites evaluated here, even in the control the cells are being exposed to some of their own cyanopeptides. In ecological scenarios, high exogenous cyanopeptides may be seen during bloom collapse. It also means that during bloom collapse, there are nutrients released which may be useful for the surviving few cells. In this scenario, the presence of exogenous cyanopeptides (by increasing the host's metabolic activity) may favour the growth of the host cells adding a competitive advantage. In a recent transcriptomics aided study, it was reported that exogenous microcystin-LR, along with light served to

maintain PSI to PSII ratio and carbon metabolism providing an advantage to the host [328]. This study also supports this view and moreover adds that apart from certain microcystins, even aerucyclamides, aeruginosamide and few anabaenopeptins may also potentially serve as signalling molecules.

The effect of cyanopeptides on the release of intracellular aerucyclamide A (Figure 5.25) was studied and it was observed that none of the peptides had any significant effect on the release of intracellular aerucyclamide A.

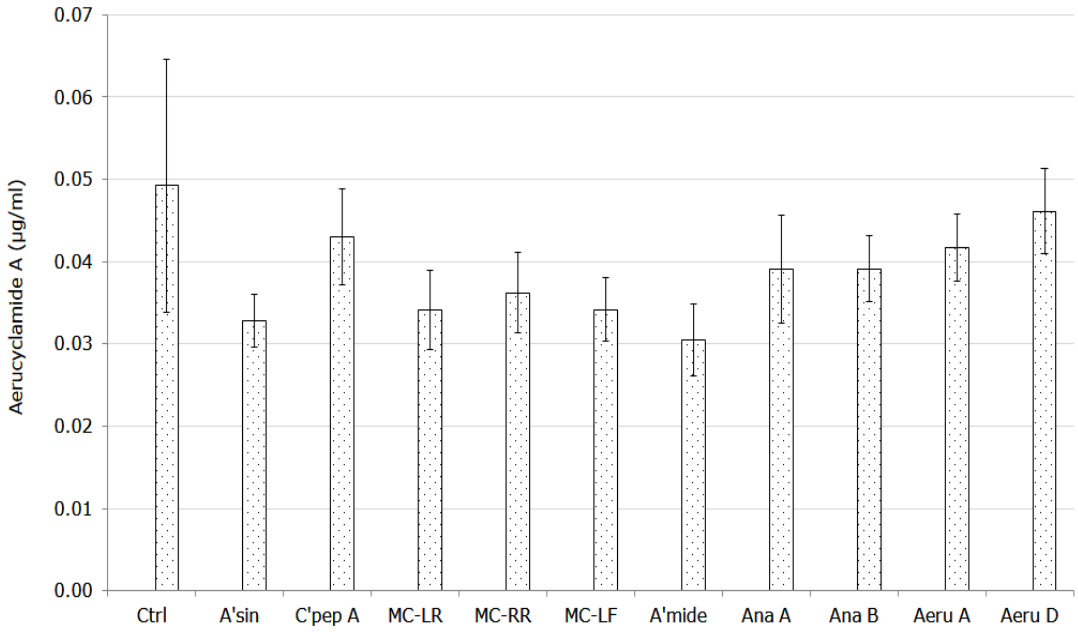


Figure 5.25: The concentration of extracellular *de-novo* Aerucyclamide A (m/z 541) when the cells of *M. aeruginosa* PCC7806 was exposed to different cyanopeptides. *M. aeruginosa* PCC7806 was exposed to different cyanopeptides and allowed to grow for 10 days. At day 10, the supernatant was analyzed in HPLC-MS/MS for de-novo microcystin. Statistical analysis was performed, however no statistically significant differences were found. Error bars = 1SD and n = 3

The *de-novo* aerucyclamide D profile was also quantified and it revealed that the treatments that received microcystin-LF, aerucyclamide A and D showed significant reduction in aerucyclamide D synthesis (Figure 5.26). Further, the production rate of aerucyclamide D, when exposed to cyanopeptides was also calculated,

but it revealed that none of the peptides showed any significant difference when compared to the control at day 10 [310]. Since aerucyclamide D is also a ribosomally synthesised molecule, this was contradicting to the picture of aerucyclamide A production rate (Figure 5.27) where the microcystins, anabaenopeptins, aeruginosamide and aerucyclamide A had shown significant increase when compared to control. However, the overall trend, i.e. the mean production in the treatments that received microcystins, aeruginosamide, anabaenopeptins and aeruginosamide A was higher than their control. This contradiction may be due to the low concentrations of aerucyclamide D produced (only 25% that of aerucyclamide A) by *M. aeruginosa* PCC7806.

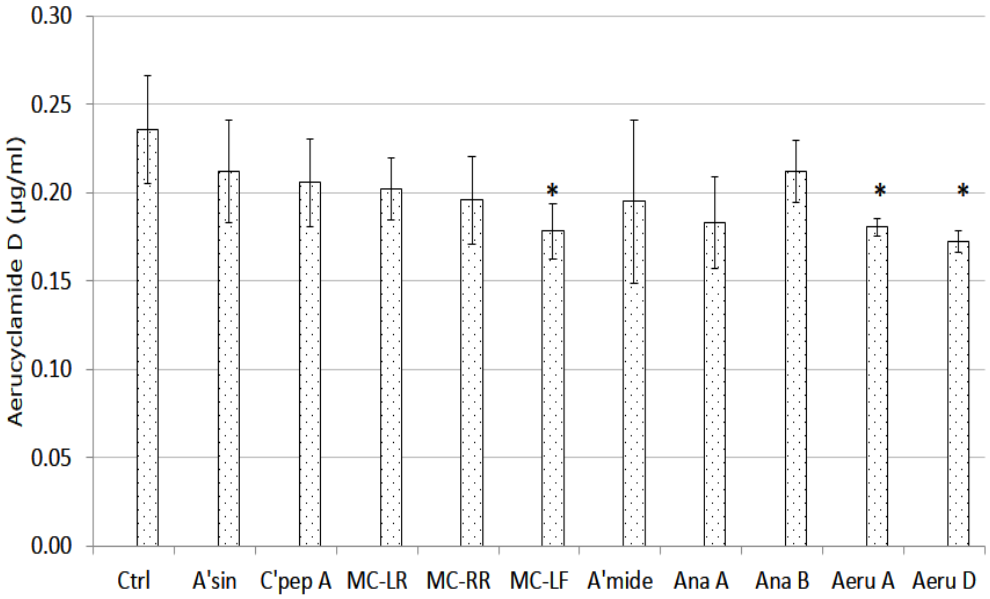


Figure 5.26: The effect of cyanopeptides on intracellular *de-novo* aerucyclamide D (m/z 593) biosynthesis. *M. aeruginosa* PCC7806 was grown in BG11-^{15}N for a period of 10 days, in the presence of exogenously added cyanopeptides. Using Anova, statistical significance was determined by comparing each treatment to its week's control. * indicates $p \leq 0.05$. Error bars = 1SD, n=3. ■ Day 0 □ Day 10

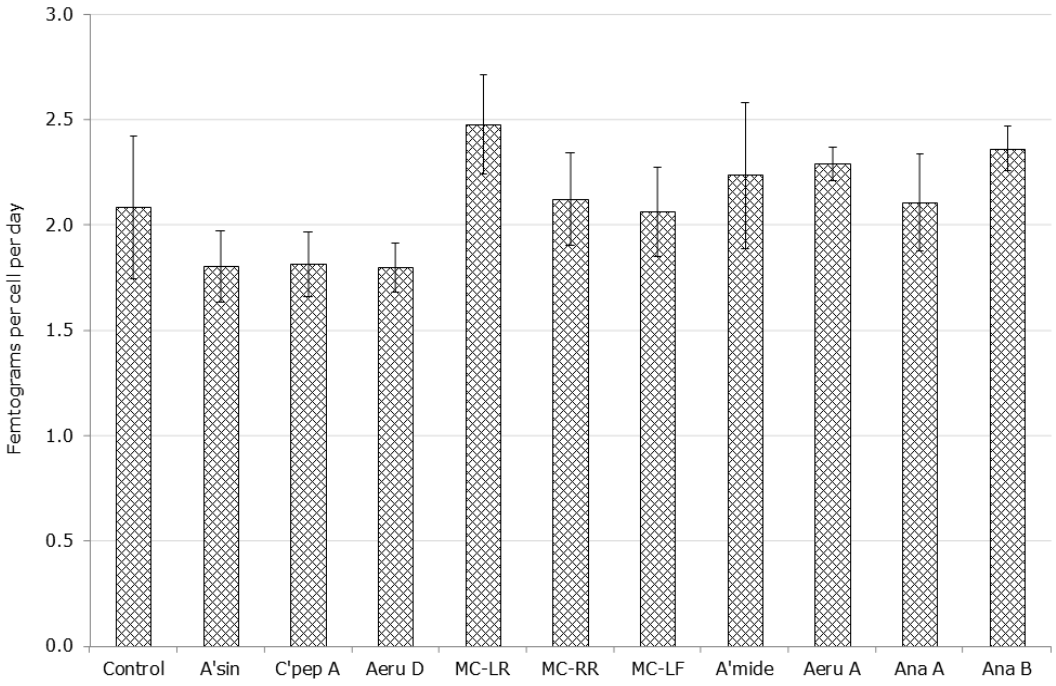


Figure 5.27: The production rate of Aerucyclamide D (m/z 593) by *M. aeruginosa* PCC7806 when exposed to exogenous cyanopeptides. The cells were grown in the presence of cyanopeptides in ^{15}N BG11 media for a period of 10 days. Statistical analysis was performed between the control and treatment using Anova. Error bars = 1SD, n=3.

The analysis of the extracellular aerucyclamide D revealed that within the time periods investigated, only 10% of aerucyclamide D is present in the extracellular region compared to that of intracellular. Also, none of the exogenously added peptides had any significant effect on the release of *de-novo* intracellular aerucyclamide D (Figure 5.28).

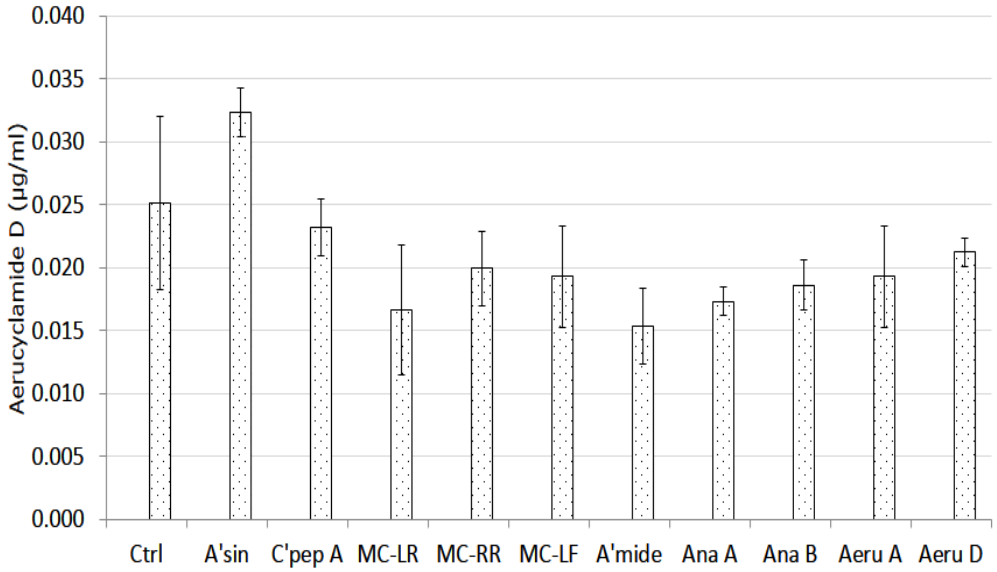


Figure 5.28: The concentration of extracellular *de-novo* Aerucyclamide D (m/z 593) when the cells of *M. aeruginosa* PCC7806 was exposed to different cyanopeptides. *M. aeruginosa* PCC7806 was exposed to different cyanopeptides and allowed to grow for 10 days. At day 10, the supernatant was analyzed in HPLC-MS/MS for de-novo microcystin. Statistical analysis was performed, however no statistically significant differences were found. Error bars = 1SD and n = 3 ■Day 0 □Day 10

5.5 Discussion

Cyanobacteria produce numerous secondary metabolites, however, their function to the host or in the ecosystem is unknown. In this study, the effect of various cyanopeptides on the growth and secondary metabolite production of *M. aeruginosa* PCC7806 was investigated. A selection of purified peptides were dissolved in MilliQ, however due to differences in their solubility, were unable to achieve the intended 10 µg/ml concentration of each of the peptides. The peptides aerucyclamide D and aeruginosamide were least soluble in MilliQ, while aeruginosin and cyanopeptolin A were readily soluble. Only for aeruginosamide, an additional sonication step was added, however, solubility was still poor. It should be noted that even after

sonication, aeruginosamide would have been added to the culture and may have contributed to some of the activity seen. In terms of stability, all the peptides studied were stable for the 10 days duration, except cyanopeptolin A whose concentration appeared to decline with time. Cyanopeptolin A's intracellular *ex-novo* concentration was also checked for the duration of the study and here again we noticed a decreasing trend.

When the cell numbers at day 10 were considered, it appeared that microcystin-LF and microcystin-RR, the two anabaenopeptins and aerucyclamides and aeruginosamide seemed to inhibit growth of *M. aeruginosa* PCC7806. However, when their corresponding doubling times were calculated, it revealed that only microcystin-LR, microcystin-LF, aerucyclamide A, aeruginosamide and anabaenopeptin B had significantly increased their doubling time (meaning slower growth rate). In 'real world' scenario, when cyanobacterial blooms collapse, the release of secondary metabolites by lysing cells, may signal surrounding cells to reduce cell division, yet prepare for re-invasion. This may be a strategy for species survival and dominance.

The effect of cyanopeptides on *de-novo* microcystins (both demethyl-microcystin-LR and microcystin-LR) was evaluated. In this study, it was observed that *M. aeruginosa* PCC7806 produces demethyl-microcystin-LR and microcystin-LR in equal concentrations and the total *de-novo* microcystin concentration at day 10 was 1.09 ± 0.09 µg/ml. Further, the daily production rate of *de-novo* microcystin was calculated and the impact of exogenous peptides was evaluated. It

revealed that during day 0 to 10, the organism was producing microcystin at the rate of 9.67 ± 0.90 fg/cell/day and only the peptides aeruginosin and anabaenopeptins B had a lasting significant enhancing effect on the production rate. It remains to be explored how these peptides induce the change and how this would affect an organism in the ecosystem. As *M. aeruginosa* PCC7806 produces cyanopeptolins as well as aeruginosamides, their production rates were also determined. It was calculated that the organism produces cyanopeptolin A at the rate of 4.16 ± 0.23 fg/cell/day (day 0 to 10) and that aeruginosin, microcystin-LF, aerucyclamide A and anabaenopeptin B had a significant effect on its production rate. From this study and previous studies, it was clear that cyanopeptolin A is an unstable molecule and hence the changes in production rate seen here may have also been the net result of cyanopeptolin biosynthesised vs degradation.

The view of the effect of cyanopeptides on *M. aeruginosa* PCC7806 was significantly changed when the production rate of aerucyclamides was determined. It was calculated that aerucyclamide A is produced at the rate of 3.01 ± 0.21 fg/cell/day (day 0 to 10) and that exogenous microcystin-LR, microcystin-LF, microcystin-RR, aeruginosamide, anabaenopeptin A & B and aerucyclamide A had an enhancing effect on the aerucyclamide A. As it is known that aerucyclamides are synthesised ribosomally and hence it is thought that they would reflect the intracellular dynamics more precisely compared to the non-ribosomally synthesised MC's or cyanopeptolins. In this view, it is inferred that many of the cyanopeptides confer a

distinct advantage to the host (by upregulating their metabolism). However, it is also noted that such high concentrations of exogenous peptides may not occur unless there is massive bloom collapse.

An analysis on the effect of cyanopeptides on the production rates of secondary metabolites (of *M. aeruginosa* PCC7806) revealed that, between day 0 to 10 the metabolite aerucyclamide A appeared to be affected by most cyanopeptides, while microcystin-LR showed least difference. It is thought that this is due to the fact that aerucyclamide A is ribosomally synthesised and hence would reflect the intracellular metabolism closely compared to its NRPS/PKS counterparts. Hence, this leads to the conclusion that the cyanopeptides (microcystin-LR, microcystin-LF, microcystin-RR, aeruginosamide, aerucyclamide A, anabaenopeptin A & B) induce significant upregulation in the metabolic activities of *M. aeruginosa* PCC7806.

Table 5.4: A summary of the effect of cyanopeptides on the secondary metabolites of *M. aeruginosa* PCC7806 between day 0 to 10.

		De-novo production rate		
	Doubling Time	Microcystin-LR	Cyanopeptolin A	Aerucyclamide A
Control	-	-	-	-
Microcystin-LF	▼	-	▲	▲
Microcystin-LR	▼	-	-	▲
Microcystin-RR	-	-	-	▲
Cyanopeptolin A	-	-	-	-
Aeruginosin	-	▼	▼	-
Aeruginosamide	▼	-	-	▲
Anabaenopeptin A	-	-		▲
Anabaenopeptin B	▼	▲	▲	▲
Aerucyclamide A	▼	-	▲	▲
Aerucyclamide D	-	-	-	-

▼ – significantly lower than control and ▲ significantly higher than control. ($p \leq 0.05$)

From the quantification of the production rate of aerucyclamide A, it appears that there is an upregulation, however, it is not known how this upregulation benefits the host. Further, it is also not known how this would affect the host in a competitive ecological scenario.

It is clear from this study, that in order to understand the role of cyanopeptides, further comprehensive studies are required. In this study, *M. aeruginosa* PCC7806, which produces several secondary metabolites (cyanopeptides), was used and its response to a selection of cyanopeptides was evaluated. In which case, the control (where no

exogenous cyanopeptide was added) would also have been exposed to some of its own cyanopeptides causing auto-induction. For future studies, it is suggested that a naturally occurring non microcystin producing strain of *M. aeruginosa* be used (e.g. *M. aeruginosa* CYA43) as this would avoid auto-induction, at least from microcystins. Further, it is recommended that the growth of the organism is monitored by measuring the gene expression of chlorophyll-a, as this would directly reflect the health of a cell. It has been reported that the DI protein (of PSII) has a half-life between 2 to 3 hrs and is constantly [335] replaced. Finally, it is also suggested that gene expressions are quantified using automated qPCR or real time qPCR as these techniques are highly sensitive and require less post reaction analysis.

In many ways this study has contributed significantly to the work on *M. aeruginosa* PCC7806 (and to the larger context of cyanobacterial research). In terms of bioassays, it can be concluded that the growth of *M. aeruginosa* PCC7806 is optimal when grown in BG11 containing 0.75 g/L of sodium nitrate as compared to 1.5 g/L. It is also better to grow the inoculum in the same nitrate concentration and light intensity. It is also suggested that a single sample withdrawn at day 10 is sufficient where rapid results are required and this is consistent even when aged inoculums are used. It was also seen that none of the exogenously added peptides significantly aided in the release of intracellular secondary metabolites of *M. aeruginosa* PCC7806.

Chapter VI

Conclusion and discussion

Cyanobacteria produce a multitude of diverse and interesting compounds of which some are harmful while others may have potential beneficial use. Of the many cyanobacteria that form blooms, *M. aeruginosa* is one of the most common in freshwater ecosystems. An interesting characteristic of some members of this genus is their ability to produce an array of cyanopeptides (secondary metabolites). Over the years of evolution, this genus has proved to be adaptable and open to accept foreign genes. It has been estimated that this species may have acquired up to 11% of its genes by horizontal gene transfer and that it may harbour about 13 secondary metabolite gene clusters. Of these clusters, the genes for microcystins (*mcy*), aeruginosins (*aer*), cyanopeptolins (*mcn*) and microviridin (*mdn*) seem to have been conserved within many of *Microcystis* species. It has been reported that the expression profile of these genes are similar in a typical day/night cycle and it is thought that the interaction between these genes may be one of the reasons for their joint conservation [217, 336] down the years. It is also clear that the cells have to expend some energy into the production of these metabolites and in the case of *M. aeruginosa* PCC7806, which has 7 different secondary metabolite gene clusters, the energy expense would be considerable. Although genetic evidence suggests that these secondary metabolites may have some function to the producing organism, to date a clear evidence has not been reported.

The primary aim of this study was to answer the question, 'Why do cyanobacteria produce secondary metabolites?'. With *M. aeruginosa* producing so many metabolites, it was essential to develop techniques

for quantifying *de-novo* production. By providing ^{15}N as the sole source of nitrogen, *de-novo* production rates could be calculated. This enabled the monitoring of both the *de-novo* and the *ex-novo* metabolites produced. It was learnt that *M. aeruginosa* PCC7806 takes about 2 days for complete biosynthesis of microcystin-LR, aerucyclamides and cyanopeptolins and that of all the metabolites produced by this organism, aerucyclamide A was the most abundant, followed by microcystin-LR. The fragmentation pattern revealed by the use of Waters® UPLC was reliable and adds to the robustness of this technique and it is suggested that this technique may be employed while studying other as yet uncharacterised metabolites. Interestingly, while microcystin-LR and cyanopeptolins showed fragmentation, aerucyclamides did not show any major fragments.

Cyanobacteria produce several secondary metabolites of which microcystins are the most studied, as they have caused several episodes of animal and human poisoning in the past. Although, microcystins have been studied for several decades and some researchers report tentative functions, a clear role has never been confirmed. When *M. aeruginosa* PCC7806 was exposed to high concentrations of microcystin-LR, an increase in the doubling time of the organism was seen. This increase caused the accumulation of more secondary metabolites intracellularly giving an impression of high production rate. Other than these changes, the presence of even high concentrations of microcystin-LR did not appear to affect the cells much. Low concentrations (0.1 µg/ml) of microcystin-LR seem to mildly benefit cell growth and metabolites production, yet the evidence

presented is insufficient to confirm a role for microcystin-LR. But interestingly, it was also learnt that the produced microcystin-LR or aerucyclamides are not recycled or degraded for the duration of (35 days) the study.

As a role for microcystin-LR could not be confirmed, it was thought that, evaluating the effect of other cyanopeptides on *M. aeruginosa* PCC7806 may shed some light on their function. The effect of several cyanopeptides on *M. aeruginosa* PCC7806 was investigated. A selection of microcystins, aerucyclamides, cyanopeptolin, anabaenopeptins, aeruginosamide and aeruginosin were used. Of the selection, it appeared that at least, microcystin-LR, microcystin-LF and anabaenopeptins A & B induced some significant differences intracellularly. While microcystin-LR and microcystin-LF induced the cells to grow two-fold slower, anabaenopeptins seems to increase the production rate of the metabolite aerucyclamide A. Although the results do not clearly confirm a role for secondary metabolites, several key lessons were learnt i.e. monitoring the production of enzymatically synthesised (NRPS/PKS) metabolites would be misleading as this would be a poor indicator of intracellular status. However, ribosomally synthesised metabolites (aerucyclamides) are more suitable candidates as their production would be a direct reflections of the expression profile of their genes, which in turn would reflect the metabolic state of the cell.

This study has also highlighted several areas of improvement, which could be incorporated into future studies on cyanobacterial

allelopathy. In terms of cell enumeration, while haemocytometry is the standard technique, advanced techniques such as Multisizer or even flowcytometry have proved to be highly reliable techniques. The labeling of metabolites with ^{15}N may be considered as a game changer, as this method enables the differentiation and accurate monitoring of *ex-novo* and *de-novo* metabolites. Further, the optimized rapid bioassay and molecular techniques such as gene expression studies coupled with sensitive qPCR techniques may be employed to study allelopathy in cyanobacteria.

Finally, although this study does not confirm a role for secondary metabolites, it makes no sense that cyanobacteria (at least some) stably maintain and express genes required for secondary metabolite biosynthesis. It is also intriguing to note that, within a cyanobacterial genus spontaneous mutants arise which lack the ability to produce secondary metabolites and that these mutants perform equally well in an ecosystem [337]. Marine ecosystems are often and always a mixed pool of many organisms, including bacteriophages. It would be very interesting to examine the role of bacteriophages (in this case cyanophages) in the transfer of NRPS/PKS genes to non-toxic strains of cyanobacteria. It is well documented that phages play a significant role in the horizontal gene transfer process and in the origin of new species [338, 339]. It is also known that lysogenic phages are stably maintained within cells conferring some advantage (or none at all) to the host and continue to be propagated infinitely. If it is hypothesised that the NRPS/PKS genes are part of a lysogenic phage genome and that sometime, due to natural processes are excised,

explains at least the development of spontaneous non-toxic mutants within in a toxic cyanobacterial population and vice-versa. The fact that cyanobacteria (compared to heterotrophic bacteria) are comparatively more difficult to transform, may suggests immunity conferred by lysogenic phages. However, further studies are required to evaluate this hypothesis.

Chapter VII

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