ISOLATION AND CHARACTERISATION OF ANTIMICROBIAL COMPOUNDS SYNTHESISED BY *MICROCYSTIS* SP.

by

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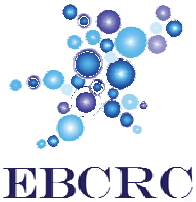


CHAPTER 5 CHEMICAL STRUCTURE ANALYSIS

5.1 Introduction

Comparison of algal peptide content data is fraught with obstacles, principally due to the choice of analytical methods available (Barbarino and Lourenco 2005). However, information gleaned from resolution of peptide concentration is essential in discerning chemical characteristics of individual species and isolating compounds with interesting structural features (Hostettmann and Wolfender 2001). Subsequently, a number of analytical chemistry techniques were applied during this study to ascertain the chemical structure of the compound demonstrating pharmacological activity during the bioassays.

High Pressure Liquid Chromatography (HPLC) is a universally accepted analytical method for applications including separation, identification, purification, and quantification of various compounds (University of Kentucky 2004). This technique has been extensively exploited for identification and purification of bioactive cyanobacterial metabolites; though Bloor and England (1989) acknowledge that only a small number have been structurally identified. This complication is often directly influenced by the minute quantities of secondary metabolite available, and consequently the high sensitivity requirements of analytical equipment. Similarly, samples containing peptide fragments or partial structures of variants with near identical masses cannot always be distinguished; Nuclear Magnetic Resonance (NMR) would be typically necessary for full structure clarification. NMR enables elucidation of compound structure with respect to the position of Hydrogen atoms within a molecule (Bailey and Bailey 1995). However, NMR requires isolation of the compound of interest in the milligram range: many secondary metabolites are not available in these quantities (Welker *et al*. 2006). Consequently, NMR was not applied during this study, and other analytical techniques were employed to elucidate the chemical structure of the bioactive metabolite.

Mass Spectrometry (MS) is an analytical tool for measuring molecular mass of a sample, and may be utilised for analysis of peptides, drug discovery and water quality (Ashcroft 2004; Beresovsky *et al.* 2006). This instrument is often coupled with chromatography apparatus to separate compounds for more accurate resolution of

components by mass spectrometry. During this study, liquid chromatography-mass spectrometry (LC/MS) and tandem mass spectrometry (MS/MS) were employed to resolve components of the bioactive compound's structure. Detailed descriptions of analytical techniques utilized throughout this study are provided below; general attributes of analytical techniques are provided in section §B.III.

5.2 Chemical Structure Identification of bioactive compounds

5.2.1 Reverse-Phase HPLC: Isolation of Bioactive Compounds

Separation of bioactive peptides of *Microcystis flos-aquae* MIC FEB05 was performed on a Vydac® C18 Protein and Peptide Column (5µm, 300Å, 4.6mm ID x 250mm; Alltech, Melbourne, Australia). The HPLC system consisted of an ICI model LC1200 UV-Vis Detector (Waters, Melbourne, Australia), and two ICI model LC1110 solvent pumps (Waters, Melbourne, Australia). The mobile phase consisted of a gradient of 95% v/v acetonitrile (0.1% TFA; solvent A) and 5% v/v acetonitrile in distilled water (0.1% TFA; solvent B), with the following linear gradient program: 15% A at 0 min, 80% A at 30 min, 100% A at 31 min, 100% A at 36 min. Sample volume was 100 µL and flow rate 2 mL min^{-1}. Fractions of approximately 2 mL were collected, corresponding to peaks in absorbance, which was monitored at 214 nm.

Eluted HPLC fractions were assessed for bioactivity against the original test organisms. Fractions that demonstrated a positive result (inhibited growth of bacteria) were further separated by HPLC, using a semi-preparative column (VYDAC® Protein and Peptide C18 Polymeric Reversed Phase 5µm, 300Å, 10 mm ID x 250 mm; Alltech, Melbourne, Australia) to accumulate large quantities of the peptide. This system consisted of a Waters 501 HPLC Pump, a Waters 510 pump, an ICI Instruments LC1200 UV/Vis Detector and a Waters Automated Gradient Controller (Waters, Melbourne, Australia). The same mobile phase was applied as described above, with the following linear gradient program: 5 % A at 0 min, 70 % A at 30 min, 100 % A at 31 min, 100% A at 35 min. Sample volume was 250 µL and flow rate 4 mL min^{-1}. Fractions of approximately 2 mL were collected, with particular focus on peaks with similar retention times to those speculated to represent bioactive compounds (approximately 15-20 min). A number of elutions were performed on the

semi-preparative column to accumulate sufficient peptidic material to enable analysis by Mass Spectrometry.

5.2.2 Mass spectrometric analysis of extracts of *M. flos-aquae*

Electrospray MS/MS data were determined using a Micromass Q-TOF-2 orthogonal acceleration time of flight mass spectrometer with a mass range to 10,000 Da. The Q-TOF-2 was fitted with an electrospray source in an orthogonal configuration with a Z-spray interface. Samples were dissolved in acetonitrile water (1:1) and infused into the electronspray source at a flow rate of 5 μL min^{-1}, under the following conditions: capillary voltage 3.10 kV; source temperature 80°C; desolvation temperature 150°C; cone voltage 50V. MS/MS data were acquired with the argon collision gas energy set between 40-80 eV to give optimal fragmentations.

The peptide was also analysed by electrospray ionisation time-of-flight mass spectrometry (LC/MS-TOF), using an Agilent 1100 series HPLC system (Agilent Technologies; Forest Hill, Victoria, Australia) coupled to a Q-STAR® Pulsar I mass spectrometer (Applied Biosystems; Melbourne, Victoria, Australia). Peptides were loaded on a Zorbax® SB-C18 column (15 cm length) (Agilent Technologies; Forest Hill, Victoria, Australia) and separated using a linear gradient of water/acetonitrile/0.1% formic acid (v/v).

Mass spectrometry analysis was conducted by the School of Chemistry and Physics, University of Adelaide, South Australia (MS and MS / MS), and Proteomics International, a subsidiary of Murdoch University, Western Australia (LC/MS-TOF). Due to commercial confidentiality, a detailed methodology was not provided by Proteomics International for LC / MS – TOF analysis; a generic description has been referred to, where appropriate.

5.3 Profile Interpretation

5.3.1 High Pressure Liquid Chromatography profiles

Extracts obtained from freeze-dried biomass of natural cyanobacterial bloom samples were separated by Reverse Phase High Pressure Liquid Chromatography on a C18 Protein and Peptide column. Chromatographic profiles of bioactive and non-bioactive

extracts were obtained to compare positional differences in peaks, which may infer a fraction containing a bioactive compound. The chromatograph illustrated by Figure 5-1 was obtained by eluting a methanol/sonication extract of *M. flos-aquae* MIC MAR05. This extract had not previously demonstrated growth inhibition of any of the test organisms. Figure 5-2 depicts an HPLC profile of a methanol/sonication extract obtained from *M. flos-aquae* MIC FEB05. The latter extract demonstrated both antibacterial and antiviral activity during bioassay screening. While the chromatograms were similar, one peak in particular was observed in the profile of the February sample (Figure 5-2) that was absent from the chromatogram of the March profile (Figure 5-1), and has been highlighted in Figure 5-2. This peak, labelled R3F6 had a retention time of approximately 18 min; there was no corresponding peak observed in the peptide profile of the chromatogram constructed from the *M. flos-aquae* MIC MAR05 extract. Given that the extracts prepared from the *M. flos-aquae* MIC MAR05 biomass sample did not exhibit any antimicrobial activity, whilst the sonicated methanol extract prepared from the *M. flos-aquae* MIC FEB05 isolate inhibited growth of both bacteria and viruses, it is implicit that differences exist between their peptide profiles.

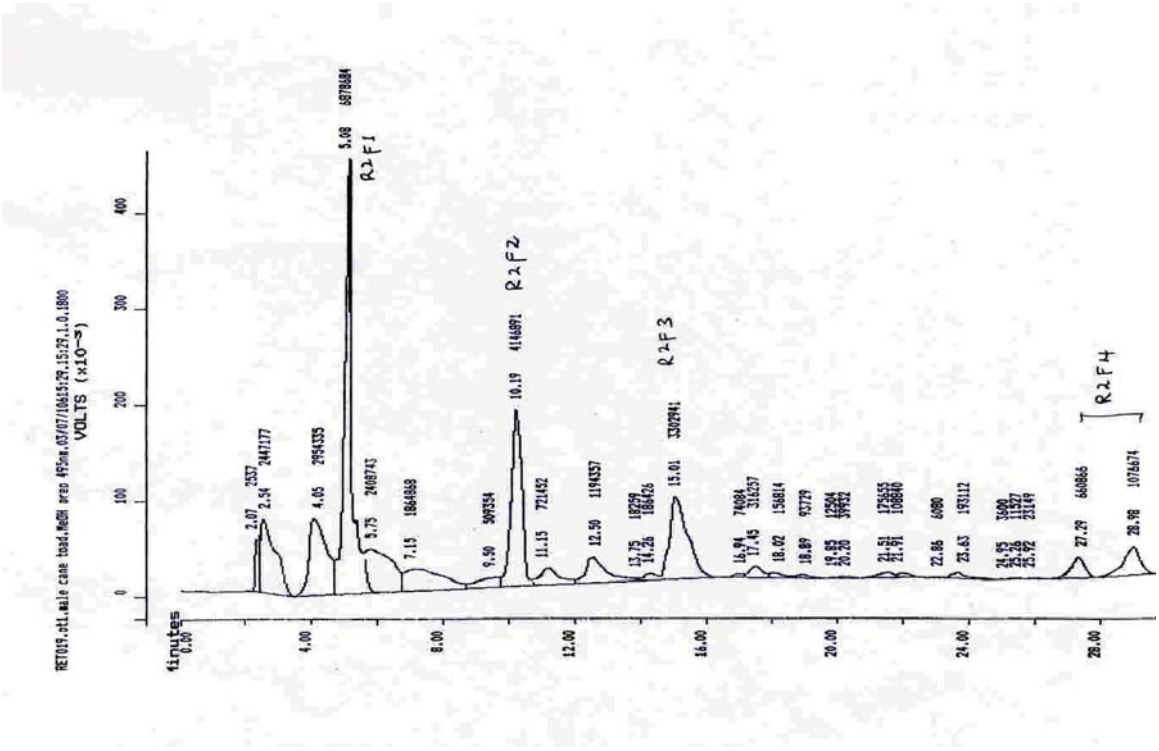


Figure 5-1: HPLC chromatogram obtained from crude methanol/sonication extract of Torrens River *M. flos-aquae* MIC MAR05 isolate. This extract did not demonstrate bioactivity against bacteria, fungi or viruses

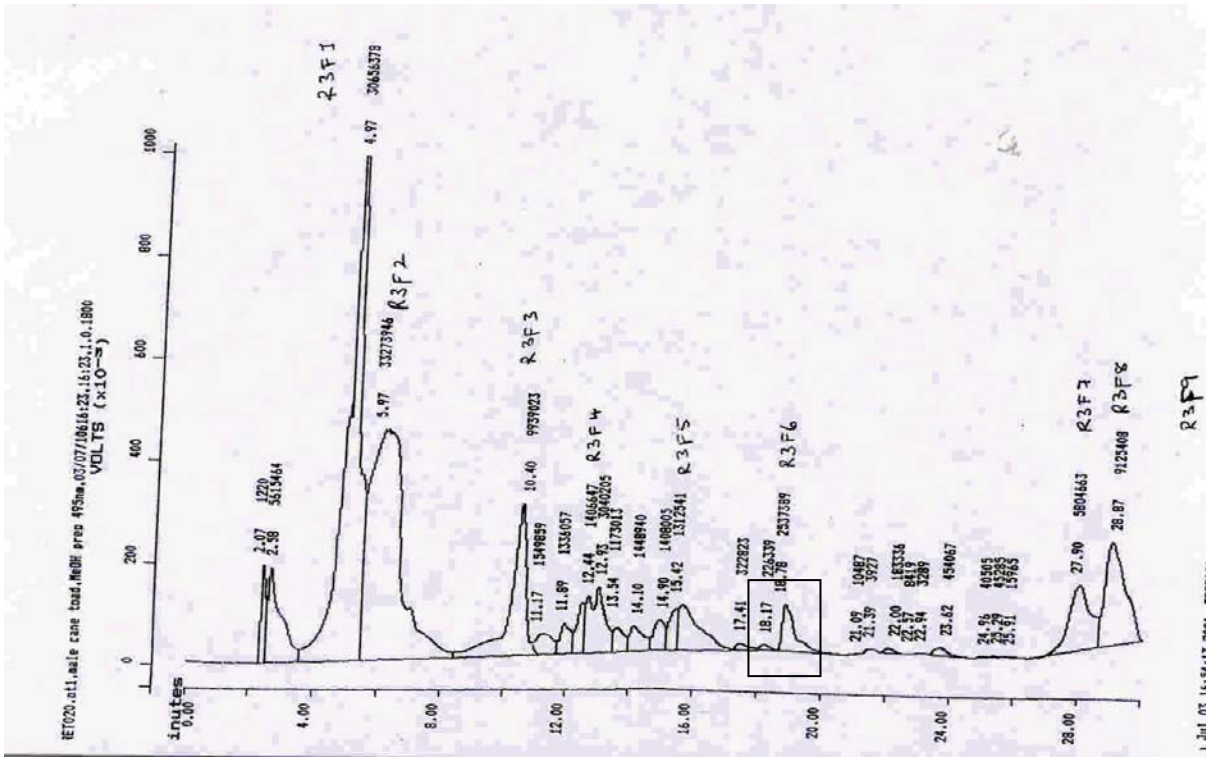


Figure 5-2: HPLC chromatogram of methanolic extract obtained from Torrens River *M. flos-aquae* MIC FEB05 isoalte. This extract has previously demonstrated antibacterial and antiviral activity. Fraction R3F6, at retention time of 18 min, was not observed in the non-bioactive extract.

5.3.2 Mass Spectrometry profiles

Mass spectrometry profiles of the extracts were constructed to identify the functional groups and amino acids that represent the bioactive peptide. The relative abundance of particular amino acids and characteristic patterns of MS profiles enables resolution of the molecular formula of a compound, and often suggests possible structures. The low mass region of MS / MS spectra often contain ions that are indicative of the presence of specific amino acid residues within the peptides (Frias *et al.* 2006).

The mass spectrometry profile presented below and labelled Figure 5-3 was created by measuring the mass to charge ratio of compounds in the methanol/sonication extract of *M. flos-aquae* MIC FEB05. A significant signal is observed on the profile with an *m/z* of 1015.7. However, the MS profile of another extract, a multiple methanol extraction of the *M. flos-aquae* MIC FEB05 biomass, did not resolve as succinctly as the methanol/sonication extract. A comparison of the profiles represented by Figure 5-4 and Figure 5-5 indicated the methanol/sonication extract

was significantly more complex than that of the multiple methanol extraction. The complexity of the profiles may be affected by the success of the individual extraction procedures. Bioactivity assay screening of the extracts indicated only the methanol/sonication extract exhibited antibacterial activity (results presented in Chapter 4).

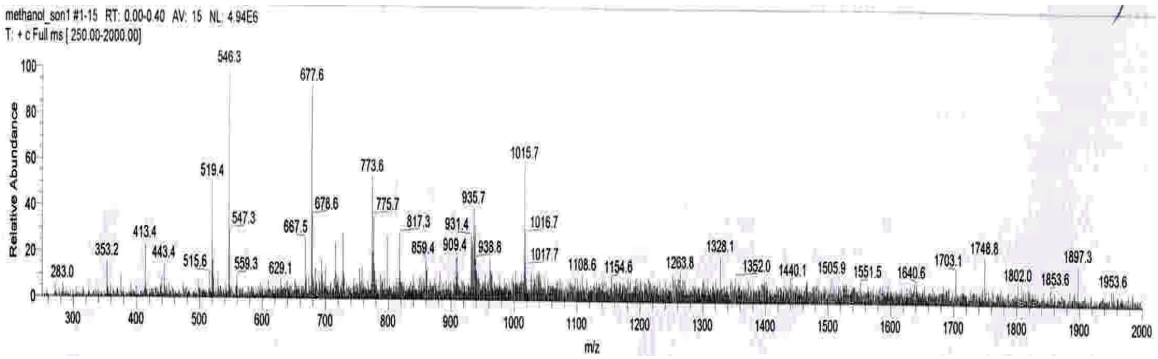


Figure 5-3: MS profile of the bioactive methanol/sonication extract obtained from Torrens River *M. flos-aquae* collected in February 2005

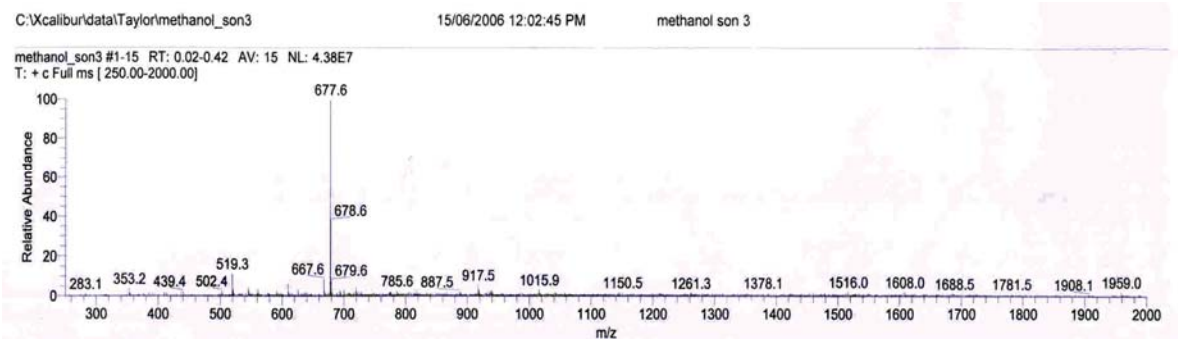


Figure 5-4: MS profile of 3 x methanol extraction of Torrens River *M. flos-aquae* MIC FEB-05

Resolution of the MS profile for the methanol/sonication extract, and removal of the background "noise" revealed a number of "daughter peaks" associated with the base peak at *m/z* 1015.7; this fragmentation is observed in Figure 5-5. This phenomenon suggested the compound had undergone a fragmentation reaction within the extract, perhaps a degree of peptide degradation. However, fragmentation patterns allow determination of the mass of an unknown compound, in addition to directing elucidation of the molecular structure. This extract was subjected to further analysis via tandem mass spectrometry and LC / MS to construct a likely molecular configuration.

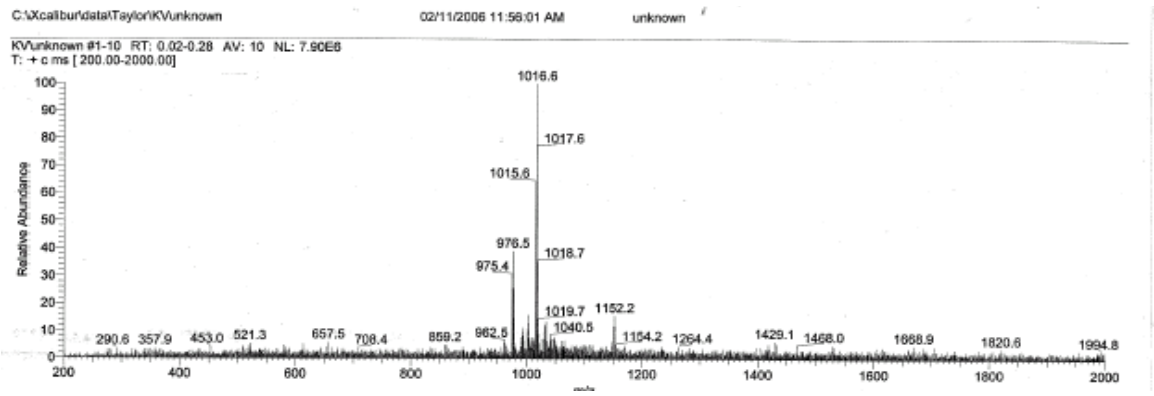


Figure 5-5: MS profile of bioactive methanol/sonication extraction of Torrens River *M. flos-aquae* MIC FEB05. Resolution of the peptide peak removal of background noise revealed several daughter peaks associated with the base peak.

5.3.3 Liquid Chromatography/Mass Spectrometry

Liquid chromatography-mass spectrometry approach measures discreet chemical fragments of bioactive compounds following separation of the peptide by chromatography (Frias *et al.* 2006). Consequently, LC / MS has proven to be a powerful tool for analysis of toxins and other bioactive metabolites at trace levels, as it provides primary amino acid sequence information for identification of the compounds of interest (Frias *et al.* 2006).

To endorse accuracy of the chemical structure analysis of the bioactive peptide forming the focus of this study, a lyophilised sample was submitted to Proteomics International, a commercial subsidiary of Murdoch University, Western Australia for *de novo* peptide sequencing analysis. HPLC profiles of the bioactive extract were supplied to Proteomics International to provide a basis for sample analysis. The eluted fraction R3F6 was analysed by LC/MS prior to *de novo* sequencing to determine potential components of the bioactive compound's structure. The peptide was analysed by electrospray ionisation time-of-flight mass spectrometry (LC/MS-TOF), using an Agilent 1100 series HPLC system (Agilent Technologies; Forest Hill, Victoria, Australia) coupled to a Q-STAR Pulsar I mass spectrometer (Applied Biosystems; Melbourne, Victoria, Australia). Peptides were loaded on a Zorbax® SB-C18 column (15 cm length) (Agilent Technologies; Forest Hill, Victoria, Australia) and separated using a linear gradient of water/acetonitrile/0.1% formic acid (v/v).

Data obtained from the LC / MS / TOF investigation was analysed to obtain *de novo* sequence using Analyst QS software (Applied Biosystems; Melbourne, Victoria, Australia). The data obtained and corresponding sequence information is provided below. The MSMS profile for the major peptide detected in the sample R3F6 with mass 1037.6 is labelled Figure 5-6.

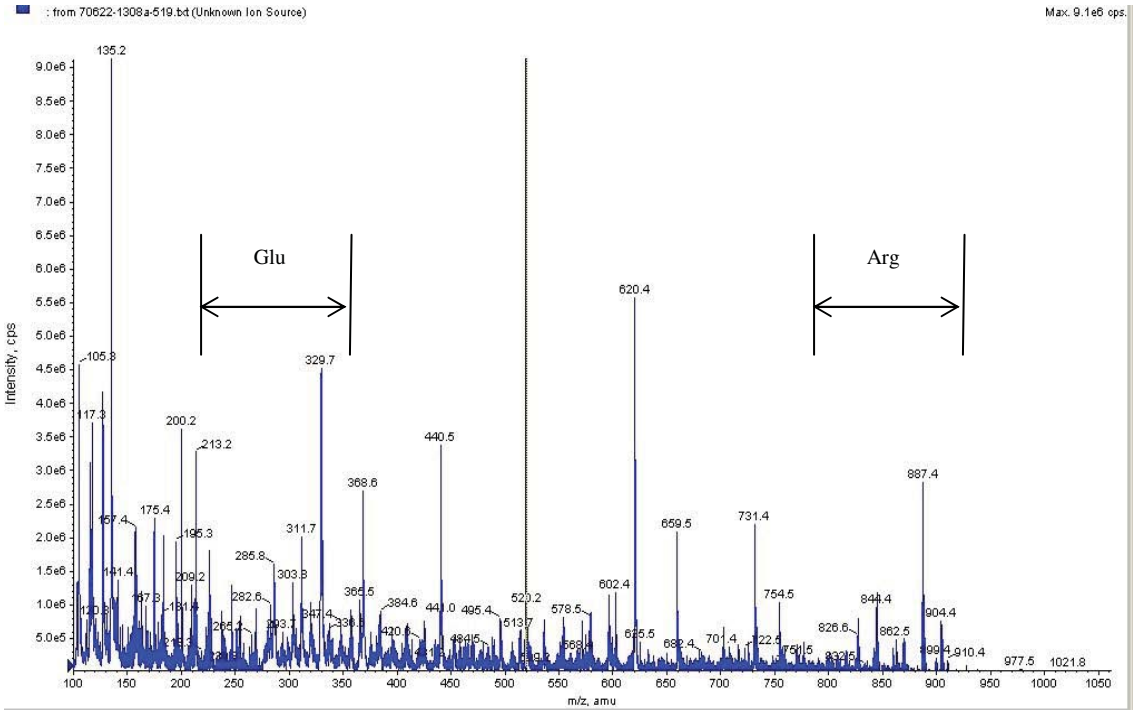


Figure 5-6: MS / MS data profile for major peptide with mass 1037.6 of the bioactive extract sample R3F6

5.3.3.1 Summary of results (Proteomics International, Murdoch University):

De novo sequencing analysis was conducted using the Analyst QS software (Applied Biosystems; Melbourne, Victoria, Australia) for the major peptide with mass (mono) 1037.6 present in sample R3F6, isolated by HPLC. Results of the analysis indicated that there was no readily interpretable, continuous amino acid sequence present, although gaps within the fragmentation ladder corresponded to known amino acids. For example, the mass gap of 156 between peaks at 887.4 and 731.4 indicated the presence of arginine (Arg); while the mass gap between peaks 329.7 and 200.2 (129) may represent glutamine (Glu); both mass gaps are highlighted in the MS / MS profile labelled Figure 5-6. Similarly, the fragmentation pattern potentially indicated the presence of modified amino acids (sulphonated; methylated; alkylated) or sugar units such as xylose (mass gap of 180 between peaks at 620.4 and 440). However, a review

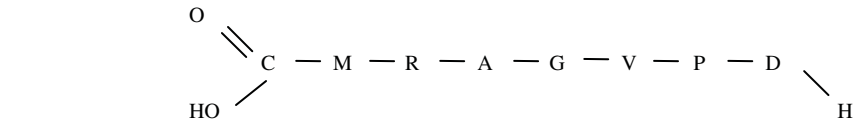
of literature suggested modified (or degraded) amino acids were more likely than sugar units, given the common structural features of cyanobacterial metabolites (Moore 1996; Tooming-Klunderud *et al.* 2007). The possibility also existed that the extract underwent some compound degradation during storage, a hypothesis which was supported by the reduction in biological activity following 2 months storage at -20°C.

5.3.3.2 Amino Acid Sequence Analysis

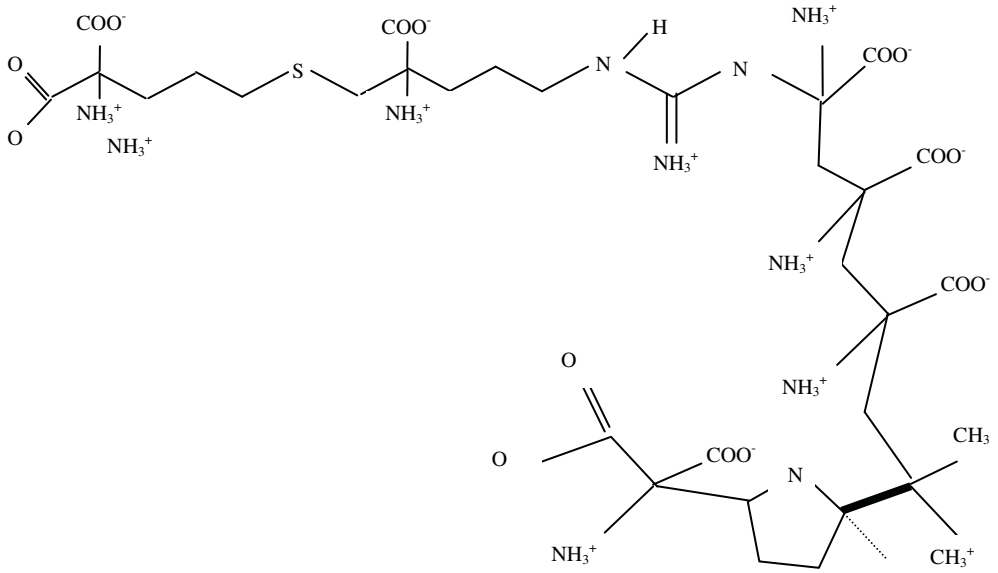
Interpretation of the amino acid sequence of the compound was also undertaken by researchers within the Discipline of Chemistry, School of Chemistry and Physics, University of Adelaide. To obtain a model of the structure, mass gaps between peaks were calculated and identified as representing particular amino acids. This process was undertaken from both limits of the mass scale to overcome the impediment identified by Proteomics International.

The following amino acid pattern was suggested based on the MSMS data provided in Figure 5-6; a proposed peptide structure has been constructed and is labelled Figure 5-7. The amino acids and associated mass gaps of the MSMS profile are illustrated in Figure 5-8.

The occurrence of Ahp (3-amino-6-hydroxy-2-piperidone) fragment at 114 Da (Tooming-Klunderud *et al.* 2007)., or Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-pheyl-4,6-decadienoic acid) at 135 Da (Frias *et al.* 2006) typically supported identification of compound as a cyanopeptolin or a variant of microcystin respectively. However, the absence of both unique amino acid fragments indicated that the bioactive compound synthesised by this strain could not be classified into either of these classes.



(a)



(b)

Figure 5-7: Proposed (a) amino acid sequence and (b) peptide structure for the bioactive compound isolated from non-toxic *M. flos-aquae*.

A comparison of the proposed amino acid sequence to a database of known peptide sequences was unable to classify this sequence and proposed structure within any known class of bioactive metabolites (B. Neilan, personal communication, April 2008). This suggests that either the compound is novel and has not previously been discovered; or the compound has been modified and is a variant of a known peptide. Further purification of the compound is necessary to conclusively identify this compound.

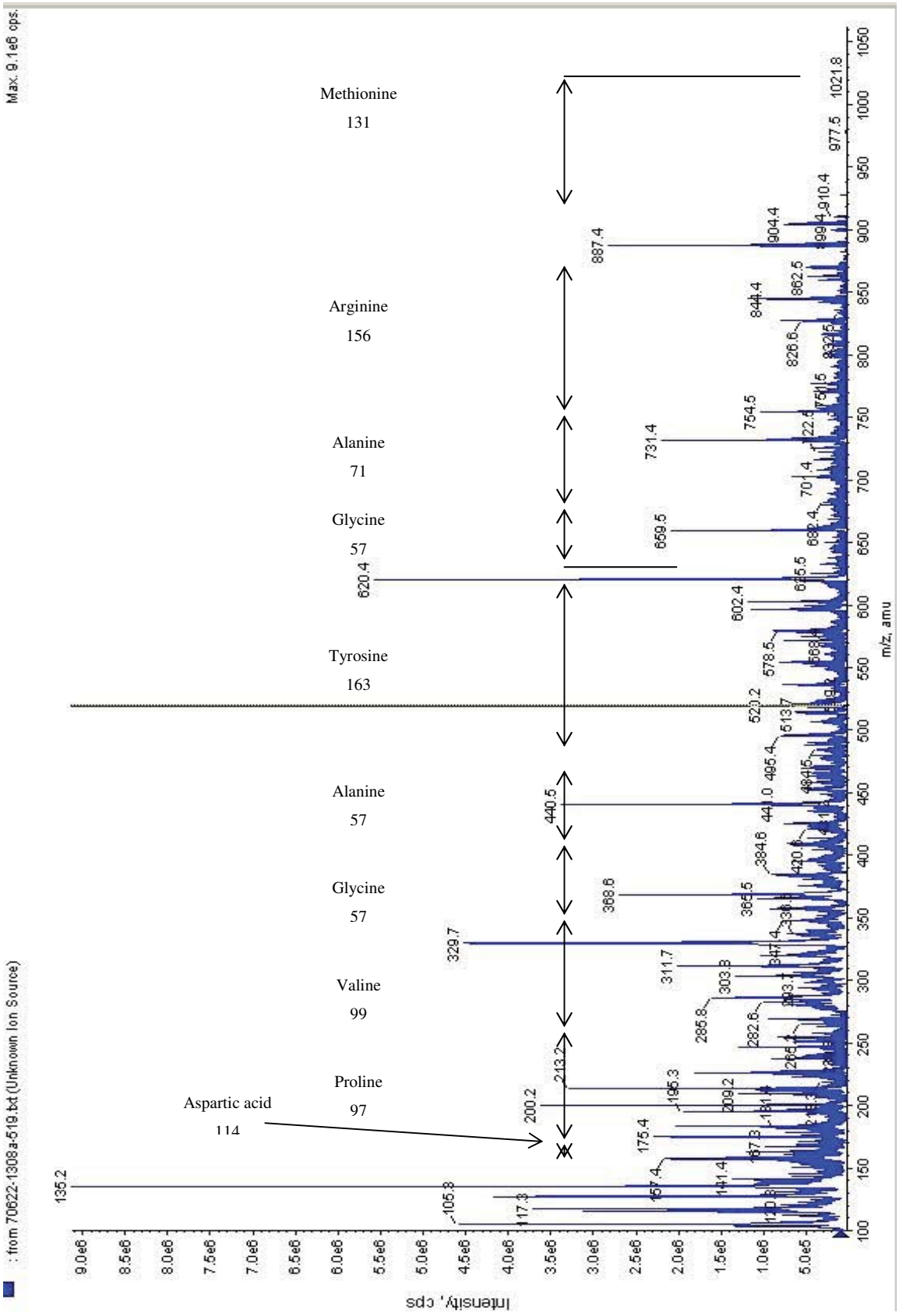


Figure 5-8: Proposed interpretation of the MS / MS profile provided by Proteomics International, to determine an amino acid sequence for major peptide with mass 1037.6 in HPLC fraction R3F6.

5.4 Summary

A methanol-based extract of *M. flos-aquae* MIC FEB05 biomass, collected from a non-toxic bloom in the Torrens River, Adelaide demonstrated antibacterial activity against *S. aureus* and *B. subtilis*, discerned through a series of agar disc diffusion assays (discussed in Chapter 4). This extract, in addition to an inert methanol extract of *M. flos-aquae* MIC MAR05 were separated into component peptides by reverse-phase HPLC. Profiles were examined for differences in the presence of peaks; a peak observed in the biologically active extract but absent in the profile of the inert sample was considered likely to represent the bioactive peptide. Fractions representing 6 peaks from both extracts were assessed for inhibitory activity of the original test organisms (described in Chapter 4). Biological activity was observed for only one fraction, collected from the peak labelled R3F6 at a retention time of approximately 18 min.

The bioactive extract was applied to a semi-preparative column to accumulate the fraction eluted at approximately 18 min. The sample was lyophilised then resuspended in methanol prior to analysis by mass spectrometry and submission to Proteomics International for analysis by LC / MS / TOF and *de novo* sequencing. Mass spectrometric profiles revealed a low resolution of amino acid peaks, with a major peptide with mass 1015.7 Da. Resolution of this peak to remove background noise revealed severe fragmentation of this peptide peak.

De novo sequencing analysis of the sample labelled R3F6 was conducted by Proteomics International to determine a likely amino acid composition of the compound, in addition to indicating possible structural features. Despite a number of gaps in the fragmentation pattern, mass gaps indicated the presence of Arginine and Glutamine, among other amino acids. Analysis of the data by a research chemist from the University of Adelaide suggested the following sequence and corresponding structure:

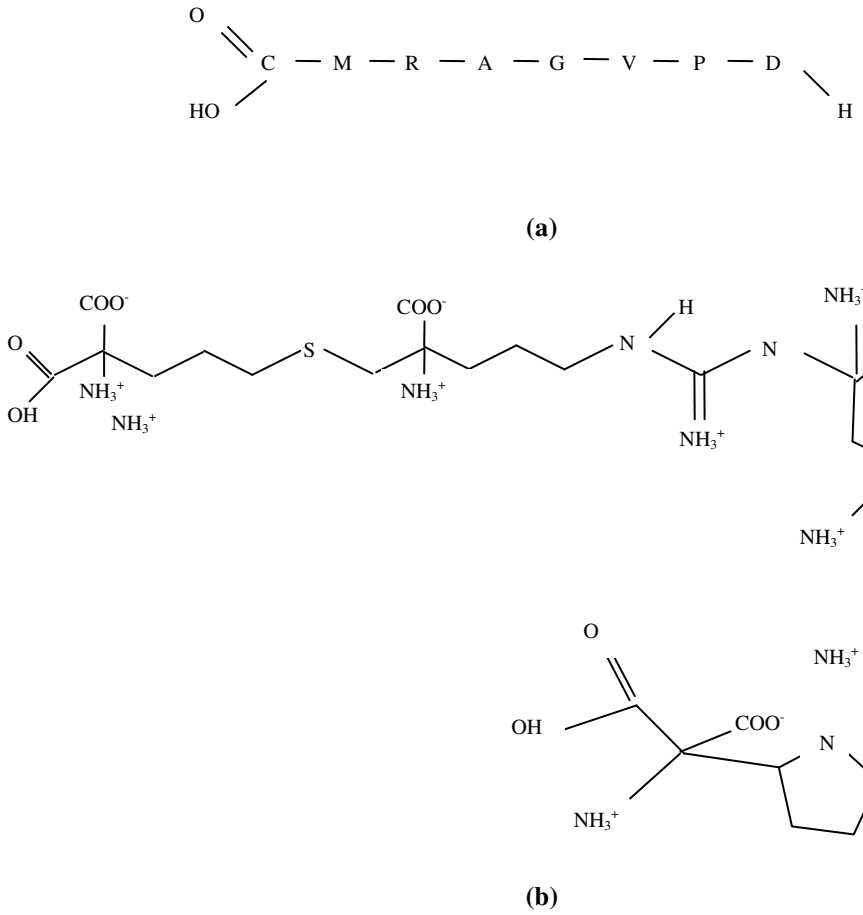


Figure 5-9: Proposed (a) amino acid sequence and (b) peptide structure for the bioactive compound isolated from non-toxic ***M. flos-aquae*****.**

Cyanobacteria are known to synthesise a number of structurally unique compounds; their often cyclic structure and the presence of unusual amino acids indicates these bioactive metabolites are produced via non-ribosomal peptide synthetases (Tooming-Klunderud *et al.* 2007), often with the assistance of polyketide synthetases (Hoffmann *et al.* 2003; Becker *et al.* 2004). Aside from microcystins, cyanopeptolins appear to be the most common peptide synthesised by *Microcystis*, and were detected in 60% of *Microcystis* colonies in a survey conducted recently by Welker *et al* (2006). A comparison of the proposed amino acid sequence to a database of known peptide sequences was unable to classify this sequence within any known class of bioactive metabolites (B. Neilan, personal communication, April 2008). This suggested that either the compound is novel and has not previously been discovered; or the compound has been modified and is a variant of a known peptide. Further purification of the compound is necessary to conclusively identify this compound.

The information acquired during this phase of the investigation divulges the presence of a bioactive compound, synthesised by a non-toxic isolate of *M. flos-aquae* MIC FEB05 and suggests a potential amino acid sequence of the peptide. However, published literature suggests many non-toxic cyanobacteria possess genes for synthesis and assembly of microcystin either with a mutation or modification, or an incomplete set of microcystin (*mcy*) genes. The presence of unusual amino acids also indicates synthesis via a non-ribosomal or hybrid pathway with polyketide synthetases; subsequently, *M. flos-aquae* MIC FEB05 was scrutinized at the molecular level, to examine the genome for the presence (or absence) of the microcystin operon or hybrid non-ribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) genes.

CHAPTER 6 MOLECULAR ANALYSIS OF BIOACTIVE COMPOUND SYNTHESIS

6.1 Introduction and Strategy

This chapter describes the development of DNA extraction methods for cyanobacterial cells; taxonomic classification of the cyanobacterial strain at the focal point of this study by 16S sequence comparison; and sequence analysis of genes potentially involved in bioactive peptide production, including microcystin type genes and peptide synthetase genes.

The following strategy was developed to meet the above objectives. Extraction of chromosomal DNA from freeze-dried biomass via a number of methods was conducted to determine the most effective procedure resulting in the highest quality DNA yield for downstream processing. Selection and development of degenerative PCR primers and cycle protocols was conducted to maximise the success of DNA amplification. PCR products were then submitted for sequence analysis. Amplification of the genome using 16S primers and subsequent analysis of the sequence within the Ribosomal Database Project website was employed for taxonomic classification, and BLASTX analysis to identify the genes present.

6.2 Isolation of genomic DNA from *M. flos-aquae*

DNA from *Microcystis* sp. was extracted and purified with varying degrees of success using a number of proprietary extraction kits, including the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), the QIAamp DNA Mini Kit and QIAamp DNA Stool Kit (QIAGEN Pty Ltd, Doncaster, Victoria, Australia), and the Mo Bio PowerPlant™ DNA Isolation Kit (Mo Bio Laboratories Inc, Carlsbad, CA, USA), typically applying the isolation protocols for Gram negative bacteria. All DNA extraction procedures are described in detail below.

6.2.1 Wizard® Genomic DNA Purification Kit

Cells from *Microcystis* sp cultures in the exponential phase of growth were centrifuged (Eppendorf Microcentrifuge 5415D, Eppendorf; 13,000 ×g, 2 min), and the supernatant discarded. The cells were lysed with 600 µL Nuclei Lysis solution and

pipetted gently to mix. This solution was incubated for 5 min at 80°C before being cooled to room temperature. RNAse solution (3 µL, 10 mg mL^{-1}) was added, and the solution mixed, then incubated at 37°C for 60 min, followed by cooling to room temperature. To aid in precipitation of the protein, 200 µL Protein Precipitation Solution was added, the solution mixed by vortex, then incubated on ice for 5 min. Undesirable proteins and other cellular debris were removed by centrifugation (13,000 ×g, 3 min), and the supernatant containing the DNA transferred to a clean microcentrifuge tube containing 600 µL RT propan-2-ol (Ajax Finechem, Seven Hills, NSW, Australia). Following centrifugation at 13,000 ×g for 2 min, the supernatant was carefully decanted and the pellet rinsed with 70 % (v/v) EtOH. The mixture was centrifuged at 13,000 ×g for a further 2 min, then the ethanol aspirated and the pellet either air dried for 10-15 min, or dried in a Speedy Vac (SAVANT SVC 100 Speed Vac; SAVANT Refrigeration Condenstion Trap; Double Stage Vacuum pump, GMI Inc, MI, USA) for 10 min. The DNA pellet was rehydrated with the addition of 100 µL Rehydration solution and incubated either at 65°C for 1 hr or at 4 °C overnight. The contents of the tubes were stored at -20 °C until required for analysis by gel electrophoresis and amplification by PCR.

6.2.2 QIAGEN QIAamp® DNA Mini Kit

Cells from a viable *Microcystis flos-aquae* 053D culture in the exponential phase of growth were harvested by centrifugation (5,000 ×g, 5 min) in a microcentrifuge tube and the supernatant discarded. A sample of freeze-dried *M. flos-aquae* MIC FEB05 cells were transferred to a clean microcentrifuge tube at a weight corresponding to the live cells harvested (approximately 0.6 g). The volume of the pellets was estimated, and an aliquot of Buffer ATL, supplied in the QIAamp® DNA Mini Kit, added to a total volume of 180 µl in each tube. Proteinase K (20 µl) was added to both tubes, which were mixed by vortex and incubated at 56 °C in a water bath for 3 hours, with regular pulse-vortexing to ensure the tissue was completely lysed. Both tubes were centrifuged briefly to remove drops from the inside of the lid, then 200 µl Buffer AL added to the samples, mixed by pulse vortex for 15 s, and incubated at 70 °C for 10 min in a heating block. A white precipitate, observed following addition of Buffer AL dissolved during incubation at 70 °C and therefore did not interfere with the remainder of the procedure. Ethanol (96-100%, 200 µl) was applied to the sample,

mixed by pulse-vortex, then centrifuged to remove drops from the inside the lid. The entire mixture was applied to a QIAamp Spin Column held in a 2 mL collection tube, without wetting the rim of the column. The collection tube and column were centrifuged (6,000 ×g, 1 min), then the spin column transferred to a clean collection tube, while the tube containing the filtrate was discarded. Buffer AW1 (500 µl) was added to the sample remaining in the spin column, and the column and tube were centrifuged (6,000 ×g, 1 min) with the cap closed to avoid aerosol formation. The filtrate was discarded and the spin column transferred to a new collection tube; this step was then repeated with the addition of Buffer AW2 (500 µl), then centrifugation at 20,000 ×g, for 3 min. A further centrifugation step (20,000 ×g, 1 min) was included to remove all traces of Buffer AW2. Final steps involved placing the QIAamp Spin Column in a fresh 1.5 mL microcentrifuge tube and discarding the collection tube containing the filtrate. Buffer AE (200 µl) was applied to the spin column and incubated at room temperature for 5 min, followed by centrifugation (6,000 ×g, 1 min). The elution step was repeated 3 times, using a fresh microcentrifuge tube for each elution, to maximise DNA extraction from the samples. The contents of the tubes were combined and stored at -20 °C until required for analysis by gel electrophoresis and amplification by PCR.

6.2.3 QIAGEN QIAamp® DNA Stool Mini Kit

Freeze-dried cells of *M. flos-aquae* MIC FEB05 were weighed into a 2 mL microcentrifuge tube and placed on ice. Buffer ASL was added to the tube and the contents vortexed until thoroughly homogenised. The suspension was heated at 70 °C for 5 min in a heating block, followed vortexing for 15s, then centrifugation at full speed (20,000 ×g) for 1 min to pellet the sample. An aliquot (1.2 mL) of the supernatant was transferred to a new microcentrifuge tube, and the pellet discarded. An InhibitEX tablet, provided in the QIAamp® DNA Stool Mini Kit was added to the tube, and vortexed continuously for 1 min to ensure the tablet was completely suspended. This suspension was then incubated for 1 min at room temperature to allow inhibitors present in the sample to adsorb to the InhibitEX matrix. To remove the inhibitors from the sample, the tube was centrifuged at full speed for 3 min, resulting in a formation of a pellet. The supernatant was then transferred to a new 1.5 mL microcentrifuge tube and the InhibitEX pellet discarded. Centrifugation was

repeated at full speed for 3 min. Proteinase K (15 µL) was added to a new tube, prior to transfer of 200 µl of the supernatant, and an addition of 200 µL of Buffer AL. The mixture was mixed by vortex for 15 s, then incubated at 70 °C for 10 min. Ethanol (96-100%, 200 µL) was applied to the lysate and mixed by vortex. A fresh QIAamp spin column placed in a 2 mL collection tube. The lysate was applied to the spin column, without moistening the rim of the column. Following centrifugation at full speed for 1 min, the spin column was transferred to a new collection tube, and the filtrate discarded. Buffer AW1 (500 µL) was added to the sample remaining in the spin column, and the column and tube were centrifuged (20,000 ×g, 1 min) with the cap closed to avoid aerosol formation. The filtrate was discarded and the spin column transferred to a new collection tube; this step was then repeated with the addition of 500 µL of Buffer AW2, then centrifugation at 20,000 ×g, for 3 min. A further centrifugation step (20,000 ×g, 1 min) was included to remove all traces of Buffer AW2. Final steps involved placing the QIAamp Spin Column in a fresh 1.5 mL microcentrifuge tube and discarding the collection tube containing the filtrate. Buffer AE (200 µL) was applied to the spin column and incubated at room temperature for 1 min, followed by centrifugation (20,000 ×g, 1 min). The elution step was repeated, using a fresh microcentrifuge tube for each elution, to maximise DNA extraction from the samples. The contents of the tubes were combined and stored at -20 °C until required for analysis by gel electrophoresis and amplification by PCR (QIAGEN 2001).

6.2.4 Mo Bio UltraClean Soil DNA Isolation Kit

To the 2 mL Bead Solution tubes provided, a sample of freeze dried *M. flos-aquae* MIC FEB05 biomass was added and the contents mixed gently by vortex. Solution S1 was added (60 µl) and the tubes inverted several times to mix. An inhibitor removal solution, Solution IRS (200 µl) was added to the tubes as the final eluted DNA would be employed as template DNA for PCR. The tubes were then secured horizontally on a flat bed vortex pad with tape and mixed at maximum speed for 10 min. Centrifugation of the tubes at 10,000 ×g for 30 s resulted in the cell debris forming a pellet at the bottom of the tube. The supernatant (approximately 400-450 µl) was transferred to a clean microcentrifuge tube, followed by addition of 250 µl of Solution S2, vortexing for 5 s, and incubation at 4 °C for 5 min. The tubes were centrifuged at

10,000 ×g for 1 min, and the entire volume of supernatant transferred to a clean microcentrifuge tube. Solution S3 was carefully applied to the supernatant at a volume of 1.3 mL and the suspension vortexed for 5 s. Of this solution, 700 µl was transferred to a spin filter and centrifuged for 1 min at 10,000 ×g. The flow through was discarded and the process repeated until all the supernatant had passed through the spin filter. Solution S4 was then applied to the filtrate (300 µl), and centrifugation repeated at 10,000 ×g for 30 s; the flow through was again discarded and an additional centrifugation for 1 min undertaken. The spin filter was transferred to a new microcentrifuge tube, and 50 µl of Solution S5 added to the centre of the white filter membrane, and both centrifuged for 30s. The spin filter was discarded, and the DNA in the tube declared application ready. Eluted DNA was stored at -20 °C prior to application as a template for PCR (Mo-Bio 2005).

6.2.5 Mo BIO PowerPlant™ DNA Isolation Kit

The PowerPlant™ DNA Isolation Kit provided a novel method for isolation of genomic DNA with a high level of purity, allowing for successful PCR amplifications from plant samples. Cell lysis and DNA liberation occurred by a combination of mechanical and chemical methods. Released genomic DNA was precipitated with isopropanol, and then captured on a silica membrane within a spin column. DNA was washed and eluted from the membrane, ready for PCR amplification or other downstream application.

Table 6-1: Cell weights used for extraction of genomic DNA from freeze-dried *Microcystis flos-aquae*

ID	Cell weights (g)
MIC FEB05 (1)	0.1535
MIC FEB05 (2)	0.0125
MIC MAR05	0.2212 g

To the PowerPlant™ Bead Tubes provided in the kit, freeze-dried cyanobacterial biomass at the cell weights recorded in Table 6-1 was added, followed by 550 µL of PowerPlant™ Bead Solution, and the mixture gently vortexed. Alternatively, 1 mL of live cyanobacterial culture was centrifuged in a PowerPlant™ Bead Tube and the supernatant removed prior to addition of 550 µL of PowerPlant™ Bead Solution. Genomic DNA from live cultures of *M. aeruginosa* PCC 7806 was also extracted; 1

mL of each culture was harvested by centrifugation (10,000 ×g, 5 min) and the supernatant discarded. Cell pellets were resuspended in the PowerPlant™ Bead Solution prior to loading in the bead tubes. Solution PB1 (60 µL) was then combined and the tubes inverted several times to mix. All tubes were incubated at 65°C for 10 min, followed by vortex for 3 runs of 30 s each at maximum speed and room temperature using a SAVANT Instruments FastPrep FP120 (SAVANT Instruments Inc., Holbrook, New York, USA). The supernatant from each sample (approximately 400-500 µL) was then transferred to clean 2 mL Collection Tubes (provided). Solution PB2 (250 µL) was transferred to each tube and the mixtures incubated on ice for 5 min, followed by centrifugation at room temperature, 10,000 ×g for 1 minute. The resultant cell pellets were retained in the tubes, and the supernatants transferred to clean 2.2 mL Collection Tubes with 1 mL of Solution PB3. Tubes were inverted 5 times to mix the contents thoroughly, followed by incubation at room temperature for 10 min, and centrifugation for 15 min at 13,000 ×g.

The resultant supernatant was discarded and the cell pellets resuspended in 100 µL of Solution PB6 and 500 µL of PB4. The contents of the tubes were mixed by vortex and loaded onto a Spin filter (provided) for centrifugation at 10,000 ×g for 1 minute. The spin filter was removed from the basket, the flow through discarded and 500 µL of solution PB5 added to the filter, replaced in the basket. Centrifugation was repeated for 30s at 10,000 ×g and the flow through discarded. The centrifugation step is repeated for another minute, and the spin filter transferred to a clean 2 mL collection tube. A 50 µL aliquot of solution PB5 was pipetted into the centre of the membrane in the spin filter, and the tubes centrifuged for 30s at 10,000 ×g to elute the DNA. The spin filters were discarded, and the DNA stored at 4 °C until required for PCR.

6.2.6 Re-precipitation of *Microcystis* DNA

Microcystis DNA, purified using the Wizard® Genomic DNA Purification Kit, contained large amounts of cellular debris and cyanobacterial pigments (e.g. chlorophyll), producing a very dark pellet. These proteins and other compounds may interfere with binding of the cyanobacterial DNA to primers during PCR, resulting in a lack of amplified DNA. Therefore, the DNA was re-precipitated using the following method (F Bell, personal communication, November 2006) to remove as much of this material as possible and "clean" the DNA product.

To the rehydrated DNA product, 2.5 µL Glycogen (20 mg/mL) per 100 µL of rehydrated DNA and an equal volume of propan-2-ol (100 µL) were added. The solution was frozen at -20 °C for 30 min, centrifuged (14,000 ×g, 20 min, 4 °C) and the supernatant discarded. The pellet was rinsed in 1 mL of 70% (v/v) ethanol, then decanted and the supernatant discarded, retaining the DNA pellet. To remove all moisture, the pellet was dried in the Speed Vac for 10 min. The pellet was then resuspended in 100 µL of DNA Rehydration solution (Wizard Genomic DNA Purification Kit), and incubated at either 65 °C for 1 hour or 4 °C overnight.

6.2.7 Rapid DNA Extraction Techniques

6.2.7.1 Rapid extraction of E coli DNA (the boiling method)

To obtain crude *E coli* DNA for exploitation as a positive control in amplification of 16s ribosomal RNA, a rapid extraction procedure known as the "boiling method" was applied. An overnight culture of *E coli* DH5-α cultured in nutrient broth was used as the bacterial DNA source. A microcentrifuge tube containing 1.5 mL of overnight culture was centrifuged (13,000 ×g, 1 min), and the supernatant discarded. The pellet was resuspended in 1 mL of 1 x PBS, centrifuged for another minute, and again the supernatant discarded. This step was repeated, and the majority of the PBS removed by pipette. The pellet was then resuspended in Milli-Q® H_2O, with 5 µL of 5% (w/v) Chelex (an ion exchange resin) added. The tube was placed in a covered boiling rack, and the rack placed in a frying pan of boiling water for 3-5 min. The tube was removed from the boiling water and placed immediately on ice for 10 min. The microcentrifuge (Eppendorf Microcentrifuge 5415D, Eppendorf) was cooled to 4 °C, and the tube spun at maximum speed (14,000 ×g) for 10 min. The supernatant was transferred to a clean tube prior to use in PCR (Metcalf and Codd 2000). (Note: the crude DNA extract was only stable for approximately 24 hrs).

6.2.7.2 Microwave lysis

Rapid cell disruption techniques have been developed for swift extraction of DNA from cyanobacteria, specifically from strains of *Microcystis*. Metcalf and Codd (2000) demonstrated that the microwave boiling treatment of field and laboratory-cultured *M. aeruginosa* was comparable to methanol extraction for toxin extraction, yet was

inherently simpler than solvent extraction and precluded the need for chemical treatment (Rasmussen *et al*. unpublished).

Cells were harvested from a viable culture of *M. flos-aquae* 053D and placed in 1.5 mL microcentrifuge tubes to a total volume of 500 µL. To improve effectiveness of microwave disruption, a detergent-based solution was included (0.5% Triton X-100, 5 mM DTT, 0.5 M Tris pH 7.5), constituting 10% of the total volume. Given the propensity for heated fluids to steam or superheat (Rasmussen *et al*. unpublished) tubes were capped securely and placed in a closed, microwave-tolerant container. Microwave lysis involved irradiation of cells (900 W min^{-1}) using a Model N-227 bench top microwave (NEC, Epping, NSW, Australia). The tubes were centrifuged (14,000 ×g, 1 min) to pellet the cell debris, and DNA contained in the supernatant applied as a template in PCR (Rasmussen *et al*. unpublished).

6.2.7.3 Probe Sonication

Sonication has been successfully applied in disruption of cyanobacterial cells, particularly those normally considered troublesome, such as filamentous or aggregating strains (Bolch *et al*. 1999). Probe sonication has been widely supported as it involves minimal sample processing and provides large amounts of cellular DNA for rapid diagnostic PCR.

Cells were directly transferred to 1.5 mL microcentrifuge tubes to a final volume of 500 µL and sonicated using a Branson Digital Sonifier 250 with a 3/16" Tapered Microtip at 70% of maximum power, to avoid destroying the tip. To remove cell debris, tubes were centrifuged (14,000 ×g, 1 min) and the supernatant applied directly as a template for PCR (Rasmussen *et al*. unpublished).

6.3 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a method used to amplify specific sequences of DNA via repeated cycles of synthesis using expressly designed primers and DNA polymerase (Brock *et al*. 1997). A schematic diagram of a PCR cycle is depicted in Figure 6-1. PCR has been extensively used in cyanobacterial genetics, to assist in determining the sequences of genes that encode specific metabolites such as toxins. Primers may also be applied to non-toxic species that may contain regions of

sequence homology, and thus be used to determine other proteins encoded by the DNA. By amplifying DNA either side of known conserved regions, such as those of peptide synthetases, it is possible to determine and predict other proteins that may also be encoded by the organism. Several sets of primers (a set consisting of the forward and reverse primer) were utilised in this research, and the primer sequences and PCR protocols are described below.

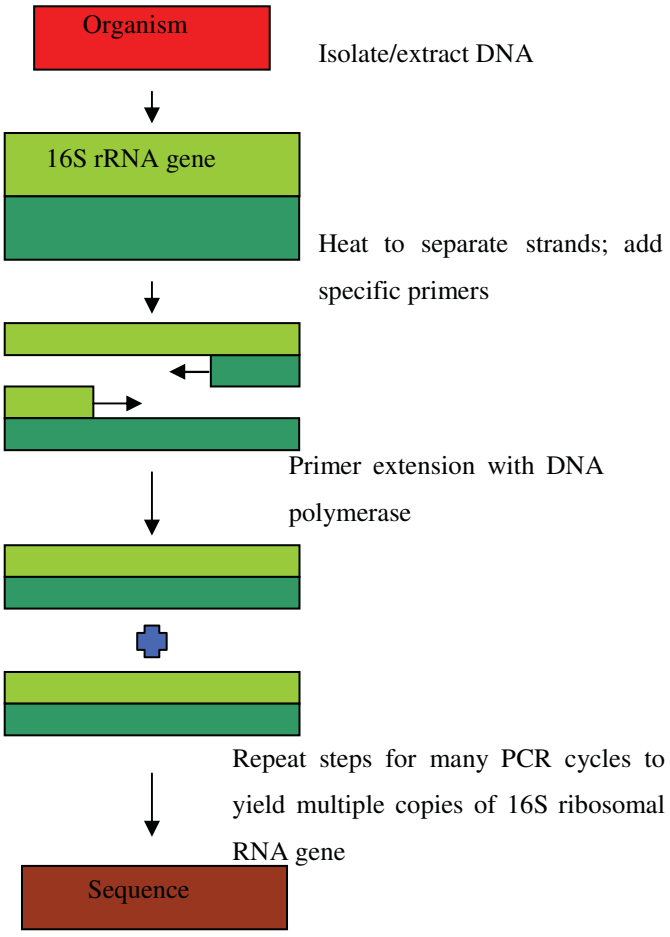


Figure 6-1: Schematic of the Polymerase Chain Reaction (PCR) (Brock *et al.* 1997)

6.3.1 Primers

Classification of an organism is often performed by amplification of 16S ribosomal DNA, a region demonstrating a high level of homology within organisms, but with significant variations to distinguish between strains or species. The PCR products from these amplifications are submitted for sequence analysis, and the resulting sequence compared to others within sequence databases. Primers used for amplification of 16S ribosomal RNA in cyanobacteria and *E coli* (included as a

positive control to validate the DNA extraction and sequence amplification procedures) are provided in Table 6-2.

Table 6-2: 16S RNA PCR Primers

Primer	T_m (°C)	Product (bp)	Oligonucleotide primer sequence, 5'→3'
16sF	48	1500	ACG GCT ACC TTG TTA CGA
16sR	48	1500	AGA GTT TGA TCC TGG CTC
27F	54	500	GAG AGT TTG ATC CTG GCT CAG
519R	52	500	ATT ACC GCG GCT GCT GG
E27F	61	500	AGA GTT TGA TCC TGG CTC AG
809R	80.1	800	GCT TCG GCA CGC CTC GGG TCG ATA

Cyanobacterial peptides are frequently assembled non-ribosomally, via an integrated NRPS/PKS system. Subsequently, primers were selected to amplify NRPS and PKS genes of cyanobacteria. Degenerative primers based on those described by Neilan *et al* (1999) were ordered from GeneWorks, for use in PCR amplification of peptide synthetase genes and polyketide synthetase genes of *Microcystis*. A directed search was also conducted for genes of the microcystin operon. Primer sequences described in Table 6-3 apply to amplification of NRPS, PKS and microcystin genes.

Table 6-3: Peptide synthetase gene consensus and specific PCR primers (Neilan *et al.* 1999)

NOTE:
This table is included on page 115 of the print copy of the thesis held in the University of Adelaide Library.

6.3.2 16s rRNA amplification

In general, rRNA genes are considered to be more conserved in function and structure than protein-coding genes, and thus the genetic diversity can be measured (Nübel *et al*. 1997). It should be noted that the primers used in microbial ecology are designed on the basis of limited sets of data; future research may reveal sequences from target organisms which do not contain the signatures necessary for efficient amplification.

6.3.2.1 Reaction mix

The reaction mix consisted of the following reagents added in the given order to a capillary or 200 µL PCR tube: 9.5 µl mQ H_2O, 1 µl Forward primer, 1 µl Reverse primer, 1 µl Template DNA and 12.5 µl AmpliTaq Gold® PCR Master Mix (Roche, New Jersey USA), a total of 25 µL. All tubes were vortexed for 10 s, to ensure the contents were well mixed prior to PCR

An alternative reaction mixture used during PCR reactions at UNSW consisted of the following reagents added to a capillary or 200 µL PCR tube: 2 µL dNTPs, 2 µL 10x buffer, 2 µL $MgCl_2$, 1 µL Forward primer, 1 µL Reverse primer, 1 µL Template DNA, 12 µL mQ H_2O and 0.2 µL of Taq polymerase enzyme, a total of 20 µL. All tubes were vortexed for 10 s, to ensure the contents were well mixed prior to PCR

6.3.2.2 PCR cycles

Due to the specificity of the primers used, the annealing temperature of the PCR cycle had to be altered accordingly. Thus, a number of PCR cycles were employed which are outlined in Table 6-4. The first cycle, as described by Neilan *et al* (1999) was applied using the MTF2 and MTR primers for amplification of peptide synthetase genes from *Microcystis*. The second cycle was developed for use with the 16S RNA primers (D May, personal communication, July 2006). Further cycles were employed during investigation of the *mcy* operon within experiments undertaken at UNSW.

Table 6-4: Polymerase Chain Reaction cycles

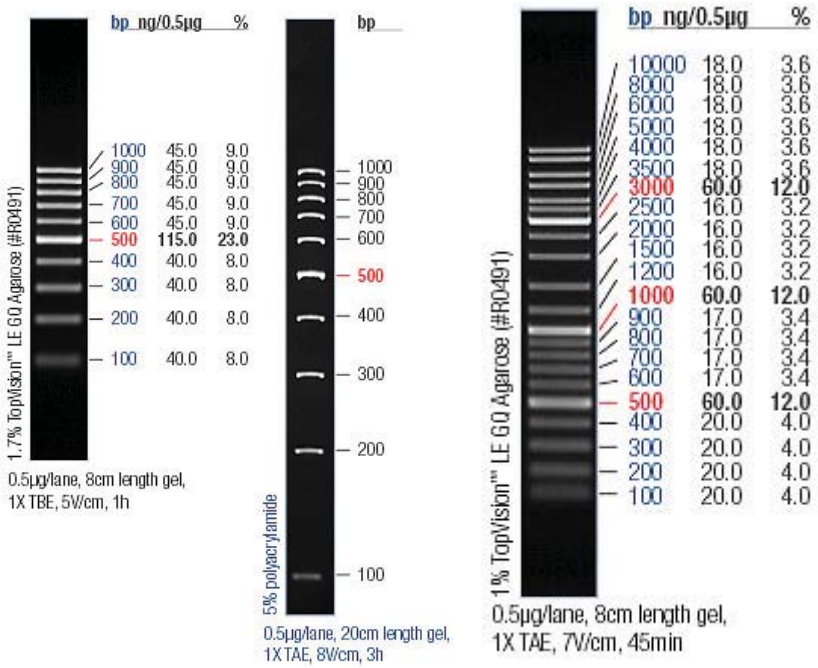
	Denaturation Temp	**Annealing Temp**	**Time**
Cycle 1			
Repeat steps 35 cycles	94°C		2 min
	93°C		10 s
		51°C	20s
		72°C	1 min
Completion		4°C	Hold
Cycle 2			
Repeat steps 35 cycles	94°C		1 min
		55°C	1 min
		72°C	1 min
Completion		4°C	Hold
Cycle 3 (JAS1 – UNSW)			
Heat	94°C		3 min
Repeat steps 30 cycles	94°C		10 s
		55 °C	20 s
		72 °C	1 min
Hold for cool down		72 °C	7 min
Completion		20°C	Hold
Cycle 4 (Gradient NRPS/PKS/*MCY* E - UNSW)			
Heat	94°C		2 min
Repeat steps 35 cycles	94°C		15 s
		52°C/55 °C	30 s
		72 °C	1 min
Hold for cool down		72 °C	7 min
Completion		20°C	Hold
Cycle 5 (*MCY* B1, C & D - UNSW)			
Heat	94°C		4 min
	92°C		15 s
Touchdown 10 cycles from 55°C 25 cycles at 45°C		55 °C	30 s
		45°C	20 s
		72 °C	1 min 30 s
		72 °C	7 min
		20°C	Hold

The "touchdown" cycle employs an initial annealing temperature of 55°C, and reduces by 1°C and 1 s each cycle until the "touchdown" temperature (i.e. 45°C) is met. The remainder of the PCR cycles occur at the minimum annealing temperature.

6.4 Agarose Gel Electrophoresis

6.4.1 Loading and Running the Gel

To determine the success of the PCR amplification, the samples were separated on an agarose gel. A 1% (w/v) solution of agarose in 1x TAE was prepared by dissolving 1 g of agarose in 100 mL of 1x TAE. The open ends of a gel casting tray were sealed with tape, and a gel comb used to create sample wells within the agarose. Molten agarose (~ 55 °C) was poured gently into the casting tray and allowed to set, and then the tape and comb were removed. The PCR products were combined with a loading buffer ["blue juice" with EDTA: 10 mg Bromophenol blue; 2 mL glycerol; 1 mg RNAse; 120 µg EDTA; make up to 10 mL with mQ H_2O. Boil 30 min], at a ratio of 5:2 (i.e. 5 µL PCR product and 2 µL of loading buffer) and mixed thoroughly. A DNA ladder was employed to visualise the relative size of the PCR product. Three DNA ladders were employed during this study; the first was a 100 bp GeneRulerTM (0.1 µg μL^{-1}; Fermentas International, Canada) run with PCR products from the amplification with peptide synthetase primers. The second ladder, based on the *SPP1* gene digested with the restriction enzyme *EcoR1* (Stackebrandt and Goodfellow 1991), provided a ruler of up to 2,500 bp and was used in combination with 16S RNA primers. A third ladder was applied during PCR amplifications of NRPS and PKS genes conducted in the Neilan laboratory at the University of New South Wales (Sydney, Australia); this ladder provided a ruler to 10 kb, and comprised three reference bands at 3,000, 1,000 and 500 bp. Figure 6-2 illustrates the distribution of DNA bands for the 100 bp DNA ladder (a) and the DNA ladder mix (b).



(a) (b)

Figure 6-2: Fermentas GeneRulerTM (a) 100 bp DNA Ruler, and (b) DNA Ladder Mix 10kb

Samples containing DNA or PCR product mixed with loading buffer were pipetted into gel wells, with the appropriate DNA ladder loaded in lane 2. The lid of the electrophoresis chamber was secured and a current (100 V) applied. The dye front was observed to monitor progression of the DNA migration. When the dye front migrated to within 1 cm of the end of the gel, the current was removed and the DNA visualised by staining with Ethidium Bromide or GelRed^{TM} and UV exposure.

6.4.2 Gel staining and UV exposure

Ethidium Bromide, a fluorescent dye that intercalates between DNA and RNA bases, was used to stain agarose gels and observe the PCR products. Following electrophoresis, the gel was placed in a container of Ethidium bromide and stained for 15 minutes. The gel was placed on a transilluminator to visualise the DNA bands by exposure to UV, and a photograph taken of the exposed gel.

GelRed^{TM} is a red fluorescent nucleic acid dye designed to replace the highly toxic ethidium bromide for gel staining (Biotium 2007). GelRed^{TM} was used for post gel staining as this method generally provides better sensitivity and eliminates any possibility for the dye to interfere with DNA migration. Gels were visualised using

the BioRad Gel Doc system and Quantity One 1-D Analysis software, allowing an electronic copy of the gel to be created.

6.5 DNA Sequencing

6.5.1 Ethanol Precipitation (Template Clean-up)

This method was employed to purify DNA from the PCR amplifications of the 16S, *mcy*A and *mcy*E genes of *M. flos-aquae* MIC FEB05. The protocol involved the addition of 2 volumes (approximately 40 µL) of ice cold (-20 °C) 100 % v/v Ethanol (AR grade) to the successful PCR amplifications in a 1.5 mL microcentrifuge tube. The solutions were left on ice for 45 min. Sodium acetate was not applied during this procedure, to avoid precipitation of the primer DNA. The tubes were centrifuged at maximum speed (14,500 ×g) for 20 min, followed by removal of the supernatant and addition of 150 µL of 70% v/v Ethanol (freshly prepared) to wash the cell pellet. After a further centrifugation step and subsequent removal of the supernatant, the pellet was dried at room temperature to remove residual ethanol, and resuspended in 15 µL of Milli-Q® H_2O. DNA was visualised on a 1% Agarose gel.

6.5.2 Sequencing PCR

An aliquot of BigDye mix, consisting of 1 µL Big Dye and 5 x Sequence Buffer 3.5 µL was thawed. Primer (3.2 pmol) was added to the dye mix, followed by PCR product at 20-50 ng, then Milli-Q® H_2O to 20 µL. The PCR was run using the cycling conditions detailed in Table 6-5.

Table 6-5: PCR cycling conditions for sequencing DNA

Cycle 5 – Sequencing PCR			
Heat	96°C		3 min
Repeat steps 30 cycles	96°C		10 s
		50 °C	5 s
		60 °C	4 min
Completion		10°C	Hold

6.5.3 Sequencing clean-up

Following the PCR reaction, the DNA was cleaned prior to submission for sequencing to remove residual primer and non-specific DNA. The sequencing reactions were transferred to a 1.5 mL tube, with 16 µL of Milli-Q® H_2O and 64 µL of 95% v/v AR grade Ethanol. The solutions were incubated at room temperature for 15 min, and then centrifuged at 14,500 ×g for 20 min. The resulting supernatant was discarded and the pellet washed with 250 µL of 70 % v/v Ethanol (centrifugation at 14,500 ×g for 5 min). DNA pellets were dried using SpeediVac following removal of the supernatant.

6.5.4 Gel Precipitation

DNA visualised in 1 % Agarose gels was recovered using the Wizard® SV Gel and PCR Clean up System (Promega Corporation, Madison, WI, USA).

The DNA bands were excised following electrophoresis using a scalpel under UV light, and placed in 1.5 mL microcentrifuge tubes with 10 µL of Membrane Binding Solution per 10 mg of gel (100 µL was applied to the gel fragments). The emulsion was mixed by vortex and incubated in a waterbath at 60°C until the gel slice dissolved. The dissolved gel mixture was transferred to an SV mini column and collection tube assembly and incubated at room temperature for 5 min, followed by centrifugation at 14,000 ×g for 1 min and the flow through discarded. The membrane within the mini column was washed with 700 µL of Membrane Wash Solution. Following centrifugation (14,500 ×g, 1 min) the flowthrough was discarded, and the wash step repeated with 500 µL of the Membrane Wash Solution. Centrifugation at 14,500 ×g for 5 min, then again for 1 min completed the washing stage. Elution of the DNA was conducted by transferring the mini column to a clean 1.5 mL microcentrifuge tube, with 20 µL of Milli-Q® H_2O, incubating the tubes at room temperature for 5 min and centrifugation at 14,500 ×g for 1 min. The mini column was discarded and the DNA visualised on a 1% Agarose gel to determine the success of the precipitation.

6.6 Comparison of DNA extraction methods

Extraction of DNA from cyanobacterial biomass via the proprietary Wizard® Genomic DNA Extraction kit and the QIAGEN QIAamp DNA Mini kit and Stool Kit had had limited success when applied to freeze dried cells of *M. flos-aquae* MIC FEB05. These kits are frequently applied to bacterial cells or cells harvested from viable cultures and are generally considered successful in achieving extraction of sufficient cellular DNA for downstream processing. Subsequently, alternative techniques were sought to increase the DNA yield from cyanobacterial cells. Four DNA extraction methods were suggested by researchers at the AWQC, and applied to quantities of freeze-dried and viable cells of *M. flos-aquae*, to verify the optimal extraction method for this study.

The results of the DNA extraction method comparison are represented within the gel photograph labelled Figure 6-3. Microwave lysis and sonication were equally successful in extraction DNA when applied to viable cultures of *M. flos-aquae* 053D*;* however, neither method successfully extraction DNA from the freeze-dried material (results not shown). The intensity of the bands visualised on the gel is proportional to the quantity of DNA extracted from the cells; the brighter the bands, the greater the yield of DNA extracted. The QIAGEN DNA Stool Kit extracted limited cellular DNA from the freeze dried biomass, while the Mo Bio UltraClean™ Soil DNA kit was considered inappropriate for extraction of cyanobacterial DNA from both freeze-dried and viable cells.

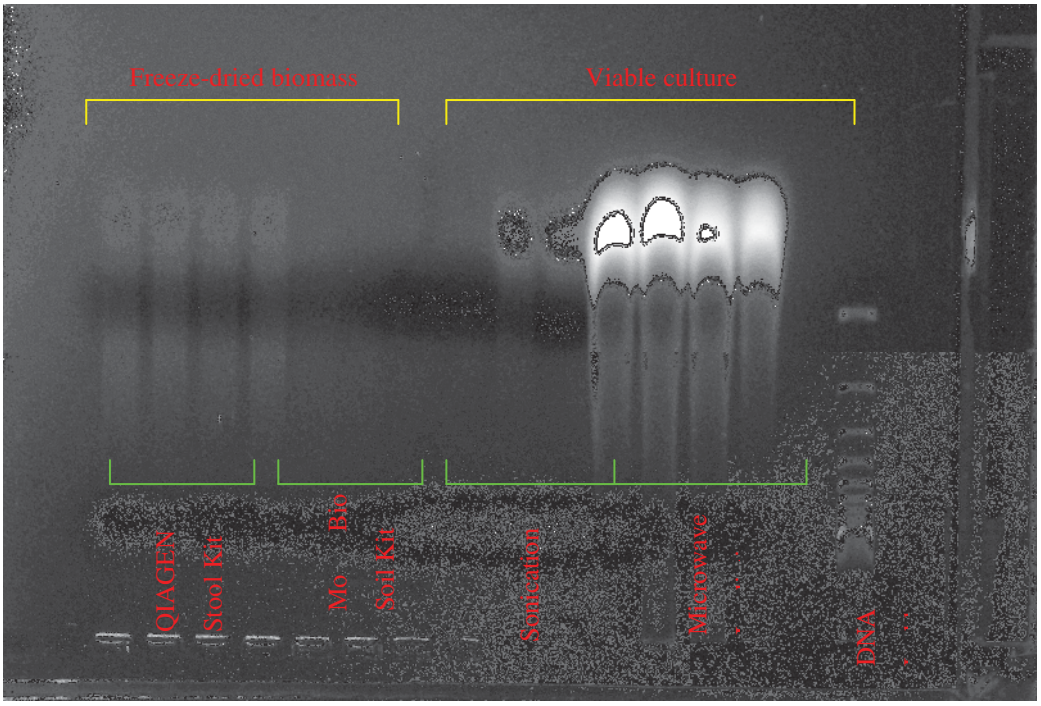


Figure 6-3: Cyanobacterial DNA extraction from *M. flos-aquae* by 4 extraction methods – QIAGEN DNA Stool Kit, Mo Bio UltraClean™ Soil DNA kit, Microwave irradiation and Sonication

PCR amplification of the extracted DNA was conducted using 2 sets of 16S RNA primers (primer sequences were defined in Table 6-2. *E coli* DNA was included as a positive control, as the primers were generically designed to amplify homologous regions of bacterial DNA and *E coli* has a high doubling time, and was able to achieve a high cell density in an overnight culture. Amplification of the cyanobacterial DNA using the 16S primers was successful for *E coli*; no amplification of *M. flos-aquae* MIC FEB05 DNA was evident when the PCR products were visualised on a gel (Figure 6-4). However, a faint band was visible for amplification of *M. flos-aquae* 053D DNA, extracted from a viable culture. Despite the appearance of three bands of PCR product using the alternate primer set, 27F and 519R, these products were suspected to be excess primer product rather than amplified DNA, as the position of the bands relative to the DNA ladder does not place them in the expected position for a PCR product using this particular primer set.

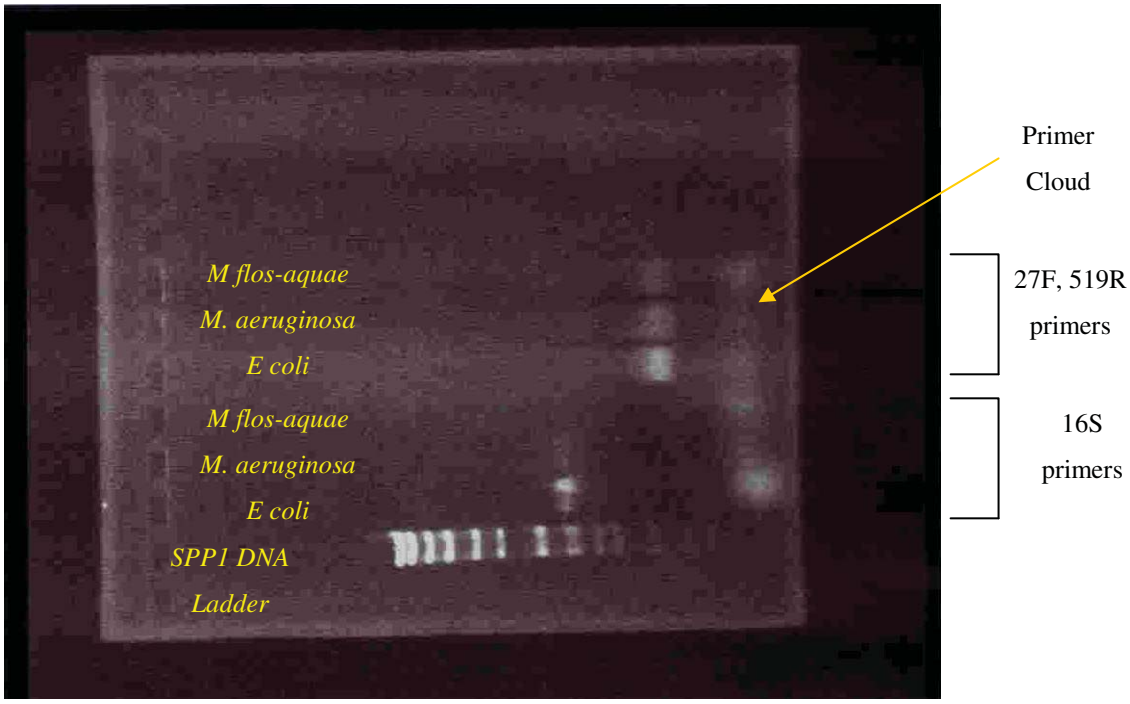


Figure 6-4: 16s RNA PCR amplification

Thus it was concluded that the 16S primers had been moderately successful in amplifying *E coli* DNA, and achieved only limited success in amplification of cyanobacterial DNA. The alternate primer set was not successful in this case.

6.6.1 DNA Extraction – Mo Bio PowerPlant™ DNA Isolation Kit

Extraction of DNA from freeze-dried cyanobacterial has previously met with limited success within this study, and subsequent downstream processing applications have similarly been futile. A short, intensive period was spent in Associate Professor Brett Neilan's laboratory at the School of Biotechnology and Biomolecular Sciences, University of New South Wales. Researchers within this group recommended the Mo Bio PowerPlant™ DNA Isolation Kit for successful extraction of DNA from freeze-dried material. The results of a DNA extraction using the Mo Bio PowerPlant™ kit are represented within the gel photograph labelled Figure 6-5. *M. flos-aquae* MIC FEB05 DNA was loaded into the 2nd and 3rd lanes, whilst *M. flos-aquae* MIC MAR05 DNA was included in lane 4. DNA extracted from a viable culture of *M. aeruginosa* 338B was included as a positive control to validate the extraction method. Although the DNA bands did not demonstrate a high resolution and was observed to smear within the gel, the extraction was considered successful and the DNA incorporated in PCR amplifications.

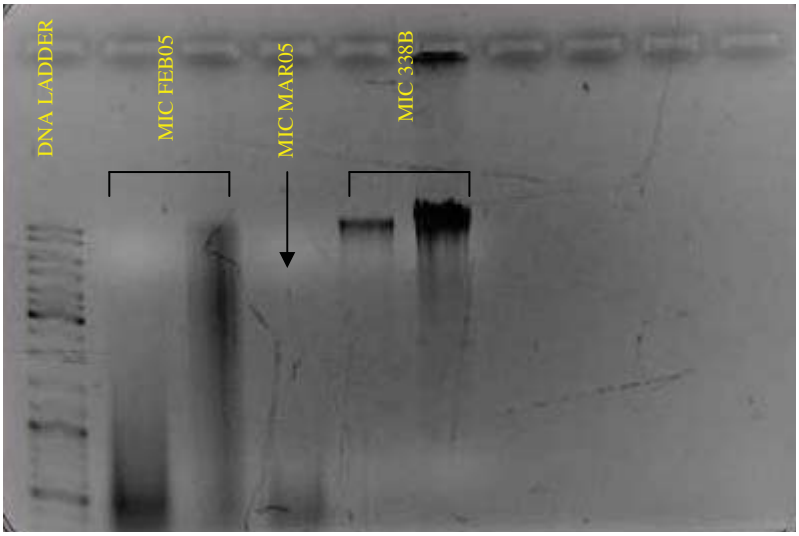


Figure 6-5: Extraction of genomic DNA using the Mo Bio PowerPlant DNA Isolation Kit. Extraction of DNA from *M. flos-aquae* MIC FEB05 and *M. flos-aquae* MIC MAR05. DNA extracted from a viable *M. aeruginosa* MIC 338B culture was included as a positive control.

6.7 DNA Sequence Analysis and Taxonomic Classification

6.7.1 Computer Analysis of DNA and Protein Sequences

DNA and protein sequences were analysed using web-based molecular analysis software programs, accessed via the School of Molecular and Biomedical Sciences webpage (http://www.microbiology.adelaide.edu.au) (accessed 14 December 2007).

DNA sequencing data was corrected with the aid of Chromas (version 1.44, http://www.technelysium.com.au/chromas.html). Species confirmation of the cyanobacteria used during this study was conducted using the Ribosomal Database Project II website (accessed 17 December 2007):

(http://rdp.cme.msu.edu/classifier/hierarchy.jsp?root=648&depth=0&confidence=0.8)

Searches for homology to known DNA and protein sequences contained in GenBank data bank was undertaken via the NCBI BLASTX search site (accessed 17 December 2007)

(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

A phylogenetic tree was calculated using the Clustal W alignment tool. This tool compares multiple alignments of DNA or protein sequences to identify conserved

sequence regions, and calculates "distances" between the sequences (accessed 11 January 2009).

(http://www.ebi.ac.uk/Tools/clustalw2/index.html).

6.7.1.1 Confirmation of genus classification by 16s RNA Comparison: Ribosomal Database Project II

The sequence obtained from the PCR cycle using 16s primers was submitted for sequence classification. The sequence was classified in the following taxonomic hierarchy (with 100% assignment detail, for a confidence threshold of 95%):

Domain: Bacteria

Phylum Cyanobacteria

Class Cyanobacteria

Family Family 1.1

Genus *Microcystis*

This classification confirms that the species investigated during this research is of the genus *Microcystis*. A gel photograph of the PCR amplification using 16S primers is labelled Figure 6-6, and the DNA sequence analysis of the 16S sequence amplified from *M. flos-aquae* (MIC FEB05) is provided in Figure 6-7.

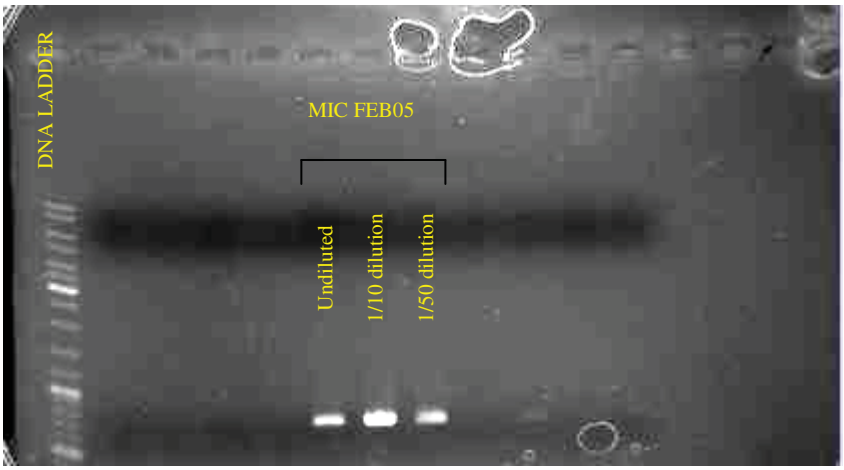


Figure 6-6: PCR amplification of extracted MIC FEB05 DNA using the 16S primers E27F and 809R.

1	5’	CCGAAGTCTA	GCATCATCGT	TTACGGCTAG	GACTACAGGG	GTATCTAATC	3’
51	5’	CCTTTCGCTC	CCCTAGCTTT	CGTCCCTGAG	TGTCAGATAC	AGCCCAGTAG	3’
101	5’	CACGCTTTCG	CCACCGATGT	TCTTCCCAAT	CTCTACGCAT	TTCACCGCTA	3’
151	5’	CACTGGGAAT	TCCTGCTACC	CCTACTGATC	TCTAGTCTGC	CAGTTTCCAC	3’
201	5’	CGCCTTTAGG	TCGTTAAGCA	ACCTGATTTG	ACGGCAGACT	TGGCTGACCA	3’
251	5’	CCTGCGGACG	CTTTACGCCC	AATAATTCCG	GATAACGCTT	GCCTCCCCCG	3’
301	5’	TATTACCGCG	GCTGCTGGCA	CGGAGTTAGC	CGAGGCTGAT	TCCTCAAGTA	3’
351	5’	CCGTCAGAAC	TTCTTCCTTG	AGAAAAGAGG	TTTACAATCC	AAAGACCTTC	3’
401	5’	CTCCCTCACG	CGGCGTTGCT	CCGTCAGGCT	TTCGCCCATT	GCGGAAAATT	3’
451	5’	CCCCACTGCT	GCCTCCCGTA	GGAGTCTGGG	CCGTGTCTCA	GTCCCAGTGT	3’
451	5’	GGCTGCTCAT	CCTCTCAGAC	CAGCTACTGA	TCGTTGCCTT	GGTAGGCCTT	3’
451	5’	TACCCCACCA	ACTAGCTAAT	CAGACGCAAG	CTCTTCTTCA	GGCCAATTAG	3’
451	5’	GTTTCACCTT	GCGGCACATC	GGGTATTAGC	AGTCGTTTCC	AACTGTTGTC	3’
451	5’	CCCGTCCTGA	AGTTAGATTC	TTACGCGTTA	CTCACCCGTC	CGCCACTAGA	3’
451	5’	ATCCTAAGAT	TCCCGTTCGA	CTTGCATGTG	TTAGGCACGC	CGCCAGCGTT	3’
451	5’	CATCCTGAGC	AGATCTTCAA	GCCTAAA	3’r		

(a)

Sequences producing significant alignments

Accession Number	**Score**	**Identity**	**Max**	**Max**	**E value**
DQ124252.1		Uncultured *Microcystis* sp. 16S ribosomal RNA gene, partial sequence	1380	96%	0.0
AJ635434.1		*Microcystis* ichthyoblabe 0BB35S01 partial 16S rRNA gene	1380	96%	0.0
AF139304.1		*Microcystis aeruginosa* strain UWOCC AubB1 16S ribosomal RNA gene, partial sequence	1360	95%	0.0
AJ635430.1		*Microcystis aeruginosa* 0BB35S02 partial 16S rRNA gene	1358	96%	0.0
AJ133170.1		*Microcystis* sp. 130 partial 16S rRNA gene, strain 130	1354	96%	0.0
AF139329.1		*Microcystis flos-aquae* strain UWOCC C3 16S ribosomal RNA gene, partial sequence	1332	95%	0.0
AF139328.1		*Microcystis flos-aquae* strain UWOCC C2 16S ribosomal RNA gene, partial sequence	1332	95%	0.0

(b)

Figure 6-7: DNA sequence analysis of the PCR product 16S from *Microcystis flos-aquae*. (a) DNA sequence of 16S obtained from *M. flos-aquae*. (b) Results of a BLASTN DNA sequence analysis of the PCR product 16S amplified from MIC FEB05. The 16S gene of *M. flos-aquae* was similar to the 16S gene of *M. flos-aquae* strain UWOCC C3.

AF139329	743	CTAGTATCCATCGTTTACGGCTAGGACTACAGGGGTATCTAATCCCTTTCGCTCCCCTAG	684
16s	8	CTAGCAT-CATCGTTTACGGCTAGGACTACAGGGGTATCTAATCCCTTTCGCTCCCCTAG	66
AF139329	683	CTTTCGTCCCTGAGTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCGATGTTCTTCC	624
16s	67	CTTTCGTCCCTGAGTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCGATGTTCTTCC	126
AF139329	623	CAATCTCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGCTCTCTAGT	564
16s	127	CAATCTCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGATCTCTAGT	186
AF139329	563	CTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACGGCAGACTTGGCTG	504
16s	187	CTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACGGCAGACTTGGCTG	246
AF139329	503	ACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCTCCCCCGTATTAC	444
16s	247	ACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCTCCCCCGTATTAC	306
AF139329	443	CGCGGCTGCTGGCACGGAGTTAGCCGAGGCTGATTCCTCAAGTACCGTCAGAACTTCTTC	384
16s	307	CGCGGCTGCTGGCACGGAGTTAGCCGAGGCTGATTCCTCAAGTACCGTCAGAACTTCTTC	366
AF139329	383	CTTGAGAAAAGAGGTTTACAATCCAAAGACCTTCCTCCCTCACGCGGCGTTGCTCCGTCA	324
16s	367	CTTGAGAAAAGAGGTTTACAATCCAAAGACCTTCCTCCCTCACGCGGCGTTGCTCCGTCA	426
AF139329	323	GGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGT	264
16s	427	GGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGT	486
AF139329	263	CTCAGTCCCAGTGTGGCTGCTCATCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTAGG	204
16s	487	CTCAGTCCCAGTGTGGCTGCTCATCCTCTCAGACCAGCTACTGATCGTTGCCTTGGTAGG	546
AF139329	203	CCTTTACCCCACCAACTAGCTAATCAGACGCAAGCTCTTCTTCAGGCCAATTAGGTTTCA	144
16s	547	CCTTTACCCCACCAACTAGCTAATCAGACGCAAGCTCTTCTTCAGGCCAATTAGGTTTCA	606
AF139329	143	CCCTGCGGCATATCGGGTATTAGCAGTCGTTTCCAACTGTTGTCCCCGTCCTGAAGTTAG	84
16s	607	CCTTGCGGCACATCGGGTATTAGCAGTCGTTTCCAACTGTTGTCCCCGTCCTGAAGTTAG	666
AF139329	83	ATTCTTACGCGTTACTCACCCGTCCGCCACTAGAATCCGAAGATTCCCGTTCGACTTGCA	24
16s	667	ATTCTTACGCGTTACTCACCCGTCCGCCACTAGAATCCTAAGATTCCCGTTCGACTTGCA	726
AF139329	23	TGTGTTAGGCACGCCGCCAGCGT	1
16s	727	TGTGTTAGGCACGCCGCCAGCGT	749

(c)

Figure 6-8: (c) DNA sequence alignment of 16S gene from *M. flos-aquae* and AF139329, *Microcystis flos-aquae* strain UWOCC C3 16S ribosomal RNA gene, partial sequence. The sequences are 95% similar.

Ribosomal DNA extracted from the freeze dried cyanobacterial biomass MIC FEB05 was similar to *M. flos-aquae strain* UWOCC C3, by DNA sequence alignment of the 16S ribosomal sequence with a known *Microcystis flos-aquae* sequence. The two sequences, with alignment demonstrated by Figure 6-8, were 95% similar.

The phylogenetic tree calculated by comparing sequences identified in the BLASTN DNA sequence analysis is illustrated in Figure 6-9.

Figure 6-9: Unrooted phylogenetic tree derived from a comparison of 16S sequences of *Microcystis* sp.

```
AF139329        ATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAACCCTGGTGAAAGC 933
AF139328        ATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAACCCTGGTGAAAGC 933
AJ635430        ATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAACCCTGGTGAAAGC 959
AJ133170        ATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAACCCTGGTGAAAGC 921
uncultured      ATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAACCCTGGTGAAAGC 958
AJ635434        ATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAACCCTGGTGAAAGC 949
AF139304        ATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAACCCTGGTGAAAGC 933
16S             CGCCCAATAATTCCGGATAACGCTTGCCTCCCCCGTATTACCGCGGCTGCTGGCA----- 320
                   * *   *   ** * ***  *  *    *  *   *  ****    ****

AF139329        TGGGGGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT 993
AF139328        TGGGGGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT 993
AJ635430        TGGGGGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT 1019
AJ133170        TGGGGGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT 981
uncultured      TGGGGGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT 1018
AJ635434        TAGGGGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT 1009
AF139304        TAGGGGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT 993
16S             CGGAGTTAGC--CGAGGCTGATTCCTCAAGTACCGTCAGAACTTCTTC--CTTGAGAAAA 376
                  * * *  *  ** *   *    * ** **   *   *    ** **  ** * *

AF139329        GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTAGTTGCCAGCATTAAG 1053
AF139328        GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTAGTTGCCAGCATTAAG 1053
AJ635430        GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTAGTTGCCAGCATTAAG 1079
AJ133170        GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTAGTTGCCAGCATTAAG 1041
uncultured      GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTAGTTGCCAGCATTAAG 1078
AJ635434        GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTAGTTGCCAGCATTAAG 1069
AF139304        GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTAGTTGCCAGCATTAAG 1053
16S             GAGGTTTACAATCCAAAGACCTTCCTCCCTCACGCGGCGTTGCT--CCGTCAGGCTTTCG 434
                *** * *    *  *    *    *   * **  *  ****  *    * ***  **  *

AF139329        TTGGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT 1113
AF139328        TTGGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT 1113
AJ635430        TTGGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT 1139
AJ133170        TTGGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT 1101
uncultured      TTGGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT 1138
AJ635434        TTGGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGT 1129
AF139304        TTGGGGACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGT 1113
16S             CCCATTGCGGAAAATTCCCCACTGCTGCCTC-CCGTAGGAGTCTGGGCCGTGTCTCAGTC 493
                       *   **     *  * * ** *   *** ****   ****       ***

AF139329        C-AGCATGCCCCTTACGTCTTGGGCGACACACGTACTACAATGGTCGGGACAAAGGGCAG 1172
AF139328        C-AGCATGCCCCTTACGTCTTGGGCGACACACGTACTACAATGGTCGGGACAAAGGGCAG 1172
AJ635430        C-AGCATGCCCCTTACGTCTTGGGCGACACACGTACTACAATGGTCGGGACAAAGGGCAG 1198
AJ133170        C-AGCATGCCCCTTACGTCTTGGGCGACACACGTACTACAATGGTCGGGACAAAGGGCAG 1160
uncultured      C-AGCATGCCCCTTACGTCTTGGGCGACACACGTACTACAATGGTCGGGACAAAGGGCAG 1197
AJ635434        C-AGCATGCCCCTTACGTCTTGGGCGACACACGTACTACAATGGTCGGGACAAAGGGCAG 1188
AF139304        C-AGCATGCCCCTTACGTCTTGGGCGACACACGTACTACAATGGTCGGGACAAAGGGCAG 1172
16S             CCAGTGTGGCTGCT-CATCCTCTCAGAC-CAGCTACTG--ATCGTTGCCTTGGTAGGCCT 549
                * **  ** *   * * ** *    *** **  ****   ** ** *        ***
```

Figure 6-10: Multiple alignments of key domains of 16S sequences from *Microcystis* sp. – partial sequence

The predicted ribosomal sequence identified in this study is labelled 16S. Protein sequence database accession numbers for the remaining sequences relate to the following strains: AF139304, *Microcystis aeruginosa* WOCC AubB1; AJ635434, *Microcystis ichthyoblabe* 0BB35S01; DQ124252, uncultured *Microcystis* sp.; AJ635430, *Microcystis aeruginosa* 0BB35S02; AJ133170, *Microcystis* sp. strain 130; AF139329, *Microcystis flos-aquae* UWOCC C3; and AF139328, *Microcystis flos-aquae* UWOCC C2.

Peptide sequences can often be inferred from broad clustering of genes according to phylogenetic grouping. Simple phylogenetic analysis contains too few representative sequences to attach any conclusive phylogenetic inferences; however, the unrooted phylogram presented in Figure 6-9 suggests the 16S sequence from *M. flos-aquae* MIC FEB05 demonstrates some similarities with published 16S sequences of *Microcystis* sp. Figure 6-10 indicates multiple alignments of key domains of partial 16S sequences from *Microcystis* sp., including the 16S sequence identified during this study.

6.8 Detection of peptide assembly genes

Researchers generally concur that cyanobacterial peptides with biological activity, including toxins, are likely products of a hybrid non-ribosomal peptide synthetase/polyketide synthetase system. Consequently, a directed search for complete or partial sequences of both NRPS and PKS genes within the *M. flos-aquae* MIC FEB05 genome was performed. Conjecture that cyanobacteria possess genes of the microcystin operon for functions other than synthesis of the toxin led to further examination of the genome for *mcy* gene sequences. The biosynthetic pathway for production of microcystin has been elucidated (Tillett *et al*. 2000), enabling development of primers specific for microcystin genes. Sequence analysis of PCR products relating to the aforementioned genes is explored below.

6.8.1 Detection of Non-ribosomal Peptide Synthetase and Polyketide Synthetase Genes in *M. flos-aquae*

Chromosomal DNA extracted from *M. flos-aquae* isolate MIC FEB05 were used as DNA templates for PCR. DNA amplification was carried out as described in section

§6.3, using the primer pairs described in Table 6-3 to detect full or partial sequences of peptide or polyketide synthetase genes. DNA from *M. aeruginosa* PCC7806 was used as a positive control, and Milli-Q® water was substituted for DNA in negative controls.

Amplifications of *M. flos-aquae* MIC FEB05 chromosomal DNA and *M. aeruginosa* PCC 7806 (Koch) produced expected peptide/polyketide synthetase gene products of 700-800 bp, depending on the gene investigated. Negative control samples did not result in any PCR products.

6.8.1.1 Non-ribosomal Peptide Synthetase (NRPS) Genes

A discussion relating to the role of peptide synthetases in synthesis of toxins and bioactive compounds is presented in section §2.5.2.1, wherein Meißner *et al* (2002) alluded to a subtle distinction in presence and function of peptide synthetases within toxic and non-toxic strains. Meißner *et al* (2002) suggested that enzymes in hepatotoxic strains involved primarily with toxin formation, while those in non-toxic strains implicated in synthesis of bioactive peptides. Meißner *et al* (1996) demonstrated that toxic and non-toxic strains of *Microcystis* differed in their genomic sequence – toxic strains possess genes for specific peptide synthetases for biosynthesis of microcystins.

A BLASTN DNA sequence analysis of the NRPS PCR product amplified from MIC FEB05 indicated that the NRPS gene of *M. flos-aquae* was most similar to the cyanopeptolin synthetase gene of *Microcystis sp,* with 93% similarity. The results of the sequence analysis are illustrated in Figure 6-11 and Figure 6-12.

1	5’	GCAAAGGAAA	TTTCCAAAGT	AAAGGTTAGC	TTCATGCTCC	GAGATACTCA	3’
51	5’	GGGTTAAAAT	GATATTAACC	TGCGAATCTT	TACAGACTTC	CCTGCCAAAT	3’
101	5’	CATAATGCTA	TTGTTGTCTG	TTTAGATAAA	GATTGGCAAG	AAATTAATCA	3’
151	5’	AACCAGTCAG	GAGAATCTTA	ACAGCGCAGT	TTCTGCGGAT	AATTTAGCCT	3’
201	5’	ACGTTATTTA	TACTTCTGGTT	CTACAGGAAT	ACCCAAGGG	TGTTATCGTC	3’
251	5’	ACTCCTCAAG	CAATTAATCG	ACTGGTATTA	AATACCAATT	ACATCCAGTT	3’
301	5’	TACTCCTGAT	GACCGCGTTG	TGCAGGCGTC	TAATATTGCT	TTTGATGCCG	3’
351	5’	CTACTTTTGA	AATTTGGGGG	GCTTTACTTA	ACGGTGCTAA	AATTATTATT	3’
401	5’	ATCGCTAAAT	CAATTTTGCT	CTCACCCCAA	GAATTGGCAC	TAAGCTTAAA	3’
451	5’	GGAAAATCAG	ATTAGTGTCT	TATTTTTAAC	CACCGCACTT	TTTAATCAGT	3’
501	5’	TAGCTAATTTA	GTTCCCCAAG	CTTTTAGTAA	CTTACGATG	CTTACTATTT	3’
551	5’	GGGGGTGAA	GCAGTTGAAC	CAAAATGGGT	ACAAGAGGTA	CTAGAAAAAG	3’
601	5’	GTGCGCCACA	ACGGTTGCTT	CATGTCTATG	GACCAACAGA	AAATACAACG	3’
651	5’	TTTTCTTCCT	GGTATTTAGT	GCAAAACGTA	GCTTCTACAG	CCACATCTAT	3’
701	5’	TCCCATTGGT	AAAGCGATTG	CCAATAGCCA	AATCTATTTGC	TGGATAAAAA	3’
751	5’	TCTGCATCCT	GTGCCGATTG	GTGTTCCAGG	AGAATTACATA	TTGGTGGCGC	3’
801	5’	AGGATTAGCG	ATAGGTTATC	TCAATCGTCC	CGAATCAACC	CTAAGCACAA	3’
851	5’	TTCATTCCTA	ATCCTTTTAG	TAATTACCC	AGATTCTCAT	CTCTATAACA	3’
901	5’	CAAGAAGACT	TAGCGCGTAT	TTACCCGATG	GTGATATTGA	ATATCTGGGA	3’
951	5’	CGCATTGATA	ATCACGCAAA	AATCCCCGGC	CG		3’

(a)

Sequences producing significant alignments

Accession Number		**Max Score**	**Max Identity**	**E value**
DQ075244.1	*Microcystis* sp. NIVA-CYA 172/5 cyanopeptolin synthetase gene cluster, complete sequence	1247	93%	0.0
DQ837301.1	*Planktothrix agardhii* NIVA-CYA 116 cyanopeptin oci gene cluster, complete sequence	1236	93%	0.0
AY768441.2	*Planktothrix* sp. PCC 7811 clone 1 non-ribosomal peptide synthetase gene cluster, partial sequence	1230	93%	0.0
AM778942.1	*Microcystis aeruginosa* PCC 7806 genome sequencing data, contig C312		91%	7e-79
AJ224718.1	*Cyanobacterium* sp. gene encoding peptide synthetase, clone Riga062, partial		85%	7e-49
AJ224712.1	*Microcystis aeruginosa* gene encoding peptide synthetase, strain EAWAG167, partial			84%

Figure 6-11: DNA sequence analysis of the PCR product NRPS from *Microcystis flos-aquae*. DNA sequence of NRPS from obtained from *M. flos-aquae* MIC FEB05. (b) Results of a BLASTN DNA sequence analysis of the PCR product NRPS amplified from *M. flos-aquae* MIC FEB05. The NRPS gene of *M. flos-aquae* was most similar to the cyanopeptolin synthetase gene of *Microcystis* sp.

```
Query 26     TTAGCTTCATGCTCCGAGATACTCAGGGTTAAAATGATATTAACCTGCGAATCTTTACA        84
             ||||||| ||||||| |||||| || ||||||||| | | ||||||||||||||||||||
Sbjct 28469  TTAGCTTTATGCTCCAAGATACCCA-GGTTAAAAT-ACTGTTAACCTGCGAATCTTTACA       28526

Query 85     GACTTCCCTGCCAAATCATAATGCTATTGTTGTCTGTTTAGATAAAGATTGGCAAGAAAT       144
              || || | |||||||||||| | |||||||||||||||||||| |||||||||||| ||||
Sbjct 28527  GAATTTCTTGCCAAATCATCAGGCTATTGTTGTCTGTTTAGACAAAGATTGGCAACAAAT       28586

Query 145    TAATCAAACCAGTCAGGAGAATCTTAACAGCGCAGTTTCTGCGGATAATTTAGCCTACGT       204
             ||||||  | |||| ||||||||| |||||| ||||||||||||||||||||||||||||
Sbjct 28587  TAATCAGGCAAGTCTGGAGAATCTGAACAGCACAGTTTCTGCGGATAATTTAGCCTACGT       28646

Query 205    TATTTATACTTCTGGTTCTACAGGAATACCCAAGGGTGTTATCGTCACTCCTCAAGCAAT       264
             |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||| |
Sbjct 28647  CATTTATACTTCTGGTTCTACAGGAATACCCAAGGGTGTTATCGTCACTCATCAAGCAGT       28706

Query 265    TAATCGACTGGTATTAAATACCAATTACATCCAGTTTACTCCTGATGACCGCGTTGTGCA       324
             |||||||||||||||||||| |||||||||||||||||||||||||||| ||||||||
Sbjct 28707  TAATCGACTGGTATTAAATACTAATTACATCCAGTTTACTCCTGATGACCGGGTTGTGCA       28766

Query 325    GGCGTCTAATATTGCTTTTGATGCCGCTACTTTTGAAATTTGGGGGGCTTTACTTAACGG       384
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 28767  AGCGTCTAATATTGCTTTTGATGCCGCTACTTTTGAAATTTGGGGGGCTTTACTTAACGG       28826

Query  385   TGCTAAAATTATTATTATCGCTAAATCAATTTTGCTCTCACCCCAAGAATTGGCACTAAG       444
             |||||| |||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  28827  TGCTAAGATTATTATTATCGCTAAATCAGTTTTGCTCTCACCCCAAGAATTGGCACTAAG      28886

Query 445    CTTAAAGGAAAATCAGATTAGTGTCTTATTTTTAACCACCGCACTTTTTAATCAGTTAGC       504
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct 28887  CTTAAAGGAAAATCAGATTAGTGTCTTATTTTTAACCACAGCACTTTTTAATCAGTTAGC       28946

Query 505    TAATTTAGTTCCCCAAGCTTTTAGTAACTTACGATGCTTACTATTTGGGGGTGAAGCAGT       564
             |||||||||||| |||||||||||| ||||||||||||||||||||||||||||||||
Sbjct 28947  CAATTTAGTTCCGCAAGCTTTTAGTAGCTTACGATGCTTACTATTTGGGGGTGAAGCAGT       29006

Query 565    TGAACCAAAATGGGTACAAGAGGTACTAGAAAAAGGTGCGCCACAACGGTTGCTTCATGT       624
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 29007  TGAACCAAAATGGGTACAAGAGGTACTAGAAAAAGGTGCGCCACAACGGTTGCTTCATGT       29066

Query 625    CTATGGACCAACAGAAAATACAACGTTTTCTTCCTGGTATTTAGTGCAAAACGTAGCTTC       684
             |||||| ||||| |||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct 29067  CTATGGGCCAACGGAAAATACAACGTTTTCTTCCTGGTATTTAGTGGAAAACGTAGCTTC       29126

Query 685    TACAGCCACATCTATTCCCATTGGTAAAGCGATTGCCAATAGCCAAATCTATTTGCTGGA       744
             |||||||||| |||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct 29127  TACAGCCACAACTATTCCCATTGGTAAAGCGATTGCCAATACCCAAATCTATTTGCTGGA       29186

Query 745    TAAAAATCTGCATCCTGTGCCGATTGGTGTTCCAGGAGAATTACATATTGGTGGCGCAGG       804
             ||||||||| || ||||| |||||||||||   ||||||||||||||||||||||    ||
Sbjct 29187  TAAAAATCTCCAACCTGTCCCGATTGGTGTAGTAGGAGAATTACATATTGGTGGTATGGG       29246

Query 805    ATTAGCGATAGGTTATCTCAATCGTCCCGAATCAACCCTAAGCACAATTCATTCCTAATC       864
             |||||| | ||||||||| || |||||||||| ||||| ||| | |||| ||||||||||
Sbjct 29247  ATTAGCCAAAGGTTATCTTAACCGTCCCGAATTAACCC-AAGAAAAATTTATTCCTAATC       29305

Query 865    CTTTT                                                              869
             |||||
Sbjct 29306  CTTTT                                                              29310
```

Figure 6-12: DNA sequence analysis of the PCR product NRPS from *Microcystis flos-aquae* MIC FEB05. DNA sequence alignment of NRPS gene from *M. flos-aquae* and DQ075244. The sequences are 93% similar

6.8.1.2 Polyketide Synthetase Genes

A search targeting the presence of polyketide synthetase genes was also conducted, to substantiate the hypothesis that the bioactive compounds were assembled via an integrated NRPS-PKS biosynthesis pathway. Sequence analysis of the PCR product created using the PKS primers described in Table 6-3 has been prepared and is included as Figure 6-13. Results of a BLASTX DNA sequence analysis demonstrated similarities between the PKS gene of *M. flos-aquae* MIC FEB05 and the PKS (*mcy*G) gene of *M. aeruginosa* PCC 7806 (Koch). However, the sequence comparison included as Figure 6-13 (c) indicates the sequences are only 63% similar, implying either only a partial PKS sequence is present in *M. flos-aquae* MIC FEB05, or the primers selected for amplification of PKS genes require a higher level of degeneracy to optimise annealing of the primer to the template DNA and subsequent DNA elongation.

1	5’	GTGGAGCACG	TTGAACGCTC	TGGAGATGCT	GGTTACATCC	CCCAGGAAGC	3’
51	5’	CGATGCCCGG	ATTGGGGTGT	TCGGGGGTGT	TGGCACTAAC	TGGCACCTGA	3’
101	5’	GCCAAGTCGC	TCAATCTAGC	GTGGCCAAGA	AATATGCCAG	CGGTGCTTCA	3’
151	5’	GTCGTGATCA	GCAACGACCA	AGACTATGTA	ACATCACGGG	TTTCCTACAA	3’
201	5’	GCTTGGGCTA	GTTGGGCCTA	GCGTTAATGT	GCAGTCGGCC	TGTTCCACCT	3’
251	5’	CCTTAGTGGC	AACTATTTTA	GGGATGACCA	GCCTGCGTTC	AAAGCAATGT	3’
301	5’	GATCTAGCCC	TTGCTGGCGG	TGCAACGATT	GAACTTCCTG	AAAGGAAAGG	3’
351	5’	CTATCTGCAT	CTGGAAGGCG	GAATGGAGTC	GCCTGATGGC	CATTGCCGGC	3’
401	5’	CGTTTGATGC	TGCCGCTAAT	GGGACGGTGT	TCAGCCGCGG	TGCCGGGGTG	3’
401	5’	GTGATACTCA	AGCGGCTGGC	TGATGCTGTG	CGCGATCGTG	ATCACATCTA	3’
401	5’	TGCTGTGCTA	GTGGATGGTG	CGGTAAACAA	CGATGGTGCT	GACAAAATTG	3’
401	5’	GCTTTACCGC	ACCAAGCATT	TCTGGACAGG	TGGCGCTGAC	CCTGGATGCT	3’
401	5’	CTTAGTCGGG	CAGGGCTTTC	GGCTGAACAA	CTCAGTTTCG	TGGAACCCAC	3’
401	5’	GGACCCGGCT	ACCGGCTGAT	GGGGGGAGCA	CAGGCGGGCC	TGAAAATATA	3’
401	5’	TAACTTCAAC	CAGCAATCTA	GTCATAATTA	GGAGGTTTTG	GGGAATTTTC	3’
401	5’	CAACCTGGCC	TGATATCACG	CCCTCCCTAG	CGTGGGCAGA	AATGTGCAGG	3’
401	5’	GGGCTTAATC	GGGGATCACA	AGAAGAAGCC	CATTAAAATA	CCGGTGTCCG	3’
401	5’	CCAAATTGTG	CCAAGGGGTC	CTTTCTTTAT	GTGGGCGCGC	TGTTCCCTCT	3’
401	5’	TTCATGGAAC	T				3’

(a)

Sequences producing significant alignments		**High Score**	**E value**
gb|AAF15892.2|AF204805_2	NosB [Nostoc sp. GSV224]	219	2e-55
ref|ZP_00110898.1| COG3321	Polyketide synthase modules	218	5e-55
ref|YP_322130.1|	Beta-ketoacyl synthase [Anabaena variabilis	215	4e-54
gb|AAX44114.1|	polyketide sythase [Leptolyngbya sp. PCC 7410]	211	4e-53
gb|AAX44111.1|	polyketide sythase [Leptolyngbya sp. PCC 73110...	211	4e-53
gb|AAX44112.1|	polyketide sythase [Leptolyngbya sp. PCC 73110]	211	5e-53
ref|ZP_01467099.1|	amino acid adenylation domain protein [Sti	210	9e-53
gb|AAF19812.1|AF188287_4	MtaD [Stigmatella aurantiaca]	210	9e-53
gb|AAX44113.1|	polyketide sythase [Leptolyngbya sp. PCC 73110]	210	9e-53
gb|AAX44119.1|	polyketide sythase [Leptolyngbya sp. PCC 7410]	210	9e-53
gb|AAX44117.1|	polyketide sythase [Leptolyngbya sp. PCC 7410]	210	1e-52
emb|CAD89775.1|	MelD protein [Melittangium lichenicola]	205	3e-51
emb|CAO90231.1|	*mcy*G [*Microcystis* aeruginosa PCC 7806]	205	4e-51
gb|AAX73195.1|	*Mcy*G [*Microcystis* aeruginosa]	205	4e-51
gb|AAF00957.1|AF183408_5	*Mcy*G [*Microcystis* aeruginosa PCC 7806]	205	4e-51

(b)

CAO90231	768	NAGYNPNTYQGSIGIFAGASMNTYLINNCYPNRGKLDSNDELQPFTLDSMGGFQTMVAND	27
PKS	25	DAGYIPQEADARIGVFGGVGTNWHLSQVAQSSVAKKYAS----------GASVVISND	168
CAO90231	828	KDYLTTRISYKLNLHGPSVNVQTACSTGLVVVHLACQSLISGESDMALAGAASINSPQKI	887
PKS	169	QDYVTSRVSYKLGLVGPSVNVQSACSTSLVATILGMTSLRSKQCDLALAGGATIELPERK	348
CAO90231	888	GYLYQEGLIMSPDGHCRAFDAEAKGTIFGSGVGVIMLKRLSDALADHDHIYAVIKGSAIN	947
PKS	349	GYLHLEGGMESPDGHCRPFDAAANGTVFSRGAGVVILKRLADAVRDRDHIYAVLVDGAVN	528
CAO90231	948	NDGGQKLGFTAPGGEGQIAAATEALAFAGVDANTISFVE	986
PKS	529	NDGADKIGFTAPSISGQVALTLDALSRAGLSAEQLSFVE	645

(c)

Figure 6-13: DNA sequence analysis of the PCR product PKS from *Microcystis flos-aquae* MIC FEB05. (a) DNA sequence of PKS from obtained from *M. flos-aquae*. (b) Results of a BLASTX DNA sequence analysis of the PCR product PKS amplified from MIC FEB05. The PKS gene of

***M. flos-aquae* was similar to the PKS (*mcy*G) gene of *M. aeruginosa* PCC 7806. (c) DNA sequence alignment of PKS gene from *M. flos-aquae* and CAO90231. The sequences are 63% similar.**

6.8.2 Detection of *mcy* genes by PCR

Chromosomal DNA extracted from *M. flos-aquae* strain MIC FEB05 were used as DNA templates for PCR. PCR was carried out as described in section §6.3, using the primer pairs described in Table 6-3 to detect full or partial sequences of genes of the microcystin operon. DNA from *M. aeruginosa* PCC 7806 (Koch) was used as a positive control, and Milli-Q® water was substituted for DNA in negative controls.

Amplifications of *M. flos-aquae* MIC FEB05 chromosomal DNA and *M. aeruginosa* PCC 7806 produced expected microcystin gene based products of 500-1500 bp, depending on the gene investigated. Negative control samples did not produce any PCR products.

6.8.2.1 DNA sequencing Results

Several researchers (Nishizawa *et al.* 1999; Fujii *et al.* 2000) have suggested that non-toxic *Microcystis* species potentially possess a partial or mutated set of genes encoding peptide synthetases for microcystin biosynthesis and assembly. Subsequently, primers specific for 5 genes of the microcystin operons were used to amplify segments of template DNA via PCR. The rationale behind this investigation was to establish the presence (or absence) of the microcystin genes and thus the potential for the strain to produce these toxins.

To amplify the amount of PCR product available for sequencing the DNA, the original PCR cycle was repeated, using the PCR products of the previous cycle as the template DNA. BLASTX DNA sequence analysis demonstrated that the 500 bp PCR product obtained using the *mcyE* primer pairs was similar to the *mcyE* gene sequences of other *Microcystis* species, with the highest homology to the *M. aeruginosa mcyE* gene (98% similarity) (Figure 6-14). Results of BLASTX DNA sequence analysis of PCR products amplified with primers based on the remaining microcystin genes examined in this study – *mcyA, mcyB, mcyC* and *mcyD* – are presented in APPENDIX D. Sequence similarity between the PCR products and known DNA sequences of *Microcystis* sp. genes ranged between 86% (*mcyA*) and 61% (*mcyB*), suggesting a

high degree of sequence homology exists between genes of *M. flos-aquae* MIC FEB05 and other *Microcystis* strains.

1	5’	AGGCACTTGT	ATTGAGCAGT	TCACGAACAA	ATGAATCGAG	GAATAGGCTT	3’
51	5’	AGGAATGCAG	TCAAATCTGG	CCGCCGAAAC	CGCCGCTTTA	ATTAGTGAAA	3’
101	5’	TGGGCCGAGT	CGAAAGAGTC	GCTTTTAGTA	ATACGGGAAC	CGAGGCGATT	3’
151	5’	ATGGCGGCTG	TTCGCATTGC	TCGCTCCCGG	ACAAAACGTC	AAAAAATCGT	3’
201	5’	TATGTTTGCC	GGCTCCTACC	ATGGAACTTT	TGACGGCATC	TTAGCACGAG	3’
251	5’	TAGGAGAAGA	TAAAACCACG	ACTCAACCCT	TAAGTTTAGG	CACTCCTTTA	3’
301	5’	GGAATGGTTG	AAGACATAAT	AGTCTTGAGT	TATGGAGTTG	AAGAAAGCCT	3’
351	5’	CGATATTATT	GCTACTCATG	CTGATGATTT	AGCTGCCGTA	TTAGTCGAAC	3’
401	5’	CAGTTCAAAG	TCGCAAACCC	GATTTACAGC	TCCAGGAATT	AACA	3’

(a)

Sequences producing significant alignments		**High Score**	**E value**
emb|CAO90229.1|	*mcy*E [*Microcystis* aeruginosa PCC 7806]	265	9e-70
gb|AAF00958.1|AF183408_6	*Mcy*E [*Microcystis* aeruginosa PCC 7806]	265	9e-70
dbj|BAB12211.1|	polykeitde synthase and peptide synthetase	263	4e-69
gb|AAX21772.1|	microcystin synthetase [*Microcystis* aeruginosa.	251	8e-66
gb|AAX21773.1|	microcystin synthetase [*Microcystis* aeruginosa UT	249	3e-65
gb|AAX21770.1|	microcystin synthetase [*Microcystis* wesenbergii	249	4e-65
gb|AAX21771.1|	microcystin synthetase [*Microcystis* viridis NIES	247	2e-64
emb|CAD29794.1|	peptide synthetase [Planktothrix agardhii NIVA-C	230	3e-59
gb|AAX21780.1|	microcystin synthetase [Phormidium sp. 4-19b]	226	4e-58
ref|ZP_01629641.1|	Amino acid adenylation [Nodularia spumigen	224	1e-57
gb|AAX21778.1|	microcystin synthetase [Phormidium sp. 2-26b3]	224	1e-57
gb|AAO64407.1|	NdaF [Nodularia spumigena]	223	2e-57
gb|AAX21776.1|	microcystin synthetase [Oscillatoria sp. 18R]	217	2e-55
gb|AAX21775.1|	microcystin synthetase [Nostoc sp. 152]	214	2e-54
gb|AAX21783.1|	nodularia synthetase [Nodularia sphaerocarpa PCC	213	3e-54
gb|AAX21769.1|	microcystin synthetase [Anabaena sp. 202]	203	3e-51

(b)

AAF00958	1592	AVQEQMNRGIGLGMQSNLAAETAALISEMGRVERVAFSNTGTEAIMAAVRIARSRTKRQK	1651
*mcy*E	16	AVHEQMNRGIGLGMQSNLAAETAALISEMGRVERVAFSNTGTEAIMAAVRIARSRTKRQK	195
AAF00958	1652	IVMFAGSYHGTFDGILARVGEDKTTTQPLSLGTPLGMVEDIIVLSYGVEESLDIIATHAD	1711
*mcy*E	196	IVMFAGSYHGTFDGILARVGEDKTTTQPLSLGTPLGMVEDIIVLSYGVEESLDIIATHAD	375
AAF00958	1712	DLAAVLVEPVQSRKPDLQPQE	1732
*mcy*E	376	DLAAVLVEPVQSRKPDLQLQE	438

(c)

Figure 6-14: DNA sequence analysis of the PCR product *mcy*E from *Microcystis flos-aquae*. (a) DNA sequence of *mcy*E from obtained from *M. flos-aquae*. (b) Results of a BLASTX DNA sequence analysis of the PCR product *mcy*E amplified from MIC FEB05. The *mcy*E gene of *M. flos-aquae* was most similar to the *mcy*E gene of *M. aeruginosa* PCC 7806. (c) DNA sequence alignment of *mcy*E gene from *M. flos-aquae* and AAF00958. The sequences are 98% similar

Routine HPLC analysis undertaken by AWQC did not detect the presence of the toxin microcystin from the isolate of *M. flos-aquae* (MIC FEB05), within the limits of detection of the HPLC system. Lack of toxin production was confirmed by researchers at UNSW by protein phosphatase inhibition assay. However, experiments conducted during this study have established that this particular strain of *M. flos-aquae* was able to synthesise an antibacterial compound, effective against *S. aureus* and *B. subtilis*, and an antiviral compound with cytotoxicity demonstrated towards RSV and Dengue virus. Detection of genes with a high degree of homology to characterised microcystin genes, combined with the lack of toxin production suggests that these genes may contribute instead to synthesis of the bioactive compound(s), an idea previously acknowledged by leading researchers (Meißner *et al.* 1996; Namikoshi and Rinehart 1996; Nishizawa *et al.* 1999; Dittmann *et al.* 2001; Fujii *et al.* 2002). The relationship between the presence of particular microcystin genes and resultant biological activity has yet to be significantly explored.

6.8.2.2 Characterisation of gene cluster

Elucidation of the structural organisation of the microcystin gene operon by Tillet *et al* (2000) suggested 10 bidirectionally transcribed open reading frames, arranged in two putative operons:

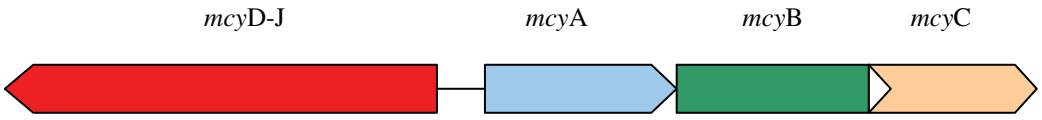


Figure 6-15: General structure of microcystin (Tillett *et al.* 2000; Mikalsen *et al.* 2003)

Amplification of DNA using primers specific for the *mcy*B gene indicated that this gene is absent or mutated within genomic DNA from *M. flos-aquae* MIC FEB05, preventing the primers binding effectively. To ascertain the presence of a region of DNA between *mcy*A and *mcy*C and the size of this region, primers were developed based on the reverse complement of the *mcy*A forward primer and the *mcy*C reverse primer; the primer sequences are provided in Table 6-6. Given the unknown properties of these primers, optimisation of the PCR cycle was undertaken to determine the most effective annealing temperature for primer binding and elongation. Applying the PCR cycle described in Table 6-7, the annealing temperature, X, was altered following gel electrophoresis of the PCR products,

staining of the gel using GelRed, and UV exposure using the BioRad Gel Doc system. Annealing temperatures assessed were: 48°C, 50°C, 52°C, 55°C, and 57°C.

Table 6-6: Primer sequences for reverse complement of *mcy*A and *mcyC* genes, developed to amplify the region of DNA between *mcy*A and *mcy*C.

Primer	T_m (°C)	Oligonucleotide primer sequence, 5'→3'
*mcy*AF	43.0	TTT GAT ACG GCT TTT AAT TTT
*mcy*CR	52.0	ACT AGA AGA TTC GCT GAT ATG CT

Table 6-7: PCR cycle for amplification of the region of DNA between *mcy*A and *mcy*C

	Denaturation Temp	Annealing Temp	Time
Cycle			
Repeat steps 35 cycles	94°C		4 min
	94°C		15 s
		X°C	1 min
		72°C	1 min
Completion		4°C	Hold

Amplification of DNA was not successful using annealing temperatures 48°C, 50°C, and 55°C. A faint band of DNA was visible at approximately 800 bp using an annealing temperature of 52°C; however, annealing appeared more successful at 57°C, with a stronger band visible at approximately 800 bp.

6.9 Summary

Chemical analysis of the isolated bioactive compound, described in Chapter 5, indicated that the peptide possessed amino acids often associated with the toxin microcystin, despite toxicity testing of the cyanobacteria by AWQC and UNSW confirming a lack of detectable toxin. However, researchers (Nishizawa *et al.* 1999; Fujii *et al.* 2000) have acknowledged that non-toxic strains often possess an incomplete microcystin operon, deficient in one or more genes, or comprising partial genes, giving rise to compounds with other alternative functions. To this end, investigations were undertaken to a) optimise extraction of DNA from freeze dried cyanobacterial cells; b) amplify regions of chromosomal DNA demonstrating homology to toxin gene sequences, through optimisation of primer pairs and PCR cycles; and c) elucidate the presence (or absence) of genes belonging to the

microcystin biosynthesis cluster. A supplementary investigation was also conducted to characterise the region of DNA between genes *mcy*A and *mcy*C.

Numerous DNA extraction methods were applied to samples of freeze dried cyanobacterial biomass, with the majority demonstrating limited or no success. Extraction techniques frequently applied to bacterial cells were unable to penetrate the structurally superior cyanobacterial cell wall. Proprietary extraction kits including the Wizard® Genomic DNA Kit and several Qiagen kits yielded very little viable DNA for downstream processing applications such as PCR. However, the Mo Bio PowerPlant™ DNA Isolation Kit was successfully able to extract DNA for use as template DNA in subsequent PCR reactions.

To confirm that the strain of interest did indeed belong to the genus *Microcystis*, a preliminary DNA amplification using 16S primers was conducted. The product obtained from the 16s PCR cycle was submitted for sequence analysis and may be classified in the following taxonomic hierarchy (with 100% assignment detail, for a confidence threshold of 95%):

Domain: Bacteria

Phylum Cyanobacteria

Class Cyanobacteria

Family Family 1.1

Genus *Microcystis*

This classification confirms that the species investigated during this research is of the genus *Microcystis*.

A comprehensive literature review of cyanobacterial genome investigations revealed many research groups had successfully developed primers specific for genes of the microcystin operon (Neilan 1995; Meißner *et al.* 1996; Dittmann *et al.* 1997; Kaebernick and Neilan 2001; Dittmann and Börner 2005). Given the similarities in structure features and amino acids between the bioactive compound and microcystin, a directed search was conducted using the aforementioned primers to ascertain the presence (or conversely, the absence) of the microcystin genes *mcyA, mcyB, mcyC, mcyD* and *mcyE,* by applying the relevant primers and using optimal PCR cycles. Complete or partial sequences were identified for 4 of the five genes investigated; amplification of the *mcyB* gene was unsuccessful.

Examination of the microcystin synthesis pathway identified the involvement of a hybrid NRPS/PKS system to assemble the components of the toxin. These systems are also suspected to be involved in synthesis of other biologically active compounds, and subsequently primers for cyanobacterial NRPS and PKS genes were applied to the template DNA for amplification of homologous regions. These investigations indicated that *M. flos-aquae* MIC FEB05 possesses an NRPS gene with 94% similarity to the cyanopeptolin synthetase gene of *Microcystis* sp. Elucidation of a PKS gene was less successful; however, a BLASTX DNA sequence analysis of the PCR product obtained using the cyanobacterial PKS primers demonstrated 63% similarity to the PKS (*mcy*G) gene of *M. aeruginosa* PCC 7806. The implications of these experiments suggest that firstly *M. flos-aquae* MIC FEB05 possesses an NRPS gene that is likely involved in synthesis of the bioactive compound (which may resemble the cyanopeptolins), given the species is non-toxic and therefore the NRPS would not be required for toxin synthesis; and secondly that either the PKS primers were not suitable for this isolate or that the species does not possess a complete PKS gene.

The absence of a conclusive *mcyB* sequence prompted examination of the region of between *mcy*A and *mcy*C to determine whether a partial (incomplete) *mcy*B gene existed, an unidentified gene was present, or whether the region was excised entirely. Reverse complement primers of *mcy*A and *mcy*C were developed and applied to amplify the region of DNA between these genes to identify the genetic sequence. Optimisation of the PCR protocol was also undertaken as the primers had not been previously tested, and therefore a recommended cycle was not available. Amplification of DNA was not successful using annealing temperatures of 48°C, 50°C, and 55°C. A faint band of DNA was visible at approximately 800 bp using an annealing temperature of 52°C; however, annealing appeared more successful at 57°C, with a stronger band visible at approximately 800 bp. The PCR product was unfortunately insufficient for further processing (i.e. DNA sequencing), and due to time constraints, the investigation ceased at this point.

Molecular analysis of DNA from *M. flos-aquae* MIC FEB05 using 16S ribosomal sequence analysis successfully confirmed the taxonomy of the cyanobacteria as genus *Microcystis*. Partial sequences of genes *mcy*A, *mcy*C, *mcy*D and *mcy*E from the

microcystin operon were identified in chromosomal DNA of *M. flos-aquae* MIC FEB05; however, a sequence homologous to *mcy*B was not identified. An NRPS gene sequence similar to that of the cyanopeptolins was also identified, though only a partial PKS sequence (63% similarity) was detected. Results of the molecular analysis of this isolate suggest the biologically active peptide may be synthesised by components of the microcystin gene operon, and that the peptide itself may resemble or be related to the cyanopeptolins.

CHAPTER 7 SUMMARY AND CONCLUDING REMARKS

7.1 Introduction

The increasing emergence of antibiotic resistant pathogens has motivated the search for alternative sources of compounds able to combat persistent infections (Burgess *et al.* 1999; Freile-Pelegrin and Morales 2004; Das *et al.* 2005), by inhibiting growth and/or infective mechanism of the pathogen. Natural therapies are considered more environmentally acceptable than synthetic drug products due to their inherent biodegradability (Ozdemir *et al.* 2004) and subsequently researchers have focused on acknowledged sources such as cyanobacteria, documented synthesisers of a diverse range of bioactive peptides and other compounds (Bloor and England 1989; Borowitzka 1995; Borowitzka 1999; Harada 2004; Dittmann and Wiegand 2006). Most commonly detected are the microcystins, hepatotoxic cyclic peptides that inhibit protein phosphatases PP1 and PP2A (Beresovsky *et al.* 2006). However, other compounds, such as the one isolated in this study, demonstrate biological activity against bacteria and viruses, though their exact biological function remains unclear.

Cyanobacteria (and microalgae) are often the preferred investigative organisms for bioactive compound detection, compared with higher plants (Mur *et al.* 1999). There are numerous reasons in support of this predilection, including rapid generation of biomass, availability of low-cost culture media, ease of cultivation in the laboratory, similarities to bacterial cells (particularly for cyanobacterial species) aiding research directions and knowledge of the organism, and diversity of environmental niches where these organisms survive whilst others fail.

Obstacles arise in identification of the responsible species, particularly with respect to field samples (Parker 1982; Ferris and Hirsch 1991). These cells often live in close association, at times forming symbiotic or parasitic relationships with other organisms and are consequently difficult to isolate for closer examination. Similarly, synthesis of the compounds is heavily influenced by environmental parameters (nutrient bioavailability, temperature, light intensity and exposure) (Skulberg 2000); strains are capable of up- or down-regulation of compound synthesis in response to external influences. Screening programmes therefore tend to focus on axenic or unialgal

laboratory cultures, produced under controlled conditions. Few of the many thousands of cyanobacterial species have been identified as bioactive (Borowitzka 1995; LeFlaive and Ten-Hage 2007), inferring either some cells are not capable of biological activity, or the combination of conditions are not optimal for that strain. Bioactive compounds are frequently the product of secondary metabolic activity (Carmichael 1992); these substances are produced only when the cell is established within its environment, and is able to afford the energy necessary for secondary compound biosynthesis.

The work described in this thesis therefore aimed to: (a) assess a field strain of cyanobacteria for bioactivity against bacterial, fungal and viral test organisms; (b) isolate the compound(s) of interest, construct a potential amino acid sequence and/or identify key structural attributes of the compound; and (c) examine the relationship between the lack of toxin synthesis and presence of toxin genes, and their potential involvement in bioactive compound synthesis.

7.2 Culture Growth and Nutrient Bioavailability

Cyanobacteria are considered one of the most robust, easily cultivable organisms and are often cultivated successfully in the laboratory following collection from the field. Whilst some species may be particularly sensitive to removal from their preferred environment and are not able to readily adapt to the sterile surroundings of a laboratory (Castenholz 1988; Andersen and Kawachi 2005), many species are successfully cultured and maintained for extended periods using techniques more frequently applied to bacteria. Provided the essential components are provided in a nutrient rich growth media, and parameters such as light intensity and exposure, temperature and agitation are optimised for the species of interest cyanobacterial cultures are relatively simple to initiate and sustain.

In particular, *Microcystis* has earned a reputation for simple cultivation using minimal media (i.e. limited essential components required) (Watanabe 2005). Isolates of two species, *M. flos-aquae* and *M. aeruginosa*, were collected from locations around Adelaide, and further cultures were obtained from the collection at AWQC for comparison of growth in minimal and enriched media, and standard and enriched river water. All tests were performed in triplicate to validate results. As expected, all

strains demonstrated strong growth in the enriched synthetic media, and also achieved satisfactory growth in minimal media. Species obtained from the field sites appeared to endure better in the enriched river water than those obtained from established laboratory cultures. This is perhaps an inherent trait of the field strains, to be able to adapt readily to changes in their immediate environment. Constant competition for nutrients and light, and deterring higher organisms that utilise cyanobacteria as a food source equip field strains with mechanisms to acclimatize to modifications in their habitat. Conversely, cells supplanted from a unialgal or "synthetic" environment where all nutrients and growth parameters are optimised may be less flexible in their response to stressors.

Optimisation of growth media and hence biomass yield will have profound effects on cell metabolism and subsequently the quantities and types of secondary compounds synthesised. Secondary metabolite production, considered non-essential to a cell's initial survival may not relate directly to high biomass yield; synthesis of these compounds is often triggered under conditions not conducive to high growth rates, such as nutrient deprivation (Olaizolá 2003). Therefore, optimisation of culture conditions should seek to establish a balance between secondary metabolism and growth rate.

During this study, species of *Microcystis* collected from various locations across Adelaide were cultured in modified growth media, in addition to samples of nutrient rich river water. Samples of the water were analysed for key nutrients – ammonia, copper, iron, lead, nitrate, phosphate, and zinc – at three stages of cyanobacterial activity within the river. Availability of specific nutrients is crucial to survival of cyanobacteria within their environment (Vonshak and Torzillo 2004; Baptista and Vasconcelos 2006); a lack of essential elements ultimately results in death of the species within a particular environmental niche. However, the nutrients must also be bioavailable; that is, existing in the environment as free ions available for uptake by the cells. These results suggest that *M. flos-aquae MIC FEB05* was potentially able to utilise particular heavy metals, limiting their impact on the environment. A significant spike in the concentration of iron was noted in the April 05 sample (refer to §4.3.1.2 Figure 4-7).

Concentrations of total Nitrogen (TKN) remained relatively high, particularly during the bloom period and generally over the entire monitoring period; however, the concentration of nitrogen-containing compounds such as ammonia (NH_3) and nitrogen oxides (NOX) were very low during the bloom suggesting that these forms were preferentially utilised by *Microcystis* cells rather than elemental nitrogen. *Microcystis* and *Anabaena* differ in their ability to fix nitrogen from the environment, as many strains of *Anabaena* are recognised "nitrogen-fixers". Similarly, low concentrations of phosphorus during the bloom period suggest that this is a vital element for growth of cyanobacteria. Conversely, continually low levels of phosphorous following the bloom may be a signal that other organisms in the environment were using this compound, or reflect upstream conditions or rainfall periods.

The combinations and relative concentrations of each of the nutrients are likely to have influenced the type and quantity of secondary metabolites synthesised by *M. flos-aquae* MIC FEB05. A reduction in concentration or bioavailability of any of essential constituents, or indeed a change in the physical parameters such as temperature and light intensity could potentially have precluded biosynthesis of bioactive compounds. Influence of physicochemical factors on synthesis of secondary, bioactive compounds requires further investigation before a conclusive argument can be constructed.

7.3 Isolation and bioactivity assessment of *Microcystis flos-aquae*

Cyanobacterial secondary metabolites exhibiting biological activity against a range of organisms are naturally synthesised, potentially as a defensive mechanism against competitors and predators in their environment (Ward and Targett 1989; Ikawa *et al.* 1996). A selection of strains collected from field sites around Adelaide and laboratory cultivated strains were examined for biological activity against bacterial, fungal and viral test organisms. To adequately assess the bioactive status of cyanobacteria, a comprehensive review of literature pertaining to extraction methods and bioactivity assays applicable to cyanobacterial cells was conducted, and preliminary investigations performed to determine the suitability and efficacy of each method. Those demonstrating successful compound isolation were chosen for examination and subsequent optimisation. Combinations of extraction techniques and solvents were

trialled, and six extraction protocols were selected for application in remaining bioactivity assays; these techniques have been outlined in section §3.4 and are described in detail in APPENDIX B; standard operating procedures are provided in APPENDIX A. As previously noted, many field strains exhibit a predisposition to form symbiotic associations in their environment (Parker 1982; Reynolds 1984; Ferris and Hirsch 1991; Andersen and Kawachi 2005), necessitating removal of these organisms prior to treatment of the cyanobacterial biomass to ensure active compounds were of cyanobacterial origins. Cyanobacterial biomass was treated by sonication and rigorous washing to remove unwanted organisms, prior to application of the selected extraction methods.

Screening programmes for bioactive cyanobacterial strains have been undertaken by research groups worldwide, applying techniques to both field and laboratory cultivated strains (Cannell *et al.* 1988; Carmichael 1992; de Caire *et al.* 1993; Borowitzka 1995; Nowotny *et al.* 1997; Cohen 1999; Kreitlow *et al.* 1999; Harada 2004; Skulberg 2004). Whilst this study has concentrated on one particular isolate of *M. flos-aquae* demonstrating dual activities, other species of *Microcystis* and cyanobacterial genera should not be ignored. The laboratory cultures assessed during this study did not exhibit growth-inhibitory properties under the chosen assay conditions; this does not imply that these strains lack bioactivity. These strains may simply be more sensitive to environmental influences, or the cultures were extracted during a less than optimal period of secondary metabolite synthesis. It is widely recognised that secondary metabolites synthesised during specific periods of development are often species and even strain unique (Armstrong *et al.* 1991; Borowitzka 1999; Skulberg 2004). Consequently, the conditions implemented for *M. flos-aquae* MIC FEB05 were optimal for bioactive compound production by this isolate, but were not suitable for other strains within the same species. Also acknowledged is the idea that laboratory cultures have a tendency to lose the ability to synthesise bioactive metabolites through repeated subculturing (Skulberg 2000; Welker *et al.* 2006); the loss of competition for nutrients and light exposure within cultures grown under “ideal” or artificial conditions eliminates the need for production of defensive compounds.

Issues related to extraction of the compounds become evident in light of the limited quantities of synthesised substance (Sasson 1991), and the potential degree of complexity of the compound, rendering chemical manufacture infeasible in many cases (Dittmann and Wiegand 2006; Welker *et al.* 2006; LeFlaive and Ten-Hage 2007). These issues can be partially overcome through optimisation of growth media components and parameters (light intensity and colour, exposure time, temperature) to prompt the onset of secondary metabolism of the cell within a "secure" environment, thus increasing yield of the metabolites of interest. Optimisation of growth conditions (and intrinsically metabolite synthesis) and extraction techniques are thus imperative for development of these compounds in commercial or medical application. The approach used in this thesis should therefore be applicable to detection of a wide range of cyanobacterial metabolites displaying antimicrobial activity.

The target species of this study, a non-toxic isolate of *Microcystis flos-aquae* MIC FEB05 was assessed for biological activity against bacterial, fungal and viral pathogens, in addition to a field strain of *A. circinalis*, and a number of cyanobacterial isolates obtained from established laboratory cultures. Freeze-dried cellular material was extracted using the methods selected, and the extracts assessed for growth inhibition. Production of antimicrobial compounds was confirmed by agar disc diffusion assays using bacterial and fungal test organisms, and by cytotoxicity assay using the viral pathogens. A microtitre plate assay was also developed to support results of the agar disc diffusion assays, based on a method described by the Australian Institute of Marine Science, Townsville, QLD (L. Llewellyn, personal communication, March 2005). Antiviral assessment was conducted via cytotoxicity assays by virologists at the IMVS, Adelaide, due to the stringent regulations surrounding handling of infectious viruses in the public domain.

One extract in particular, a methanol-based extraction of *M. flos-aquae* MIC FEB05 collected from the Torrens River, demonstrated inhibitory activity for the bacteria *S. aureus, B. subtilis* and to a lesser extent, *C. albicans*. Virologists at the IMVS confirmed that this extract also displayed cytotoxic effects for Dengue virus and RSV. Neither the laboratory cultured isolates, nor the *A. circinalis* sample collected from the same field site inhibited growth of any of the test organisms. On the basis of these properties, only the extract and corresponding *M. flos-aquae* MIC FEB05 isolate was

selected for further investigation, including chemical analysis and molecular examination of biosynthetic genes.

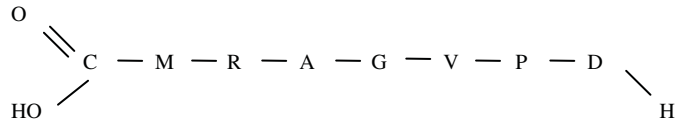
7.4 Chemical structure analysis of the bioactive metabolite

In this study, chromatographic profiles of extracts were constructed via reverse phase HPLC to visualise the components isolated from the biomass. HPLC is a universally accepted analytical method for separation, identification, purification, and quantification of various compounds (University of Kentucky 2004), and has been extensively exploited for identification and purification of cyanobacterial bioactive metabolites (Voloshko *et al.* 2008), though Bloor and England (1989) acknowledge that only a small number have been structurally identified. Chromatographic profiles were constructed of the extract with proven bioactive characteristics, and a non-bioactive extract of the *M. flos-aquae* MIC FEB05 biomass collected at a later stage of growth but developed using the same technique. Differences in peaks were interpreted to indicate the presence (or absence) of components between the extracts, and subsequently comparison of the profiles highlighted a number of peaks that could have represented the bioactive compound. Using a semi-preparative column fractions were accumulated at the retention times of the chosen peaks and were re-examined for bioactivity using the original organisms. One fraction, collected at a retention time of approximately 18 min successfully inhibited growth of *S. aureus* and *B. subtilis*; this fraction was observed to be absent from the chromatographic profile of the non-bioactive extract, suggesting it was likely to be the compound of interest, and warranted further investigation. Fractionated samples were also submitted for antiviral analysis; however, these samples have not been assessed when this manuscript was submitted, due to the limitations on resources of the IMVS to dedicate to non-essential projects.

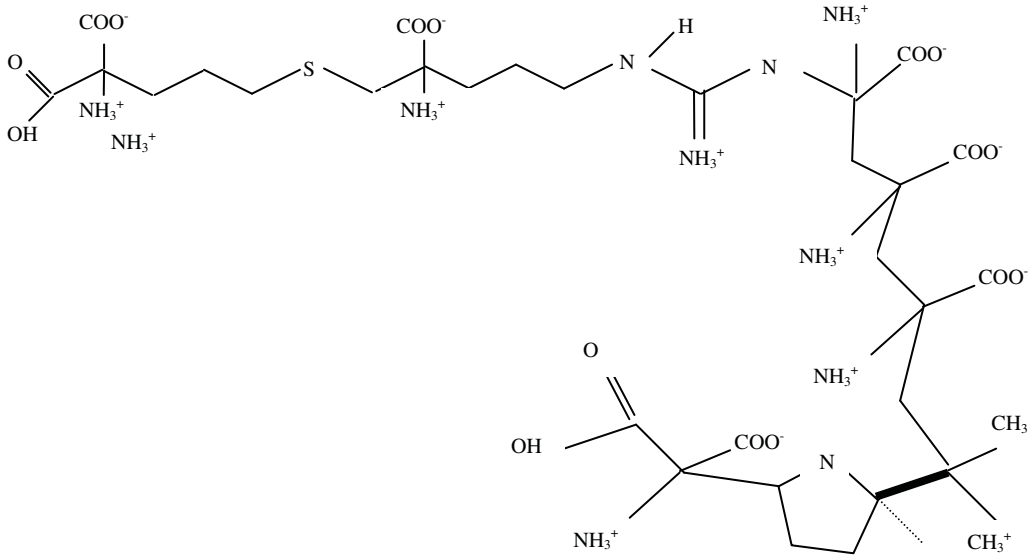
A key issue in this study was isolation of the biologically active peptides and structure analysis to elucidate a likely amino acid sequence and structure. Mass Spectrometry (MS) is an analytical tool often utilised for analysis of peptides, drug discovery and water quality (Ashcroft 2004; Beresovsky *et al.* 2006). Tandem Mass spectroscopy (MS/MS) and liquid chromatography-mass spectrometry (LC/MS) was employed during this study to resolve components of the bioactive compound structure. LC/MS has proven to be a powerful tool for analysis of toxins and other bioactive metabolites

at trace levels, as it provides primary amino acid sequence information for identification of the compounds of interest (Frias *et al.* 2006).

Results of the chemical analysis indicated that there was no readily interpretable, continuous amino acid sequence present, although gaps within the fragmentation ladder were perceived to correspond to known amino acids. Interpretation of the amino acid sequence of the compound undertaken by a research chemist suggested the following amino acid pattern and related structure based on the MS/MS and LC/MS data generated:



(a)



(b)

Figure 7-1: Proposed (a) amino acid sequence and (b) peptide structure for the bioactive compound isolated from non-toxic *M. flos-aquae*.

The fragmentation pattern also indicated the presence of modified amino acids (sulphonated; methylated; alkylated) or sugar units such as xylose (mass gap of 180 between peaks at 620.4 and 440). A review of literature suggested modified (or degraded) amino acids were more likely than sugar units, given the common structural features of cyanobacterial metabolites (Moore 1996; Tooming-Klunderud *et*

al. 2007). The possibility also existed that the extract underwent a degree of compound degradation during storage, a hypothesis which was supported by a reduction in biological activity of the extract following 2 months storage at -20°C.

Although this thesis concentrated on the antibacterial property of *M. flos-aquae* MIC FEB05, other antimicrobial properties, particularly the antiviral trait, should not be discounted. Fractionated extract sample were reassessed only for antibacterial and antifungal activity; any of the remaining fractions may have contained the antiviral compound (it has been assumed that the antibacterial and antiviral activity displayed by this strain are caused by 2 discrete, though potentially related, compounds, given the divergence in infection mechanism between the pathogens). Thus, future work must examine this cyanobacterium as a source of diverse biologically active metabolites. Justification for this approach is inherent in the previously demonstrated antiviral activity, and the commercial potential for development of the compounds into vaccines or as constituents of rigorous treatment regimes.

7.5 Characterisation of NRPS, PKS and *mcy* genes present in bioactive *M. flos-aquae*

Classification of an organism is often confirmed by comparing the 16S ribosomal RNA sequence of the target strain with known 16S sequences stored in genome databases. These sequences are known to possess regions of highly conserved homology within species, and often with other organisms of the same domain (e.g. bacteria and cyanobacteria). The DNA sequence obtained from the PCR amplification using 16S primers was submitted for sequence classification, and was classified according to the following taxonomic hierarchy (with 100% assignment detail, for a confidence threshold of 95%):

Domain: Bacteria

Phylum Cyanobacteria

Class Cyanobacteria

Family 1.1

Genus *Microcystis*

This classification confirms that the species investigated during this research was of the genus *Microcystis*.

In this study, genomic DNA extracted from the bioactive *M. flos-aquae* MIC FEB05 was examined for the presence of genes involved in biosynthesis of the peptides. Given the propensity for cyanobacterial strains to possess genes for the toxin microcystin, the search was initiated using primers specific for conserved regions of these genes. In order to detect the presence of *mcy* biosynthesis genes in this isolate, a PCR method was devised to detect the presence of genes with sequence homology to known *mcy*A, *mcy*B, *mcy*C, *mcy*D and *mcy*E. Similarity to NRPS and PKS genes was also sought, to support the theory that bioactive metabolites are assembled via a hybrid NRPS-PKS system.

Tooming-Klunderud *et al* (2007) remarked that ". . . despite the large number of cyclic peptides found in cyanobacteria, relatively few classes of NRPS operons have been characterised . . ." Investigating the sequence variation in *mcy* genes of *Microcystis* strains, Mikalsen *et al* (2003) suggested that frequent recombination event between domains, in addition to gene deletions may account for the inconsistency in the *mcy*ABC operon between strains. *Microcystis* has demonstrated co-production of microcystin and cyanopeptolin in several strains (Birk *et al.* 1989; Ishida *et al.* 1995; Murakami *et al.* 1997). Cyanopeptolins are a class of protease-inhibiting peptides, characterised by the presence of the amino acid Ahp (3-amino-6-hydroxy-2-piperidone), and the cyclic configuration of the peptide. These compounds appear to be the second most common peptide synthesised by *Microcystis*, following microcystin (Tooming-Klunderud *et al.* 2007), and Welker *et al* (2006) detected cyanopeptolins in 60% of *Microcystis* colonies studied.

Alignment of the DNA sequences with equivalent sequence data from different cyanobacterial sources confirmed the presence of a non-ribosomal peptide synthetase

gene, and a number of genes from the *mcy* operons, known to be involved in synthesis of the enzymes responsible for assembly of the toxin, microcystin. Specifically, *mcyA*, *mcyC*, *mycD* and *mcyE* were detected, with sequence similarity to previously characterised *mcy* genes between 63% and 98%. Although a sequence was returned following analysis of the PCR product amplified by the *mcyB* primers, it was concluded that a non-specific product had been amplified with these primers. The *mcyB* gene sequence of *M. flos-aquae* MIC FEB05 did not resemble any known sequences of *mcyB* genes of other *Microcystis* sp. but demonstrated 61% homology to a type 1 site-specific deoxyribonuclease from *Shewanella baltica* OS155 (Ziemke *et al* 1998). This suggests either *mcyB* is absent from the operon; only a partial gene sequence is present with low sequence similarity to the primer set used; or degeneracy of the primer sets was insufficient for this strain.

The presence of microcystin genes *mcyA, mcyC, mcyD* and *mcyE* supported the hypotheses previously presented, that toxin genes present in non-toxic strains are potentially involved in biosynthesis of metabolites with alternative bioactivities such as antibacterial or antiviral. Disruption mutations in genes *mcyA*, *mycD* (Tillett *et al.* 2000) and *mcyB* (Dittmann *et al.* 1997) confirmed the specific involvement of these genes in microcystin production. The disrupted genes were alleged to encode peptide synthetases specifically involved in biosynthesis of microcystin, and chemical and enzymatic analysis by the respective research groups confirmed the absence of any partial, truncated or linear forms of microcystin (Tillett *et al.* 2000), though the strains were able to maintain synthesis of smaller peptides (Dittmann *et al.* 1997). Applying these principles to the isolate investigated during this study, the lack of *mcyB* indicated that the combination of genes was predicted to encode a non-toxic metabolite and the bioactive compound was regarded as novel. Chromosomal DNA from *M. flos-aquae* MIC FEB05 was also examined for the presence of NRPS and PKS genes, thought to play a primary role in assembly of the bioactive or toxic compound. NRPS and a partial PKS gene with similarity to those of the cyanopeptolin family were also identified during this phase of research.

7.6 Future work

Microcystis flos-aquae isolate MIC FEB05 produced both an antibacterial and an antiviral compound within a natural environment under bloom-forming conditions,

potentially as a deterrent to grazers feeding on the organism, or to competitors for essential nutrients. Elucidation of optimal synthesis conditions for these compounds may stimulate function of *M. flos-aquae* MIC FEB05 as a primary producer for large scale production of alternative drug therapies. However, further experiments are required to improve production and extraction of bioactive metabolites, and to develop assessment techniques with high sensitivity to minute quantities of the compound. Numerous extraction methods were examined during this study to obtain sufficient material for bioactivity assessment; for development into commercial-grade products more refined techniques are required. Bioactive compound production was not optimised during this investigation, as the primary objective was to isolate and characterise bioactive compounds synthesised by the isolate.

The implication that environmental parameters including light exposure, temperature, agitation and nutrient bioavailability, influence the metabolism of cyanobacterial cells has been well documented (Huisman *et al.* 1999; Vonshak and Torzillo 2004; Baptista and Vasconcelos 2006; LeFlaive and Ten-Hage 2007), though the relationship between a change in environment and the resultant cellular response is not well understood. Subsequently, an in-depth assessment into the affect of environmental stressors on the metabolic activities of cyanobacterial cells is essential to further understand algal ecology, and potentially to identify mechanisms for up-regulation of production of desirable compounds. Conversely, these same principles may be utilised in minimising synthesis of harmful substances such as toxins.

HPLC analysis of extracts of *M. flos-aquae* MIC FEB05 indicated the presence of a number of compounds other than the antibacterial metabolite isolated. It is feasible that under different combinations of growth conditions (temperature, light exposure and intensity, nutrient concentration) peptides with other biological properties may be synthesised in sufficient quantities for bioassay detection. For a broader perspective of the ecological functions of this strain, further information should be obtained by analysing the molecular structures of the remaining peptides, particularly that of the antiviral compound.

Due to stringent requirements imposed on handling of viral particles, assessment of the antiviral status of the extracts was outsourced to virologists at the Institute of Medical and Veterinary Science (IMVS). The methanolic extract that inhibited

growth of the bacterium *S. aureus* also demonstrated antiviral effects towards Dengue virus and RSV. Currently, treatment for RSV is primarily supportive care. Antiviral drugs such as ribavirin, which is the only approved drug for treatment of RSV in infants, have been severely limited by the mode of administration, cost and limited efficacy (Krilov 2002). Other treatment regimes based on monoclonal antibodies are suitable for prevention of severe RSV in premature infants and those with chronic lung disease; however the drug has not proven effective for treatment of established RSV disease (Krilov 2002). Similarly, there is an urgent need to provide a solution to the escalating global public health problems caused by dengue infections (Stephenson 2005). Better disease management, vector control and improved public health measures will help reduce the current disease burden, but a safe and effective vaccine is probably the only long-term solution (Stephenson 2005). The commercial potential for a naturally synthesized antiviral compound able to reduce or prevent infection by both Dengue virus and RSV is enormous. However, significant research is required to isolate the compound, develop techniques to increase yield, and examine the action of the compound against the virus itself to determine the most effective target within the viral infection and replication cycle.

A number of authors (Birk *et al.* 1989; Meißner *et al.* 1996; Namikoshi and Rinehart 1996; Nishizawa *et al.* 1999; Dittmann *et al.* 2001; Fujii *et al.* 2002) have observed that the presence of genes of the *mcy* operon within non-toxic strains of cyanobacteria, generally responsible for synthesis of the toxin microcystin, may serve alternative functions. As suggested by Dittman *et al* (2001) in section §2.5.2, some cyanobacterial genera appear to possess the biosynthetic genes but do not synthesise the corresponding metabolites, raising the question of function of peptide synthetases in toxic and non-toxic strains. Despite the recent investigations undertaken by these authors and the research undertaken as part of this study, a clear understanding of the role of the *mcy* operon in bioactive metabolite synthesis is still forthcoming. Significant research is required to provide valid information regarding biosynthetic pathways of cyanobacteria. Ziemert et al (2008) suggests conducting a comparative analysis of related gene clusters to provide new insights into the versatility of these pathways, and hence allow discovery of novel natural products and the functional role of the peptides.

The commercial potential of these compounds, particularly the antiviral metabolites, is substantial, and deserves further investigation to maximise the yield of the compounds and simplify efficient extraction and purification processes to enable rapid development. Genetic examination of the strains will contribute significantly to this development, and while alternatives such as transformation of the metabolic genes into organisms with a higher growth rate (e.g. *E coli*) may have potential, the key to successful extraction of the compounds is to ultimately identify the responsible genes and perform directed investigations involving mutation or up-regulation of the genes.

7.7 Concluding Remarks

The overall objectives of this study were to (a) assess a field isolate of cyanobacteria for bioactivity against bacterial, fungal and viral test organisms; (b) isolate the compound(s) of interest, construct a potential amino acid sequence and/or identify key structural attributes of the compound; and (c) examine the relationship between the lack of toxin synthesis and presence of toxin genes, and their potential involvement in bioactive compound synthesis.

Historically, identification of biologically-active strains of cyanobacteria has halted at the detection phase following rigorous screening programs; few studies have pursued the metabolite to the extent of determining chemical structure (Bloor and England 1989; Borowitzka 1995; Luescher-Mattli 2003), and fewer still have attempted to ascertain the relationship between biosynthetic pathway and secondary metabolite (Meißner *et al.* 1996; Fujii *et al.* 2000; Volk and Furkert 2006; Tooming-Klunderud *et al.* 2007).

The results presented in this thesis describe a bioactive strain of *M. flos-aquae* MIC FEB05 with cytotoxic activity for *S. aureus*, *B. subtilis*, RSV and the Dengue virus. The focus of the study followed the isolation and analysis of the antibiotic compound, and the subsequent molecular investigations to identify genes potentially involved in synthesis of the bioactive compounds, and the relationship of these genes to those involved in microcystin synthesis. Publication of this research will contribute significant knowledge to the limited data surrounding cyanobacterial secondary metabolites, and direct further studies toward development of these compounds as alternative, novel therapies for treatment of drug resistant pathogens.

CHAPTER 8 REFERENCES AND BIBLIOGRAPHY

Acreman, J. (1994). "Algae and cyanobacteria: isolation, culture and long-term maintenance." Journal of Industrial Microbiology **13**: 193-194.

Agrawal, M. K., Zitt, A., Bagchi, D., Weckesser, J., Bagchi, S. N. and von Elert, E. (2005). "Characterization of Proteases in Guts of *Daphnia magna* and Their Inhibition by *Microcystis aeruginosa* PCC 7806." Environmental Toxicology **20**: 314–322.

Allen, E. A. D. and Gorham, P. R. (1981). Culture of planktonic cyanophytes on agar. The water environment: algal toxins and health. Carmichael, W. W. New York, Plenum Publishing Corporation. **20:** 185-192.

Andersen, R. A. and Kawachi, M. (2005). Traditional Microalgae Isolation Techniques. Algal Culturing Techniques. Andersen, R. A., Elsevier Academic Press**:** 83-132.

Angel, D. L., Eden, N., Breitstein, S., Yurman, A., Katz, T. and Spanier, E. (2002). "*In situ* biofiltration: a means to limit the dispersal of effluents from marine finfish cage aquaculture." Hydrobiologia **469**: 1-10.

Antonopoulou, S., Oikonomou, A., Karantonis, H. C., Fragopoulou, E. and Pantazidou, A. (2002). "Isolation and structural elucidation of biologically active phospholipids from *Scytonema julianum* (cyanobacteria)." Biochemistry Journal **367**: 287-293.

Apt, K. E. and Behrens, P. W. (1999). "Commercial Developments in Microalgal Biotechnology." Journal of Phycology **35**: 215-226.

Armstrong, J. E., Janda, K. E., Alvarado, B. and Wright, A. E. (1991). "Cytotoxin production by a marine *Lyngbya* strain (cyanobacterium) in a large-scale laboratory bioreactor." Journal of Applied Phycology **3**: 277-282.

Asayama, M., Kabasawa, M., Takahashi, I., Aida, T. and Shirai, M. (1996). "Highly repetitive sequences and characteristics of genomic DNA in unicellular cyanobacterial strains." FEMS Microbiology Letters **137**: 175-181.

Ashcroft, A. E. (2004). An Introduction to Mass Spectrometry. Leeds, The University of Leeds. **2007**.

Asker, D. and Ohta, Y. (2002). "Production of canthaxanthin by *Haloferax alexandrinus* under non-aseptic conditions and a simple, rapid method for its extraction." Applied Microbiological Biotechnology **58**: 743–750.

Bailey, P. S. J. and Bailey, C. A. (1995). Organic Chemistry. A brief survey of concepts and applications. New Jersey, Prentice Hall.

Baker, J. A., Entsch, B., Neilan, B. A. and McKay, D. B. (2002). "Monitoring Changing Toxigenicity of a Cyanobacterial Bloom by Molecular Methods." Applied and Environmental Microbiology **68**(12): 6070–6076.

Baker, J. A., Neilan, B. A., Entsch, B. and McKay, D. B. (2001). "Identification of Cyanobacteria and their Toxigenicity in Environmental Samples by Rapid Molecular Analysis." Environmental Toxicology **16**: 472–482.

Baker, P. D. and Humpage, A. R. (1994). "Toxicity Associated with Commonly Occurring Cyanobacteria in Surface Waters of the Murray-Darling Basin, Australia." Australian Journal of Marine and Freshwater Research **45**: 773-786.

Ballantine, D. L., Gerwick, W. H., Velez, S. M., Alexander, E. and Guevara, P. (1987). "Antibiotic activity of lipid-soluble extracts from Caribbean marine algae." Hydrobiologia **151/152**: 463-469.

Bansemir, A., Just, N., Michalik, M., Lindequist, U. and Lalk, M. (2004). "Extracts and Sesquiterpene Derivatives from the Red Alga *Laurencia chondriolides* with Antibacterial Activity against Fish and Human Pathogenic Bacteria." Chemistry and Biodiversity **1**: 463-467.

Baptista, M. S. and Vasconcelos, M. T. (2006). "Cyanobacteria Metal Interactions: Requirements, Toxicity, and Ecological Implications." Critical Reviews in Microbiology **32**: 127–137.

Barbarino, E. and Lourenco, S. O. (2005). "An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae." Journal of Applied Phycology **17**: 447–460.

Barclay, W. R., Meager, K. M. and Abril, J. R. (1994). "Heterotrophic production of long chain omega-3 fatty acid utilising algae and algae-like microorganisms." Journal of Applied Phycology **6**: 123-129.

Basch, H. and Gadebusch, H. H. (1968). "*In vitro* Antimicrobial Activity of Dimethylsulfoxide." Applied Microbiology **16**(12): 1953-1954.

Becker, J. E., Moore, R. E. and Moore, B. S. (2004). "Cloning, sequencing and biochemical characterisation of the nostocyclopeptide biosynthesis gene cluster: molecular basis for imine macrocyclization." Gene (Amsterdam) **325**: 35-42.

Becker, W. (2004). Microalgae in Human and Animal Nutrition. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Richmond, A. Oxford, Blackwell Science Ltd**:** 312-351.

Benedetti, S., Benvenuti, F., Pagliarani, S., Francogli, S., Scoglio, S. and Canestrari, F. (2004). "Antioxidant properties of a novel phycocyanin extract from the blue-green alga *Aphanizomenon flos-aquae*." Life Sciences **75**: 2353-2362.

Benemann, J. R. (1992). "Microalgae aquaculture feeds." Journal of Applied Phycology **4**: 233-245.

Bennamara, A., Abourriche, A., Berrada, M., Charrouf, M., Chaib, N., Boudouma, M. and Garneau, F. X. (1999). "Methoxybifurcarenone: an antifungal and antibacterial meroditerpenoid from the brown alga *Cystoseira tamariscifolia*." Phytochemistry **52**: 37-40.

Beresovsky, D., Hadas, O., Livne, A., Sukenik, A., Kaplan, A. and Carmeli, S. (2006). "Toxins and Biologically Active Secondary Metabolites of *Microcystis sp*. isolated from Lake Kinneret." Israel Journal of Chemistry **46**: 79–87.

Biotium (2007). Product Data Sheet: GelRed™ Nucleic Acid Gel Stain, 3X in H_2O, Biotium**:** 1-6.

Birk, I. M., Dierstein, R., Kaiser, I., Matern, U., Konig, W. A., Krebber, R. and Weckesser, J. (1989). "Nontoxic and toxic oligopeptides with D-amino acids and unusual residues in *Microcystis aeruginosa* PCC 7806." Archives of Microbiology **151**: 411-415.

Bister, B., Keller, S., Baumann, H. I., Nicholson, G., Weist, S., Jung, G., Sussmuth, R. D. and Juttner, F. (2004). "Cyanopeptolin 963A, a Chymotrypsin Inhibitor of *Microcystis* PCC 7806." Journal of Natural Products **67**: 1755-1757.

Bloor, S. and England, R. R. (1989). "Antibiotic production by the cyanobacterium *Nostoc muscorum*." Journal of Applied Phycology **1**: 367-372.

Bokesch, H. R., O'Keefe, B. R., McKee, T. C., Pannell, L. K., Patterson, G. M. L., Gardella, R. S., Sowder, R. C., Turpin, J., Watson, K., Buckheit Jr., R. W. and Boyd, M. R. (2003). "A Potent Novel Anti-HIV Protein from the Cultured Cyanobacterium *Scytonema varium*." Biochemistry **42**: 2578-2584.

Bolch, C. J. S. and Blackburn, S. I. (1996). "Isolation and purification of Australian isolates of the toxic cyanobacterium *Microcystis aeruginosa* Kutz." Journal of Applied Phycology **8**: 5-13.

Bolch, C. J. S., Orr, P. T., Jones, G. J. and Blackburn, S. I. (1999). "Genetic, morphological and toxicological variation among globally distributed strains of *Nodularia* (Cyanobacteria)." Journal of Phycology **35**: 339-355.

Bold, H. C. and Wynne, M. J. (1985). Introduction to the Algae: Structure and Reproduction. New Jersey, Prentice Hall Inc.

Börner, T. and Dittmann, E. (2005). Molecular Biology of Cyanobacterial Toxins: *Genetic basis of microcystin production*. Harmful Cyanobacteria. Huisman, J., Matthijs, H. C. P. and Visser, P. M. Dordrecht, The Netherlands, Springer. **3:** 25-40.

Borowitzka, M. A. (1988). Vitamins and fine chemicals from microalgae. Micro-algal biotechnology. Borowitzka, M. A. and Borowitzka, L. J. Cambridge, Cambridge University Press**:** 153-196.

Borowitzka, M. A. (1992). "Algal biotechnology products and processes - matching science and economics." Journal of Applied Phycology **4**: 267-279.

Borowitzka, M. A. (1995). "Microalgae as sources of pharmaceuticals and other biologically active compounds." Journal of Applied Phycology **7**: 3-15.

Borowitzka, M. A. (1997). "Microalgae for aquaculture: Opportunities and constraints." Journal of Applied Phycology **9**: 393–401.

Borowitzka, M. A. (1999). Pharmaceuticals and agrochemicals from microalgae. Chemicals from Microalgae. Cohen, Z. London, Taylor & Francis**:** 313–352.

Botes, D., Tuinman, A., Wessels, P., Viljoen, C., Kruger, H., Williams, D., Santikarn, S., Smith, R. and Hammond, S. (1984). "The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*." Journal of the Chemistry Society Perkins Transactions **1**: 2311-2318.

Botes, D., Wessels, P., Kruger, H., Runnegar, M., Santikarn, S., Smith, R., Barna, J. and Williams, D. (1985). "Structural studies on cyanoginosins-LR, -YR, -YA and -YM, peptide toxins from *Microcystis aeruginosa*." Journal of the Chemical Society Perkin Transactions **1**: 2747-2748.

Boyd, M. R., Gustafson, K. R., McMahon, J. B., Shoemaker, R. H., O'Keefe, B. R., Mori, T., Gulakowski, R. J., Wu, L., Rivera, M. I., Laurencot, C. M., Currens, M. J., Cardellina II, J. H., Buckheit JR., R. W., Nara, P. L., Pannell, L. K., Sowder II, R. C. and Henderson, L. E. (1997). "Discovery of Cyanovirin-N, a Novel Human Immunodeficiency Virus-Inactivating Protein That Binds Viral Surface Envelope

Glycoprotein gp120: Potential Applications to Microbicide Development." Antimicrobial Agents and Chemotherapy **41**(7): 1521–1530.

Brock, T. D., Madigan, M. T., Martinko, J. M. and Parker, J. (1997). Biology of Microorganisms. New Jersey, Prentice Hall International Inc.

Brown, M. R., Mular, M., Miller, I., Farmer, C. and Trenerry, C. (1999). "The vitamin content of microalgae used in aquaculture." Journal of Applied Phycology **11**: 247–255.

Buikema, W. J. and Haselkorn, R. (1991). "Isolation and Complementation of Nitrogen Fixation Mutants of the Cyanobacterium *Anabaena* sp. Strain PCC 7120." Journal of Bacteriology **173**(6): 1879-1885.

Burgess, J. G., Jordan, E. M., Bregu, M., Mearns-Spragg, A. and Boyd, K. G. (1999). "Microbial antagonism: a negleced avenue of natural products research." Journal of Biotechnology **70**: 27-32.

Burjà, A. M., Abou-Mansour, E., Banaigs, B., Payri, C., Burgess, J. G. and Wright, P. C. (2002). "Culture of the marine cyanobacterium, *Lyngbya majuscula* (Oscillatoriaceae) for bioprocess intensified production of cyclic and linear lipopeptides." Journal of Microbiological Methods **48**: 207-219.

Burjà, A. M., Banaigs, B., Abou-Mansour, E., Burgess, J. G. and Wright, P. C. (2001). "Marine Cyanobacteria - a prolific source of natural products." Tetrahedron **57**: 9347-9377.

Cannell, R. J. P., Owsianka, A. M. and Walker, J. M. (1988). "Results of a Large-Scale Screening Programme to Detect Antibacterial Activity from Freshwater Algae." British Phycological Journal **23**: 41-44.

Canter-Lund, H. and Lund, J. W. G. (1995). Freshwater Algae: Their microscopic world explored. Bristol, Biopress Ltd.

Carmichael, W. W. (1986). "Algal Toxins." Advances in Botanical Research **6**: 47-101.

Carmichael, W. W. (1992). "Cyanobacteria secondary metabolites - the cyanotoxins." Journal of Applied Bacteriology **72**: 445-459.

Carr, N. G. and Whitton, B. A., Eds. (1982). The Biology of Cyanobacteria. Botanical monographs. Oxford, Blackwell Scientific.

Castenholz, R. W. (1988). Culturing methods for cyanobacteria. Methods in Enzymology: Cyanobacteria. Packer, L. and Glazer, A. N. San Diego, Academic Press. **167:** 68-93.

Chen, F., Li, H.-B., Wong, R. N.-S., Ji, B. and Jiang, Y. (2005). "Isolation and purification of the bioactive carotenoid zeaxanthin from the microalga *Microcystis aeruginosa* by high-speed counter-current chromatography." Journal of Chromatography A **1064**: 183-186.

Codd, G. A., Lindsay, J., Young, F. M., Morrison, L. F. and Metcalf, J. S. (2005). Harmful Cyanobacteria: *From mass mortalities to management measures*. Harmful Cyanobacteria. Huisman, J., Matthijs, H. C. P. and Visser, P. M. Dordrecht, The Netherlands, Springer. **3:** 1-23.

Cohen, Z. (1999). Chemicals from Microalgae. London, Taylor and Francis.

Court, G. J., Kycia, J. H. and Siegelman, H. W. (1981). Collection, Purification and Culture of Cyanobacteria. The Water Environment: Algal toxins and health. Carmichael, W. W. New York, Plenum Press. **20:** 173-183.

Cuvin-Aralar, M. L., Fastner, J., Focken, U., Becker, K. and Aralar, E. V. (2002). "Microcystins in Natural Blooms and Laboratory Cultured *Microcystis aeruginosa* from Laguna de Bay, Philippines." Systematic and Applied Microbiology **25**: 179–182.

Cyanosite (1997). *Microcystis aeruginosa* 100x. Lafayette, Indiana, Department of Biological Sciences at Purdue University. **2007**.

Dagnino, D. and Schripsema, J. (2005). "^{1}H NMR quantification in very dilute toxin solutions: application to anatoxin-a analysis." Toxicon **46**: 236–240.

Das, B. K., Pradhan, J., Pattnaik, P., Samantaray, B. R. and Samal, S. K. (2005). "Production of antibacterials from the freshwater alga *Euglena viridis* (Ehren)." World Journal of Microbiology & Biotechnology **21**: 45–50.

Day, J. G., Benson, E. E. and Fleck, R. A. (1999). "*In vitro* culture and conservation of microalgae: applications for aquaculture, biotechnology and environmental research." In Vitro Cellular & Developmental Biology Plant **35**: 127-136.

de Caire, G. Z., de Cano, M. M. S., de Mule, M. C. Z. and de Halperin, D. R. (1993). "Screening of cyanobacterial bioactive compounds against human pathogens." International Journal of Experimental Botany - Phyton **54**(1): 59-65.

de la Noüe, J., Laliberté, G. and Proulx, D. (1992). "Algae and waste water." Journal of Applied Phycology **4**: 247-254.

Dignum, M., Matthijs, H. C. P., Pel, R., Laanbroek, H. J. and Mur, L. R. (2005). Nutrient Limitation of Freshwater Cyanobacteria: *Tools to monitor phosphorus limitation at the individual level*. Harmful Cyanobacteria. Huisman, J., Matthijs, H. C. P. and Visser, P. M. Dordrecht, The Netherlands, Springer. **3:** 65-86.

Dittmann, E. and Börner, T. (2005). "Genetic contributions to the risk assessment of microcystin in the environment." Toxicology and Applied Pharmacology **203**: 192–200.

Dittmann, E., Neilan, B. A. and Börner, T. (2001). "Molecular biology of peptide and polyketide biosynthesis in cyanobacteria." Applied Microbiology and Biotechnology **57**: 467–473.

Dittmann, E., Neilan, B. A., Erhard, M., von Döhren, H. and Börner, T. (1997). "Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806." Molecular Microbiology **26**(4): 779-787.

Dittmann, E. and Wiegand, C. (2006). "Cyanobacterial toxins – occurrence, biosynthesis and impact on human affairs." Molecular Nutrition and Food Research **50**: 7 – 17.

Dokulil, M. T. and Teubner, K. (2000). "Cyanobacterial dominance in lakes." Hydrobiologia **438**: 1–12.

Eloff, J. N. (1981). Autecological studies on *Microcystis*. The Water Environment: Algal Toxins and Health. Carmichael, W. W. New York, Plenum Press. **20:** 71-96.

Environment-ACT (2003). What are blue-green algae?, ACT Urban Services. **2004**.

Etchegaray, A., Rabello, E., Dieckmann, R., Moon, D. H., Fiore, M. F., von Döhren, H., Tsai, S. M. and Neilan, B. A. (2004). "Algicide production by the filamentous cyanobacterium *Fischerella* sp. CENA 19." Journal of Applied Phycology **16**: 237–243.

Falconer, I. R. and Humpage, A. R. (1996). "Tumour promotion by cyanobacterial toxins." Phycologia **35**: 75-79.

Fastner, J., Erhard, M. and von Dohren, H. (2001). "Determination of Oligopeptide Diversity within a Natural Population of *Microcystis* spp. (Cyanobacteria) by Typing Single Colonies by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry." Applied and Environmental Microbiology **67**(11): 5069–5076.

Fastner, J., Flieger, I. and Neumann, U. (1998). "Optimised Extraction of Microcystins from Field Samples - A Comparison of Different Solvents and Procedures." Water Research **32**(10): 3177-3181.

Ferrao-Filho, A. S., Azevedo, S. M. F. O. and DeMott, W. R. (2000). "Effects of toxic and non-toxic cyanobacteria on the life history of tropical and temperate cladocerans." Freshwater Biology **45**: 1–19.

Ferris, M. J. and Hirsch, C. F. (1991). "Method for Isolation and Purification of Cyanobacteria." Applied and Environmental Microbiology **57**(5): 1448-1452.

Fiore, M. F., Moon, D. H., Tsai, S. M., Lee, H. and Trevors, J. T. (2000). "Miniprep DNA isolation from unicellular and filamentous cyanobacteria." Journal of Microbiological Methods **39**: 159–169.

Fiore, M. F. and Trevors, J. T. (1994). "Cell composition and metal tolerance in cyanobacteria." BioMetals **7**: 83-103.

Fistarol, G. O., Legrand, C. and Granéli, E. (2005). "Allelopathic effect on a nutrient-limited phytoplankton species." Aquatic Microbial Ecology **41**: 153–161.

Fogg, G. E. (1973). The Blue-green algae. London; New York, Academic Press.

Freile-Pelegrin, Y. and Morales, J. L. (2004). "Antibacterial activity in marine algae from the coast of Yucatan, Mexico." Botanica Marina **47**: 140–146.

Frias, H. V., Mendes, M. A., Cardozo, K. H. M., Carvalho, V. M., Tomazela, D., Colepicolo, P. and Pinto, E. (2006). "Use of electrospray tandem mass spectrometry for identification of microcystins during a cyanobacterial bloom event." Biochemical and Biophysical Research Communications **344**: 741–746.

Fujii, K., Sivonen, K., Naganawa, E. and Harada, K.-i. (2000). "Non-Toxic Peptides from Toxic Cyanobacteria, *Oscillatoria agardhii*." Tetrahedron **56**: 725–733.

Fujii, K., Sivonen, K., Nakano, T. and Harada, K.-i. (2002). "Structural elucidation of cyanobacterial peptides encoded by peptide synthetase gene in *Anabaena* species." Tetrahedron **58**: 6863–6871.

Gale, R. J. B., Gale, S. J. and Winchester, H. P. M. (2006). "Inorganic pollution of the sediments of the River Torrens, South Australia." Environmental Geology (Berlin) **50**: 62–75.

Gantar, M., Obreht, Z. and Dalmacija, B. (1991). "Nutrient Removal and Algal Succession during the Growth of *Spirulina platensis* and *Scenedesmus quadricauda* on Swine Wastewater." Bioresource Technology **36**: 167-171.

Gerloff, G. C., Fitzgerald, G. P. and Skoog, F. (1950). "The Isolation, Purification and Culture of Blue-Green Algae." American Journal of Botany **37**(3): 216-218.

Gerwick, W. H., Roberts, M. A., Proteau, P. J. and Chen, J.-L. (1994). "Screening cultured marine microalgae for anticancer-type activity." Journal of Applied Phycology **6**: 143-149.

Ghadouani, A., Pinel-Alloul, B., Plath, K. and Codd, G. A. (2004). "Effects of *Microcystis aeruginosa* and purified microcystin-LR on the feeding behavior of *Daphnia pulicaria*." Limnology and Oceanography **49**(3): 666–679.

Gladue, R. M. and Maxey, J. E. (1994). "Microalgal feeds for aquaculture." Journal of Applied Phycology **6**: 131-141.

Google-Earth (2007). Google Earth. Google, Google. **2007**.

Gress, C. D., Treble, R. G., Matz, C. J. and Weger, H. G. (2004). "Biological availability of iron to the freshwater cyanobacterium *Anabaena flos-aquae*." Journal of Phycology **40**: 879–886.

Guillard, R. R. L. (2005). Purification Methods for Microalgae. Algal Culturing Techniques. Andersen, R. A., Elsevier Academic Press**:** 117-132.

Guven, B. and Howard, A. (2006). "A review and classification of the existing models of cyanobacteria." Progress in Physical Geography **30**(1): 1–24.

Guzzetta, A. (2001). Reverse Phase HPLC Basics for LC/MS: An IonSource Tutorial, IonSource. **2007**.

Harada, K.-i. (2004). "Production of Secondary Metabolites by Freshwater Cyanobacteria." Chemical & Pharmaceutical Bulletin (Tokyo) **52**(8): 889—899.

Haselkorn, R. (1978). "Heterocysts." Annual Review of Plant Physiology and Plant Molecular Biology **29**: 319-344.

Hoeger, S. J., Hitzfeld, B. C. and Dietricha, D. R. (2005). "Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants." Toxicology and Applied Pharmacology **203**: 231– 242.

Hoffmann, D., Hevel, J. M., Moore, R. E. and Moore, B. S. (2003). "Sequence analysis and biochemical characterization of the nostopeptolide: a biosynthetic gene cluster from *Nostoc* sp. GSV224." Gene (Amsterdam) **311**: 171-180.

Hoffmann, J. P. (1998). "Wastewater treatment with suspended and nonsuspended algae." Journal of Phycology **34**: 757–763.

Hogan, J. (1995). A Catchment Journey to Integrated Natural Resources Management. Adelaide, Torrens Catchment Water Management Board, Government of South Australia**:** 1-36.

Hostettmann, K. and Wolfender, J.-L. (2001). Applications of Liquid Chromatography/UV/MS and Liquid Chromatography/NMR for On-line Identification of Plant Metabolites. Bioactive Compounds from Natural Sources. Tringali, C. London, Taylor & Francis**:** 32-67.

Huisman, J., Jonker, R. R., Zonneveld, C. and Weissing, F. L. (1999). "Competition for Light between Phytoplankton Species: Experimental Tests of Mechanistic Theory." Ecology **80**(1): 211-222.

Ibelings, B. W. (1996). "Changes in photosynthesis in response to combined irradiance and termperature stress in cyanobacterial surface waterblooms." Journal of Phycology **32**: 549-557.

Ikawa, M., Haney, J. F. and Sasner, J. J. (1996). "Inhibition of *Chlorella* growth by the lipids of cyanobacterium *Microcystis aeruginosa*." Hydrobiologia **331**: 167-170.

Imai, A., Fukushima, T. and Matsushig, K. (1999). "Effects of iron limitation and aquatic humic substances on the growth of *Microcystis aeruginosa*." Canadian Journal of Fisheries and Aquatic Sciences **56**: 1929–1937.

Ishida, K., Matsuda, H. and Murakami, M. (1998). "Micropeptins 88-A to 88-F, Chymotrypsin Inhibitors from the Cyanobacterium *Microcystis aeruginosa* (NIES-88)." Tetrahedron **54**: 5545-5556.

Ishida, K., Matsuda, H., Murakami, M. and Yamaguchi, K. (1996). "Kawaguchipeptin A, a novel cyclic undecapeptide from cyanobacterium *Microcystis aeruginosa* (NIES-88)." Tetrahedron **52**(27): 9025-9030.

Ishida, K., Matsuda, H., Murakami, M. and Yamaguchi, K. (1997). "Kawaguchipeptin B, an Antibacterial Cyclic Undecapeptide from the Cyanobacterium *Microcystis aeruginosa*." Journal of Natural Products **60**: 724-726.

Ishida, K., Matsuda, H., Murakami, M. and Yamaguchi, K. (1997). "Microginins 299-A and -B, Leucine Aminopeptidase Inhibitors from the Cyanobacterium *Microcystis aeruginosa* (NIES-299)." Tetrahedron **53**(30): 10281-10288.

Ishida, K., Matsuda, H., Murakami, M. and Yamaguchi, K. (1997). "Micropeptins 478-A and -B, Plasmin Inhibitors from the Cyanobacterium *Microcystis aeruginosa*." Journal of Natural Products **60**: 184-187.

Ishida, K., Matsuda, H., Okita, Y. and Murakami, M. (2002). "Aeruginoguanidines 98-A–98-C: cytotoxic unusual peptides from the cyanobacterium *Microcystis aeruginosa*." Tetrahedron **58**: 7645–7652.

Ishida, K. and Murakami, M. (2000). "Kasumigamide, an Antialgal Peptide from the Cyanobacterium *Microcystis aeruginosa*." Journal of Organic Chemistry **65**: 5898-5900.

Ishida, K., Murakami, M., Matsuda, H. and Yamaguchi, K. (1995). "Micropeptin 90, a Plasmin and Trypsin Inhibitor from the Blue-Green Alga *Microcystis aeruginosa* (NIES-90)." Tetrahedron Letters **36**(20): 3535-3538.

Ishida, K., Nakagawa, H. and Murakami, M. (2000). "Microcyclamide, a Cytotoxic Cyclic Hexapeptide from the Cyanobacterium *Microcystis aeruginosa*." Journal of Natural Products **63**: 1315-1317.

Jacoby, J. M., Collier, D. C., Welch, E. B., Hardy, F. J. and Crayton, M. (2000). "Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*." Canadian Journal of Fishery and Aquatic Sciences **57**: 231-240.

Janse, I., Kardinaal, W. E. A., Meima, M., Fastner, J., Visser, P. M. and Zwart, G. (2004). "Toxic and Nontoxic Microcystis Colonies in Natural Populations Can Be Differentiated on the Basis of rRNA Gene Internal Transcribed Spacer Diversity." Applied and Environmental Microbiology **70**(7): 3979–3987.

Jiménez-Pérez, M. V., Sánchez-Castillo, P., Romera, O., Fernández-Moreno, D. and Pérez-Martinez, C. (2004). "Growth and nutrient removal in free and immobilized planktonic green algae isolated from pig manure." Enzyme and Microbial Technology **34**: 392–398.

Kaebernick, M. and Neilan, B. A. (2001). "Ecological and molecular investigations of cyanotoxin production." FEMS Microbiology Ecology **35**: 1-9.

Kardinaal, W. E. A. and Visser, P. M. (2005). Dynamics of Cyanobacterial Toxins: Sources of variability in microcystin concentrations. Harmful Cyanobacteria. Huisman, J., Matthijs, H. C. P. and Visser, P. M. Dordrecht, The Netherlands, Springer. **3:** 41-64.

Kellam, S. J. and Walker, J. M. (1989). "Antibacterial Activity from Marine Microalgae in Laboratory Culture." British Phycological Journal **24**: 191-194.

Kelly, M. G., Cazaubon, A., Coring, E., Dell'Uomo, A., Ector, L., Goldsmith, B., Guasch, H., Hurlimann, J., Jarlman, A., Kawecka, B., Kwandrans, J., Laugaste, R., Lindstrom, E.-A., Leitao, M., Marvan, P., Padisak, J., Pipp, E., Prygiel, J., Rott, E., Sabater, S., van Dam, H. and Vizinet, J. (1998). "Recommendations for the routine sampling of diatoms for water quality assessments in Europe." Journal of Applied Phycology **10**: 215-224.

Keren, N., Aurora, R. and Pakrasi, H. B. (2004). "Critical Roles of Bacterioferritins in Iron Storage and Proliferation of Cyanobacteria." Plant Physiology (Rockville) **135**: 1666–1673.

Kerr, M. K. and Churchill, G. A. (2001). "Statistical Design and the Analysis of Gene Expression Microarray Data." Journal of Computational Biology **unpublished**.

Kodani, S., Suzuki, S., Ishida, K. and Murakami, M. (1999). "Five new cyanobacterial peptides from water bloom materials of lake Teganuma (Japan)." FEMS Microbiology Letters **178**: 343-348.

Kreitlow, S., Mundt, S. and Lindequist, U. (1999). "Cyanobacteria - a potential source of new biologically active substances." Journal of Biotechnology **70**: 61-63.

Krilov, L. R. (2002). "Recent developments in the treatment and prevention of respiratory syncytial virus infection." Expert Opinion on Therapeutic Patents **12**(3): 441-449.

Krishnamurthy, T., Szafraniec, L., Hunt, D. F., Shabanowitz, J., Yates, J. R., Hauer, C., Carmichael, W. W., Skulberg, O. M., Codd, G. A. and Missler, S. (1989). "Structural characterisation of toxic cyclic peptides from blue-green algae by tandem mass spectrometry." Proceedings of National Academy of Science **86**: 770-774.

Kulik, M. M. (1995). "The potential for using cyanobacteria (blue-green algae) and alga in the biological control of plant pathogenic bacteria and fungi." European Journal of Plant Pathology **101**: 585-599.

LeBlanc, S., Pick, F. R. and Aranda-Rodriguez, R. (2005). "Allelopathic Effects of the Toxic Cyanobacterium *Microcystis aeruginosa* on Duckweed, *Lemna gibba* L." Environmental Toxicology **20**: 67–73.

Lee, S. J., Jang, M.-H., Kim, H.-S., Yoon, B.-D. and Oh, H.-M. (2000). "Variation of microcystin content of *Microcystis aeruginosa* relative to N:P ratio and growth stage." Journal of Applied Microbiology **89**: 323-329.

Lee, Y.-K. and Shen, H. (2004). Basic Culturing Techniques. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Richmond, A. Oxford, Blackwell Science**:** 40-56.

LeFlaive, J. and Ten-Hage, L. (2007). "Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins." Freshwater Biology **52**: 199–214.

Luescher-Mattli, M. (2003). "Algae, A possible source for New Drugs in the Treatment of HIV and Other Viral Diseases." Current Medical Chemistry - Anti Infective Agents **2**: 219-225.

Lurling, M. (2003). "Effects of Microcystin-Free and Microcystin-Containing Strains of the Cyanobacterium *Microcystis aeruginosa* on Growth of the Grazer *Daphnia magna*." Environmental Toxicology **18**: 202–210.

MacIntyre, H. L. and Cullen, J. J. (2005). Using Cultures to Investigate the Physiological Ecology of Microalgae. Algal Culturing Techniques. Andersen, R. A., Elsevier Academic Press**:** 287-326.

Matern, U., Oberer, L., Erhard, M., Herdman, M. and Weckesser, J. (2003). "Hofmannolin, a cyanopeptolin from *Scytonema hofmanni* PCC 7110." Phytochemistry **64**: 1061–1067.

Matsuda, H., Okino, T., Murakami, M. and Yamaguchi, K. (1996). "Aeruginosins 102-A and B, New Thrombin Inhibitors from the Cyanobacterium *Microcystis viridis* (NIES-102)." Tetrahedron **52**(46): 14501-14506.

Meißner, K., Dittmann, E. and Börner, T. (1996). "Toxic and non-toxic strains of the cyanobacterium *Microcystis aeruginosa* contain sequences homologous to peptide synthetase genes." FEMS Microbiology Letters **135**: 295-303.

Metcalf, J. S. and Codd, G. A. (2000). "Microwave oven and boiling waterbath extraction of hepatotoxins from cyanbacterial cells." FEMS Microbiology Letters **184**: 241-246.

Micro*scope (2006). *Microcystis flos-aquae*. Star sites for biologists. Massachusetts, Micro*scope. **2007**.

Mikalsen, B., Boison, G., Skulberg, O. M., Fastner, J., Davies, W., Gabrielsen, T. M., Rudi, K. and Jakobsen, K. S. (2003). "Natural Variation in the Microcystin Synthetase Operon *mcy*ABC and Impact on Microcystin Production in Microcystis Strains." Journal of Bacteriology **185**(9): 2774–2785.

Mo-Bio (2005). UltraClean Soil DNA Isolation Kit. Carlsbad, Mo Bio Laboratories, Inc**:** 1-8.

Monaghan, R. L. and Tkacz, J. S. (1990). "Bioactive Microbial Products: Focus upon mechanism of action." Annual Review Microbiology **44**: 271-301.

Moore, A. (2001). "Blooming prospects?" European Molecular Biology Organization **2**(6): 462-464.

Moore, R. E. (1996). "Cyclic peptides and depsipeptides from cyanobacteria: a review." Journal of Industrial Microbiology **16**: 134-143.

Morrison, L. F., Parkin, G. and Codd, G. A. (2006). "Optimization of anabaenopeptin extraction from cyanobacteria and the effect of methanol on laboratory manipulation." peptides **27**: 10–17.

Morton, S. L. and Bomber, J. W. (1994). "Maximizing okadaic acid content from *Prorocentrum hoffmannianum* Faust." Journal of Applied Phycology **6**: 41-44.

Mugavin, D. (2004). "Adelaide's greenway: River Torrens Linear Park." Landscape and Urban Planning **68**: 223–240.

Muller-Feuga, A. (2000). "The role of microalgae in aquaculture: situation and trends." Journal of Applied Phycology **12**: 527-534.

Mundt, S., Kreitlow, S. and Jansen, R. (2003). "Fatty acids with antibacterial activity from the cyanobacterium *Oscillatoria redekei* HUB 051." Journal of Applied Phycology **15**: 263–267.

Mundt, S., Kreitlow, S., Nowotny, A. and Effmert, U. (2001). "Biochemical and pharmacological investigations of selected cyanobacteria." International Journal of Hygiene and Environmental Health **203**: 327-334.

Mur, L. R., Skulberg, O. M. and Utkilen, H. (1999). Cyanobacteria in the Environment. Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management, World Health Organisation.

Murakami, M., Kodani, S., Ishida, K., Matsuda, H. and Yamaguchi, K. (1997). "Micropeptin 103, a Chymotrypsin Inhibitor from the Cyanobacterium *Microcystis viridis* (NIES-103)." Tetrahedron Letters **38**(17): 3035-3038.

Namikoshi, M. and Rinehart, K. (1996). "Bioactive compounds produced by cyanobacteria." Journal of Industrial Microbiology **17**: 373-384.

Neilan, B. A. (1995). "Identification and Phylogenetic Analysis of Toxigenic Cyanobacteria by Multiplex Randomly Amplified Polymorphic DNA PCR." Applied and Environmental Microbiology **61**(6): 2286–2291.

Neilan, B. A., Dittmann, E., Rouhainen, L., Bass, R. A., Schaub, V., Sivonen, K. and Börner, T. (1999). "Nonribosomal Peptide Synthesis and Toxigenicity of Cyanobacteria." Journal of Bacteriology **181**(13): 4089–4097.

Neri, P. and Tringali, C. (2001). Applications of Modern NMR Techniques in the Structural Elucidation of Bioactive Natural Products. Bioactive Compounds from Natural Sources: Isolation, characterisation and biological properties. Tringali, C. London, Taylor and Francis**:** 69-128.

Nishizawa, T., Asayama, M., Fujii, K., Harada, K.-i. and Shirai, M. (1999). "Genetic Analysis of the Peptide Synthetase Genes for a Cyclic Heptapeptide Microcystin in *Microcystis* spp." Journal of Biochemistry (Tokyo) **126**: 520-529.

Nixon, S. W. (1995). "Coastal Marine Eutrophication: A Definition, Social Causes and Future Concerns." Ophelia **41**: 199-219.

Nowotny, A., Mentel, R., Wegner, U., Mundt, S. and Lindequist, U. (1997). "Antiviral Activity of an Aqueous Extract of the Cyanobacterium *Microcystis aeruginosa*." Phytotherapy Research **2**: 93-96.

Nübel, U., Garcia-Pichel, F. and Muyzer, G. (1997). "PCR Primers To Amplify 16S rRNA Genes from Cyanobacteria." Applied and Environmental Microbiology **63**(8): 3327–3332.

Ohta, S., Ono, F., Shiomi, Y., Nakao, T., Aozasa, O., Nagate, T., Kitamura, K., Yamaguchi, S., Nishi, M. and Miyata, H. (1998). "Anti-Herpes Simplex Virus substances produced by the green alga *Dunaliella primolecta*." Journal of Applied Phycology **10**: 349-355.

Ohtake, A., Shirai, M., Aida, T., Mori, N., Harada, K.-I., Matsuura, K., Suzuki, M. and Nakano, M. (1989). "Toxicity of *Microcystis* Species Isolated from Natural Blooms and Purification of the Toxin." Applied and Environmental Microbiology **55**(12): 3202-3207.

Olaizolá, M. (2003). "Commercial development of microalgal biotechnology: from the test tube to the marketplace." Biomolecular Engineering **20**: 459-466.

Ördög , V., Stirk, W. A., Lenobel, R., Bancírová , M., Strnad, M., van Staden, J., Szigeti, J. and Németh, L. (2004). "Screening microalgae for some potentially useful

agricultural and pharmaceutical secondary metabolites." Journal of Applied Phycology **16**: 309-314.

Ostensvik, O., Skulberg, O. M., Underdal, B. and Hormazabal, V. (1998). "Antibacterial properties of extracts from selected planktonic freshwater cyanobacteria - a comparative study of bacterial bioassays." Journal of Applied Microbiology **84**: 1117-1124.

Oudra, B., Loudiki, M., Vasconcelos, V., Sabour, B., Sbiyyâa, B., Oufdou, K. and Mezrioui, N. (1998). "Detection and Quantification of Microcystins from Cyanobacteria Strains Isolated from Reservoirs and Ponds in Morocco." Environmental Toxicology **17**: 32-39.

Ouellette, A. J. A. and Wilhelm, S. W. (2003). "Toxic Cyanobacteria: The Evolving Molecular Toolbox." Frontiers in Ecology and the Environment **1**(7): 359-366.

Ozdemir, G. K., N. Ulku , Dalay, M. C. and Pazarbasi, B. (2004). "Antibacterial Activity of Volatile Component and Various Extracts of *Spirulina platensis*." Phytotherapy Research **18**: 754–757.

Paczuska, L. and Kosakowska, A. (2003). "Is iron a limiting factor of *Nodularia spumigena* blooms?" Oceanologia **45**(4): 679–692.

Parker, B. C., Radmer, R. J., T, A. F. C. and Chen, H. (1994). "Microalgal Biotechnology and Commercial Applications: Introduction." Journal of Applied Phycology **6**: 91-92.

Parker, D. L. (1982). "Improved procedures for the cloning and purification of *Microcystis* cultures (Cyanophyta)." Journal of Phycology **18**: 471-477.

Patterson, G. M. L., Baker, K. K., Baldwin, C. L., Bolis, C. M., Caplan, F. R., Larsen, L. K., Levine, I. A., Moore, R. E., Nelson, C. S., Tschappat, K. D., Tuang, G. D., Boyd, M. R., Cardellina II, J. H., Collins, R. P., Gustafson, K. R., Snader, K. M., Weislow, O. S. and Lewin, R. A. (1993). "Antiviral Activity of Cultured Blue-Green Algae (Cyanophyta)." Journal of Phycology **29**: 125-130.

Patterson, G. M. L., Baldwin, C. L., Bolis, C. M., Caplan, F. R., Karuso, H., Larsen, L. K., Levine, I. A., Moore, R. E., Nelson, C. S., Tschappat, K. D., Tuang, G. D., Raybourne, R. B., Furusawa, E., Furusawa, S. and Norton, T. A. (1991). "Antineoplastic Activity of Cultured Blue-Green Algae (Cyanophyta)." Journal of Phycology **27**: 530-536.

Patterson, G. M. L. and Bolis, C. M. (1997). "Fungal cell wall polysaccharides elicit an antifungal secondary metabolite (phytoalexin) in the cyanobacterium *Scytonema ocellatum*." Journal of Phycology **33**: 54-60.

Patterson, G. M. L., Larsen, L. K. and Moore, R. E. (1994). "Bioactive natural products from blue-green algae." Journal of Applied Phycology **6**: 151-157.

Pergament, I. and Carmeli, S. (1994). "Schizotrin A; a Novel Antimicrobial Cyclic Peptide from a Cyanobacterium." Tetrahedron Letters **35**(45): 8473-8476.

Phoolcharoen, W. and Smith, D. R. (2004). "Internalization of the Dengue Virus is Cell Cycle Modulated in HepG2, But Not Vero Cells." Journal of Medical Virology **74**: 434–441.

Prescott, L. M. (1999). Bacteria: The Deinococci and Nonproteobacteria Gram Negatives. Microbiology. Prescott, L. M., Harley, J. P. and Klein, D. A. New York, McGraw Hill**:** 438-457.

Prescott, L. M. (1999). Metabolism: The Generation of Energy. Microbiology. Prescott, L. M., Harley, J. P. and Klein, D. A. New York, McGraw Hill**:** 163-189.

Prescott, L. M. (1999). Metabolism: The Use of Energy in Biosynthesis. Microbiology. Prescott, L. M., Harley, J. P. and Klein, D. A. New York, McGraw Hill**:** 190-210.

Prescott, L. M. (1999). Prokaryotic Cell Structure and Function. Microbiology. Prescott, L. M., Harley, J. P. and Klein, D. A. New York, McGraw Hill**:** 36-72.

Pulz, O. and Gross, W. (2004). "Valuable products from biotechnology of microalgae." Applied Microbiological Biotechnololgy **65**: 635-648.

QIAGEN (2001). QIAamp DNA Stool Mini Kit Handbook: For DNA purificaiton from stool samples. QIAGEN. Clifton Hill, QIAGEN Worldwide**:** 1-40.

Radmer, R. J. and Parker, B. C. (1994). "Commercial applications of algae: opportunities and constraints." Journal of Applied Phycology **6**: 93-98.

Rasmussen, J. P., Barbez, P. H., Burgoyne, L. A. and Saint, C. P. (unpublished). Rapid Preparation of Cyanobacterial DNA for real-time PCR Analysis.

Ray, S. and Bagchi, S. N. (2001). "Nutrients and pH regulate algicide accumulation in cultures of the cyanobacterium *Oscillatoria laetevirens*." New Phytologist **149**: 455-460.

REDNOVA (2003). Allelopathy in phytoplankton - biochemical, ecological and evolutionary aspects, REDNOVA News. **2005**.

Regan, D. L. (1988). Other Microalgae. Micro-algal Biotechnology. Borowitzka, M. A. and Borowitzka, L. J. Cambridge, Cambridge University Press**:** 135-150.

Reshef, V. and Carmeli, S. (2006). "New microviridins from a water bloom of the cyanobacterium *Microcystis aeruginosa*." Tetrahedron **62**: 7361–7369.

Reynolds, C. S. (1984). The Ecology of Freshwater Phytoplankton. Cambridge, Cambridge University Press.

Reynolds, C. S. (1997). Vegetation Processes in the Pelagic: a Model for Ecosystem Theory. Oldendorf/Luhe, International Ecology Institute.

Richlen, M. L. and Barber, P. H. (2005). "A technique for the rapid extraction of microalgal DNA from single live and preserved cells." Molecular Ecology Notes **5**: 688-691.

Rinehart, K. l., Namikoshi, M. and Choi, B. W. (1994). "Structure and biosynthesis of toxins from blue-green algae (cyanobacteria)." Journal of Applied Phycology **6**: 159-176.

Rippka, R. (1988). "Isolation and Purification of Cyanobacteria." Methods in Enzymology **167**: 3-27.

Robles-Centeno, P. O., Ballantine, D. L. and Gerwick, W. H. (1996). "Dynamics of antibacterial activity in three species of Carribean marine algae as a function of habitat and life history." Hydrobiologia **326/327**: 457-462.

Rouhiainen, L., Paulin, L., Suomalainen, S., Hyytiainen, H., Buikema, W., Haselkorn, R. and Sivonen, K. (2000). "Genes encoding synthetases of cyclic depsipeptides, anabaenopeptilides, in *Anabaena* strain 90." Molecular Microbiology **37**(1): 156-167.

Safonova, E. and Reisser, W. (2005). "Growth promoting and inhibiting effects of extracellular substances of soil microalgae and cyanobacteria on *Escherichia coli* and *Micrococcus luteus*." Phycological Research **53**: 189–193.

Saker, M. L., Fastner, J., Dittmann, E., Christiansen, G. and Vasconcelos, V. M. (2005). "Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river." Journal of Applied Microbiology **99**: 749–757.

Saker, M. L., Jungblut, A.-D., Neilan, B. A., Rawn, D. F. K. and Vasconcelos, V. M. (2005). "Detection of microcystin synthetase genes in health food supplements containing the freshwater cyanobacterium *Aphanizomenon flos-aquae*." Toxicon **46**: 555–562.

Sandsdalen, E., Haug, T., Stensvåg, K. and Styrvold, O. B. (2003). "The antibacterial effect of a polyhydroxylated fucophlorethol from the marine brown alga, *Fucus vesiculosus*." World Journal of Microbiology & Biotechnology **19**: 777-782.

Sasson, A. (1991). "Production of useful biochemicals by higher-plant cell cultures: biotechnological and economic aspects." Options Méditerranéennes - Série Séminaires **14**: 59-74.

Schaeffer, D. J. and Krylov, V. S. (2000). "Anti-HIV Activity of Extracts and Compounds from Algae and Cyanobacteria." Ecotoxicology and Environmental Safety **45**: 208-227.

Schepetiuk, S. K. and Kok, T. (1993). "The use of MDCK, MEK and LLC-MK2 cell lines with enzyme immunoassay for the isolation of influenza and parainfluenza viruses from clinical specimens." Journal of Virological Methods **42**: 241-250.

Schlegel, I., Doan, N. T., de Chazal, N. and Smith, G. D. (1999). "Antibiotic activity of new cyanobacterial isolates from Australia and Asia against green algae and cyanobacteria." Journal of Applied Phycology **10**: 471–479.

Schmidt, N. and Emmons, R. (1989). General principles of laboratory diagnostic methods for viral, rickettsial and chlamydial infections. Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections. Lennette, E. H. and Schmidt, N. J. Washington DC, American Public Health Association**:** 1-36.

Schwartz, R. E., Hirsch, C. F., Sesin, D. F., Flor, J. E., Chartrain, M., Fromtling, R. E., Harris, G. H., Salvatore, M. J., Liesch, J. M. and Yudin, K. (1990). "Pharmaceuticals from cultured algae." Journal of Industrial Microbiology **5**: 113-I24.

Shafee, N. and AbuBakar, S. (2002). "Zinc accelerates dengue virus type 2-induced apoptosis in Vero cells." FEBS Letters **524**: 20-24.

Shamsudin, L. (1992). "Lipid and fatty acid composition of microalgae used in Malaysian aquaculture as live food for the early stage of penaeid larvae." Journal of Applied Phycology **4**: 371-378.

Shimizu, Y. (1996). "Microalgal Metabolites: A New Perspective." Annual Review Microbiology **50**: 431-465.

Shirai, M. (1989). "Development of a solid medium for growth and isolation of axenic *Microcystis* strains (cyanobacteria)." Applied and Environmental Microbiology **55**(10): 2569-2571.

Shirai, M., Ohtake, A., Sano, T., Matsumoto, S., Sakamoto, T., Sato, A., Aida, T., Harada, K.-i., Shimada, T., Suzuki, M. and Nakano, M. (1991). "Toxicity and Toxins of Natural Blooms and Isolated Strains of *Microcystis* spp. (Cyanobacteria) and Improved Procedure for Purification of Cultures." Applied and Environmental Microbiology **57**(4): 1241-1245.

Shirai M., M. K., *et al*, (1989). "Development of a solid medium for growth and isolation of axenic *Microcystis* strains (cynaobacteria)." Applied and Environmental Microbiology **55**(10): 2569-2571.

Shukla, B. and Rai, L. C. (2007). "Potassium-induced inhibition of nitrogen and phosphorus metabolism as a strategy of controlling *Microcystis* blooms." World Journal of Microbiology & Biotechnology **23**: 317–322.

Singh, S., Pradhan, S. and Rai, L. C. (1998). "Comparative assessment of Fe^{3+} and Cu^{2+} biosorption by field and laboratory-grown *Microcystis*." Process Biochemistry **33**(5): 495-504.

Sinoven, K. and Jones, G. J. (1999). Cyanobacterial Toxins. Toxic Cyanobacteria in Water. Chorus, I. and Bartram, J. London, E & FN Spon**:** 41-111.

Skulberg, O. M. (2000). "Microalgae as a source of bioactive molecules - experience from cyanophyte research." Journal of Applied Phycology **12**: 341-348.

Skulberg, O. M. (2004). Bioactive Chemicals in Microalgae. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Richmond, A. Iowa, Blackwell Science Ltd**:** 485-512.

Smith, C. D., Craft, D. W., Shiromoto, R. S. and Yan, P. O. (1986). "Alternative Cell Line for Virus Isolation." Journal of Clinical Microbiology **24**(2): 265-268.

Smith, G. D. and Doan, N. T. (1999). "Cyanobacterial metabolites with bioactivity against photosynthesis in cyanobacteria, algae and higher plants." Journal of Applied Phycology **11**: 337–344.

South, G. R. and Whittick, A. (1987). Algae, human affairs and environment**:** 263-278.

Spoof, L., Vesterkvist, P., Lindholm, T. and Meriluoto, J. (2003). "Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatography–electrospray ionisation mass spectrometry." Journal of Chromatography A **1020**: 105–119.

Stackebrandt, E. and Goodfellow, M. (1991). Nucleic acid techniques in bacterial systematics. New York, Wiley.

Stanier, R. Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. (1971). "Purification and Properties of Unicellular Blue-Green Algae (Order *Chrococcales*)." Microbiology and Molecular Biology Reviews **35**(2): 171-205.

Stephenson, J. R. (2005). "Understanding dengue pathogenesis: implications for vaccine design." Bulletin of the World Health Organization **83**(4): 308-314.

Tan, C. K. and Johns, M. R. (1996). "Screening of diatoms for heterotrophic eicosapentaenoic acid production." Journal of Applied Phycology **8**: 59-64.

Thajuddin, N. and Subramanian, G. (2005). "Cyanobacterial biodiversity and potential applications in biotechnology." Current Science (Bangalore) **89**(1): 47-57.

Tillett, D., Dittmann, E., Erhard, M., Börner, T. and Neilan, B. A. (2000). "Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system." Chemistry & Biology (London) **7**: 753-764.

Tooming-Klunderud, A., Rohrlack, T., Shalchian-Tabrizi, K., Kristensen, T. and akobsen, K. S. J. (2007). "Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from *Microcystis*: implications for evolution of cyanopeptolins." Microbiology **153**: 1382–1393.

University of Kentucky (2004). High Performance Liquid Chromatography (HPLC): A User's Guide. Kentucky, The University of Kentucky. **2004:** Online guide to HPLC operation.

Vairappan, C. S., Kawamoto, T., Miwa, H. and Suzuki, M. (2004). "Potent Antibacterial Activity of halogenated Compounds against Antibiotic resistant bacteria." Planta Medica **70**: 1087-1090.

van der Westhuizen, A. J. and Eloff, J. N. (1985). "Effect of temperature and light on the toxicity and growth of the blue-green alga *Microcystis aeruginosa* (UV-006)." Planta **163**: 55-59.

Vezie, C., Brient, L., Sivonen, K., Bertru, G., Lefeuvre, J.-C. and Salkinoja-Salonen, M. (1998). "Variation of Microcystin Content of Cyanobacterial Blooms and Isolated Strains in Lake Grand-Lieu (France)." Microbial Ecology **35**: 126–135.

Vezie, C., Rapala, J., Vaitomaa, J., Seitsonen, J. and Sinoven, K. (2002). "Effect of Nitrogen and Phosphorus on Growth of Toxic and Nontoxic *Microcystis* strains and on Intracellular Microcystin concentrations." Microbial Ecology **43**: 443-454.

Visser, P. M., Ibelings, B. W., Mur, L. R. and Walsby, A. E. (2005). The Ecophysiology of the Harmful Cyanobacterium *Microcystis*: *Features explaining its success and measures for its control*. Harmful Cyanobacteria. Huisman, J., Matthijs, H. C. P. and Visser, P. M. Dordrecht, The Netherlands, Springer. **3:** 109-142.

Vlietinck, A. J. and Apers, S. (2001). Biological Screening Methods in the Search for Pharmacologically Active Natural Products. Bioactive Compounds from Natural Sources: Isolation, characterisation and biological properties. Tringali, C. London, Taylor and Francis**:** 1-30.

Voet, D. and Voet, J. G. (2004). Biochemistry. New York, J. Wiley & Sons.

Volk, R.-B. and Furkert, F. H. (2006). "Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth." Microbiological Research **161**: 180—186.

Voloshko, L., Kopecky, J., Safronova, T., Pljusch, A., Titova, N., Hrouzek, P. and Drabkova, V. (2008). "Toxins and other bioactive compounds produced by cyanobacteria in Lake Ladoga." Estonian Journal of Ecology **57**(2): 100-110.

Vonshak, A. and Torzillo, G. (2004). Environmental Stress Physiology. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Richmond, A. Oxford, Blackwell Science Ltd**:** 57-83.

Walker, T. L., Collet, C. and Purton, S. (2005). "Algal Transgenics in the Genomic Era." Journal of Phycology **41**: 1077–1093.

Wang, Q., Deeds, J. R., Place, A. R. and Belas, R. (2005). "Dinoflagellate community analysis of a fish kill using denaturing gradient gel electrophoresis." Harmful Algae **4**: 151-162.

Ward, J. E. and Targett, N. M. (1989). "Influence of marine microalgal metabolites on the feeding behaviour of the blue mussel *Mytilus edulis*." Marine Biology **101**: 313-321.

Watanabe, M. F., Harada, K.-I., Matsuura, K., Watanabe, M. and Suzuki, M. (1989). "Heptapeptide toxin production during the batch culture of two *Microcystis* species (Cyanobacteria)." Journal of Applied Phycology **1**: 161-165.

Watanabe, M. M. (2005). Freshwater Culture Media. Algal Culturing Techniques. Andersen, R. A., Elsevier Academic Press**:** 13-20.

Waters (2007). High Performance Liquid Chromatography (HPLC) Primer, Waters. **2007**.

Waters (2007). Mass Spectrometry (MS) Primer. **2007**.

Welker, M., Brunke, M., Preussel, K., Lippert, I. and von Dohren, H. (2004). "Diversity and distribution of *Microcystis* (Cyanobacteria) oligopeptide chemotypes from natural communities studied by single-colony mass spectrometry." Microbiology **150**: 1785–1796.

Welker, M., Marsalek, B., Sejnohova, L. and von Dohren, H. (2006). "Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: Toward an understanding of metabolic diversity." peptides **27**: 2090 – 2103.

White, D. O. and Fenner, F. J. (1994). Medical Virology. San Diego, Academic Press.

Wicks, R. J. and Thiel, P. G. (1990). "Environmental Factors Affecting the Production of Peptide Toxins in Floating Scums of the Cyanobacterium *Microcystis aeruginosa* in a Hypertrophic African Reservoir." Environmental Science & Technology **24**: 1413-1418.

Wiegand, C. and Pflugmacher, S. (2005). "Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review." Toxicology and Applied Pharmacology **203**: 201– 218.

Wilson, A. E., Sarnelle, O., Neilan, B. A., Salmon, T. P., Gehringer, M. M. and Hay, M. E. (2005). "Genetic Variation of the Bloom-Forming Cyanobacterium *Microcystis aeruginosa* within and among Lakes: Implications for Harmful Algal Blooms." Applied and Environmental Microbiology **71**(10): 6126–6133.

Wilson, A. E., Wilson, W. A. and Hay, M. E. (2006). "Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium, *Microcystis aeruginosa*." Applied and Environmental Microbiology **Published online ahead of print**: 1-14.

Wolfe, G. V. (2000). "The Chemical Defense Ecology of Marine Unicellular Plankton: Constraints, Mechanisms, and Impacts." Biological Bulletin **198**: 225–244.

Woods, G. L. and Young, A. (1988). "Use of A-549 Cells in a Clinical Virology Laboratory." Journal of Clinical Microbiology **26**(5): 1026-1028.

Wu, X. Q., Zarka, A. and Boussiba, S. (2000). "A Simplified Protocol for Preparing DNA from Filamentous Cyanobacteria." Plant Molecular Biology Reporter **18**: 385–392.

Xue, L., Zhang, Y., Zhang, T., An, L. and Wang, X. (2005). "Effects of Enhanced Ultraviolet-B Radiation on Algae and Cyanobacteria." Critical Reviews in Microbiology **31**: 79–89.

Yamamoto, Y. and Nakahara, H. (2005). "Competitive dominance of the cyanobacterium *Microcystis aeruginosa* in nutrient-rich culture conditions with special reference to dissolved inorganic carbon uptake." Phycological Research **53**: 201–208.

Zainuddin, E. N., Mundt, S., Wegner, U. and Mentel, R. (2002). "Cyanobacteria a potential source of antiviral substances against influenza virus." Medical Microbiology and Immunology **191**: 181-182.

Ziemert, N., Ishida, K., Quillardet, P., Bouchier, C., Hertweck, C., Tandeau de Marsac, N. and Dittmann, E. (2008). "Microcyclamide Biosynthesis in Two Strains of *Microcystis aeruginosa*: from Structure to Genes and Vice Versa." Applied and Environmental Microbiology **74**(6): 1791–1797.