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

by

Kyleigh Jane Victory

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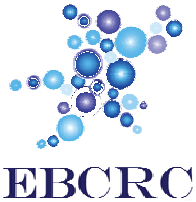
School of Chemical Engineering,

Faculty of Engineering, Computer and Mathematical Sciences

The University of Adelaide,

Australia



CHAPTER 1 INTRODUCTION

1.1 Gap Statement

The increasing emergence of drug-resistant pathogens (bacteria and viruses) has prompted research into novel treatments to counter the prevalence of persistent infections. Natural products from microalgae and cyanobacteria have long been utilised in health food supplements, to exploit synthesis of beneficial secondary compounds including ß-carotene and carotenoids that have demonstrated health enhancing properties. Further investigations into secondary metabolite products of cyanobacteria identified biologically active compounds with antimicrobial properties. However, while screening programs to identify the bioactive strains have been implemented by researchers world-wide, few have progressed to characterisation of the responsible compound and fewer still have sought to investigate the relevant genes, particularly in relation to species collected from the field.

A species of non-toxic *M. flos-aquae* (Wittr. Elenkin) was isolated from a cyanobacterial bloom in the Torrens River, a local estuary in Adelaide, South Australia and assessed for antimicrobial activity against bacterial, fungal and viral organisms. Following identification of the related bioactive properties, the compounds underwent chemical isolation and analysis to determine a likely amino acid sequence of the metabolite, which was compared to a database of known peptides to elucidate the chemical class. Investigations were also conducted to identify a relationship between the presence of genes known to control assembly of the toxin microcystin, and synthesis of non-toxic compounds.

1.2 Project Appreciation

Excessive growth of cyanobacteria, broadly referred to as “blooms”, represents a serious threat to aquatic ecosystems (Ghadouani *et al*. 2004). In freshwater lakes, the combined effects of ambient temperature fluctuations, water movement and overloading of nutrients (referred to as “eutrophication”) provide ideal conditions for disproportionate growth of selected species (Nixon 1995; Jacoby *et al.* 2000). Cyanobacteria appear to employ factors such as chemical agents that influence survival and position of other organisms, to aid in their dominance of an environment

(Ray and Bagchi 2001). Bloom formation contributes to degradation of water quality and recreational value of lakes and reservoirs (Skulberg 2000; Ghadouani *et al*. 2004; Fistarol *et al*. 2005).

Freshwater cyanobacteria are acknowledged synthesisers of biologically-active and structurally diverse secondary metabolites (Morrison *et al*. 2006; LeFlaive and Ten-Hage 2007). These compounds exhibit activity against organisms including viruses, bacteria, fungi and other algal species (Carmichael 1992; Patterson *et al*. 1994; Skulberg 2000; Mundt *et al*. 2001). Selected inhibitory agents may serve as prospective candidates for development of natural biopharmaceuticals (Gerwick *et al*. 1994; Sandsdalen *et al*. 2003; Vairappan *et al*. 2004). Currently, only a small proportion of bioactive metabolites have been chemically defined and investigated for mode of action (Skulberg 2000); for many known compounds, no biological nor physiological function has been ascribed (LeFlaive and Ten-Hage 2007). Research into these compounds has primarily focused on screening of laboratory cultures for biological activity; species from local environments are rarely analysed for pharmacological properties (Mundt *et al*. 2001), and assessment of these strains for *in vitro* biological activity is severely underexploited. These compounds are often produced in minimal quantities, creating obstacles in isolation and elucidation of the metabolites (LeFlaive and Ten-Hage 2007). Therefore, a number of disparities exist in knowledge of these compounds and few have undergone development for commercial applications.

The research undertaken and reported in this PhD thesis presents a comprehensive study of techniques developed to isolate and identify biologically active metabolites from a locally acquired species of *Microcystis flos-aquae*. Biomass from a cyanobacterial bloom was collected from the Torrens Lake, South Australia, and evaluated for antimicrobial compound synthesis and inhibitory activity against bacteria, fungi and viruses. Active compounds were isolated from crude extracts of the biomass and submitted for chemical analysis by High Pressure Liquid Chromatography (HPLC) and Liquid chromatography-mass spectrometry (LC/MS) to identify structure and molecular formula. A series of investigative experiments was developed to examine the biosynthetic pathway and genes responsible for assembly of these bioactive secondary metabolites.

1.3 Objectives and Achievements

The primary objective of this research was to evaluate a field strain of cyanobacteria obtained from a local bloom for antimicrobial activity against representative organisms of different genera, and to isolate and classify structure and functional groups of identified bioactive metabolites. Additionally, a series of experiments focussed on understanding the molecular biology of the organism were undertaken to examine the biosynthetic pathway responsible for secondary metabolite assembly. 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).

Traditionally, evaluation of cyanobacteria for inhibitory activity has been restricted to laboratory strains, cultivated under controlled conditions. Mundt (2001) observed that local strains of cyanobacteria were rarely investigated for their antimicrobial properties. *Microcystis* is one of the most common bloom-forming species, comprising more than 85% of most bloom populations (Shukla and Rai 2007). Cells of this genus often secrete toxins into the aquatic ecosystem; consequently *Microcystis* is considered a "nuisance" species. Cyanobacterial blooms are frequently comprised of both toxic and non-toxic forms, though often toxins are so potent they may mask the actions of non-toxic peptides. However, a number of *Microcystis* strains have been examined for bioactivity and the inhibitory agent identified (Ishida *et al*. 1995; Ishida *et al*. 1996, 1997; Ishida *et al*. 1998; Ishida and Murakami 2000; Ishida *et al*. 2002). Luescher-Mattli (2003) remarked in a review paper recognising microalgae as a source of new drugs, that a water bloom of *Microcystis aeruginosa* (Kuetz Elenkin) had exhibited anti-influenza activity, though the active compound had not been elucidated at the time of publication. Similarly, novel compounds are continually discovered in strains of *Microcystis* (Baker and Humpage 1994; Bister *et al.* 2004; Chen *et al.* 2005; Beresovsky *et al.* 2006; Reshef and Carmeli 2006; Welker *et al.* 2006). Thus, assessment of a species of non-toxic *Microcystis flos-aquae*

isolated from a cyanobacterial bloom in a local river in Adelaide, South Australia for antimicrobial activity contributes to the concept that naturally occurring cyanobacteria are a rich source of bioactive secondary metabolites.

Efficient extraction techniques are vital for successful isolation of secondary metabolites, as they accumulate in the cell in minute quantities (Sasson 1991), and their distribution is highly restricted compared to that of primary metabolites. Further complications arise from the lack of morphological distinctions between toxic and non-toxic strains (Spoof *et al*. 2003), limiting the probability that the compound isolated will exhibit activity unrelated to the toxin. Hence, a number of extraction techniques were developed during this study, and were customised to improve efficacy of extraction of potentially bioactive compounds. Following compound extraction, analytical chemistry techniques including HPLC and MS were applied to resolve the metabolite molecular formula, structure and functional groups.

Recently, microalgal biotechnology has evolved in colossal strides. Fujii *et al* (2000) compared secondary metabolite products from toxic and non-toxic cyanobacteria to identify the biosynthetic relationship between non-toxic and hepatotoxic peptides. Results from these studies indicated that toxic strains possess synthetase genes for peptides other than hepatotoxins, and that production of non-toxic peptides is closely related to that of toxic peptides (Fujii *et al*. 2000). Further studies undertaken by Neilan *et al* (1999) and Börner (2005) have identified genes responsible for hepatotoxin synthesis by *Microcystis*. In view of Fujii's conjecture that synthesis of toxic and non-toxic peptides is closely related, a series of directed molecular investigations were undertaken to examine this relationship.

1.4 Thesis Overview

A literature review is presented in Chapter 2 that summarises pertinent aspects of cyanobacterial dominance in freshwater environments and laboratory cultivation techniques. A brief review of existing secondary metabolite applications is included, and a description of the field site is provided. Screening of cyanobacteria for bioactive secondary metabolites is discussed extensively; with specific reference to *Microcystis* sp. Chapter 3 outlines the instrumentation, and field and laboratory procedures used to assess bioactive properties of *Microcystis flos-aquae* collected from the Torrens

River, South Australia. Selection of a field species of *M. flos-aquae* for further investigation was confirmed following preliminary bioassays involving laboratory-cultivated and field cyanobacteria; *M. flos-aquae* isolate MIC FEB05 demonstrated the strongest inhibitory activity against the test organisms employed in the assays, and did not synthesise hepatotoxins (P. Hobson; personal communication, 2005). This latter property, confirmed by the University of New South Wales by protein phosphatase bioassay (A Knight; personal communication, 2007), enabled the study to focus on non-toxic secondary metabolite production, removing complications involved with cytotoxic compounds. Data obtained from the bioactivity assays, both antibacterial and antiviral, are presented in Chapter 4, in conjunction with a comprehensive discussion.

Isolation of bioactive compounds from cyanobacteria proceeds with two primary objectives: to discover new compounds that may be used in biotechnological applications; and to better understand interactions of these compounds with the environment (Schlegel *et al*. 1999). To accomplish each of these objectives, screening programs to examine frequency and distribution of (cultivable) bioactive strains from natural populations should be established (Schlegel *et al*. 1999). However, Borowitzka (1995) noted that often non-axenic cultures are screened (Borowitzka 1995), and further remarked that assumptions regarding activity belonging to the abundant organism in the sample are not necessarily accurate. Isolation and chemical analysis of the bioactive compound(s) constituted a significant phase in this study, and the results of the investigations are presented (Chapter 5). Though cyanobacteria have been screened extensively for biological activity, many studies have not proceeded to the next stage – identifying those compounds responsible for growth inhibition (Borowitzka 1995). Chemical investigations indicate most of the cyanobacterial secondary metabolites isolated belong to one of two classes: cyclic peptides, and depsipeptides (Moore 1996). A review of the analytical techniques applied during this study is provided in APPENDIX B.

A number of authors (Neilan *et al*. 1999; Dittmann *et al*. 2001; Börner and Dittmann 2005) have established that the hepatotoxin, microcystin, is non-ribosomally synthesised by peptide synthetases (Fujii *et al*. 2000). Nishizawa *et al* (1999) concedes that very little is known regarding peptide synthetase genes in

cyanobacteria, despite the large number of peptidic compounds previously isolated. However, investigations by Meißner *et al* (1996) have determined a high degree of homology between DNA sequences of *Microcystis* and known peptide synthetase genes, though the sequences differ in their complement of genes encoding specific peptide synthetases. Isolation experiments by Fujii *et al* (2000) resulted in the preposition that ". . . toxic strains producing hepatotoxic peptides have synthetase genes for other groups of peptides. . ." Chapter 6 describes the DNA purification techniques employed to elucidate genes involved in secondary metabolite biosynthesis of *M. flos-aquae*. A number of Polymerase Chain Reaction (PCR) protocols were developed to amplify specific DNA sequences, applying primers designed for homologous regions of peptide synthetase DNA. Sequencing of the amplified DNA and comparison with genome databases (GenBank) identified the presence of peptide synthetases present in this field strain, and allowed speculation of the classes of peptides likely to be transcribed from these genes.

This thesis is concluded (Chapter 7) with a discussion of the implications surrounding the bioactive compound elucidated in this study. *De novo* synthesis of the compound for commercial applications as a novel pharmaceutical is highly plausible following further purification of the bioactive metabolite. To contribute to the scope of knowledge regarding cyanobacterial secondary metabolites isolated from field samples, this research was presented at three international conferences and four national conferences, and the manuscripts included in the conference proceedings.

1.4.1 Research Program

The progression and achievements of the research presented in this thesis is outlined below:

- Literature Review (Chapter 2);
- Field sampling and cultivation of bloom-forming cyanobacteria from a local habitat (Chapter 3);
- Project Methodology (Chapter 3);
- Development and application of bioactivity assays (Chapter 4);
- Comparison of bioactivity of field samples and laboratory cultured strains, and evaluation of bioactive compound production at different stages of the cell life cycle (Chapter 4);

- Isolation and elucidation of the chemical structure and molecular formula of the bioactive metabolite (Chapter 5);
- Investigations of biosynthesis of cyanobacterial secondary metabolites using regions of DNA homology from toxic strains of cyanobacteria (Chapter 6);
- Design of primer sequences to amplify regions of cyanobacterial DNA (Chapter 6); and
- Summary and concluding remarks (Chapter 7)

1.5 Background

Cyanobacteria, a morphologically diverse class of prokaryotic, photosynthetic organisms (Fogg 1973; Carr and Whitton 1982), that flourish in static, eutrophicated water bodies, dominating the microbial assemblage through formation of "blooms" (Ghadouani *et al*. 2004), particularly at warmer temperatures. Bloom-forming species include *Microcystis*, *Anabaena* and *Nostoc*, species acknowledged for excretion of toxins such as microcystin and anatoxin into the surrounding environment (Falconer and Humpage 1996; LeBlanc *et al*. 2005). Despite seasonal recurrence of toxic blooms, not all species are noxious, nor are all strains within a species capable of synthesising toxic compounds (Spoof *et al*. 2003). For example, Dittmann *et al* (1997) detected a number of non-microcystin peptides in several strains of *Microcystis* and other bloom-forming cyanobacteria. Production of these metabolites is highly species and even strain dependent (LeFlaive and Ten-Hage 2007), and synthesis often confers a unique and important selective advantage to the producing organism (Wolfe 2000).

Natural products research, traditionally a source for discovery of novel therapeutics (Schwartz *et al*. 1990), has frequently been directed toward isolation of compounds from higher plant species (Shimizu 1996; Smith and Doan 1999). Screening of extracts or isolated compounds from natural sources is a universal approach to identify biologically active metabolites (Patterson *et al*. 1993; Volk and Furkert 2006). The enduring importance of natural products is illustrated by the high proportion (25%) of pharmaceuticals derived from plants (Freile-Pelegrin and Morales 2004). Recently, microalgae and cyanobacteria have emerged as a veritable pharmacopeia of bioactive substances (Morrison *et al*. 2006). Though toxins have been the dominant metabolites isolated from cyanobacteria (Burjà *et al*. 2001), non-

toxic peptides demonstrate potential to serve as lead compounds in pharmaceutical, agricultural or other industrial applications (Smith and Doan 1999). Among those secondary metabolites synthesised by cyanobacteria, a number of non-toxic bioactive peptides share biosynthetic pathways with familiar toxins (LeFlaive and Ten-Hage 2007); this trait suggests cyanobacteria are ideal candidates for a dual roles of investigating: 1) motives for production of a complex spectrum of metabolites; and 2) implications of the natural function of the compounds (Morrison *et al*. 2006).

The term "bioactive molecule" is a colloquial expression, applied to substances which ". . . at low concentrations may influence organisms in the immediate environment. . . " (Skulberg 2000). Similarly, Rice (1984) defined allelopathy as ". . . the direct or indirect (harmful or beneficial) effect of one organism on another organism through release of compounds (allelochemicals). . .". Substances that manipulate microbial assemblages encompass both positive and negative regulators that promote or dampen growth or function of neighbouring cells (Ostensvik *et al*. 1998; Smith and Doan 1999). A number of physiological questions are raised when considering the function of secondary metabolites: what are the effects of environmental conditions on production of the compounds?; how bioactive substances are elicited?; are bioactive compounds liberated from the cells, and how are they released?; and how does the producer organism engage self protection mechanisms to elude auto-suppression? (Smith and Doan 1999).

Despite the ideological function of these metabolites remaining undetermined, research has demonstrated they may assist in deterring predatory organisms in the environment (Burjà *et al*. 2001; Volk and Furkert 2006). Speculation beyond this role is precarious, as observed biological activity may not have an obvious role in the life cycle of the organism (Bokesch *et al*. 2003; Beresovsky *et al*. 2006). Peptide discovery is in an exponential phase, with many peptides found in environmental cyanobacterial bloom samples previously unknown (Fastner *et al*. 2001; Harada 2004; Welker *et al*. 2004).

1.5.1 Screening of Cyanobacteria for Bioactivity

Establishment of high throughput screening programs have resulted in detection of chemical compounds demonstrating biological activities including antibacterial,

antiviral, anti-cancer, immunosuppression and neurological activities (Patterson *et al*. 1994; Kulik 1995; Namikoshi and Rinehart 1996; Skulberg 2000; Burjà *et al*. 2001; Matern *et al*. 2003). A relatively high proportion of cyanobacteria accumulate peptide metabolites in the form of cyclic depsipeptides (Matern *et al*. 2003), alkaloids, polyketides and non-ribosomal peptides (Zainuddin *et al*. 2002). However, few of these compounds have been chemically defined to date, despite the increasing number of allelopathic interactions between cyanobacterial species, and little is known regarding mechanisms that regulate production and excretion of the compounds (LeFlaive and Ten-Hage 2007).

Most authors adopt the view that allelopathy evolved as a secondary process or by-product of other ecological processes (LeFlaive and Ten-Hage 2007). Allelopathic interactions are not always detectable under laboratory conditions as organisms adapt to one another through long term co-existence; however, interactions may become evident under physicochemical stress (LeFlaive and Ten-Hage 2007). Though organisms in the same environment are subject to the same stressors, these factors may manipulate the balance in favour of a strain excreting bioactive compounds (Ghadouani *et al*. 2004), leading to dominant growth of a particular species, or bloom formation. Natural function of these substances presumably relates to regulation or succession of the microalgal population, symptomatic of formation of metabolites with biological activity (Mundt *et al*. 2001). Inderjit and del Moral (1997) emphasise that the impact of stress factors on the allelopathic interactions within a microbial community remain unclear, though Smith and Doan (1999) propose that allelopathy should explain, in part, species succession.

Growth phase significantly influences secondary metabolite synthesis and hence interactions with other organisms (LeFlaive and Ten-Hage 2007). Some species attain maximal metabolite synthesis during the stationary phase, other during the exponential phase (Skulberg 2000; LeFlaive and Ten-Hage 2007). Microbial secondary metabolites are usually produced during the stationary phase (Namikoshi and Rinehart 1996); however, Repka *et al* (2004) proposed that cyanobacteria contain secondary metabolites at all stages of growth. Volk and Furkert (2006) concur, maintaining that cyanobacteria accumulate secondary metabolites in cyanobacterial biomass, and are excreted into the environment. Chetsumon *et al* (1995) observed

cells of the cyanobacterium *Scytonema* sp. TISTR 8208 accumulated large amounts of antibiotic peptide intracellularly during the growth phase. However, Cannell *et al* (1988) dispute this proposition, supporting Namikoshi and Rinehart's (1996) premise of secondary metabolite synthesis during the stationary phase. Borowitzka (1995) also observed that ". . . [Secondary metabolites] are usually most abundant in stationary phase or in slow-growing cultures. . . ". Several studies have also highlighted the influence of growth phase and culture conditions of production of secondary metabolites by cyanobacteria (van der Westhuizen and Eloff 1985; Armstrong *et al*. 1991; Patterson *et al*. 1991; Morton and Bomber 1994).

To successfully explore biosynthesis, occurrence and function of cyanobacterial secondary metabolites, reliable extraction techniques are paramount (Morrison *et al*. 2006). Efficiency of extraction solvents appears to be a complex issue, related to factors including water content of cells (freeze-dried vs. freshly frozen and thawed), variants present in cells, and differences between field and laboratory cultures (morphological variations, matrix effects) (Fastner *et al*. 1998). Consequently, a number of extraction solvents, both aqueous and inorganic, combined with a variety of extraction techniques were evaluated during this study to ascertain an optimal method for isolation of bioactive metabolites. Fastner *et al* (1998) established that pure methanol did not quantitatively extract microcystin from freeze dried biomass, though 75% v/v methanol, or sequential extraction with methanol and water proved efficient. Thus, a series of dilutions of methanol were employed initially to extract secondary metabolites. A more detailed discussion regarding solvent selection is presented in Chapter 3.

Selection of an appropriate screening method is a crucial aspect of drug discovery (Monaghan and Tkacz 1990), and bioassays employed during screening programs must be sensitive, straightforward and environmentally relevant (Monaghan and Tkacz 1990; Smith and Doan 1999). Many bioassays employ target organisms unrelated to those for which the metabolites originally evolved (Smith and Doan 1999); thus to validate results of the screen, choice of indicator or target organism is vital (LeFlaive and Ten-Hage 2007). Additionally, understanding reasons behind the organism's production of biologically active metabolites afford a rational basis for selection of organisms to screen (Monaghan and Tkacz 1990). Strain selection of

cyanobacteria is also imperative; biological activity is highly variable between strains of the same species (Ördög *et al*. 2004). Bioassay screens based upon growth inhibition as the primary mechanism of action present inherent advantages of simplicity and reduced time to find compounds with the desired characteristics (Monaghan and Tkacz 1990).

A large number of metabolites are difficult or impossible to synthesize at economic values (Sasson 1991), due to the significant energy input required for synthesis. However, compounds produced naturally rather than synthetically are generally considered more environmentally acceptable (Ozdemir *et al*. 2004). To establish an acceptable balance between economics and environment, Luescher-Mattli (2003) proposes that microalgal metabolites form the basis of novel therapy system for treatment of a number of enveloped viruses, while Burja *et al* (2001), as previously discussed, believes bioactive metabolites may serve as lead compounds in a number of industrial applications (Burjà *et al.* 2001; Bokesch *et al.* 2003).

Biomass from a local cyanobacterial bloom was recovered and assessed following solvent extraction for bioactivity against bacterial, fungal and viral test organisms. Crude extracts with confirmed growth inhibition were separated by reverse phase HPLC to isolate the bioactive fraction. Accumulation of the bioactive fraction using a semi-preparative chromatography column was followed by chemical structure identification using LC / MS. Further investigations involved DNA amplification of specific regions to identify peptide synthetase genes responsible for assembly of the bioactive secondary metabolites.

1.5.1.1 Bioactivity of identified compounds

Extensive screening of cyanobacteria has identified a number of species able to synthesise a variety of secondary metabolites with biological activity (Borowitzka 1995). Cyanobacteria are attractive as sources of bioactive metabolites as the compounds can be synthesised in culture, enabling accumulation of complex molecules which may be difficult to chemically synthesise (Patterson *et al*. 1993; Borowitzka 1995). Isolation of these compounds requires some understanding of conditions favouring their production (Borowitzka 1995; Ördög *et al*. 2004), since biosynthesis is influenced by external factors (de Caire *et al*. 1993). As observed by

Cannell *et al* (1988) and Borowitzka (1995), metabolites of interest are generally products of secondary metabolism. Patterson *et al* (1993) established that the occurrence of antiviral compounds is widely distributed among cyanophytes, and that particular taxonomic groups are more likely to display specific activities. Conversely, Cannell *et al* (1988) remarked that no particular group of organisms appeared to be prolific producers. However, this may be a reflection of the species assessed during their study, or the extraction methods employed; the authors state that for practical reasons, every culture could not be harvested at individual optimum times of production, and that no organic solvent extracts were performed from "young" cultures (Cannell *et al*. 1988). Screening programs have led to elucidation of more than 400 bioactive secondary metabolites (Burjà *et al*. 2001; Bokesch *et al*. 2003); examples of these compounds, their target organism or effect, the species responsible are provided in Table 1-1.

Table 1-1: Cyanobacterial metabolites, their target organism or effect and the species responsible for synthesis.

Organism	Active Compound	Effect/target organism	References
Microcystis aeruginosa	lipid	algaecide	(Ikawa *et al*. 1996)
Microcystis aeruginosa	micropeptin 478-A micropeptin 478-B microginin 299-A, B Kawaguchipeptin B	Plasmin inhibitor Leucin aminopeptidase inhibitor Bactericide	(Ishida *et al*. 1997)
Microcystis aeruginosa	Aqueous extract	Antiviral (influenza A)	(Nowotny *et al*. 1997)
Microcystis viridis	aeruginosin 102-A aeruginosin 102-B	Thrombin inhibitor	(Matsuda *et al*. 1996)
Microcystis viridis	Micropeptin 103	Chymotrypsin inhibitor	(Murakami *et al*. 1997)
Microcystis aeruginosa (NIES-88)	Cyclic undecapeptide	*S. aureus*	(Ishida *et al*. 1997)
Chlorella stigmatophora	N/R	*S. aureus; B. subtilis*	(Kellam and Walker 1989)
Synechococcus leopoliensis	N/R	*B. subtilis*	(Cannell *et al*. 1988)
Laurencia chondrioides	sesquiterpene	*S. aureus; B. subtilis, M. flavus*	(Bansemir *et al*. 2004)
Cystoseira tamariscifolia	meroditerpenoid	*A. tumafaciens, E. coli*	(Bennamara *et al*. 1999)
Fucus vesiculosus	Polyhydroxylated fucophlorethol	*S. aureus, E. coli, P. aeruginosa*	(Sandsdalen *et al*. 2003)
Scenedesmus sp	N/R	*Alternaria sp*	(Ördög *et al*. 2004)
Scytonema julianum	Acetyl-sphingomyelin;	Causes platelet aggregation	(Antonopoulou *et al*. 2002)
Scytonema varium	Scytovirin; Cyanovirin N	Anti HIV protein	(Boyd *et al*. 1997; Bokesch *et al*. 2003)
Haloferax alexandrinus	Canthaxanthin	carotenoid	(Asker and Ohta 2002)
Microcystis PCC 7806	Cyanopeptolin 963A	Chymotrypsin inhibitor	(Bister *et al*. 2004)
Aphanizomenon flos-aquae	phycocyanin	antioxidant	(Benedetti *et al*. 2004)
Microcystis aeruginosa	Zeaxanthin	carotenoid	(Chen *et al*. 2005)
Oscillatoria redekei HUB 051	Unsaturated fatty acids	Antibacterial	(Mundt *et al*. 2003)
Fischerella sp CENA 19	Fischerellin A; 12-epi-hapalindole	algaecide	(Etchegaray *et al*. 2004)
Microcystis sp	NR	Antiviral (Influenza A)	(Zainuddin *et al*. 2002)
Microcystis aeruginosa	Microviridin	Serine protease inhibitor	(Reshef and Carmeli 2006)
Schizotrix sp	Schizotrin A	antimicrobial	(Pergament and Carmeli 1994)

N/R – Not Recorded at time of publication

Emergence of multi-drug-resistant pathogens (e.g. “Golden Staph”, Multi Resistant *Staphylococcus aureus* (Rosenbach 1884) (MRSA)) requires urgent discovery of new classes of antibiotics (Burgess *et al*. 1999; Freile-Pelegrin and Morales 2004; Das *et al*. 2005). However, rediscovery of known natural products presents a significant obstacle in the search for novel, biologically active molecules (Schwartz *et al*. 1990). Many researchers in the pharmaceutical industry have largely avoided the use of algae as a source of pharmaceutical lead compounds to circumvent the technical problems associated with collection of biomass from nature and achieving viable cultures in the laboratory (Schwartz *et al*. 1990). In any natural products screening program, identification of promising compounds requires a critical evaluation of the biological activity and efficacy of the compounds (Schwartz *et al*. 1990). Development of selective bioassays to investigate new therapeutic areas may overcome this problem.

Most studies focus on *in vitro* assessment of bioactive metabolites; few *in vivo* studies are carried out using compounds isolated from cyanobacteria due to their acute toxicity or inactivity *in vivo* (Borowitzka 1995; Schaeffer and Krylov 2000; Burjà *et al*. 2002). Despite the extensive research into cyanobacterial metabolites, significant gaps remain regarding identification of compounds, elucidation of biosynthesis genes, and factors influencing metabolite production. Indeed, Borowitzka (1995) emphasises that knowledge of physiological control of secondary metabolite formation in algae and the relevant biosynthetic pathways is extremely limited. To build on the comprehension of algal metabolites, continued screening and isolation of algae is required to identify bioactive strains, in addition to research in algal physiology (Borowitzka 1995). Schaeffer and Krylov (2000) take particular interest in the lack of antiviral metabolites identified, though this is not unexpected, given the limited number of classes of microalgae and cyanobacteria investigated. A number of studies (Carmichael 1986; Borowitzka 1995) have verified that the desired bioactivity may decline or be lost entirely during culture, though reasons for this phenomenon are so far inexplicable. Investigations during this study involving laboratory-cultured strains of cyanobacteria alluded to the trait described above by Borowitzka (1995); strains that had been repeatedly subcultured in the laboratory did not inhibit growth of any test organism.

1.5.2 Torrens River Catchment system

The Torrens River is an urban catchment system passing through the city of Adelaide, South Australia, from the Mt Lofty Ranges to an outlet at West Beach. The Torrens Lake, formed by construction of a weir on the western side of the central business district (CBD) in the 1890's, generally provides a picturesque area for recreational activities. However, negligible river flow and high nutrient loading upstream results in a primarily static, eutrophicated water body, prone to cyanobacterial blooms during temperate periods. Excessive growth of cyanobacteria has detrimental effects on domestic, industrial and recreational uses of the water (Dokulil and Teubner 2000). The lake is regularly closed from February to April by toxic, malodorous blooms, principally comprised of *Microcystis* or *Anabaena*. Monitoring of the biomass by the Australian Water Quality Centre (AWQC) has identified a toxic form of *Anabaena circinalis* (Rabenhorot ex Bornet & Flanhault), and an intermittently toxic form of *M. flos-aquae* as the most common causative organisms. Preliminary screening of samples of both species collected from the same bloom (the bloom was first observed in mid February 2005, and a species succession occurred after 4-5 weeks) for antibacterial activity revealed only the non-toxic form of *M. flos-aquae* demonstrated growth inhibition of the test organisms; despite synthesis of a potent toxin, *A. circinalis* did not exhibit bioactive characteristics against organisms selected for assessment. Subsequently, non-toxic *M. flos-aquae* was selected for application in this study. Identification of the bioactive compound and investigation into the genes responsible for synthesis of this metabolite may lead to development of a novel, natural drug therapy for resistant pathogens.

CHAPTER 2 ENVIRONMENT AND APPLICATION

2.1 Algae

2.1.1 Classification

Algae are a diverse group of eukaryotic organisms that typically carry out oxygenic photosynthesis and may be located in freshwater, marine and terrestrial environments (Brock *et al*. 1997). They are distinguished from other chlorophyll-containing plants by aspects of reproduction (Bold and Wynne 1985). Algae are simply constructed, ranging from unicellular forms to aggregations of cells (Canter-Lund and Lund 1995), trichomes (filaments of cells) or tubular structures (Prescott 1999). Many unicellular forms are also motile, and are able to move freely though liquid suspensions (South and Whittick 1987). Most algae are microscopic, though some (known as macroalgae) may grow to over 30 m in length. The majority of freshwater algae are of microscopic size – smallest inland algae consist of single cells about one micron in diameter (Canter-Lund and Lund 1995). Among the beneficial aspects of algae is their use as tools to study "systems" in biological research (Bold and Wynne 1985). They are ideal experimental organisms, due to their small size and easy manipulation in liquid media.

Algae are highly important as primary producers of organic matter in aquatic ecosystems through their photosynthetic capabilities (Bold and Wynne 1985; Walker *et al*. 2005). During daylight hours, algae contribute to the oxygenation of water in their environment, which aids in maintaining the delicate balance in the ecosystem. The structural materials of cells and the rigid cell walls serve as a primary food source for many other organisms, while secreted and excreted products are also significant (Bold and Wynne 1985). All algae contain photosynthetic pigments such as chlorophyll-*a*, a pigment causing the green coloration commonly observed, and accessory pigments which act as traps for light energy. However, many species also contain other pigments (e.g. carotenoids) that mask the effect of chlorophyll (Brock *et al*. 1997). Algae are grouped into 6 classes, based on their colour (Reynolds 1984; Bold and Wynne 1985; South and Whittick 1987):

- Chlorophytae – green algae
- Rhodophytae – red algae
- Phaeophytae – brown algae
- Chrysophytae – yellow/green algae
- Cyanophytae –cyanobacteria (also known as blue-green algae)
- Xanthophytae - yellow/green algae

Species from the above algal classes are widely distributed in marine and freshwater environments, from tropical to arctic regions (Stanier *et al.* 1971; Fogg 1973; Reynolds 1984; Bold and Wynne 1985; South and Whittick 1987). Prokaryotic forms of microalgae are classed together under Cyanophyceae.

2.1.2 Phytoplankton/cyanobacteria

Microalgae and cyanobacteria constitute the majority of the world's biomass; however there is considerable disparity between the two families. "Microalgae" refers to microscopic, eukaryotic phytoplankton, possessing attributes and properties commonly associated with algae. Cyanobacteria exhibit microalgal morphology but possess characteristics more frequently observed in photosynthetic bacteria (South and Whittick 1987). The cellular structure and biochemistry of cyanobacteria resembles that of bacteria; however, the chlorophyll-α pigment differs between cyanobacteria and bacteria, and oxygen is liberated during cyanobacterial photosynthesis (Bold and Wynne 1985). Recent publications have estimated the number of cyanobacterial species at more than 2000 (Saker *et al.* 2005; Guven and Howard 2006)

The morphology of cyanobacteria ranges from unicellular (*Microcystis*) to filamentous (*Anabaena*, *Nodularia* and *Oscillatoria*), illustrated in Figure 2-1. The cell wall is a four-tiered structure (2 membrane bilayers) with the major structural layer composed of peptidoglycan (South and Whittick 1987; Fiore and Trevors 1994). The fine structure of cyanobacteria is similar to that of gram-negative bacteria, though the peptidoglycan layer has been shown to be many times thicker in cyanobacterial cells (Fiore and Trevors 1994). The outer membrane comprises lipopolysaccharides, proteins, lipids and carotenoids, and many species have additional layers external to the outer membrane, known as a sheath, capsule, slime or external cell wall layer. The

sheath layer is composed predominately of polysaccharides, normally of at least one uronic acid and several neutral sugars, and may be in combination with proteins (Fiore and Trevors 1994).

Cyanobacteria lack the structural organisation of chromosomes within a separate nucleus typical of eukaryotic cells, and the discrete pigment-containing organelles (plastids or chromatophores) (Reynolds 1984; Mur *et al*. 1999). Photosynthetic thylakoids are distributed in the cytoplasm rather than in a membrane-bounded chloroplast (Fiore and Trevors 1994; Mur *et al*. 1999). Antenna system for photosystem 1 (PS1) differs from photosystem 2 (PS2); PS1 consists of chlorophyll-*a*, while PS2 is comprised of phycobilisome. Cyanobacteria generally have more PS1 reaction centres than PS2, though this ratio changes in response to altered light intensity (Vonshak and Torzillo 2004). The genome size of cyanobacteria ranges from 1.6×10^9 to 8.6×10^9 Da (Thajuddin and Subramanian 2005), they contain a 70s rather than 80s ribosome, and differ in fatty acid composition, frequently possessing unsaturated fatty acids with two or more double bonds (Brock *et al*. 1997). Plasmids have also been detected in many species of cyanobacteria, however their role is unclear. Many researchers have suggested that they carry extrachromosomal genes involved in conferring resistance to heavy metals and antibiotics, and in the synthesis of gas vacuoles (Fiore and Trevors 1994).

NOTE:
This figure is included on page 18
of the print copy of the thesis held in
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Figure 2-1: Structures of common cyanobacteria (Environment-ACT 2003)

Cyanobacteria are able to colonise a wide range of ecological niches as a result of their ability for continuous photosynthetic growth in the presence of O_2, and having water as their electron donor for CO_2 reduction (Mur *et al*. 1999). These prokaryotes have been found in extreme habitats, from hot springs to desert rocks and in some cases Antarctic glaciers (Pulz and Gross 2004; Hoeger *et al*. 2005; Thajuddin and

Subramanian 2005). They are ubiquitous in surface waters worldwide, and many species including *Microcystis*, *Nodularia*, *Cylindrospermopsis*, *Anabaena* and *Aphanizomenon* are known as producers of toxins (Hoeger *et al*. 2005). A variety of metabolites, including compounds toxic to other cyanobacteria, bacteria, fungi, algae and zooplankton are synthesised by many species (Sinoven and Jones 1999). These "reserve products" are accumulated under conditions of excess nutrients (Mur *et al*. 1999).

Cyanobacteria have a higher affinity for nitrogen and phosphorous than other photosynthetic organisms (Mur *et al*. 1999; Kardinaal and Visser 2005) and have a remarkable ability to store essential nutrients and metabolites in their cytoplasm (Mur *et al*. 1999). Some species are able to fix atmospheric nitrogen under conditions of nitrogen limitation, while others produce gas vesicles, allowing them to alter their location within a vertical water column by regulating buoyancy (Mur *et al*. 1999; Dokulil and Teubner 2000; Pulz and Gross 2004). Cyanobacteria cannot reach the maximum growth rates of green algae; however, at very low light intensities their growth rate is higher. In water of high turbidity or low CO_2/high pH, they have better a chance of out-competing other species (Mur *et al*. 1999), particularly under conditions of N or P limitation. Cyanobacteria often exploit a combination of these traits to dominate their environment (Kardinaal and Visser 2005).

2.1.3 Applications – environmental and commercial uses of microalgae

Microalgae have 3 fundamental attributes that can be converted into technical and commercial advantages (Radmer and Parker 1994):

- are a genetically diverse group of organisms;
- are able to incorporate stable isotopes ^{13}C, ^{15}N and ^{2}H into their biomass; and
- comprise a large, unexplored group of organisms, providing an untapped source of molecules.

The industry market for microalgal biomass achieves at least 5000 ton/yr of dried material, with an annual turnover of approximately USD 1.3 billion (Pulz and Gross 2004). Successful application of microalgal biotechnology relies predominantly on selection of an appropriate species for a specific task, and applying optimum culture conditions to achieve the desired outcome.

Despite the recognized existence of more than 2000 species of cyanobacteria, relatively few are utilised for biotechnology applications (Borowitzka 1995; LeFlaive and Ten-Hage 2007). However, the diversity of microalgal species has led to a number of significant advances in recent years, resulting in an expansion of microalgal biotechnology into new areas (Parker *et al.* 1994; Apt and Behrens 1999; Harada 2004). Foodstuffs for humans and aquaculture, industrial chemicals, pharmaceuticals and neutraceuticals, and renewable fuels are among the numerous products that may potentially be developed through microalgal biotechnology (Olaizolá 2003). Table 2-1 provides examples of microalgal genera already utilised for their products or inherent traits such as nutritional value, and further examples of microalgal applications are provided below.

Table 2-1: Microalgal species for biotechnological applications (Borowitzka 1992; Day *et al*. 1999; Pulz and Gross 2004)

Species	Group	Product	Application	Culture system
Spirulina platensis	Cyanobacteria	Phycocyanins, biomass	Health food, cosmetics	Open ponds, natural lakes
Chlorella vulgaris	Chlorophyta	Biomass, Ascorbic acid	Health food, food supplement, food surrogate	Open ponds, basins, glass tube PBR
Dunaliella salina	Chlorophyta	Carotenoids, β-carotene	Health food, food supplement, feed	Open ponds, lagoons
Haematococcus pluvialis	Chlorophyta	Carotenoids, astaxanthin	Health food, pharmaceuticals, feed additives	Open ponds, PBR
Odontella aurita	Bacillariophyta	Fatty acids	Pharmaceuticals, cosmetics, baby food	Open ponds
Porphyridium cruentum	Rhodophyta	Polysaccharides	Pharmaceuticals, cosmetics, nutrition	Tubular PBR
Isochrysis galbana	Chlorophyta	Fatty acids	Animal nutrition	Open ponds
Phaedactylum tricornutum	Bacillariophyta	Lipids, fatty acids	Nutrition, fuel production	Open ponds, basins
Lyngbya majuscule	Cyanobacteria	Immune modulators	Pharmaceuticals, nutrition	Open ponds

2.1.3.1 Nutritional Products

Microalgae have been consumed as part of human and animal diets since 850 BC (South and Whittick 1987). Reasons for consumption of microalgal species include flavour, protein content and nutritional value (Becker 2004). Protein content may reach as high as 20-25% dry weight (South and Whittick 1987), and microalgae also produce essential vitamins and omega-3 fatty acids. EPA and DHA are a vital dietary component with nutritional and pharmaceutical benefits (Barclay *et al*. 1994; Becker 2004), but cannot be synthesised de novo by humans or animals (Regan 1988). Microalgae are primary producers of omega-3 fatty acids, stimulating interest in growth of microalgae to obtain higher yields of the acids for dietary supplements (Barclay *et al*. 1994). There is evidence that small amounts of microalgal biomass, most often of the genera *Chlorella, Scenedesmus*, and *Spirulina*, have a beneficial effect on the physiology of animals, in particular eliciting a non-specific immune response (Pulz and Gross 2004). Addition of biomass or extracts to pet food also demonstrates health enhancements, such as shinier coats or more ornate feathers (Pulz and Gross 2004). Nutritional supplements produced from microalgae have been the primary focus in past decades, with dried biomass or cell extracts dominating commercial opportunities (Apt and Behrens 1999). Today, microalgae is marketed as health foods, and sold as tablets, capsules, powders or liquids (Becker 2004). Algae are also added to pasta, snack foods and drinks either as nutritional supplements or natural food colourants (Becker 2004).

2.1.3.2 Aquaculture

Microalgae play a key role in aquaculture, forming the basis of the natural food chain (Day *et al*. 1999; Muller-Feuga 2000). Feeds containing microalgae are used primarily for shell- and fin-fish rearing at the larval and juvenile stages (Gladue and Maxey 1994; Brown *et al*. 1999; Pulz and Gross 2004), particularly those containing elevated concentrations of high value proteins and lipids (omega-3 fatty acids) (Benemann 1992). Examples include oysters, which utilise microalgae at all 3 growth stages (larval, juvenile and adult); abalone at the larval and juvenile stages; and prawns and lobsters that only consume microalgae at the larval stage; other examples are provided in Table 2-2. Size, palatability, nutrition profile, ease of culture, rapid growth rate and toxicity are important factors when selecting a species of microalgae

for aquaculture feed (Borowitzka 1997; Day *et al*. 1999), and must be maintained for optimal growth and survival of cultured animals (Shamsudin 1992). Improvement in the water quality of hatchery ponds through oxygen production and pH stabilization has been observed when microalgae are present. Fish display an improved condition as a result of regulation of bacterial populations, probiotic effects and stimulation of their immune systems (Muller-Feuga 2000). Most microalgal requirements are supplied by firms in-house, grown in specialized units or within the larvae rearing tanks (Muller-Feuga 2000) due to the need for immediate availability of live microalgae.

Table 2-2: Marine animals farmed in Australia requiring microalgae feedstock (Olaizolá 2003)

Species	Larvae	Juveniles	Adult (grow out)
Oysters	Yes	Yes	Yes
Scallops	Yes	Yes	Yes
Mussels	Yes	Yes	Yes
Abalones	Yes	Yes	-
Prawns	Yes	-	-
Finfish	Yes	-	-
Lobsters	Yes	-	-

For some genera of microalgae, heterotrophic growth may be a favourable option for economic cultivation. Heterotrophic growth eliminates the requirement for light (either natural or artificial) and therefore light limitation is no longer an issue (Gladue and Maxey 1994). Whilst growth using light may result in higher yields of lipids for most species, few are able to efficiently utilise organic carbon sources in the absence of light and return a higher proportion of long chain polyunsaturated fatty acids than if they were grown photoautotrophically (Tan and Johns 1996; Borowitzka 1997).

2.1.3.3 Wastewater Treatment & Agriculture

Declining water quality caused by pollution through human and agricultural practices has raised environmental and public health concerns (Hoffmann 1998). The combined nitrogen content of effluent water from agricultural or industrial origin is up to three orders of magnitude higher than that of natural concentrations found in lakes (de la Noüe *et al*. 1992). Additional problems result through discharge of heavy metals and other organics (Angel *et al*. 2002). Chemical and physical based technologies are

available to remove excess nutrients and metals from wastewater but both techniques require excessive energy and/or chemicals (Hoffmann 1998). Biological processes have performed well compared with chemical and physical treatment protocols, and are less likely to result in a secondary pollution problems such as eutrophication (Gantar *et al*. 1991; de la Noüe *et al*. 1992). Microalgae are a key component of an alternative wastewater treatment, offering a tertiary biotreatment combined with production of valuable biomass (de la Noüe *et al*. 1992; Hoffmann 1998). High rate oxidation ponds that favour microalgal growth are employed to remove heavy metals and excess nutrients, while simultaneously producing valuable biomass for animal feed (Day *et al*. 1999; Jiménez-Pérez *et al*. 2004). Microalgae also perform a number of secondary functions, including disinfection of effluent as the conversion of light energy to heat increases water temperature, killing enteric bacteria (Day *et al*. 1999).

2.1.3.4 Medical Applications

Microalgae have been used as "traditional" medicines for centuries (Olaizolá 2003). Anaesthetics and ointments for treatment of coughs, wounds, and hypertension originated from natural products (South and Whittick 1987), while the vitamin and mineral content of some species plays a key role in prevention of diet-deficiency diseases. *Spirulina* has been shown to support digestive tract function by promoting growth of *Lactobacilli* species and maintaining the balance of intestinal bacteria (Moore 2001). Many toxins are action-specific and may be utilised in biotechnological applications. For example, neurotoxins such as homoanatoxin-a target specific ion channels in nerve and muscle membranes (Skulberg 2000) and nerve endings similarly to neurodegenerative diseases, leading to an application in medical research (Luescher-Mattli 2003). A number of bioactive actions have been detected in microalgae and cyanobacteria, including anticancer, antibacterial, and antiviral (Apt and Behrens 1999; Mundt *et al*. 2001). However, despite the number of compounds detected, few have been developed as commercially useful pharmaceuticals. A summary of the claimed beneficial applications of microalgal extracts (Moore 2001) is presented in Table 2-3; some of these remedies have no scientific merit, while others demonstrate basis in their bioactive compounds.

Table 2-3: Claimed beneficial effects and applications of microalgal extracts (Moore 2001)

NOTE:
This table is included on page 24
of the print copy of the thesis held in
the University of Adelaide Library.

2.1.3.5 Renewable Fuel

Considerable attention has been directed at cultivating unicellular microalgae for the production of oils and fatty acids (Apt and Behrens 1999). As the omega-3 fatty acids in fish oils derive from marine phytoplankton, production of marine microalgae, particularly of biomass with a high content of omega-3 fatty acids would be of valuable interest. Certain species of microalgae produce large quantities of oils and fats containing both long chain omega-3 and omega-6 fatty acids (Radmer and Parker 1994). Technology for production of oils containing specific long chain polyunsaturated fatty acids (LCPUFAs) has been developed by identifying, manipulating and culturing microalgae which produce large quantities of specific LCPUFAs (Radmer and Parker 1994). Extensive research has been carried out over past decades on cultivation of low-cost microalgae biomass high in lipids for purposes of fuel production (Benemann 1992). Cultivation of algae is also being investigated for the production of biomass energy through fermentation of various genera to produce methane (South and Whittick 1987).

2.2 Metabolism

Metabolism is defined as the "total of all chemical reactions occurring within a cell" (Prescott 1999) and is divided into catabolism and anabolism. Catabolism involves a release of energy coinciding with a degradation of large, complex molecules (Prescott 1999). Cells use this free energy to construct more complex molecules and structures

from smaller, simpler precursors during anabolism. Biosynthetic pathways are organised to optimise production efficiency by employing multifunctional enzymes and conserving energy (Prescott 1999). Mature cells use energy to synthesise enzymes and other metabolites for release into their environment (Prescott 1999), and for continual degradation and synthesis of cellular molecules. Metabolic processes are carefully regulated so that the rate of biosynthesis is approximately balanced by the rate of catabolism.

For photosynthetic organisms such as cyanobacteria, the reduction and incorporation of carbon dioxide as a nutrient source requires large amounts of energy (Prescott 1999). Phototrophic CO_2 fixation is a crucial process as it generates organic matter that is used as an energy source by heterotrophic organisms. Cyanobacteria assimilate CO_2 via the Calvin cycle, a specialised metabolic pathway that occurs in eukaryotic autotrophs and prokaryotes (Prescott 1999). Many organisms undergo the Tricarboxylic acid cycle (TCA; also known as citric acid or Kreb's cycle) for aerobic oxidation of nutrients to carbon dioxide (Brock *et al*. 1997; Prescott 1999). Cyanobacteria do not possess a fully functional TCA cycle, as these organisms lack a necessary enzyme; however this pathway still plays an important role in carbohydrate metabolism (Prescott 1999). Metabolic processes in cyanobacteria, with particular reference to synthesis of secondary metabolites, will be discussed in the following section.

2.2.1 Secondary metabolite production

The study of secondary metabolism in microalgae and cyanobacteria has undergone a progression from isolation and recognition of bioactive compounds to investigations into the physiological control mechanisms involved in their biosynthesis (Skulberg 2004). Secondary metabolism is part of normal cell growth and is directly related to interactions with the cell's environment; secondary biosynthetic pathways are highly regulated systems (Skulberg 2000). Formation of secondary metabolites is an inherent feature of the overall development of the producing culture, and synthesis is initiated or proceeds most efficiently at particular development stages (Armstrong *et al*. 1991; Borowitzka 1999; Skulberg 2004). For example, Watanabe *et al* (1985) found that synthesis of particular secondary metabolites identified as toxins was highest at the beginning of the stationary phase of growth and that they are constitutively

synthesised; however, Ohtake *et al* (1989) detected the highest level of toxin production in the early-mid exponential phase during their research. Mason *et al* (1982) observed that the metabolite cyanobacterin is constitutively expressed by *Scytonema hofmanni* UTEX B1581 (Argardh *ex* Bornet *et* Flahault). Bioactivity was not detected in *Nostoc muscorum* MAC (Vaucher *ex* Bornet *et* Flahault) until the post-exponential phase of growth; though onset of biosynthesis was rapid, the timing implies the bioactive compound is a secondary metabolite (Bloor and England 1989). Toxin synthesis is discussed in section §2.3 as an aspect of cyanobacterial bioactivity. Level of secondary metabolite production is determined by the availability of precursor molecules and activity of biosynthesis enzymes (Skulberg 2000).

Many species of microalgae and cyanobacteria have a high primary productivity, but desirable compounds suitable for biotechnological development are often products of secondary metabolism (Carmichael 1992). These compounds are generally a build up of intermediate precursor components that accumulate during primary metabolism (Sasson 1991; Skulberg 2004). Depending on the species, secondary pigments may be present in the cell in greater quantities than primary pigments (Borowitzka 1988). Synthesis of these compounds is often triggered under conditions not conducive to high growth rates, such as nutrient deprivation (Olaizolá 2003), and may act as a mediator or cue for physiological or behavioural processes of some planktonic invertebrates (Ward and Targett 1989). Effects of these processes may be either stimulatory or inhibitory towards growth, development and feeding practices of higher organisms such as zooplankton and developing larvae (Ward and Targett 1989; REDNOVA 2003). Many microalgal species are known to release compounds that inhibit growth of other algae (Ikawa *et al*. 1996). For example, the release by some species of polyunsaturated fatty acids (PUFA's) linoleic acid and linolenic acid play an important role in the establishment of species dominance and succession in lake communities, and are recognised inhibitors of *Chlorella* growth (Ikawa *et al*. 1996).

Microalgae and cyanobacteria demonstrate great metabolic diversity (Borowitzka 1988), and are known to excrete substances including amino acids, sugars, fatty acids, vitamins, and steroids into their surrounding medium (Wolfe 2000). Of those strains that synthesise acknowledged secondary metabolites, many produce more than one type of compound, while other morphologically identical strains do not produce any

secondary metabolites (Shimizu 1996). Cyclic peptides and depsipeptides are the most commonly observed structural types, but linear peptides, guanidines, purines and macrolides are also found (Namikoshi and Rinehart 1996). The chemical nature and ecological relevance of these compounds is quite diverse (Schlegel *et al*. 1999; Skulberg 2004; Safonova and Reisser 2005), however ecological function is for the most part unknown (Bokesch *et al*. 2003). A directed search for biologically active compounds during microalgal culture requires solid understanding of external conditions favouring production. Some of these high value products may be limited to chemicals so complex that they are not able to be synthesised artificially, and thus may require culture conditions to be manipulated to increase yields of metabolites by providing excess nutrients or stressors. Genetic and biochemical instability may also limit the usefulness of microalgal culture for production of secondary metabolites (Skulberg 2004); some species show a high degree of variation in metabolite production, and activity may decline or be lost altogether if a species is maintained in culture for long periods (Borowitzka 1999).

2.2.2 External influences on metabolite production

Secondary metabolite production is affected by a multitude of external factors, particularly culture conditions, natural or artificial (Skulberg 2000). For example, several species of *Microcystis* and *Oscillatoria* synthesise elevated quantities of toxic secondary metabolites under high nitrogen conditions (Borowitzka 1999), while some antibiotic compounds may be synthesised throughout the growth cycle but are favoured by low temperatures. Production of microcystin, the most recognizable cyanobacterial toxin, may be enhanced by culturing cells in green or red light. Competition for limiting nutrients and light sources within a microbial population requires organisms to fight for survival (Schlegel *et al*. 1999). This competition has led to the supposition that organisms produce inhibitory compounds to heighten their chance of survival, and is yet another example of the influence of external factors on metabolite synthesis.

2.2.3 Metabolite Applications

Natural products from microalgae and cyanobacteria have traditionally been a rich source for the discovery and development of novel therapeutics (Bokesch *et al*. 2003).

Investigations into secondary metabolite production by cyanobacteria have revealed effects such as antibacterial and antiviral activity are caused by substances distinct from the cyanotoxins (Ostensvik *et al*. 1998). Isolation of bioactive compounds is done with 2 objectives in mind (Schlegel *et al*. 1999):

- To discover new compounds for pharmaceutical, agricultural or bio-control application; and
- To better understand the interactions between individual organisms within natural communities.

These factors will contribute to a better understanding of the frequency and distribution of bioactive strains, and design of effective screening programs for species in natural habitats (Schlegel *et al*. 1999). Identification of the precursors of secondary, bioactive metabolites is of particular practical importance for elucidation of regulatory phenomena related to metabolite synthesis (Wiegand and Pflugmacher 2005). Further characterisation of the bioactive compounds then becomes essential for practical exploitation in biotechnology applications (Skulberg 2000).

Functionality of secondary metabolites is as diverse as the compounds. The unicellular green algae *Chlorella vulgaris* (Beijerinck) and *Chlamydomonas pyrenoidosa* (Dangeard) demonstrated *in vitro* antibacterial activity against both gram positive and negative bacteria (Bokesch *et al*. 2003). The antiviral compounds scytovirin and cyanovirin-N, isolated from *Scytonema varium* (Argardh *ex* Bornet *et* Flahault) and *Nostoc ellipsosporum* (Vaucher *ex* Bornet *et* Flahault) respectively, have demonstrated protection of T cells from HIV-induced killing (Boyd *et al*. 1997; Bokesch *et al*. 2003). The increasing prevalence of antibiotic-resistant pathogens has made efforts to identify novel substitute compounds a priority (Skulberg 2000; Bokesch *et al*. 2003). Organisms identified as bioactive provide a finite pool of secondary metabolites for application. However, generation of candidate drugs from natural resources, though potentially laborious, appears promising through development of microbial gene engineering (Skulberg 2000).

Microalgae also generate vitamins and accessory pigments which are considered secondary metabolites (Borowitzka 1988) and are required by cells for growth. Vitamin B-12 and vitamin E are synthesised by many species of cyanobacteria and are of interest for commercial applications (Borowitzka 1988). Vitamin content varies

greatly between and within a species, and is influenced by genotype, light intensity, stage in growth cycle, and nutritional status: all factors that affect microalgal metabolism. Water soluble vitamins are often detected in supernatants of microalgal culture, and may be actively excreted or released through death and disintegration of the cell (Borowitzka 1988). There are extensive markets for vitamins in human health and animal husbandry with a basic requirement for commercial production defining the potential for mass culture of the species (Borowitzka 1988). Accessory pigments including phycobiliproteins and carotenoids (β-carotene, astaxanthin and cantaxanthin) are used as natural food colourings or feed additives to enhance flesh colour of farmed animals. Colour-enhancing effects of phycocyanins from *Spirulina*, asthaxanthin from *Haematococcus* or carotenoids produced by *Dunaliella* have been exploited in ornamental fish with the addition of these species to the fish diet (Olaizolá 2003; Pulz and Gross 2004).

2.3 Bioactivity

2.3.1 Toxins

Cyanobacteria synthesise a wide spectrum of toxic compounds as secondary metabolites (Borowitzka 1999; Harada 2004; Börner and Dittmann 2005), exerting a cytopathic effect on algae, fungi, bacteria and mammalian cell lines (Carmichael 1992). With particular reference to production by cyanobacteria, Carmichael (1992) defines toxins as ". . . secondary compounds that have a harmful effect on other tissues, cells or organisms . . ." Cyanobacteria synthesise a variety of neuro-, hepato- and cyto-toxic metabolites (Börner and Dittmann 2005) collectively termed phycotoxins (Skulberg 2000). *Microcystis aeruginosa*, *Anabaena flos-aquae* (Richt) and *Aphanizomenon flos-aquae* ([Linné] Ralfs *ex* Bornet *et* Flahault) are confirmed toxin sources (Kulik 1995; Patterson and Bolis 1997) and all species are abundant in surface waters used as drinking supplies and for recreation (Hoeger *et al*. 2005). Phycotoxins are characterised by potency (ie μg toxin/kg body mass) (Skulberg 2000) and are divided into 5 principal categories (Sinoven and Jones 1999; Wiegand and Pflugmacher 2005):

- Hepatotoxins (Cyclic peptides)
- Neurotoxins (Alkaloids)

- Cytotoxins
- Dermatotoxins, and
- Irritant toxins (Lipopolysaccharides)

Microcystin and nodularin are the most frequently encountered hepatotoxins, primarily targeting the liver (Dittmann *et al*. 1997; Sinoven and Jones 1999), and are more common than neurotoxins anatoxin-a and saxitoxin. Alkaloid toxins have been identified in a number of species, detailed in Table 2-4, and affect the liver, skin and nerve system. Synthesis of lipopolysaccharides has been detected in all species of cyanobacteria, many of which act as skin irritants (Sinoven and Jones 1999). Maximum toxin quantities are synthesised under optimum growth conditions (Sinoven and Jones 1999; Kardinaal and Visser 2005). For example, at high phosphate concentrations, hepatotoxins will dominate the environment; similarly under nitrogen rich conditions, non-nitrogen fixing species increase toxin production (Sinoven and Jones 1999).

Table 2-4: Toxins produced by cyanobacteria and their target organ (Sinoven and Jones 1999)

Toxin Group	Cyanobacterial Genera	Primary Target Organ
Cyclic Peptides		
Microcystin	*Microcystis*, *Anabaena*, *Planktothrix, Nostoc, Hapalosiphon, Anabaenopsis*	Liver
Nodularin	*Nodularia*	Liver
Alkaloids		
Anataxin-a	*Anabaena, Planktothrix, Aphanizomenon*	Nerve synapse
Anatoxin-a (S)	*Anabaena*	Nerve synapse
Aplysiatoxins	*Lyngbya, Schizothrix, Planktothrix*	Skin
Cylindrospermopsins	*Cylindrospermopsis, Aphanizomenon, Umezakia*	Liver
Lyngbyatoxin-a	*Lyngbya*	Skin, gastrointestinal tract
Saxitoxins	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*	Nerve axons
Lipopolysaccharides (LPS)	All	Potential irritant; affects any exposed tissue

Exposure to toxins, and subsequent poisoning, may occur through recreational activities, agricultural products, or ingestion of cyanobacterial health foods or contaminated fish and shellfish (Sinoven and Jones 1999; Ferrao-Filho *et al*. 2000;

Hoeger *et al*. 2005; Saker *et al*. 2005); thus they have been specifically highlighted as potential hazards to human health (South and Whittick 1987; Schlegel *et al*. 1999). Acute hepatotoxicosis targets the liver hepatocytes and may be induced following absorption of toxins into the blood (Carmichael 1992). Neurotoxins exert their effects on specific ion channels in nerve and muscle membranes (Skulberg 2000). Cyanobacterial lipopolysaccharides (LPS), while less potent than bacterial LPS, often elicits an allergenic response resulting from exposure to blooms (Sinoven and Jones 1999). Though microcystin and other toxins may cause fatal illnesses in mammals, it is likely that toxin synthesis evolved as a defence mechanism against grazing by zooplankton by inhibiting filtering rates (Ferrao-Filho *et al*. 2000). Feed inhibition may occur following ingestion of the cyanobacterial cells and release of toxic compounds on digestion, or by chemical deterrence, causing behavioural avoidance of toxic cells (Ferrao-Filho *et al*. 2000).

2.3.1.1 Toxic vs. non-toxic strains

Identification of a cyanobacterial genus within an environmental sample by microscopic or molecular analysis does not confer any indication of the potential for toxin production (Baker *et al*. 2001; Kaebernick and Neilan 2001; Baker *et al*. 2002). Morphological characteristics repeatedly vary between samples, and strains may appear morphologically identical (Baker *et al*. 2001). For example, *M. aeruginosa* is known to possess toxigenic and non-toxigenic variants, and both phenotypes have been detected within a water body, suggesting the phenotypes are able to exist concurrently (Vezie *et al*. 1998; Baker *et al*. 2002). It is presently unclear whether toxic and non-toxic strains differ with respect to their gene content. Production of toxins may be feasible in all strains, but down-regulated in non-toxic subspecies (Meißner *et al*. 1996). However, Nishizawa *et al* (1999) described two alternative scenarios for non-toxigenic strains: those that do not possess genes required for toxin production (the *mcy* genes); and those that possess the *mcy* genes, but may have a mutated gene or lack a complete set of genes.

Growth responses of toxigenic and non-toxigenic strains to external stimuli are not implicit (Vezie *et al*. 2002). Environmental factors (temperature, nutrient availability, and trace metals) influence toxin production and transcriptional responses of the gene cluster responsible for microcystin biosynthesis (Meißner *et al*. 1996; Kaebernick and

Neilan 2001; Vezie *et al*. 2002; Janse *et al*. 2004). Toxin quotas may vary up to 5-fold in response to changes (Sinoven and Jones 1999), though there is much debate concerning the relative significance of each of the environmental factors listed above. Vèzie *et al* (2002) speculated that when resources essential for growth are limited, non-toxic strains may be superior competitors to toxic strains. Wicks and Thiel (1990) observed non-toxic strains tended to dominate lakes and reservoirs during winter months, while toxic strains prevailed during summer. Field and laboratory studies have yet to reveal the major factor responsible for up-regulation of toxin synthesis (Vezie *et al*. 2002).

2.3.1.2 Secondary metabolite action

Secondary metabolites of cyanobacteria are often action specific (Skulberg 2000). Under selected combinations of physicochemical conditions metabolites will act only on explicit receptor sites on a target organism. For example, the neurotoxin homoanatoxin-a targets specific ion channels in nerve and muscle membranes (Skulberg 2000); while microcystin binds to and inhibits protein phosphatases 1 and 2A, the control enzymes for metabolism (Lurling 2003). Secondary biosynthetic pathways, both *in vitro* and *in vivo*, are highly regulated systems (Skulberg 2000). In some circumstances biological activity may be manipulated, either constitutively or induced (Safonova and Reisser 2005). Constitutive antimicrobial activity is always present in the algal culture medium, while inducible activity is only expressed when microalgae is in contact with bacteria or other pathogens (Safonova and Reisser 2005).

2.3.1.3 Toxin Detection

Toxicity of cyanobacteria has previously been monitored by mouse bioassay; however this method is not always suitable, as it is unable to measure low concentrations of toxin in populations of cyanobacteria that either do not form scums, or have not yet accumulated enough biomass to form a scum (Sinoven and Jones 1999). Development of HPLC and Enzyme Linked Immunosorbent Assays (ELISA) have contributed greatly to detection and quantification of total and individual toxins present in an ecosystem (Sinoven and Jones 1999). The first molecular biological investigations in cyanobacteria focused on the possible involvement of plasmids in toxin production.

However, this was proven unlikely, as plasmids were found in both toxin and non-toxin producing strains of one species, and several toxic strains contained no detectable plasmids (Sinoven and Jones 1999). More recently, biochemical and genetic studies have identified a hybrid polyketide/non-ribosomal peptide synthetase origin of several toxins, including microcystin (Tillett *et al.* 2000). However, relatively few metabolites of mixed origin have been genetically characterised (Tillett *et al.* 2000), and significant research is required to identify biosynthetic pathways for production of bioactive or cytotoxic secondary metabolites.

2.4 Growth Environment

2.4.1 Response to changes in environment (stressors)

Cyanobacteria often dominate summer communities of phytoplankton in eutrophic reservoirs (Yamamoto and Nakahara 2005). Assemblages comprise several species, and each community is unique to the particular water body and environmental conditions (Sinoven and Jones 1999). Coexistence may be attributable to species experiencing different limiting conditions simultaneously (Reynolds 1984), implying that the species are not adapted to perform identical functions. Resources for which microalgae compete extend beyond nutrients (e.g. light), and these parameters influence the competitive status of the species present (Reynolds 1984). Cells face significant stress when exposed to solar irradiation for long periods; UVB in particular affects growth, pigmentation, photosynthesis and metabolism of cells (Xue *et al*. 2005). Consequently, cyanobacteria have evolved various repair and avoidance mechanisms to combat photooxidative damage to cells.

Metal cations are essential to maintain cellular metabolism of cyanobacteria (Baptista and Vasconcelos 2006); however, different species rely on the bioavailability of selective cations, at highly variable concentrations. Bioavailability of ions in the immediate environment will considerably influence the dominating species in the water body (Baptista and Vasconcelos 2006). Changes in environmental conditions may be defined based on the response that cells undergo as a result of sensing that change, either a limiting condition or a stress (Vonshak and Torzillo 2004). A limiting condition determines the rate of growth or biochemical reaction taking place within the cell; a change in nutrient concentration will result in a change in rate, without the

need for an acclimation process. Conversely, a stress is defined as a change in environmental conditions that requires both metabolic and biochemical adjustments before a new steady state may be achieved (Vonshak and Torzillo 2004).

Availability of growth factors, particularly light, exerts a major impact on structure of microbial communities (Huisman *et al*. 1999). Competition for nutrients between members of an aquatic community creates a dynamic relationship between those members. Legrand *et al* (2003) suggests that ". . . natural selection favours [allelopathic] compound production, given that this reduces competition and improves resource availability. . . ". The inherent implication is that biosynthesis of compounds able to influence survival of other organisms in the environment will greatly enhance the likelihood of a single cyanobacterial species dominating the environment. Le Flaive and Ten-Hage (2007) acknowledge that long term co-existence within a habitat leads to necessary adaptation of organisms to each other. However, a change in environmental conditions, such as those described above, will favour survival of a particular organism determined by the resources affected. The persistence of a species depends on its competitive capabilities – species succession is often explained as a direct consequence of competition (LeFlaive and Ten-Hage 2007)

2.4.2 Bloom formation

Investigations into bloom-forming cyanobacterial taxa are gaining interest due to their potential to disrupt marine food chains and cause toxicity to humans (Wolfe 2000). Excessive growth of cyanobacteria creates severe water quality and health hazards (Rinehart *et al*. 1994; Skulberg 2000). High cell density increases water turbidity, thus lowering light penetration, conditions favourable to further cyanobacterial growth (Regan 1988; Mur *et al*. 1999). Blooms occur in eutrophic and polluted water bodies and are recognised as instigators of poisonings to wildlife and domestic animals via toxin synthesis (Ward and Targett 1989; Lee *et al*. 2000). The field site selected for this study is prone to cyanobacterial blooms; the photograph labelled Figure 2-2 illustrates characteristic traits of a bloom in the Torrens Lake, a eutrophicated waterway, including surface scum and colonisation of the cells. Those species which form surface blooms or "scums", appear to have an elevated tolerance for high light intensities; this trait is characteristic of carotenoid synthesisers, a protein contributing to protection of cells from photoinhibition (Mur *et al*. 1999; Dignum *et al*. 2005).

Bloom formation is enhanced by a neutral or slightly alkaline pH, light winds (gentle mixing) and low nitrogen to phosphorous ratio (Wicks and Thiel 1990; Mur *et al*. 1999; Paczuska and Kosakowska 2003; Yamamoto and Nakahara 2005). Timing and duration of cyanobacterial blooms is heavily dependent on climatic conditions of the region (Vezie *et al*. 2002). In temperate zones blooms are most prominent during late summer and early autumn, with the bloom season lasting 2-4 months. However, in regions with Mediterranean or subtropical weather patterns, the bloom season starts earlier and persists for longer periods (Sinoven and Jones 1999).

Figure 2-2: A cyanobacterial bloom on the Torrens Lake, Adelaide, February 2005

Colony formation has several ecologically important factors relating to the survival of bloom-forming cyanobacteria. Cells within a colony are protected from grazing by zooplankton and other predators due to the indigestible size of the colony (Reynolds 1997). Colonies of cells that possess gas vacuoles undergo rapid upward movements to maximise exposure to light; however, this passage may result in photodamage to cells due to prolonged exposure to full sunlight (Ibelings 1996; Mur *et al*. 1999). Toxicity of some cyanobacterial species to grazers is thought to be another component of their survival mechanism, favouring bloom formation and persistence (Mundt *et al*. 2001; Lurling 2003; Agrawal *et al*. 2005; Wilson *et al*. 2005). The role and mechanism of action of these "anti-predator" defences in bloom maintenance is poorly understood (Wolfe 2000; Codd *et al*. 2005) as bloom species may vary from

toxic to non-toxic from one season to the next within the same water body (Wilson *et al*. 2005).

2.5 Target Species: *Microcystis*

2.5.1 Genus *Microcystis*

Microcystis is a unicellular, colony-forming, non-nitrogen fixing photosynthetic cyanobacterium (Asayama *et al*. 1996; Visser *et al*. 2005), notorious for blooms in water bodies high in nutrients (Sinoven and Jones 1999; Welker *et al*. 2006). Two of the most widespread species of *Microcystis* are *M. aeruginosa* and *M. flos-aquae*. Cells of *M. flos-aquae*, observed in Figure 2-3, are approximately 3-4 µm in diameter, possess gas vesicles for buoyancy regulation (discussed below) and form spherical or lens-shaped colonies. Similarly, cells of *M. aeruginosa* aggregate to form colonies such as those evident in Figure 2-4 and also possess gas vesicles to regulate their position in the vertical water column, thus maximising light exposure.

Growth of *Microcystis* is strongly influenced by a variety of environmental factors; however, this species is ranked amongst the poorest resource competitors and is one of the slowest growing species of phytoplankton (Reynolds 1997; Mur *et al*. 1999). Maximum growth rates occur at temperatures 25-28 °C, (Mur *et al*. 1999; Kardinaal and Visser 2005), whilst the presence of gas vesicles within the cells allows *Microcystis* to alter position in the vertical water column to attain optimum light intensity (Mur *et al*. 1999). Cell buoyancy is also affected by nutrient concentration and availability, particularly phosphate concentration (Visser *et al*. 2005). Manipulation of cellular position in the vertical water column to maximise light absorption provides cyanobacteria with a distinct advantage over other species (Kardinaal and Visser 2005; Yamamoto and Nakahara 2005); however, regulation is only possible in lakes with a shallow euphotic depth (Mur *et al*. 1999) where *Microcystis* can benefit fully from buoyancy regulation (Visser *et al*. 2005)

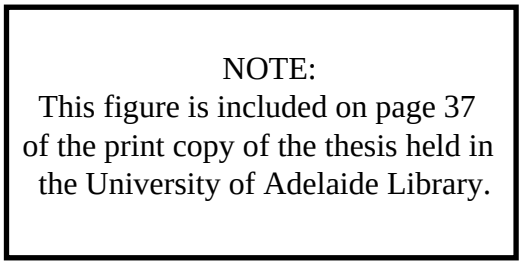

Figure 2-3: *Microcystis flos-aquae*. The individual cells in a *M. flos-aquae* colony are 3-4 µm in diameter; the colony is spherical or lens-shaped, with varying degree of spacing between cells within a colony. Dark stains visble in the individual cells are due to reflection of light from the gas vesicles. A number of dinoflagellates are attached to the upper left side of the colony (Micro*scope 2006).

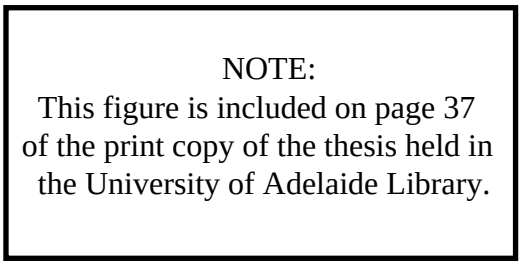

Figure 2-4: *Microcystis aeruginosa*. Cells aggregate to form colonies, a characteristic feature of bloom-forming species (Cyanosite 1997).

The slow growth rate (average doubling time, t_d, 3-4 days (Wilson *et al*. 2006); c.f. generation time of *E coli* 15 min) of *Microcystis* requires a long water retention time to establish a bloom, thus *Microcystis* is rarely able to maintain a bloom in highly turbulent or dynamic waterways (Mur *et al*. 1999). Bloom formation is also affected by nutrient loadings, interactions between light and water, pH and the tolerance of cells to low O_2 concentrations and a low redox potential (Visser *et al*. 2005). However, as loss rates of this species are generally low once a population is established, their slow growth is compensated for by maintaining high cell numbers

(Mur *et al*. 1999). This genus is most commonly recognised for excretion of the toxin microcystin, though not all strains are toxigenic (Ferrao-Filho *et al*. 2000). Synthesis of microcystin is coupled to the cell cycle, with maximum toxin synthesis occurring in the late logarithmic and early stationary phase of growth (Watanabe *et al*. 1989; Vezie *et al*. 2002). Many strains are able to synthesise several variations of microcystin simultaneously, though usually only one or two variants dominate (Sinoven and Jones 1999). *Microcystis*, most often *M. aeruginosa* (Watanabe *et al*. 1989; Wicks and Thiel 1990; Sinoven and Jones 1999; Lee *et al*. 2000) is frequently present in or responsible for cyanobacterial blooms (Singh *et al*. 1998; Janse *et al*. 2004).

Microcystis has a high requirement for iron (Paczuska and Kosakowska 2003) - availability is essential for cell proliferation, enzymatic and metabolic processes, and to maintain a functional photosystem 1 complex (Paczuska and Kosakowska 2003; Keren *et al*. 2004). Synthesis of siderophores, specific iron-complexing compounds, enables cyanobacteria to solubilize Fe^{3+} ions, recycling them through the cell population to maintain a high biomass (Imai *et al*. 1999). Iron uptake by siderophores is thought to be a contributing factor to the dominance of *Microcystis* over eukaryotic algae (Gladue and Maxey 1994; Imai *et al*. 1999; Gress *et al*. 2004), and increases in iron concentrations have been reported to selectively stimulate cyanobacterial growth (Imai *et al*. 1999). However, the presence of natural chelators significantly effects growth of *Microcystis* by forming complexes with Fe ions, reducing bioavailability to cyanobacteria (Imai *et al*. 1999; Paczuska and Kosakowska 2003; Gress *et al*. 2004). Consequently these compounds may play an essential role in formation and dispersion of *Microcystis*-dominated blooms in eutrophic reservoirs (Imai *et al*. 1999).

2.5.2 Bioactive peptides of *Microcystis* sp.

Microcystis is an exceptionally rich source of peptide metabolites (Namikoshi and Rinehart 1996; Dittmann *et al*. 1997; Saker *et al*. 2005). The presence of bioactive compounds in *Microcystis* cultures, in addition to microcystin, was first reported by Weckesser and co-workers (1989) with the isolation of cyanopeptolins A-D from *Microcystis* aeruginosa PCC 7806 (Namikoshi and Rinehart 1996)[1]. Peptides of

[1]Note that the reference cited for this statement did not affirm responsibility of Weckesser as first reporting this phenomenon; however, Weckesser was included as an author on the paper concerned

Microcystis are often classified according to shared structural properties, for example microcystins, cyanopeptolins, and aeruginosins (Welker *et al*. 2006); however there is limited knowledge available on the occurrence of individual peptides and peptide classes in environmental samples. Production of these compounds appears advantageous to *Microcystis*, regardless of the geographic location and trophic state of their natural habitat (Welker *et al*. 2006), and many peptides, such as micropeptin, microginin and anabaenopeptin are produced in the natural environment and under laboratory conditions (Kodani *et al*. 1999; Harada 2004). *Microcystis* has been extensively screened for bioactive peptide synthesis (Bister *et al*. 2004); however studies driven by pharmacological interests have revealed little regarding physiological and ecological functions (Kodani *et al*. 1999; Welker *et al*. 2006).

Structural variation in peptides can significantly influence the bioactivity of the compound. Mass spectrometric (MS) analysis of *Microcystis* colonies frequently detects new structural variants of known peptide classes, with variations differing by changes in amino acids, or by modifications including methylation, chlorination and glycosilation (Welker *et al*. 2006). Figure 2-5 illustrates the structure of microcystin-LR, possessing the amion acid leucine at position X, and arginine at position Y (Dittmann and Wiegand 2006).

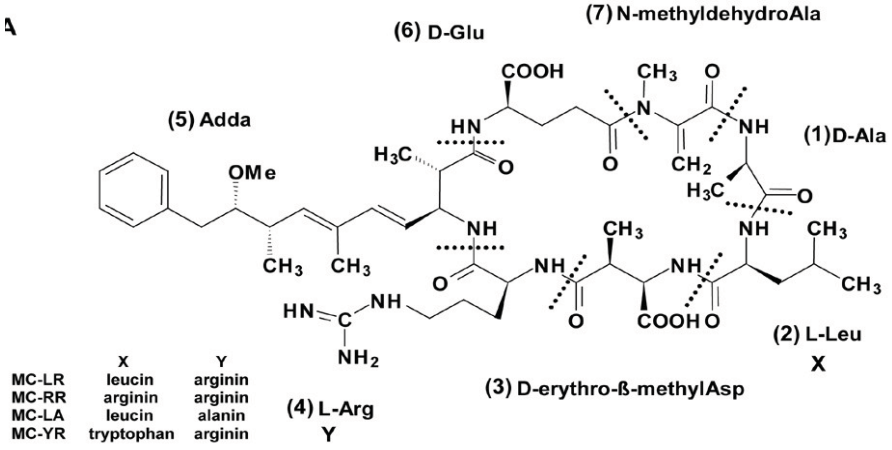

Figure 2-5: Structure of the hepatotoxin microcystin-LR. Amino acids at positions X and Y are variable (Dittmann and Wiegand 2006)

Diversity appears to be achieved *in vivo* by combinatorial biochemistry, with amino acid sequences seemingly determined by individual peptide synthetases giving rise to new peptides (Welker *et al*. 2006). Illustrating this assertion, more than 80

microcystin-type peptides have been reported, with varying levels of toxicity (Kodani *et al*. 1999; Dittmann and Wiegand 2006); examples of these modifications are supplied in Table 2-5. This variation also applies to other oligopeptides. For example, daphnid trypsin inhibition by cyanopeptolins is dependent on the presence of Lys or Arg in position 2 of the cyclic portion of the peptide; this inhibition is lost if these amino acids are replace by Tyr (Welker *et al*. 2006). Similarly, particular modifications to peptides occur within one structural class, but do not necessarily apply to other classes. Chlorination is often noted in aeruginosins, but not in microginin-type peptides (Welker *et al*. 2006), while methylation is frequently observed in microcystins (Rinehart *et al*. 1994).

Table 2-5: Microcystin-type peptides and variants reported in literature

Species/strain	Microcystin variant	Modification	Original Source
M. aeruginosa, M. viridis	microcystin-LR	Leucine-Arginine	(Watanabe *et al*. 1989)
M. aeruginosa, M. viridis	microcystin-YR	Tyrosine-Arginine	(Watanabe *et al*. 1989)
M. viridis	microcystin-RR	Arginine-Arginine	(Watanabe *et al*. 1989)
Microcystis sp.	microcystin-YM	Tyrosine-Methionine	(Watanabe *et al*. 1989)
	microcystin-YA	Tyrosine-Alanine	(Watanabe *et al*. 1989)
	microcystin-LA	Leucine-Alanine	(Watanabe *et al*. 1989)
M. aeruginosa	cyanoginosin-LA	Leucine-Alanine	(Botes *et al*. 1984)
	Cyanoginosin-LR	Leucine-Arginine	(Botes *et al*. 1985)
	Cyanoginosin-YR	Tyrosine-Arginine	(Botes *et al*. 1985)
	Cyanoginosin-YA	Tyrosine-Alanine	(Botes *et al*. 1985)
	Cyanoginosin-YM	Tyrosine-Methionine	(Botes *et al*. 1985)
	microcystin-LY	Leucine-Tyrosine	(Cuvin-Aralar *et al*. 2002)
	microcystin -LW	Leucine-Tryptophan	(Cuvin-Aralar *et al*. 2002)
	microcystin -LF	Leucine-Phenylalanine	(Cuvin-Aralar *et al*. 2002)
	microcystin -WR	Tryptophan-Arginine	(Cuvin-Aralar *et al*. 2002)
	[D-Asp] microcystin-RR	D-Aspartic acid	(Cuvin-Aralar *et al*. 2002)
	[D-Asp] microcystin-LR	D-Aspartic acid	(Cuvin-Aralar *et al*. 2002)

A number of *Microcystis* strains have been recognised for synthesis of peptidic metabolites in varying quantities, and with diverse structural characteristics, and consequently inhibitory activities. *M. aeruginosa* NIES-98 (Kützing Lemmermann) is known to produce unusual peptides in large quantities, including aeruginoguanidines 98-A-98-C (Ishida *et al*. 2002). These compounds have a highly hydrophobic and a strong hydrophilic component, are not soluble in H_2O or organic solvents, and have demonstrated moderate cytotoxicity against P388 leukaemia cells (Namikoshi and Rinehart 1996; Ishida *et al*. 2002). Microcyclamide, the first example of a cyclic hexapeptide produced by *M. aeruginosa*, has also confirmed cytotoxicity towards P388 leukaemia cells (Ishida *et al*. 2000), and these compounds have also been recognised for antibacterial and antifungal activity (Ishida *et al*. 2000). Demonstrating that compounds with similar structures do not necessarily exhibit the same biological activity, two microcyclamide compounds isolated from *M. aeruginosa* PCC 7806 were found to be inactive against P388 leukaemia cells and produced negative results when subjected to antibacterial and antifungal assays (Ziemert *et al.* 2008).

Okino *et al* (1993) isolated micropeptins A and B from *M. aeruginosa* NIES-100 (Kützing Lemmermann), a strain already recognised for production of the enzyme inhibitor, microginin. Both micropeptin A and B inhibit the serine proteases plasmin and trypsin. A micropeptin structural variant, micropeptin 90 was isolated from *M. aeruginosa* NIES-90 (Kützing Lemmermann), and again inhibited activity of serine proteases (Ishida *et al*. 1995). Cyanopeptolin 963A is a known compound of *Microcystis* sp., and was found to be an inhibitor of chymotrypsin (Bister *et al*. 2004). Aeurginopeptin 228A was isolated from a Japanese strain of *M. aeruginosa* M228 by Harada's research group, while cyanopeptolin A was detected in a bloom of *M. aeruginosa* PCC 7806 (Harada 2004). Recently, Voloshko *et al* (2008) isolated 9 protease and chymotrypsin inhibitors within natural samples, including aeruginopeptin 917S-A, anabaenopeptin F, microcin SF608, micropeptin T-20, nodulapeptin B, planktopeptin BL 1061, oscillapeptilide 97-A, and oscillapeptilide 97-B (Voloshko *et al.* 2008). Other examples of secondary metabolites synthesised by *Microcystis* sp and their activities were compiled by Skulberg (2000) and are shown in Table 2-6.

In section §2.3.1 it was noted that cyanobacteria ". . . possess both toxigenic and non-toxigenic strains within a species and both strains may exist concurrently" *(*Vezie *et al*. 1998; Sinoven and Jones 1999). Harada (2004) hypothesised that non-toxic peptides are co-produced with microcystin, and may assist in facilitating activity of the toxins. Conversely, Saker *et al* (2005) detected anabaenopeptin and aeruginosamide only in strains that did not synthesise microcystin, while microviridins have been detected in both toxic and non-toxic cyanobacteria (Harada 2004). This may due to the potency of the toxins overwhelming any biological activity of other peptides, consequently making non-toxic peptides difficult to detect. Birk *et al* (1989) found an interesting phenomenon in production of non-toxic peptides with D-amino acids and unusual amino acid residues in addition to the toxin synthesised by *M. aeruginosa* PCC 7806.

Table 2-6: Examples of secondary metabolites isolated from strains of *Microcystis* sp. (Skulberg 2000)

Organism	Active Compound	Effect	References
Microcystis aeruginosa	lipid	algicide	(Ikawa *et al*. 1996)
Microcystis aeruginosa	micropeptin 478-A micropeptin 478-B	Plasmin inhibitor	(Ishida *et al*. 1997)
Microcystis aeruginosa	microginin 299-A microginin 299-B	Leucin aminopeptidase inhibitor	(Ishida *et al*. 1997)
Microcystis aeruginosa	Kawaguchipeptin B	Bactericide	(Ishida *et al*. 1997)
Microcystis aeruginosa	Aqueous extract	Antiviral (influenza A)	(Nowotny *et al*. 1997)
Microcystis viridis	aeruginosin 102-A aeruginosin 102-B	Thrombin inhibitor	(Matsuda *et al*. 1996)
Microcystis viridis	Micropeptin 103	Chymotrypsin inhibitor	(Murakami *et al*. 1997)

2.5.2.1 Peptide Synthetases

Research into microcystin production has elucidated a metabolic pathway involving non-ribosomal synthesis via a multienzyme complex of peptide synthetases, encoded by the *mcy* genes (Meißner *et al*. 1996). The small size, cyclic structure and inclusion of unusual amino acids, such as the ADDA motif, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, suggest microcystin is assembled by an operon of peptide synthetase enzymes (Tillett *et al.* 2000); cyanobacterial peptides with similar structures, containing non-protein amino acids are also likely to be products of a multifunctional peptide synthetase (Rouhiainen *et al*. 2000; Fujii *et al*. 2002). Dittman *et al* (2001) has demonstrated the presence of non-ribosomal peptide synthetase (NRPS) genes for bloom-forming strains of *Anabaena flos-aquae*, *Oscillatoria agardhii* (Argardh) and *Nodularia spumigena* (Mertens); however, the ability to synthesise non-ribosomal peptides is not considered a general feature of cyanobacteria.

Opposing views arise concerning the presence and function of peptide synthetase genes in toxic and non-toxic cyanobacteria. Contrary to Nishizawa's (1999) speculation that non-toxic strains "may lack a complete set of *mcy* genes". Meißner *et al* (1996) suggests both phenotypes possess relevant genes, though they may be down-regulated in non-toxic strains or involved in synthesis of other secondary metabolites. Namikoshi *et al* (1996) recognises ". . . toxic and non-toxic strains

produce similar peptides . . .", and Dittman *et al* (2001) suggests some cyanobacterial genera 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.

Isolation of microcystin and bioactive metabolites from a toxigenic strain of *M. aeruginosa* leads Harada (2004) to question the relationship between biotoxin and bioactive peptide production. Fujii *et al* (2002) supports Harada's view that toxic strains contain peptide synthetase genes for other classes of peptides, and that ". . . production of these peptides is related to that of hepatotoxic peptides . . ." However, Meißner *et al* (2002) draws a subtle distinction in presence and function of peptide synthetases in toxic and non-toxic strains, with enzymes in hepatotoxic strains involved primarily with toxin formation, and those in non-toxic strains implicated in synthesis of bioactive peptides. Overall expression of biosynthetic proteins may first depend on possession of relevant genes, then restrictions imposed by environmental conditions, which remain to be elucidated (Meißner *et al*. 1996; Dittmann *et al*. 2001). The presence, and function, of *mcy* genes within a non-toxic isolate of *Microcystis* will form a significant aspect of this research.

2.6 Summary

Microalgae and cyanobacteria are recognised as a rich source of pharmacologically active secondary metabolites. However, while numerous screening programmes have identified species that synthesise these compounds, only a small number of metabolites have been structurally characterised and fewer still have been examined at the molecular level (Tillett *et al.* 2000). Many programmes primarily screen laboratory-cultivated strains, overlooking the potential of natural or "wild" species. Assessment of the bioactive properties of a locally-obtained, non-toxic strain of *Microcystis flos-aquae*, and the subsequent structural elucidation of the compound and molecular investigation reported in this thesis will make a significant contribution to closing these gaps and establish the potential of this species for development of natural therapeutic agents.

Currently there has been minimal research undertaken regarding function of peptide synthetases in toxic and non-toxic strains of cyanobacteria. Identification of the

enzymes involved in non-toxic bioactive compound synthesis and their genetic code will allow rapid comparison of toxic and non-toxic genomes to discern their respective role. The following chapter provides a detailed discussion of selected techniques available for microalgal cultivation, bioactivity assessment, structure determination and molecular investigation. A comprehensive literature review of these topics is available in APPENDIX B. A more directed approach is presented in Chapter 4, outlining the techniques developed and applied during this research.

CHAPTER 3 PROJECT METHODOLOGY

3.1 Introduction

Techniques available for collection and cultivation of microalgae, assessment of bioactivity, and structure elucidation of interesting compounds are as diverse as the research teams that develop them. A comprehensive review of literature related to these techniques is presented in APPENDIX B, highlighting a number of issues that require consideration when undertaking such an investigation. Subsequently, several of these techniques were selected based on their merits, and the resources available, and were developed for application in this study. General project methodology and the project field site are discussed within this chapter. Specific procedures are detailed in following chapters.

3.2 Field Site: Torrens River Catchment System, Adelaide. South Australia

The Torrens River is a freshwater urban run-off, and is the major water system in the Adelaide region (Mugavin 2004). The river is approximately 85 km long, rising from the Mt Lofty Ranges in the Adelaide Hills, passing west across the Adelaide Plains through the City of Adelaide before discharging through the Gulf of St Vincent outlet at West Beach (Gale *et al.* 2006). Prior to 1937, the river flowed into lagoonal swamps dammed by the coastal dune ridge; however to alleviate seasonal flooding of the coastal suburbs, an artificial channel was constructed (Gale *et al*. 2006). Erection of a series of weirs, sluice gates and reservoirs along the main river conduit served to maintain water levels (Gale *et al*. 2006). A weir constructed on the perimeter of the CBD in 1881 created the Torrens Lake (Mugavin 2004), a popular site for recreational activities and wildlife. The current path of the river is identified in Figure 3-1, and the field site for this study is highlighted in Figure 3-2. The Torrens River and adjacent parks were eventually recognised as a "multi-objective greenway", providing recreation and refuge, but also emphasising habitat conservation, water quality improvement, alleviation of flood flow resulting from urbanisation and conservation of heritage (Mugavin 2004).

There is strong evidence that human activity has significantly influenced levels of phosphorous and other contaminants in the Torrens River, though much of this appears to be a direct result of past pollution practices (Gale *et al*. 2006). However, cyanobacterial blooms are often prominent following summer rainfall due to an increased nutrient load. Prior to major settlement, the river was utilised as both a source of town water and a sewer, with many industrial companies along the banks of the river discharging waste into the water (Gale *et al*. 2006). Sand, gravel and clay were mined from the river bed, and natural vegetation stripped from the banks leading to an outbreak of noxious weeds and a regression in species diversity due to loss of habitat (Mugavin 2004). Sluice gates and weirs along the river have also had an inadvertently negative effect, by restricting free flow of water and consequently preventing flushing of contaminated bed sediments or dilution with relatively uncontaminated water (Gale *et al*. 2006). River flow during summer is negligible or non-existent, and combined with release of nutrients from the sediment and an increased nutrient loading from the upstream urban section of the catchment, has contributed to occurrence of major cyanobacterial blooms in the river (Gale *et al*. 2006). These blooms are most often of the species *Microcystis* or *Anabaena*, may be toxic or non-toxic and undergo several species successions during the bloom season. Non-toxic *Microcystis flos-aquae* collected from the Torrens Lake in February 2005 was selected as the species of interest in this study, following encouraging results during preliminary assessments of bioactivity.

NOTE:
This figure is included on page 47 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3-1: The Torrens River rises from the Mt Lofty Ranges in the east and discharges into the Gulf of St Vincent through an outlet at West Beach (Hogan 1995)

NOTE:
This figure is included on page 48 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3-2: The Torrens River, Adelaide, as it passes through the CBD. The Torrens Lake (circled) was formed by the construction of the weir circa 1881 (Google-Earth 2007)

3.3 Universal Methods of Microalgal Cultivation

Successful application of microalgal biotechnology relies on selection of a species with pertinent properties for specific culture conditions and products (Pulz and Gross 2004). Alternatively, the culture environ may be manipulated to promote growth of a species for a specific purpose. A basic knowledge of microalgal physiology, ecology and taxonomy is thus imperative (Pulz and Gross 2004), as initial cultivation conditions will implicitly influence the success of the final application. However, there are no definitive rules for purification and culture of cyanobacteria, and intuition is required of the researcher (Court *et al*. 1981; Castenholz 1988). A comprehensive review of methods undertaken to determine those suitable for the work conducted during this research is presented in APPENDIX B. Strategies applied during this study are presented below.

3.3.1 Microalgal Cultivation

The aim of microalgal cultivation is to optimise growth and product yield of a species for a specific application by manipulating the culture environment. Principles of microbial cultivation are applicable to cyanobacteria; however, the added capability

of microalgae to harvest light energy for growth yields discrete differences in specific culturing environments (Lee and Shen 2004).

The method of cultivation will be determined by species requirements, application and the growth state (balanced or unbalanced) of the cells (MacIntyre and Cullen 2005). If conditions are constant (within reasonable limits), cells will eventually acclimatise to their environment; growth will be balanced, and physiological responses will be in equilibrium. Similarly, if cultures respond to repeatable and predictable changes, equilibrium may be dynamic but growth is still balanced. Cultures subjected to step changes in conditions will not achieve equilibrium and growth will be unbalanced (MacIntyre and Cullen 2005). The latter scenario of unbalanced growth is characteristic of natural conditions which may be subject to numerous step changes in a short period.

Early attempts to obtain data on the nutrition and physiology of cyanobacteria were instigated by Gerloff *et al* (1950) during the late 1940's. Gerloff *et al* (1950) had acknowledged that despite a natural dominance of microalgae, particularly cyanobacteria, in the field, minimal work had been undertaken to determine their nutritional requirements and physiology when grown under controlled conditions Microalgae are recognised as sensitive indicators of ecological change in their immediate environment (Kelly *et al*. 1998), and under natural conditions exist as mixed assemblages which include other algal taxa and microorganisms (Day *et al*. 1999). Maintenance of *in vitro* cultures are thus important in gaining an understanding in microbial ecology (Day *et al*. 1999). Castenholz (1988) urges compilation of as much ecological information as possible about the field population when establishing new cultures of cyanobacteria. Eloff (1981) suggested that cells in natural and laboratory cultures are not significantly dissimilar, but there is an increased resistance of natural cells to light. However, research undertaken initially by Gerloff *et al* (1950), and later by Reynolds (1984), Sly (1994), Neilan (1995) and Kondo *et al* (2000) refute this claim, recognising that there are considerable difficulties in matching wild cyanobacteria to cultured material, whose morphology may alter so radically under laboratory conditions from that of the original isolate as to be scarcely recognizable.

An experiment by Eloff (1981) established the importance of bacteria present in natural samples as protectors of *Microcystis* from photoinhibition. The absence of bacteria in laboratory samples rendered cyanobacterial cells notably light sensitive. However, Gerloff *et al* (1950) claimed during early experiments that removal of bacteria is essential in nutrition experiments to ensure the data is specific for microalgae. Scott *et al* (1981) observed that a natural population of *Microcystis aeruginosa* reverted from its colonial form to a unicellular form during laboratory cultivation. Further, Vance (1966) had found that following a number of purification steps, only cultures of *Microcystis* contaminated by bacteria survived. Thus results achieved in the laboratory do not always contribute valid data on growth requirements of bloom-forming cyanobacteria. Conversely, Reynolds *et al* (1981) believed a laboratory approach to be suitable, but highlights the need to relate laboratory studies to the behaviour of natural populations. Similarly, environmental variations will influence metabolite production which may not be reproducible in a stable laboratory atmosphere. For example, toxin production by *Microcystis* was observed by Shirai *et al* (1991) to be inconsistent during the bloom, and surmised that synthesis is influenced by environmental factors. A clear comprehension of the mechanisms that drive natural cycles of microalgal productivity will come chiefly from quantitative comparisons between natural assemblages and cultures (MacIntyre and Cullen 2005).

3.3.2 Media for Microalgal Cultivation

The distribution of microalgal species in freshwater is influenced by the selective action of the physicochemical environment and the ability of the organism to survive and colonise in that environment (Watanabe 2005). Consequently, the composition of synthetic media selected for cultivation of freshwater microalgae in the laboratory will be implicitly defined by the species to be cultured and essential nutrient requirements observed for that species within a natural environment. Variations to the accepted media recipes may be necessary to optimise growth of cells, particularly those collected from unique habitats. Chemical constituents for media preparation should be of the highest quality available; care should be taken to observe the manufacturer's specifications as some chemicals may contain trace metals or contaminants that may inhibit growth of the microalgae (Watanabe 2005). Similarly, tap water and some distilled waters may contain high levels of heavy metals;

constituting a significant proportion of the media, water should be double-distilled to remove all impurities, though single-distilled or deionised water are also acceptable. The level of quality of both chemical constituents and water is dictated by the sensitivity of the microalgae to impurities (Watanabe 2005). Attention should also be given to the pH of the media; adjustment of the pH is often required, and should be conducted prior to sterilisation.

Culturing media are often composed of macronutrients (e.g. a phosphate, carbon or nitrate source), trace elements (e.g. ferrous or potassium ions) and vitamins (e.g. Vitamin B12 - cyanocobalamin). Stock solutions of the trace elements and vitamins may be prepared at concentrations 100 to 1,000 times the required level; a small quantity (e.g. 1 mL) is removed aseptically and used in preparation of the media. Stock solutions provide a convenient source of nutrients required at low doses, avoiding the laborious task of repeatedly weighing individual components (Watanabe 2005). Solutions containing elements that encourage growth of bacteria or fungi must be sterilised prior to use.

Synthetic or artificial media are prepared to provide a simplified, defined media for routine culture maintenance and experimental investigations (Watanabe 2005). Examples of synthetic media commonly used include BG_{11}, Bold's Basal media and WC media; all can be prepared in a liquid or solid form (Watanabe 2005). BG_{11} media was specifically designed for cultivation of cyanobacteria to provide adequate concentrations of nitrate and phosphate (Watanabe 2005). Bold's Basal medium has also been used successfully to cultivate species of cyanobacteria, though is unsuitable for species that require vitamins. Recipes are provided in APPENDIX C for media utilised during this study. Enriched media, created by the addition of essential nutrients to natural lake or river waters, are not as well defined due to the unknown chemical composition of the field samples. Enriched media are discussed further in section §B.II.2 as a component of single cell isolation techniques.

3.4 Bioactivity assessment of non-toxic *M. flos-aquae*

A variety of direct and indirect methods may be applied to detect the presence of secondary metabolites with antimicrobial activity in microalgae and cyanobacteria. Direct assays seek detection of specific target compounds; indirect assays evaluate the

biological activity of desired products (Lee and Shen 2004). Development of analytical methods and instrumentation (HPLC, GC, MS and NMR) provide rapid and highly sensitive analytical techniques to detect pharmacologically active compounds (Cohen 1999). Indirect assays, or bioassays, employ cultured cells or pathogenic microorganisms to screen for antimicrobial activity (Lee and Shen 2004). Both direct and indirect assays were employed in this study to isolate and identify bioactive secondary metabolites synthesised by *M. flos-aquae*; these methods are described in detail in the remainder of this chapter.

Screening programmes for bioactivity of cyanobacterial metabolites are undertaken worldwide, and consequently utilise a variety of different extraction techniques, cell disruption methods and bioactivity assessment protocols. Following a comprehensive literature review, presented in APPENDIX B, the methods outlined in Table 3-1 were selected for extraction of (potential) bioactive metabolites. Preliminary investigations identified the most successful techniques which were applied for all future extractions.

Table 3-1: Cell disruption techniques and extraction methods applied to *Microcystis* biomass

Extract Method	Solvent	Disruption method	Centrifugation	Other	Source
Distilled water	distilled water	agitation 30 min, RT	2241 ×g 10 min @ 4°C	-	(Ostensvik *et al.* 1998)
dH_2O/ Sonication	distilled water	sonication on ice, 2 min: 15s on, 10s off 45% amp	2241 ×g 10 min @ 4°C	-	(Ördög *et al.* 2004)
MeOH/ Sonication	methanol	sonication 2 min on ice: 15s on, 10s off, 45% amp	2241 ×g 10 min @ 4°C	Filtration 0.22µm (Millipore)	(Ohta *et al.* 1998)
Methanol/ Acetone/ Hexane	Methanol, acetone, hexane	2 hrs shaker table 80 rpm	2241 ×g 10 min @ 4°C	Further extract pellet with acetone, hexane	(Cannell *et al.* 1988)
Combined methanol	80 % & 100% v/v Methanol	Agitation 30 min, RT	2241 ×g, 10 min @ 4°C	Repeat extraction with 100% v/v MeOH	(Ishida *et al.* 1997)
Chloroform / Methanol/	2:1 chloroform : methanol	Extraction over low heat for 30 min	Extract portioned with water; aqueous phase discarded	Chloroform removed by flash evaporation	(Ballantine *et al.* 1987)
Methanol/ Hexane	Methanol, Hexane	20 min shaker table, 80 rpm	2500 ×g 10 min @ 4°C	Repeat extraction with hexane	(Kellam and Walker 1989)
3 times MeOH extraction	3 x 100 % v/v MeOH	Agitation 1 hr, 80 rpm, RT	2500 ×g 10 min @ 4°C	Repeat extraction twice. Dilute extracts with Milli-Q® water (final conc. 20% v/v)	(Oudra *et al.* 1998)

3.4.1 Assessment of the antiviral activity of non-toxic *M. flos-aquae*

Stringent requirements to meet explicit criteria have limited the number of antiviral drugs available (Vlietinck and Apers 2001). Compounds ideally should inhibit at least one propagation step of viral infection, demonstrate broad spectrum activity, and must not be immunosuppressive. Evaluation of *in vitro* antiviral activity is governed by the different capacities of viruses to replicate in cell cultures (Vlietinck and Apers 2001). Plaque formation, cytopathic effect and cell transformation are characteristic of a number of viruses and may be used as foundations for bioassay development. Viral replication in cultured cells can be monitored by detection of viral products – DNA, RNA or polypeptides (Vlietinck and Apers 2001).

To ascertain the level of viral infectivity in cultured cells in the presence of a test substance, a number of *in vitro* antiviral screening assays are available. Vlietinck and Apers (2001) describe assays involving plaque inhibition or reduction, inhibition of virus-induced cytopathic effect, virus yield reduction, and quantification of specialised viral function or product synthesis. A variation of the virus yield reduction assay was selected for this study, involving calculation of the 50% tissue culture dose end point, or $TCID_{50}$. In biological quantification, the endpoint is usually taken as "the dilution at which a certain proportion of the test animals [or cultured cells] reacts or dies" (Schmidt and Emmons 1989). However, while 100% endpoint can be used, accuracy is greatly affected by chance variations in the cell culture or test animal. Subsequently, the desirable endpoint is that in which 50% of the animals or cells react, while the other half remain unaffected (Schmidt and Emmons 1989). To avoid the impracticality of the large numbers of test animals required for every dilution point, cell cultures are routinely employed to determine the $TCID_{50}$.

Vlietinck and Apers (2001) explain the virus yield reduction assay as ". . . determination of virus yield in tissue cultures infected with a given amount of virus, and treated with a non-toxic dose of the test substance . . . ". Schmidt and Emmons (1989) elaborated further, stating that $TCID_{50}$ represents the ". . . dose giving rise to cytopathic changes in 50% of inoculated cultures . . ." This technique may be applied in the presence of a single compound or a mixture of compounds, and provides rapid and accurate results (Vlietinck and Apers 2001). Potentially lethal effects of the antiviral agent against the chosen cell line must be evaluated; the substance may exhibit apparent bioactivity via cytopathic effects on the cells. Cytotoxicity of the antiviral agent may be assessed by cell viability or cell growth rate assays performed in the presence of the test substance (Vlietinck and Apers 2001).

3.4.1.1 Cell lines

Traditional approaches for culture of viruses in diagnostic virology involve inoculation of susceptible cell lines (Woods and Young 1988; Schepetiuk and Kok 1993); the type of cells used in viral assays is implicitly determined by the nature of the virus. Woods and Young (1988) observed that virus isolation is both time consuming and expensive, and emphasized the necessity of selecting cell lines that are sensitive and versatile, allowing detection of a number of viruses. Primary cell lines

are the most sensitive tissue culture systems available for initial recovery of particular viruses; however, cultures may vary significantly, and primary cell lines are costly (Woods and Young 1988). Thus, it is imperative that a balance between sensitivity and expense be resolved.

Madin-Darby canine kidney (MDCK) cells are considered the optimal mammalian cell system for growth and isolation of many viruses, particularly influenza, though Govorkova *et al* (1996) noted the line had not yet been licensed for use in commercial vaccine production. However, research instigated by Schepetiuk and Kok (1993) established that use of MDCK cell lines provided an effective medium for routine isolation of influenza viruses, and subsequently were selected as the cell cultures for assessment of the antiviral agent against Influenza virus types A and B for this study. Though the nature of receptor molecules utilized by Dengue virus remains largely unknown (Phoolcharoen and Smith 2004), research undertaken by de Jesús Martínez-Barragán and Angel (2001) identified highly sulphated heparans (HS) on the cell surface, required for Dengue virion binding and entry. Vero cells, an African green monkey kidney cell line (Shafee and AbuBakar 2002; Phoolcharoen and Smith 2004), possess large amounts of HS on the cell surface and are therefore highly susceptible to Dengue virus infection. Vero cells were chosen in this study for assessment of the antiviral agent against Dengue virus.

A continuous cell line derived from human lung carcinoma cells, labeled A-549, has been established as a proficient and economical cell line for isolation of Adenovirus and Herpes Simplex Virus (HSV) (Smith *et al*. 1986). Woods and Young (1988) detected 96% of Adenovirus isolates tested in their study in A-549 cells, and 97.5% of HSV isolates. However, Woods and Young (1988) did note there were some limitations in the use of this cell line, as RSV, rhinovirus and Influenza type A were not recoverable from A-549 cell cultures. Hughes *et al* (1988) also had partial success detecting RSV in cultures of A-549 cells, attaining only 29% culture positive results. Conversely, Rabalais *et al* (1992) found RSV was adequately detectable in A-549 cells. Halstead *et al* (1990) also achieved moderate success culturing RSV in A-549 cells, attaining culture positive results for approximately 48% of specimens. Based on findings by Smith (1986), Woods and Young (1988), Halstead *et al* (1990) and

Rabalais *et al* (1992), A 549 cells were chosen to assess the antiviral activity of the cyanobacterial extracts against Adenovirus, RSV and HSV.

3.4.1.2 $TCID_{50}$ of virus

The Tissue Culture Infection Dose (50%), defined by Vlietinck and Apers (2001), and Schmidt and Emmons (1989) in section §3.4.1, was selected to evaluate the antiviral properties of the cyanobacterial extracts in this study. Extracts were assessed for antiviral activity by Dr TuckWeng Kok, a virologist at the Institute of Medical and Veterinary Science (IMVS; Adelaide, South Australia) against the following viruses: Influenza A, Influenza B, Herpes simplex Virus type 1 (HSV-1), Adenovirus, Respiratory Syncytial (RSV) and Dengue viruses (DV). To ascertain any apparent cytopathic effects of the extracts on the cells, both extracts and solvents were tested against un-inoculated cell lines. Dilutions (10^{-1} to 10^{-8}) of extracts were tested against 200 $TCID_{50}$/200 µl of virus, and cytopathic effects in the presence of the extracts recorded.

3.4.2 Establishment of bioactivity of non-toxic *M. flos-aquae*

3.4.2.1 Optimisation of secondary metabolite extraction

Solvent extraction is often applied to isolate secondary metabolites from cyanobacterial samples, and is frequently used in conjunction with freeze-drying of cell biomass (Cannell *et al.* 1988; Mundt *et al.* 2001). A number of solvents were examined during this study, in combinations and at varying concentrations to optimise extraction of secondary metabolites from *M. flos-aquae* biomass. Moderate success has been achieved by some research groups using n-hexane, acetone and chloroform, due to the suspected non-polar nature of bioactive compounds (Cannell *et al*. 1988; Kellam and Walker 1989; Ishida *et al*. 1997); subsequently these solvents were selected during preliminary screening of cyanobacteria to extract secondary metabolites from biomass. Methanol has been recommended by Fastner *et al* (1998) as a preferred solvent for cyanobacterial samples (Fastner *et al*. 1998) and was included accordingly for the extraction of bioactive compounds in this study. Similarly, Cannell *et al* (1988) predominantly employed methanol for extracting algal pellets, though sequential extractions using methanol, acetone and *n*-hexane were also applied to some cell pellets.

3.5 Chemical Structure Analysis

Chemical structure analysis techniques were selected based on their efficacy in isolating the compound of interest and resolution of the metabolite constituents. A brief overview of the two primary techniques, High Pressure Liquid Chromatography and Mass Spectrometry is provided below, and a more detailed explanation is provided in section §B.III, APPENDIX B.

High Pressure (Performance) Liquid Chromatography is one of the most powerful analytical tools available for separation, identification and quantification of components within an aqueous sample (Waters 2007). HPLC can be applied to diverse industries, including pharmaceuticals, food, forensic science and environmental assessment. Reverse phase HPLC is applied to separate compounds based on their hydrophobic character, making it an ideal instrument for isolation of peptides (Guzzetta 2001). Columns are commonly packed with silica particles, and may be of varying lengths and diameters (Guzzetta 2001); column selection will depend on the analysis to be conducted and sample type involved.

Mass spectrometry (MS) is an analytical tool used to measure the molecular mass or molecular formula of a sample (Bailey and Bailey 1995; Ashcroft 2004), or more specifically, the mass-to-charge ratio of ions in a sample. Mass spectrometry is generally utilized to resolve the composition of a physical sample, by generating a mass spectrum representing the masses of sample components. The technique encompasses diverse applications, including: identification of unknown compound molecules or their fragments; structure elucidation of a compound by observing the fragmentation pattern; quantifying the amount of a compound in a sample using a mass spectrometer with multiple analysers (mass spectrometry is not intrinsically quantitative); and determination of other physical, chemical and biological properties of compounds when combined with other techniques (Ashcroft 2004).

3.6 DNA Extraction Techniques

DNA-based detection techniques have increased in popularity due to the potential specificity, sensitivity and speed with which they can be performed (Ouellette and Wilhelm 2003). However, preparation of high quality genomic DNA from microalgae

and cyanobacteria is often time intensive, given the requirement to remove contaminating material that may interfere with downstream processing (Wu *et al*. 2000). Elimination of contaminants is a vital step in DNA extraction, particularly for cyanobacteria and microalgae that are rich in polysaccharides and polyphenols, substances known to interfere with enzymatic manipulation of genomic DNA (Wu *et al*. 2000). Consequently, emphasis has been placed on development of technology and methods for extraction and quantification of genomic DNA (Richlen and Barber 2005). However, limited success of DNA extraction from cyanobacterial cells has imposed restrictions on the species investigated and the hypotheses examined (Richlen and Barber 2005).

Previously, the vast majority of techniques required cultivable species to provide sufficient biomass; the alternative involved DNA extraction and polymerase chain reaction (PCR) amplification followed denaturing gradient gel electrophoresis. (DGGE) (Wang *et al*. 2005). Wu *et al* (2000) refer to the caesium chloride gradient centrifugation technique, highlighting the specialized equipment required to perform the extraction, and the delay in obtaining results of 1 to 2 days. Porter (1988) observes that established methods for physical, chemical and enzymatic lysis of cyanobacterial cells to expose DNA often involve the use of toxic substances; collectively, the above techniques are time consuming and expensive. Field samples, potentially containing few cells, have been near impossible to study, though are of ecological significance given the occurrence of harmful blooms. Ethanol precipitation is also applied frequently, though care must be taken as the addition of ethanol and the subsequent drying stages of the procedure may enhance association of the DNA with contaminants; removal of these substances prior to ethanol precipitation may increase efficacy of the technique (Wu *et al*. 2000). Some species may also possess surface structures that further complicate DNA extraction (Fiore *et al*. 2000). A major obstacle is efficient cell lysis and subsequent access to the genomic DNA (Fiore *et al*. 2000).

The tendency of some species of cyanobacteria, including *Microcystis* sp., to form compact aggregations as a survival mechanism also complicates extraction of DNA, interfering with cell disruption and subsequently altering the final yield of DNA (Fiore *et al*. 2000). Cell disruption methods such as cavitation, homogenization and

sonication are often necessary to suspend the cells prior to extraction. Fiore *et al* (2000) noted that DNA extraction from *M. aeruginosa* was difficult due to their inherent resistance to lysis. However, a review of several different lysis methods by Fiore *et al* (2000) determined the most effective extraction was obtained by pre-treatment of cells with proteinase K.

3.6.1 Polymerase Chain Reaction (PCR)

The principle behind the Polymerase Chain Reaction (PCR) is amplification of DNA molecules *in vitro*, yielding large amounts of DNA for application in cloning, sequencing and mutagenesis investigations (Brock *et al*. 1997). Short, oligonucleotide primers (segments of DNA with a known sequence, complementary to the DNA to be amplified) are used to amplify specific sections of DNA extracted from an organism of interest (Brock *et al*. 1997; Ouellette and Wilhelm 2003). DNA primers are added in excess to heat-denatured target DNA; as the mixture cools, primers anneal to a denatured DNA strand with a complementary sequence. Following a suitable incubation period (modified for a particular primer set), the mixture is heated to separate the primer and DNA strands, then cooled to allow the primers to hybridise with newly synthesized DNA and the cycle is repeated, up to 35 cycles for some primer sets. Thus original DNA concentration doubles each cycle, yielding a 106 to 109-fold increase in target DNA for use in future applications (Brock *et al*. 1997).

PCR cycles can be used for a variety of applications, including amplification of small fragments of DNA (Brock *et al*. 1997; Wang *et al*. 2005) or small quantities present in a sample. PCR is also routinely used for comparative studies to amplify genes from one organism, and yields large amounts of specific DNA sequences for use as templates in further reactions (Brock *et al*. 1997). By targeting particular genes of the chromosome which are independent of cultivation and growth conditions, sequence information can be retrieved by PCR from very small amounts of DNA; this capability is particularly useful when determining the genotype of an unknown sample. By combining PCR with techniques such as DGGE, sequence data can be generated without time consuming molecular cloning procedures (Nübel *et al*. 1997). PCR has been used extensively during the molecular investigations of this study; the precise PCR cycles and primers are presented in Chapter 6.

3.6.2 The Microcystin Gene Cluster

Microcystins were originally purified by Botes *et al* in 1982, characterized in the unicellular *M. aeruginosa* (Botes *et al*. 1985). The cyclic structure and presence of unusual amino acids in microcystin suggests the toxin is synthesised non-ribosomally (Tooming-Klunderud *et al*. 2007) by the microcystin synthetase enzyme complex, involving both polyketide synthases and non-ribosomal peptide synthases (Dittmann and Börner 2005). Non-ribosomal peptide synthetase genes typically comprise modules built up of domains; each module activates one amino acid which is incorporated into the growing peptide chain (Mikalsen *et al*. 2003; Tooming-Klunderud *et al*. 2007). A condensation (C) domain, adenylation (A) domain and thiolation (T) domain are required for addition of amino acids to the peptide. The most intensively studied NRPS operon is the microcystin class of genes, the *mcy* operon (Tooming-Klunderud *et al*. 2007); synthetases encoded by these genes contain both NRPS and PKS modules.

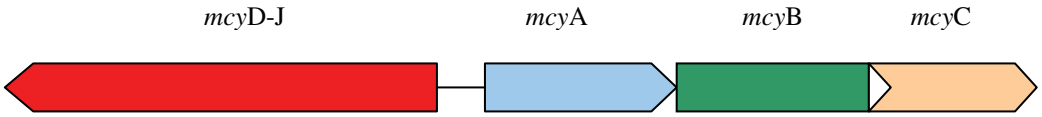

Figure 3-3: Structure of the microcystin (*MCY*) gene cluster (Mikalsen *et al*. 2003)

Challis *et al* (2000) defines non-ribosomal peptide synthetases (NRPSs) as ". . . large modular proteins that facilitate selective binding, activation and condensation of amino acids in an ordered manner". The small size, cyclic structure and content of unusual amino acids suggests that microcystins are non-ribosomally synthesized via peptide synthetases (Krishnamurthy *et al*. 1989).

Dittman *et al* (1997) identified a basic difference between a toxic and a non-toxic strain of *Microcystis* is the presence of one or more genes coding for microcystin synthetases. Tillet *et al* (2000) progressed further, by characterizing the *mcy* gene cluster responsible for microcystin biosynthesis illustrated in Figure 3-3. The cluster spans 55 kb, and comprises 10 bi-directionally transcribed open reading frames (ORF) in 2 putative operons, *mcy* D-J and *mcy* A-C. Sequence analysis of *mcy* gene cluster revealed the larger of the two putative operons encodes the PKS-NRPS modules, catalyzing the formation of the pentaketide-derived Adda and its linkage to D-

glutamate; the smaller operon encodes NRPS modules for extension of a peptidyl intermediate to the heptapeptidyl step and subsequent peptide cyclisation.

3.7 Summary

Procedures are developed for cultivation of specific strains of microalgae, perhaps for a defined application; for detection and extraction of compounds with beneficial properties; and for investigation into the molecular mechanisms responsible for production of these compounds. Each of the techniques has implicit advantages and disadvantages. Given the diversity available, assessment of all methods would be a task of immeasurable proportions. Therefore, prospective options were selected and developed for application during this research project, based on the resources available for the project, and more specifically, the relative success of the technique where applied to a strain of cyanobacteria from similar circumstances. The techniques developed and applied during this investigation are detailed within each chapter.

CHAPTER 4 CULTIVATION OF *MICROCYSTIS* & BIOACTIVITY ASSESSMENT

4.1 Establishment of a non-toxic *Microcystis* culture

Isolation of *Microcystis* sp., as previously noted by Ferris *et al* (1991), is often an arduous and time consuming practice, achieving only modest success. Axenic cultures in particular have been difficult to establish (Ohtake *et al.* 1989; Bolch and Blackburn 1996). Parker (1982) describes several obstacles associated with the purification of *Microcystis* cultures. Firstly, contaminants often significantly outnumber *Microcystis* cells in most cultures. Secondly, the capsule surrounding the *Microcystis* colonies traps contaminants (Gerloff *et al*. 1950; Shirai *et al*. 1991), often leading to recontamination of cultures once purified. Finally, encapsulated *Microcystis* has been observed to grow poorly in the absence of contaminants (Parker 1982). However, many isolation and purification techniques, including those applied below, have been developed successfully for the isolation of *Microcystis* cells and the establishment of unialgal and / or axenic cultures.

4.1.1 Reagents and Cyanobacterial strains

All chemicals and reagents, unless otherwise specified were purchased from the following suppliers: Sigma-Aldrich, BDH, Ajax, and Crown Scientific. Luria Bertani (LB) agar and LB nutrient broth used for bioactivity assays and cultivation of bacterial strains for testing were obtained from the Central Services Unit, School of Molecular and Biomedical Sciences, University of Adelaide. Recipes for preparation of cultivation media are provided in APPENDIX C. Synthetic medium BG_{11} was selected as the primary cultivation media, as it was designed for culturing cyanobacteria (Watanabe 2005) and has been used to successfully culture *Microcystis* sp. The species of cyanobacteria assessed for bioactivity during this study are recorded in Table 4-1. The original source of the strains is provided, and each isolate or culture has been assigned a code, which will serve as the reference for the remainder of this document. Prior to bioactivity assessment, all field species were cultivated in BG_{11} medium for a minimum of 2 generations to establish unialgal cultures; reference codes apply to cultured strains, originating from the blooms. All

biomass preparations, extractions, assays and chemical analysis procedures were conducted using aseptic techniques.

Table 4-1: Cyanobacteria assessed for biological activity against selected test organisms. Original source and toxicity status are provided, and species are coded for ease of reference.

Code	Species	Source	Collection Date	Toxicity (if known)
MIC FEB05	*Microcystis flos-aquae*	Torrens Lake, SA	14 February 2005	No toxin detected by HPLC
MIC MAR05	*Microcystis flos-aquae*	Torrens Lake, SA	4 March 2005	No toxin detected by HPLC
ANA APR05	*Anabaena circinalis*	Torrens Lake, SA	13 April 2005	Toxic
MIC 053D	*Microcystis flos-aquae*	Torrens Lake, SA	5 December 2000	Toxicity Unknown
MIC 054A	*Microcystis flos-aquae*	Farm Dam, Birdwood	4 December 2000	Toxicity Unknown
MIC 306B	*Microcystis aeruginosa*	Mt Bold, VIC	10 February 1988	No toxin detected by HPLC
MIC 046E	*Microcystis aeruginosa*	Oak billabong, Yarra Flat, VIC	7 March 1995	No toxin detected by HPLC
SCE DEC05	*Scenedesmus sp.*	Torrens Lake, SA	December 2005	Toxicity unknown
MIC BOL	*Microcystis aeruginosa*	Bolivar WWTP, SA	24 January 2006	Toxicity unknown
ANA FEB06	*Anabaena circinalis*	Torrens Lake, SA	21 February 2006	Toxicity unknown

4.1.2 Sample collection of *M. flos-aquae* from the Torrens Lake

An outbreak of cyanobacteria dominated the Torrens Lake from early February to April 2005. Three samples of the bloom were collected approximately 3 weeks apart by skimming the surface of the lake between the Albert Bridge, Frome Rd, and the University Footbridge (34°54'50.73" S; 138°36'15.23" E. Elevation 34 m (Google-Earth 2007)). The samples were observed under a microscope for taxonomic identification, and concurred with the observations of the Australian Water Quality Centre (AWQC) at Bolivar, South Australia. Initially, the bloom consisted predominantly of *M. flos-aquae*, with some *A. circinalis* and green algae also present. Approximately 4-5 weeks following the initial harvest of biomass, the dominating

species of the bloom was observed to be *A. circinalis*, with traces of *M. flos-aquae* still detectable. AWQC routinely analyses the blooms for the presence of toxins by High Pressure Liquid Chromatography (HPLC). Table 4-1 above outlines the collection dates, species identified for each sample, and the results of the toxicity tests undertaken by AWQC, if known.

Biomass was harvested by centrifugation (Sorvall RC5C, HB-6 Rotor; 3772 ×g, 10 min at 4 °C), rinsed thoroughly with RO water to remove contaminants and isolated as unialgal cultures in BG_{11} medium via the serial dilution technique described in §B.II.5 and the agar streak plate procedure in section §B.II.4. Liquid and solid media cultures of all species were maintained under low light for use in future investigations.

4.1.3 Establishment of field cyanobacteria enrichment cultures

A water sample from the Torrens Lake was collected on 14th December 2005 and algal biomass scraped from the concrete perimeter of the river. Sterile conical flasks containing 100 mL of fresh BG_{11} medium were inoculated with 10 mL of Torrens water, or 1 g of biomass in 10 mL of Torrens water. Flasks were placed on an orbital shaking table (RATEK Orbital Mixer, 1 ×g, 22 °C) under cool white fluorescent lights (12:12 hr light-dark cycle). Following 5 days of incubation, cultures were observed under an inverted confocal microscope to identify species present. The flask inoculated with water from the Torrens Lake revealed the presence of small, unicellular colonies speculated to be *Microcystis* sp.; clusters of 4 - 8 green cells (characteristic of *Scenedesmus* sp. (Shihira *et* Krauss) Kessler *et al*) and a significant amount of mucilage. The second flask inoculated with biomass revealed trichomes of green cells, few single cells and organic matter.

Serial dilutions were performed on both flasks to isolate single cells. Cell counts were performed for both flasks to determine the approximate concentration; a simple calculation was then performed to determine the number and size of dilutions required to isolate a single cell. Cell enumeration for the flask containing Torrens Lake water had a concentration of 5.85×10^6 cells mL^{-1}, while the second flask had a concentration of 3.45×10^6 cells mL^{-1}. To achieve a single cell isolation several dilutions of 1/100 were made, followed by a 1/5 dilution for the first flask and a 1/3 dilution for the second flask to reach a final theoretical cell concentration of 1 cell

mL^{-1}. Three flasks containing fresh BG_{11} medium were inoculated from the each of the third 1/100 dilution (the 1/1,000,000 dilution) while another 3 flasks were inoculated with the 1/5 or 1/3 dilution; this series of inoculations provided a greater probability that only one or two cells would be present in the inoculum and that the cells would represent a single species. All flasks were placed on an orbital shaking table at 80 rpm, room temperature under a 12:12 hour light/dark cycle.

Samples of each flask were taken to AWQC for taxonomic identification. Both flasks contained green microalgae, including *Scenedesmus*, *Chlamydomonas* and *Arthrodesmus* but no cyanobacteria. Those cells thought to be *Microcystis* were found to be elliptical green cells standing "on end" in the sample, causing them to appear spherical. Due to the lack of cyanobacterial cells present in these samples, no further investigations were conducted using these cultures.

4.1.4 Inoculation of an environmental water sample with laboratory cultured *Microcystis*

Cyanobacteria subjected to repeated subculture, or maintained for long periods in a laboratory environment have demonstrated a loss of toxicity or bioactivity over time (Borowitzka 1999). The premise behind this decline is that in an artificial environment, free of competitors and grazers, cells are not required to synthesise deterrent molecules to survive. However, by using laboratory cultured *Microcystis* to inoculate samples of Torrens Lake water containing competitors and infectious agents, it may be feasible to stimulate cyanobacterial cells to produce bioactive compounds. Two species of non-toxic *Microcystis*, *M. flos-aquae* 053D and *M aeruginosa* 046E were selected from cultures recorded in Table 4-1, to determine the practicality of this supposition. Prior to inoculation in water, the cultures were revived in fresh BG_{11} media to promote viability.

Water collected from the Torrens River on 14 December 2005 (refer to §4.1.3) was filtered using a Buchner funnel and Whatman no. 1 filter paper (0.45 µm) to remove debris and organic matter. Aliquots of 100 mL filtered water were added to 250 mL sterilised conical flasks. To encourage growth of *Microcystis,* additional nitrate (1.5 g L^{-1}) and phosphate (0.03 g L^{-1}) corresponding to nutrient levels in BG_{11} medium were added to half of the flasks. Inoculates of 10 mL were added to the flasks, with one

nutrient-enriched flask and one "standard" flask per strain. All flasks were placed under a 12:12 hr light/dark cycle on an orbital shaker (RATEK, 80 rpm, 22 °C). Flasks were observed daily for signs of growth; 7 days following inoculation the enriched flasks displayed a dark green hue, while the standard flasks were pale green. Cultures were maintained under low light prior to bioactivity assessment.

4.1.5 Establishment of laboratory cultures of *Microcystis* in enriched culture medium (AWQC cultures)

Four cultures of *Microcystis*, isolated from various sites around Australia, were donated by AWQC for bioactivity testing. The species are recorded in Table 4-2 with their source, isolation date and toxicity status. Samples were received in tissue cultures flasks, and placed in a growth cabinet at 25 °C to maintain viability. However, these cultures experienced a prolonged adaptation phase, possibly due to the length of time spent in long-term storage. The cultures had been maintained under low light conditions, and were on-grown in fresh media for several generations prior to assessment. As each culture exhibited a significant increase in cell density, it was used to inoculate two 100 mL samples of filtered Torrens Lake water, one of which was enriched with nitrate (1.5 g L^{-1}) and phosphate (0.03 g L^{-1}) corresponding to nutrient levels in BG_{11} medium. All flasks were incubated on an orbital shaker (RATEK, 80 rpm, 22 °C) under a 16:8 hr light/dark cycle. Cultures were observed daily for cell growth, and cultures that had been nutrient-enriched achieved a higher cell density. Flasks that had been inoculated with *M. aeruginosa* MIC 046E displayed a darker green colour than all other flasks, both for the enriched and standard culture. Cultures were also used to inoculate BG_{11} medium and maintained at low light intensity for future applications.

Table 4-2: Cultures of *Microcystis* sp. donated by AWQC, isolation dates, original source and toxicity, if known

Lab Code	Species	Source	Date Isolated	Toxicity
MIC 053D	*Microcystis flos-aquae*	Torrens Lake	5/12/2000	Toxicity Unknown
MIC 054A	*Microcystis flos-aquae*	Farm Dam, Birdwood	4/12/2000	Toxicity Unknown
MIC 306B	*Microcystis aeruginosa*	Mt Bold	10/2/1988	No toxin detected by HPLC
MIC 046E	*Microcystis aeruginosa*	Oak billabong, Yarra Flat, VIC	7/3/1995	No toxin detected by HPLC

4.1.5.1 Field Samples of Microcystis obtained from the Bolivar Wastewater Treatment Ponds

A sample of a cyanobacterial bloom was collected from wastewater treatment ponds (Lagoon 3, site 1) at AWQC, Bolivar and identified as a non-toxic species of *M. aeruginosa*. Cells exhibited expected *Microcystis* properties, typically including colony formation and migration to the sample surface. Applying the micropipette isolation technique described in section §B.II.3, aggregates of *Microcystis* cells were transferred to sterile tubes containing artificial seawater media (ASM); ASM was recommended for cell isolation in preference to BG_{11} as the latter has a low phosphate content and is poorly buffered (Stanier *et al.* 1971); subsequently, it was assumed BG_{11} would be unable to provide sufficient nutrients for the cells to thrive. Due to the diminutive size of this species, it was not possible to isolate a single cell in each sequence; however, aggregates were disrupted during transfer between water droplets and only a few cells used to inoculate each tube. Six tubes were inoculated, and incubated on an orbital shaking table (RATEK, 1 x g, 22 ºC) under a 16:8 hr light/dark cycle. Once established, cells were used to inoculate 100 mL conical flasks containing 50 mL of BG_{11} medium and maintained under low light intensity, prior to harvest for bioactivity assays.

4.2 Bioactivity assessment of non-toxic *M. flos-aquae*

A variety of direct and indirect methods may be applied to detect the presence of secondary metabolites with antimicrobial activity in microalgae and cyanobacteria. Direct assays seek detection of specific target compounds; indirect assays evaluate the biological activity of desired products (Lee and Shen 2004). Development of analytical methods and instrumentation (HPLC, GC, MS and NMR) provide rapid and highly sensitive analytical techniques to detect pharmacologically active compounds (Cohen 1999). Indirect assays, or bioassays, employ cultured cells or pathogenic microorganisms to screen for antimicrobial activity (Lee and Shen 2004). Both direct and indirect assays were employed in this study to isolate and identify bioactive secondary metabolites synthesised by *M. flos-aquae*; these methods are described in detail in the remainder of this chapter. Table 4-3 provides a summary of the extraction techniques applied to each of the cyanobacterial strains investigated in this study; extraction techniques have been numbered and are provided below the table for ease of reference.

Table 4-3: Extraction techniques applied to cyanobacterial cultures in this study.

Code	Species	Extraction Techniques applied
MIC FEB05	*M. flos-aquae*	1, 3, 4, 6, & 7
MIC MAR05	*M. flos-aquae*	1, 2, 3, 4 & 5
ANA APR05	*A. circinalis*	1, 4, 5, 7 & 9
MIC 053D	*M. flos-aquae*	4, 5 & 9
MIC 054A	*M. flos-aquae*	4, 5 & 9
MIC 306B	*M. aeruginosa*	4, 5 & 9
MIC 046E	*M. aeruginosa*	4, 5 & 9
MIC BOL	*M. aeruginosa*	3, 4, 7 & 9

1 – Methanol/Acetone/Hexane; 2 – Methanol/Hexane; 3 – Three times methanol; 4 - Methanol/Sonication; 5 – Combined Methanol; 6 – distilled water; 7 – dH_2O/Sonication; 8 – Chloroform/methanol; 9 – 75% Methanol.

4.2.1 Sample preparation and solvent extraction

Biomass was rinsed thoroughly in distilled water to remove organic debris and contaminants, then harvested by centrifugation (Sorvall RC5C, HB-6 Rotor; 3772 ×g,

10 min at 4 °C) to form cell pellets. Pellets were freeze-dried overnight (Dynavac FD3 freeze dryer, Javac Double Stage High Vacuum Pump) and stored at -18 °C until required for solvent extraction and bioactivity testing. A number of cell disruption techniques were employed to ensure complete lysis of the cells prior to or during extraction. Pellets were subjected to solvent extraction using distilled water, methanol, hexane, acetone or sequential extractions utilising combinations of solvents. Following a comprehensive literature review, 9 methods were selected for extraction of (potential) bioactive metabolites. Preliminary investigations identified the most successful of these techniques, which were applied for all future extractions. The extraction solvents and cell disruption techniques applied during this study were outlined in Table 3-1, and detailed in Chapter 8APPENDIX A. Extracts were stored at -18 °C until required for antimicrobial assays.

4.2.2 Development of an assay to determine antibacterial activity

4.2.2.1 Agar Disc Diffusion assay of antimicrobial metabolites

An agar disc diffusion assay was developed, based on the assay described by Cannell *et al* (1988), to assess extracts for antibacterial and antifungal activity. Extracts were tested against *Staphylococcus aureus* (Rosenbach), *Bacillus subtilis* (Ehrenberg & Cohn) and *Escherichia coli* (Migula ex Castellani & Chalmers), *Aspergillus fumigatus* (Fresenius) and *Candida albicans* ([Robin] Berkhout). All test organisms were cultured in Luria Bertani broth overnight at 37 °C. Luria Bertani agar plates were seeded with 100 µl of overnight bacterial or fungal culture and pre-incubated at 25 °C for 1 hr. Sterile filter discs (Whatman #1 filter paper, 8 mm diameter) were impregnated with 25 µL of extract and dried at room temperature for 20 min. Discs saturated with Ampicillin at 10 mg/mL were included as a positive control; all solvents and a sample of lake water were incorporated as negative controls. The discs were placed on the surface of the agar plates, as illustrated by the schematic diagram in Figure 4-1, and all plates incubated at 25 °C for 24 hrs. Plates were examined for growth inhibition of the test organism surrounding the discs and the diameter of inhibition zones measured. During each bioactivity assessment, extracts were assessed in triplicate.

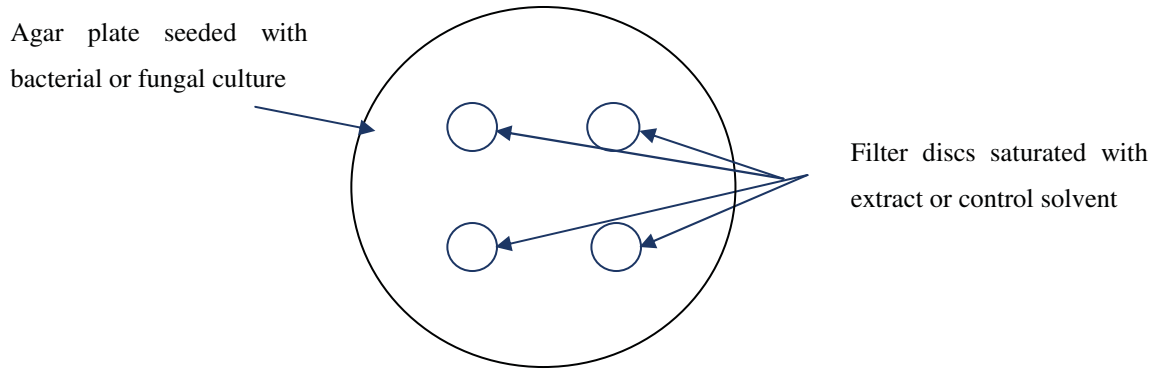

Figure 4-1: Agar disc diffusion assay developed to assess the antibacterial and antifungal activity of the cyanobacterial extracts.

4.2.2.2 Microtitre Plate Assay

A microtitre plate assay was developed to corroborate results obtained from the agar disc diffusion assays for assessing antimicrobial activity of extracts. Microtitre plates allow a large number of conditions to be imposed within each test. A microtitre plate consists of 96 sample wells arranged in an 8 x 12 matrix configuration. The predetermined plate format described by Table 4-4 was applied for the assessment of the cyanobacterial extracts by microtitre plate assay. Columns 1, 2, 11 and 12 were allocated as controls; wells in columns 1 and 2 received 5 µL of solvent (negative controls). Each well in column 11 received 5 µL of dimethyl sulphoxide (DMSO; a chemical known to possess bacteriostatic properties, commonly used to increase the sensitivity of antibiotic-resistant bacteria to antimicrobial agents (Basch and Gadebusch 1968)). Wells in column 12 received 5 µL aliquots of Ampicillin, the antibiotic employed to validate results of the agar disc diffusion assays.

Table 4-4: Microtitre plate format for bioactivity assessment of cyanobacterial extracts.

B. subtilis	Acetone	Hexane	1a	1b	1c	2a	2b	3a	3b	3c	DMSO	Amp
	Methanol	dH_2O	4	5a	5b	T1 MeOH	T1 dH_2O	T2 MeOH	T2 dH_2O		DMSO	Amp
C. albicans	Acetone	Hexane	1a	1b	1c	2a	2b	3a	3b	3c	DMSO	Amp
	Methanol	dH_2O	4	5a	5b	T1 MeOH	T1 dH_2O	T2 MeOH	T2 dH_2O		DMSO	Amp
S. aureus	Acetone	Hexane	1a	1b	1c	2a	2b	3a	3b	3c	DMSO	Amp
	Methanol	dH_2O	4	5a	5b	T1 MeOH	T1 dH_2O	T2 MeOH	T2 dH_2O		DMSO	Amp
E coli	Acetone	Hexane	1a	1b	1c	2a	2b	3a	3b	3c	DMSO	Amp
	Methanol	dH_2O	4	5a	5b	T1 MeOH	T1 dH_2O	T2 MeOH	T2 dH_2O		DMSO	Amp

Extracts were defined by the following numbers; 1 – Methanol/Acetone/hexane (3 supernatants); 2 – Methanol/Hexane (2 supernatants); 3 – 3 x Methanol (3 supernatants); 4 – Methanol/Sonication; 5 – combined Methanol (2 supernatants). T1 – Torrens water collected 14 February 2005, re-hydrated in either Methanol (MeOH) or dH2O. T2 – Torrens water collected 4 March 2005 re-hydrated in either Methanol (MeOH) or dH2O. Shaded wells were left blank

Test organisms were cultured overnight at 37 °C then diluted to 1 in 10,000 in LB broth and an aliquot of 195 µL added to all wells in 2 rows of the microtitre plate. Columns 3 to 10 inclusive received 5 µL aliquots of the extracts according to the plate format constructed in Table 4-4. The microtitre plate was incubated overnight on an orbital shaking table (RATEK; 1 x g, 22 °C). Absorbance was read at 600 nm on a microtitre plate reader (Dynatech MR5000). A positive result for bioactivity was identified as absorbency lower than that of the corresponding solvent control when assessed against the same organism.

4.3 Analysis of non-toxic *M. flos-aquae* growth

The influence of environmental factors (field samples) or culture conditions (laboratory strains) on growth rate and metabolic processes of cyanobacteria has been discussed at length in Chapter 2. Temperature, light exposure and intensity, bioavailability of nutrients and the presence (or absence) or grazers and competitors significantly impacts on survival rates and metabolic activity of cells in culture or blooms. This series of experiments was designed to establish cultures of *Microcystis* sp. from field and laboratory culture collections in assorted media; to determine the effect of the aforementioned parameters on the rate of culture growth by monitoring cell number or absorbance; and to identify biological activity of a non-toxic field isolate of *M. flos-aquae* (MIC FEB05) against a number of bacterial, fungal and viral test organisms. A comprehensive analysis of the data obtained during these investigations is presented.

4.3.1 *Microcystis* sp. growth determination

To establish a baseline for *Microcystis* growth, nutrient-rich medium (BG_{11}) was inoculated with a laboratory-cultivated strain of *M. flos-aquae* 053D, cultured under optimal growth conditions (16:8 hr light/dark cycle; 25°C, gentle agitation) and monitored daily for cell enumeration and culture absorbance at 675 nm (Shimadzu UV-1601 UV Visible Spectrophotometer). Figure 4-2 illustrates the correlation between absorbance and cell number for *M. flos-aquae* 053D cultivated in synthetic media under controlled conditions; as expected, a near linear relationship exists between cell density and chlorophyll absorption. The correlation gleaned from this plot was applied to cultures to determine cessation of the exponential phase of organism growth, and consequently the most likely period for secondary metabolite synthesis and optimum period for cell harvest.

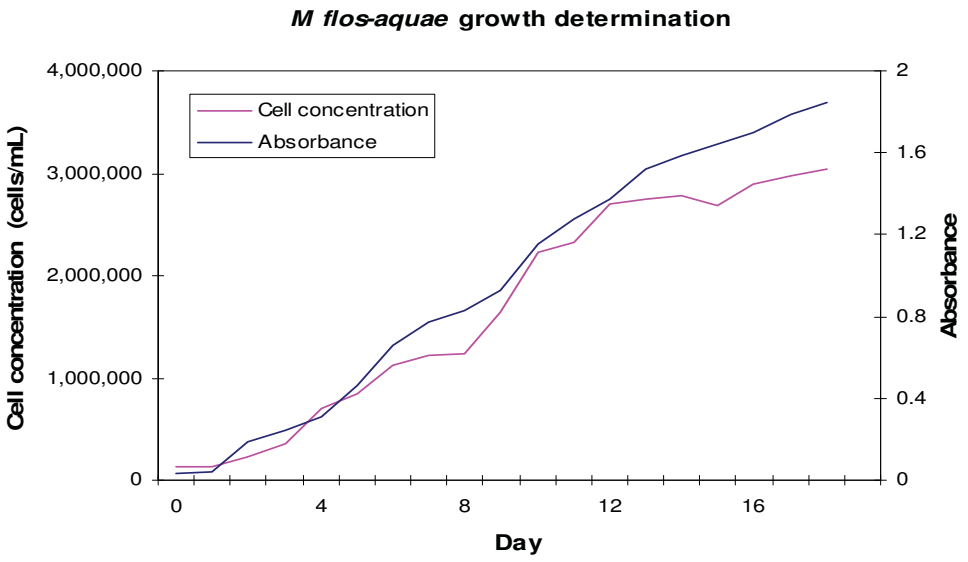

Figure 4-2: Growth of *M. flos-aquae* 053D in BG_{11} media. A similar growth curve is observed for absorbance and cell enumeration of the culture

4.3.1.1 Torrens Water Inoculations

Bioactivity of cyanobacteria maintained in laboratory cultures is acknowledged to decline following repeated subculturing (Borowitzka 1999), potentially due to the absence of grazers or competitors that would require cells to synthesise "survival" compounds. Awareness of this decline prompted design of experiments to ascertain whether synthesis of bioactive compounds could be induced in strains of *Microcystis* sp. obtained from long-term laboratory cultures, by inoculating samples of water from a local river containing natural microorganisms (e.g. bacteria, zooplankton, and other algal species). A second set of experiments examined the effect of enrichment of culture media with essential nutrients (nitrate, phosphate) on metabolic processes, and ultimately bioactive compound synthesis. The third group of cultures were grown in a synthetic nutrient medium, BG_{11} to provide a benchmark for comparison, and all modifications were repeated in triplicate for experimental validation and to demonstrate scientific rigour. To assess the significance of the results, the standard error was calculated for each triplicate set and displayed on the plots as error bars at a 95% confidence interval for the median of the absorbance readings.

Growth rates of three species of *Microcystis* (*M. flos-aquae* 053D, *M. aeruginosa* 046E and *M. aeruginosa* MIC BOL) cultured in 3 modified media were compared by

monitoring chlorophyll-α absorbance and cell number. Media examined were a standard BG_{11} media; filtered water collected from the Torrens River; and filtered Torrens River water enriched with nitrate and phosphate (designated Torrens +) at concentrations equivalent to those in BG_{11}. Culture conditions including culture media, temperature, light exposure, and agitation method (if applicable) are summarised in Table 4-5. Enriched culture media "Torrens +" was created by addition of 100 µL K_2HPO_4, 100 µL Trace metals mix, and 0.15g $NaNO_3$ to filtered Torrens water; the concentrations were selected based on those required for BG_{11} medium.

Despite a few anomalies, all strains demonstrated strong growth in either the standard BG_{11} media, or in enriched Torrens River water. Species cultured in filtered Torrens River water did not achieve successful growth, and often swiftly entered senescence. Figure 4-3 illustrates the growth response of *M. flos-aquae* 053D to the different media compositions. Cells cultured in enriched Torrens River water (Torrens +) demonstrated a more stable increase in cell number compared with those grown in BG_{11}, a standard algal cultivation media. Cells cultured in filtered Torrens River water, while exhibiting a reasonably stable growth, entered senescence more rapidly than those in the enriched medium.

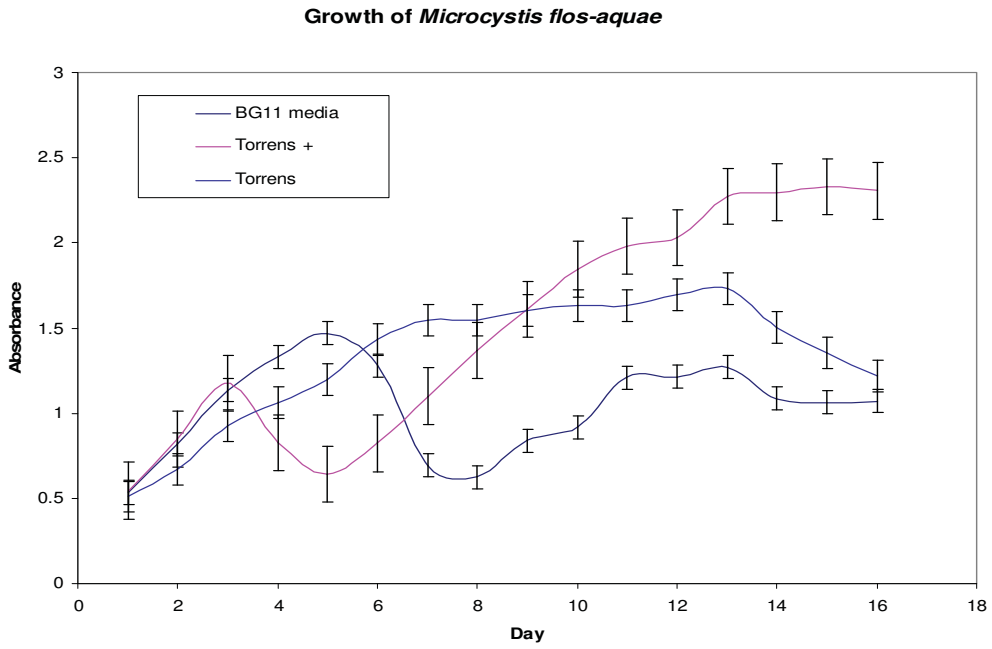

Figure 4-3: Growth of *M. flos-aquae* 053D in BG_{11}, filtered Torrens water, and Torrens + media.

Table 4-5: Culture conditions imposed on *M. flos-aquae 053D, M. aeruginosa 046E and Microcystis (MIC BOL)* cultivated in BG_{11}, filtered Torrens River water, and filtered river water enriched with nitrate and phosphate

Flask	Date Started	Volume	Culture Volume	Base	Inoculum Species	Inoculum Volume	Additional Dosing	Growth conditions Light	Temp	Agitation
1	11-May	250mL	100mL	BG_{11} media	Bolivar *Microcystis*	10mL		14:6 hr	24.9C	70rpm
2	11-May	250mL	100mL	BG_{11} media	Bolivar *Microcystis*	10mL		14:6 hr	24.9C	70rpm
3	11-May	250mL	100mL	BG_{11} media	Bolivar *Microcystis*	10mL		14:6 hr	24.9C	70rpm
4	11-May	250mL	100mL	BG_{11} media	*Microcystis flos-aquae*	10mL		14:6 hr	24.9C	70rpm
5	11-May	250mL	100mL	BG_{11} media	*Microcystis flos-aquae*	10mL		14:6 hr	24.9C	70rpm
6	11-May	250mL	100mL	BG_{11} media	*Microcystis flos-aquae*	10mL		14:6 hr	24.9C	70rpm
7	11-May	250mL	100mL	BG_{11} media	*Microcystis aeruginosa*	10mL		14:6 hr	24.9C	70rpm
8	11-May	250mL	100mL	BG_{11} media	*Microcystis aeruginosa*	10mL		14:6 hr	24.9C	70rpm
9	11-May	250mL	100mL	BG_{11} media	*Microcystis aeruginosa*	10mL		14:6 hr	24.9C	70rpm
10	11-May	250mL	100mL	Torrens +	Bolivar *Microcystis*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
11	11-May	250mL	100mL	Torrens +	Bolivar *Microcystis*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
12	11-May	250mL	100mL	Torrens +	Bolivar *Microcystis*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
13	11-May	250mL	100mL	Torrens +	*Microcystis flos-aquae*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
14	11-May	250mL	100mL	Torrens +	*Microcystis flos-aquae*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
15	11-May	250mL	100mL	Torrens +	*Microcystis flos-aquae*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
16	11-May	250mL	100mL	Torrens +	*Microcystis aeruginosa*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
17	11-May	250mL	100mL	Torrens +	*Microcystis aeruginosa*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
18	11-May	250mL	100mL	Torrens +	*Microcystis aeruginosa*	10mL	N, P, Fe-EDTA	14:6 hr	24.9C	70rpm
19	11-May	250mL	100mL	Torrens	Bolivar *Microcystis*	10mL		14:6 hr	24.9C	70rpm
20	11-May	250mL	100mL	Torrens	Bolivar *Microcystis*	10mL		14:6 hr	24.9C	70rpm
21	11-May	250mL	100mL	Torrens	Bolivar *Microcystis*	10mL		14:6 hr	24.9C	70rpm
22	11-May	250mL	100mL	Torrens	*Microcystis flos-aquae*	10mL		14:6 hr	24.9C	70rpm
23	11-May	250mL	100mL	Torrens	*Microcystis flos-aquae*	10mL		14:6 hr	24.9C	70rpm
24	11-May	250mL	100mL	Torrens	*Microcystis flos-aquae*	10mL		14:6 hr	24.9C	70rpm
25	11-May	250mL	100mL	Torrens	*Microcystis aeruginosa*	10mL		14:6 hr	24.9C	70rpm
26	11-May	250mL	100mL	Torrens	*Microcystis aeruginosa*	10mL		14:6 hr	24.9C	70rpm
27	11-May	250mL	100mL	Torrens	*Microcystis aeruginosa*	10mL		14:6 hr	24.9C	70rpm

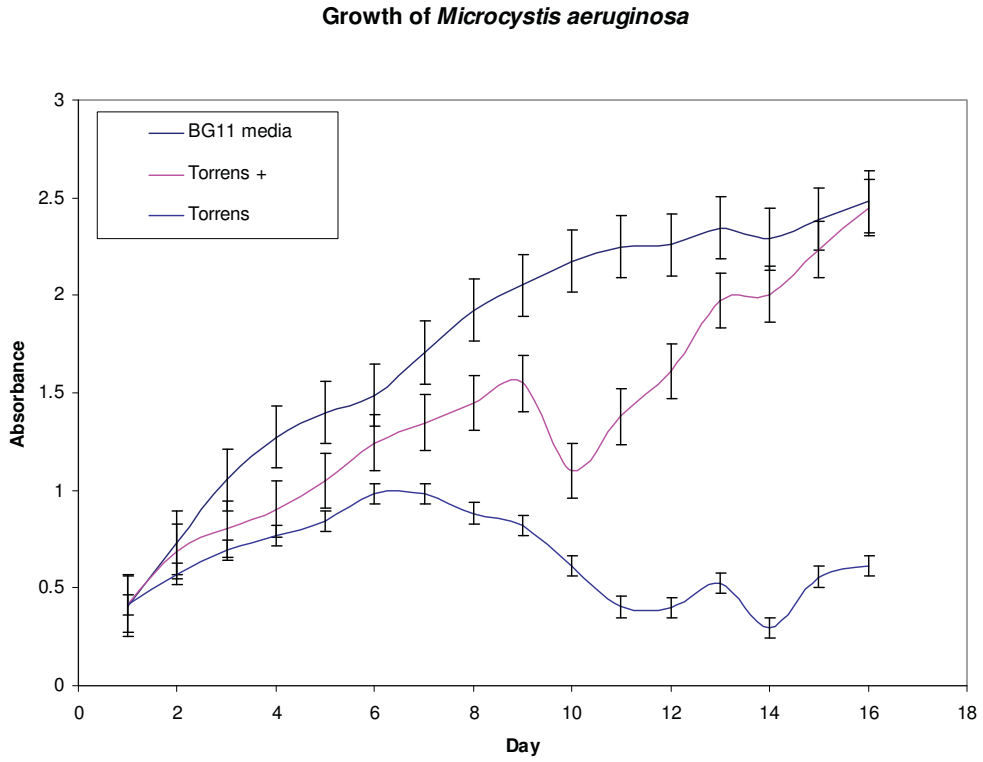

Figure 4-4: Growth of *M. aeruginosa* 046E in BG_{11}, filtered Torrens River water, and enriched filtered Torrens River water.

M. aeruginosa 046E performed exceptionally well in BG_{11} media, and demonstrated strong growth in the enriched Torrens water; however, when cultured in unmodified, filtered river water *M. aeruginosa* 046E was not able to maintain viability and all flasks containing this media resulted in rapid death of the cells (generally around Day 6). Figure 4-4 illustrates the growth response of *M. aeruginosa* 046E to the variations in media composition. Similarly the growth trends of *Microcystis* MIC BOL collected from the Bolivar Wastewater treatment lagoons follow those of *M. aeruginosa 046E*, and are depicted by the graph labelled Figure 4-5. *Microcystis* MIC BOL achieved remarkable cell numbers in the enriched Torrens + medium; this may be a reflection of the similarities in environments between the cyanobacteria and the media base. *Microcystis* MIC BOL cells were obtained from a wastewater lagoon potentially containing high levels of nutrients and natural organisms, such as bacteria and viruses. Consequently, the level of adaptation required by these cells to acclimatise to the Torrens River water would be lower than that required from the laboratory-cultured *M. flos-aquae* 053D and *M. aeruginosa* 046E. This notion is supported by the relatively stable growth of *Microcystis* MIC BOL cultured in unenriched river water,

compared to growth of *M. flos-aquae* 053D and *M aeruginosa* 046E. The laboratory-cultivated strains entered senescence very early during culture (generally around Day 6-7), and while several flasks demonstrated an attempt to reinitiate growth, this was not maintained and cultures re-entered the death phase.

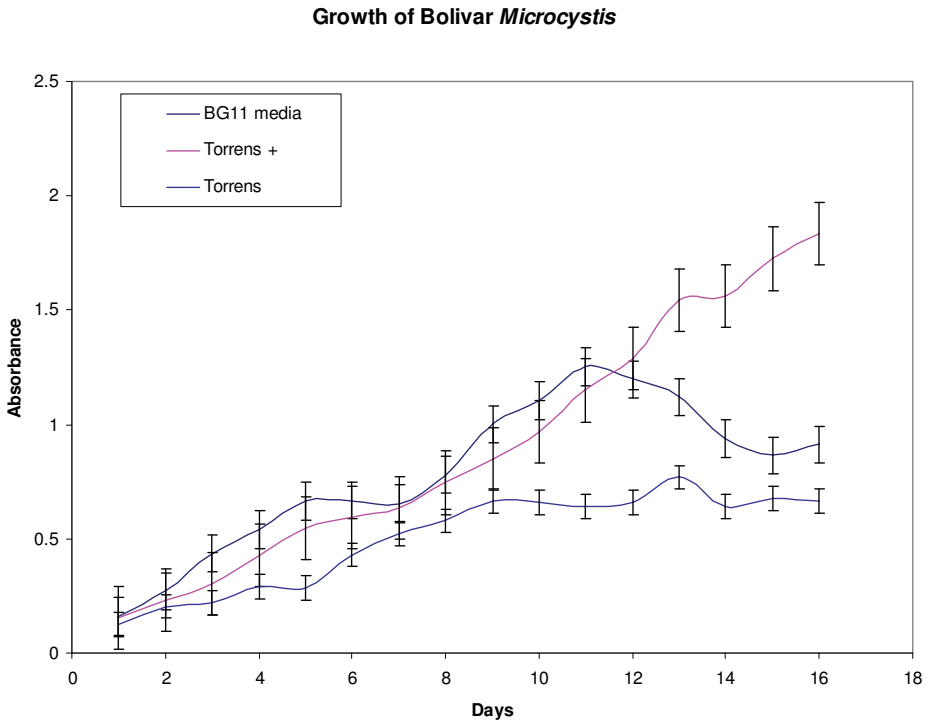

Figure 4-5: Growth of *Microcystis* (MIC BOL), obtained from the wastewater treatment ponds at Bolivar in BG_{11}, filtered Torrens River water, and enriched filtered Torrens River water

4.3.1.2 Nutrient Analysis of the Torrens Lake – a likely cause of a species succession?

Bioavailability of nutrients and metal ions in the environment can significantly influence the composition of algal assemblages. Domination of a water body by a particular species of cyanobacteria suggests concentrations of nutrients and trace elements will be more or less abundant during the bloom period than others, as species utilise different metals and complexes at different rates. Some metals may be bioaccumulated by the cells, reducing their concentration in the ecosystem. Fluctuations in concentration or bioavailability of one element through external natural or artificial interference may lead to a shift in the dominating species, or "species succession", as the nutrient is depleted. The bloom considered in this study underwent a species succession during late March 2005, the dominant species

progressing from a non-toxic form of *M. flos-aquae*, to a toxic form of *Anabaena circinalis* (P. Hobson, personal communication 2005). This prompted an investigation into the nutrient composition of the lake water on each of the three bloom collection dates, to ascertain any striking disparities within the cyanobacterial environment. Figure 4-6 illustrates the variation in total and complexed nitrogen and phosphorus in the Torrens Lake through the bloom season, beginning in mid-December 2004, and monitoring through to June 2005. Results of analysing Torrens Lake water for metal ions are depicted in Figure 4-7.

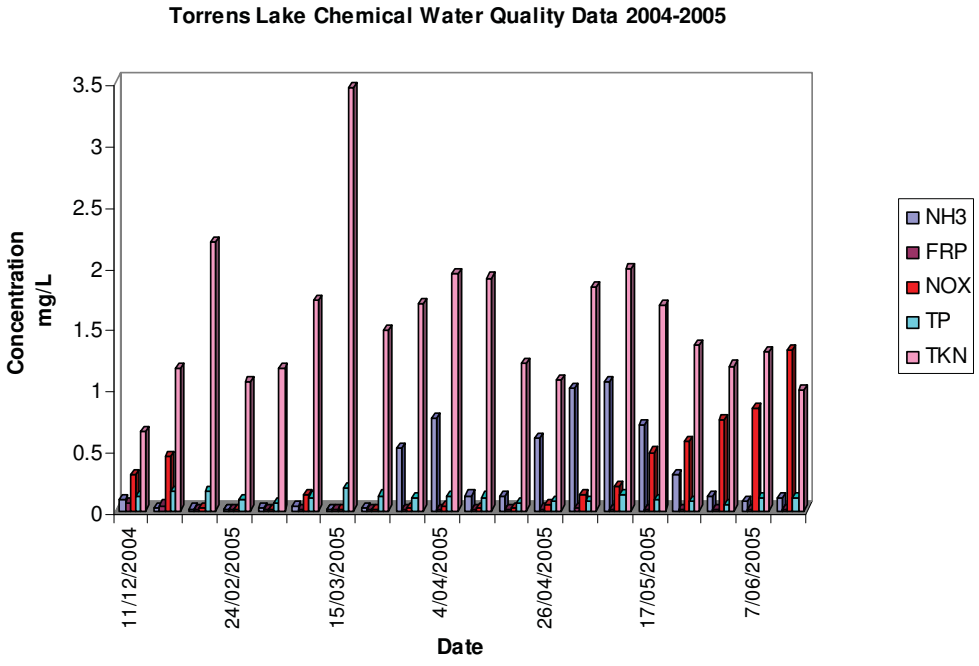

Figure 4-6: Analysis of Torrens Lake chemical water quality, sampled at the surface of the lake, CBD (AWQC, personal communication, February 2005)

Nitrogen and phosphorus are acknowledged as significant elements required for cyanobacterial growth, though species may preferentially sequester one nutrient over the other. The data presented by the plot labelled Figure 4-6 indicated that 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 may be preferentially utilised by *Microcystis* cells. *Microcystis* and *Anabaena* differ in their ability to fix nitrogen from the environment as many strains of *Anabaena* are

recognised "nitrogen-fixers" (Haselkorn 1978; Buikema and Haselkorn 1991). An increase in TKN was observed during early March, followed by a reduction in concentration. This may be a reflection of the preference of *Microcystis* for complexed forms of Nitrogen (e.g. Ammonia) rather than the elemental form, particularly when compared to the low concentrations of Ammonia and NOX in the samples. Similarly, low concentrations of phosphorus during the bloom period suggest that this was a vital element for growth of cyanobacteria. However, continually low levels of phosphorus following the bloom may be a signal that other organisms in the environment are using this compound, or reflect upstream conditions or rainfall periods.

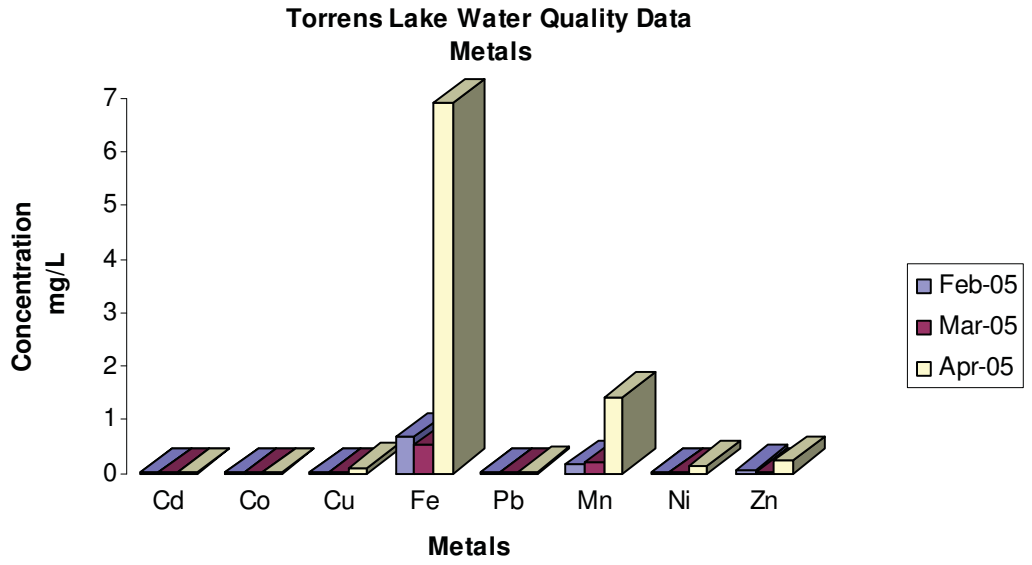

Figure 4-7: Analysis of metal ions in Torrens Lake water. Samples were collected on 14/2/05 (FEB-05); 4/3/05 (MAR-05); and 4/4/05 (APR-05). Analysis performed by AWQC.

Figure 4-7 illustrates the water quality data obtained by analysing for metals and major cations in the water samples. Samples Feb-05 and Mar-05 were both obtained during domination of *Microcystis*; concentrations of metals were generally similar for these samples, with the exception of Zinc and Manganese. Trace metals are recognised as significant for cyanobacterial growth, though concentration and metal is species-dependent. It could be envisaged that bioaccumulation of metals was higher in cells in later phases of growth, corresponding to low metal concentrations in sample Apr-05.

4.4 Review

All species of *Microcystis* from laboratory cultures and field siolates demonstrated strong growth in either BG_{11} or enriched "Torrens +" media. However, *M. aeruginosa* 046E and *M. flos-aquae* 053D obtained from laboratory stocks rapidly entered senescence when grown in non-enriched Torrens water, and were generally not able to survive post 6-7 days. *M. aeruginosa* acquired from wastewater treatment lagoons at Bolivar, isolate labelled MIC BOL, achieved very high cell numbers when cultured in enriched lake water; potentially a reflection of the limited adaptation required by the cells to their alternative environment. *M. aeruginosa* MIC BOL also performed satisfactorily in non-enriched lake water. Growth experiments were repeated in triplicate to demonstrate scientific rigour, and data sets analysed statistically using error bars to assess the significance of the results. Ignoring potential sources of experimental bias can yield misleading results; error bars provided a basis to decide which data points likely represented consistent values and which are likely to have arisen by chance (Kerr and Churchill 2001).

A significant increase in the concentration of iron was detected in the water sample collected during late March, corresponding to the shift in dominant species from *Microcystis* to *Anabaena*. Low metal concentrations in sample Mar-05 may be cognizant of utilisation by the *Microcystis* cells early in the growth cycle. Analysis of cells would be required to confirm suggested bioaccumulation of metals.

4.5 Establishment of bioactivity of non-toxic *M. flos-aquae*

4.5.1 Evaluation of Antibacterial Assay

Assessment of crude extracts of *M. flos-aquae* MIC FEB05 for bioactivity against bacteria and fungi was performed by agar disc diffusion assays described by Cannell *et al* (1988), with slight modifications in accordance with available resources. A variety of conditions were imposed on the assays to determine the efficacy of the extracts in response to incubation temperature, concentration and organism. Primarily, gram positive (*Staphylococcus aureus, Bacillus subtilis*) and gram negative (*Escherichia coli*) bacteria were employed as test organisms; two fungi (*Candida albicans* and *Aspergillus fumigatus*) were also included in preliminary investigations. However, as no antifungal control was included in this study; positive results obtained for the crude extracts against either of the fungi require further investigation. Two incubation temperatures were examined to assess efficacy of the extracts. Initially, assays were incubated at 25 °C; duplicate assays were assessed at 37 °C, the optimum growth temperature for many bacteria and fungi. Extracts were also diluted to determine the minimum concentration required for biological activity.

All solvents employed for extraction were assessed for cytotoxic activity against the chosen test organisms, to ensure bioactivity was attributed to the extract rather than the solvents. Samples of water collected with the cyanobacterial biomass were retained following cell harvest and were also included in the assay. No activity was observed for either solvents or the water samples. Ampicillin, a commonly prescribed antibiotic, was included as a positive control against bacterial test organisms to validate results. The photograph labelled Figure 4-8 illustrates an example of the results obtained for the control experiments included as part of this study. The intrinsic antibacterial activity of Ampicillin is visualised by clear zones surrounding filter discs on the left of the photograph, indicating inhibition of growth of the organism (*S. aureus*). Similarly, Ampicillin significantly inhibited growth of *B. subtilis*, observed in the photograph provided as Figure 4-9, and also inhibited growth of *E coli* (results not presented). However, no growth inhibition was visible for discs infused with methanol (right hand side of Figure 4-8), a primary extraction solvent.

Suppression of bacterial or fungal growth was not evident for any of the extraction solvents.

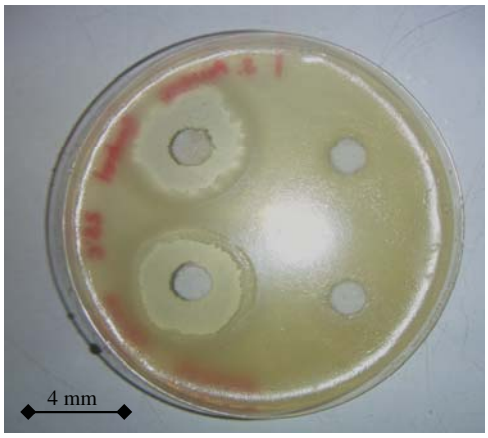

Figure 4-8: *S. aureus* control plate following 24 hrs incubation at 25 °C. Inhibition by Ampicillin (left) but not Methanol (right)

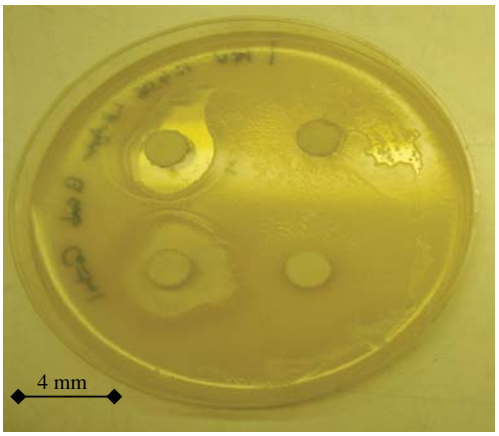

Figure 4-9: Agar disc diffusion assay control plate. Agar was seeded with an overnight culture of *B. subtilis* and incubated for 48 hrs with saturated discs of Ampicillin (left) and methanol (right)

A methanolic extract of *M .flos-aquae* MIC FEB05 isolated from the Torrens River, SA inhibited growth of *S. aureus*, observed by agar disc diffusion assay. The photograph presented as Figure 4-10 represents this particular assay after 24 hrs incubation at 25 °C. The discs on the left of the photograph were saturated with methanol/sonication extracts of the February sample; discs on the right were imbued with extracts of isolate *M. flos-aquae* MIC MAR05. Growth inhibition was clearly visible for *M. flos-aquae* MIC FEB05, given the 3-4 mm clear zones observed surrounding the filter discs on the left of the figure. Zones created in the *S. aureus* bacterial lawn were comparable to those produced by Ampicillin; the antibiotic inhibited growth of all bacterial test organisms creating zones of up to 5 mm diameter. This extract also inhibited growth of *B. subtilis*, though to a lesser extent, indicated by clear zones of approximately 1-3 mm.

A sample of *M. flos-aquae* isolate MIC MAR05 was subjected to the same extraction protocols as previously described, and assessed for biological activity against cultures of the original test organisms. However, extracts of these cells did not demonstrate cytotoxic effects against either the bacterial or fungal cultures. No evidence of bacterial inhibition is visible surrounding discs to the right of the agar plate in the

photograph labelled Figure 4-10. Similarly, no indication of growth suppression was apparent by the April isolate, a toxic form of *A. circinalis* ANA APR05, against any of the test organisms. Extracts of *M. flos-aquae* MIC FEB05 with solvents other than methanol did not demonstrate any discernible inhibition of bacterial or fungal growth.

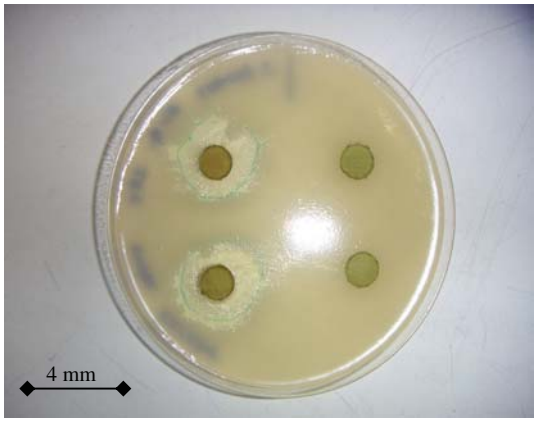

Figure 4-10: *S. aureus* extract plate incubated at 25 °C. Inhibition is evident around discs infused with the extract of M. flos-aquae MIC FEB05, but not the *M. flos-aquae* MIC MAR05 extract (right).

The lack of activity by either the extraction solvents or lake water indicates that growth inhibition of *S. aureus*, and to a lesser extent *B. subtilis*, was related to the methanolic extract of *M. flos-aquae* MIC FEB05. Discrepancies in potential bioactivity may be allied with variation in growth phase between the two *M. flos-aquae* samples. Algal ecologists at the Australian Water Quality Centre (AWQC) confirmed that the cyanobacterial bloom underwent a species succession during early-mid March, suggesting that at the time of the second biomass harvest (early March) *M. flos-aquae* was nearing the end of the stationary phase, and other species were beginning to dominate the bloom. Synthesis of cyanobacterial metabolites generally occurs during the late exponential and early stationary phase of organism growth (Borowitzka 1995); thus, the inference that the species was no longer producing antimicrobial compounds.

Results of the bioactivity assessment of extracts of *M. flos-aquae* MIC FEB05 utilising bacterial and fungal test organisms are summarised in Table 4-6, with a key defining symbols used in the table provided below. Extent of growth inhibition by the extracts has been quantified below the table, in line with the scale bars displayed on each of the figures above. For example, an extract exhibiting strong inhibition of the

organism in question has been identified with the symbol "++", indicating a clear zone of 3-5 mm was visible surrounding the saturated filter disc.

Table 4-6: Inhibition of growth of test organisms by extracts of *M. flos-aquae (MIC FEB05)*

	Extract Technique			
Test organism	**Distilled water**	**dH_2O / Sonication**	**MeOH / Sonication**	**Methanol / Acetone / Hexane**
E. coli	-	-	-	-
B. subtilis	-	-	+	-
S. aureus	-	-	++	-
A. fumigatus	-	-	-	-
C. albicans	-	-	+	-

- No inhibition; + Mild inhibition (1-2mm clear zone); ++ strong inhibition (3-5mm clear zone)

4.5.2 Long-term efficacy of antibacterial metabolites

To determine the efficacy of the methanolic extract following storage at -20 °C for 2 months, the agar disc diffusion assay was repeated using the methanol / sonication extract (identified in Table 4-6) of *M. flos-aquae* MIC FEB05, examining biological activity against the original bacterial and fungal test organisms. Following an incubation period of 48 h at 25 °C, the assay confirmed bioactivity of the extract for *S. aureus*, indicated by clear zones of approximately 3 mm around saturated discs. Antibacterial activity was also observed for *B. subtilis* (zones of growth inhibition of 2 mm), in addition to a weak response to *C. albicans* (clear zones of <1 mm). However, an antifungal agent was not applied during these experiments; consequently, the results can not be considered decisive for antifungal activity.

4.5.2.1 Effect of Incubation Temperature and Extract Concentration on Antibacterial Activity

To determine the efficacy of the methanol / sonication extracts of *M. flos-aquae* MIC FE05 in response to temperature and extract concentration, the agar disc diffusion assays were repeated using bacteria *E coli, B. subtilis* and *S. aureus*. Duplicates of extract plates and control plates were incubated at both 25°C and 37°C. The latter temperature is routinely applied in microbiology experiments to encourage rapid growth of bacterial cultures. Plates were observed following 24 hrs and 48 hrs

incubation at the selected temperature; plates incubated at 25°C reaffirmed the original assay results, with the extract exhibiting growth inhibition of *S. aureus* and *B. subtilis*. However, no disruption of bacterial growth was evident on plates incubated at 37°C. Ampicillin exhibited growth inhibition at both temperatures, though methanol did not demonstrate cytotoxic activity at either temperature. It is plausible that growth of the bacterial test organisms at 37°C was significantly stronger than at 25°C, and consequently the methanol / sonication extract was unable to inhibit cell growth.

The above supposition directed a further set of assays towards the minimum dilution of extract at which bacterial growth was disrupted. Dilutions of the methanol / sonication extract were performed in BG_{11} media at 1/10, 1/100 and 1/1,000 and assessed for biological activity at 25°C against *S. aureus, E. coli* and *B. subtilis*. Figure 4-11 presents a photograph of the agar disc diffusion assay using discs infused with the 1/10 dilution of the extract. Plates seeded with *E coli* (left) and *B. subtilis* (right) were unaffected by the diluted extract, as no growth inhibition is apparent. However, the plate seeded with *S. aureus* (centre) was not conclusive; interruptions to the bacterial lawn are visible in close proximity to the saturated discs, though these clear areas may be attributed to inadequate spreading of the bacterial culture. This assay was repeated but did not demonstrate bacterial growth inhibition. Similar assays were performed for dilutions of 1/100 and 1/1,000 of the extract.

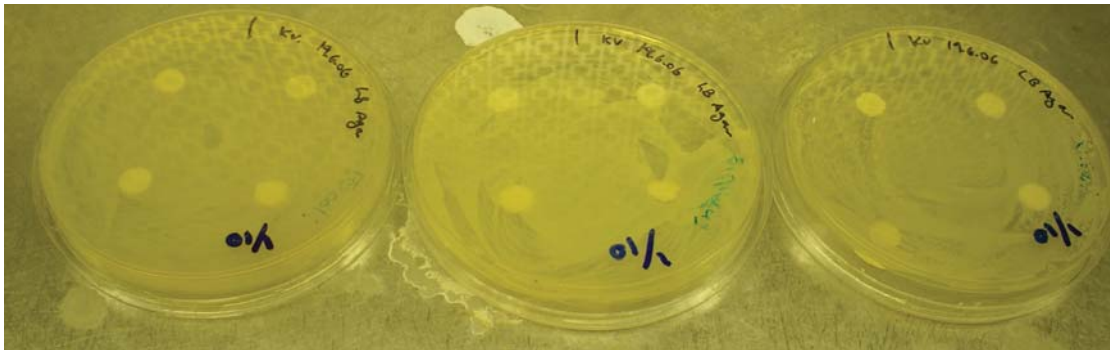

Figure 4-11: Agar disc diffusion assay assessing efficacy of diluted extracts. Methanol/sonication extracts have been diluted 1/10 in BG_{11} media. No inhibition of bacterial lawns is visible (L-R: *E coli, S. aureus,* and *B. subtilis*)

The photograph labelled Figure 4-12 illustrates the 1/100 dilution assay employing *B. subtilis* as the test organism. Growth inhibition is not evident for this dilution, nor for

the other bacteria, or the 1/1,000 dilutions. It was concluded that dilutions of this extract were not adequate to hinder growth of bacterial cells.

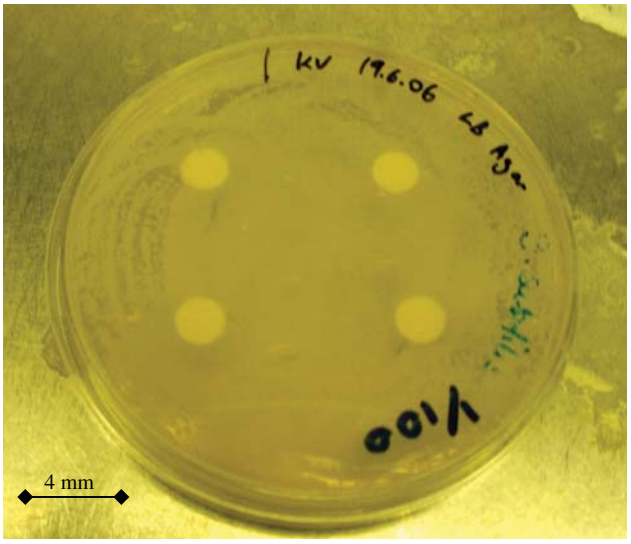

Figure 4-12: Methanol/sonication extract of *M. flos-aquae* MIC FEB05 dilution 1/100 assessed for bioactivity against *B. subtilis*. No growth inhibition was visible

4.5.3 Bioactivity Assessment of HPLC fractions

The methanolic / sonication extract of *M. flos-aquae* MIC FEB05 identified in section §4.5, demonstrating growth inhibition of *S. aureus, B. subtilis* and *C. albicans* was separated by high pressure liquid chromatography to isolate peptide peaks. The fractions collected, representing specific peaks on the chromatogram, were assessed for bioactivity by agar disc diffusion assay against the bacterial and fungal organisms identified in section 4.2. Figure 4-13 is a photograph of the agar disc diffusion assay of an HPLC fraction labelled R3F6 (a description of the HPLC process and identified fractions are provided in Chapter 5). Two of the discs impregnated with fraction R3F6 demonstrated mild growth inhibition of *B. subtilis*. No cytotoxicity was observed for this fraction against any of the other test organisms, nor for any of the other HPLC fractions tested against the bacteria (results not shown). Ampicillin and Methanol were included as positive and negative controls respectively, producing similar results to previous agar disc diffusion assays and are displayed in the photograph labelled Figure 4-14. Ampicillin strongly inhibited growth of all bacteria, while methanol did not exhibit any growth inhibitory effects on the bacterial lawns.

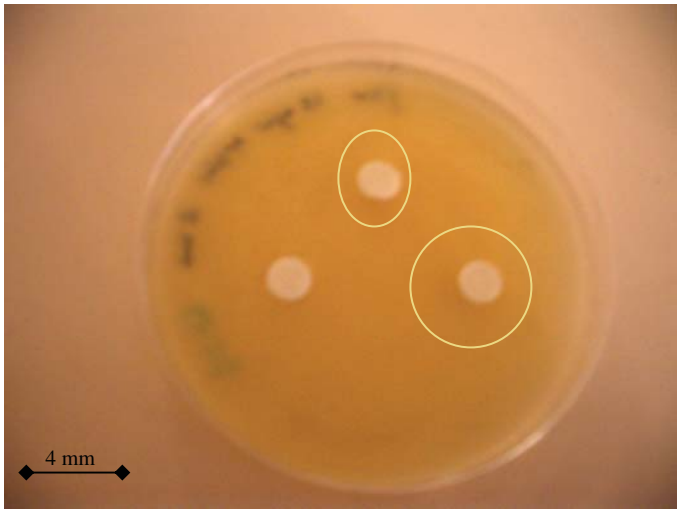

Figure 4-13: Bioactivity assessment of R3F6 fraction against *B subtilis*

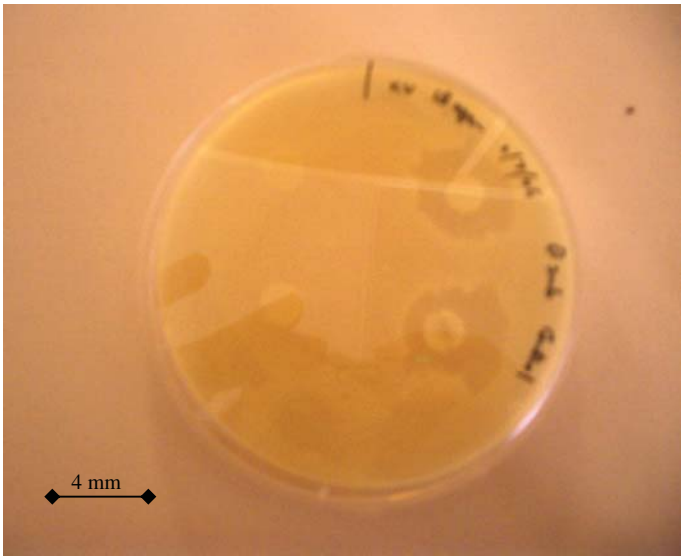

Figure 4-14: Bioactivity assessment of HPLC fractions. This control plate was seeded with a culture of *B. subtilis* and incubated with discs of Ampicillin (right) and Methanol (left).

4.5.4 Assessment of cyanobacterial extracts for antiviral activity

Extracts of *M. flos-aquae* MIC FEB05 and *M. flos-aquae* MIC MAR05 biomass obtained from the Torrens Lake were assessed for antiviral activity by Dr TuckWeng Kok, a virologist at the Institute of Medical and Veterinary Science (IMVS), Adelaide, SA. A number of viral families and subfamilies were represented by the selected viruses and these are recorded in Table 4-7. Cell lines infected with Dengue virus, Respiratory Syncytial Virus (RSV), Influenza virus types A and B, Herpes Simplex Virus Type 1 (HSV-1) and Adenovirus were examined for indications of inhibition of viral growth by the extracts.

Table 4-7: Viruses selected as test pathogens for assessment of antiviral activity of the extracts, and the virus families represented. (White and Fenner 1994)

Family	Subfamily	Virus
Adenoviridae	*(Adenovirus)**	Adenovirus
Herpesviridae	*Alphaherpesvirinae*	Herpes Simplex Virus type 1
Orthomyxoviridae	*(Influenzavirus)**	Influenza virus A Influenza virus B
Paramyxoviridae	*Pneumovirinae*	Respiratory Syncytial Virus (RSV)
Flaviviridae	*(Flavivirus)**	Dengue Virus

*() denotes genus; no subfamily specified

The data presented in Table 4-8 summarises the results of the antiviral activity assays. No growth inhibition was observed for the Influenza strains, HSV-1 or Adenovirus by any of the extracts assessed, judged by cytopathic effects on the cells. However, Dengue virus and RSV were both inhibited by the methanol / sonication extract, and the methanol fraction of the methanol/hexane/acetone extract; growth of RSV was also inhibited by distilled water extracts. Previous antiviral activity has been identified in *Nodularia spumigena* HU 280 and *Synechocystis aquatilis* 428 against HSV-1, while no activity was detected against adenovirus by Mundt *et al* (Mundt *et al.* 2001). A water bloom of *M. aeruginosa* SPH 01 has shown strong antiviral activity for Influenza A (Nowotny *et al*. 1997); however, the mechanism of antiviral activity has yet to be determined.

Table 4-8: Antiviral activity of extracts of *M. flos-aquae*

	Extract Technique			
Virus	**Distilled water**	**dH_2O / Sonication**	**MeOH / Sonication**	**Methanol / Acetone / Hexane**
Influenza A	-	-	-	-
Influenza B	-	-	-	-
Herpes Simplex Virus (1)	-	-	-	-
Adenovirus	-	-	-	-
RSV	+	+	+	+
Dengue Virus	-	-	+	+

- denotes no antiviral activity; + denotes antiviral activity

Dissimilarities in infection mechanisms and cellular target receptors for bacteria and viruses suggest that the activity detected in this study is likely to be affected by 2 different compounds. This theory requires further investigation following antiviral testing of the HPLC-eluted fractions.

4.6 Summary

A summary of the antimicrobial activity, including antibacterial, antifungal and antiviral activity, demonstrated by extracts of cyanobacterial biomass assessed during this investigation is provided in Table 4-9.

A methanolic extract of *M. flos-aquae* MIC FEB05 collected from the Torrens Lake, South Australia demonstrated antibacterial activity against *S. aureus* and *B. subtilis*. Zones of growth inhibition observed during agar disc diffusion assays, employed to assess antibacterial activity, were comparable to those produced by Ampicillin, a commercially available antibiotic applied in this study at 10 mg mL^{-1}. Some disruption to culture growth was also observed for *C. albicans*; however, as an antifungal agent was not included in these assays, the experiments should be repeated with appropriate control conditions to validate this result.

The methanol / sonication extract of *M. flos-aquae* MIC FEB05 also demonstrated antiviral activity against Dengue virus and RSV, as indicated by the lack of cytopathic effects on the cells, indicating the virus had been unable to infect the cell. The remaining cyanobacterial extracts provided to the IMVS did not exhibit toxicity towards either of the Influenza strains, HSV or Adenovirus, as indicated by the cytopathic effect of the viruses on the cell lines assessed.

Bacterial infections are caused by independent reproduction of bacterial cells within a "host", causing damage to the host organism's cells through toxin synthesis. Conversely, viruses require a living host to multiply; viruses are essentially capsules of genetic material, and are unable to reproduce without the aid of their host. Viral infections result in invasion of the host's cells where the virus takes over the cellular machinery, redirecting it to synthesise more viral particles. The virus, unless treated, may eventually kill the host. The distinction between "infection mechanisms" of viral and bacterial pathogens is essential, as medications that are effective against one do

not necessarily affect the other. Due to the intrinsic differences in infection mechanisms between bacteria and viruses, it is likely that the biological activity detected during this study is affected by 2 different compounds. Further analysis of the antiviral component of the methanol / sonication extract of *M. flos-aquae* MIC FEB05 is recommended to determine the compound responsible.

Table 4-9: Summary of antimicrobial activity against bacterial, fungal and viral test organisms

Code	Extraction Techniques applied	Bioactivity Assessment		Comment
		Antibacterial	**Antiviral**	
MIC FEB05	1, 3, 4, 6, & 7	*S. aureus, B. subtilis* - extract 4	Dengue, RSV – extract 4	Strong activity against bacteria and viruses.
MIC MAR05	1, 2, 3, 4 & 5	None	None	No bioactivity against test organisms.
ANA APR05	1, 4, 5, 7 & 9	*B subtilis* (weak) – extract 1 & 5	None	Weak activity against bacteria. Potential toxin synthesiser
MIC 053D	4, 5 & 9	None	None	No bioactivity against test organisms.
MIC 054A	4, 5 & 9	None	None	No bioactivity against test organisms.
MIC 306B	4, 5 & 9	None	None	No bioactivity against test organisms.
MIC 046E	4, 5 & 9	None	None	No bioactivity against test organisms.
MIC BOL	3, 4, 7 & 9	*B subtilis* (weak) – extract 4 & 9	None	Weak activity against bacteria.

1 – Methanol/Acetone/Hexane; 2 – Methanol/Hexane; 3 – Three times methanol; 4 - Methanol/Sonication; 5 – Combined Methanol; 6 – distilled water; 7 – dH_2O/Sonication; 8 – Chloroform/methanol; 9 – 75% Methanol.