BIODEGRADATION OF CYANOBACTERIAL HEPATOTOXINS [Dha^7]MC-LR AND MC-LR BY NATURAL AQUATIC BACTERIA

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Abstract

The aims of this doctoral study were to: isolate naturally occurring bacteria, able to degrade microcystins (MCs), from New Zealand waterbodies; to understand the biological processes of microcystin degradation by bacteria; and to develop small scale biofilm technology for testing the effectiveness of bacteria for microcystin degradation and/or remediation.

A significant amount of microcystins were required for biodegradation experiments. A modified method, using DEAE and Strata-X cartridge chromatography, was optimized for purifying microcystin variants from lyophilized bloom samples of the cyanobacterium *Microcystis aeruginosa*, collected en masse from Lake Horowhenua. Seven microcystin variants, MC-RR, MC-dMe-RR, MC-YR, MC-LR, [Dha^7]MC-LR, MC-FR, and MC-AR were purified by chromatography and then identified by reverse-phase High Performance Liquid Chromatography (HPLC) with UV detector (UVD) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). A mixture of [Dha^7]MC-LR and MC-LR, the main microcystin variants present, was used for examining biodegradation of microcystins by degrading bacteria.

Three isolates of bacteria—NV-1, NV-2 and NV-3—purified from Lake Rotoiti, New Zealand were capable of degrading [Dha^7]MC-LR and MC-LR. Among these isolates, NV-3 demonstrated the strongest degradative activity and was identified as a member of the genus *Sphingomonas.* On the basis of 16S rRNA sequencing, and 100% nucleotide sequence homology, it aligned most closely to strain MD-1. Based on the detection of two intermediate by-products (linearized peptides and a tetrapeptide) and the identification of four genes (*mlr*A, *mlr*B, *mlr*C and *mlr*D), that encode four putative proteins (enzymes) involved in microcystin degradation, it was suggested that the degradation of [Dha^7]MC-LR and MC-LR by the *Sphingomonas* isolate NV-3 occurred by a similar mechanism previously described for *Sphingomonas* strain MJ-PV (ACM-3962).

The bacterium *Sphingomonas* isolate NV-3 was examined for its ability to inhibit the growth of the cyanobacterium *Microcystis aeruginosa* strain SWCYNO4. It was found that the bacterium did not have any significant affect on the growth of the cyanobacterium, either by means of secretion of bacterial extracellular products or cell-to-cell contact between bacterial and cyanobacterial cells.

It was established that *Sphingomonas* isolate NV-3 was a moderate biofilm former, based on two types of biofilm formation assays, namely, microtiter plate assays and coupon biofilm assays. This was carried out in preparation for using the bacterium in a bioreactor for biodegradation of [Dha^7]MC-LR and MC-LR. The bacterium attached most effectively to ceramic, followed by PVC, polystyrene, stainless steel, and finally glass coupons. Biodegradation of MCs by the bacterium, in an internal airlift loop ceramic honeycomb support bioreactor (IAL-CHS bioreactor), was established in batch and continuous-flow experiments. In the batch experiment, NV-3 degraded a combination of [Dha^7]MC-LR and MC-LR at an initial concentration of 25 µg/ml at 30^{o}C in 30 hours, whereas in the continuous-flow experiment, NV-3 degraded the same concentration of [Dha^7]MC-LR and MC-LR in 36 hours with an hydraulic retention time (HRT) of 8 hours.

This study has demonstrated that microcystin-degrading bacteria are present in New Zealand waterbodies and that these bacteria could be used, potentially on a larger scale, for removing microcystins from water.

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Chapter 1
Introduction, chapter summary, and objectives

Cyanobacteria (Blue-green algae) are a primitive and omnipresent family of microorganisms, many with photosynthetic abilities. When environmental conditions are favorable, the cyanobacteria can proliferate and form algal blooms or cyanobacterial blooms (Carmichael, 1994; Dow & Swoboda, 2000; Haider, Naithani, Viswanathan, & Kakkar, 2003; Svrcek & Smith, 2004). Cyanobacteria are well recognized for their ability to produce a variety of bioactive secondary metabolites, and the rapid growth of toxin producing strains create severe problems in natural water (Bell & Codd, 1994). Cyanobacteria generate a wide range of toxins including hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and endotoxins (Carmicheal, 1994; Wiegand & Pflugmacher, 2005).

The most commonly encountered cyanobacterial toxins in freshwater are hepatotoxins referred to as microcystins (MCs) (Carmichael, 1994; Chorus & Bartram, 1999). These toxins are generated by a diverse range of species from the genera *Microcystis*, *Anabaena*, *Planktothrix*, *Oscillatoria*, and *Nostoc* (Carmichael, 1992; Carmichael & Falconer, 1993; Skulberg, Carmichael, Codd, & Skulberg, 1993; Sivonen & Jones, 1999; Chorus, 2001). To date, around 80 different structural variants of MCs have been chemically identified (Haider et al., 2003; Zurawell, Chen, Burke, & Prepas, 2005).

Microcystins are extremely stable and resistant to chemical hydrolysis or oxidation at neutral pH (Harada, 1996). The toxins can remain potent even after boiling (Lawton & Robertson, 1999). In natural waters and in the dark, MCs may persist for months or years (Sivonen & Jones, 1999). Dissolved MCs have been documented as highly resistant to conventional water treatment processes (Himberg Keijola, Hiisvirta, Pyysalo, & Sivonen, 1989; Lahti & Hiisvirta, 1989). Therefore, most drinking water treatment plants are typically ineffective in the complete destruction of MCs. A combination of sophisticated water treatments e.g., coagulation, chlorination, adsorption with activated carbon and ozonation can reduce the concentration of MCs, but during cyanobacterial blooms the ability of the water

treatment plants can be overwhelmed (Hoffman, 1976; Keijola, Himberg, Esala, Sivonen, & Hiisvirta, 1988; Lambert, Holmes, & Hrudey, 1996; Lawton & Robertson, 1999; Hitzfeld, Höger, & Dietrich 2000; Hoeger, Hitzfeld, & Dietrich, 2005).

Biodegradation is one of the safe and natural treatments for removing MCs from water. This process involves indigenous bacteria and complex communities of microorganisms in lake water (Jones & Orr, 1994; Inamori et al., 1998). Biodegradation can reduce both intracellular toxins within cyanobacterial cells and extracellular toxins released into waterbodies (Zurawell et al., 2005). Variation in the MC degradation rates among natural water probably stems from the bloom history of the lake and environmental factors that affect biological metabolic rates e.g., temperature and pH. Lakes with a history of cyanobacterial blooms containing MCs may have indigenous bacteria that are capable of using cyanobacterial cells and MCs as a food source and also rapidly degrading the toxins (Christoffersen, Lyck, & Winding, 2002).

Cyanobacterial blooms are frequently observed in eutrophic, brackish and fresh water, worldwide as well as in New Zealand waterbodies (Carmichael & Falconer, 1993; Zurawell et al., 2005). Wood (2004) detected MCs in 102 samples from 54 different locations around New Zealand. The samples collected from Lake Rotoiti, Lake Hakanoa, Lake Horowhenua, and Lake Waitawa in April 2003 contained high levels of MCs. Some of these samples were higher than the WHO provisional drinking-water guideline value of 1 µg/l for MC-LR (WHO, 1998). The MC levels ranged from 0.02 µg/l to a maximum of 36,500 µg/l. The latter collected from scum samples at Lake Horowhenua, Levin.

This study will determine whether or not bacteria isolated from New Zealand lakes (e.g. Lakes Rotoiti and Horowhenua) are able to degrade MCs. Small scale biofilm systems will be tested to determine the potential of bacterial degradation of MCs on a larger scale within water treatment plants. The MC-degrading genes and by-products will be characterized in order to understand the biodegradation mechanism. The research will establish whether or not the MC-degrading bacteria can also inhibit growth and/or lyse intact *Microcystis* cells which normally produce MCs, allowing complete removal of both intracellular and extracellular toxins from New Zealand waterbodies.

Chapters and Objectives:

The thesis is divided into seven chapters as follows:

Chapter 1: Introduction, chapter summary and objectives. It sets out to respond to the following objective:

- To provide general information, introduction and objectives of the thesis.

Chapter 2: Literature review. It sets out to respond to the following objectives:

- To provide general knowledge of cyanobacteria, cyanobacterial blooms, cyanotoxins, MCs and other aspects of the toxins e.g. structure and toxicity.
- To place this doctoral study in the wider context of cyanobacterial research.

Chapter 3: Extraction, purification, and identification of microcystins from a cyanobacterial bloom in Lake Horowhenua, New Zealand. It sets out to respond to the following objectives:

- To extract MC from cyanobacterial bloom material, using a modified method for MC isolation and purification.
- To identify and characterize purified toxins using reversed-phase HPLC and LC-MS/MS.

Chapter 4: Isolation and characterization of microcystin-degrading bacteria from New Zealand lakes. It sets out to respond to the following objectives:

- To isolate and identify natural aquatic bacteria capable of degrading MCs from New Zealand water bodies.
- To characterize the biotransformation of MCs using the most active MC-degrading bacteria isolated.
- To elucidate the genes that encode the MC-degrading enzymes of the bacterium.

Chapter 5: Effect of the bacterium *Sphingomonas* isolate NV-3 on the cyanobacterium *Microcystis aeruginosa* strain SWCYNO4. It sets out to respond to the following objective:

- To determine whether or not the MC-degrading bacterium *Sphingomonas* isolate NV-3 can inhibit growth and/or lyse *Microcystis aeruginosa* strain SWCYNO4.

Chapter 6: Biodegradation of [Dha^{7}]MC-LR and MC-LR by a microcystin-degrading bacterium *Sphingomonas* isolate NV-3 in an internal airlift loop ceramic honeycomb support bioreactor. It sets out to respond to the following objectives:

- To assess biofilm formation of the MC-degrading bacterium, so as to optimize bacterial attachment on carrier substrates within the bioreactor.
- To establish whether or not the internal airlift loop ceramic honeycomb support bioreactor is capable of effectively removing MCs under batch and continuous mode operation.

Chapter 7: General discussion and conclusions. This chapter draws together the discussion of this study.

Chapters 3 to 6, contain an abstract, a review of literature specific to the section, methodology, results, discussion, and references. The appendices for all chapters are at the end of the thesis.

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Chapter 2
Literature review

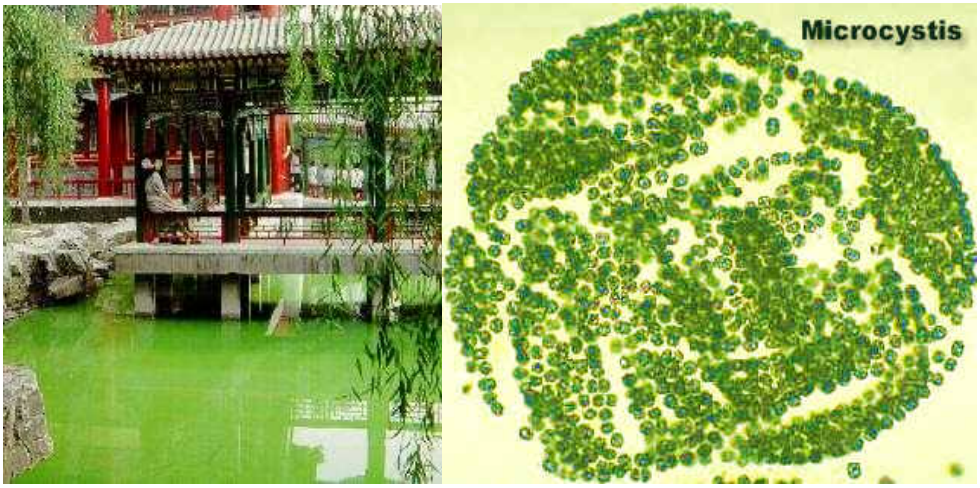
2.1 Cyanobacteria

Cyanobacteria, also referred to as blue-green algae, are members of the Domain Bacteria, Kingdom Monera (or Prokaryotae), Phylum Cyanobacteria, according to the second edition of the Bergey's manual of systematic bacteriology (Garrity, 2001). They are the largest and most diverse group of photosynthetic bacteria. Cyanobacteria are a primitive microorganism that can survive in a wide range of extreme environments such as the Antarctic coastal waters, volcanic hot springs, and dry deserts where no other vegetation can exist (Svrcek & Smith, 2004). Although cyanobacteria are prokaryotes, their photosynthetic system is closely associated with that of the eukaryotic algae because they have chlorophyll-*a* and photosystem II, and carry out oxygenic photosynthesis (Prescott, Harley, & Klein, 2005), but unlike eukaryotic microalgae, cyanobacteria do not possess membrane-bound subcellular organelles like chloroplasts. The photosynthetic pigments of cyanobacteria are located in thylakoids lying free in the cytoplasm near the cell periphery (Mankiewicz, Tarzyńska, Walter, & Zalewski, 2003).

Most cyanobacteria appear blue green due to the presence of the accessory pigment phycocyanin, a bluish phycobilin, although other pigments especially carotenoids and phycoerythrin, may also be present yielding red or brown colour to some species.

Cyanobacteria are also diverse in form and shape. They range in diameter from about 1-10 μm, and may be unicellular, colonies of many shapes, or as filaments. The unicellular forms may be spherical, ovoid, or cylindrical in shape and proliferate by binary fission. Cells growing in colonies may be packed in a mucilaginous sheath like *Microcystis* sp. or, in the case of filamentous species, grow as floating mats or as free-floating strands (Hitzfeld, Höger, & Dietrich, 2000; Haider, Naithani, Viswanathan, & Kakkar, 2003).

Figure 2.1 *Microcystis* bloom and *Microcystis* cells embedded in mucilage (Webb, 2001)



A filamentous form is a unique feature of some cyanobacterial genera. Repeated cell divisions which occur in a single plane at right angles to the main axis of the filament give rise to a multicellular structure known as a trichome. Distinctions in trichome morphology, such as straight or coiled forms, are important, in cyanobacterial identification (Carmichael, 2001). Filamentous structures reproduce by trichome fragmentation, or by formation of special hormogonia. Vegetative cells may develop into akinetes (large thick-walled cells, containing reserve material, enabling survival under adverse conditions), and heterocysts (a thick walled cell, capable of nitrogen fixation) (Chorus & Bartram, 1999).

Some cyanobacteria are able to form symbiotic associations with other organisms including protozoa, fungi, and plants (e.g. *Anabaena azollae* inside the leaves of *Azolla*). Some cyanobacterial species can survive on rocks and in soil and grow as terrestrial organisms (e.g. *Stigonema hormoides* and *Scytonema crassum*) and many of the cyanobacterial species (e.g. *Anabaena flos-aquae* and *Microcystis aeruginosa*) contain specialized intracellular gas vesicles, which allow the organism to regulate its buoyancy and therefore to actively seek water depth with optimal growth conditions (Prescott et al., 2005).

Many cyanobacterial genera, particularly filamentous forms such as *Gloeothece*, *Synechococcus*, *Plectonema*, and *Oscillatoria*, are capable of fixing nitrogen from the atmosphere. In most cases this activity is associated with

specialized cells “heterocysts”, which contain a nitrogenase enzyme that can fix nitrogen from dinitrogen (N_2) into ammonium (NH_4) to provide the cells with nitrogen for biosynthesis (Tortora, Funke, & Case, 2004).

2.2 Cyanobacterial blooms

The rapid growth of human population and the significant increase in agricultural and industrial activities along with lack of water management have resulted in the intensification of eutrophication (nutrient-rich water) in surface freshwater bodies used for recreational purposes and as drinking water sources (Figueiredo, Azeiteiro, Esteves, Gongalves, & Pereira, 2004). In eutrophic temperate lakes during summer or perennially eutrophic subtropical lakes, cyanobacteria can grow rapidly to create “blooms”, which may be located throughout the water column owing to buoyancy regulation mechanisms of cyanobacteria, or to waterbody-mixing processes. Furthermore, the cyanobacteria on occasion may accumulate at the water surface to form a scum due to a rapid increase in cyanobacterial buoyancy after calm weather conditions, or may develop as cyanobacterial mats and biofilms on the surface of the sediment in shallow lake or on rocks at the water’s edge (Codd, Morrison, & Metcalf, 2005).

Favorable cyanobacterial growth conditions that often lead to blooms are a combination of:

- ample sunlight;
- balmy water temperature (15-30ºC);
- neutral to alkaline conditions (pH 6-9);
- stagnant water conditions and little or no wind, leading to stratified waterbodies; and
- eutrophic water conditions, namely the increased concentration of the nutrients, particularly phosphorus and nitrogen, which may arise from household, industrial, or agricultural waste (Carmichael, 1994; Dow & Swoboda, 2000; Haider et al., 2003; Svrcek & Smith, 2004).

In New Zealand, a combination of favorable growth conditions together with global warming and intensive farming using a wide range of materials (e.g. fertilizers and inorganic matter) have resulted in an increase of cyanobacterial blooms throughout New Zealand over the last 20 years (Pridmore & Etheredge, 1987; Hamill, 2001; Stirling & Quilliam, 2001; Ryan, Hamilton, & Barnes, 2003; Wood & Stirling, 2003, Wood, 2004; Wood et al., 2006a, Wood et al., 2006b, Kouzminov, Ruck, & Wood, 2007; Wood et al., 2007; Selwood, Holland, Wood, Smith, & McNabb, 2007; Wood, Rueckert, Cowan, & Cary, 2008).

Although many species of freshwater eukaryotic algae grow very well in eutrophic waters, under such circumstances, cyanobacteria seem to proliferate more effectively than do true algae (Carmichael, 1994).

Cyanobacterial blooms may have a great impact on society, the economy, and the environment. Blooms and accumulated scum, with the production of secondary metabolite cyanotoxins, are a major concern in terms of animal deaths (e.g. birds, ducks, and fish) and aesthetic nuisances (e.g. odors, tastes, and color), as well as a health risk when contaminated water is used for recreation and as drinking water sources. Toxic bloom-forming cyanobacteria consist of the genera *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia*, *Planktothrix*, and *Raphidiopsis* with the most common scum production by *Microcystis*, *Anabaena*, *Anabaenopsis*, *Planktothrix*, and *Aphanizomenon*, and less so with the remaining genera. In addition, toxic cyanobacteria, which form biofilms and dense benthic mats in fresh water lakes, ponds, and rivers, belong to *Lynbya*, *Phormidium*, and *Oscillatoria* (Codd et al., 2005). Cyanobacterial blooms may be dominated by a single species or be composed of a variety of species, of which some may be toxic. Even in a single-species bloom there can be mixtures of toxic and non-toxic strains (Sivonen & Jones, 1999; Svrcek & Smith, 2004).

2.3 Occurrence of cyanobacterial blooms

Blooms of toxic cyanobacteria have been reported as occurring under a wide range of environmental conditions and in nearly all parts of the world (Table 2.1). The first toxic cyanobacterial bloom was documented by Francis in 1878 occurring in Lake Alexanderina in South Australia. The lake was contaminated by an overgrowth of toxic species *Nodularia spumigena*. This bloom made the water harmful for livestock and other animals that consumed the water, causing rapid death. Toxic cyanobacterial blooms have also been widely found in China. The bloom-forming cyanobacteria, growing in the Grandview Garden Park (Beijing), are mainly members of the genus *Microcystis* producing potent liver toxins. Carmichael (1994) reported that the toxins resulted in animal death, and the consumption of low doses in drinking water is suspected of contributing to a high risk of human liver cancer in certain parts of China. Cyanobacterial toxins have been the cause of lethal acute poisonings of humans. More than 50 patients at a haemodialysis unit in Caruaru, Brazil (1996), died from the use of contaminated water source containing a bloom of *Anabaena* and *Microcystis* spp. (Pouria et al., 1998; Hitzfeld et al., 2000; Park et al., 2001; Figueiredo et al., 2004).

Table 2.1 Known occurrences of toxic cyanobacterial blooms, scum, or mats

Europe	Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Norway, Poland, Portugal, Russia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Ukraine, United Kingdom
Americas	Argentina, Bermuda, Brazil, Canada, Chili, Mexico, USA (at least 27 States), Venezuela
Middle East and Asia	Bangladesh, India, Israel, Japan, Jordan, Malaysia, Nepal, Peoples' Republic of China, Philippines, Saudi Arabia, Sri Lanka, South Korea, Thailand, Turkey, Vietnam
Australasia	Australia, New Caledonia, New Zealand
Africa	Botswana, Egypt, Ethiopia, Kenya, Morocco, South Africa, Zimbabwe

From: Hitzfeld et al., 2000; Figueiredo et al., 2004; Svrcek & Smith, 2004; Codd et al., 2005.

2.4 Cyanobacterial blooms in New Zealand

With regards to cyanobacteria in New Zealand, Hamill (2001) published the first report of animal deaths in New Zealand caused by a species probably belonging to *Oscillatoria.* Five dogs were reported dead within three days at Edgewood Park near the Waikanae River, Northland in December 1998 due to licking a mat or consumption of contaminated water from the river. Anatoxin-a was the most likely toxin responsible for the dog deaths.

During the same summer (between January-February 1999) the death of five dogs was reported along the Mataura River, near Mataura, Southland. The following summer, a dog died at the Mataura River, near Mataura Island Bridge. As with the first incidence, the dog deaths along Mataura River are thought to be caused by ingestion of toxic cyanobacteria from the river. In each incidence, samples of river water and mats were subsequently collected, analyzed for toxicity, and examined to identify the cyanobacteria. The results showed that all samples of cyanobacterial mats were highly toxic in mouse bioassays due to the presence of anatoxin-a. The cyanobacterium suspected as being the toxic agent in the dog deaths was identified as an *Oscillatoria*-like species (Hamill, 2001).

In early June 1999, cyanobacteria accumulated as scum at the water surface of Lake Waitawa, a recreational lake north of Wellington. Stirling and Quilliam (2001) reported the presence of several cyanobacterial toxins in water from the lake. The cyanotoxins detected from the lake included cylindrospermopsin and microcystins (MCs) (e.g. MC-LR, MC-RR, MC-WR and MC-LA). The causative cyanobacterium was initially identified as a *Cylindrospermum* species. However, a rigorous identification was not carried out, and a sample collected two weeks later contained *Microcystis aeruginosa* as the dominant species.

During the summer of 2002/2003, Kouzminov et al. (2007) reported a cyanobacterial bloom in the Waikato River, a major drinking-water supply for Hamilton City (Northland), and other smaller towns along the river. This bloom was found to be dominated by toxic genera, *Anabaena*, *Oscillatoria*, and *Microcystis*. In January 2003, saxitoxins were detected at low levels in raw and treated water at the Hamilton City drinking-water intake. The occurrence of cyanobacterial blooms led to taste and odor problems in Waikato drinking-water supplies. However, cases of

cyanotoxin poisoning and/or illness as a result of consumption of the contaminated water or contact with the river were not observed.

In March 2003, a cyanobacterial bloom was reported by Wood and Stirling (2003) as containing the cyanotoxin cylindrospermopsin in Lake Waahi, a shallow mesotrophic lake in the lower Waikato River basin. The cylindrospermopsin-producing species was identified as *Cylindrospermopsis raciborskii*. The discovery of *C. raciborskii* bloom in this lake is the first reported occurrence of this species in New Zealand.

In the uncommonly dry summer of 2005/2006, the Hutt River (Wellington) experienced cyanobacterial mat formation with *Phormidium* sp. dominating. Two neurotoxic cyanotoxins, anatoxin-a and homoanatoxin-a, detected from the mats caused the deaths of some birds and at least five dogs after contact with the mats (Wood et al., 2007).

2.5 Cyanotoxins

Cyanobacteria are capable of producing a wide range of bioactive secondary metabolites, some of which can be used for medical and pharmaceutical purposes such as antiviral, antifungal and antitumor compounds, as well as compounds toxic to other tissues, cells, or organisms (Sivonen & Jones, 1999; Singh, Kate, & Banerjee, 2005). The toxic compounds produced from cyanobacteria (i.e. cyanotoxins) are secondary metabolites, which are not essential for primary metabolism but synthesized within the cells of some species. While it is unclear why certain strains of cyanobacteria are able to produce toxins, it has long been speculated that they may have a role as protective compounds, since there have been reports that some cyanotoxins are potent inhibitors of invertebrate grazers in the aquatic environment (Carmichael, 1992; Svrcek & Smith, 2004).

In general, cyanobacteria (e.g. *M. aeruginosa*) are able to produce cyanotoxins during all stages of their growth with the greatest amount of the toxins at the end of their logarithmic growth phase. The toxins remain within active cells (intracellular toxin) and are released into the surrounding water (extracellular toxin) when the cells age or stress (Sivonen & Jones, 1999; Svrcek & Smith, 2004). Cyanobacterial species

known for their potential to produce toxins are shown in Table 2.2 (Dow & Swoboda, 2000).

Table 2.2 Confirmed toxin-producing species of cyanobacteria

Species	
Anabaena circinalis Rabenh.	*Nodularia spumigena* Mertens
A. flos-aquae (Lyngb) Breb.	*Nostoc linckia* (Roth) Born. et Flah.
A. hassallii (Kütz.) Wittr.	*N. paludosum* Kütz.
A. lemmermanni Richter	*N. rivulare* Kütz.
A. spiroides var. contracta Kleb.	*N. zetterstedtii* Areschoug
A. variabilis Kütz.	*Oscillatoria acutissima* Kuff.
Anabaenopsis milleri Woron.	*O. agardhii/rubescens* group
Aphanizomenon flos-aquae (L.) Ralfs	(= *Planktothrix*)
Coelosphaerium kuetzingianum Näg.	*O. Formosa* Bory
Cylindrospermum sp.	*O. nigroviridis* Thwaites
Cylindrospermopsis raciborskii (Wolos.)	*Oscillatoria* sp.
Seenaya et Subba Raju	*Pseudanabaena catenata* Lauterb.
Fischerella epiphytica Ghose	*Schizothrix calcicola* (Ag.) Gom
Gloeotrichia echinulata Richter	*Scytonema pseudohofmanni* Bharadw.
Gomphosphaeria lacustris Chod.	*Synechococcus* strains Miami BCII 6S
G. naegliana (Unger) Lemmerm.	and ATCC 18800
Hormothamnion enteromorphoides Grun.	*Synechocustis* sp.
Lyngbya majuscula Harvey	*Tolypothrix byssoidea* (Hass.) Kirchn.
Lyngbya wollei	*Trichodesmium erythraeum* Ehrb.
Microcystis aeruginosa Kütz.	*Umezakia natans* Harada
M. wesenbergii (Komárek in Kondrateva)	
M. botrys	
M. viridis	

From: Dow & Swoboda, 2000.

Cyanotoxins have adverse effects on wild and domestic animals, including cattle, dogs, birds, amphibians, and fish, and also on humans, ranging from mild to fatal illness depending on the degree of exposure (Hitzfeld et al., 2000; Mankiewicz et al., 2003; Codd et al., 2005). Hepatotoxicosis and neurotoxicosis are the most common syndromes of animal illness resulted from consumption of cyanotoxins. The symptoms of hepatoxicosis include weakness, inability to walk, anorexia, pallor of the extremities, mucous membranes, vomiting, cold extremities, hypovolaemic shock and occasionally mental derangement whereas the clinical signs of neurotoxins included a progression of muscle fasciculation, decreased movement, abdominal breathing, cyanosis, convulsions, and even death (Carmichael & Falconer, 1993).

Cyanobacterial toxins can be categorized into three large groups according to their chemical structures: cyclic peptides, alkaloids, and lipopolysaccharides (Sivonen & Jones, 1999; Hitzfeld et al., 2000; Svrcek & Smith, 2004) (see Table 2.3). However, since cyanotoxins are very diverse in their toxicity, they can also be classified into five major groups on the basis of the physiological systems, organs, tissues, or cells which are primarily affected: neurotoxins, hepatotoxins, cytotoxins, dermatotoxins, and irritants and gastrointestinal toxins (Sivonen & Jones, 1999; Mankiewicz et al., 2003; Svecek & Smith, 2004; Codd et al., 2005) (see Table 2.3).

Table 2.3 Cyanotoxins: general features and producer organisms (adapted from Sivonen & Jones, 1999; Mankiewicz et al., 2003; Svrcek & Smith, 2004)

Toxin group	Primary target organ in mammals	Mechanism of toxicity	Producer cyanobacteria genera*
Cyclic peptides			
Hepatotoxins			
Microcystins (MCs)	Liver	Inhibition of protein phosphatase activity, hemorrhaging of the liver	*Anabaena, Anabaenopsis, Aphanocapsa, Hapalosiphon, Microcystis, Nostoc, Oscillatoria, Nodularia* (mainly brackish water)
Nodularins	Liver		
Alkaloids			
Neurotoxic alkaloids			
Anatoxin-a	Nerve synapse	Blocking of post-synaptic depolarization	*Anabaena, Aphanizomenon, Oscillatoria*
Anatoxin-a (s)	Nerve synapse	Blocking of acetylcholinesterase	*Anabaena, Oscillatoria*
Saxitoxins	Nerve axons	Blocking of sodium channels	*Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya*
Cytotoxic alkaloids			
Cylindrospermopsins	Liver, kidney, spleen, intestine, heart, thymus	Inhibition of protein synthesis	*Anabaena, Aphanizomenon, Cylindrospermopsis, Umezakia*
Dermatotoxic alkaloids			
Aplysiatoxins	Skin		*Lyngbya, Oscillatoria, Schizothrix*
Debromoaplysiatoxins	Skin		*Lyngbya, Oscillatoria, Schizothrix Lyngbya*
Lyngbyatoxin-a	Skin, gastrointestinal tract	Protein kinase C activators, inflammatory activity	All
Lipopolysaccharides (LPS) (*Irritant toxins*)	Any exposed tissue	Potential irritant and allergen	

* Not produced by all species of the particular genus

The hepatotoxic cyclic peptides, the neurotoxic alkaloids, and the cytotoxic alkaloid cylindrospermopsin are the most frequently detected in freshwater (Svrcek & Smith, 2004). However, among these toxin groups, the hepatoxins and especially microcystins (MCs) are the most commonly observed and also the most extensively studied (Zurawell, Chen, Burke, & Prepas, 2005). The focus of this study is the MCs, and only brief mention of alkaloid toxins and hepatotoxic nodularins will be made.

2.5.1 Neurotoxic alkaloids

The neurotoxic alkaloids are heterocyclic nitrogenous compounds with at least one nitrogen-carbon bond. They are the most rapidly acting toxins with highly specific effects on the neuromuscular system of animals and humans. The toxins act by paralyzing first peripheral skeletal muscles then respiratory muscles, causing paralysis, respiratory failure, or sudden death in exposed animals within a few minutes to a few hours (Carmichael, 1981). However, since cyanobacteria responsible for production of neurotoxin are rare, and not so many populations of the potential producers actually produce the toxins, they are not as common as the cyclic peptide hepatotoxins in freshwater (Mankiewicz et al., 2003; Svrcek & Smith, 2004). The cyanobacteria that produce each neurotoxin are listed in Table 2.3. The cyanobacterial neurotoxins can be classified into three distinct groups (Sivonen & Jones, 1999):

- anatoxin-a and homoanatoxin-a, which mimic the effect of acetylcholine,
- anatoxin-a (S), which inhibits enzyme acetylcholinesterase, and
- saxitoxins, also known as paralytic shellfish poisons (PSPs) in the marine literature, which block nerve cell sodium channels. There have been reports of PSP toxins in shellfish within New Zealand, which cause severe health, problems and serious damage to the economic viability of shellfish industry (Garthwaite et al., 2001).

2.5.2 Cytotoxic alkaloids

One of the most recently discovered cyanotoxins in New Zealand, is the cytotoxic alkaloid cylindrospermopsin. The name is derived from the cyanobacterium *Cylindrospermopsis raciborskii*, the first organism from which it was isolated (Falconer, 2005). The toxin is a tricyclic guanidine alkaloid combined with hydroxymethyl uracyl with a molecular weight of 415 Daltons (Sivonen & Jones, 1999). Although the toxin is basically hepatotoxic in its pure form (Svrcek & Smith, 2004), cylindrospermopsin inhibits protein synthesis in general (Mankiewicz et al., 2003). The toxin is thus not specific to the liver as it also damages a wide variety of body organs. The initial clinical signs of the toxin are kidney and liver failure, but there are also affects on the spleen, intestine, heart, and thymus (Codd, 2000; Mankiewicz et al., 2003). In New Zealand, *C. raciborskii* was also reported in waterbodies by Wood et al. (2003), and the presence of *C. raciborskii* and its toxin in New Zealand has become a potential threat to animals and humans.

2.5.3 Hepatotoxic cyclic peptides

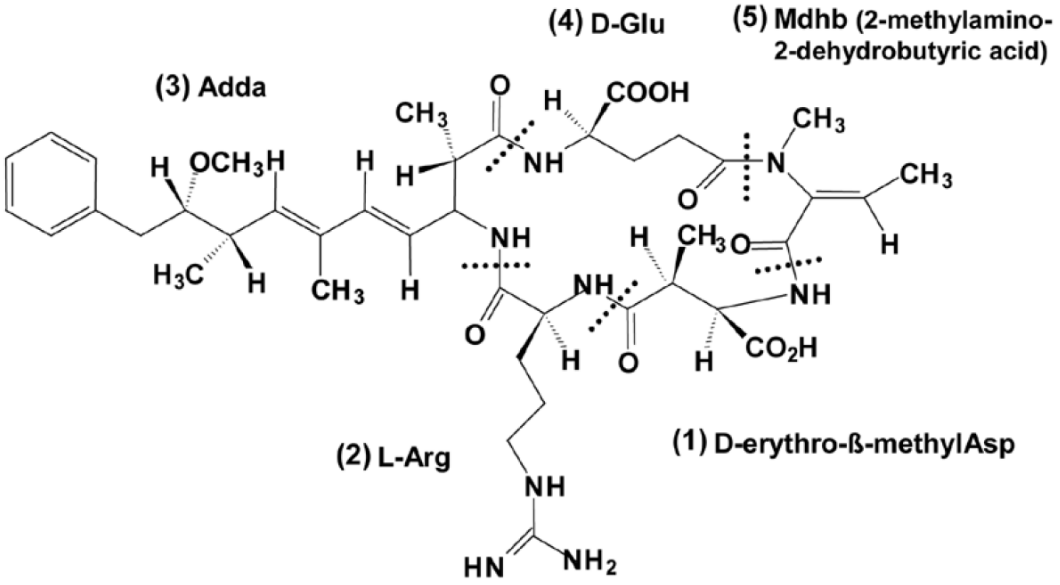
Hepatotoxic cyclic peptides are the most frequently found cyanotoxins in fresh and brackish waters worldwide (Sivonen & Jones, 1999). This group includes MCs and nodularins. These two hepatotoxins are both cyclic peptides, containing the same basic cyclic structure, a unique hydrophobic amino acid known as Adda; (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; plus six (in MCs) or four (in nodularins) other amino acids (Briand, Jacquet, Bernard, & Humbert, 2003). They possess similar toxicity mechanisms, and are preferentially taken up by hepatocytes (functional cells of the liver), causing a wide range of adverse effects (Zurawell et al., 2005).

2.5.3.1 Nodularins

Nodularins are potent hepatotoxins produced by the bloom forming cyanobacterium *Nodularia spumigena*, which occurs in brackish waters, particularly in the Baltic Sea, Australia, and New Zealand (Briand et al., 2003). They are cyclic pentapeptides, containing five amino acids. The chemical structure is cyclo-(D-MeAsp^{1}-L-Arg^{2}-Adda^{3}-D-Glu^{4}-Mdhb^{5}), in which the D-MeAsp is D-*erythro-β*-

methylaspartic acid, and Mdhb is 2-(methylamino)-2-dehydrobutyric acid (Figure 2.2). The structure of nodularins are different from that of MCs (heptapeptide hepatotoxins) by lacking two amino acids (D-Ala and L-amino acids) and having Mdhb in place of Mdha (*N*-methldehydroalanine) (Sivonen & Jones, 1999). So far eight variants of nodularin have been identified (Mazur-Marzec, Meriluoto, Pliński, & Szafranek, 2006). The mode of action of nodularins is similar to that of MCs in spite of their different chemical structures. They are hepatotoxic to vertebrates, cause disruption of liver structure and eventual death of the animal by haemorrhagic (internal bleeding) shock or liver failure (Briand et al., 2003; Mankiewicz et al., 2003; Svrcek & Smith, 2004).

Figure 2.2 Chemical structure of nodularins (Dittmann & Wiegand, 2006)



2.5.3.2 Microcystins (MCs)

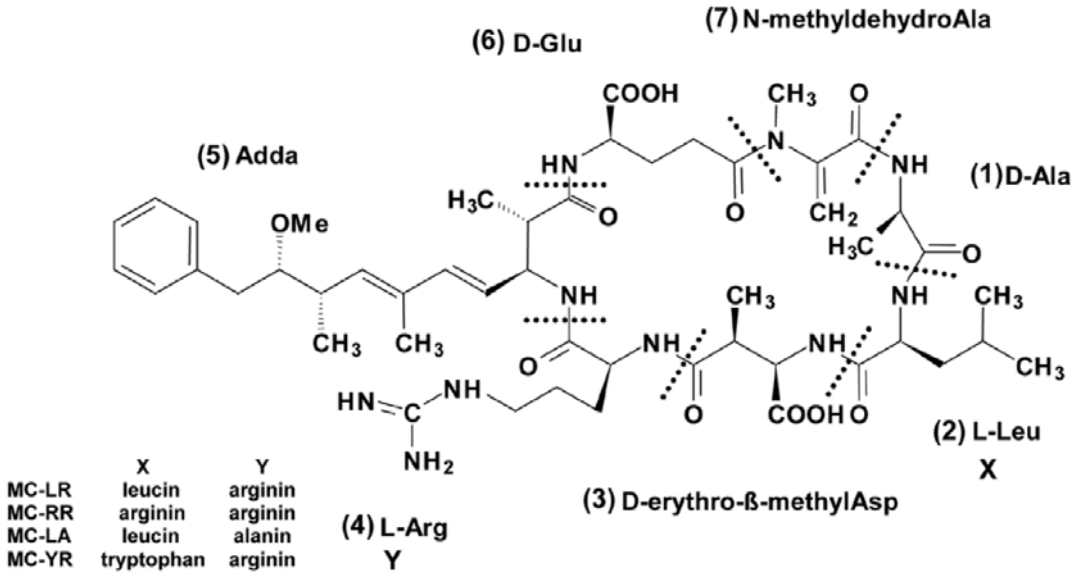
The most frequently occurring and widespread of the cyanotoxins are MCs. They are a family of monocyclic heptapeptide hepatotoxins produced by different genera of freshwater cyanobacteria *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktothrix*), *Nostoc*, and *Anabaenopsis* (Sivonen & Jones, 1999; Chorus, 2001). The name of these toxins is derived from the genus of cyanobacterium from which it

was first extracted, *Microcystis aeruginosa* strain (Carmichael et al., 1988). Bishop Anet, and Gorham (1959) reported the first isolation of these hepatotoxins from *M. aeruginosa* strain NRC-1. The toxins were identified as peptides referred to as "fast-death factor". Various alternate names for these toxins were given including cyanoginosin, cyanoviridin, and cyanogenosin. However, microcystin is the term most frequently used for naming monocyclic heptapeptide hepatotoxins of cyanobacteria (Carmichael et al., 1988).

2.5.3.2.1 Structure of MCs

Microcystins (MCs) are small monocyclic peptides with a molecular weight of about 1000 Daltons. They consist of seven variable amino acids in their structure. The chemical structure (Figure 2.3) is cyclo(-D-Ala^{1}-X^{2}-D-MeAsp^{3}-Y^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}) in which X and Y are variable L-amino acids, D-MeAsp is D-*erytho*-*β*-methylaspartic acid, and Mdha is *N*-methldehydroalanine. The Adda, (2s, 3s, 8s, 9s)-3-amino- 9- methoxy-2, 6, 8-trimethyl- 10-phenyldeca- 4, 6-dienoic acid, is the most unique structure in this group of cyanobacterial cyclic peptide toxins and essential for expression of biological activity.

Figure 2.3 Chemical structure of MCs (Dittmann & Wiegand, 2006)



Structural variation has been encountered at all seven amino acid positions, but the highest degree of structural variation is found with the substation of L-variable amino acids at positions 2 and 4 (denoted X and Y, respectively) (Figure 2.3), and demethylation of D-MeAsp and/or Mdha at positions 3 and 7, respectively (Sivonen & Jones, 1999). The variation of MC names is due to the variable L-amino acid at two non-conserved positions in the molecule. X is commonly leucine (L), arginine (R), or tyrosine (Y), and Y is usually arginine (R), alanine (A), or methionine (M) (Carmichael et al., 1988). For example, MC-LR contains the amino acid leucine (L) and arginine (R) at these positions and is one of the most common MC variants (Carmichael, 1992; Sivonen & Jones, 1999).

As mentioned above, structural variations have been reported, giving approximately 80 variants of MCs to date (Mankiewicz et al., 2003; Svrcek & Smith, 2004; Zurawell et al., 2005; Leflaive & Ten-Hage, 2007). Variations in other constituent amino acids include methyl esterification of D-Glu at position 6 or substitution (modification) of Mdha at position 7, with/without demethylation of D-MeAsp at position 3 (Zurawell et al., 2005). Furthermore MC variants have recently been found by modification in Adda, including geometric isomerization at the C-7 position, and demethylation or substitution of the methoxyl group with an acetyl group at the C-9 position, with or without variation in amino acids at positions 1, 3, and/or 7 (Zurawell et al., 2005).

2.5.3.2.2 Property and toxicity of MCs

Microcystins are recognized to be physically and chemically stable compounds over a range of pH, temperature, and irradiation possibly due to their cyclic structure (Lawton & Robertson, 1999; Svrcek & Smith, 2004). They are reported to persist for several hours in boiling water and remain stable for many years when stored dry at ambient temperature (Lawton & Robertson, 1999).

The invariable and variable amino acid components of MCs contain both polar (hydrophilic) and nonpolar (hydrophobic) residues. In spite of the variable amino acids, MCs are soluble in water to varying degrees (Harada, 1996). Due to their molecular weight and high solubility in water, the toxins have a low volatility and do

not readily penetrate lipid membranes of animal, plant, and bacterial cells (Sivonen & Jones, 1999; Svrcek & Smith, 2004).

As mentioned earlier, most variations occur in L-amino acids at positions 2 (X) and 4 (Y) in the MC structure (Sivonen & Jones, 1999), L-amino acid variants normally contain hydrophobic amino acids at X and hydrophilic acids at Y (Falconer, 2005). The most toxic of the MCs are those with the more hydrophobic L-amino acid. Substitution of hydrophobic L-amino acid in the first variable position with another hydrophobic L-amino acid (e.g. alanine, phenylalanine, or tryptophan) maintains toxicity, but replacement with a hydrophilic amino acid (e.g. arginine) significantly decreases toxicity (Namikoshi et al., 1992). Therefore, MCs composed of polar (hydrophilic) replacements in both variable amino acid positions, such as MC-RR (arginine-arginine), and MC-M(O)R (methionine sulfoxide, arginine), are the least toxic (Zurawell et al., 2005). Why hydrophobic L-amino acids increase toxicity is unclear, but may relate to the principal mode of toxic action of MC, that of binding to the enzyme protein phosphatase and blocking enzyme function (see paragraph below).

In addition, variations in invariable residues may or may not affect toxicity. For example, absence of the methyl group from D-MeAsp or from Mdha results in minor change in toxicity, however, esterification at the free carboxyl group of D-Glu diminishes toxicity (Zurawell et al., 2005). More importantly, modification in the Adda moiety is vital for toxicity. Isomerization of the Adda diene 6(E) to 6(Z) results in nontoxic MC analogues (Sivonen & Jones, 1999; Zurawell et al., 2005; Falconer, 2005). However, the methoxy group at C-9 of the Adda seems less significant, as substitution of the methoxy group with an acetoxy group or demethylation at this position has no major impact on toxicity (Namikoshi, Rinehart, Sakai, Sivonen, & Carmichael, 1990; Zurawell et al., 2005; Falconer, 2005).

At the molecular level, MCs are potent and specific inhibitors of protein phosphatase type 1 and 2A (PP1 and PP2A) (Mackintosh, Beattie, Klumpp, Cohen, & Codd, 1990; Dawson, 1997; Codd et al., 1999). Protein phosphatases are crucial enzymes in catalyzing dephosphorylation of serine/threonine residues in phosphoproteins and regulating several cellular processes in animals, including carbohydrate metabolism, muscle contraction, and cell division (Lawton & Codd, 1991; Babica, Bláha, & Maršálek, 2006). Inhibition of the enzymes, therefore, results

in various responses such as protein hyperphosphorylation, morphological change of hepatocytes (liver cells) and tumor promotion (Mazur-Marzec, 2006).

The toxicity of MCs is mediated by their uptake into hepatocytes by means of a carrier-mediated transport system (Oberholster, Batha, & Grobbelaar, 2004). Since MCs are unable to move across cell membranes, the entry of the toxins into the hepatocytes and other target tissue is achieved by the multi-specificity anion transport bile carrier (Oberholster et al., 2004; Zurawell et al., 2005).

The MCs can cause illness or death in animals. It is known that the toxins cause hepatocytes to shrink. As a result, the cells which are normally compacted together firmly, separate and other cells forming the so-called sinusoidal capillaries of the liver also separate. Afterward the blood carried by the vessels seeps out (intrahepatic hemorrhage) and accumulates in liver tissue, resulting in tissue damage and, frequently hypovolemic shock - the direct cause of death (Carmichael, 1994).

Although the major effect of MCs is liver damage, less severe effects are also observed in the gastrointestinal tract and kidneys. Gastrointestinal effects consist of gastroenteritis and enterocyte deformation, hemorrhage, and may be a direct effect of toxin uptake through intestinal epithelia. Kidney effects, which may involve indirect toxicity through the liver, include lesions and cell damage in both the glomeruli and collection tubules, with resultant increases in blood urea-nitrogen, creatinine, and potassium (Zurawell et al., 2005).

Microcystins can induce tumor formation by the inhibition of PP1 and PP2A, which involve cell cycle regulation (Falconer, 1993; Carmichael, 1994; Haider et al., 2003). It is believed that MCs function as tumor promoters similar to the fatty acid polyketal okadaic acid - a marine algal toxin produced by dinoflagellates, causing diarrheic shellfish poisoning (Falconer, 2005). Okadaic acid is one of the strongest liver tumor promoters and a phosphatase inhibitor. The tumor promotion occurs as a result of the stimulation of cell protein phosphorylation by protein kinase C, which phosphorylates serine and threonine hydroxyl groups on specific proteins; or by inhibition of dephosphorylation by PP1 and PP2A, which normally remove phosphate groups from serine and threonine hydroxyls of specific proteins. Both of these mechanisms lead to hyperphosphorylation of particular proteins causing a range of actions in the cell cycle (Falconer, 1993). Similarly, MCs, when added to a mouse

liver cytosol fraction, result in a significant increase in protein phosphorylation (Falconer, 2005).

It is expected that the loss of cell-cell contact with MCs could reduce the normal contact inhibition of cell replication in organs, which is also associated with tumor growth. Studies in which mice consume MCs with their drinking water showed increased weight of carcinogen-initiated skin tumors. Direct intraperitoneal (i.p.) injection of MC-LR into rats promoted liver tumor cell growth after chemical initiation of the tumors. This is entirely consistent with the parallel mechanism of enzyme inhibition by MC and okadaic acid described earlier (Falconer, 1993).

The risk of tumor promotion by chronic exposure of MCs in drinking water and the occurrences of animal and human death caused by the toxins have led to the introduction of guideline values for drinking water by the World Health Organization (WHO, 1998), with a recommended limit of 1 µg of MC-LR per liter. In New Zealand, the Ministry of Health has also developed national criteria for assessing the risk of toxic cyanobacteria, including MCs in drinking-water supplies (DWSNZ 2005) with maximum acceptable values (MAV) of 1 µg of MC-LR per liter (Ministry of Health, 2005).

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Chapter 3
Extraction, purification and identification of microcystins from a cyanobacterial bloom in Lake Horowhenua, New Zealand

3.1 Abstract

Microcystins (MCs) were purified with DEAE anion exchange chromatography and Strata-X solid phase extraction cartridge and identified by reversed-phase High Performance Liquid Chromatography (HPLC) with UV detector (UVD) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Chemical characterization revealed [Dha^7]MC-LR was the major variant extracted from the lyophilized cyanobacterial bloom material (20 grams) with a purity of 93% and yield of 51.84 mg. MC-FR (94% purity, 3.16 mg), MC-RR (95% purity, 2.97 mg), MC-AR (92% purity, 0.04 mg) and MC-YR (94% purity, 0.01 mg) were also purified by chromatography. This study proved the use of the DEAE and Strata-X cartridge chromatography is an effective method for the purification of various MCs. It is a cost effective way to obtain MCs, for further studies, from freeze-dried bloom samples of the cyanobacterium *Microcystis aeruginosa* collected en masse from Lake Horowhenua. It provided a mix of naturally occurring MC variants potentially present in New Zealand drinking water sources.

3.2 Keywords: microcystins, purification, identification

3.3 Introduction

Microcystins (MCs) are the most commonly detected cyanobacterial toxins in freshwater (Chorus & Bartram, 1999) and have been implicated in animal and human illness or even deaths worldwide (Carmichael, 1994; Codd et al., 1999; Chorus & Bartram, 1999). Increasing interest in these toxins (such as investigations into their toxicology, their biodegradation and provision of analytical standards for environmental analysis) has greatly increased the demand for pure MCs (Edward, Lawton, Coyle, & Ross, 1996a). It is therefore important to develop methodologies

which can accommodate the purification of milligram to gram quantities of MCs from natural bloom and/or cultured cells.

In this study, significant quantities of MCs were required for use in experiments testing the effectiveness of biodegradation as a means of purifying drinking water. Lake Horowhenua was known to produce *Microcystis* blooms, which had high concentrations of MCs (Wood, 2004). The extraction and purification method was modified from that used by Saito et al. (2002) which employed anion exchange chromatography as the first purification step. However, a different type of secondary purification or clean-up step with Strata-X cartridges was used. The toxins were further identified and characterized by reversed-phase HPLC and LC-MS/MS.

3.3.1 Extraction and purification of MCs

A wide variety of methods have been developed for isolation and purification of MCs (Gregson & Lohr, 1983; Brooks & Codd, 1986; Krishnamurthy, Carmichael, & Sarver, 1986; Meriluoto & Eriksson, 1988; Lawton, Edwards, & Codd, 1994; Tsuji et al., 1994; Edwards et al., 1996a; Edwards, Lawton, Coyle, & Ross, 1996b; Fastner, Flieger, & Neuman, 1998; Lawton, McElhineya, & Edwards, 1999; Ramanan, Tang, & Velayudhan, 2000; Pyo & Lee, 2002; Saito et al., 2002; Aranda-Rodriguez, Kubwabo, & Benoit, 2003; Barco, Lawton, Rivera, & Caixach, 2005). The number of steps and procedures used vary widely. No consensus on the most proficient method for purifying MCs has been reported (Lawton & Edwards, 2001). It is practicable to use properties of MCs such as molecular size, net or potential charge, and adsorption capability to isolate and purify the toxins. The toxins have molecular weights of about 1000 Daltons and contain polar (hydrophilic) and nonpolar (hydrophobic) residues in the structure. They are soluble in methanol and relatively soluble in water but unable to dissolve in non-polar solvents such as diethyl ether. In general, the extraction and purification of MCs from toxic cyanobacterial cells involves many steps as follows: the MCs contained in lyophilized cyanobacterial cells are extracted several times with organic solvents involving stirring, shaking, or sonication; then the extract is concentrated in smaller volumes of solvent; and finally separated and cleaned up with multiple-steps of chromatography (Pyo & Lee, 2002).

3.3.1.1 Extraction of MCs

Typically cyanobacterial extracts are protein-rich solutions (Meriluato, 1997). The high concentration of the protein presents a difficulty in subsequent purification and identification processes; for example, proteins easily degrade on reversed-phase packing of HPLC column and make the column ineffective (Benedek, Dong, & Karger, 1984). The extraction and purification should therefore remove proteins as well as pigments, inorganic and organic compounds. Harada et al. (1988) promoted 5% aqueous acetic acid as superior to extraction with water, and it resulted in three times less protein in the extract compared to using water. An advantage of using aqueous acetic acid extraction is that the extract can be directly applied onto a C_{18} solid phase extraction cartridge (a typical step after solvent extraction), therefore rotary evaporation is no longer required to concentrate the toxins into smaller volumes of solvent, reducing a step in the purification process. Krishnamurthy et al. (1986) introduced extraction of cyanobacteria *M. aeruginosa* and *Anabaena flos-aquae* freeze-dried cells with a water-methanol-butanol solution (75:20:5%). However Meriluoto and Eriksson (1988) discovered no significant differences between extractions of freeze-dried cyanobacteria using water alone or a water-methanol-butanol solution. Lawton et al. (1994) demonstrated that methanol extracted MC variants slightly better than a water-methanol-butanol solution (75:20:5) and significantly better than 5% acetic acid. A 5% acetic acid solution was particularly weak in extracting the more hydrophobic MCs (e.g. MC-LY, MC-LW, and MC-LF) and the yields were only 10-20% compared to those of methanol extraction. The water-methanol-butanol extraction was about 15% less efficient than methanol extraction and methanol was therefore recommended for the extraction of freeze-dried samples of cyanobacterial cells (Lawton et al., 1994). However, the best extraction was observed at 50–80% methanol with 70% aqueous methanol selected as the most suitable (Lawton & Edwards, 2001).

3.3.1.2 Sample concentration of MCs

Extraction of cyanobacterial cells usually requires large volumes of solvent, giving very dilute solutions of MCs which need further concentration prior to purification. Examples of techniques used for concentration of MCs have been

evaporation and solid phase extraction (Lawton & Edwards, 2001). Evaporation is commonly performed using a rotary evaporator at 40^{o}C under air or nitrogen compression (Lawton & Edwards, 2001; Saito et al., 2002). Some scientists also employed a combination of rotary evaporation and solid phase extraction to decrease the volume of the extract and eliminate some impurities in the sample (Bateman, Thibault, Douglas, & White, 1995; Namikoshi et al., 1998). C_{18} solid phase extraction (SPE) is one of the most commonly used methods for concentration and sample clean-up. Aqueous acetic acid extracts can be used directly with C_{18} cartridges then eluted with aqueous methanol (typically about 70%) or absolute methanol, leaving some impurities on the cartridges. The use of C_{18} solid phase extraction can also be the primary step of sample purification if the absorbed toxins are eluted from the cartridges in an appropriate manner, such as using a methanol step gradient. For example, a proportion of impurities may be washed out from the cartridges using pure distilled water and/or a low concentration of aqueous methanol of about 10-30% (some impurities are also still retained on the cartridges), then the toxins are eluted using a linear gradient of methanol from 40 to 100% (Harada et al., 1988; Lawton et al., 1994; Lawton & Edwards, 2001).

3.3.1.3 Separation of the toxins

Without using any chromatography technique, MCs were first isolated from *M. aeruginosa* NRC-1 and reported as "fast-death factor" by Bishop et al. (1959). However, the first effective procedure for isolation and purification of MCs from cyanobacterial cells was documented by Murthy and Capindale (1970) where they used a combination of solvent extraction, dialysis and ion-exchange (DEAE Sephadex A-25) column chromatography. This protocol has been widely used and modified over the last 25 years to purify and obtain significant amounts of MC variants. Elleman, Falconer, Jackson, and Runnegar (1978) used a new combination of two chromatographies, size exclusion (Sephadex G-25) and ion exchange (DEAE Sephadex A-25) to isolate MC-YM from cyanobacterial cells. The size exclusion step is to remove pigments and large interfering molecules whereas the ion-exchange is to clean up the toxins (Lawton & Edwards, 2001). Using Sephadex G-50 and DEAE-cellulose DE-52 chromatography followed by paper electrophoresis and paper

chromatography, Botes, Anet, and Gorham (1982) first successfully purified four MC variants (called cyanoginosins) and obtained adequate pure materials for determining the complete structure of the toxins. Since then, a wide range of chromatographic approaches have been developed for further isolation, purification and identification of MC variants.

HPLC is also used for purification of MCs as the final polishing step, typically referred to as preparative reversed-phase HPLC, and commonly using C_{18} bonded to silica as the predominant stationary phase (Lawton & Edwards, 2001). Gregson and Lohr (1983) were among the first to purify 'aeruginosin' and another hepatotoxic peptide (probably MC) from *M. aeruginosa* using a combination of DEAE-Sepharose, Sephadex G-25 and C_{18} HPLC. Brooks and Codd (1986) and Krishnamurthy, Butalla, and Sprinkle (1986) purified hepatotoxic peptides from *Microcystis* and *Anabaena* using similar protocols in which the lyophilized cyanobacteria were extracted with water-methanol-butanol (75:20:5), concentrated on C_{18} solid phase extraction cartridges and first purified on Sephadex G-25 column and then on a C_{18} HPLC. Krogman et al. (1986) purified two 'fast-death-factor peptides' (probably MC-LR and another MC) using a C_{18} column and acetonitrile gradient in water containing 0.1% trifluoroacetic acid (TFA) as the final purification step (Krogman et al., 1986). Kungsuwan et al. (1988) purified two variants of MCs (MC-YR and MC-LR) using aqueous extraction combined with DEAE-5PW ion-exchange and C_{18} HPLC purification (Kungsuwan et al., 1988; Harada, 1996; Lawton & Edwards, 2001).

Edwards et al. (1996a) introduced a powerful and simplified methodology called C_{18} flash chromatography (pre-packed columns with reversed-phase media widely used in HPLC instruments) to purify MCs from laboratory cultures and bloom samples of cyanobacteria. This method comprised methanol extraction, followed by concentration of diluted supernatant onto a C_{18} flash chromatography cartridge and further purification by preparative C_{18} HPLC, enabling extracts containing about 500 mg of MCs to be partially purified in a single run within a day. They also revealed that the concentration of various minor MCs was improved making subsequent purification easier. The purity of MC-LR increased from 9% purity in the initial extract to 75% after this single step. Pyo and Lee (2002) reported a novel, rapid and efficient procedure for extraction and purification of MC-LR involving supercritical

fluid extraction (SFE) using aqueous methanol, modified supercritical carbon dioxide fluid for the fast extraction, and final purification by silica gel flash chromatography and preparative HPLC. Typical purity was up to 95% with the yield of 3.15 mg (±200 mg) from 5 g of freeze-dried cyanobacterial cells.

A final step of desalting is required if the toxins are contaminated with salts (e.g. ammonium acetate) used in the mobile phase of column chromatography. This may be achieved by using the same mobile phase column or a new commercial purification kit (e.g. C_{18} solid phase extraction cartridges). In general, the purified sample is applied to the column which is then washed with high purity water and finally the pure toxin subsequently eluted in organic solvent, commonly methanol or aqueous methanol (Lawton & Edwards, 2001; van Apeldoorn, Egmond, Speijers, & Bakker, 2007).

3.3.2 Identification of MCs

After purification is accomplished, it is crucial to identify the pure MCs. The analytical techniques for MC determination range from bioassays (e.g. mouse assay and brine shrimp assay), biochemical or immunological techniques (e.g. enzyme linked immunosorbent assay (ELISA) and protein phosphatase inhibition assays), to quantitative chromatographic approaches (e.g. high performance liquid chromatography (HPLC) (Svrcek & Smith, 2004). However; bioassay and biochemical techniques are normally used for toxicity assessment in terms of MC-LR equivalence and identification of MCs as total toxins. They do not have adequate sensitivity to distinguish between homologues of different toxins (Carmichael, 1997). Therefore, biochemical assays as well as bioassays are normally used as screening methods (Apeldoorn et al., 2007). Analytical reversed-phase HPLC coupled with UV detection is the most widespread method used for identification and quantification of MCs (Luukkainen et al., 1993; Lawton & Edwards, 2001). The International Organization for Standardization (ISO) has therefore published a standard procedure for the extraction, separation, and identification of MC-LR, MC-RR, and MC-YR using reversed-phase HPLC with UV detection (ISO 20179, 2005).

3.3.2.1 High performance liquid chromatography (HPLC)

HPLC is an expensive, time consuming and technically demanding method. It normally relies on the availability of toxin standards for determination and identification of MC variants (Pyo, Lee, & Choi, 2005). However, this technique also provides a powerful and sensitive approach for identification of MCs, giving both quantitative and qualitative data (de Figueiredo, Azeiteiro, Esteves, Goncalves, & Pereira, 2004; McElhiney & Lawton, 2005). The identification of toxins using HPLC involves separation of MC variants as well as closely related compounds from each other, allowing individual detection using ultraviolet (UV), photo-diode-array (PDA) or mass spectrometry (MS) detectors (Nicholson & Burch, 2001).

3.3.2.1.1 Separation

The separation of MC variants is largely dependent on the stationary phase employed in the analysis and the composition of the mobile phase (McElhiney & Lawton, 2005). The stationary phase most commonly used over the last several decades is the reversed-phase C_{18} packed column. The reversed-phase C_{18} HPLC is typically used for separating several kinds of small peptides, e.g. peptides in enzymatic digests of proteins (Meriluoto, 1997). MCs are separated from each other and other co-extracted compounds using a reversed-phase C_{18} HPLC column (Nicholson & Burch, 2001). Spoof, Karlsson, and Meriluoto (2001) carried out valuable research in which a novel amide C_{16} reversed-phase column was compared with C_{18} reversed-phase columns. The amide C_{16} column exhibited the best overall performance and unique selectivity properties. It separates desmethylated from the non-desmethylated MCs and may prove useful in the separation of certain stereoisomers. Anion-exchange HPLC has also been used for separation and identification of MCs. Gathercole and Thiel (1987) identified MC-LR, MC-FR, MC-YR and MC-LA (in this elution order) using diethylaminoethyl anion-exchange HPLC (Spherogel-TSK DEAE 2-SW). They demonstrated anion-exchange as superior to reversed-phase in resolving the different MC variants. For example, MC-LR and MC-YR coeluted in their C_{18} system but were separated by anion exchange HPLC (Meriluoto, 1997).

Microcystins can be separated using both gradient elution and isocratic mobile phases on reversed-phase C_{18} HPLC, although a wider range of toxin variants can be resolved when a gradient is employed (McElhiney & Lawton, 2005). The mobile phases appropriate for the separation are aqueous methanol or acetonitrile with a gradient of water and organic modifier (van Apeldoorn et al., 2007). One of the most common gradient elutions used is a combination of water and acetonitrile, containing 0.05% TFA, which acts as an ion-pairing agent. This technique was shown to be successful for separation of more than ten MC variants of varying polarity (Lawton et al., 1994). Although the gradient elution provides a great resolving power, many chemists tend to use the isocratic mobile phases due to the simpler and less error prone instrumentation (Meriluoto, 1997).

3.3.2.1.2 Detection

Following toxin separation comes detection, a very important step in that it must unambiguously respond to different toxins with high sensitivity (McElhiney & Lawton, 2005). MCs are composed of an interesting family of closely related peptides. The number of toxin variants is high (approximately 80 MC variants), and therefore the detection and identification requires high resolution and good sensitivity (van Apeldoorn et al., 2007).

Microcystins are amorphous colourless solids and normally demonstrate negative specific rotation in methanol or water. In the UV spectra of MCs, there is a strong absorption maximum due to the conjugated diene located in the Adda moiety, which can be detected in chromatography such as HPLC (Harada, 1996). The MC toxins show two typical spectra, one with an absorption maximum of 238 nm, exhibited by all except a few MCs that contain tryptophan (W) (such as MC-LW and MC-WR). The latter provide an absorption maximum at 222 nm. These characteristic spectra, therefore, are used in the identification of MCs even in the absence of a wide array of standards (Lawton et al., 1994). The wavelength of the UV detector of the HPLC equipment is normally set at 238 nm to record the responses of MCs in sample extracts separated on the HPLC column. However other components in the sample extract can also possess some absorbance at this wavelength and such an approach can only be regarded as producing a tentative result as to the presence of a toxin

(Nicholson & Burch, 2001). Ikawa, Phillips, Haney, and Sasner (1999) discovered that common additives in plastics could contaminate water samples, coelute with MCs and have sufficient UV absorbance at 238 nm to produce erroneous results. Therefore, plastic materials must be avoided, or their suitability checked in the determination of these toxins using HPLC-UVD. In order to improve specificity for the identification of MCs, a photo-diode array (PDA) detector can be used (van Apeldoorn et al., 2007). The use of a PDA detector allows the identification of MCs from their characteristic spectra and enables other peaks to be eliminated (Lawton et al., 1994). The PDA detector which not only records the UV response but also the spectrum of a separated MC variant provides much better evidence of the presence of the toxin than using single wavelength detection (Nicholson & Burch, 2001). However, its ability to distinguish different MCs is limited as most MC variants have a similar absorption profile between 200 and 300 nm (Lawton et al., 1994; Harada, Kondo, & Lawton, 1999). This is because the main chromophore of MCs is a conjugated diene in the Adda residue, which absorbs at 238 nm. Variants containing tryptophan, such as MC-LW, can be more easily identified by PDA detection, as they possess a second absorption maximum at 222 nm. HPLC with a single-wavelength UV detector, therefore, should be avoided in critical MC identification and even HPLC-PDA results should be examined carefully (Meriluoto, 1997) and re-confirmed by mass spectrometry (Lawton et al., 1994; McElhiney & Lawton, 2005).

A specific and accurate technique for MC identification can be achieved using HPLC coupled with mass spectrometry (LC/MS) (McElhiney & Lawton, 2005). The LC/MS approach measures discreet chemical fragments of the toxins after a chromatographic separation step. Various MS techniques have been used for the identification of MCs in different matrices, including fast atom bombardment (FAB/MS), (Kondo et al., 1995; Luukkainen, Namikoshi, Sivonen, Rinehart, & Niemelä, 1994), electrospray ionization (ESI-MS, Zweigenbaum et al., 2000; Barco et al., 2002, Spoof, Vesterkvist, Lindholm, & Meriluoto, 2003) and Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) (Welker, Fastner, Erhard, & von Dohren, 2002). The mass spectrometry profiles obtained from these techniques serve as a fingerprint for individual MC variants and play an important role in the characterization of MCs as it provides structural (primary amino acid sequence)

information (Frias et al., 2006). The information of mass spectrometry is very useful to confirm the existence of the toxins after tentative identification with HPLC-UV or PDA detector (McElhiney & Lawton, 2005).

Fast atom bombardment mass spectrometry (FAB/MS) has been a significant complementary technique to nuclear magnetic resonance (NMR) spectroscopy in the structural analysis, molecular weights determination and identification of MCs (Meriluoto, 1997; Zweigenbaum et al., 2000). After isolation and purification by HPLC, Sivonen (1994) assigned structures to many MCs and their variants by FAB/MS, FAB/MS/MS and NMR. Kondo et al. (1992) developed frit micro liquid chromatography/mass spectrometry with fast atom bombardment (LC/MS frit-FAB) in the analysis of MCs. In LC/MS frit-FAB, the effluent (a few µl/min) containing an ionization matrix is subjected to a frit surface and ionized using FAB. LC/MS frit-FAB produces not only molecular ion species $[M+H]^+$ but also characteristic fragment ions. One drawback with the frit-FAB LC/MS system is the low sensitivity (partially because of the inherent peak broadening in the technique). Microgram amounts are required in conventional HPLC but the detection limit can be very much lowered if a microbore column is employed. The detection limit was 400 pg for MC-LR in a frit-FAB microbore LC/MS system using selected ion monitoring (Kondo et al., 1995; Meriluoto, 1997).

Recently, HPLC-ESI-MS has become more reliable and is now extensively used in the identification and characterization of MCs (Dahlmann, Budakowski, & Luckas, 2003; Spoof et al., 2003; McElhiney & Lawton, 2005). The technique is a valuable and powerful hyphenated technique (chromatographic separation technique coupled with a sensitive, element-specific detector) for establishing sequence structure of the toxins (Cong et al., 2006). ESI-MS generates principally a protonated $[M+H]^+$ ion and depending on structure, a $[M+2H]^{2+}$ ion, and a few fragment ions, yielding molecular weight details which are able to identify the structure of toxin variants detected by HPLC-PDA (McElhiney & Lawton, 2005). HPLC-ESI-MS information is relatively easy to interpret, and this method is considered to be a promising technique for monitoring MCs in water samples. Recent research involving the use of microbore LC with ESI-MS has facilitated the detection of pg quantities of MC-LR and has allowed trace amounts of MCs to be identified in water samples and

cyanobacterial blooms (Zweigenbaum et al., 2000; Barco et al., 2002; McElhiney & Lawton, 2005).

A current study of HPLC-ESI-MS includes characterization of the breakdown products of MCs after degradation of the toxins by titanium dioxide photocatalysis, which has enabled the transformation pathways to be elucidated (Liu, Lawton, & Robertson, 2003). The HPLC-ESI-MS approach has also been developed which enables simultaneous detection of a wide range of compounds in a single chromatographic operation (Dahlmann et al., 2003). This method offers both quantification and detection of a range of cyanotoxins including MCs, anatoxin-a, nodularin and saxitoxin and shows great potential for rapid screening of bloom samples for cyanotoxins.

Mass spectral analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a relatively recent analytical method, generated in the 1980s (Karas & Hillenkamp, 1988). It has been used for the analysis of many peptides, including MCs (Fastner et al., 2001; Welker et al., 2002) and has proven to be a reliable and rapid tool to detect and identify MC variants in very small samples such as single *Microcystis* colonies or microgram quantities of cell material (not milligram quantities like other techniques such as HPLC-UV detector or bioassays) (Welker et al., 2002). The detection is rapidly made, without the need for time-consuming extraction or purification processes, allowing the identification of known MC variants and other unknown metabolites which can be further characterized (Fastner et al., 2001). Mass signals presumptive of MC variants can be characterized further and identified by postsource-decay fragmentation and comparison of observed fragment spectra with theoretical ones or with those of pure reference compounds. MALDI-TOF MS also provides considerable support to HPLC by identifying MC variants not available as purified standards, especially in the samples in which demethylated variants are predominant (Welker et al., 2002).

3.3.3 Rationale for choice of methods used in this study

The freeze-dried material of cyanobacterial bloom was extracted using 70% methanol (MeOH)-0.1% trifluoroacetic acid (TFA) as it has proved one of the best extraction methods (Ward, Beattie, Lee, & Codd, 1997; Meriluoto, 1997; Fastner et al., 1998; Hyenstrand et al., 2001; Lawton & Edwards, 2001). The large volumes of solvent generated (70% MeOH-0.1% TFA) were concentrated using a rotary evaporator at 40^{o}C under air compression. Anion exchange chromatography was chosen for the first purification step as it has proved successful in purification of MC variants in many studies (Murthy & Capindale, 1970; Elleman et al., 1978; Botes et al., 1982; Gregson & Lohr, 1983; Martin et al., 1990; Cremer & Henning, 1991; Saito et al., 2002). The anion exchanger used was Toyopearl DEAE-650M resin = 1000 Å pore size, medium particle size 'M' = 40-90 μm, from Toyopearl Co Ltd., Japan. A Strata-X SPE cartridge was used for the secondary purification step since it has been proved valuable for clean-up of polar and non-polar compounds (Ghanem, Bados, Kerhoas, Dubroca, & Einhorn, 2007; Hanke, Singer, & Hollender, 2008). The purified toxins were identified using the HPLC-UVD system with toxin standards (MC-RR and MC-LR). LC-MS/MS (ESI-MS) was used if the toxins could not be identified using HPLC-UVD as the mass spectrometry profiles can provide essential details for identification and characterization of MCs (Frias et al., 2006). Finally, where MC standards were unavailable for particular toxins (e.g. [Dha^{7}]MC-LR), it was necessary to estimate the concentration of these MCs against MC-LR as the analytical standard, based on the assumption that individual variants have similar responses with the UV or PDA detector, since work to date has shown this to be so (Nicholson & Burch, 2001).

3.4 Objectives of the chapter

The objectives of this study were to (1) extract MC from cyanobacterial bloom material, using a modified method for MC isolation and purification and (2) identify and characterize purified toxins using reversed-phase HPLC and LC-MS/MS.

3.5 Methods

3.5.1 History of cyanobacterial blooms and sampling collection

Lake Horowhenua is a small (2.9 km^2) shallow (< 2 m deep), coastal dune lake on the west coast of the North Island of New Zealand, fed by various small streams, springs, and associated swamps. Previous study (Wood, 2004) revealed the presence of the cyanobacterium *M. aeruginosa* in Lake Horowhenua producing a wide variety of MCs such as MC-RR, MC-dMe-RR, MC-LR, [Dha^7]MC-LR, MC-YR, MC-FR, MC-WR, MC-AR, MC-LA, and MC-LY. Toxic cyanobacterial blooms had closed the lake for three consecutive years (2003-2005). A large mass of cyanobacterial cell material was harvested from the littoral zone of Lake Horowhenua with a 300-µm plankton-net in May, 2005, when the bloom was at its peak. The material was lyophilized using a freeze drier at -18ºC. The lyophilized material was stored at -20^{o}C until extraction.

3.5.2 Extraction of the lyophilized material

Microcystins were extracted and purified using a modification of a method used by Saito et al. (2002). For each extraction twenty grams of the freeze-dried material was mixed with 200 ml of 70% methanol (MeOH)-0.1% trifluoroacetic acid (TFA) solution (Appendix 1) and sonicated for 30 min. The extract was centrifuged at 10,000 rpm for 5 min (4^{o}C) and the pellets were further extracted twice (resuspended in the acidic-methanol solution and centrifuged again). The supernatants were filtered through a GF/C glass filter (Whatman), and initial determination of the quantity of the toxins and the number of variants was carried out using LC-MS/MS at Cawthron Institute (Nelson, New Zealand), before evaporating at 40°C to dryness. The residue was resuspended in 500 ml of milli-Q water (ultra-pure water, Millipore) and precipitated with 55% saturation of ammonium sulphate (175 g). The suspension was stirred at 4°C overnight, then centrifuged (10,000 rpm, 30 min), and decanted. The pellet was re-suspended in absolute MeOH and filtered through the GF/C glass filter. The filtrate was evaporated and then dissolved and adjusted in 0.05M 2-morpholinoethanesulfonic acid (MES)–potassium hydroxide (KOH) (pH 5)–20% (v/v) ethanol (EtOH) (solution A) (Appendix 1) to a concentration of 50 mg/ml. A

portion (10 ml) of the extract (50 mg/ml) was loaded onto a Toyopearl DEAE-650M column.

3.5.3 Packing and equilibrating Toyopearl DEAE-650M column

Two hundred grams of Toyopearl DEAE-650M resin (Toyopearl) was allowed to swell in solution A at room temperature overnight. The gel slurry was then poured carefully into a 4 x 30 cm glass column, connected to a peristaltic pump operating at a flow rate of 1 ml/min. The column was equilibrated with solution A using 5-10 times the total column volume.

3.5.4 First purification with DEAE anion exchange chromatography

The extract from Section 3.5.2 was added to a column packed with a Toyopearl DEAE-650M resin. The toxins were separated by a gradient from solution A to solution B, which is 0.05 M MES-KOH (pH 5.5)-20% EtOH to 0.05 M MES-KOH (pH 5.5)-20% EtOH-1M sodium chloride (solution B; Appendix 1), starting at 100% of solution A for 50 min, and then solution B was linearly increased from 0 to 100% at 230 min. A flow rate was obtained at 1 ml/min by a peristaltic pump. The fractions (10 ml each) were collected in test tubes placed in a fraction collector, and their absorbances were read at 238 nm using a spectrophotometer. Eluates were then pooled into fractions 1-5 (M-1 to M-5)(see Section 3.6.2 and Figure 3.2)

3.5.5 Secondary purification with solid phase extraction (SPE) cartridges

3.5.5.1 Optimization of toxin elution

In order to elute impurities and the toxins from the cartridge efficiently, optimal concentrations of methanol were investigated to determine an appropriate washing and eluting solution. Twenty ml of M-3 fraction, obtained from DEAE chromatography, was applied through a Strata-X SPE cartridge (200 mg/ 6 ml, Phenomenex) that had been conditioned with 90% and 20% MeOH. A sequentially stepped gradient from 30% to 80% aqueous MeOH in 10% increments was used to elute the impurities and the toxins under a compression module (SPE system, Baker Co Ltd.) at a flow rate of 1 ml/min. The toxins corresponding to each elution step

were collected and analyzed separately by HPLC-UV detector (HPLC/UVD; Appendix 1).

3.5.5.2 The clean-up of the purified toxins with the SPE cartridges

Two optimal concentrations of MeOH from 3.5.5.1 were used as the washing solution (30% MeOH) and the eluting solution (70% MeOH). The M-1 to M-5 pooled fractions from DEAE anion exchange chromatography (Section 3.5.4) were applied through the Strata-X cartridges conditioned with 90% and 20% MeOH, according to the manufacturers specifications. Impurities were successively washed with 30% (v/v) aqueous MeOH and the toxins were eluted with 70% MeOH at a flow rate of 1 ml/min under the compression module.

3.5.6 Identification of the purified toxins

3.5.6.1 Identification of MCs with HPLC-UV detector

The eluted toxins from the SPE cartridges (see Section 3.5.5.2) were first identified using HPLC-UV detector by comparing their retention time (RT) to that of authentic standards at 238 nm. However; in this study, only two authentic standards were available, MC-LR and MC-RR (Sigma), therefore using the HPLC, purified toxins MC-LR and MC-RR were identified. For further identification of MC variants, LC-MS/MS was used (see Section 3.5.6.2).

The HPLC system consists of a 515 HPLC pump (Waters), a SPD-20A UV/VIS detector (Shimadzu) and a Peak Simple Data System (SRI Instruments). Separation was accomplished under reversed-phase isocratic conditions with a TSK-GEL ODS-80Ts column (150x4.6 mm, Tosoh) and a mobile phase of acetonitrile: 0.05M phosphate buffer (pH 3.0) (3:7) (Appendix 1) with a flow rate at 1.0 ml/min.

3.5.6.2 Identification and characterization with LC-MS/MS

The unknown eluted toxins referred to in Section 3.5.6.1 were further identified using LC-MS/MS at Cawthron Institute, Nelson, New Zealand. The protocol used is presented in Appendix 2.

3.5.7 The yield and purity of the purified toxins in the pooled fractions

3.5.7.1 The yield of the purified toxins

The eluted toxins described in Section 3.5.5.2 in each pooled fraction (M-1 to M-5), were collected individually, and evaporated for dryness under reduced pressure of nitrogen gas at 40°C. If the purified toxin was identified as MC-RR or MC-LR (see Section 3.5.6.1), the dried toxin yield was determined by using the area peak value from the HPLC analysis described in Section 3.5.6.1 and that of their authentic standards as follows:

$$\text{The yield (mg)} = \frac{\text{area peak value of purified toxin}}{\text{area peak value of its authentic standard}} \times \text{concentration of authentic standard (µg/ml)} \times \text{total volume of diluent} \times \text{dilution factor / 1000}$$

For example:

(a) The purified and dried MC-LR was dissolved with a known volume of absolute MeOH (15 ml).

(b) The dissolved toxin was diluted in the MeOH (1:100)

(c) The diluted sample was analyzed with HPLC as described in Section 3.5.6.1 using area peak values. For example, the area peak of purified MC-LR is 445 whereas that of authentic MC-LR standard is 369 (from the concentration of the authentic standard at 9.5 µg/ml).

The yield (mg) = (445/369) x 9.5 x 15 x 100 / 1000 = 17.18 mg

However, if the purified toxins are unknown (not MC-RR or MC-LR), the area peak value of the authentic standard of MC-LR is used and applied to other MC variants.

3.5.7.2 Purity of the purified toxins

Using the HPLC system at waveledge of 238 nm, the amount of each MC was determined as a peak area percentage of the total MCs in pooled fractions of the purified toxins. For example in the M-5 fraction, there were only two peaks from the HPLC chromatogram (identified as [Dha^7]MC-LR and MC-LR).

- peak area value of [Dha^7]MC-LR = 90
- peak area value of MC-LR = 10

Therefore, M-5 fraction contains 90% of [Dha^7]MC-LR and 10% of MC-LR

3.5.7.3 The concentration of the purified toxins

The concentration of the toxins was determined using HPLC analysis (Section 3.5.6.1) using area peak with pure analytical standards such as MC-LR from Sigma. In case of unavailable standards such as [Dha^7]MC-LR, the instrument was calibrated with authentic standard of MC-LR at concentrations of 1, 10, 50 and 100 µg/ml (Appendix 3). The toxin was diluted and calculated under linear calibration curves of the MC-LR standard.

3.6 Results

3.6.1 First identification of MCs from crude extracts with LC-MS/MS (before purification)

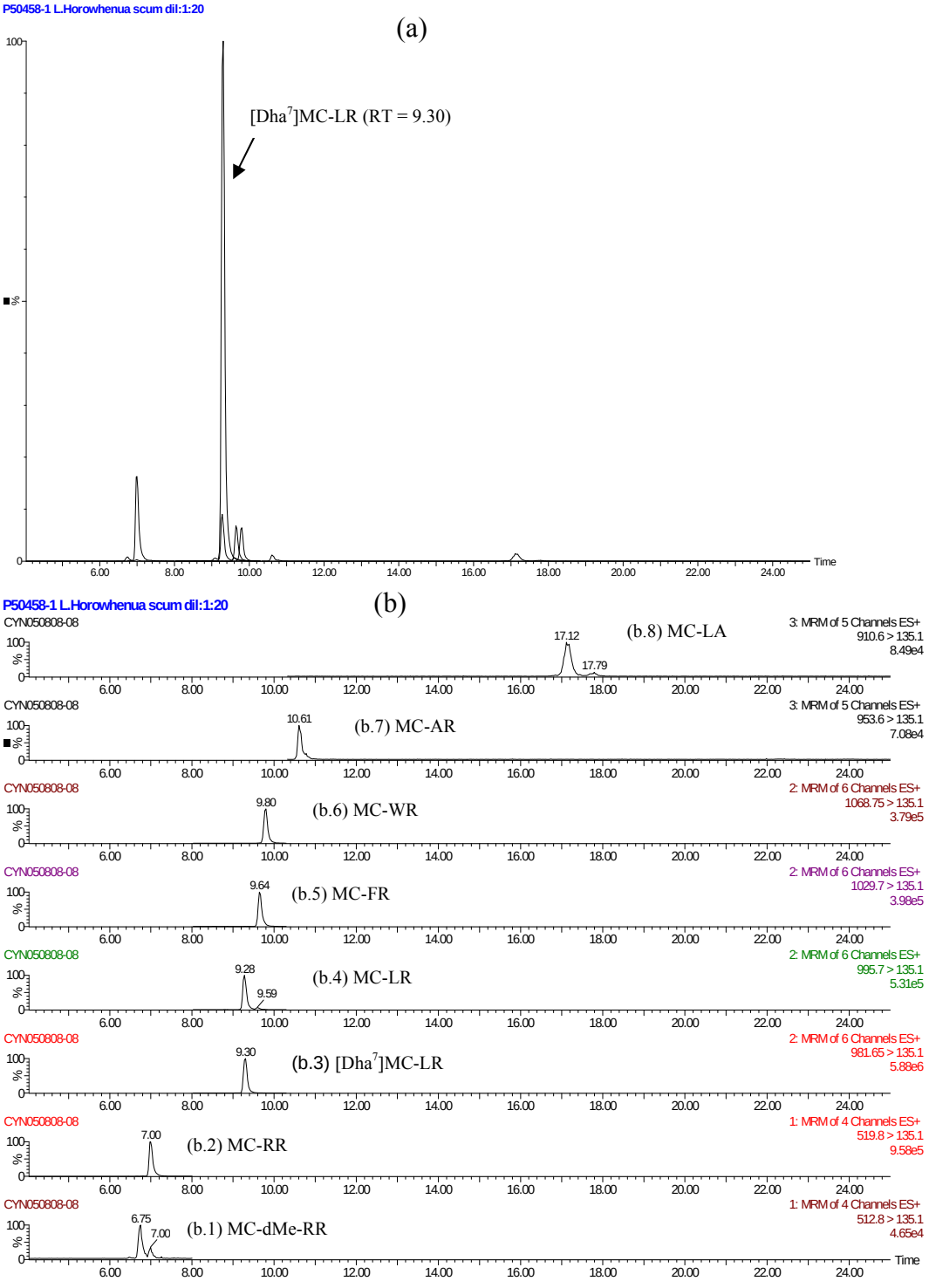
Nine variants of MCs, MC-RR, dMe-RR, MC-LR, MC-YR, [Dha^7]MC-LR, MC-FR, MC-WR, MC-AR, and MC-LA were detected in crude extracts of cyanobacterial bloom from Lake Horowhenua by LC-MS/MS before purification with DEAE chromatography and Strata-X SPE cartridges (Table 3.1).

Table 3.1 LC-MS/MS parameters and concentration of MCs from the lyophilized material before purification.

Retention time (RT)	Ion	MC Traces	Molecular Weight (Dalton)	MC identified	Area peak	Concentration (mg/kg dry weight)
6.74	$[M+2H]^{2+}$	512.8 >135.1	1024	MC-dMe-RR	5190	9.6
7.00	$[M+2H]^{2+}$	519.8 >135.1	1037	MC-RR	107100	199.1
9.15	$[M+H]^{+}$	1045.7 >135.1	1045	MC-YR	1393	10.0
9.31	$[M+H]^{+}$	981.65>135.1	981	[Dha^7]MC-LR	651501	3555.5
9.28	$[M+H]^{+}$	995.7>135.1	995	MC-LR	61391	335.0
9.67	$[M+H]^{+}$	1029.7>135.1	1029	MC-FR	43708	238.5
9.80	$[M+H]^{+}$	1068.75>135.1	1068	MC-WR	42339	231.1
10.63	$[M+H]^{+}$	953.6>135.1	953	MC-AR	8661	47.3
17.14	$[M+H]^{+}$	910.6>135.1	910	MC-LA	17837	97.3

Figure 3.1a and Figure 3.1b show that [Dha^7]MC-LR was predominant in the material with approximately 75% or 3555.5 mg/kg dry weight (Table 3.1). There were moderate amounts of MC-LR, MC-FR, MC-WR, and MC-RR, and small amounts of MC-LA, MC-AR, MC-YR, and MC-dMe-RR.

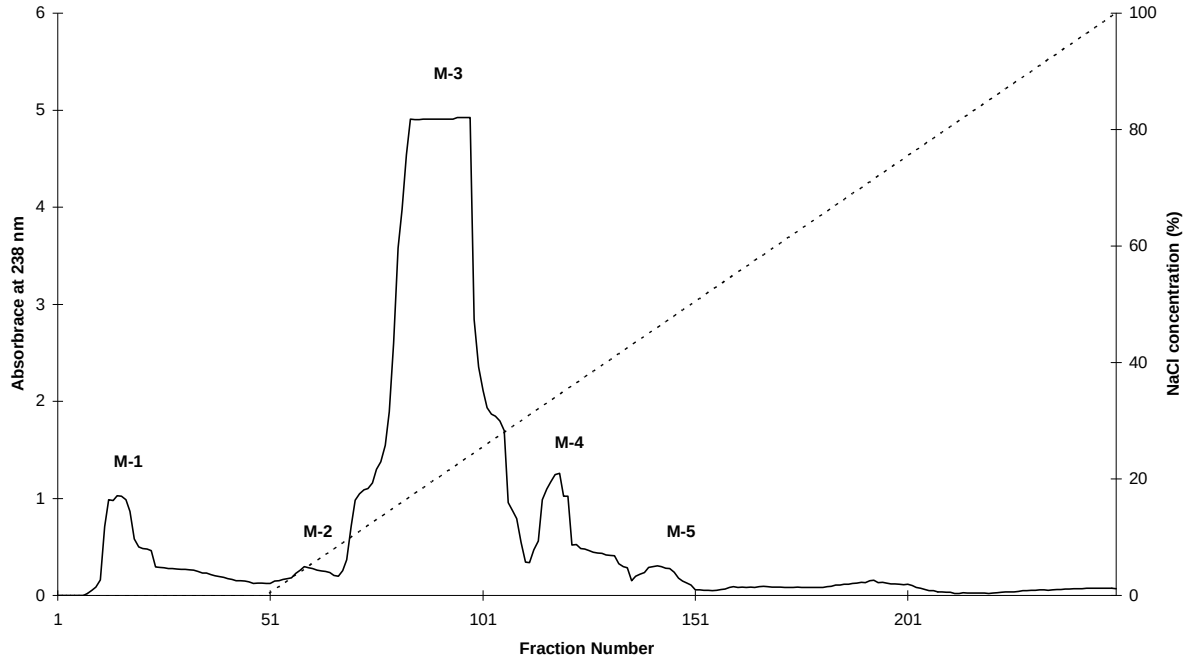
Figure 3.1 Base peak intensity (a), and reconstructed ion chromatograms of MCs (b) in the lyophilized material before purification. MC traces from bottom to top (MRM = multiple reaction monitoring): (b.1) MRM *m/z* 512>135.1; (b.2) MRM *m/z* 519.8>135.1; (b.3) MRM *m/z* 981.65>135.1; (b.4), MRM *m/z* 995.7>135.1; (b.5), MRM *m/z* 1029.7>135.1; (b.6), MRM *m/z* 1068.75>135.1; (b.7), MRM *m/z* 53.6>135.1; (b.8), MRM *m/z* 910.6>135.1. Time scale is in minutes.



3.6.2 Extraction and first purification with anion exchange chromatography

The lyophilized cyanobacterial bloom material (20 g) provided the successive extractive values of 3.52 g (17.6% yield by weight from the lyophilized cyanobacterial material) after acidic-methanol extraction and precipitation with ammonium sulfate. The crude extract loaded onto a Toyopearl DEAE-650M column gave the elution profile as shown in Figure 3.2. The chromatographic profile shows five maxima at 238 nm (5 fractions), designated M-1 to M-5.

Figure 3.2 Chromatograms of DEAE measured at 238 nm. Broken line shows concentrations of NaCl (%). The toxins were categorized into five fraction groups (designated by M-1 to M-5).

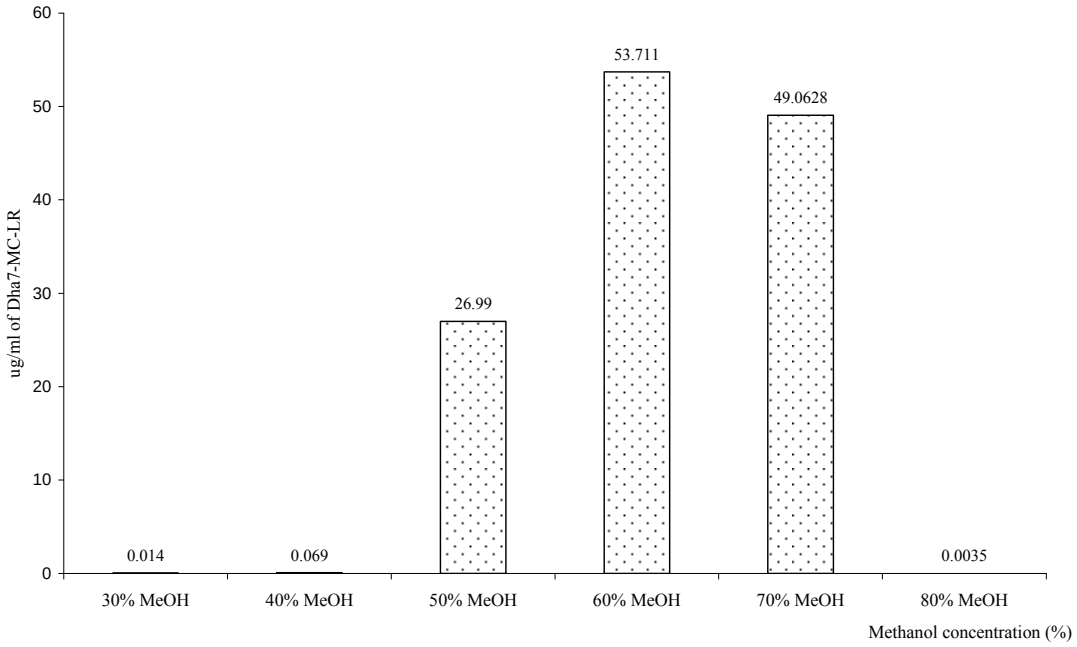


3.6.3 Optimization of toxin elution using Strata-X SPE cartridges

Appendix 4 shows HPLC chromatograms of MC elution from the Strata-X SPE cartridges using aqueous methanol gradient from 30% to 80% (10% increments) (Figure 3.3). The concentration of eluted toxin (later identified as [Dha^7]MC-LR) was calculated using the area peak of the toxin compared to that of MC-LR standard in calibration plot of Appendix 3 (Section 3.5.7.3) since the standard of [Dha^7]MC-LR was commercially unavailable (Nicholson & Burch, 2001). Results revealed that the

aqueous methanol, less than 30%, proved to be a suitable washing solution as it strips only tiny amount of the toxins from the cartridge (Figure 3.3 and in Appendix 4), whereas 50% methanol eluted large amounts of the toxins (Figure 3.3 and in Appendix 4). Therefore, 30% aqueous methanol was used as the washing solution and 70 % aqueous methanol proved appropriate for eluting MCs from Strata-X cartridges.

Figure 3.3 [Dha^7]MC-LR eluted from Strata-X cartridges with aqueous methanol from 30 to 80%



3.6.4 Identification of purified MCs with LC-MS/MS

The compounds from M-1 to M-5 fractions were identified using LC-MS/MS at Cawthron Institute. Fraction M-3 with most of the toxin material contains mainly [Dha^7]MC-LR with small amounts of MC-LR. Reversed-phase HPLC at retention time (RT) of 9.57, MS/MS spectrum of *m/z* 981.75 in ESI$^+$ with parent ion spectrum (Figure 3.4a and Figure 3.4c) and a major product the daughter ion spectrum (Figure 3.5) with $[M+H]^+$ at *m/z* 135.14 (characteristic Adda fragment) confirmed that the toxin is [Dha^7]MC-LR whereas the retention time of 9.51 and that of *m/z* 995.75 proved that the other toxin is MC-LR (Figure 3.4b and Figure 3.4d).

Figure 3.4 Reversed-phase HPLC (a) RT 9.57 and MS/MS spectrum (c) (*m/z* 981.75) of [Dha^7]MC-LR and (b) RT 9.51 and (d) *m/z* 995.75 of MC-LR in ESI$^+$ with parent ion spectrum for MS-MS channels set up

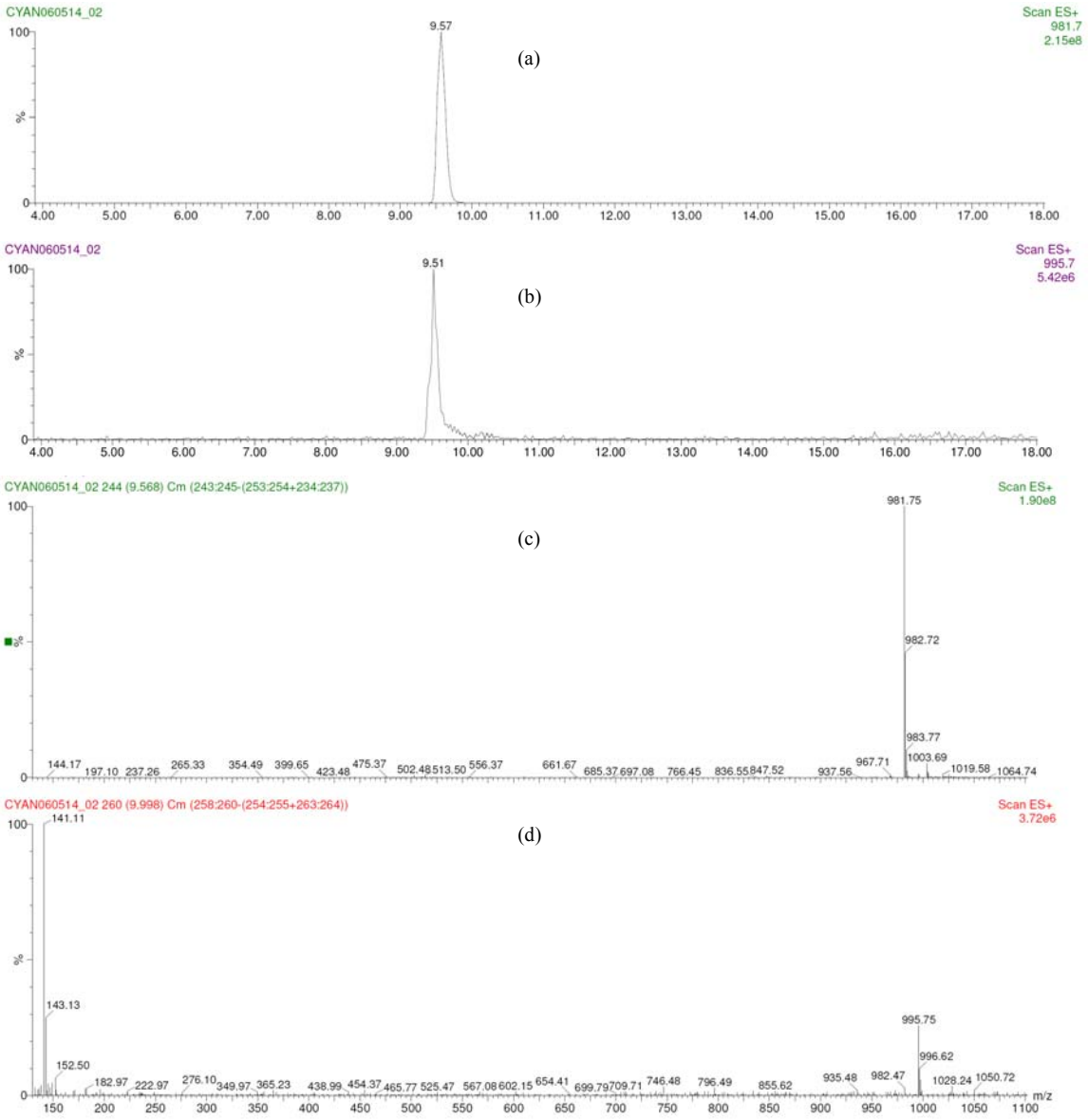
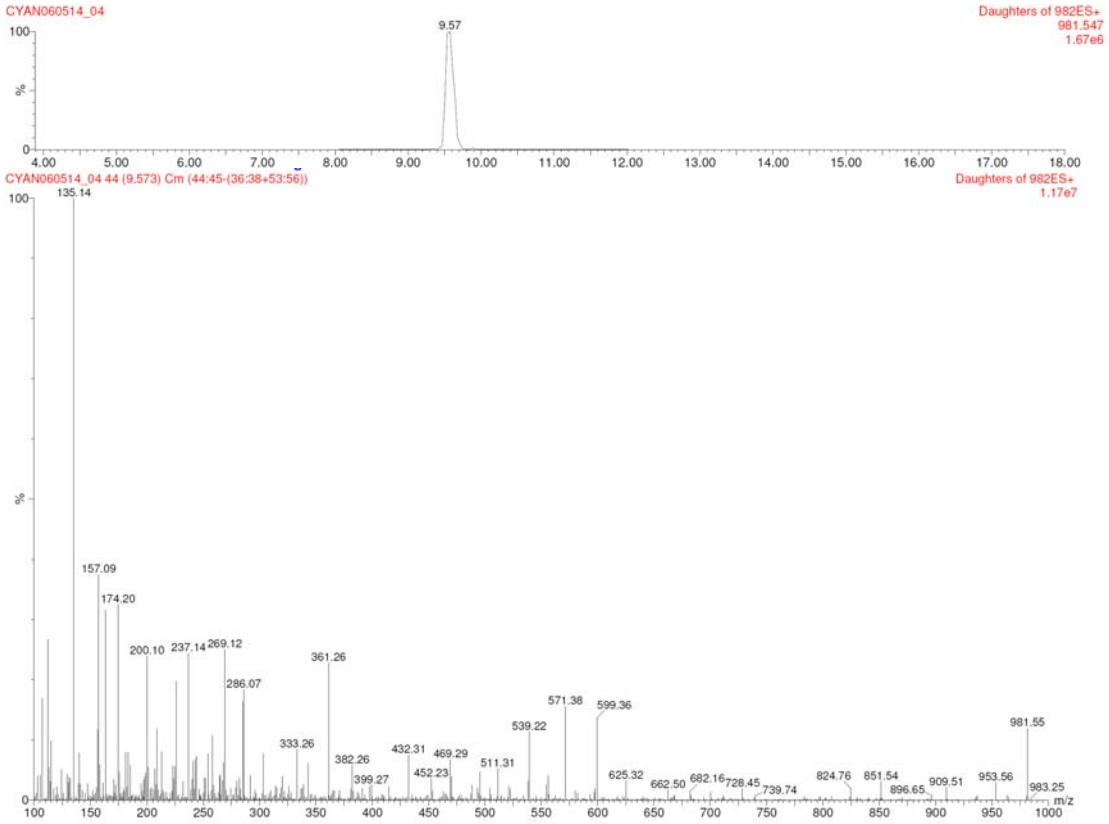


Figure 3.5 Reversed-phase HPLC (RT 9.57) and MS/MS spectrum of [Dha^7]MC-LR at *m/z* 981.55 in ESI$^+$ with daughter ion spectrum for MS-MS channels set up



[Dha^7]MC-LR (M-3 fraction) is 14 mass units less than MC-LR due to the loss of a methyl group which occurs at Mdha. Therefore the toxin has Dha (dehydroalanine) instead of Mdha (*N*-Methyldehydroalanine). Table 3.2 represents predicted fragment ions observed in the mass spectra of [Dha^7]MC-LR in Figure 3.5. In the MS^2 experiment by fragmenting the protonated molecule at *m/z* 981 it revealed the base peak at *m/z* 135.1, which is heterolytic cleavage of the C8-C9 bond of the Adda residue. The *m/z* 135 ion is a valuable diagnostic ion for identifying MCs because it is generally present in spectra of MCs with unmodified Adda residues (Chris, Scott, & William, 2005). The existence of Adda in the structure was revealed by fragment ion peaks at *m/z* 135 and 847 (M+H-135). Ions *m/z* 114 and 157 were assigned to a fragment of the amino acid leucine and arginine at positions 2 and 4, respectively. The ion *m/z* 200 appears to be a fragment of the amino acid pair Glu-Dha (position 6 and 7; Glu = D-glutamic acid; Dha = dehydroalanine) whereas the

fragment ion at *m/z* 270 consists of a series of amino acids Glu-Dha-Ala (Ala = alanine). The sequence Adda-Glu-Dha-Ala was shown by the fragment ion peaks for [Dha^7]MC-LR at *m/z* 361, 199 and 141 (Namikoshi et al., 1992). The ion at *m/z* 539 represents a loss of Adda and Glu moiety from the protonated molecule, whereas the fragment ion at *m/z* 599 consists of a series of amino acids MeAsp-Arg-Adda (MeAsp is erythro-3-methyl-D-aspartic acid, Arg = arginine; Adda = (2s, 3s, 8s, 9s)-3- amino- 9- methoxy-2, 6, 8- trimethyl- 10- phenyldeca- 4, 6- dienoic acid). In this study, therefore, the structure of [Dha^7]MC-LR is cyclo(-D-Ala^1-Leu^2-D-MeAsp^3-Arg^4-Adda^5-D-Glu^6-Dha^7).

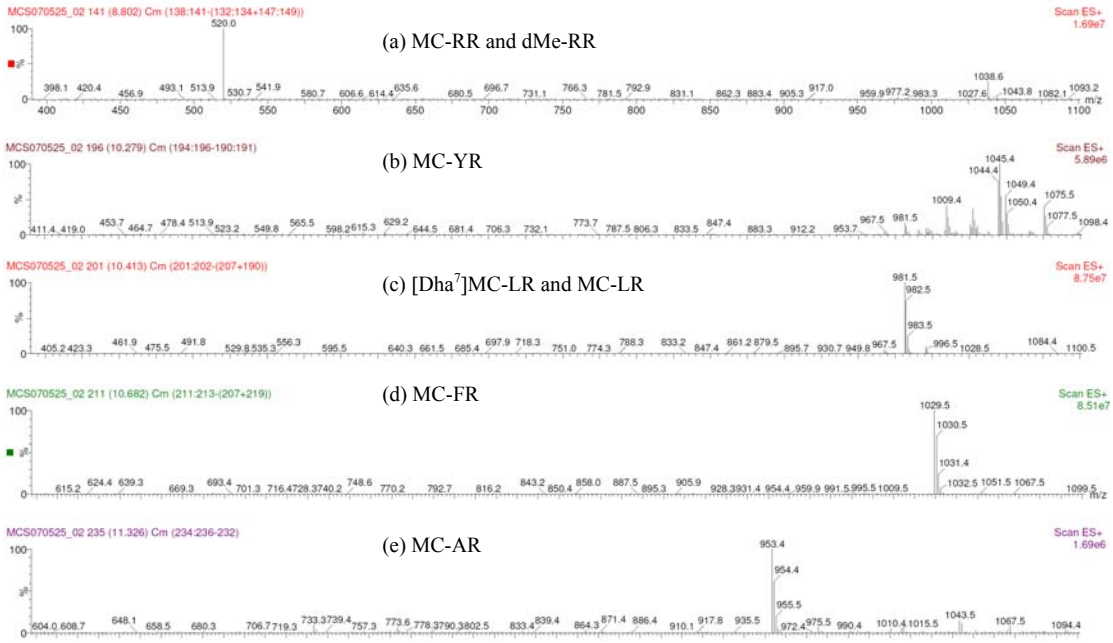
Table 3.2 Predicted fragment ion observed in mass spectra of [Dha^7]MC-LR

Observed	*m/z*	Predicted MS/MS fragment ion	Observed	*m/z*	Predicted MS/MS fragment ion
**	114	Leu +H	**	539	Dha Ala Leu MeAsp Arg +H
***	130	Glu +H		545	$C_{11}H_{14}O$ + Glu Dha Ala Leu +H
***	130	MeAsp +H		583	Adda Glu Dha Ala +H
*****	135	Characteristic Adda Fragment	**	599	Arg Adda Glu +H
	141	Dha Ala +H	*	599	MeAsp Arg Adda +H
****	157	Arg +H		668	Glu Dha Ala Leu MeAsp Arg +H
	185	Ala Leu +H	*	668	Arg Adda Glu Dha +H
***	199	Glu Dha +H		674	$C_{11}H_{14}O$ + Glu Dha Ala Leu MeAsp +H
	243	Leu MeAsp +H		696	Adda Glu Dha Ala Leu +H
	254	Dha Ala Leu +H		712	Leu MeAsp Arg Adda +H
***	270	Glu Dha Ala +H	*	728	MeAsp Arg Adda Glu +H
**	286	MeAsp Arg +H		739	Arg Adda Glu Dha Ala +H
	292	$C_{11}H_{14}O$ + Glu +H		783	Ala Leu MeAsp Arg Adda +H
	314	Ala Leu MeAsp +H		797	MeAsp Arg Adda Glu Dha +H
	314	Adda +H	*	825	Adda Glu Dha Ala Leu MeAsp +H
***	361	$C_{11}H_{14}O$ + Glu Dha +H		830	$C_{11}H_{14}O$ + Glu Dha Ala Leu MeAsp Arg +H
	383	Dha Ala Leu MeAsp +H		841	Leu MeAsp Arg Adda Glu +H
*	383	Glu Dha Ala Leu +H		847	[M+H] - Adda Fragment
	399	Leu MeAsp Arg +H	*	852	Arg Adda Glu Dha Ala Leu +H
*	432	$C_{11}H_{14}O$ + Glu Dha Ala +H	*	852	Dha Ala Leu MeAsp Arg Adda +H
	443	Adda Glu +H		868	MeAsp Arg Adda Glu Dha Ala +H
*	470	Arg Adda +H	*	910	Leu MeAsp Arg Adda Glu Dha +H
*	470	Ala Leu MeAsp Arg +H		912	Ala Leu MeAsp Arg Adda Glu +H
*	512	Glu Dha Ala Leu MeAsp +H	**	981	[M+H]+
*	512	Adda Glu Dha +H			

Note: Alanine (Ala); Leucine (Leu); Erythro-3-methyl-D-aspartic acid (MeAsp); arginine (Arg); 2s, 3s, 8s, 9s-3- amino- 9- methoxy-2, 6, 8- trimethyl- 10- phenyldeca- 4, 6- dienoic acid (Adda); D-Glutamic acid (Glu); Dehydroalanine (Dha).

The M-1 fraction contains mainly MC-RR and small amounts of MC-dMe-RR. MC-RR revealed both $[M+2H]^{2+}$ and $[M+H]^{+}$ ions at *m/z* 520 and 1038, respectively (Figure 3.6a) (molecular weight of MC-RR = 1037; see Table 3.1). In addition, a very poor signal intensity of MC-dMe-RR at *m/z* 513 ($[M+2H]^{2+}$) also was found due to the low concentration in the fraction. The MRM ion pairs *m/z* 1045>135 were used to trace MC-YR in the M-2 fraction (Table 3.1). The $[M+H]^{+}$ at *m/z* 1045 (Figure 3.6b) indicated the presence of MC-YR. The M-3 fraction has already been described (p 53). The $[M+H]^{+}$ ion at *m/z* 1029 (Figure 3.6d) revealed that the M-4 fraction contained MC-FR, and finally $[M+H]^{+}$ *m/z* 953 (Figure 3.6e) indicated the existence of MC-AR in the M-5 fraction.

Figure 3.6 MS/MS spectrum of M-1 to M-5 fractions after DEAE and Strata-X purification.
M-1 to M-5 are from top to bottom: (a) M-1, MC-RR and dMe-RR (b) M-2, MC-YR (c) M-3, [Dha^{7}]MC-LR and -LR (d) M-4, MC-FR and (e) M-5, MC-AR



3.6.5 The yields and purity of MCs

Seven MC variants, MC-RR, MC-dMe-RR, MC-YR, MC-LR, [Dha^7]MC-LR, MC-FR, and MC-AR were purified from the samples extracted from Lake Horowhenua. [Dha^7]MC-LR was the major variant of MCs in the freeze-dried material (approximately 89.34%) in the M-3 fraction (Table 3.3).

The M-1 fraction contained predominantly MC-RR (95%) and small amounts of MC-dMe-RR (5%), giving a total yield of 2.97 mg; whereas M-2 was mainly MC-YR (94%) total yield of 0.01 mg; M-3 mainly [Dha^7]MC-LR (93%) with some MC-LR (7%) total yield of 51.84 mg; M-4 with MC-FR (94%) total yield of 3.16 mg; and M-5 contained MC-AR (92%) being 0.04 mg of the total yield.

Table 3.3 The yield and purity of MCs (mg) from DEAE and Strata-X cartridges chromatography

Fractions	Yield (mg)	Purity of MC variants in the fraction (%)
M-1	2.97	95% of MC-RR and 5% of MC-dMe-RR
M-2	0.01	94% of MC-YR
M-3	51.84	93% of [Dha^7]MC-LR and 7 % of MC-LR
M-4	3.16	94% of MC-FR
M-5	0.04	92% of MC-AR

3.7 Discussion

To obtain significant amounts of MC variants for the biodegradation experiments of this study, a modified method of Saito et al. (2002) was used for further extraction and purification of MCs from cyanobacterial bloom samples of Lake Horowhenua rather than purchase expensive MCs. Prior to commencing purification of MCs, Lawton and Edwards (2001) recommended performing preliminary analysis of toxic cyanobacterial material to determine the quantity of MC, the number of variants and their potential identity. These assist in determining the efficiency of the purification and predict the potential yield of variants, preventing wasted effort in purifying samples containing little or no toxins. Normally MC-LR is the most common and the most toxic variant detected in cyanobacterial blooms worldwide (Gupta, Pant, Vijayarghavan, & Rao, 2003). In this study, however, nine

variants of MCs, predominantly [Dha^7]MC-LR, were detected in the large mass of cyanobacterial bloom in Lake Horowhenua before purification. It is obvious the cyanobacterial bloom material from Lake Horowhenua provides variants of the toxins in suitable quantities for further purification. Interestingly, the cyanobacterial bloom contains significant quantities of the toxic variants, MC-LR with an LD_{50} value (ip) of 50 µg/kg in mice, and moderately toxic variants, [Dha^7]MC-LR, MC-AR, and MC-FR all with the LD_{50} (ip) of 250 µg/kg in mice (Zurawell, Chen, Burke, & Prepas, 2005). Given the presence of the MC variants in significant quantities and their known toxicity, Lake Horowhenua water is a potential health risk for people who use the lake for recreation.

Preliminary data about MC variants and concentration of each in the lyophilized material confirmed the feasibility of using samples from Lake Horowhenua as a source of MCs. Extraction and purification of MCs used a modified method of Saito et al. (2002).

In this study the lyophilized cells were extracted with 70% MeOH-0.1% TFA solution and precipitated with 55% saturation ammonium sulfate to eliminate some contaminants from the toxins. The secondary clean-up step is strongly recommended for elimination of trace impurities as well as desalting where fractions contain salts that were used in the mobile phase such as sodium chloride in this study (Tsuji et al., 1994; Lawton & Edwards, 2001). The Strata-X cartridges were used for the second clean-up process, in which MCs are retained in the cartridge during the process of removing contaminants using pure water and low concentration of aqueous methanol, and then the toxins are readily eluted from the cartridge under high concentration of methanol (Tsuji et al., 1994). One MC fraction was passed through the Strata-X cartridge using different concentrations of methanol to establish optimal elution. It revealed that the aqueous methanol, less than 30%, proved to be a suitable washing solution since it did not strip any MCs from the cartridge. In contrast, the solutions containing greater than 50% methanol eluted too many MC variants (Figure 3.3). Therefore, for this study 30% aqueous methanol was used as the washing solution and 70 % aqueous methanol was used for eluting MCs from Strata-X cartridges.

This is the first study that a combination of the anion exchange DEAE chromatography and the Strata-X SPE cartridges was used and proved to be very

effective for cleaning up the cyanobacterial samples and MC purification. Seven MC variants, MC-RR, MC-dMe-RR, MC-YR, MC-LR, [Dha^7]MC-LR, MC-FR, and MC-AR were purified and characterized from the freeze-dried material. [Dha^7]MC-LR was the major MC variant, confirming the initial analysis of toxic cyanobacterial material before purification. In this study, it proved difficult to separate MC-LR from its desmethylated variants ([Dha^7]MC-LR), even using a more shallow gradient of sodium chloride between 5% and 30% to improve the resolution. Therefore, an additional purification step using preparative HPLC might provide more pure toxins in future research.

In Saito's study, employing DEAE and Sep-Pak C_{18} cartridges chromatography for MCs purification, they purified four known variants of MCs, namely MC-LR, MC-LY, MC-LF, and MC-LW and an unknown MC. In their study MC-LR was revealed as the major variant of MCs, giving a total yield of 50 mg with 96% purity (Saito et al., 2002). They also demonstrated that hydrophobic MCs (MC-LW and MC-LF) could be purified with DEAE as well as hydrophilic ones (MC-LR) in only one step of chromatography (DEAE column), whereas Edwards et al. (1996a) reported that it was difficult to purify MC-LW and MC-LF (W = tryptophan and F = phenylalanine) in preparative reversed-phase HPLC. As a result, they concluded that DEAE chromatography was superior to reversed-phase chromatography for purification of MCs, especially hydrophobic MCs (Saito et al., 2002).

For MC identification and characterization, LC-MS offers unsurpassed selectivity and specificity, with MS–MS techniques such as multiple reaction monitoring (MRM) mode. In MRM mode, low detection limits (0.1–1.5 ng) can be achieved and only specified structural variants will be detected (Hiller, Krock, Cembella, & Luckas, 2007). In general, LC–MS/MS full scan of most MCs created a single charged protonated molecular ion ($[M+H]^+$) as the base peak but MC-RR and MC-dMe-RR containing two basic arginine residues (R = a**r**ginine whereas A = alanine), produced a base peak $[M+2H]^{2+}$ ion at *m/z* 520 and 513, respectively (Wang, Pang, Ge, & Ma, 2007). Therefore, in the present study, the MRM ion pairs were selected as *m*/*z* 520>135 and 513>135 for detection and identification of MC-RR and MC-dMe-RR (Table 3.1).

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Chapter 4

Isolation and characterization of microcystin-degrading bacteria from New Zealand lakes

4.1 Abstract

Three isolates of bacteria—NV-1, NV-2 and NV-3—purified from Lake Rotoiti were able to break down [Dha^7]MC-LR and MC-LR. Among these isolates, NV-3 demonstrated the strongest degradative activity whereas isolate NV-1 exhibited the weakest activity. Therefore, NV-3 and NV-1 were investigated further. NV-3 and NV-1 were found to have identical 16S rRNA sequences but differed in several of their biochemical characteristics. On the basis of phylogenetic analysis of their 16S rRNA sequence, both isolates are probably members of the genus *Sphingomonas*, and they share 100% nucleotide sequence homology with the MC-degrading bacterial strain MD-1 from Japan. It was established that the optimal degradative activity for NV-3 was 25 μg/ml [Dha^7]MC-LR and MC-LR at 30^{o}C within just 3 days, whereas at 30^{o}C NV-1 decomposed the same concentration of [Dha^7]MC-LR and MC-LR within 5 days. Using liquid chromatography-tandem mass spectrometry (LC/MS-MS) intermediate by-products of [Dha^7]MC-LR and MC-LR degradation were detected. Using polymerase chain reaction (PCR) the presence of three genes (*mlr*A, *mlr*B and *mlr*C), that encode three enzymes involved in the degradation of MC-LR, were identified in NV-3 and NV-1. These enzymes sequentially hydrolyze Arg-Adda, Ala-Leu and then any peptide bonds within MC-LR to give two intermediate degradation products, linearized and tetrapeptide products of MC-LR and undetected products. The linearized and tetrapeptide by-products identified from the [Dha^7]MC-LR variant, indicate that the enzymes that degrade MC-LR can also degrade [Dha^7]MC-LR. The gene *mlr*D that encodes a putative transport protein, considered able to carry digested peptides from the biodegradation process into the bacterial cell, was also identified in NV-3 and NV-1.

4.2 Keywords: microcystin-LR, [Dha^7]MC-LR, degradation, *Sphingomonas*, microcystin-degrading genes, intermediate degradation products

4.3 Introduction

The most frequently encountered cyanobacterial toxins in freshwater are microcystins (MCs) (Chorus & Bartram, 1999). These toxins are stable and undergo hydrolysis only in the presence of very strong acids or extremely high temperatures (Botes et al., 1984; Lawton & Robertson, 1999). MCs can exist for months or years in distilled water in the absence of light. MCs have also been reported to persist for up to 6 months as cyanobacterial dry scum**,** and can be released from the crust material to the surrounding water after rewetting (Jones, Falconer, & Wilkins, 1995; Chorus & Bartram, 1999). There have, however, been reports that decomposition of MCs can be caused by naturally-occurring bacteria. Jones and Orr (1994) revealed that MCs began to disappear a few weeks after the algicidal treatment of cyanobacterial bloom on Lake Centenary, Australia. Such biodegradation by bacteria could, therefore, be a potentially safe and natural method for removing MCs from water.

In this study, MC-degrading bacteria were isolated and characterized. The biodegradation rates of the isolated bacteria were also investigated to identify the bacterium with the highest degrading-ability for further study. The genes that encode the enzymes responsible for the degradation of MCs and by-products from the biodegradation process were also examined to further understand the degradation pathway.

4.3.1 Stability of MCs

As mentioned above, MCs are chemically stable over a wide range of temperatures and pH, possibly because of their cyclic structure (Lawton & Robertson, 1999). They have been reported to withstand extreme temperatures (>300^{o}C) for several hours or survive under dry and dark conditions at ambient temperature for many years (Tsuji et al., 1994; Harada, Tsuji, Watanabe, & Kondo, 1996; Svrcek & Smith, 2004; de Figueiredo, Azeiteiro, Esteves, Goncalves, & Pereira, 2004). The toxins are also generally resistant to enzymatic hydrolysis by common proteases, such as pepsin, trypsin, collagenase and chymotrypsin (Takenaka, 1998; Saitou, Sugiura, Itayama, Inamori, & Matsumura, 2003), and complete degradation, therefore, requires the presence of strong acidic conditions such as 6N-hydrochloric acid and trifluoroacetic acid under reflux in the laboratory (Harada, 1996).

The toxins are also resistant to irradiation by sunlight; however, the existence of pigments such as chlorophyll-*a* and phycocyanin within the cyanobacterial cells can lead to photosensitization and decomposition of the MC secondary to the isomerization of a double bond in the Adda moiety. The half-life for this decomposition was about 10 days (Tsuji et al., 1995). MC-LR and MC-RR degraded more rapidly when the toxins were exposed to UV light at wavelengths around their absorption maxima (238–254 nm), and the process depended on the intensity of the light (Tsuji et al., 1995). The effect of pH on thermal decomposition of MC-LR was investigated by Harada et al. (1996). Harada and co-workers demonstrated the half-life of MC-LR at pH 1 and pH 9 was 3 and 10 weeks, respectively. Linearized peptides of the toxins were detected as by-products of the degradation produced by hydrolysis of the toxin at an Mdha residue. Esterification was common as a result of hydrolysis under these acidic conditions.

A study by Cousins et al. (1996) reported that MC-LR at a concentration of 10 µg/l was stable for over 27 days in deionized water and for over 12 days in sterilized reservoir water. In the presence of reservoir water, however, the degradation of MC-LR occurred in less than 7 days. This phenomenon was believed to be the result of biological degradation of the toxin by indigenous bacteria living in natural waters (Cousins, Bealing, James, & Sutton, 1996).

4.3.2 Natural processes of MC degradation

Microcystins are endotoxins and, therefore, remain within viable cyanobacterial cells throughout growth (Zurawell, Chen, Burke, & Prepas, 2005). The release of toxins to the surrounding water occurs during the senescence and decomposition of the cyanobacteria, and as a result, concentrations of the toxins increase after a cyanobacterial bloom (Mazur-Marzec, 2006). However, some natural processes can lead to the reduction of toxin levels in water. These include dilution by uncontaminated water masses, photodegradation, adsorption on suspended particles and sediments and biodegradation (Mazur-Marzec, 2006). The focus of this study is the mechanism of microbial degradation of MCs, and, therefore, other mechanisms of toxin degradation will only be mentioned briefly.

4.3.2.1 Degradation of MCs by water-dilution, photodegradation and adsorption on suspended particles and sediments

Algicides are normally used to treat water bodies that are contaminated with toxic cyanobacteria. This treatment results in undesirable outcomes, which are cell lysis and release of intracellular toxins into the surrounding water. It is generally presumed that the toxins are rapidly diluted by uncontaminated water from the main body of water (Jones & Orr, 1994; Harada, 1996). One report demonstrated the persistence of high concentrations of MC (100-1800 μg/l) for 9 days after algicide treatment of a *M. aeruginosa* bloom, after which almost all toxins started to disappear by dilution and microbial degradation.

As mentioned earlier, MCs are readily degraded at wavelengths close to their absorption maxima. In the presence of natural sunlight and in distilled water, no significant change in MC-LR concentration is observed over 26 days (Cousins et al., 1996). On the other hand, as mentioned above, degradation of MC occurred by sunlight irradiation in presence of various pigments from cyanobacterial cells**,** indicating that in the presence of pigments and natural sunlight, MC degradation can occur (Tsuji et al., 1995).

Morris et al. (2000) found that >81% of the MC-LR toxins could be removed from water by using fine grained particles containing a high concentration of clay minerals. Similarly Miller, Critchley, Hutson, & Fallowfield (2001) showed that the clay particles are the most likely to be active binding components that will adsorb MC-LR. The ability to scavenge MC using fine-grained particles might, therefore, provide an effective method to strip these toxins from drinking water supplies.

The reduction of MC concentrations in water and sediment of a shallow lake (Lake Taihu, China) was investigated by Chen et al. (2008). They reported that MC degradation occurred in the water column and sediment, however, degradation in sediments was greater than in water and has a critical role in the degradation of MCs in aquatic systems. The relatively low levels of MCs found in the environment are most likely due to bacterial degradation. Sediments are, in fact, an important source of MC-degrading bacteria, providing the water column with bacteria for metabolization of MCs.

4.3.2.2 Biological degradation of MCs

In freshwater, the mechanism of degradation of MCs is most likely to be by biodegradation process (Chen et al., 2008). Other mechanisms for MC degradation discussed above (Section 4.3.2.1), are limited in their magnitude of impact. For instance, eutrophic water is normally covered with thick cyanobacterial scum and macrophyte beds, which limit visible light penetration; limiting the effects of photolysis. MCs adsorb only weakly on lake sediments, and usually no more than 20% of toxins can be adsorbed (Rapala, Lahti, Sivonen, & Niemela, 1994; Lahti, Rapala, Fardig, Niemela, & Sivonen, 1997; Chen et al., 2008). Biodegradation, therefore, is probably the main means of MC removal in natural water.

Biological degradation is associated with the presence of complex populations of indigenous bacteria in water bodies (Jones & Orr, 1994). Biodegradation can reduce the intracellular toxins present within the viable toxic cyanobacterial cell and the extracellular toxins in the water (Zurawell et al., 2005). Variation in the toxin degradation rates among different bodies of natural water probably relates to the bloom history of the lake and environmental factors, for example temperature and pH that affect metabolic rate of degrading bacteria. Lakes with a prior history of MC blooms may contain bacteria that are capable of exploiting toxic cyanobacterial cells and MCs as a food source and may rapidly degrade the toxins (Christoffersen, Lyck, & Winding, 2002).

4.3.2.2.1 MC-degrading bacterial strains

Jones and Orr (1994) were among the first who investigated bacterial degradation of MCs. They revealed a biphasic degradation process, comprising a rapid phase that lasted 3 days (90%–95% degradation) and a slower phase that continued until a flashflood occurred on day 21. A further study resulted in the isolation of *Sphingomonas* strain MJ-PV (ACM-3962), which was initially identified as a *Pseudomonas* sp (Jones, Bourne, Blakely, & Doelle, 1994). This strain was the first bacterium identified as able to utilize MC-LR as its sole source of carbon and nitrogen needed for growth. After addition of the MC-degrading bacterium to surface water samples, the lag phase of MC degradation (at a concentration of 1 mg/l) lasted for a few days followed by a log phase from day 2 until day 8. However, at

concentrations greater than 1 mg/l an initial slow removal of the toxins occurred before rapid degradation commenced. The lag phase was absent upon re-addition of MC-LR for a subsequent batch experiment (Jones et al., 1994).

Various research groups have since reported other bacterial strains that are able to utilize MCs as a carbon and/or nitrogen source (Table 4.1). More than four strains of *Sphingomonas* that are able to degrade MCs, have now been isolated and studied (Park et al., 2001; Ishii, Nishijima, & Abe, 2004; Saito et al., 2003; Tsuji, Asakawa, Anzai, Sumino, & Harada, 2006; Valeria, Ricardo, Stephan, & Alberto, 2006).

Table 4.1 MC-degrading bacteria (adapted from Ho, Lewis, Brookes, & Newcombe, 2007)

Bacterium	**Source**	**Degraded MC variants**	**Reference(s)**
Sphingomonas strain ACM-3962 (or strain MJ-PV)	Murrumbidgee River, Australia	MC-LR and MC-RR	Jones et al., 1994
Pseudomonas aeruginosa	A Japanese reservoir	MC-LR	Takenaka and Watanabe,1997
Paucibacter toxinivorans	Lake Vihnusjärvi, Finland	MC-LR and MC-YR	Lahti et al., 1998 and Rapala et al., 2005
Sphingosinicella microcystinivorans (or *Sphingomonas* sp. Y2)	Lake Suwa, Japan	MC-LR, MC-RR and MC-YR and 6(Z)-Adda-MC-LR	Park et al., 2001 and Maruyama et al., 2007
Sphingomonas strain MD-1	Lake Kasumigaura, Japan	MC-LR, MC-RR and MC-YR	Saitou et al., 2003
Sphingomonas strain 7CY	Lake Suwa, Japan	MC-LR, MC-RR, MC-LY, MC-LW and MC-LF	Ishii, et al., 2004
Sphingomonas sp. strain B-9	Lake Tsukui, Japan	MC-LR, MC-RR, 3-DMMC-LR, DHMC-LR and MC-LR-Cys	Tsuji et al., 2006
Sphingomonas sp. strain CBA4	San Roque reservoir, Argentina	MC-RR	Valeria et al., 2006
Sphingopyxis strain LH21	Biological sand filter, Australia	MC-LR and MC-LA	Ho et al., 2007
Burkholderia sp.	Patos Lagoon, Brazil	MC–LR and [D-Leu]MC–LR	Lemes et al., 2008

Note: 6(Z)-Adda-MCLR = Isomer 6(Z)-Adda microcystin-LR; 3-DMMC-LR =3-desmethyl microcystin-LR; DHMC-LR = Dihydro microcystin-LR; MC-LR-Cys = microcystin-LR cysteine conjugate.

Sphingomonas strain 7CY was isolated from the eutrophic Lake Suwa in Japan, and classified as *Sphingomonas* on the basis of 16S rRNA sequencing (Ishii et al., 2004). This strain has the ability to degrade MC-LR, MC-RR, MC-LY, MC-LW

and MC-LF extracted from *Microcystis* PCC7820 cells. Degradation of each of these MCs (at a concentration of 6 μg/ml each) began on the first day of treatment. During the first 3 days, 70% of MC-LR was degraded whereas the degradation of MC-LY, MC-LW, and MC-LF occurred more gradually. All MCs were, however, completely degraded after 4 days (Ishii et al., 2004).

Another strain of bacteria was isolated from Lake Kasumigaura by Saitou and co-workers in 2003. The 16S rRNA sequence demonstrated 98.5% homology with that of *Sphingomonas stygia*; and therefore, the bacterium was named *Sphingomonas* strain MD-1. The bacterium was capable of completely degrading MCs as a sole carbon source for its own growth and proliferation. The ability of *Sphingomonas* MD-1 to degrade at least three variants of MCs (MC-LR, MC-YR and MC-RR) has been investigated. MC-LR (1 mg/l) was degraded by 95% in 2.5 hours whereas MC-YR and MC-RR were degraded by 96% and 98%, respectively. The bacterium, therefore, had the highest degradation activity using MC-RR as a substrate. The degradation activity and growth rate of the bacterium was also found to decrease under alkaline pH conditions (Saitou et al., 2003).

Tsuji et al. (2006) investigated bacteria that were active against the cyanobacterial hepatotoxin MCs. Eleven active bacteria were isolated from samples taken from Lakes Tsukui and Sagami, Japan. Strain B-9 from Lake Tsukui exhibited the strongest degradative activity, and was able to degrade MC-RR and MC-LR (2 mg/l) within 24 hours. The 16S rDNA sequence of the strain B-9 showed the highest similarity to that of *Sphingomonas* sp. Y2 (AB084247, 99% similarity), therefore, the bacterium was tentatively named *Sphingomonas* sp. strain B-9 (Tsuji et al., 2006).

Aerobic biodegradation of MC-RR by *Sphingomonas* strain CBA4 was studied by Valeria et al. (2006). This bacterium was isolated from San Roque reservoir (Cordoba, Argentina) and was able to degrade MC-RR at a concentration of 200 μg/l within 36 hours, resulting in an increase in bacterial density from $1.2x10^6$ to $5.75x10^6$ CFU/ml. These studies demonstrated an absence of inhibition of bacterial growth, and proved that MC-RR was used as the sole carbon and nitrogen source (Valeria et al., 2006).

Sphingomonas is not the only bacterial genus capable of degrading MCs. Other genera of bacteria (*Pseudomonas*, *Paucibacter*, *Sphingosinicella*, *Sphingopyxis*

and *Burkholderia*) have also been reported to degrade MCs (Takenaka & Watanabe, 1997; Rapala et al., 2005; Maruyama et al., 2006; Ho et al., 2007; Lemes et al., 2008).

Takenaka and Watanabe (1997) isolated a strain of *Pseudomonas aeruginosa* from a Japanese reservoir that had the ability to degrade MC-LR. The concentration of MC (initially 50 µg/ml) significantly decreased to 4.5% of original in over 21 days.

Lahti, Niemi, Rapala, and Sivonen (1998) isolated a total of 17 bacterial strains with an ability to breakdown MCs—16 from sediment and one from water from two eutrophic lakes in southern Finland. One of the 17 strains was tentatively identified as a *Sphingomonas* strain whereas the two other strains investigated could only be classified as members of the *β*-Proteobacteria a group of aerobic or facultative bacteria that are often highly versatile in their degradative capacities. Thirteen of these 17 MC-degrading strains were, however, further characterized at a later date by Rapala and co-workers in 2005. Rapala et al. (2005) were the first research group, using chemotaxonomic (e.g. analysis of the cell components etc.), phenotypic (e.g. morphological and biochemical characteristics etc.), genotypic (e.g. the G-C content etc.) and phylogenetic analyses (e.g. 16S rRNA sequencing etc.), to classify MC-degrading bacteria, whereas other research groups commonly used biochemical and 16S rRNA sequencing approaches (Jones et al., 1994; Takenaka & Watanabe, 1997; Park et al., 2001; Maruyama et al., 2003; Saitou et al., 2003; Harada et al., 2004; Ishii et al., 2004; Rapala et al., 2005; Maruyama et al., 2006; Valeria et al., 2006; Lemes et al., 2008). According to phenotypic characteristics (carbon utilization, fatty acid and enzyme activity profiles), the G+C content of the genomic DNA (66.1–68.0 mol%), and 16S rRNA sequences (98.9–100% similarity), the strains formed a single microdiverse genospecies that was most closely related to *Roseateles depolymerans* (95.7–96.3% 16S rRNA nucleotide sequence similarity). The cellular fatty acids identified from all strains were C16:0 (9.8–19 %) and C17:1ω7c (<1–5.8 %). A more detailed analysis of two strains indicated that C16:1ω7c was the primary fatty acid. The phylogenetic and phenotypic features separating this strain from recognized bacteria supported the creation of a novel genus and species. Rapala et al. (2005) therefore re-classified and named these 13 bacterial strains as *Paucibacter toxinivorans* gen. nov., sp. nov. All bacteria from this family could degrade a mixture of MC-LR and MC-YR, with maximum rates of 4-16 µg/l/h (Rapala et al., 2005).

A MC-degrading bacterium was isolated from Lake Sawa (the same location as strain 7CY) by Park and colleagues in 2001. The bacterium was tentatively identified as *Sphingomonas* by manual chemotaxonomy, but 16S rRNA sequence analysis suggested that it was, in fact, a new species or potentially a new genus (Park et al., 2001). Maruyama and co-workers re-classified this strain 5 years later, as Y2 (Maruyama et al., 2006). These authors also classified two other strains of MC degradative bacteria (MDB2 and MDB3) isolated from the Tenryu River, Japan. Phylogenetic analyses revealed that the three strains formed a tight cluster within the family Sphingomonadaceae but were obviously separate from established genera of this family such as *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. Sequence similarities between the three strains and type strains from established genera ranged from 90.9 to 94.9%. Chemotaxonomic and phenotypic data supported the conclusion that these strains were members of the family Sphingomonadaceae (Maruyama et al., 2007). The major components of the cellular fatty acids of these bacteria were 18:1ω7c (36–41 %) and 16:1ω7c (33–36 %). Hydroxy fatty acids were mainly 2-OH 14:0 (11–13 %), whereas 3-OH fatty acids were absent. Glycosphingolipids were detected. Ubiquinone-10 and homospermidine were present as the major quinine and polyamine, respectively. Maruyama and co-workers, in 2006, therefore proposed the three strains formed part of a new genus and species of the family Sphingomonadaceae with the name *Sphingosinicella microcystinivorans* gen. nov., sp. nov (Maruyama et al., 2006).

MC degradation by the strain Y2 was examined by Park and colleagues in 2001. Strain Y2 began degradation of MC-RR and MC-LR (20 mg/l) on day 1. After incubation for 3 days, the MC concentrations were below detectable levels. The greatest rates of degradation were 13 and 5.4 mg/l/day for 18 mg/l MC-RR and MC-LR, respectively. The same study also tested the ability of the strain Y2 to degrade MC-YR. The rate of degradation was 61 mg/l/day using an initial concentration of MC-YR of 22 mg/l. Similar to previous studies, the degradation rates were strongly dependent on temperature, with the maximum rate achieved at 30^{o}C (Park et al., 2001).

A novel bacterium, LH21, with the ability to degrade two MCs (MC-LR and MC-LA) was isolated from a biological sand filter. The sand filter itself had an ability

to eliminate MCs from source waters. Based on phylogenetic analysis of the 16S rRNA gene sequence, the LH21 bacterium most likely belonged to the genus *Sphingopyxis* and was, therefore, designated *Sphingopyxis* strain LH21. This strain could decompose MC-LA and MC-LR in batch experiments with complete removal of the MCs observed within 5 hours after re-exposure of the toxins to the bacteria. Again, the degradation rate was dependent upon bacterial numbers and temperature of environment (Ho et al., 2007).

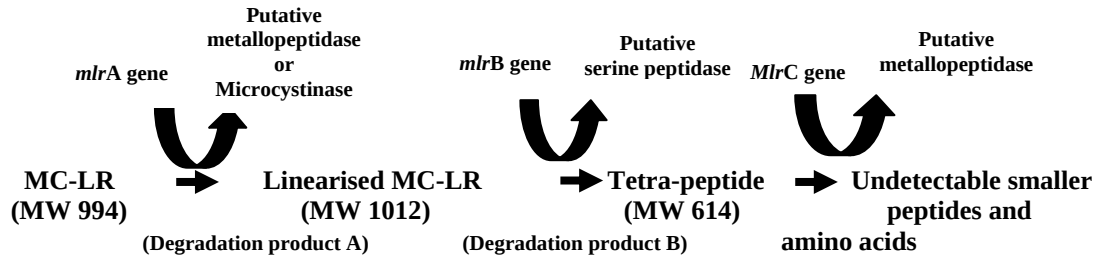
Recently, a novel bacterium with MC-degrading activity was isolated from the estuarine water of the Patos Lagoon, in the southernmost region of Brazil (Lemes et al., 2008). Analysis of the partial 16S rRNA sequence revealed 96% homology to the *Burkholderia* genus. The bacterium, therefore, was classified as a *Burkholderia* species, belonging to the beta subdivision on proteobacteria (*β*-proteobacteria). This report is, therefore, the first to demonstrate a member of the genus *Burkholderia* to have a cyanobacterial toxin degradative activity. This bacterium exhibited an ability to degrade a commercial MC (MC–LR) as well as a semi-purified MC ([D-Leu^{1}]MC–LR) extracted from *Microcystis* RST9501 cells at initial toxin concentration of 1 µg/ml. The toxins were no longer detectable after 15 days with a biodegradation rate of 0.05 µg/ml/day.

4.3.2.2.2 Bacterial process and intermediate products of MC degradation

Numerous groups have investigated the process by which bacteria degrade MCs and the intermediate products formed. Very few research groups, however, have successfully characterized the process completely (Bourne et al., 1996; Harada et al., 2004; Imanishi, Kato, Mizuno, Tsuji, & Harada, 2005). A number of studies have detected just one biodegradation by-product (Takenaka & Watanabe, 1997; Edwards, Graham, Fowler, & Lawton, 2008), and some studies have only reported unidentified peaks obtained from High Performance Liqiud Chromatography (HPLC) analyses (Park et al., 2001; Ishii et al., 2004; Tsuji et al., 2006; Valeria et al., 2006; Lemes et al., 2008). These peaks were presumed to represent the main by-product in the biodegradation pathway, but was insufficient to identify the complete enzymatic pathway.

The enzymatic pathway of MC degradation was first successfully characterized by Bourne and colleagues in 1996, who investigated the pathway by which the *Sphingomonas* strain ACM-3962 (MJ-PV) degraded MC-LR (Bourne et al., 1996; Bourne, Riddles, Jones, Smith, & Blakeley, 2001). Two intermediate degradation products were identified, suggesting that at least three intracellular hydrolytic enzymes were involved in the degradation of MC-LR (Figure 4.1). The first enzyme in the degradation pathway, a metalloprotease named microcystinase, cleaves the aromatic ring of MC-LR at the Arg-Adda peptide bond (see Figure 2.3, p.21). This step yields a linearized MC-LR (also called 'degradation product A') (Figure 4.1), which has a 160-fold reduction in toxic activity compared with the parent MC-LR (NH_2-Adda-D-Glu-Mdha-D-Ala-L-Leu-D-MeAsp-L-Arg-OH). Next, a serine peptidase catalyzes the linearized MC-LR at the Ala-Leu peptide bond, producing a tetrapeptide, NH_2-Adda-D-Glu-Mdha-D-Ala-OH (also called 'degradation product B') (Figure 4.1). Finally, the third enzyme, another metalloprotease, cuts the peptide bonds randomly resulting in undetectable peptide fragments and amino acids (Bourne et al., 1996).

Figure 4.1 MC-LR degradation pathway of *Sphingomonas* strain ACM-3962 (Bourne et al., 1996, 2001)



In 2004, Harada and co-workers showed that Adda was one of the degradation products of MC-LR after degradation by *Sphingomonas* strain B-9. Adda was the product by way of two intermediates—linearized MC-LR and a tetrapeptide. In contrast to the native toxin, neither the purified Adda, nor the two intermediates were toxic to mice (Harada et al., 2004).

Imanishi and colleagues (2005) further investigated the bacterial degradation process by *Sphingomonas* strain B-9, and proposed that the process by which *Sphingomonas* strain B-9 and *Sphingomonas* strain ACM-3962 degraded MC-LR is

similar, except that Adda was detected as an additional by-product in strain B-9 whereas in strain ACM-3962 the Adda was undetectable. The process of MC degradation by strain B-9, therefore, consists of the sequential enzymatic hydrolysis of Arg-Adda, Ala-Leu and then Adda-Glu peptide bonds, to produce two nontoxic intermediate degradation products (linearized MC-LR and tetrapeptide) and Adda.

As mentioned above, some studies of MC degradation have detected only one intermediate and, therefore, the complete biotransformation process of MC could not have been achieved. For example, Edwards et al. (2008) were able to detect two linear peptides of MC-LR (NH_2-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-OH) and MC-LF (NH_2-Adda-Glu-Mdha-Ala-Leu-MeAsp-Phe-OH), from a total of four MC variants ([D-Asp^3]MC-RR, MC-LR, MC-LW and MC-LF) degraded by indigenous bacteria from Loch Rescobie, UK. The findings from this study suggested that the enzymatic degradation of the MCs used in this study was identical to those of *Sphingomonas* strain ACM-3962, characterized by Bourne et al. (1996).

Another incomplete degradation of MC-LR by bacteria was reported by Takenaka and Watanabe (1997), who used bacterium *Pseudomonas aeruginosa* isolated from a Japanese Lake. Interestingly, they suggested that an alkaline protease enzyme from *P. aeruginosa* was the enzyme responsible for MC-LR degradation, by hydrolyzing the peptide bond of MC-LR to produce (2S, 3S, 8S)-3-amino-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid (DmAdda), as an intermediate by-product. In addition, they also suggested that two additional compounds produced by this bacterium, pyochelin (a small, high-affinity iron chelating compound) and pyocyanin (a blue-green antibiotic pigment) might stimulate the generation of superoxide radicals, implicated in the hydrolysis of MC.

4.3.2.2.3 Characterization of genes that encode the enzymes responsible for degradation of MC

Only one research team, to date, has completely characterized the genes that encode the enzymes responsible for the bacterial degradation of MC (Bourne et al., 2001). Other studies have only characterized one gene or identified genes in isolated MC-degrading bacteria (Saito et al., 2003; Bourne, Blakeley, Riddles, & Jones, 2005; Geueke, Busse, Fleischmann, Kämpfer, & Kohler, 2007; Ho et al., 2007).

(i) Complete characterization of the genes, encoding MC degradation enzymes

After identification of the bacterial enzymatic pathway for MC-LR degradation in 1996, Bourne and co-workers performed cloning and molecular characterization of a gene cluster (*mlr*A, *mlr*B, *mlr*C and *mlr*D) from *Sphingomonas* strain ACM-3962 (MJ-PV) in 2001. Genes *mlr*A, *mlr*B, and *mlr*C encoded the three hydrolytic enzymes involved in the bacterial degradation pathway, and *mlr*D encoded a putative oligopeptide transporter. The DNA sequence was analyzed by comparison with DNA and protein databases in GenBank. The *mlr*A gene was found to encode a putative metalloprotease that cuts an aromatic ring of MC-LR to produce the linear MC-LR product. The enzyme is a 336-residue endopeptidase. The residues that are most likely to chelate an active-site transition metal ion are in the sequence HXXHXE [a short segment with two histidines (H) and a glutamate residue (E)] (25% sequence identity and a 54% sequence similarity), and would be unique for a metalloproteinase. The *mlr*B gene encodes the 402 residue putative serine protease that hydrolyzes the linear peptide of MC-LR to produce a tetrapeptide degradation product. This enzyme was classified into the 'penicillin-binding enzyme' family of active-site serine hydrolases. The *mlr*C gene encodes another putative metalloprotease of 507 residues that cleaves the tetrapeptide to produce peptide fragments and amino acids. This protein exhibits greater than 30% sequence identity and 54% sequence similarity to a hypothetical protein from *Streptomyces coelicolor*. The *mlr*D gene encodes 442-residue protein and demonstrates considerable sequence identity and similar putative transmembrane spanning regions to the PTR2 family of oligopeptide transporters. On the basis of their gene and protein analyses, Bourne et al. (2001) suggested that the genes *mlr*A, *mlr*B and *mlr*C may normally be involved in peptidoglycan cycling in the cell wall of the degrading bacteria and then perform fortuitously in hydrolysis of MC-LR, whereas *mlr*D is predicted to carry small peptides of MC-LR or its degradation products across the bacterial cell wall (Bourne et al., 2001).

(*ii*) *Characterization of a single gene that encodes an MC degradation enzyme*

Saito et al. (2003) performed PCR assays to detect *mlr*A from two *Sphingomonas* strains (strain MD-1 and Y2; strain Y2 was reclassified later by Maruyama et al. (2007) to *Sphingosinicella microcystinivorans*). The results revealed that both strains contained *mlr*A homologues. Nucleotide BLAST analyses

demonstrated the similarity of the *mlr*A gene homologue of strains MD-1 and Y2 with the strain ACM-3962 (MJ-PV) (98% and 84% similarity, respectively). Amino acid sequences of the putative MlrA protein from strains MD-1 and Y2 were 91% and 87% similar to the amino acid sequence of strain ACM-3962 and identical homology of 97% and 91%. Saito et al. (2003) also found that the putative or predicted amino acid sequence from positions 421-724 of the *mlr*A gene of strain MD-1 was closely identical to the CAAX amino-terminal protease family of *Bacillus* spp. (unknown function) with 35% similarity and 50% identical homology (Saito et al., 2003).

(*iii*) *Determining the presence of genes that encode MC degradation enzymes in MC-degrading bacteria*

Ho and colleagues detected the gene cluster involved in bacterial degradation of MC (*mlr*A, *mlr*B, *mlr*C and *mlr*D) in a MC-degrading bacterium *Sphingopyxis* sp. strain LH21 (Ho et al., 2006, 2007). The *mlr*A gene sequence of strain LH21 was 98% similar to that of *Sphingopyxis* sp. C-1 (NCBI accession number AB161685) (Ho et al., 2006) whereas *mlr*B, *mlr*C and *mlr*D sequences were 92%, 89% and 88% similar to their respective genes from *Sphingomonas* sp. ACM-3962 (AF411069, AF411070 and AF411071, respectively) (Ho et al., 2007). These authors also suggested that isolate LH21 may degrade MC via a similar pathway to that of *Sphingomonas* sp. ACM-3962 (Ho et al., 2007).

Using PCR, Geueke et al. (2007) determined the presence of the genes, *mlr*A, *mlr*B, *mlr*C and *mlr*D, in two newly-proposed species of bacteria that had highest levels of similarity (99%) to each other, according to 16S rRNA sequence analysis (*Sphingosinicella microcystinivorans* gen. nov., sp. nov., a new bacterium able to degrade MCs and *Sphingosinicella xenopeptidilytica* sp. nov., a new bacterium able to degrade β-peptide but unable to degrade MCs). All of the genes involved in MC-degradation were detected in the genome of *S. microcystinivorans*, but were not present in *S. xenopeptidilytica*. The absence of *mlr*A in *S. xenopeptidilytica* was confirmed by southern hybridization using prime DNA labelling with a probe composed of 806 base pairs (bp) amplified by PCR from genomic DNA of *S. microcystinivorans* (Geueke et al., 2007). The results from this study suggest that the genes that encode enzymes responsible for degradation of MC are conserved and unique to MC degrading bacteria.

4.3.3 Bacterial identification

Bacterial identification is one of three separate but interrelated areas of bacterial taxomony: classification, nomenclaclature, and identification (Brenner, Staley, & Krieg, 2005). Bacterial classification involves the catagorization of bacteria into groups or taxa based on their similarity or relationship. Bacterial nomenclature involves the assignment of names to the taxonomic group on the basis of International Code of Nomenclature of Bacteria and bacterial identification involves the practical use of a classification scheme to determine the identity of an isolate as a member of an established taxon or as a member of a previously unidentified species (Brenner, Staley, & Krieg, 2005). A key aspect of this study was identification of MC-degrading bacteria isolated from Lake Rotoiti, therefore a consideration of the principles of bacterial classification and identification was necessary (Busse, Denner, & Lubitz, 1996; Brenner, Staley, & Krieg, 2005; Staley, 2006).

Early bacterial taxonomy was based on metabolic (biochemical) and physiological characteristics of bacteria. These characteristics combined with morphological characteristics under light microscopy formed the basis for the initial bacterial classification such as those found in Bergey's Manual of Deterinative Bacteriology culminating in the seventh edition in 1957 (Breed et al., 1957). Since the 1960s, a number of technical innovations such as use of electron microscope to observe cell fine structure and analysis of DNA, including mol% G-C values, DNA-DNA hybridization and 16S rDNA sequencing have been used to better define taxonomic entities (Vandamme et al., 1996; Busse et al., 1996; O'Hara, Brenner, & Miller, 2000; Schloter, Lebuhn, Heulin, & Hartmann, 2000, Krieg & Garrity, 2001; Owen, 2004; Staley, 2006). Many bacteriologists agree that bacterial identification should be defined by a polyphasic taxonomic approach integrating different kinds of data and information (phenotypic, and genotypic) on microorganisms (Colwell, 1970, Willems et al., 1991; Gillis et al., 1995; Vandamme et al., 1992, 1996). A polyphasic approach was used in this study for establishing NV-1 and NV-3 as belonging to *Sphingomonas* (see Section 4.3.2.2.1, pp 75-76). Genotypic methods are those that are directed toward DNA or RNA molecules such as DNA-DNA hybridization, 16S rDNA sequencing and DNA fingerprinting whereas phenotypic methods comprise all those that are associated with physiological, biochemical and chemotaxonomic

characteristics such as catalase test, pH range and whole-cell protein analysis (Vandamme et al., 1996; Busse et al., 1996).

Various ranks or levels are used in bacterial classification. The highest rank is called a Domain. All bacteria are assigned to one or other of two Domains, *Archaea* and *Bacteria*. Phylum, class, order, family, genus, species, and subspecies are successively smaller, non-overlapping subsets within the Domain (Brenner, Staley, & Krieg, 2005).

The basic and most important taxonomic rank in modern bacterial taxomony is the species, however there is no consensus on a definition of what a bacterial species actually is (Rossellσ-Mora, & Amann, 2001). It is enough to focus on a functional definition of a bacterial genus and species for this study, and therefore phylum, class, order and family will not be considered further. Bacterial taxomony is inconsistent and sometimes confusing because of the use of different concepts (taxonomic, phylogenetic and biological approaches) (Wayne et al., 1987; Schloter et al., 2000; Brenner, Staley, & Krieg, 2005). However, bacterial species is defined broadly in Bergey's Manual of Systematic Bacteriology (Krieg & Holt, 1984) as the following:

"*A bacterial species may be regarded as a collection of strains that share many features in common and differ considerably from other strains. One strain of a species is designated as the type strain; this strain serves as the name-bearer strain of the species and is the permanent example of the species, i.e. the reference specimen for the name. The type strain has great importance for classification at the species level, because a species consists of the type strain and all other strains that are considered to be sufficiently similar to it as to warrant inclusion with it in the species*"

Furthermore, Wayne et al. (1987) also provide a definition of bacterial species using DNA reassociation (DNA-DNA hybridization) between the strains within a purported species as follows:

"Bacterial species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m". [ΔT_m = melting temperature]

Therefore, if an unknown strain demonstrates 70% or greater hybridization to a designated 'type strain' that is deposited in a culture collection, then the strain is considered to be a member of the same species; however, if the unknown strain demonstrates a lower level of hybridization, it can be named as a separate species (Staley, 2006). *(Note: "A strain is a clonally proliferating organism. It is made up of the descendants of a single isolation in pure culture, and usually is made up of a succession of cultures ultimately derived from an initial single colony" Brenner, Staley, & Krieg, 2005*).

The definition of bacterial genus is also defined in Bergey's Manual of Systematic Bacteriology (Krieg, & Holt, 1984) as follows:

"*The bacterial genus is usually a well-defined group that is clearly separated from other genera, and the thorough descriptions of genera in the 1984 edition of Bergey's Manual exemplify the depth to which this taxonomic group is usually known. However, there is so far no general agreement on the definition of a genus in bacterial taxonomy, and considerable subjectivity is involved at the genus level. Indeed, what is perceived to be a genus by one person may be perceived as being merely a species by another systematist.*"

The use of 16S rRNA gene sequencing in bacterial identification has been widely accepted as a primary method to identify and assign unknown bacteria to recognised families, genera and species (Vandamme et al., 1996; Busse et al., 1996; O'Hara, Brenner, & Miller, 2000; Schloter, Lebuhn, Heulin, & Hartmann, 2000; Krieg & Garrity, 2001; Owen, 2004; Staley, 2006). However, it is also well known that bacteria can have identical 16S rRNA gene sequences, yet can still belong to different species based on DNA–DNA hybridization (Fox et al., 1992). One advantage of using the 16S rRNA gene sequence analysis is that it allows a rapid determination of whether there is a need to carry out DNA–DNA hybridization. This is due to a known demarcation within existing species that have been analysed by both procedures. If a strain shows less that 97% 16S rRNA gene homology with its

highest match-described species, then it can be declared a novel species without carrying out DNA–DNA hybridization (Stackebrandt & Goebel, 1994). The establishment of this boundary condition has resulted in the naming of many more species without the need for DNA–DNA hybridization (Staley, 2006).

4.4 Objectives of this chapter

The objectives addressed in this chapter were (1) to isolate and identify natural aquatic bacteria capable of degrading MCs from New Zealand water bodies, (2) to characterize the biotransformation of MCs using the most active MC-degrading bacteria isolated, and (3) to elucidate the genes that encode the MC-degrading enzymes of the bacterium. The information gained from realizing these objectives, prepared for the later stages of this doctoral study, which involved testing of small-scale biofilm technology to establish the effectiveness of MC degradation by the bacterium, and to simulate bioremediation on a small scale for possible application to water treatment plants.

4.5 Methods

4.5.1 Isolation of MC-degrading bacteria and their biodegradation

Water samples taken from Lake Rotoiti, Lake Rotoehu, Lake Rotorua and Lake Horowhenua during a bloom of *M. aeruginosa* were used as sources for isolation of MC-degrading bacteria. Lake water (10 ml) was transferred into a Erlenmeyer flask containing 190 ml of a sterile mineral salts medium (MSM) broth (Appendix 5) plus [Dha^7]MC-LR and MC-LR, purified as described in Chapter 3 (final concentration 1 µg/ml) as the main source of carbon and nitrogen. The flask was incubated in a shaking incubator (200 rpm) at 30^{o}C in the dark for 5 days. Five subcultures were established from each water sample. It was presumed that bacteria with an ability to use MCs as a food source would survive in the culture. After final inoculation, bacteria were isolated using the cross-streak technique onto peptone-yeast extract medium agar plates (PYEM) (Appendix 5) and were incubated at 30^{o}C for 72 h. When colonies appeared on the plates, morphologically different colonies were selected and re-streaked onto new plates until pure cultures were obtained.

A single colony of each bacterial isolate was then grown onto 5 ml of PYEM broth overnight in a shaking incubator at 200 rpm at 30°C. The overnight culture (1 ml) was inoculated into 19 ml of fresh MSM broth containing MCs at a final concentration of 25 µg/ml, and incubated in a shaking incubator at 30^{o}C and 200 rpm for 7 days. The MC degradation products were analysed individually for each bacterial isolate. Every day for 7 days, an aliquot (1 ml) of sample was withdrawn and centrifuged at 12,000 rpm for 10 min. The MC concentration was then analyzed in the supernatant using the HPLC-UV detector as previously described (Section 3.5.6.1).

4.5.2 Preservation of bacterial culture

The bacterial isolates that had the ability to degrade MCs (designated NV-1, NV-2 and NV-3) were kept as a working stock as PYEM agar slants at 4°C and were also stored in the Mast Cryobank™ (Mast Diagnostics Co Ltd.) at -80^{o}C. For the cryopreservation of the isolates, a pure culture of the isolate was picked from PYEM agar (1-4 colonies) using an inoculation loop and a thick suspension was then made in the cryopreservative fluid provided by the manufacturer (Mast Diagnostics). The suspension was then transferred to tubes containing beads, provided by the manufacturer (Mast Diagnostics). The tubes were capped, and inverted briskly and as much cryopreservative fluid as possible was then withdrawn using a sterile pipette before storing at -80°C - storage beads remaining were coated with the bacterium.

4.5.3 Identification and characterization of the MC-degrading bacteria

4.5.3.1 Preliminary characterization of the bacteria

Gram stain, bacterial size, and oxidase tests were carried out using standard procedures according to Chan, Pelczar, and Krieg (1993). Colony size, morphology and colour were examined after 72 h incubation on PYEM agar at 30^{o}C.

4.5.3.2 Bacterial morphology under scanning and transmission electron microscopy

Cell morphology was investigated using electron microscopy as per standard procedures at Manawatu Microscopy and Imaging Centre, Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand.

4.5.3.3 Biochemical and nutritional characteristics

The characterization of gram-negative rods, oxidase-positive bacteria was achieved using the API 20 NE test kit (BioMerieux), according to the manufacturers instructions. The kit includes 8 conventional tests and 12 assimilation tests.

Each bacterial isolate (1-4 colonies) was inoculated into 0.85% NaCl and the turbidity was adjusted to 0.5 MacFarland standard (BioMerieux). For identification of nitrate oxidation (NO_3), indole (TRP) production, the anaerobic utilization of glucose (GLU), arginine (ADH), urea (URE), β-glucosidase (ESC) production, protease or gelatin (GEL) and β-galactosidase (PNPG), the inoculum was immediately distributed into the tubes provided.

For measurements of the utilization of glucose [GLU], arabinose [ARA], mannose [MNE], mannitol [MAN], N-acetyl-glucosamine [NAG], maltose [MAL], gluconate [GNT], caprate [CAP], adipate [ADI], malate [MLT], citrate [CIT] and phenyl-acetate [PAC], 200 µl of the remaining 0.85% NaCl plus AUX medium (BioMerieux) was distributed into the tubes and cupules. Sterile mineral oil was then added to the cupules of tests for the utilization of GLU, ADH and URE. The strips were incubated at 30^{o}C, and read after 48 h. Interpretation of readings from the API 20 NE identification kit are presented in Appendix 6 as per the manufacturers instructions.

Additional morphological, biochemical and nutritional assays were performed by New Zealand's Reference Laboratory at Environmental and Scientific Research (ESR), Wellington, New Zealand, including gram staining, cell morphology, colonies growth, colonies pigment, flagella stain, motility at 25^{o}C, growth on TSA (tryptic soy agar), growth on 5% sheep blood agar, growth on MacConkey, growth at 25^{o}, 37^{o} and 42^{o}C, catalase test, oxidase test, simmons citrate test, urea test, Christensens test, nitrate test, TSI test, gelatin hydrolysis, aesculin hydrolysis, lysine decarboxylase test, arginine dihydrolase test, ornithine decarboxylase test, basal decarboxylase test, and utilization of glucose, xylose, mannitol, lactose, sucrose, maltose and fructose.

4.5.3.4 16S rRNA sequencing

Sequencing of 16S rRNA was also carried out by ESR, Wellington, New Zealand. The sequencing protocol is presented in Appendix 7. DNA Sequencing was performed using an ABI PRISM BigDye terminator DNA sequencing kit and analyzed using a model 3730XL ABI DNA sequencer (Applied Biosystems). The obtained sequences were compared with those in the GenBank, EMBL, and DJB prokaryote databases using the default settings of the Fasta3 alignment program through the EBI server.

4.5.4 Optimum temperatures of bacterial growth

A single colony of the bacterium isolate NV-3 (the strongest degradative bacterium) was inoculated into 5 ml of PYEM broth and grown overnight in a shaking incubator at 200 rpm and 30°C. The overnight culture was inoculated (2% v/v) into 100 ml of PYEM broth in Erlenmeyer flasks and incubated in the shaking incubator at 200 rpm at constant temperatures of 20°, 25°, 30° and 35°C for 72 h. The growth of the bacteria for each temperature was followed every 6 h by optical density measurements at a wavelength of 600 nm (OD_{600}). In order to get accurate measurement using the spectrophotometer, it was necessary to dilute the bacterial culture with distilled water to the range of optical density 0.2-0.8.

4.5.5 Bacterial growth curve experiments

The temperature for optimum bacterial growth (30°C) identified in Section 4.5.4 was used to establish the growth curve of the bacterium isolate NV-3. The protocol followed was identical to that used in Section 4.5.4 except that bacterial growth, as measured by an OD_{600}, was followed every 2 h. Growth was also examined using the viable plate count method. For this technique, serial tenfold dilutions were made by initially diluting 1 ml of cell suspension (obtained every 2 h), with 9 ml of 0.1% (w/v) peptone water. This was considered to be the 10^{-1} dilution. Further dilutions were achieved by transferring 1 ml of each dilution into 9 ml of fresh peptone water. One hundred µl of each dilution was aseptically spread over the entire surface of PYEM agar and incubated at 30°C. The plates were quantified after

48 h incubation by counting colony-forming units (CFU), which ranged from 30–300 colonies per plate. The mean CFU number was obtained from triplicate cultures.

4.5.6 Effect of temperature, bacterial and MC concentration on MC degradation by the bacterium isolate NV-3

To obtain sufficient quantities of bacteria for the degradation experiments, the pure bacterial isolate NV-3 was cultured in a PYEM broth for 36 h (late exponential growth phase determined from the experiment outlined in Section 4.5.5) in a shaking incubator at 30^{o}C and 200 rpm. The bacterium was centrifuged at 12,000 rpm for 5 min (4^{o}C). The supernatant was decanted and the pellet was resuspended in 0.05 M phosphate buffer, pH 7.0. This washing process was repeated three times. The final pellet was resuspended in 5 ml of sterile MSM broth and this culture was used in Sections 4.5.6.1 to 4.5.6.3.

4.5.6.1 Temperature

The addition of sterile MSM broth to the bacterial culture adjusted the concentration of the stock culture to give an optical density of 1.0 (OD_{600} using wavelength at 600 nm), which translated to a bacterial concentration of approximately 1.0x10^{8} CFU/ml. To establish the effect of temperature on MC degradation, 9 ml of the bacterial culture was mixed with 1 ml of [Dha^{7}]MC-LR and MC-LR, which provided the sole carbon and nitrogen sources and yielded a final cyanotoxin concentration of 25 µg/ml. The experiment was carried out in triplicate at 6 different temperatures, 10^{o}, 15^{o}, 20^{o}, 25^{o}, 30^{o} and 35^{o}C, in a shaking incubator at 200 rpm. The biodegradation of MCs was monitored for each experiment over a period of 28 days. An aliquot (1 ml) of the bacterial/cyanotoxin mix was withdrawn after 0, 1, 3, 5, 7, 10, 14, 21 and 28 days of incubation and centrifuged at 12,000 rpm for 10 min. The MC concentration was determined in the supernatant using HPLC as described in Section 3.5.6.1.

4.5.6.2 Bacterial concentration

Using the optimum temperature for MC degradation derived from Section 4.5.6.1, experiments to determine the effect of bacterial concentration on MC

degradation were carried out at different bacterial concentrations with a fixed MC concentration of 25 µg/ml. Five bacterial concentrations were prepared, when OD_{600} = 0.1 (bacterial concentration is approximately $7.9x10^6$ CFU/ml), OD_{600} = 0.3 ($2.5x10^7$ CFU/ ml), OD_{600} = 0.5 ($4.9x10^7$ CFU/ml), OD_{600} = 1.0 ($1.0x10^8$ CFU/ml) and OD_{600} = 1.5 ($1.45x10^8$ CFU/ml), using the stock bacterial culture from 4.5.6 above, by adding sterile MSM broth until the required OD at 600 nm was obtained.

As described previously (Section 4.5.6.1), 9 ml of sample of each bacterial concentration was mixed with 1 ml of [Dha^7]MC-LR and MC-LR, giving a final cyanotoxin concentration of 25 µg/ml. The experiments were carried out in a shaking incubator at the optimum temperature (30°C) obtained from 4.5.6.1 and at 200 rpm. Progressive biodegradation of MCs was monitored over 3 days as described in Section 4.5.6.1.

4.5.6.3 MC concentration

The optimum temperature and bacterial concentration determined in Sections 4.5.6.1 and 4.5.6.2 were used in subsequent experiments to establish optimum MC concentration for bacterial degradation. Nine ml of the bacterial culture (as described in Section 4.5.6.1 with an OD_{600} = 1.0) was mixed with 1 ml of [Dha^7]MC-LR and MC-LR, yielding final MC concentrations of 1, 10, 25 and 50 µg/ml, and incubated in a shaking incubator at 30°C and 200 rpm. The progressive biodegradation of the MCs was monitored over 6 days as previously described (Section 4.5.6.1).

4.5.7 Detection of MC degradation by-products with LC/MS-MS

The degradation by-products obtained by [Dha^7]MC-LR (mainly) and MC-LR breakdown with the isolate NV-3 was examined. A single colony of the bacterial isolate was grown in 5 ml PYEM broth in a shaking incubator at 200 rpm and 30°C for 30 h. The culture (1 ml) was inoculated into 19 ml of MSM broth plus MCs at a final concentration of 25 µg/ml and incubated in a shaking incubator at 30°C and 200 rpm. Samples (1 ml) were taken every 6 h until 48 h, and the MC degradation by-products analyzed using LC/MS-MS at Cawthron Institute, Nelson, New Zealand. The protocol for determination of the by-products is described in Appendix 2.

4.5.8 Detection of genes that encode MC-degrading enzymes

The detection of genes for MC-degrading enzymes involved extraction and purification of DNA from NV-3 and NV-1, determination of genomic DNA purity and yield, amplification of fragments of the *mlr*A, *mlr*B, *mlr*C and *mlr*D genes by PCR and subsequent DNA sequencing of the PCR product.

4.5.8.1 Genomic DNA isolation and purification

The genomic DNA of the isolate NV-3 and NV-1 was isolated and purified using the wizard® genomic DNA purification kit (Promega), in accordance with the manufacturers instructions. The bacterium was cultured in PYEM broth with shaking at 200 rpm and 30°C for 36 h. Cells were pelleted by centrifugation at 16,000 *g* for 2 min and resuspended completely in 600 µl of nuclei lysis solution. The resuspended cells were incubated at 80°C for 5 min and then cooled to room temperature. This suspension mixed with 3 µl of RNAse solution, incubated at 37°C for 60 min and cooled to room temperature. The sample was then mixed with 200 µl of protein precipitation solution, incubated on ice for 5 min and centrifuged at 16,000 *g* for 3 min. The supernatant was transferred to a clean microcentrifuge tube containing 600 µl of room temperature isopropanol and gently mixed by inversion until the thread-like strands of DNA formed a visible mass. The DNA was then pelleted by centrifugation at 16,000 *g* for 2 min and resuspended in 600 µl of room-temperature 70% ethanol. The sample was centrifuged at 16,000 *g* for 2 min and the ethanol was carefully aspirated. The DNA pellet was then dried for 15 min, mixed with 100 µl of DNA rehydration solution and incubated overnight at 4°C. Purified DNA was stored at 4°C.

4.5.8.2 Determination of DNA purity and concentration

The purity and concentration of DNA were measured using a biophotometer (Eppendorf) according to the manufacture's protocol. The DNA was diluted 100-fold with ultra-pure DNAse/RNAse-free distilled water (Invitrogen) and the absorbance measured at wavelengths 260 nm (A_{260}) and 280 nm (A_{280}). DNA purity was determined by the A_{260}/A_{280} ratio and DNA concentration was recorded in units of µg/ml. The measurements were performed in triplicate.

4.5.8.3 Amplification of fragments of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes using PCR

All PCR reactions in this study were performed in a total volume of 20 µl in 0.2 ml PCR tubes. Negative controls of no DNA or no primer (ultra-pure distilled water in place of DNA or primers, respectively) were included in each experiment. PCR fragments of expected size for each gene examined were subsequently purified and then sequenced at the Allan Wilson Centre, Institute of Molecular BioSciences, Massey University at Palmerston North, New Zealand.

4.5.8.3.1 Oligonucleotide primers

The primers used for amplification are listed in Table 4.2. Lyophilized primers, from Invitrogen, Auckland, New Zealand were resuspended in ultra-pure DNAse/RNAse-free distilled water to a stock concentration of 100 pmol/µl. The primers were diluted to a final concentration of 5 pmol/µl for PCR reactions and 3.2 pmol/µl for sequencing reactions. All primers were stored at -20^{o}C.

Table 4.2 Oligonucleotide primers used for amplification of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes

Gene	Primer	Sequence (5′–3′)	Expected size of PCR products (bp)	Reference(s)
*mlr*A	MF (forward1)	GACCCGATGTTCAAGATAC	807	Saito et al., 2003
	MF2 (forward2)	TCGCCATTTATGTGATGGCT	453	
	MR (reverse)	CTCCTCCCACAAATCAGG		
*mlr*B	mlrBf (forward)	CGACGATGAGATACTGTCC	448	Ho et al., 2007
	mlrBr (reverse)	CGTGCGGACTACTGTTGG		
*mlr*C	mlrCf (forward)	TCCCCGAAACCGATTCTCCA	666	Ho et al., 2007
	mlrCr (reverse)	CCGGCTCACTGATCCAAGGCT		
*mlr*D	mlrDf (forward)	GCTGGCTGCGACGGAAATG	671	Ho et al., 2007
	mlrDr (reverse)	ACAGTGTTGCCGAGCTGCTCA		

4.5.8.3.2 Reagents and cycling conditions for amplification of the *mlr*A gene

Amplification of *mlr*A was based on Saito et al. (2003) and involved two sets of PCR reactions. The first reaction used the forward and reverse primers, MF and MR, and second reaction used the primers MF2 and MR. The product of the first PCR was used as template for the second PCR to ensure that fragments detected from the first PCR were *mlr*A homologues.

PCR reaction and components were modified from Saito et al. (2003) using a 1x PCR buffer (20mM Tris-HCl pH 8.3, 50mM KCl), 2.5mM $MgCl_2$, 250µM deoxyribonucleotide triphosphates (dNTPs), 1 pmol each primer, 1 U Amplitaq gold DNA polymerase and 1ng DNA template (or 1 µl of first PCR product), in a final reaction volume of 20 µl (the manufacturers are listed in Appendix 5), and performed in 0.2 ml thin-walled tubes using a Thermo Hybaid PCR system under the following conditions: initial denaturation step at 94°C for 10 min; 30 cycles of denaturation at 94°C for 20 seconds; annealing at 55°C for 10 seconds and extension at 72°C for 30 seconds; and 72°C for 10 min.

4.5.8.3.3 Reagents and cycling conditions for amplification of the *mlr*B, *mlr*C and *mlr*D genes

The primer pairs, mlrBf and mlrBr, mlrCf and mlrCr and mlrDf and mlrDr were used to detect the *mlr*B, *mlr*C and *mlr*D genes, respectively. The amplification reaction composition and conditions were the same as for the first PCR for amplification of *mlr*A described in Section 4.5.8.3.2.

4.5.8.3.4 Agarose gel electrophoresis of PCR products

Gel electrophoresis was used to visualize and purify the amplified PCR products. Horizontal gels of 1.5% agarose were made in 1X TAE buffer (Appendix 5). Five µl of each PCR product plus 1 µl of loading dye (Appendix 5) was electrophoresed at 100V for 1-2 h in 1X TAE buffer. Following electrophoresis, the gels were stained into ethidium bromide solution (Appendix 5) for 15-20 min and then destained in Milli-Q water. The bands on the gels were visualized on a UV transilluminator and photographed with Uvipro gel documentation system (Appendix 5). PCR product sizes on the gel were then determined by comparison with a 1 Kb plus DNA ladder.

4.5.8.3.5 Purification of PCR products

The PCR products were purified either directly from the PCR reaction (Section 4.5.8.3.2 and Section 4.5.8.3.3) or from the agarose gel (Section 4.5.8.3.4) using PCR clean up kit and wizard SV gel (Promega) respectively, according to the

manufacturers protocols. After visualization of the amplified products under the UV light PCR products of the expected sizes were excised using a clean scalpel blade and placed in a 1.5 ml microcentrifuge tube. The gel slice was melted at 60^{o}C and completely dissolved with membrane binding solution (10 µl of membrane binding solution per 10 mg of the gel). If the PCR products were purified directly from the PCR reaction, an equal volume of membrane binding solution was added to the PCR reaction. One SV minicolumn was placed in a collection tube for each dissolved gel slice or PCR product. The dissolved gel mixture or prepared PCR product was transferred to the SV minicolumn assembly and incubated for 1 min at room temperature. The SV minicolumn assembly was centrifuged at 16,000 *g* for 1 min, the liquid in the collection tube discarded, and the SV minicolumn reinserted into the collection tube. The column was washed 2 times with 700 µl membrane wash solution by centrifuging at 16,000 *g* for 1 min. The liquid in the collection tube was discarded and the SV minicolumn assembly was transferred to a clean microcentrifuge tube. Fifty µl of nuclease-free water was applied to the column, incubated at room temperature for 1 min and then centrifuged at 16,000 *g* for 1 min. The SV minicolumn assembly was discarded and the microcentrifuge tube containing the eluted DNA was stored at 4°C or -20°C. The concentration and purity of the amplified PCR product was determined using the method described in Section 4.5.8.2.

4.5.8.3.6 DNA sequencing

All PCR products were sent to the Allan Wilson Centre, Institute of Molecular BioSciences, Massey University at Palmerston North, New Zealand, for sequencing. An automatic sequencing reaction was performed using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Perkin-Elmer) with 2 ng/100 bp PCR product, 3.2 pmol of each primer and 8 µl of terminator ready reaction mix in a total volume of 20 µl. Products were sequenced using an ABI Prism 377 automatic sequencer (Perkin-Elmer).

4.5.8.4 Bioinformatic analysis

DNA sequence data were displayed and edited using the Chromas software programs version 1.45 (Retrieved December 2, 2008, from http://www.technelysium.

com.au/chromas14x.html). The forward and reverse primer sequences were aligned to obtain the expected DNA (nucleotide) sequences of each gene using the BLAST2 SEQUENCES program (Tatusova & Madden, 1999) at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The DNA sequence comparison was performed using the NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul, Gish, Miller, Myers, & Lipman, 1990). For comparative sequence analysis, multiple sequence alignments were performed using the Molecular Evolutionary Genetics Analysis (MEGA) software program version 4.0 (Retrieved December 10, 2008, from http://www.megasoftware.net/) (Tamura, Dudley, Nei, & Kumar, 2007).

For further characterization of the DNA sequences, the putative amino acid sequence for each gene was obtained by using a translation program at the PROSITE site (http://ca.expasy.org/tools/dna.html). The translated amino acid (polypeptide) sequence was searched against a wide range of protein databases to investigate protein homology, protein family or domain and protein patterns or motifs. For example, Pfam was used to scan amino acid sequences against collections of protein families (http://pfam.sanger.ac.uk/), PROSITE was used to scan amino acid sequences against PROSITE or to scan protein patterns against the UniProt knowledgebase at the Swiss Institute of Bioinformatics (http://www.expasy.ch/prosite). InterProScan is an integrated search that combines PROSITE, Pfam and PRINTS databases at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/InterProScan/), and BLASTP was used to search protein homology in GeneBank databases (htpp://www.ncbi.nlm.nih.gov/).

4.6 Results

4.6.1 Isolation of MC-degrading bacteria

To obtain bacteria that were capable of degrading MCs, water samples obtained from Lake Horowhenua, Lake Rotoehu, Lake Rotoiti and Lake Rotorua were plated on MSM broth with [Dha^7]MC-LR and MC-LR as the sole carbon and nitrogen source. A total of 27 isolates of different types, shapes and colors of colony were selected (Table 4.3), however, only three isolates that were obtained from Lake Rotoiti—designated NV-1, NV-2 and NV-3—were truly able to breakdown the MCs.

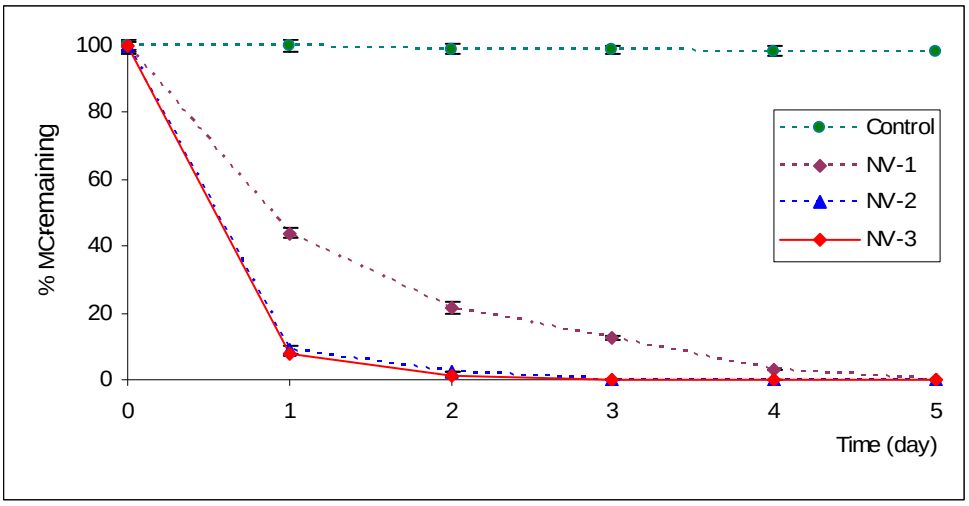
Table 4.3 Bacterial isolates from Lake Horowhenua, Lake Rotoehu, Lake Rotoiti and Lake Rotorua.

NO.	Isolate	Source	Colony pigment	Colony morphology	Colony size (48 h)
1	AS-1	Lake Horowhenua	White	circular, raised, entire	3.0 mm
2	AS-2	Lake Horowhenua	White	circular, convex, entire	2.5 mm
3	AS-3	Lake Horowhenua	White	irregular, raised, undulate	3.0 mm
4	AS-4	Lake Horowhenua	Yellow	circular, convex, entire	1.5 mm
5	AS-5	Lake Horowhenua	Yellow	circular, raised, entire	4.0 mm
6	AS-6	Lake Horowhenua	Yellow	irregular, convex, undulate	3.0 mm
7	AS-7	Lake Horowhenua	Red	circular, convex, entire	3.5 mm
8	AS-8	Lake Horowhenua	Red	circular, raised, entire	3.0 mm
9	KT-1	Lake Rotoehu	White	circular, convex, entire	2.5 mm
10	KT-2	Lake Rotoehu	White	circular, raised, entire	1.5 mm
11	KT-3	Lake Rotoehu	Yellow	circular, raised, entire	3.5 mm
12	KT-4	Lake Rotoehu	Yellow	irregular, convex, undulate	4.0 mm
13	KT-5	Lake Rotoehu	Yellow	circular, convex, entire	3.5 mm
14	KT-6	Lake Rotoehu	Red	circular, raised, entire	2.5 mm
15	KT-7	Lake Rotoehu	Red	circular, convex, entire	3.0 mm
16	NV-1	Lake Rotoiti	Yellow	circular, convex, entire	1.0 mm
17	NV-2	Lake Rotoiti	Yellow	circular, convex, entire	1.5 mm
18	NV-3	Lake Rotoiti	Yellow	circular, convex, entire	1.5 mm
19	NV-4	Lake Rotoiti	White	circular, raised, entire	2.5 mm
20	NV-5	Lake Rotoiti	White	irregular, raised, undulate	3.0 mm
21	NV-6	Lake Rotoiti	Red	circular, convex, entire	3.0 mm
22	TS-1	Lake Rotorua	Yellow	circular, convex, entire	3.0 mm
23	TS-2	Lake Rotorua	Yellow	irregular, raised, undulate	2.5 mm
24	TS-3	Lake Rotorua	White	circular, raised, entire	1.5 mm
25	TS-4	Lake Rotorua	White	circular, convex, entire	4.0 mm
26	TS-5	Lake Rotorua	Red	circular, raised, entire	2.5 mm
27	TS-6	Lake Rotorua	Red	circular, convex, entire	3.0 mm

4.6.2 MC-degradation by isolated bacteria

The isolates NV-2 and NV-3 completely degraded [Dha^7]MC-LR and MC-LR within 3 days, whereas complete degradation was achieved by NV-1 within 5 days (Figure 4.2). Comparison of MC-degradation by isolates NV-2 and NV-3 revealed that NV-3 had the higher rate of toxin degradation. The isolates with the fastest and slowest degradation rates, NV-3 (8.33 µg/10^8 CFU/day) and NV-1 (5 µg/ml/day) were selected for further study. This allowed a comparison of performance of the slowest and fastest MC-degrading bacteria.

Figure 4.2 Degradation of [Dha^7]MC-LR and MC-LR over a 5-day period by the isolates NV-1, NV-2 and NV-3. Control represents culture medium without bacteria.



4.6.3 Characterization of MC-degrading bacteria

4.6.3.1 Characterization of bacterial isolate NV-3

The bacterial isolate NV-3 had the greatest ability to degrade the MCs at a concentration of 25 mg/l, degrading the toxins completely in 3 days. It is an aerobic, gram-negative, slow-growing bacterium and formed pale yellow-coloured colonies aproximately 1 mm in diameter after 48-h incubation at 30^{o}C on PYEM. The colonies were particularly adhesive to the agar medium and could not be easily scraped off. The bacterial cell was rod-shaped, 1.0–1.2 µm in length and 0.5-0.7 µm in width, and possessed bipolar flagella (Figure 4.3 and Figure 4.4). The results of routine biochemical tests and microbial techniques performed by ESR and using the API 20 NE system are shown in Table 4.4 and 4.5, respectively.

Figure 4.3 Transmission electron micrographs of the bacterium isolate NV-3 showing flagella and pilli

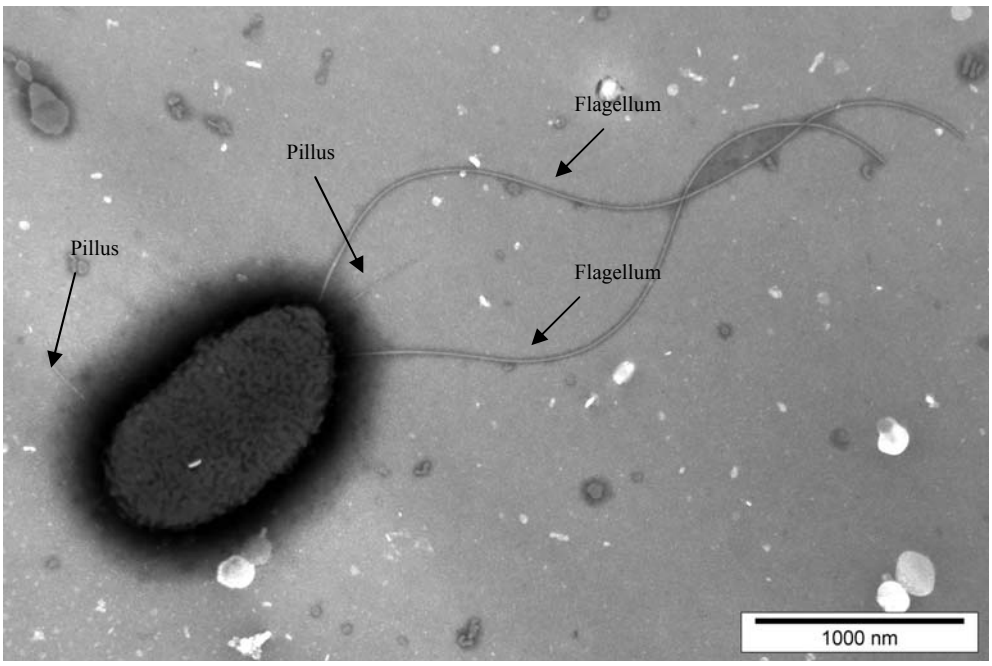


Figure 4.4 Scanning electron micrograph of the bacterium isolate NV-3

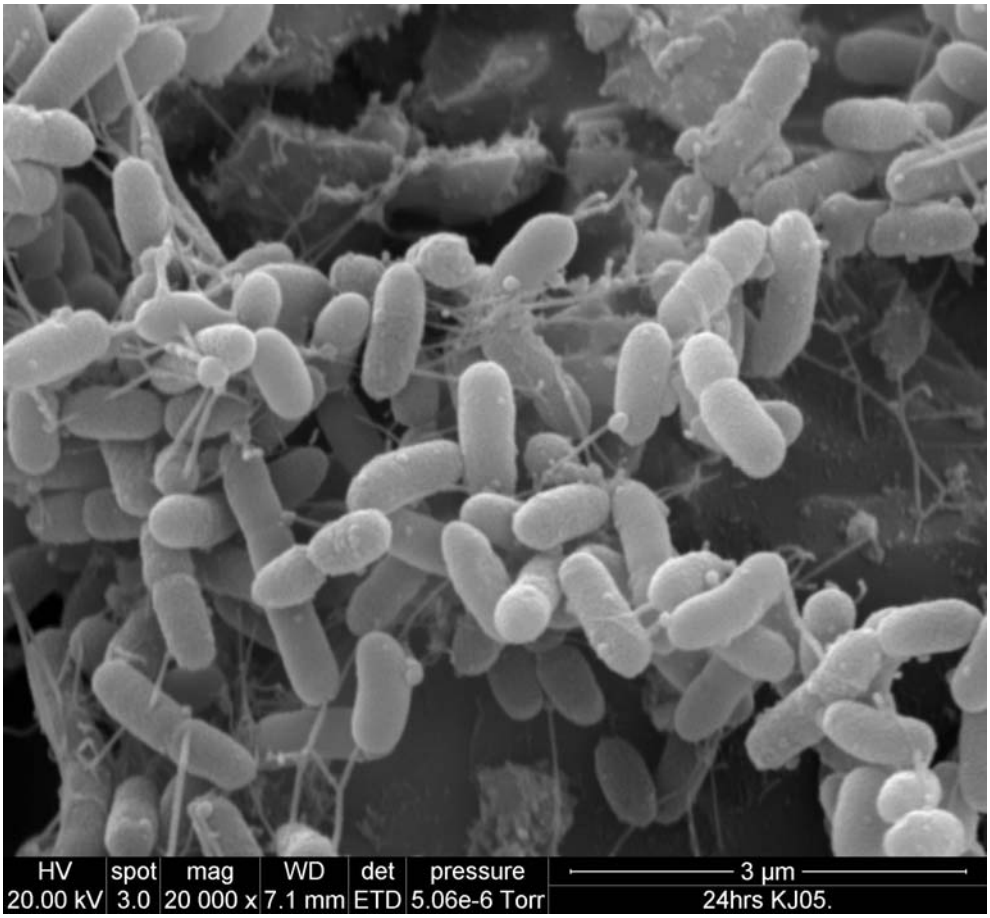


Table 4.4 Morphological and biochemical characteristics of the isolates NV-3 and NV-1 from ESR

Morphological and biochemical characteristics	NV-3	NV-1
Gram staining	Gram negative	Gram negative
Cell morphology	Thin, rods, quite short	Thin, rods, filament
Colonies growth	Slow growing	Slow growing
Colonies pigment	Yellow	Yellow
Flagella stain	Bi-polar at lateral	None seen
Motility at 25° C	Positive	Negative
Growth on TSA	Positive	Positive
Growth on 5% sheep blood agar	Positive	Positive
Growth on MacConkey	Negative	Negative
Catalase test	Positive	Positive
Oxidase test	Positive	Positive
Simmons citrate	Negative	Negative
Urea, Christensens	Negative	Negative
Nitrate	Negative	Negative
TSI	Negative	Negative
Gelatin hydrolysis	Negative	Negative
Growth at 25° C	Positive	Positive
Growth at 37° C	Positive, reluctant	Positive, reluctant
Growth at 42° C	Negative	Negative
Aesculin hydrolysis	Negative	Negative
Lysine decarboxylase	Negative	Negative
Arginine dihydrolase	Negative	Negative
Ornithine decarboxylase	Negative	Negative
Basal decarxylase	Negative	Negative
Utilization of;		
Glucose	Negative	Negative
Xylose	Negative	Negative
Mannitol	Negative	Negative
Lactose	Negative	Negative
Sucrose	Negative	Negative
Maltose	Negative	Negative
Fructose	Negative	Negative

The isolate NV-3 tested negative in all tests performed at the ESR, except in the oxidase and catalase reactions (Table 4.4). The isolate could not utilize glucose, xylose, mannitol, lactose, sucrose or maltose as a carbon source. It grew on TSA and sheep blood agar but not on MacConkey agar. The temperature range permissive for growth was 10–37°C and the optimum temperature for growth was between 25–30°C. The growth was reluctant at 37°C with no growth observed at 45°C.

The API 20 NE database system revealed positive results for assimilation of L-arabinose and adipate only (Table 4.5). On the basis of these findings, the isolate NV-3 closely matched the profile of *Methylobacterium mesophilicum*, with 96.0% similarity between their biochemical and morphological characteristics. The NV-3 isolate could not assimilate glucose, D-mannose, D-maltose, N-acetyl-glucosamine, D-maltose, potassium gluconate, caproate, malate, citrate or phenylacetate. It also exhibited negative reactions for all other phenotypic tests: reduction of nitrate, indole production, glucose fermentation, hydrolysis of aesculin, hydrolysis of gelatin, arginine dihydrolase, urease and *β*-galactosidase activity.

Table 4.5 Biochemical characteristics of the bacterium strain NV-3 and NV-1 using API 20 NE

Biochemical tests from API 20 NE kit	**Results of NV-3**	**Results of NV-1**
Reduction of nitrate	Negative	Negative
Acid from glucose	Negative	Negative
Arginine dihydrolase	Negative	Negative
Urease	Negative	Negative
Hydrolysis of aesculin	Negative	Negative
Hydrolysis of gelatin	Negative	Negative
pNP-b-d-galactopyranoside	Negative	Negative
Utilization of:	Negative	Negative
Glucose	Negative	Negative
L-Arabinose	**Positive**	**Positive**
Mannose	Negative	Negative
Mannitol	Negative	Negative
N-Acetyl-glucosamine	Negative	Negative
Maltose	Negative	Negative
Potasium gluconate	Negative	Negative
Caprate	Negative	Negative
Adipate	**Positive**	**Positive**
Malate	Negative	Negative
Citrate	Negative	Negative
Tetramethyl-p-phenylene diamine	Negative	Negative

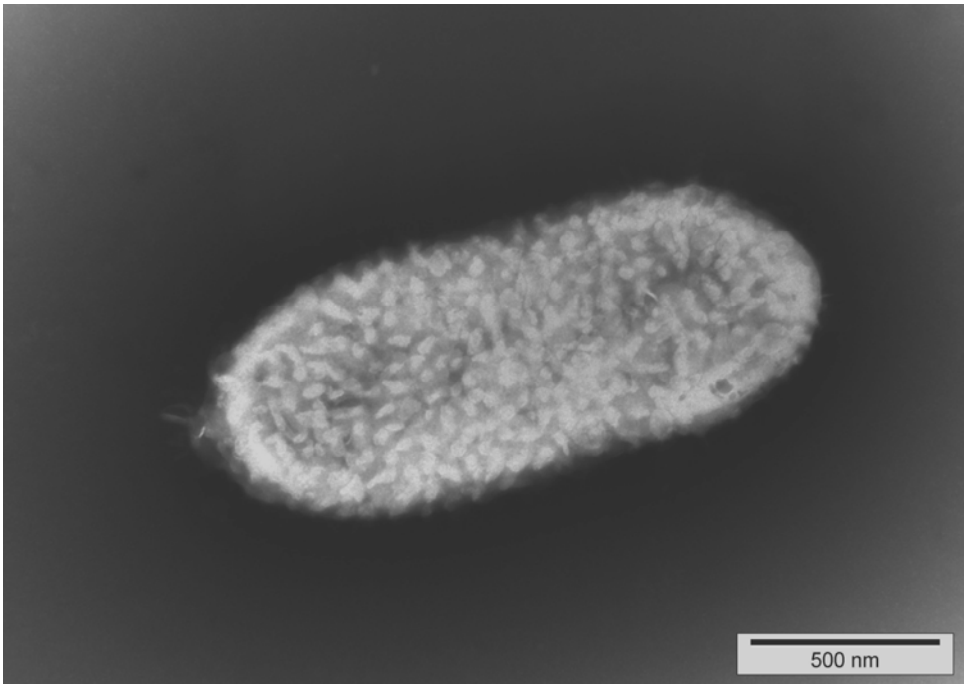
The 16S rRNA sequence of the isolate NV-3 was determined and compared with the GenBank, EMBL and DJB prokaryote databases. The databases revealed that the 16S rRNA sequences of the bacterium resembled the sequence of *Sphingomonas* strain MD-1 (AB110635), with 100% sequence homology for 1436 continuous nucleotides (Appendix 8). The *Sphingomonas* strain MD-1, a MC-degrading bacterium isolated from Lake Kasumigaura, Japan, in 1999 (Saitou et al., 2003), is

classified as a *Sphingomonas* sp. and exhibits 98.5% homology with the 16S rRNA sequence of *Sphingomonas stygia* (AB025013). The isolate NV-3 was, therefore, at this stage tentatively classified as a *Sphingomonas* species, indistinguishable from the *Spingomonas* strain MD-1 and referred to as *Sphingomonas* isolate NV-3.

4.6.3.2 Characterization of bacterial isolate NV-1

Figure 4.5 shows the bacterium isolate NV-1 by transmission electron microscopy. The isolate NV-1 with the weakest ability to degrade MCs is almost identical to the isolate NV-3 in terms of bacterial identification, with the exception of a few characteristics (Table 4.4 and Table 4.5). As for NV-3, analyses, using the API 20 NE database system also identified the isolate NV-1 as *Methylobacterium mesophilicum*, with 96.0% similarity. The growth of isolate NV-1 was slower compared with the isolate NV-3. A colony size of 1 mm in diameter was reached by isolate NV-1 after 72 h incubation at 30^{o}C on PYEM whereas a similar colony size was reached by isolate NV-3 within just 48 h. Furthermore, the bacterial cell of isolate NV-1 contained no flagellum (Figure 4.5) whereas that of NV-3 possessed bipolar flagella.

Figure 4.5 Transmission electron micrograph of the bacterium isolate NV-1

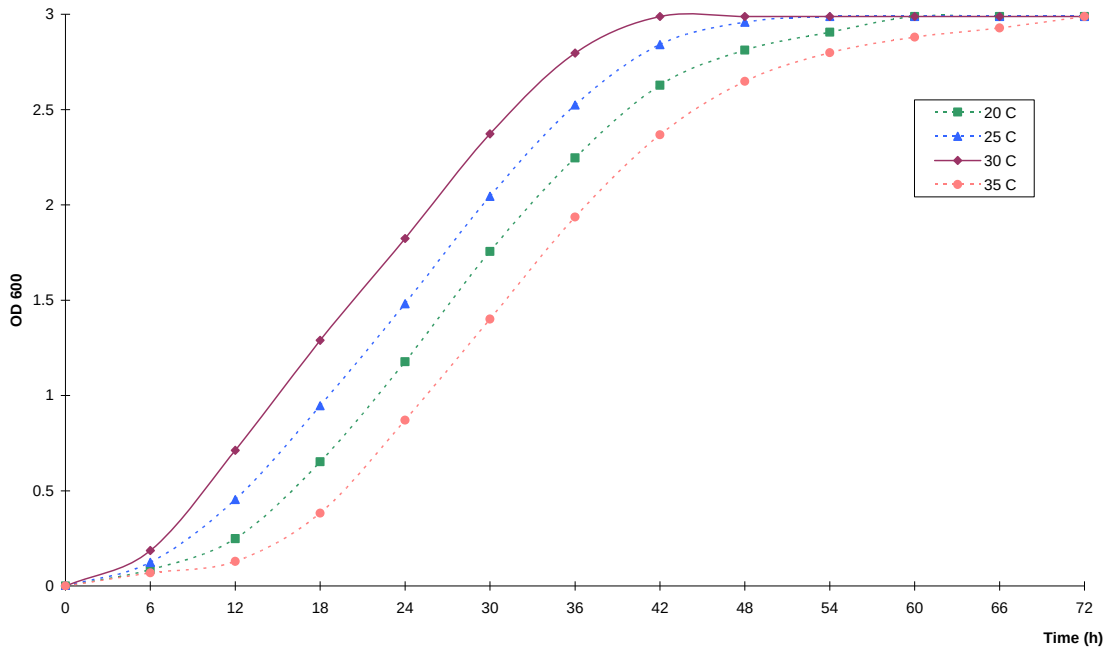


Like NV-3, however, the 16S rRNA sequence of the isolate NV-1 also resembled the sequence of *Sphingomonas* strain MD-1 (AB110635), with 100% sequence homology for 1437 continuous nucleotides (Appendix 9). The NV-1 isolate was, therefore, tentatively designated as a *Sphingomonas* isolate NV-1.

4.6.4 Optimum temperatures for NV-3 growth

As demonstrated above, the isolate NV-3 had the greatest ability to breakdown MCs, therefore, this isolate was selected for further study. Growth of the NV-3 isolate was noted to increase immediately after incubation in PYEM medium in all temperatures tested, however, the growth rates of the bacterium varied over the range of temperatures tested (Figure 4.6). Bacterial growth was low at 20°C and gradually increased with increasing temperatures up to 30°C. At 35°C NV-3 growth decreased slightly, and maximum growth was, therefore, found to occur at 30°C. The late exponential phase of the bacterial growth reached its maximum value after 42 h at 30°C, while this stage was reached after 48 h at 25°C, 60 h at 20°C or 72 h at 35°C (Figure 4.6).

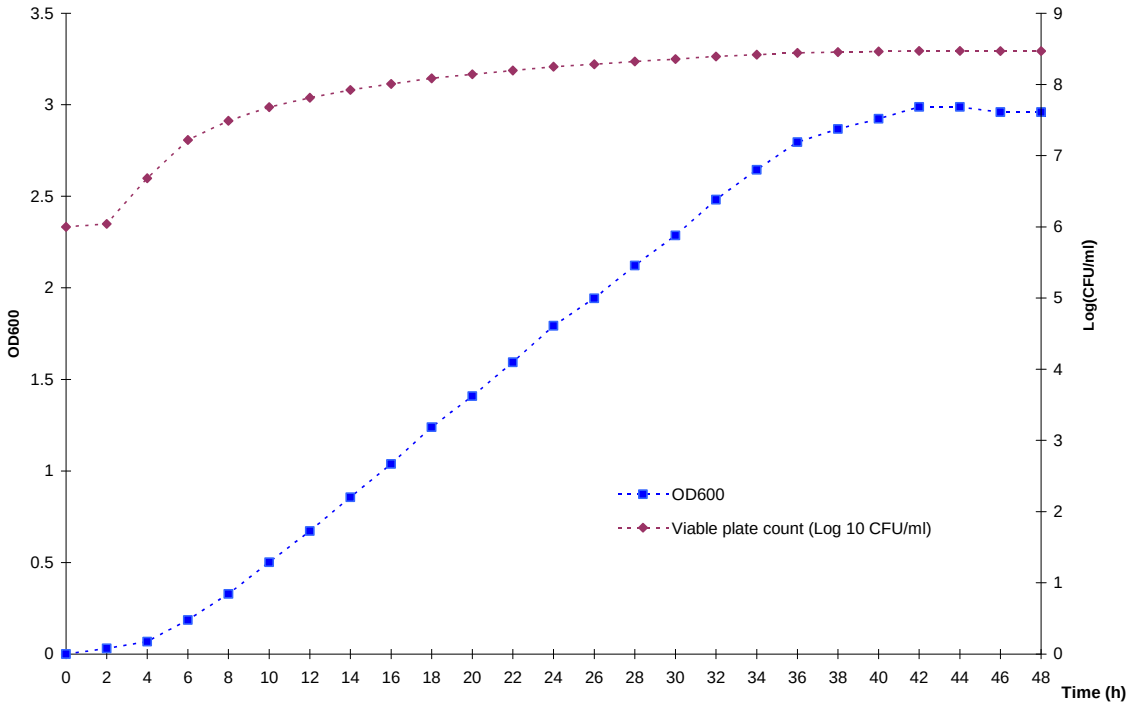
Figure 4.6. Growth curves of the bacterial isolate NV-3 at varying temperatures



4.6.5 Bacterial growth curve assays of the bacterial isolate NV-3

Figure 4.7 shows the bacterial growth curves of NV-3 measured by OD_{600} and viable plate count (log 10 CFU/ml). Changes in OD_{600} generated the classic s-shaped growth curve, whereas that of viable plate count (log 10 CFU/ml) did not provide the typical pattern. Therefore, change in OD_{600} was used to describe the bacterial growth curve. At 30°C the OD_{600} of NV-3 slowly increased over the first 6 h of the assay, corresponding to the lag phase of growth (Figure 4.7). From 6 h to 40 h, the OD_{600} increased exponentially corresponding to the exponential or log phase of growth, and from 42 h to 48 h, the OD_{600} remained relatively constant, indicative of the stationary phase.

Figure 4.7 Demonstrates bacterial growth curve from absorbance measurements (OD_{600}) and viable Plate count (log10 CFU/ml).



4.6.6 Effect of temperature, bacterial and MC concentrations on degradative activity of the isolate NV-3

The effect of temperature on the ability of NV-3 ($1.0x10^8$ CFU/ml) to degrade 25 µg/ml MCs was investigated. MC degradation by NV-3 began on day 1 under all

temperatures tested (Figure 4.8), however, the rate of MC degradation varied with temperature. The degradation rate was lowest at 10°C, and steadily increased with increasing temperatures to 35°C (0.89, 3.57, 5.00, 8.30, 8.33 and 8.30 µg/10^8 CFU/day, respectively). Microcystin concentrations rapidly decreased at temperatures from 20° to 35°C, with complete degradation occuring within 5 days (Figure 4.8). However, at these temperatures a noticeable drop off in degradation rate occurred after the initial rapid phase. The highest degradation rate was reached with temperatures of 30°C, however the rate of degradation during the initial rapid phase was similar between temperatures of 25°, 30° and 35°C and was calculated to be approximately 8.30 µg/10^8 CFU/day. These experiments demonstrated that the optimum temperature for biodegradation was 30°C.

Figure 4.8 Biodegradation of [Dha^7]MC-LR and MC-LR with bacterial isolate NV-3 at temperatures of 10°, 15°, 20°, 25°, 30°, and 35°C

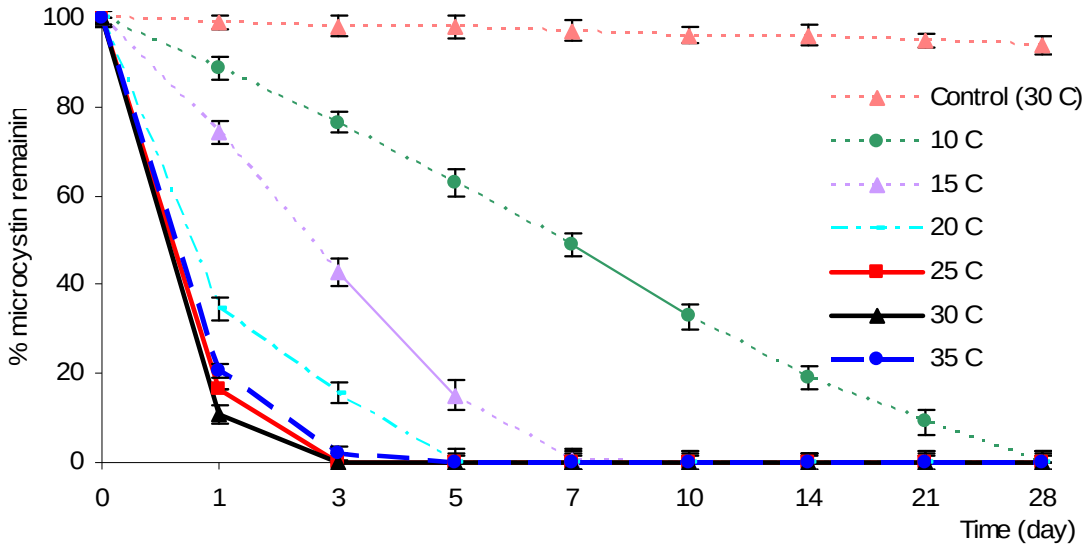
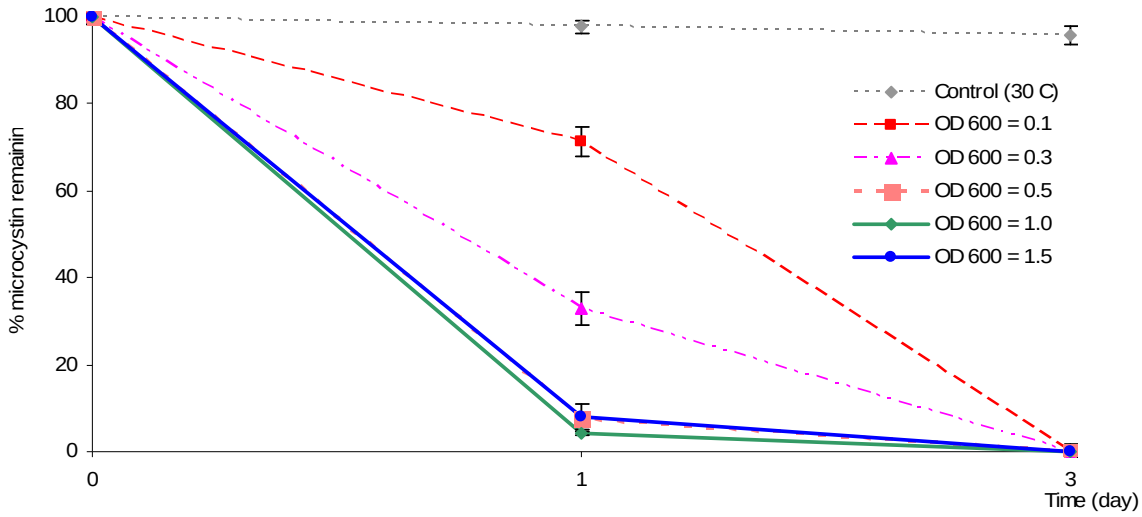


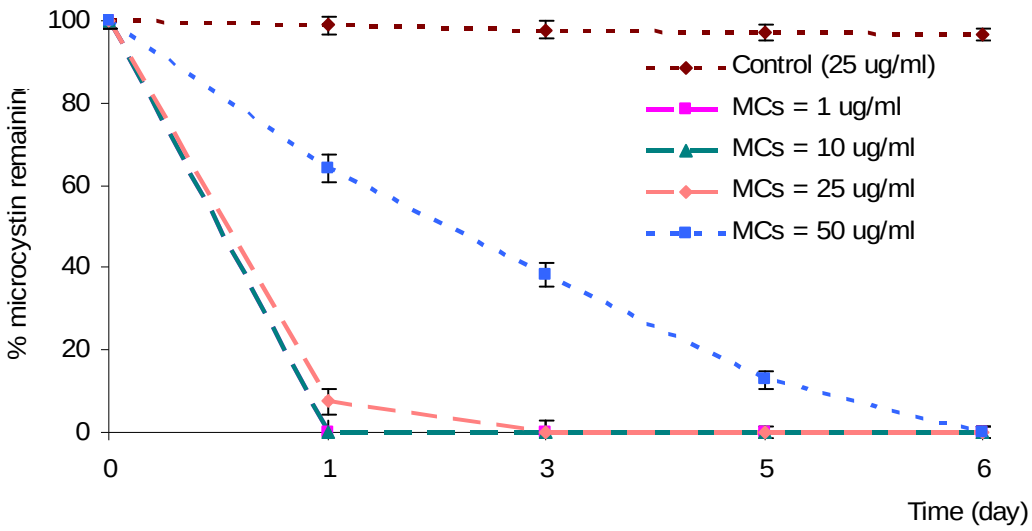
Figure 4.9 Varying rates of [Dha^7]MC-LR and MC-LR biodegradation with varying bacterial concentrations. Bacterial concentrations were 7.9x10^6 CFU/ml (or using optical density of bacteria at 600 nm = 0.1), 2.5x10^7 CFU/ml (OD_{600} = 0.3), 4.9x10^7 CFU/ml (OD_{600} = 0.5), 1.0x10^8 CFU/ml (OD_{600} = 1.0) and 1.45x10^8 CFU/ml (OD_{600} = 1.5)



The optimum concentration of NV-3 for degradation of 25 µg/ml MCs was then investigated at 30^{o}C (being the optimum temperature for MC degradation by NV-3). Bacterial concentrations of 7.9x10^6, 2.5x10^7, 4.9x10^7, 1.0x10^8 and 1.45x10^8 CFU/ml, completely degraded the MCs within 3 days (Figure 4.9). However; the degradation rate was slow with the lowest bacterial concentrations (7.9x10^6 and 2.5x10^7 CFU/ml). After one day of incubation with 7.9x10^6 and 2.5x10^7 CFU/ml NV-3, the amount of [Dha^7]MC-LR remaining was 80% and 40%, respectively. By contrast, after incubation with the higher NV-3 concentrations of 4.9x10^7, 1.0x10^8 and 1.45x10^8 CFU/ml, only about 5-8% of [Dha^7]MC-LR remained. The MC degradation rate in general increased with increasing bacterial concentration. At bacterial concentrations of 4.9x10^7, 1.0x10^8 and 1.45x10^8 CFU/ml, the degradation rates were not significantly different with the same degradation rate of 8.33 µg/10^8 CFU/day. However; the degradation rate actually decreased slightly at the highest bacterial concentration of 1.45x10^8 CFU/ml. To ensure that the bacterial density was adequate for degradation processes, a bacterial concentration of 1.0x10^8 CFU/ml was chosen for further studies.

The effect of variation in MC concentration on biological degradation was also investigated. At low concentrations (1 and 10 µg/ml), the toxins were entirely degraded in one day, whereas at higher concentrations of toxins (25 and 50 µg/ml) degradation took significantly longer, reaching undetectable levels by day 3 and 6, respectively (Figure 4.10). Nevertheless, the rate of degradation for concentrations of 25 and 50 µg/ml were equal at 8.33 µg/10^8 CFU/day. Therefore it was decided to carry out further MC degradation experiments using 25 µg/ml.

Figure 4.10 [Dha^7]MC-LR and MC-LR biodegradation at varying MC concentrations (1, 10, 25 and 50 µg/ml)

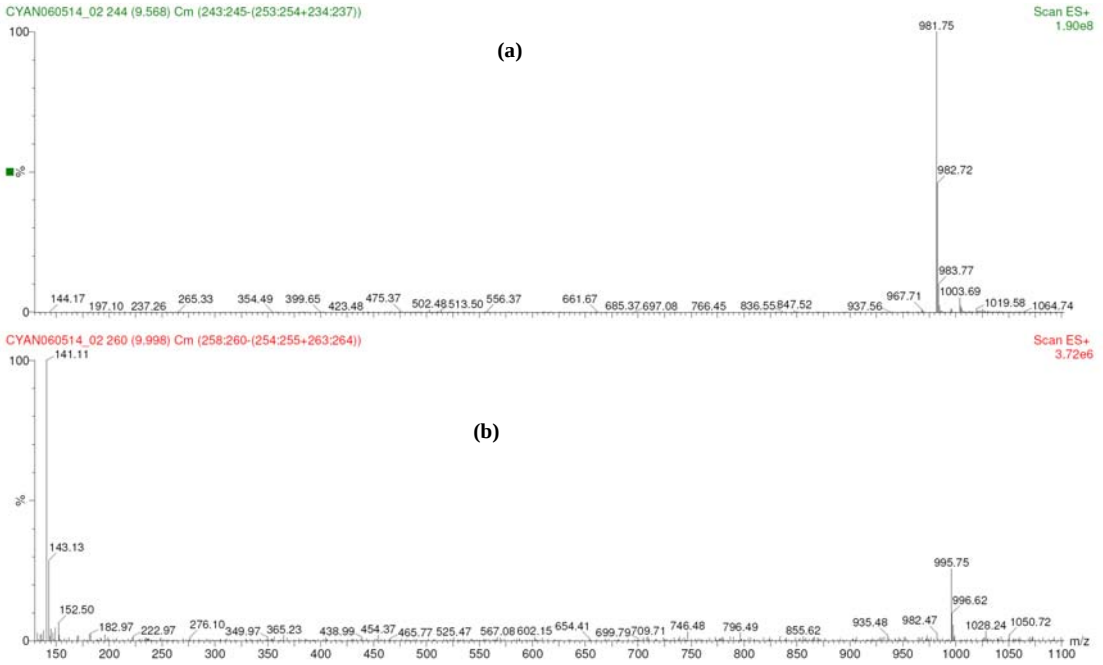


4.6.7 Detection of MC-degraded by-products from the isolate NV-3

The biodegradation of MCs was induced using a mixture of [Dha^7]MC-LR and MC-LR (at final concentration of 25 µg/ml) with cell suspensions of the isolate NV-3 (concentration of 1.0x10^8 CFU/ml). Upon incubation of the MCs with NV-3, the toxin concentration began to decline and two peaks, referred to as by-products A and B, were detected. The peaks corresponding to the by-products increased gradually while the peaks corresponding to the parent toxins decreased. The by-products from microbial catabolism were further analyzed using LC/MS-MS.

The $[M+H]^+$ observed at *m/z* 981.75 and 995.75 confirmed [Dha^7]MC-LR and MC-LR were present in the samples (Figure 4.11). [Dha^7]MC-LR, the major source of the biodegradation, was 14 mass units less than MC-LR due to the loss of a methyl group at Mdha. Therefore, [Dha^7]MC-LR possesses Dha (dehydroalanine) instead of Mdha in the structure.

Figure 4.11 MS/MS spectrum of [Dha^7]MC-LR at *m/z* 981.75 (a), and MC-LR at *m/z* 995.75 (b) in ESI^+ with parent ion spectrum for MS-MS channels set up



The MS^2 fragmentation of [Dha^7]MC-LR is shown in Figure 4.12 and the predicted fragment ion observed in the mass spectra of the toxin is shown in Table 4.6. The *m/z* 981.75 (molecular weight of [Dha^7]MC-LR) and *m/z* 135.14 (cleavage of the Adda residue in [Dha^7]MC-LR) confirmed that the toxin is [Dha^7]MC-LR.

Figure 4.12 MS/MS spectrum of [Dha^7]MC-LR at *m/z* 981.55 in ESI^+ with daughter ion spectrum for MS-MS channels set up

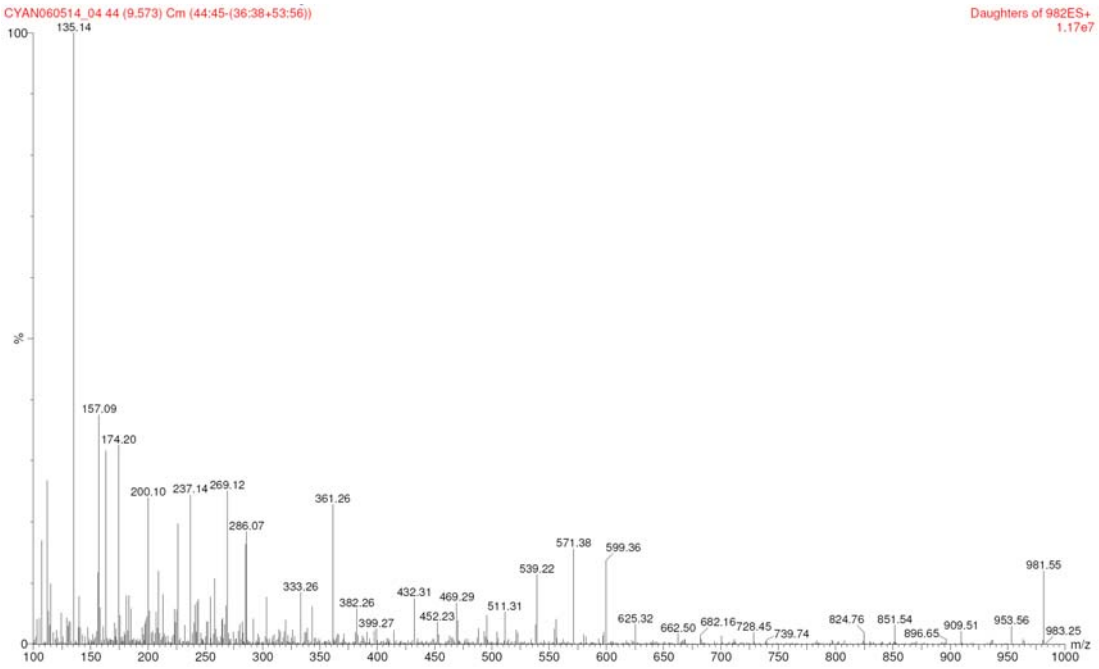
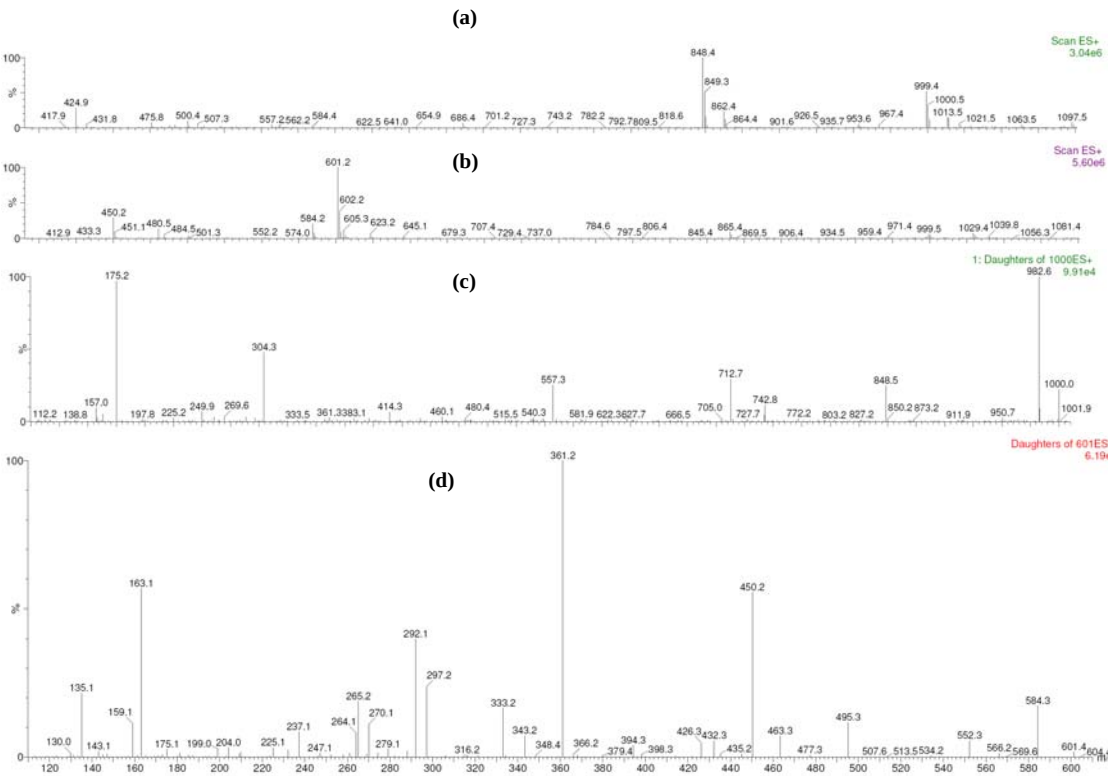


Table 4.6 Predicted fragment ion observed in mass spectra of [Dha^7]MC-LR

m/z	Identity	*m/z*	Identity
130	MeAsp +H	539	Dha Ala Leu MeAsp Arg +H
135	$PhCH_2CHOMe$	599	Arg Adda Glu +H
141	Dha Ala +H	668	Glu Dha Ala Leu MeAsp Arg +H
157	Arg +H	668	Arg Adda Glu Dha +H
199	Glu Dha +H	728	MeAsp Arg Adda Glu +H
270	Glu Dha Ala +H	825	Adda Glu Dha Ala Leu MeAsp +H
286	MeAsp Arg +H	852	Arg Adda Glu Dha Ala Leu +H
314	Adda +H	852	Dha Ala Leu MeAsp Arg Adda +H
361	$C_{11}H_{14}O$ + Glu Dha +H	910	Leu MeAsp Arg Adda Glu Dha +H
432	$C_{11}H_{14}O$ + Glu Dha Ala +H	912	Ala Leu MeAsp Arg Adda Glu +H
470	Arg Adda +H	981	M +H (cyclo Ala Leu MeAsp Arg Adda Glu Dha)
470	Ala Leu MeAsp Arg +H		

Two components at *m/z* 1013 and 999 that gave strong in-source fragments at *m/z* 862 and 848 were detected from by-product A (Figure 4.13a). The weaker *m/z* 1013 was analogous to the linearized MC-LR previously identified after MC biodegradation by *Sphingomonas* sp. ACM-3962 (Bourne et al., 1996) while the *m/z* 999 was a fragment of the linearized peptides of [Dha^7]MC-LR (NH_2-Adda-D-Glu-Dha-D-Ala-L-Leu-D-MeAsp-L-Arg-OH). The ion spectra of the prominent ions at *m/z* 862 and 848, which were from the loss of NH_2 and $PhCH_2CHOMe$ from the linearized peptides of MC-LR and [Dha^7]MC-LR, confirmed that they were related to the ring opening of MCs (the same fragment ions).

Figure 4.13 MS/MS spectrum in ESI^+ with parent scan and daughter ion spectrum of the degradation product A at *m/z* 1000 (a and c) and the degradation product B at *m/z* 601 (b and d)



The biodegradation by-product B revealed a base peak at *m/z* 601 (Figure 4.13b). The $[M+H]^+$ ion 601 is the tetrapeptide of [Dha^7]MC-LR (NH_2-Adda-D-Glu-Dha-D-Ala-OH), 14 mass units less than the tetrapeptide of MC-LR microbial degradation reported by Bourne et al. (1996). The by-product B also provided

fragment ion peaks at *m/z* 450, 552 and 584 (Figure 4.13b), related to the tetrapeptide. However; by-product B from the MC-LR degradation, which would be expected at *m/z* 615, was undetectable in this experiment.

The MS-MS fragmentation of the protonated molecules at *m/z* 999 and 601 was further carried out to validate the proposed structures of the by-products A and B. The MS^2 experiment performed by fragmenting *m/z* 999 exhibited a base peak at *m/z* 175 and a strong peak at *m/z* 982 (Figure 4.13c). The *m/z* 175 and *m/z* 982 peaks were indicative of a linear peptide with a C-terminal arginine, and facile loss of the NH_3 from the linearized peptides, respectively. In addition, a series of ions at *m/z* 304, 540, 557, 712 and 742 also confirmed that arginine was in the C-terminal position while, ions at *m/z* 361, 712 and 742 revealed that Adda was in the N-terminal position of the linearized peptides (Figure 4.13c and Table 4.7). The ions at *m/z* 383, 540, 557, 712 and 742, all containing the residues Dha-Ala-Leu-MeAsp, strongly suggested that these residues were connected to each other in the centre part of the molecule.

Table 4.7 Predicted fragment ion observed in mass spectra of the by-product A

m/z	Identity
135	$PhCH_2CHOMe$
157	Arg +H
175	Arg OH + 2H
304	Masp Arg OH + 2H
361	$C_{11}H_{14}O$ + Glu Dha +H
383	Dha Ala Leu MeAsp +H
540	Dha Ala Leu MeAsp Arg +2H
557	Dha Ala Leu Masp Arg OH + 2H
712	CO Glu Dha Ala Leu Masp Arg OH - 2H
742	CH_3CHCO Glu Dha Ala Leu Masp Arg OH + 2H
848	M + H - NH_2 - PhCH2CHOMe
982	M + H - NH_3
999	M + H (Adda Glu Dha Ala Leu Masp Arg OH + H)

The MS/MS spectrum of *m/z* 601 produced a base peak at *m/z* 361, which was identified to be $C_{11}H_{14}O$-Glu-Dha +H (Figure 4.13d). Several ions also resulted from Adda fragmentation (Table 4.8). For example, *m/z* 135 ($PhCH_2CHOMe$) was generated by cleavage of the C8–C9 bond of the Adda residue and the ion series (*m/z* 163, 292 and 361) contains a unit of $C_{11}H_{14}O$ (163 Da) derived from the Adda moiety by loss of the 135 unit and C-N bond cleavage. The loss of MeOH from the Adda residue was also found at *m/z* 463 and 552 and the loss of ammonia was observed at *m/z* 297, 426, 463, 495 and 552 (Table 4.8).

Table 4.8 Predicted fragment ion observed in mass spectra of the by-product B

m/z	Identity
130	MeAsp +H
135	$PhCH_2CHOMe$
159	Dha Ala -OH + 2H
163	$C_{11}H_{14}O$ + H
175	Arg -OH + 2H
199	Glu Dha + H
270	Glu Dha Ala +H
292	C11H14O Glu + H
297	Adda (- NH_2) + H
361	$C_{11}H_{14}O$ Glu Dha + H
426	Adda (- NH_2) Glu + H
432	$C_{11}H_{14}O$ + Glu Dha Ala +H
450	M + H - $PhCH_2CHOMe$ - NH_2
463	Adda (- NH_2 - MeOH) Glu Dha + H
495	Adda (- NH_2)-Glu-Dha + H
552	Adda (- NH_2 - MeOH) Glu Dha Ala -OH + H
584	M + H - NH_3
601	M + H (Adda Glu Dha Ala -OH + H)

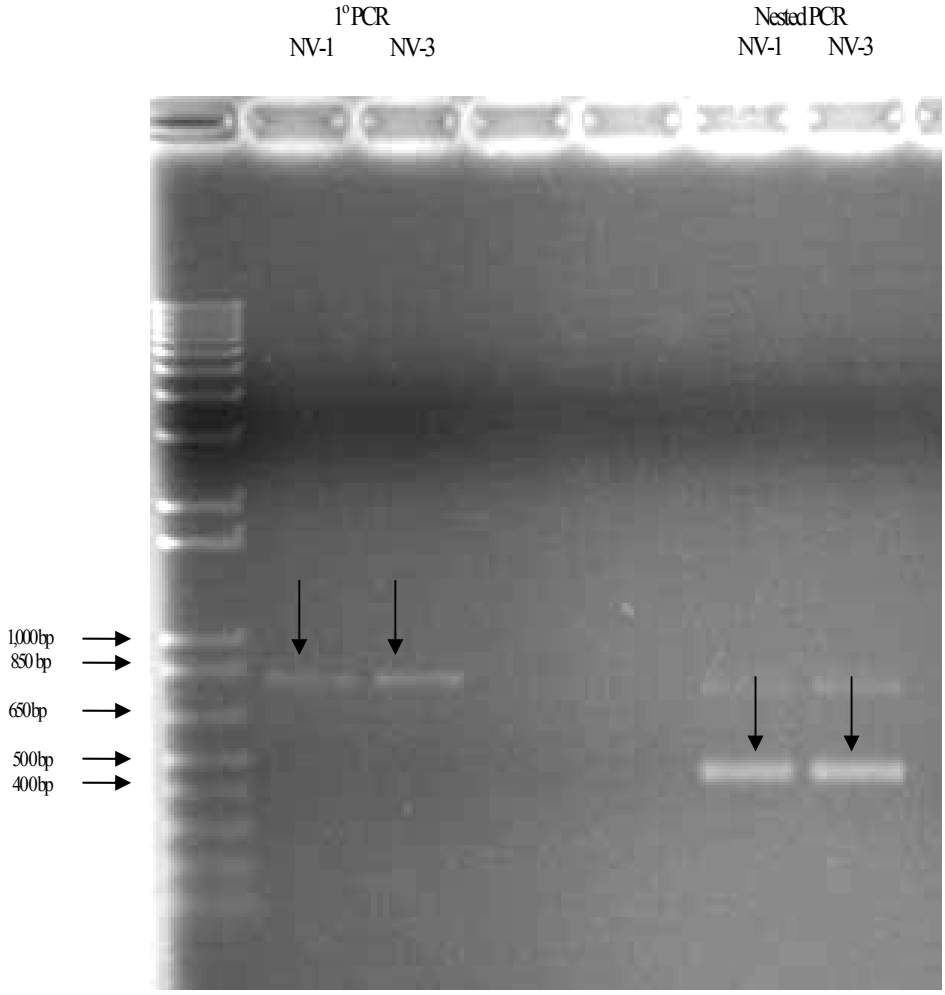
4.6.8 Detection of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes of NV-3 and NV-1

PCR was employed to detect the presence of the *mlr*A, *mlr*B, *mlr*C and *mlr*D genes that encode the MC-degrading enzymes that have been previously identified (Bourne et al., 2001; Ho et al., 2006, 2007). PCR products were visualized by gel electrophoresis (Figure 4.14 and Figure 4.15), extracted, purified and directly sequenced. The expected size of the PCR products are shown in Table 4.2 (see Section 4.5.8.3.1).

4.6.8.1 Detection of *mlr*A

After extraction of DNA from the bacterial isolates NV-1 and NV-3, *mlr*A was detected using nested PCR and two sets of primers. The PCR products from the first set of primers (MF-MR) were of the expected size (807 bp) (Figure 4.14) from NV-1 and NV-3. The second PCR products from primers MF2-MR gave a band of approximately 453 bp, as expected, from NV-1 and NV-3 (Figure 4.14).

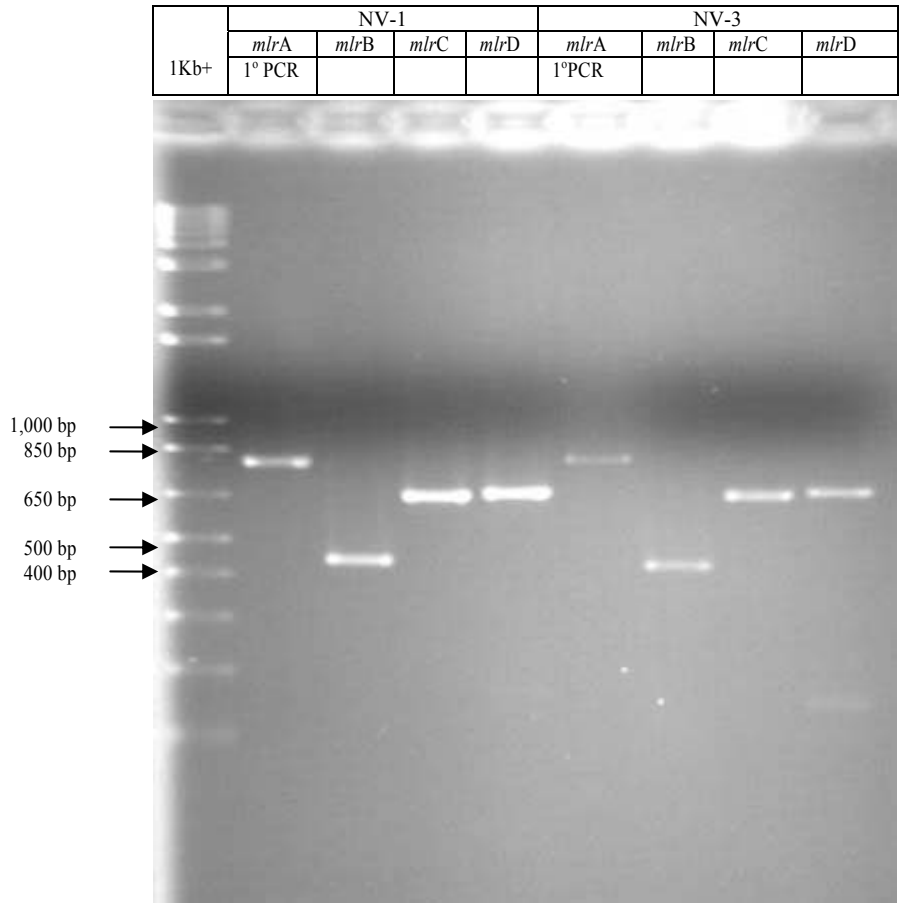
Figure 4.14 Detection of *mlr*A gene by first PCR and nested PCR in the isolates NV-1 and NV-3



4.6.8.2 Detection of *mlr*A, *mlr*B, *mlr*C and *mlr*D

Amplification of *mlr*A, *mlr*B, *mlr*C and *mlr*D was performed using the primer sets for each gene as shown previously in Table 4.2. Each amplification produced one clear amplification product and was visualized by using gel electrophoresis. The size of the bands were about 807 bp for *mlr*A, about 448 bp for *mlr*B, about 666 bp for *mlr*C and about 671 bp for *mlr*D from NV-1 and NV-3.

Figure 4.15 Detection of MC-degrading gene cluster, *mlr*A, B, C and D of the isolates NV-1 and NV-3



4.6.8.3 Homology of MC-degrading genes of the isolates NV-1 and NV-3

Direct sequencing of the amplification PCR products from the isolates NV-1 and NV-3 (obtained from Section 4.6.8.2) provided partial nucleotide sequences and putative (predicted) polypeptide sequences of *mlr*A, *mlr*B, *mlr*C and *mlr*D (Appendix 10 and Appendix 11). Alignment of the nucleotide sequences obtained for the PCR products from the two isolates revealed that the *mlr*A, *mlr*B, *mlr*C and *mlr*D genes of the isolate NV-1 were very similar to those of isolate NV-3 (99-100% homology) (Table 4.9). The partial *mlr*A nucleotide sequence (756 bp) and *mlr*D nucleotide sequence (585 bp) of the isolate NV-1 had 100% identity to the 721 bp nucleotide sequence of the *mlr*A gene and 597 bp nucleotide sequence of the *mlr*D gene of the isolate NV-3. The *mlr*B nucleotide (348 bp) and *mlr*C nucleotide (585 bp) of the isolate NV-1 had 99% identity to the 337 bp of the *mlr*B gene and the 588 bp of the *mlr*C gene of the isolate NV-3.

Table 4.9 Homology of MC-degrading genes of the isolate NV-1 and NV-3

Gene	Partial nucleotide of the isolate NV-1	Partial nucleotide of the isolate NV-3	% identity of the isolate NV-1 and NV-3
*mlr*A	756 bp	721 bp	100% (721/721)
*mlr*B	348 bp	337 bp	99% (327/329)
*mlr*C	585 bp	588 bp	99% (582/585)
*mlr*D	585 bp	597 bp	100% (583/583)

4.6.8.4 Analysis of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes of NV-1 and NV-3

4.6.8.4.1 Nucleotide analysis of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes

The nucleotide sequence of *mlr*A, *mlr*B, *mlr*C and *mlr*D from the isolates NV-1 and NV-3 were investigated using BLASTN. The nucleotide sequence of *mlr*A from the isolates NV-1 and NV-3 exhibited 99% identity to the 806 base pair (bp) nucleotide sequence of the *mlr*A gene from the *Sphingomonas* strain MD-1 (NCBI accession number AB114202) (Table 4.10 and 4.11). The *mlr*B nucleotide sequence of NV-1 and NV-3 showed close similarity to *mlr*B of *Sphingopyxis* sp. strain LH21 (DQ423530) with 94% identity (Table 4.10 and Table 4.11), whereas the nucleotide sequence of *mlr*C and *mlr*D from NV-1 and NV-3 were 99% and 97% identitical respectively to the corresponding genes from *Sphingomonas* sp. ACM-3962 (AF411070 and AF411071).

Table 4.10 BLASTN analysis of the *mlr* nucleotide sequences from NV-1 (identities of top five matches shown only)

Gene	Organism and homologous gene	Accession number	% identity
*mlr*A	*Sphingomonas* sp. MD-1, mlrAMD-1 gene for MlrA (806 bp)	AB114202	99% (753/756)
(756 bp)	*Sphingomonas* sp. ACM-3962, MlrA (mlrA) gene (1008 bp)	AF411068	98% (749/758)
	Sphingopyxis sp. C-1 gene for putative MlrA gene (740 bp)	AB161685	91% (675/7380
	Sphingopyxis sp. LH21, MlrA gene (731 bp)	DQ112243	91% (667/731)
	Sphingomonas sp. Y2, mlrAY2 gene for MlrA gene (806 bp)	AB114203	82% (625/755)
*mlr*B	*Sphingopyxis* sp. LH21, MlrB gene (367 bp)	DQ423530	94% (319/339)
(348 bp)	*Sphingomonas sp.* ACM-3962, MlrB (mlrB) gene (1206 bp)	AF411069	93% (327/348)
	Sphingomonas sp. ACM-3962 MlrD-like and MlrB-like genes (807 bp)	DQ423537	96% (25/26)
	Candidatus (Methanoregula) boonei 6A8, complete genome. (2542943 bp)	CP000780	86% (31/36)
	Roseiflexus sp. RS-1, complete genome (5801598 bp)	CP000686	92% (25/27)
*mlr*C	*Sphingomonas* sp. ACM-3962 MlrC (mlrC) gene (1521 bp)	AF411070	99% (583/585)
(585 bp)	*Sphingopyxis* sp. LH21 MlrC gene (638 bp)	DQ423531	88% (520/585)
	Magnetospirillum magneticum AMB-1, complete genome (4967148 bp)	AP007255	100% (22/22)
	Jannaschia sp. CCS1, complete genome (4317977 bp)	CP000264	92% (26/28)
	Chlamydomonas reinhardtii strain CC-503 (4989 bp)	XM_001701102	100% (21/21)
*mlr*D	*Sphingomonas* sp. ACM-3962 MlrD (mlrD) gene (1266 bp)	AF411071	97% (570/585)
(585 bp)	*Sphingopyxis* sp. LH21 MlrD gene (645 bp)	DQ423532	89% (519/583)
	Sphingomonas sp. ACM-3962 MlrA and MlrD genes (569 bp)	DQ423535	99% (262/263)
	Sphingopyxis sp. LH21 MlrA and MlrD genes (530 bp)	DQ423536	96% (218/225)
	Phenylobacterium zucineum HLK1, complete genome (3996255 bp)	CP000747	82% (47/57)

Table 4.11 BLASTN analysis of the mlr nucleotide sequences from NV-3 (identities of top five shown only)

Gene	Organism and homologous gene	Accession number	% identity
*mlr*A	*Sphingomonas* sp. MD-1, mlrAMD-1 gene for MlrA (806 bp)	AB114202	99% (719/721)
(721 bp)	*Sphingomonas* sp. ACM-3962, mlrA (mlrA) gene (1008 bp)	AF411068	98% (715/723)
	Sphingopyxis sp. C-1 gene for putative mlrA gene (740 bp)	AB161685	91% (649/712)
	Sphingopyxis sp. LH21, mlrA gene (731 bp)	DQ112243	90% (641/705)
	Sphingomonas sp. Y2, mlrAY2 gene for mlrA gene (806 bp)	AB114203	82% (85/708)
*mlr*B	*Sphingopyxis* sp. LH21, mlrB gene (367 bp)	DQ423530	94% (319/337)
(337 bp)	*Sphingomonas sp.* ACM-3962, mlrB (mlrB) gene (1206 bp)	AF411069	93% (315/337)
	AGAP005569-PA (AgaP_AGAP005569) mRNA (1189 bp)	XM_315576	100% (21/21)
	Candidatus Methanoregula boonei 6A8, complete genome (2542943 bp)	CP000780	86% (31/36)
	Roseiflexus sp. RS-1, complete genome (5801598 bp)	CP000686	92% (25/27)
*mlr*C	*Sphingomonas* sp. ACM-3962 mlrC gene (1521 bp)	AF411070	99% (585/588)
(588 bp)	*Sphingopyxis* sp. LH21 mlrC gene (638 bp)	DQ423531	88% (521/588)
	Magnetospirillum magneticum AMB-1, complete genome (4967148 bp)	AP007255	100% (22/22)
	Jannaschia sp. CCS1, complete genome (4317977 bp)	CP000264	92% (26/28)
	Chlamydomonas reinhardtii strain CC-503 (4989 bp)	XM_001701102	100% (21/21)
*mlr*D	*Sphingomonas* sp. ACM-3962 mlrD gene (1266 bp)	AF411071	97% (582/597)
(597 bp)	*Sphingopyxis* sp. LH21 mlrD gene (645 bp)	DQ423532	89% (533/597)
	Sphingomonas sp. ACM-3962 mlrA and mlrD genes (569 bp)	DQ423535	99% (276/277)
	Sphingopyxis sp. LH21 mlrA and mlrD genes (530 bp)	DQ423536	97% (232/239)
	Phenylobacterium zucineum HLK1, complete genome (3996255 bp)	CP000747	82% (47/57)

4.6.8.4.2 Protein analysis of translated polypeptide sequences from *mlr*A, *mlr*B, *mlr*C and *mlr*D

To investigate the protein homology, identity and possible function of the translated peptide sequences of *mlr*A, *mlr*B, *mlr*C and *mlr*D, the putative amino acids (Appendices 10 and 11) were analyzed against protein databases including BLASTP, Pfam, PROSITE and IntroPro.

The results from protein analyses of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes from NV-3 and NV-1 were identical (Tables 4.12 – 4.14), therefore, only NV-3 will be considered, this point on, when assessing implication of multiple sequence alignment.

Table 4.12 BLASTP analyses of translated polypeptide (amino acids) sequences of *mlr*A, B, C and D genes from the isolate NV-1

Gene	Translated amino acid of *mlr* genes	Homologous protein and organism	Accession number	% identity
mlrA	252 aa	MlrA [*Sphingomonas* sp. MD-1] (268 aa)	BAC82712	99% (250/252)
		MlrA [*Sphingosinicella microcystinivorans*] (268 aa)	BAC82713	86% (217/252)
		MlrA [*Sphingomonas* sp. ACM-3962] (336 aa)	AAL10286	98% (210/213)
		MlrA [*Sphingopyxis* sp. LH21] (244 aa)	AAZ16519	90% (214/236)
		Abortive infection protein [*Solibacter usitatus* Ellin6076] (252 aa)	ABJ85866	34% (72/209)
		CAAX amino terminal protease family protein [*Bacillus cereus* AH1134] (306 aa)	ZP_02525124	28% (62/219)
mlrB	116 aa	MlrB [*Sphingomonas* sp. ACM-3962] (402 aa)	AAL10287	93% (108/116)
		MlrB [*Sphingopyxis* sp. LH21] (122 aa)	ABD72228	94% (107/113)
		Beta-lactamase [*Solibacter usitatus* Ellin6076] (394 aa)	ABJ83531	33% (30/89)
		Beta-lactamase precursor [*Robiginitalea biformata* HTCC2501] (363 aa)	EAR14819	34% (27/780
		Beta-lactamase [*Anabaena variabilis* ATCC 29413] (537 aa)	ABA21143	33% (23/68)
mlrC	195 aa	MlrC [*Sphingomonas* sp. ACM-3962] (507 aa)	AAL10288	99% (194/195)
		MlrC [*Sphingopyxis* sp. LH21] (212 aa)	ABD72229	92% (181/195)
		hypothetical protein BRADO6169 [*Bradyrhizobium* sp. ORS278] (491 aa)	CAL79819	37% (73/197)
		MlrC domain-containing protein [*Silicibacter pomeroyi* DSS-3] (482 aa)	AAV94505	35% (69/193)
		Hypothetical protein Rmet_5087 [*Ralstonia metallidurans* CH34] (482 aa)	ABF11946	36% (70/193)
mlrD	195 aa	MlrD [*Sphingomonas* sp. ACM-3962] (422 aa)	AAL10289	96% (189/195)
		MlrD [*Sphingopyxis* sp. LH21] (214 aa)	ABD72230	91% (179/195)
		Amino acid/peptide transporter [*Sphingomonas wittichii* RW1] (445 aa)	ABQ70951	37% (74/195)
		Amino acid/peptide transporter [*Phenylobacterium zucineum* HLK1] (447 aa)	ACG78424	41% (70/168)
		Peptide transporter [*Gloeobacter violaceus* PCC 7421] (469 aa)	NP_925208	36% (62/169)

aa = amino acid

Table 4.13 BLASTP analyses of translated polypeptide (amino acids) sequences of *mlr*A, B, C and D genes from the isolate NV-3

Gene	Translated amino acid of *mlr* genes	Homologous protein and organism	Accession number	% identity
*mlr*A	240 aa	MlrA [*Sphingomonas* sp. MD-1] (268 aa)	BAC82712	99% (239/240)
		MlrA [*Sphingomonas* sp. ACM-3962] (336 amino acid; aa)	AAL10286	98% (209/212)
		MlrA [*Sphingopyxis* sp. LH21] (244 aa)	AAZ16519	91% (214/235)
		MlrA [*Sphingosinicella microcystinivorans*] (268 aa)	BAC82713	85% (206/240)
		Abortive infection protein [*Solibacter usitatus* Ellin6076] (252 aa)	ABJ85866	34% (72/209)
		CAAX amino terminal protease family protein [*Bacillus cereus* AH1134] (306 aa)	ZP_02525124	28% (62/219)
*mlr*B	112 aa	MlrB [*Sphingomonas* sp. ACM-3962] (402 aa)	AAL10287	92% (104/112)
		MlrB [*Sphingopyxis* sp. LH21] (122 aa)	ABD72228	95% (107/112)
		Beta-lactamase [*Solibacter usitatus* Ellin6076] (394 aa)	ABJ83531	33% (30/89)
		Beta-lactamase precursor [*Robiginitalea biformata* HTCC2501] (363 aa)	EAR14819	34% (27/78)
		Beta-lactamase [*Anabaena variabilis* ATCC 29413] (537 aa)	ABA21143	32% (26/79)
*mlr*C	195 aa	MlrC [*Sphingomonas* sp. ACM-3962] (507 aa)	AAL10288	99% (194/195)
		MlrC [*Sphingopyxis* sp. LH21] (212 aa)	ABD72229	91% (179/195)
		Hypothetical protein BRADO6169 [*Bradyrhizobium* sp. ORS278] (491 aa)	CAL79819	36% (71/194)
		MlrC domain-containing protein [*Silicibacter pomeroyi* DSS-3] (482 aa)	AAV94505	38% (75/197)
		Hypothetical protein H16_B1129 [*Ralstonia eutropha* H16] (484 aa)	ABF11946	36% (71/196)
*mlr*D	199 aa	MlrD [*Sphingomonas* sp. ACM-3962] (422 aa)	AAL10289	96% (193/199)
		MlrD [*Sphingopyxis* sp. LH21] (214 aa)	ABD72230	92% (184/199)
		Amino acid/peptide transporter [*Phenylobacterium zucineum* HLK1] (447 aa)	ACG78424	41% (72/172)
		Amino acid/peptide transporter [*Sphingomonas wittichii* RW1] (445 aa)	ABQ70951	38% (76/198)
		Proton/peptide symporter family protein [*Myxococcus xanthus* DK 1622] (462 aa)	ABF87785	37% (72/191)

aa = amino acid

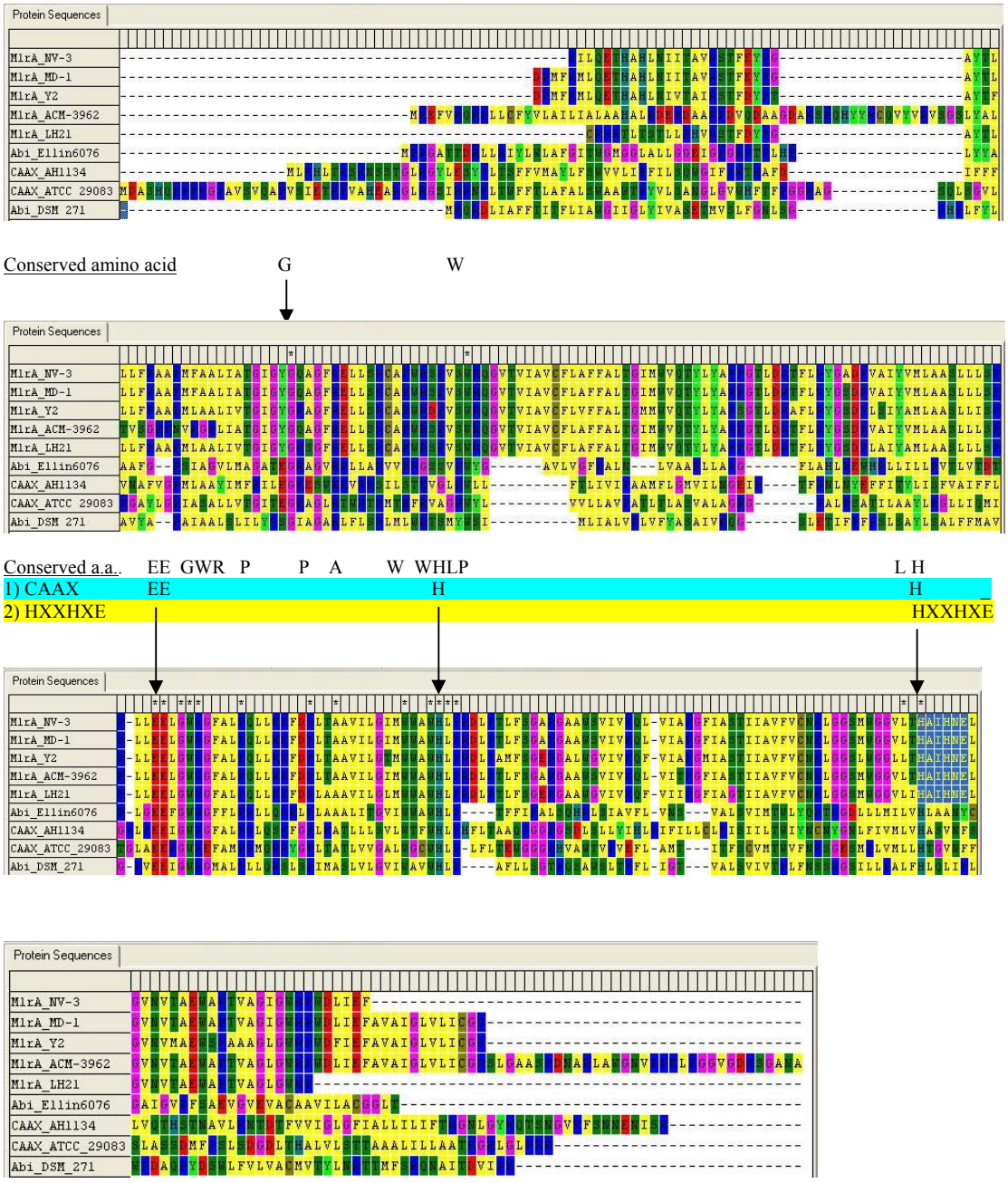
Table 4.14 Protein analysis of translated polypeptide sequences of *mlr*A, B, C and D genes from the bacterial isolate NV-1 and NV-3

Gene	Translated amino acids from *mlr* genes	Analysis database	Homologous protein and description	Note
*mlr*A	252 aa (NV-1) / 240 aa (NV-3)	Pfam	CAAX amino terminal protease family (Family) (Abi; PF02517)	Significant Pfam-A Match
		InterPro	Signal-peptide	Unintegrated IntroPro
		InterPro	Transmembrane regions	Unintegrated IntroPro
*mlr*B	116 aa (NV-1) / 112 aa (NV-3)	Pfam	Beta-lactamase (Domain) (PF00144)	Significant Pfam-A Match
*mlr*C	195 aa (NV-1) / 199 (nv-3)	Pfam	MC-LR degradation protein *mlr*C, C-terminal (Family) (PF07171)	Significant Pfam-A Match
		InterPro	MC LR degradation protein MlrC, C-terminal	IntroPro links to Pfam (IPR010799)
*mlr*D	195 aa (NV-1) / 199 aa (NV-3)	Pfam	Proton-dependent oligopeptide transport family (PTR2; PF00854)	Significant Pfam-A Match
		Prosite	MFS_Major facilitator superfamily (MFS) profile (PS50850)	
		Prosite	PTR2 family proton/oligopeptide symporters signature 1 (PTR2_1; PS01022)	

aa = amino acid

BLASTP analysis demonstrated that the putative MlrA, B, C and D proteins of the bacterial isolate NV-3 had highest similarity to corresponding proteins of the *Sphingomonas* strain MD-1 and *Sphingomonas* sp. ACM-3962 (Tables 4.12 and 4.13). The MlrA protein of the isolate NV-3 (as well as the isolate NV-1) was 99% identical to the MlrA protein of the *Sphingomonas* strain MD-1 (NCBI accession number BAC82712). The amino acid residues 109 to 204 of the putative MlrA protein were identified as the abortive infection protein (Abi) or the putative CAAX amino terminal protease family protein from Pfam analysis (http://pfam.sanger.ac.uk/family?acc=PF02517) (Table 4.14). Multiple sequence alignment of the putative MlrA protein from isolate NV-3 with its homologue proteins and selected Abi proteins (top six from BLASTP analysis) revealed conserved amino acids, containing G_{37}-W_{57}-**EE**$_{123}$-GWR_{127}-P_{132}-P_{140}-A_{143}-W_{151}-W**H**$_{155}$LP_{157}-L_{293}-**H**$_{295}$ (the letters = a type of amino acid; number = position of the amino acid on putative protein sequence) (Figure 4.16). From this alignment, it could be determined that all proteins contained 2 glutamic acids and 2 histidines, corresponding to a highly conserved Glu-Glu and histidine motif of the Abi or CAAX proteins. In addition, the MlrA proteins also contained a short segment of amino acid sequences with two histidines and a glutamate residue (**H**$_{210}$Al**H**$_{213}$N**E**$_{215}$), which relates to a variant of the classic zinc-binding motif (HEXXH) found in a number of families of metalloproteases (in which X is any amino acid). Furthermore, the proposed active-site region of the putative MlrA protein of *Sphingomonas* sp. ACM-3962—a sequence very similar to the extended consensus region (region containing similar amino acids on the protein sequence) of other metallopeptidases—was also found in the putative MlrA protein of isolate NV-3 (**V**LT**H**AI**H**N**E**$_{215}$). The putative MlrA protein of isolate NV-3 also showed the typical features of a hypothetical signal-peptide (Table 4.14) and transmembrane regions as revealed by analysis of the amino acid sequence using IntroProScan.

Figure 4.16 Multiple sequence alignment of MlrA protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis

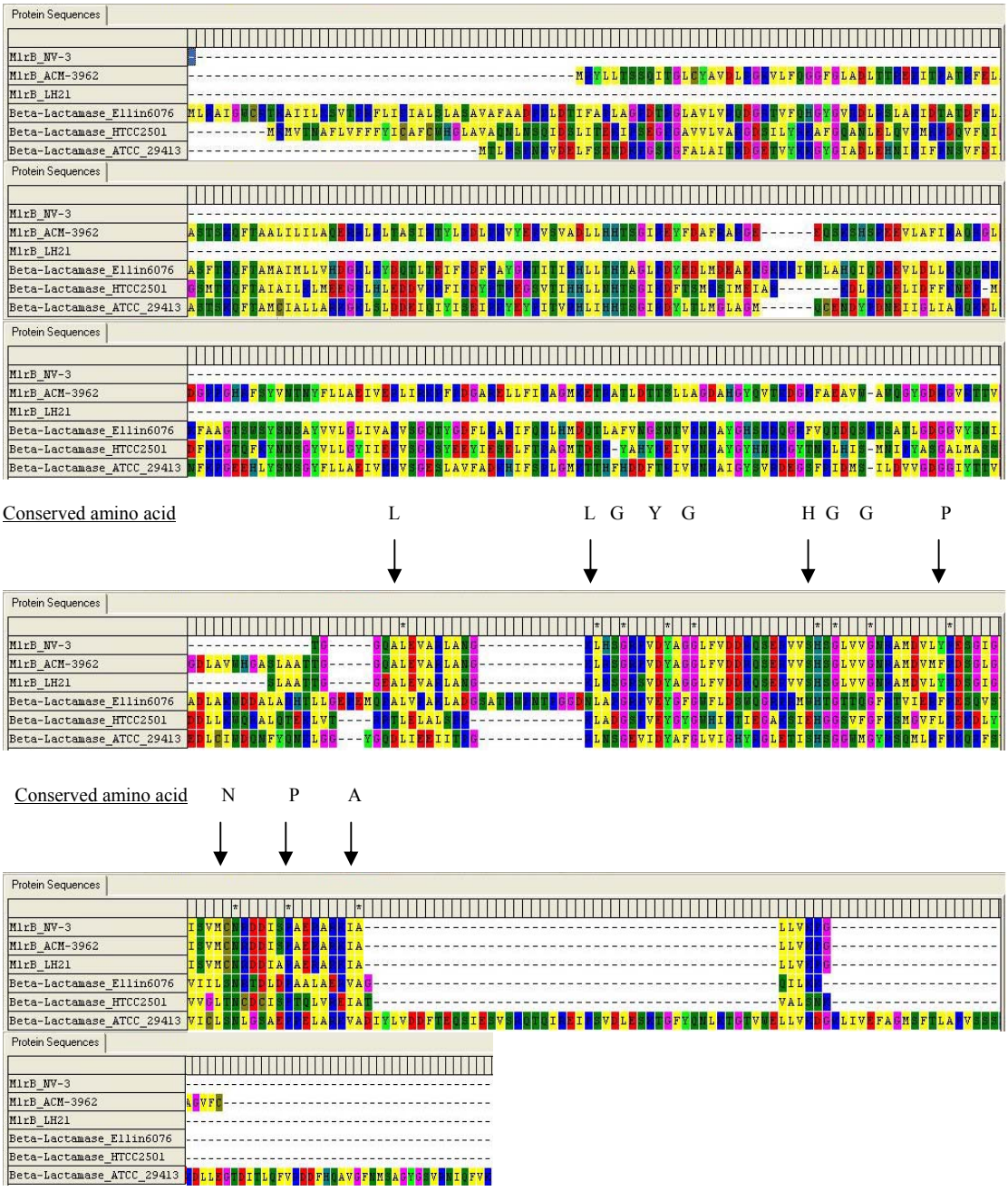


- MlrA_NV-3 = putative MlrA protein of isolate NV-3; MlrA_MD1 = MlrA protein of *Sphingomonas* sp. MD-1 (NCBI accession number BAC82712); MlrA_Y2 = MlrA of *Sphingomonas* sp. Y2 (BAC82713); MlrA_ACM-3962 = MlrA of *Sphingomonas* sp. ACM-3962 (AAL10286); MlrA_LH21 = MlrA of *Sphingopyxis* sp. LH21 (AAZ16519); Abi_Ellin6076 = Abortive infection protein of *Solibacter usitatus* Ellin6076 (ABJ85866); Caax_AH1134 = CAAX amino terminal protease family protein of *Bacillus cereus* AH1134 (ZP_02525124); Caax_ATCC_29083 = Caax amino terminal protease family protein of *Streptomyces sviceus* ATCC 29083 (YP_002206943); Abi_DSM_271 = Abortive infection protein of *Prosthecochloris aestuarii* DSM 271 (ACF46029).
- This alignment was constructed using MEGA 4.0 program

Sequence analysis of the putative MlrB protein from the isolate NV-3 (112 translated amino acids, Appendix 11) using BLASTP, revealed this protein was most closely related to the MlrB protein of *Sphingomonas* sp. strain ACM-3962 (AAL10287), the MlrB protein of *Sphingopyxis* sp. strain LH21 (ABD72228) and beta-lactamase from *Solibacter usitatus* Ellin6076 (ABJ83531). In addition, by using the Pfam database, the amino acid residues 4 to 85 of the putative MlrB protein were shown to closely match the beta-lactamase domain (PF00144) (Table 4.14). Multiple sequence alignment of the putative protein together with its homologue proteins and beta-lactamase (top five from BLASTP analysis) revealed conserved amino acids containing $L_6L_{16}G_{19}Y_{24}G_{27}H_{41}G_{43}G_{47}P_{56}N_{67}P_{73}A_{81}$ (Figure 4.17). From this alignment, the putative MlrB protein of isolate NV-3 also contains a short series of amino acid sequences with a histidine, serine and glycine (HSG_{43}) that has been identified as the third active site domain of the MlrB protein in *Sphingomonas* sp. strain ACM-3962.

The *mlr*C gene of the isolate NV-3 provided a partial nucleotide sequence (588 bp) encoding a putative protein of 195 amino acid residues referred to as the putative MlrC protein. The putative MlrC protein has marked similarity to the MlrC protein of *Sphingomonas* sp. strain ACM-3962 (99% identity), and the putative MlrC protein of *Sphingopyxis* sp. LH21 (92% identity) (Tables 4.12 and 4.13). Furthermore, the residues 62 to 195 of the putative amino acid sequences of the putative MlrB protein were identified by Pfam analysis as the C-terminus of the MlrC protein family involved in degradation of MCs (http://pfam.sanger.ac.uk/family?acc=PF07171) (Table 4.14). The putative MlrC protein of isolate NV-3 was aligned with a selection of its hypothetical homologues. The multiple sequence alignment shows conserved amino acids containing $D_{65}DN_{69}$-$G_{73}P_{101}AG_{111}G_{113}G_{121}$ $P_{131}G_{165}$ (Figure 4.18).

Figure 4.17 Multiple sequence alignment of MlrB protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis



- MlrB_NV-3 = putative MlrB protein of isolate NV-3; MlrB_ACM-3962 = MlrB of *Sphingomonas* sp. ACM-3962 (AAL10287); MlrB_LH21 = MlrB of *Sphingopyxis* sp. LH21 (ABD72228); Beta-lactamase_Ellin6076 = Beta-lactamase of *Solibacter usitatus* Ellin6076 (ABJ83531); Beta-lactamase_HTCC2501 = Beta-lactamase precursor of *Robiginitalea biformata* HTCC2501 (EAR14819); Beta-lactamase_ATCC 29413 = Beta-lactamase of *Anabaena variabilis* ATCC 29413 (ABA21143).
- This alignment was constructed using MEGA 4.0 program

Figure 4.18 Multiple sequence alignment of MlrC protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis

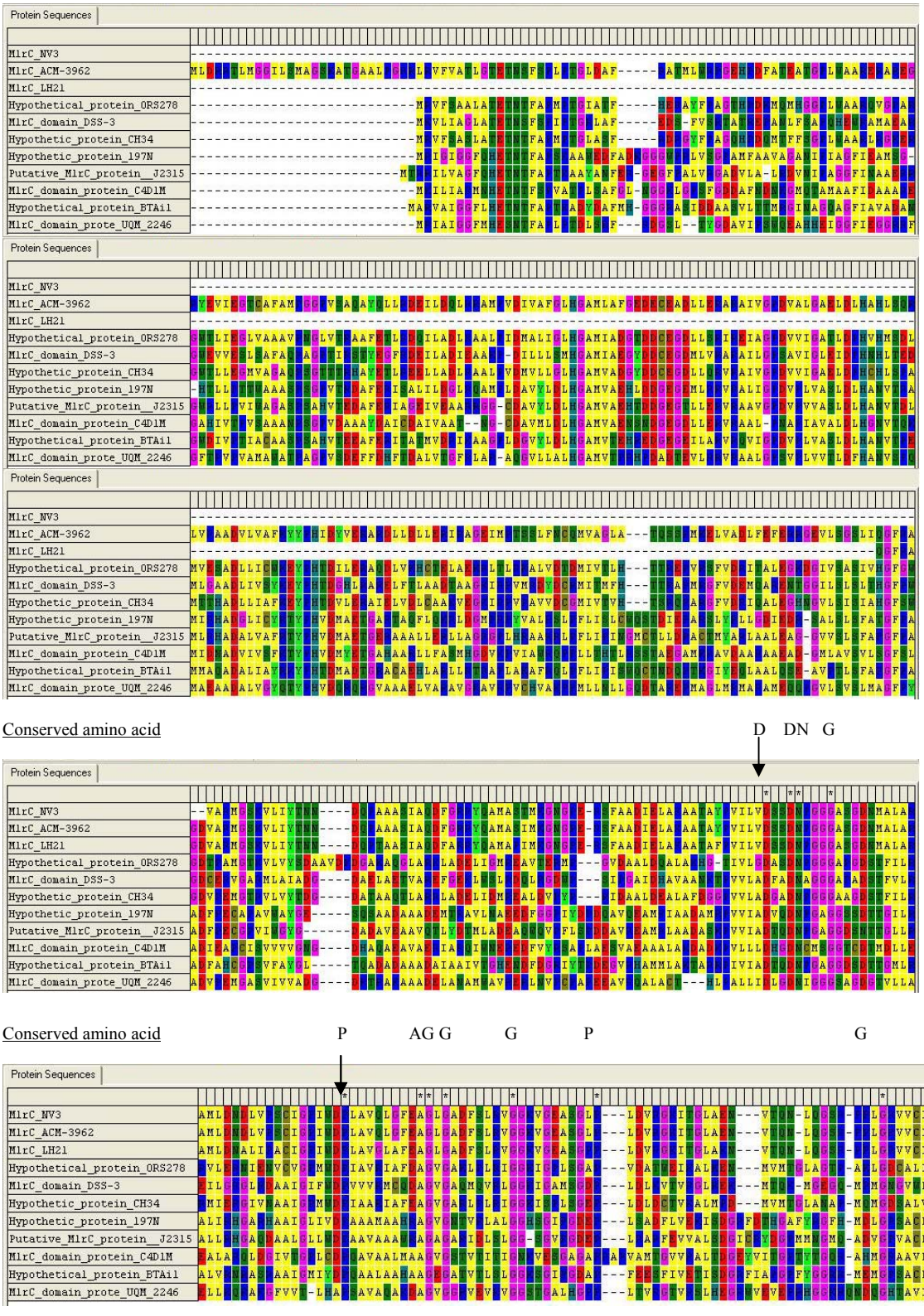
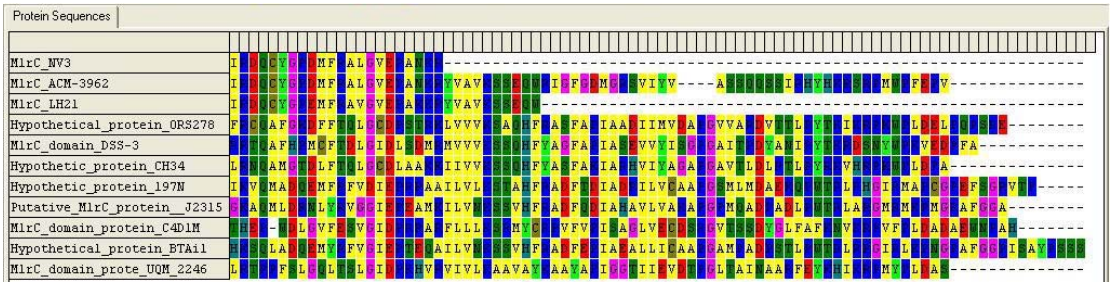


Figure 4.18 (continued) Multiple sequence alignment of MlrC protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis

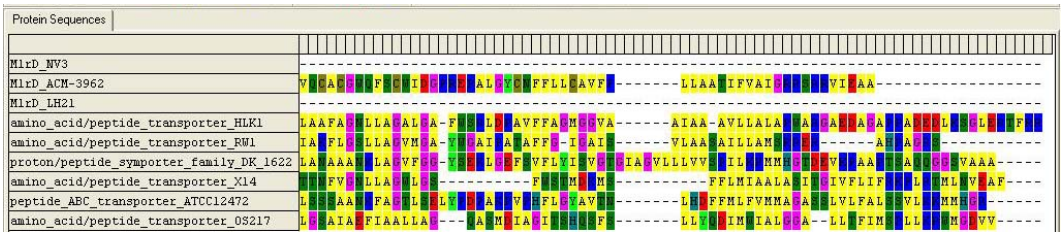


- MlrC_NV-3 = putative MlrC protein of isolate NV-3; MlrC_ACM-3962 = MlrC of *Sphingomonas* sp. ACM-3962 (AAL10288); MlrC_LH21 = MlrC of *Sphingopyxis* sp. LH21 (ABD72229); Hypo_protein_ORS278 = conserved hypothetical protein; putative MlrC domain [*Bradyrhizobium* sp. ORS278] (CAL79819); Hypo_protein_DSS-3 = MlrC domain protein [*Silicibacter pomeroyi* DSS-3] (AAV94505); Hypo_protein_CH34 = Uncharacterised conserved protein UCP012702 [*Ralstonia metallidurans* CH34] (ABF11946); Hypothetical_protein_197N = conserved hypothetical protein [*Bordetella avium* 197N] (CAJ48107); putative_MlrC_protein_J2315 = putative MlrC family protein [*Burkholderia cenocepacia* J2315] (CAR56411); MlrC_domain_protein_C4D1M = MlrC domain protein [*Burkholderia graminis* C4D1M] (EDT10361); Hypothetical_protein_BTAi1 = Hypothetical protein BBta_5259 [*Bradyrhizobium* sp. BTAi1] (ABQ37246); MlrC_domain_protein_UQM_2246 = MlrC domain protein [*Gemmata obscuriglobus* UQM 2246] (ZP_02731807).
- This alignment was constructed using MEGA 4.0 program

Finally, the 195 amino acid sequence predicted from the 585 nucleotides of *mlr*D had highest similarity to the putative MlrD proteins of *Sphingomonas* sp. strain ACM-3962 and *Sphingopyxis* sp. strain LH21 (Table 4.12 and Table 4.13), the amino acid/peptide transporter of *Phenylobacterium zucineum* HLK1 and of *Sphingomonas wittichii* RW1 and the proton/peptide symporter family protein of *Myxococcus xanthus* DK 1622 by BLASTP analysis. From the protein motif library (PROSITE), the putative MlrD protein from NV-3 closely matched the PTR2 family proton/ oligopeptide symporters signature 1 (PTR2_1; PS01022), a family of prokaryotic and eukaryotic proteins that are mainly involved in the uptake of small peptides with the concomitant uptake of a proton (http://ca.expasy.org/ cgi-bin/nicedoc.pl?PS01022). The consensus pattern of PTR2—[**G**A] - [**G**AS] - [LIVMFY**W**A] - [L**I**VM] - [G**A**S] - **D** - x - [LIVM**F**YWT] - [L**I**VMFYW] - **G** - x(3) - [T**A**V] - [**I**V] - x(3) - [GST**A**V] - x - [**L**IVMF] - x(3) - [**G**A] where X is any amino acid, was found between amino acid residues 40 to 64 (GGWIA**D**RFI**G**RSAAIVGGAILKMAG) of the putative amino acid sequences of putative MlrD protein from NV-3 (Figure 4.19). Pfam anlaysis also identified the putative amino acid residues 51 to 162 of the putative protein as PTR2 (PF00854), belonging to the proton-dependent oligopeptide transport (POT) family and the MFS clan (CL0015).

Figure 4.19 Multiple sequence alignment of MlrD protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis

Figure 4.19 (continued) Multiple sequence alignment of MlrD protein from bacterium isolate NV-3 and its homologue proteins from BLASTP analysis



- MlrD_NV-3 = putative MlrD protein of isolate NV-3; MlrD_ACM-3962 = MlrD of *Sphingomonas* sp. ACM-3962 (AAL10289); MlrD_LH21 = MlrD of *Sphingopyxis* sp. LH21 (ABD72230); amino acid/peptide transporter_ HLK1 = amino acid/peptide transporter [*Phenylobacterium zucineum* HLK1](ACG78424); amino acid/peptide transporter_ RW1 = amino acid/peptide transporter [*Sphingomonas wittichii* RW1] (ABQ70951); proton/peptide symporter family protein_DK 1622 = proton/peptide symporter family protein [*Myxococcus xanthus* DK 1622] (ABF87785); Amino acid/peptide transporter_ X14 = Amino acid/peptide transporter [*Nitrobacter hamburgensis* X14] (ABE64494); peptide_ABC_transporter_ATCC12472 = di-tripeptide ABC transporter [*Chromobacterium violaceum* ATCC 12472 (AAQ58837); Amino acid/peptide_transporter_OS217 = Amino acid/peptide transporter [*Shewanella denitrificans* OS217] (ABE56579).
- This alignment was constructed using MEGA 4.0 program

4.7 Discussion

The presence of MCs in freshwater is an increasing problem and poses a potential threat to human and animal health worldwide. These toxins are well recognized as a stable and persistent compound, however, reports have shown that MCs are vulnerable to breakdown by indigenous bacteria (Jones et al., 1994; Rapala et al., 1994; Lam, Prepas, Spink, & Hrudey, 1995; Cousins et al., 1996; Lahti et al., 1997; Chorus & Bartram, 1999). The bacterium *Sphingomonas* sp. strain ACM-3962 (MJ-PV) was one of the first MC-degrading bacteria to be identified (Jones et al., 1994). Since then the number of known MC-degrading bacteria has increased. So far, these bacteria have been identified to belong to genera *Sphingomonas* (5 strains), *Pseudomonas* (1 strain), *Sphingosinicella* (1 strain), *Sphingopyxis* (1 strain), *Paucibacter* (1 strain) and *Burkholderia* (1 strain) (Jones et al., 1994; Takenaka & Watanabe, 1997; Park et al., 2001; Maruyama et al., 2003; Saitou et al., 2003; Harada et al., 2004; Ishii et al., 2004; Rapala et al., 2005; Maruyama et al., 2006; Valeria et al., 2006; Maruyama et al., 2007; Lemes et al., 2008). In this study, MC degrading bacteria were isolated from Lake Rotoiti, and designated as isolates NV-1, NV-2 and NV-3. The ability of these bacteria to utilise [Dha^7]MC-LR and MC-LR as their sole source of carbon and energy was demonstrated.

Two approaches used in this study to identify the two isolates, NV-1 and NV-3, were biochemical assays and 16s RNA sequencing. Biochemical tests are important and valuable for the delineation of bacterial taxa and are now available as commercial kits, such as the API 20 NE kit (BioMérieux). The API 20 NE has previously been used to identify a wide range of bacteria (Vandamme et al., 1996), however, the results obtained by such tests can be false or ambiguous because there is a high possibility that a new species might be detected that has a substrate utilization profile similar to an unrelated strain within the database (Busse et al., 1996). Indeed, in this study, the isolates were initially identified using the API 20 NE kit as *Methylobacterium mesophilicum*, demonstrating that caution must be taken when interpreting results from such kits. To prevent the mis-identification of the isolates, ESR 16S RNA phylogenetic tests were performed and ESR biochemical tests to obtain additional information of bacterial motility and substrate utilization.

The 16S rRNA undergoes change very slowly possibly due to its critical function for the life of a cell and is, therefore, conserved to a greater extent than the bulk of the bacterial genome (Vandamme et al., 1996; Krieg, 2005; Krieg & Garrity, 2005). Recently, it became generally accepted that bacterial classification should reflect as closely as possible the natural relationships among bacteria, which are reflected as the phylogenetic relationships as encoded by the 16S rRNA sequence (Vandamme et al., 1996). On the basis of 16S rRNA sequences the isolates NV-1 and NV-3 were indistinguishable from the *Sphingomonas* strain MD-1 from Japan (Saitou et al., 2003). There were, however, some minor biochemical differences, for example, in their utilization of xylose and arginine dihydrolase, which might be expected to occur among bacterial isolates or strains isolated from different locations (Busse et al., 1996). Further comparisons of NV-1 and NV-3 with the strain MD-1, will require full genomic sequence studies such as mol% G-C values and DNA-DNA hybridization for indication of their DNA homology. According to Wayne et al. (1987), if NV-1 and NV-3 demonstrate 70% or greater DNA-DNA hybridization to strain MD-1, then NV-1 and NV-3 can be considered the same species as strain MD-1, or NV-1 and/or NV-3 can be called *Sphingomonas* strain MD-1 isolated from New Zealand waterbodies. The isolates NV-1 and NV-3 are, however, the first MC-degrading bacteria isolated from New Zealand waterbodies and at this stage they can be tentatively classified into the genus of *Sphingomonas*, most closely resembling the Japanese strain, MD-1.

As noted earlier, for future research to confirm that these isolates belong to the genus of *Sphingomonas*, a series of taxonomic studies will also need to be conducted, for example, to establish that the G-C content of their genomic DNA is approximately 61.6 mol% to 67.8 mol%, the presence of ubiquinone Q-10 as the major respiratory lipoquinone, 2-hydroxymyristic acid (14:02-OH) as the major hydroxylated fatty acid, and sphingoglycolipids (typical feature of the genus *Sphingomonas*) as the major components in their cellular lipids. In addition, most species of the genus *Sphingomonas* have carotenoid pigment, but do not contain 3-hydroxy fatty acids and the lipopolysaccharide characteristic of gram negative bacteria (Yabuuchi et al., 1990; Takeuchi, Kawai, Shimada, & Yokota, 1993; Takeuchi et al., 1995; Yabuuchi, Kosako, Naka, Suzuki, & Yano, 1999; Stolz et al., 2000; Yun et al., 2000).

It is interesting to note that all MC-degrading genera *Pseudomonas, Sphingomonas*, *Sphingosinicella*, *Sphingopyxis*, *Paucibacter* and *Burkholderia* are gram-negative, obligate aerobic rod-shaped bacteria and are oxidase and catalase-positive. Saito et al. (2003) suggested the ability to degrade MCs is present only in specific strains of the genus *Sphingomonas*, and in some strains of related genera, therefore, it is plausible that the bacteria that belong to these genera may be derived from common ancestors in genetic evolution that had the ability to breakdown MCs. The genera *Sphingomonas* and *Burkholderia* were previously part of the genus *Pseudomonas*. Based on their 16S rRNA sequences, cellular lipid and fatty acid composition, the genus *Sphingomonas* was reclassified by Yabuuchi and colleagues in 1990, and *Burkholderia* was defined as a new genus by Yabuuchi and colleagues in 1992. The genus *Sphingopyxis*, previously a member of genus *Sphingomonas*, was assigned by Takeuchi and colleagues in 2001 as a new genus within the family Sphingomonadaceae on the basis of phylogenetic, chemotaxonomic and phenotypic studies. *Sphingosinicella* forms a tight cluster within family Sphingomonadaceae on the basis of 16S rRNA sequencing, but it is clearly separate from other established genera of this family (e.g. *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*) (Maruyama et al., 2006). The genus *Paucibacter*, was proposed to be a novel genus in 2005, however, there is no genetic evidence to link this bacterium to other MC-degrading genera.

The focus of this doctoral study is the development of a small scale biodegradation bioreactor for possible application in water treatment plants. Therefore, the bacterial isolate able to degrade MCs at the fastest rate, NV-3, was further investigated, in terms of temperature, bacterial cell concentration and mode of possible enzymatic action etc, to establish optimum conditions when used in a bioreactor (see Chaptor 6). The bacterial isolate NV-3 was found to grow optimally on PYEM at 30^{o}C, as evidenced by the higher cell number achieved at this temperature than other temperatures examined.

The rate of MC degradation by any bacterial isolate, including NV-3, relies mainly on the incubation temperature as well as bacterial concentration. Biodegradation of MCs by the bacterium occurred under a wide range of temperatures between 10^{o}C and 35^{o}C. At 10^{o}C, the biodegradation was very slow and identical to

that of the MC degrading-bacterium *Sphingopyxis* strain LH21, isolated from Australia, suggesting that it would similarly not be able to degrade MCs at 4^{o}C (Ho et al., 2007). The ability of bacteria to degrade toxins in low temperatures is relevant to the degradation of toxins in winter, for example, when the water temperature is low. For NV-3, increases in water temperature from 15^{o} to 30^{o}C, were associated with an increase in biodegradation rate, however; temperatures higher than the optimum temperature (30^{o}C) for bacterial growth resulted in slightly decreased degradation ablity. It is possible that at high temperatures, NV-3 bacterial cells are unable to produce MC-degrading enzymes or the cells might be rendered inactive or growing slowly. The highest rate of MC-degradation by the isolate NV-3 was achieved at 30^{o}C, similar to temperature at which maximum MC-degradation was achieved by the *Sphingomonas* strain Y2, isolated from Lake Suwa, Japan (Park et al., 2001). At the optimum temperature of bacterial growth, the bacterial metabolism is very active, producing a lot of the enzymes responsible for MC degradation. By contrast, at temperatures that are lower or higher than that for optimum growth, bacterial metabolism and the production of biodegradation enzymes is presumably less active, slowing the rate of MC degradation.

Biodegradation of toxins is also dependent on bacterial concentration. In this study, the degradation rate increased with increasing bacterial cell concentration; however, concentrations above 1.0x10^{8} CFU/ml resulted in a decrease in degradative ability. This decrease might be due to competition or inhibition between the cells at very high cell numbers and, therefore, the optimum cell density is also crucial for toxin degradation. Exploration of the exact mechanisms at play were beyond the intent and scope of this study. The optimum cell density of the bacterium isolate NV-3, required for MC degradation experiments, was between 4.9 x 10^{7} and 1.0 x 10^{8} CFU/ml and the minimum number of cells required was approximately 7.9x10^{6} CFU/ml. It is interesting that at the minimum cell density MCs were completely degraded within 3 days, the same length of time taken with higher concentrations of bacteria. This confirms the ability of the bacterium isolate NV-3 to utilise the toxins as a primary food source, and for it to multiply to a cell density sufficient to completely degrade the MCs.

The effect of toxin concentrations on MC degradation by NV-3 was also examined. In this study, at the optimum temperature (30^{o}C) and bacterial concentration (1.0x10^{8} CFU/ml), the biodegradation rate increased with increasing concentrations of the toxins (mixure of [Dha^{7}]MC-LR and MC-LR). The degradation rate by the bacterial isolate was identical (i.e. 8.33 μg/10^{8} CFU/day) for moderate to high concentrations of the toxins (25 μg/ml to 50 μg/ml). It is possible that as the sole carbon and nitrogen sources for bacterial growth, high concentrations of MCs were beneficial to growth of the isolated bacteria. However, the apparent inhibitory effects of very high toxin concentrations (i.e. >50 μg/ml) and the limit to the amount of MCs that NV-3 can degrade, needs to be further investigated, as the effects on bacterial growth and metabolism are unknown.

To compare MC degradation ability among MC-degrading bacteria, the bacteria need to degrade the same variant of MCs since the ability to degrade MCs is most likely to vary depending on MC variants (or substrate specificity), possibly because of their structural variation (Imanishi et al., 2005). It has been shown in several studies that MC-degrading bacteria yielded different degradation rates on different MC variants (Park et al., 2001; Saitou et al., 2003; Ishii et al., 2004; Imanishi et al., 2005). For example, Park et al. (2001) revealed for *Sphingomonas* strain Y2, that the biodegradation rate of MC-LR was 5.4 μg/l/day, whereas that of MC-RR was 13 μg/ml/day. It would be interesting, therefore, in future study to investigate biodegradation of MC-LR only by NV-3, to compare degradation ability with the strain MD-1 (NV-3 is most likely the same species as strain MD-1), and other MC-degrading bacteria. Saitou et al. (2003) demonstrated that *Sphingomonas* strain MD-1 had degradation rates for MC-LR, MC-RR and MC-YR of 0.66, 1.25 and 1.90 μg/ml/hour, respectively. Park and co-workers used initial MCs at 20 μg/l, whereas Saitou and colleagues used initial MCs at 1 μg/l, therefore future work to establish optimum initial concentrations, would be important in the design of practical systems for toxin removal.

Studies of MC biodegradation have mainly focused on the degradation of the MC-LR analogue since it is found worldwide and is highly toxic (LD_{50} (ip) = 50 μg/kg in mice) (Rinehart et al., 1988; Carmichael, 1992; Bell & Codd, 1996; Zurawell

et al., 2005). The degradation of other MC analogues such as MC-RR, MC-YR, MC-LW and MC-LF have also been examined (Jones et al., 1994; Takenaka & Watanabe, 1997; Park et al., 2001; Maruyama et al., 2003; Saitou et al., 2003; Harada et al., 2004; Ishii et al., 2004; Rapala et al., 2005; Maruyama et al., 2006; Valeria et al., 2006; Maruyama et al., 2007 and Lemes et al., 2008). In this study, [Dha^7]MC-LR was used in addition to MC-LR as a substrate to characterize the biological degradation pathway by which the bacterium isolate NV-3 degrades MCs. Based on the by-products detected (Section 4.6.7), at least three hydrolytic enzymes are involved in this pathway. The first enzyme catalyses the aromatic ring of [Dha^7]MC-LR at the Adda-arginine peptide bond, yielding linearized peptides (NH_2-Adda-D-Glu-Dha-D-Ala-L-Leu-D-MeAsp-L-Arg-OH; MW = 999) (by-product A). The second enzyme cuts linearized (acryclo-) [Dha^7]MC-LR at the alanine-leucine peptide bond, producing a tetrapeptide (NH_2-Adda-D-Glu-Dha-D-Ala-OH; MW = 601) (by-product B); and finally, the third enzyme presumably cleaves other bonds, producing undetectable or unidentified peptide fragments. This enzymatic pathway is identical to that previously identified by Bourne et al. (1996).

It is important to note that the intermediate MC-degradation products, the linearized peptides, the tetrapeptide and digested amino acids, are significantly less toxic than the parent MC molecules. Bourne et al. (1996) demonstrated that the toxicity of the linearized peptides of MC-LR is reduced 160-fold compared with the parent compound, while other studies have demonstrated that the tetrapeptide and digested amino acids are non-toxic (Harada et al., 2004; Imanishi et al., 2005). These findings strongly indicate that microbial degradation is a potentially safe and natural treatment for removing MCs from water (Tsuji et al., 2006).

MC-degrading bacterium strain B-9 isolated from Lake Tsukui, Japan was characterized by Imanishi et al. (2005) and it was shown that the process by which B-9 degades MC-LR is similar to that of the strain ACM-3962, but that their degradation enzymes are not identical. The enzymatic pathway of MCs by the strain B-9 involves sequential enzymatic hydrolysis of Arg-Adda, Ala-Leu and Adda-Glu peptide bonds, yielding the linearized MC-LR, tetrapeptide and Adda residue as by-products whereas that of strain ACM-3962 involved hydrolysis of the Arg-Adda, Ala-Leu, and unknown peptide bonds, giving only linearized MC-LR and tetrapeptide.

Imanishi et al. (2005) also demonstrated that the strain B-9 can hydrolyze MCs at other peptide bonds. For example, the first enzyme is able to hydrolyze not only the Arg-Adda peptide bond but also the Arg-6(*Z*)-Adda peptide bond of 6(*Z*)-MC-LR and 6(*Z*)-MCRR and the Phe-Adda peptide bond of MC-LF. The second enzyme can hydrolyze the Ala-Arg peptide bond in addition to the Ala-Leu peptide bond. Similarly, the third enzyme is able to hydrolyze the 6(*Z*)-Adda-Glu peptide bond in addition to the Adda-Glu peptide bond. Moreover, the ability of strain B-9 to degrade other non-toxic cyanobacterial cyclic peptides, such as nostophycin, microcyclamide, microviridin I, aeruginopeptin 95-A, and anabaenopeptin A was demonstrated by Kato, Imanishi, Tsuji, and Harada (2007), by hydrolysis of their peptide bonds. These results suggest that MC-degrading bacteria possess a high potential to degrade a wide range of cyclic peptides.

To further understand the mechanism of MC-degrading enzymes, Bourne et al. (2001) performed the cloning and molecular characterization of four genes from the *Sphingomonas* strain ACM-3962, to identify the MC-degrading gene cluster, *mlr*A, *mlr*B, *mlr*C and *mlr*D. The *mlr*A, *mlr*B, *mlr*C genes express three peptidases (two metalloproteases and a serine protease) while *mlr*D gene is predicted to be a transport protein (Bourne et al., 2001). In this study, the *mlr*A, *mlr*B, *mlr*C and *mlr*D genes in the genomic DNA of the bacterial isolates NV-1 and NV-3, were detected indicating that the MC-degrading bacteria isolated have the ability to degrade MCs in the same way as outlined by Bourne et al. (1996). The amplifid nucleotide sequences of the *mlr* genes from NV-1 and NV-3 were identical and therefore discussion of further analyses, here on in, is related to isolate NV-3 only.

These studies revealed that the predicted amino acid sequences of the putative MlrA protein from isolates NV-1 and NV-3 were identical to the putative MlrA protein of *Sphingomonas* strain MD-1 and the putative MlrA protein of *Sphingomonas* strain ACM-3962, respectively (see Section 4.6.8.4.2) (Saito et al., 2003; Bourne et al., 2001). The putative MlrA protein of isolate NV-3 was identified as a zinc-dependent metalloprotease on the basis of two histidines and a glutamate residue (HXXHXE) in similar sequence to the putative MlrA protein of the *Sphingomonas* strain ACM-3962 (Bourne et al., 2001). The putative MlrA protein of isolate NV-3 was also identified as a putative endometalloprotease, on the basis of

protein homology to the putative CAAX amino terminal protease family protein (a novel superfamily of putative membrane-bound metalloproteases) similar to the putative MlrA protein of the *Sphingomonas* strain MD-1 (Saito et al., 2003). Therefore, the putative MlrA protein of isolate NV-3 is most likely to be a zinc endometalloprotease similar to the MlrA protein of strain ACM-3962 and MD-1, which hydrolyze the endopeptide bond of the aromatic ring of [Dha^7]MC-LR and MC-LR at the Adda-arginine position, producing linearized peptide intermediate products, by-product A, as demonstrated in Section 4.6.7.

The mechanism by which the putative MlrA protein of isolate NV-3 hydrolyzes MCs is probably similar to that of the MlrA protein of strain ACM-3962, because they contain identical active sites (VLT**H**AI**H**N**E**) (where the 2 Hs are zinc ligands and E is the active site residue involved in the catalytic activity) (Bourne et al., 2001). It is interesting that among the different amino acids of the two MlrA proteins there is some similarity in which two of the nonpolar amino acids, Isoleucine and Leucine and two of the polar amino acids, alanine and glycine, align. The amino acids that differ between the two proteins may not affect the ability of the proteins to hydrolyze MCs because the conserved active site is unchanged. Moreover, the presence of a signal peptide sequence, that is conserved between the ACM-3962 and NV-3 strains implies that the degradation of peptides by these bacteria occurs in the periplasmic space of the cells (Bourne et al., 2001).

Protein analysis revealed the putative MlrB protein of isolate NV-3 was a member of the beta-lactamases or penicillin-recognising enzyme family whereas the MlrB protein of strain ACM-3962, which had closest identity (93%) to the putative MlrB protein of isolate NV-3, belong to a specific group of beta-lactamase, which is beta-lactamase class-A or referred to as serine hydrolase (see Section 4.6.8.4.2). The predicted amino acid sequence of the putative MlrB protein of NV-3 obtained from direct DNA sequencing was only partial (approximately 116 residues), and did not cover the active site domain I, to enable identification of the protein as beta-lactamase class-A. It is important to note that three active site domains of beta-lactamase were detected in strain ACM-3962, consisting of active site I ($SXXK_{53}$), active site II (SYN_{144}) and active site III (HSG_{282}), whereas only one active site domain was detected in the putative MlrB protein of isolate NV-3: HSG_{43}, where the

first amino acid of putative MlrB protein of isolate NV-3 started at amino acid 240 of the MlrB protein of strain ACM-3962. The predicted protein sequence of the putative MlrB protein from isolate NV-3 was identical to the putative MlrB protein from strain ACM-3962, therefore, the putative MlrB protein of isolate NV-3 is most likely to be a serine beta-lactamase, which enables cleavage of linearized [Dha^7]MC-LR and linearized MC-LR at the alanine-leucine peptide bond to produce the second intermediate by-product of MC degradation, by-product B, as demonstrated in Section 4.6.7.

Members of the beta-lactamase family contain diverse sequence proteins such as D-alanyl-D-alanine carboxypeptidase B, aminopeptidase (DmpB), alkaline D-peptidase, animal D-Ala-D-Ala carboxypeptidase homologues and the class A and C beta-lactamases (Knox & Moews 1991). Interestingly, most of these family members hydrolyze peptides containing two D-amino acids, therefore, the putative MlrB protein of isolate NV-3 is unique in cleaving between D-amino acids and an L-amino acid at the D-alanine-L-leucine peptide bond of linearized [Dha^7]MC-LR and linearized MC-LR, providing undetectable amino acids. Asano and Luebbehuesen (2000) reported that D-aminopeptidase can also utilize peptides containing either D- or L-amino acids (with a preference for D-amino acids) with D-alanine being the best substrate for the enzyme. This may suggest that the putative MlrB protein is more closely related to the D-aminopeptidase than other proteins in the family.

Bourne et al. (2001) suggested that characterization of the MlrB protein provides crucial evidence as to the actual function of the *mlr*A, *mrl*B, *mlr*C, *mlr*D gene cluster in MC-degraders. One of the best known examples of peptides that contain D-amino acids are the building blocks of the bacterial cell wall. The bacterial cell envelope is stabilized by an exoskeleton consisting of murein (peptidoglycan), a long-chain polymer of alternating N-acetylglucosamine and D-acetyl muramic acid (Asano & Luebbehuesen, 2000). Adjacent macromolecules are linked by peptide bridges, where D-Glu and D-Ala are found. The general structure of MCs is also comprised of three D-amino acid; D-Glu, D-Ala and D-MeAsp. Therefore, the consecutively-acting putative proteins, MlrA and MlrB and MlrC act to break down MCs into small amino acids to build up their bacterial cell wall.

Protein database analysis revealed that the putative MlrC protein of isolate NV-3 correlated with the C-terminus of a MlrC protein belonging to the MlrC protein family (Section 4.6.8.4.2). Many numbers of this family are hypothetical proteins and have not been further characterized (http://pfam.sanger.ac.uk/family?acc=PF07171). The MlrC protein from the *Sphingomonas* strain ACM-3962 is believed to mediate the last step of peptidolytic degradation of the MC and is suspected to be a metallopeptidase on the basis of its inhibition by metal chelators. No classic conserved protein motif of the metallopeptidases was observed in the MlrC putative protein sequence from the isolate NV-3 (Bourne et al., 1996, 2001). The putative MlrC protein of isolate NV-3 was identical to the MlrC protein of the strain ACM-3962; therefore, the function of the putative MlrC protein of isolate NV-3 will most likely degrade the tetrapeptide of [Dha^7]MC-LR and MC-LR to produce smaller peptides or single amino acids. It was found that the MlrC protein of strain ACM-3962 was only a MC-degrading protein in the gene cluster that exists in protein databases and identifies as a protein family whereas other MC-degrading proteins (MlrA, MlrB and MlrD protein) were recognized as individual proteins.

The putative MlrD protein of the isolate NV-3 belongs to the PTR2 transport protein family that is mainly involved in the intake of small peptides and the concomitant uptake of a proton. This is consistent with the findings of Bourne et al. (2001), who predicted that the putative MlrD protein of strain ACM-3962 is involved in the transport of digested peptides or small peptides across the bacterial cell wall. Therefore, the putative MlrD protein of isolate NV-3 probably functions in the final step of MC degradation, to carry peptides into the cell for the synthesis of the bacterial cell wall and for bacterial metabolism. It is noteworthy that the putative sequence of the MlrD protein from isolate NV-3 matched the amino acid/peptide transporter of *Sphingomonas wittichii* RW1, a dioxin degrading bacterium (Wilkes, Wittich, Timmis, Fortnagel, & Francke, 1996). This protein is involved in carrying glycerol-3-phosphate (the triose sugar backbone of triglycerides and glycerophospholipids) into the cell, which may indirectly imply that MlrD proteins indeed have a role in the transport of small molecules across the cell membrane for bacterial metabolism.

In summary, the key findings from this section of the study include: (1) bacteria were found with the ability to degrade [Dha^7]MC-LR and MC-LR as the sole carbon and nitrogen sources; (2) 16S RNA of the MC-degrading bacteria isolated were indistinguishable from a previously identified bacterium MD-1; (3) optimal degradation by NV-3 occured at 30^{o}C with bacterial concentration of 1.0x10^8 CFU/ml; (4) a MC concentration of 25 μg/ml was found optimum for degradation experiments; (5) the by-products A and B from biodegradation of [Dha^7]MC-LR and the detection of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes in NV-3 and NV-1, indicate that degradation of [Dha^7]MC-LR is via a similar mechanism for degradation of MC-LR as described by Bourne et al. (1996, 2001).

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Chapter 5

Effect of the bacterium *Sphingomonas* isolate NV-3 on the cyanobacterium *Microcystis aeruginosa* strain SWCYNO4

5.1 Abstract

A bacterium, *Sphingomonas* isolate NV-3, known to degrade the cyanobacterial hepatotoxin microcystin (MC) by enzyme action, was tested to determine whether or not it could also directly affect the cyanobacterium *Microcystis aeruginosa* strain SWCYNO4, in terms of growth inhibition and/or lysis of cells. The impact on the cyanobacterium, was evaluated by determining changes in chlorophyll-*a* (Chl-*a*) concentration of infected cultures, which would indicate possible death of *M. aeruginosa* cells, and/or growth inhibition. The bacterial isolate NV-3 did not have a direct lytic effect, nor growth inhibition, by either secretion of bacterial extracellular products or cell-to-cell contact between bacterial and cyanobacterial cells. Experiments also revealed that a control for testing bacterial media is crucial as the media alone may have an adverse effect on *M. aeruginosa* cells.

5.2 Keywords: inhibition growth effect, *Sphingomonas*, *Microcystis aeruginosa*

5.3 Introduction

The cyanobacterium *Microcystis* is well recognized as one of the most common bloom-forming cyanobacteria in freshwater environments. *Microcystis* has been reported to produce a wide range of toxic MC analogues, resulting in animal or human illness or even death, worldwide (Carmichael, 1992; Carmichael, 1994; Carmichael, 1996; Codd et al., 1999; Chorus & Bartram, 1999; Manage, Kawabata, & Nakano, 2000; Svrcek & Smith, 2004; Zurawell, Chen, Burke, & Prepas, 2005). Chemical algicides such as copper sulphate are commonly used to control *Microcystis*, but this approach is potentially damaging to the environment as the chemicals are non-selective and destroy other freshwater organisms in addition to *Microcystis*, and there is a risk of accumulation of MCs released into the environment

from lysed cells (Jones & Orr, 1994; Sigee et al., 1999; Manage, Kawabata, & Nakano, 2001). Therefore, the application of biological control, in the form of algicidal breakdown of cyanobacteria by naturally occurring aquatic bacteria is potentially a safer and more natural way to eliminate *Microcystis* cells (Sigee, 2005). The *Sphingomonas* isolate NV-3, known to be capable of degrading MCs, was tested to see whether or not it was able to directly or indirectly inhibit the growth, or destroy the cells of *M. aeruginosa* strain SWCYNO4.

5.3.1 *Microcystis*

Cyanobacteria from the *Microcystis* genus are unicellular, planktonic, gram-negative, and oxygenic photosynthetic organisms (Zurawell et al., 2005). Individual cells are cocci, normally united as colonies packed together by mucilage and the cells appear blackish under high bright field magnification and range from 3.2 to 6.6 μm in diameter (Skulberg, Carmichael, Codd, & Skulberg, 1993). The colonies are more or less firm, up to several millimeters across, sub-spherical and reticulated, elongated or lobated.

Microcystis is a non-nitrogen-fixing genus which usually causes cyanobacterial bloom under warm, calm, nutrient enriched water, although it may also form blooms in less polluted waters (Chorus & Bartram, 1999). In eutrophic to hyper-eutrophic waters, *Microcystis* blooms and surface scums are regularly extensive, persistent and in certain latitudes, dominant throughout the year (Watanabe, 1996). *Microcystis* is the most cosmopolitan of the cyanobacteria (Carmichael, 1996) and, especially *M. aeruginosa*, has been connected to human and animal illness worldwide. *Microcystis* was the first genus (from *M. aeruginosa* strain NRC-1) shown to produce the cyclic-peptide hepatotoxins (Bishop, Anet, & Gorham, 1959), later referred to as microcystins (MCs) (Carmichael et al., 1988). To date, more than 80 MC variants have been isolated (Zurawell et al., 2005) and about 60 variants of the toxins have been produced from species and strains of *Microcystis* (Rinehart, Namikoshi, & Choi, 1994; Park, Namikoshi, Brittain, Carmichael, & Murphy, 2001a). However, unlike other potentially toxigenic planktonic cyanobacteria, *Microcystis* is almost always toxic and produces more than one variant of MCs (Watanabe & Oishi, 1985). The occurrence of cyanobacterial blooms, and background information on cyanotoxins,

including the hepatotoxin MC produced by *Microcystis* have been discussed in detail in Chapter 2.

5.3.2 Control of *Microcystis* in freshwater bodies

The control of toxic cyanobacterial blooms, especially those dominated by *Microcystis* species, has become a major issue in water management around the world, especially where change in water quality, shift of composition and biomass of phytoplankton populations, and the acceleration of eutrophication and cyanobacterial blooms, has resulted from mainly human activities. Currently, there are three major approaches used to control *Microcystis* blooms in freshwater environments; nutrient limitation; direct eradication; and biological control (Manage et al., 2000; Sigee, 2005).

5.3.2.1 Nutrient limitation

In water management programmes, decreasing the inflow of dissolved inorganic (phosphorus and nitrogen) and organic nutrients (external nutrient loading) results in a low accumulation of *Microcystis* cells as well as other toxic cyanobacteria (Sigee, 2005). Reduction of nutrient loading is always the first step in attempting to reduce the effects of eutrophication, effectively removing the root cause of the cyanobacterial blooms, and signaling a return toward restoration of water quality. The process involves (1) catchment control by minimizing use of fertilizers (mainly nitrogen and phosphorus), increasing forestation and buffer vegetation and minimizing household pollution and soil erosion adjacent to water systems (2) use of wastewater treatment plants to diminish nutrient loading into water bodies and (3) application of registration to control nutrient concentration from agricultural and industrial sectors into natural waterbodies (Carmichael & Falconer, 1993). However, in some circumstances, reduction in external nutrient loading does not result in reduced cyanobacterial blooms due to internal loading of the nutrient accumulated in the sediment. For example, in Lake Trummen, USA, only a moderate change in *Microcystis* bloom took place after diminishing the external phosphorus-load, but the bloom considerably reduced after sediment removal by dredging (Cronberg, Gelin, & Larsson, 1975). In many water bodies where eutrophication is diminished, the bloom

of *Microcystis* may be replaced by other algae such as diatoms (*Asterionella* and *Fragillaria*), chrysophytes (*Dinobryon* and *Synura*) and dinoflagellates (*Ceratium*). With further nutrient-reduction, *Microcystis* blooms may disappear or remain at a reduced biomass after lake restoration (Visser, Walsby, Ibelings, & Mur, 2005).

5.3.2.2 Direct eradication

A wide range of methods can be used for the direct removal, destruction or growth inhibition of toxic cyanobacteria as well as *Microcystis*. One of the most common methods is to flush them out of the water system. Water flushing has been successful in reducing the amount of cyanobacteria, including *Microcystis* in lakes by draining the water or reducing the water retention time using alteration of the hydrology to increase the inflow and outflow rates (Sigee, 2005; Visser et al., 2005).

A rapid and efficient way to eradicate *Microcystis* blooms is the application of chemical algicides using copper salts (e.g. copper sulphate). This method is normally used to reduce cyanobacteria in small water bodies such as ponds and small lakes. The disadvantage of using copper sulphate is that it results in unfavorable release of intracellular toxins into the surrounding water (Carmichael & Falconer, 1993). The control of toxic cyanobacteria using copper sulphate was demonstrated by Jones and Orr (1994). The treatment of a *M. aeruginosa* bloom on Lake Centenary, Australia caused cell lysis and the release of MC into the water. Dissolved MC was detected within 24 hours of spraying and the toxins persisted at high levels (1300–1800 μg/l) for 9 days before they began to disappear.

Artificial mixing is used to inhibit cyanobacterial growth. In this process, a stream of air is released into the hypolimnion, breaking water stratification and circulating water as well as the colonies of *Microcystis* within the entire water column. Consequently, *Microcystis* colonies are no longer able to accumulate at the water surface and the reduced exposure to light results in lower rates of photosynthesis and growth rates and a shift of phytoplankton composition from *Microcystis* spp. to other algae such as diatoms or green algae (Visser et al., 2005; Sigee, 2005).

5.3.2.3 Biological control

Biological control or biocontrol is a short-term strategy to eradicate or inhibit the growth of toxic cyanobacteria, including *Microcystis* species. This strategy involves adding natural organisms (algicidal/algistatic organisms) into the surface waters where the *Microcystis* bloom is developing or has already developed. The algicidal/algistatic organisms have been shown to act as antagonists and thus play an important role in reducing *Microcystis* blooms in various aquatic ecosystems (Sugiura et al., 1993; Sigee et al., 1999).

The use of naturally-occurring organisms to control *Microcystis* blooms means that use of such organisms can be viewed simply as an alteration in balance of biota within the freshwater environment. When biological control agents are used in natural water, they inhibit the growth or lyse cells of *Microcystis* and will also themselves become subject to a wide range of environmental conditions resulting in the reduction of their populations to levels normally present. One benefit of using biological control (in contrast to chemical control) is that the organisms are specific to their target without any general adverse effect on other organisms in aquatic ecosystem (Sigee, 2005).

The action of these control organisms, ranges from highly specific parasitism and predation to non-specific release of extracellular lytic products. A wide range of aquatic organisms, including bacteria, viruses, fungi and zooplankton, have been shown to have the potential to control *Microcystis* blooms (Sugiura et al., 1993; Sigee et al., 1999) and the most well studied are the algicidal bacteria.

5.3.2.3.1 *Microcystis* control with bacteria

Aquatic bacteria are numerous and abundant in waterbodies especially when *Microcystis* is blooming (Imai, Ishida, & Hata, 1993). These bacteria, referred to as algicidal bacteria, can inhibit, or lyse *Microcystis* cells. Various genera of algicidal bacteria have been documented, including *Myxococcus* (Yamamota & Suzuki, 1990), *Cytophaga* (Daft & Stewart, 1971; Daft, McCord, & Stewart, 1975), Bdellovibrio-like bacterium (Caiola & Pellegrini, 1984), *Alcaligenes* (Manage et al., 2000), *Streptomyces* (Yakamoto et al., 1998; Choi, Kim, Kim, & Han, 2005), *Pseudomonas* (Kang, Kim, Kim, Kong, & Han, 2005), *Bacillus* (Nakamura, Nakano, Sugiura, &

Matsumura, 2003; Shunyu, Yongding, Yinwu, Genbao, & Dunhai, 2006; Mu, Fan, Pei, Yuan, Liu, & Wang, 2007) and *Sphingomonas* (Imamura et al., 2001; Hibayashi & Imamura, 2003).

Yamamota and Suzuki (1990) reported the lytic effect of the bacterium *Myxococcus fulvus* (S-1-8) on *Microcystis aeruginosa*, *M. wesenbergii* and *M. viridis*. This particular bacterium was able to lyse the *Microcystis* cells in both the water and sediments of Lake Suwa (eutrophic lake), Japan. Lytic activity occurred during growth of the organism with the maximum amount of activity produced at the end of the log phase of bacterial growth. However, detailed information about the relationship between the cyanobacteria and algicidal bacteria was not given.

Daft and Stewart (1971) isolated four strains of *Cytophaga* (strain CP 1-4) able to lyse over 40 strains of cyanobacteria, including strains of *Microcystis*. No extracellular products from the algicidal bacteria were detected during the experiment. Therefore, the bacterial surface contact was implicated as a significant mechanism for causing lysis of *Microcystis* cells. Lysis developed within 20 minutes of initial contact.

A study by Caiola and Pellegrini (1984) revealed that *M. aeruginosa* in a lake bloom was extensively infected by a Bdellovibrio-like bacterium. After attachment and penetration of *Microcystis* cells, the bacterium was localized mainly in the periplasmic space between host cell wall and cytoplasmic membrane. This was followed by decrease in the amount of the cytoplasm, disruption of internal membrane systems (including gas vacuoles and granular inclusion) and multiplication of the endoparasite to form infective daughter cells. These would ultimately be released into the environment upon fragmentation of the cell wall and bursting of the host cell.

A gliding bacterium, *Alcaligenes denitrificans* isolated from a hyper-eutrophic pond in Japan was able to cause cell lysis and death in some cyanobacterial species (e.g. *M. aeruginosa*, *M. wesenbergii* and *M. viridis*). The cyanobacteria were vulnerable to attack by the bacterium and the growth inhibition of *M. aeruginosa* was significant, especially when the cyanobacterium was in the exponential growth phase. *Alcaligenes denitrificans* was initially inoculated at low density of 10^3 cells/ml, together with the *Microcystis* species, and then the bacterium propagated to a bacterial density of 10^8 cells/ml, resulting in cyanobacterial cell lysis. *Microcystis aeruginosa*

died when *A. denitrificans* was added to the cyanobacterial culture, but not when only the filtrate from the bacterial culture was added. It suggests that normal extracellular products are not inhibitory to *M. aeruginosa* and that only direct contact between *A. denitrificans* and *M. aeruginosa* was lethal. However, this does not exclude the possibility, that when the two are together, *Microcystis* may induce the release of some soluble chemical from *A. denitrificans* which is lethal to *Microcystis.* Whatever the exact mechanism, it is clear that *A. denitrificans* plays an important role in influencing the growth of *Microcystis* species, and contributes to the death of *Microcystis* species in freshwater environments (Manage et al., 2000).

Eighty-three isolates of actinomycetes (a group of bacteria) were isolated from sediments of Lake Suwa, Japan. Approximately half of these isolates were able to lyse cyanobacteria and *Streptomyces phaeofaciens* strain S-9 demonstrated the highest ability. The strain S-9 grew well on lawns of living cyanobacteria and rapidly lysed *Microcystis* cells by producing compounds causing extensive lysis. The amino acid, L-lysine secreted by the isolate was found to be one cause of lysis by inducing severe damage to the cell wall (Yamamoto et al., 1998).

Another isolate of actinomycetes capable of eliminating the cyanobacterium *M. aeruginosa* was isolated from the sediment of a eutrophic lake (Lake Juam, Korea). This bacterium was identified as *Streptomyces neyagawaensis* on the basis of 16S rDNA sequences and biochemical and morphological characteristics. In the presence of *S. neyagawaensis*, the biomass of cyanobacteria *M. aeruginosa* NIES-298 and *M. aeruginosa* NIES-44 was greatly reduced. The anticyanobacterial activity of *S. neyagawaensis* depended on which growth phase the cyanobacterium was in, but not the growth phase of the algicidal bacterium. The anticyanobacterial activity of *S. neyagawaensis* was also effective against a wide range of toxic cyanobacteria from other genera, including *Anabaena cylindrica*, *Anabaena flos-aquae*, and *Oscillatoria sancta*. *Streptomyces neyagawaensis* indirectly attacked *M. aeruginosa* by secretion of extracellular anticyanobacterial substances that were localized in the bacterial periplasm (Choi et al., 2005).

Kang et al. (2005) isolated 76 strains of algicidal bacteria from surface water of the Pal'tang Reservoir, Korea using the algal lawn method. Isolate HYK0203-SK02 exhibited the strongest algicidal activity and was identified as *Pseudomonas*

putida based on 16S rDNA sequence (92% homology). The isolate inhibited the growth of *M. aeruginosa* as well as a diatom, *Stephanodiscus hantzschii*. The algicidal activity of the bacterium relies on cell-to-cell contact, with the bacteria rapidly besieging the cyanobacterial cells within 12 hours of initial contact.

At least three strains of algicidal bacteria from the genus *Bacillus* have been reported (Nakamura et al., 2003; Shunyu et al., 2006; Mu et al., 2007). *Bacillus cereus* (strain N-14), was isolated by the double layer method using the cyanobacterium, *Microcystis*, as a sole nutrient. This strain showed the highest cyanobacteriolytic activity of the three, by production of extracellular products to lyse *Microcystis* cells. Lytic assay tests of culture supernatants indicated that the main substances for lytic activity were non-proteinaceous, hydrophilic, heat stable agents with a molecular weight of less than 2 kDa. The highest lytic activity was obtained under alkaline conditions, which would be good for practical application of water bloom control in eutrophic lakes where the pH is usually in the alkaline region. The lytic substances of *B. cereus* N-14 were compared with enterotoxins, and an emetic toxin, produced by a pathogenic strain of *B. cereus*, and also with a known algicide produced by *Bacillus brevis*, namely gramicidin. Indications are that the lytic substance is a novel algicide. The second strain of *Bacillus cereus* (strain DC22) isolated from Lake Dianchi of Yunnan province, China, showed lytic activities against a wide range of cyanobacteria including *M. aeruginosa*, *M. viridis*, *M. wesenbergi*, *Aphanizomenon flos-aquae*, *Oscillatoria tenuis*, *Nostoc punctiforme*, *Anabaena flos-aquae*, *Spirulina maxima*, and the algae *Chlorella ellipsoidea* and *Selenastrum capricornutum*. *Bacillus cereus* rapidly attacked cyanobacterial cells by cell-to-cell contact mechanisms. It was shown that lysis began with the cell wall, and the cyanobacterial cell took on an abnormal appearance, becoming swollen with large vacuoles (Shunyu et al., 2006). *Bacillus fusiformis*, another species and strain, was able to lyse *M. aeruginosa* by secreting metabolites, which could withstand significant heat treatment. The efficiency of algicidal activity was dependant on the population size of *B. fusiformis*. When introduced into laboratory cultures or field samples, at least 3.6×10^7 cells/ml of *B. fusiformis* were required to cause significant cyanobacterial cell lysis (Mu et al., 2007). The reason for this is not well understood.

5.3.2.3.2 *Microcystis* control with fungi

Fungi also produce extracellular compounds which cause cyanobacterial lysis. The fungi *Acremonium*, *Emericellopis* and *Verticillium* produce an antibiotic substance called cephalosporin C, which lyses *Anabaena flos-aquae* and other cyanobacteria, including *Microcystis* species (Redhead & Wright, 1978). Species of planktonic cyanobacteria from the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Lyngbya* and *Gomphospaeria* are known to be attacked by chytridiaceous fungi (Fogg, Stewart, Fay, & Walsby, 1973; Raynolds, 2006). The seasonal variation in fungal pathogens of cyanobacteria is correlated directly with the abundance of the cyanobacteria themselves, that is, the fungi appear and disappear with the cyanobacteria.

5.3.2.3.3 *Microcystis* control with viruses

A large number of viruses exist in natural freshwater and it is suspected that a significant number of these viruses are infectious for cyanobacteria and other organisms (Bergh, Borsheim, Bratbak, & Heldal, 1989). Mortality induced by virus attack is one of the important factors that control *Microcystis* blooms (Brussaard, 2004; Yoshida, Yoshida, Takashima, Hosoda, & Hiroishi, 2007). The study of cyanophages (viral pathogens infecting cyanobacteria) has long been recognized but only a few cyanophage strains, including SM-1 (Safferman, Schneider, Steere, Morris, & Diene, 1969), SM-2 (Fox, Booth, & Martin, 1976) and MA 1 (Phlips, Monegue, & Aldridge, 1990), were reported to be lytic for *M. aeruginosa*. However, the *M. aeruginosa* RC-1 strain reported to be sensitive to SM-1 and SM-2 was later found to be a *Synechococcus* strain, so SM-1 and SM-2 are phages that also infect *Synechococcus* sp. Phlips et al. (1990) reported isolation of a lytic agent that formed plaques on lawns of an *M. aeruginosa* strain, however, the agent was not identified. Manage, Kawabata, & Nakano (1999) observed that an increase in cyanophage titers (the numbers of particles forming plaques on an *M. aeruginosa* lawn) was accompanied by a large decrease in the abundance of *M. aeruginosa* in a natural freshwater environment. Recently, two strains of podovirus-like phages (Tucker &

Pollard, 2005) and one strain of T4-like phage (Yoshida et al., 2006), inhibiting growth of *M. aeruginosa*, were isolated from natural lake waters collected during an *M. aeruginosa* bloom. In these reports, it was suggested that phage play an important role in regulating blooms of *Microcystis*.

5.3.2.3.4 *Microcystis* control with zooplankton

In aquatic ecosystems, control of *Microcystis* blooms using zooplankton predators, seems to be more effective than direct contact and production of extracellular inhibitory substances by algicidal bacteria (Sigee et al., 1999). Sugiura, Imamori, Sudo, Ouchiyama, & Miyoshi (1990) examined degradation characteristics of *M. aeruginosa* by the mastigophoran, *Manas guttula*, isolated from sewage in the biological oxidation facility, Kasumigaura, Japan. The removal of *M. aeruginosa* was 90% over 48 hours of cultivation. Moreover, Watanabe, Zhang, & Kaya (1996) found a chrysomonad *Poterioochromonas malhamensis* which had effectively ingested and digested all *Microcystis* cells as its main food source within 5 days.

Biological control of *Microcystis* cells by zooplankton, has also been demonstrated in experimental batch and continuous bioreactors, and within a water treatment plant. Inamori et al. (1998) established biological control of *M. viridis* by using predaceous zooplankton in batch culture experiments. They found that *M. viridis* were predated by zooplankton, namely the oligochaete *Aeolosoma hemiprichi* and the rotiferan *Philodina erythrophthalma*. The two zooplankton *A. hemprichi* (100 cells/l) and *P. erythrophthalma* (1000 cells/l) can remove 60% of 100 mg/l of the viable *M. viridis* cells in 14 days. Further research reported a species of the zooplankton which was responsible for *Microcystis* removal in a water treatment plant, Japan. Saito, Sugiura, Itayama, Inamori and Matsumura (2003) isolated a strain of the mastigophoran *Monas guttula* that could effectively predate the toxic genus of *Microcystis* sp. as a food source. In batch culture experiments, *Monas* spp. can grow and prey on *M. viridis* in spite of mixed culture conditions with many other organisms. The reduction of MC-RR, MC-YR and MC-LR produced by *M. viridis* was associated with the direct ingestion by *Monas* spp.

5.4 Objective of this chapter

The objective of this study, as reported in this chapter, was to determine whether or not the MC-degrading bacterium *Sphingomonas* isolate NV-3 could inhibit growth and/or lyse *Microcystis aeruginosa* strain SWCYNO4.

5.5 Methods

5.5.1 Microorganisms and culture conditions

The cyanobacterium *M. aeruginosa* strain SWCYNO4 was provided by Dr Susanna Wood from Cawthron Institute, Nelson, New Zealand. Cultures of the strain were grown in BG-11 broth medium (Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979) (see Appendix 12) in a shaking incubator at 25^{o}C and 100 rpm with fluorescent illumination at 1500 Lux, and under a 12L/12D cycle. To maintain the culture, the cyanobacterium was re-incubated (2% inoculum) in fresh BG-11 broth every 14 days under the same conditions. A 14-day culture of *M. aeruginosa* strain SWCYNO4 is typically in late exponential growth phase (see Section 5.5.2).

The bacterium *Sphingomonas* isolate NV-3 was cultured and maintained on PYEM medium at 30^{o}C as described in Sections 4.5.1 and 4.5.2.

5.5.2 Cyanobacterial growth curve

To obtain late exponential phase *M. aeruginosa* strain SWCYNO4 cyanobacteria for later experiments (in late exponential phase the cells are abundant and active), the cyanobacterial growth curve was determined. A cyanobacterial inoculum was prepared from a 14-day old culture, by diluting with sterile BG-11 broth until the culture had an optical density, at 750 nm, of 0.1 (OD_{750}). Two ml of the diluted culture (OD_{750} = 0.1) were transferred into 500 ml Erlenmeyer flasks containing 198 ml of BG-11 broth (2% v/v), and incubated in the shaking incubator under the same conditions described in 5.5.1. To obtain growth curves, each culture was sampled every 2 days for 20 days, and growth was monitored as a function of the optical density at 750 nm using a spectrophotometer (Appendix 12) with a 10 mm light path glass cuvette. At a wavelength of 750 nm, the pigments of cyanobacteria

exhibit negligible absorbance, and optical density obtained from this wavelength can be used as a measure of biomass (Ernst, Deicher, Herman, & Wollenzien, 2005). The experiment was carried out in triplicate.

5.5.3 Preparation of bacterial cells for growth inhibition assays

The *Sphingomonas* isolate NV-3 was prepared using two bacterial media, 1/10 nutrient broth (NB) (Imamura, et al., 2001) (see Appendix 12) and PYEM broth (medium previously used for culture of the bacterial isolate NV-3, see Chapter 4) (Jones, Bourne, Blakely, & Doelle, 1994). A single colony of the bacterium was inoculated into 5 ml of each broth and grown overnight in a shaking incubator at 200 rpm and 30°C. The overnight culture (2% v/v) was then inoculated into fresh lots of each broth, and incubated in the shaking incubator at 200 rpm and constant temperature of 30°C, for a further 36 h (36-h bacterial isolate NV-3 culture is typically in late exponential growth phase, see Fig. 4.6, Chapter 4). The 36-h culture from both 1/10 NB and PYEM broth, was subdivided and treated in three different ways in preparation for experiments to examine growth inhibition.

a) The 36 h culture derived from each medium (1/10 NB and PYEM) was directly harvested and later used without further modification (Section 5.5.5). This solution, labeled 'bacterial culture', contained bacterial cells, bacterial medium, and any extracellular products released from the *Sphingomonas* isolate NV-3.

b) The 36 h culture derived from each medium was centrifuged at 12,000 rpm for 5 min (4^{o}C). The supernatant was then filtered using 0.2 μm sterile filter to remove cellular material, and retained for later use (Section 5.5.5). This solution was labeled as 'bacterial supernatant', and contained bacterial medium and bacterial extracellular products.

c) The 36 h culture derived from each medium was centrifuged at 12,000 rpm for 5 min. The supernatant was decanted and the pellet resuspended with 0.05 M phosphate buffer, pH 7.0 (See Appendix 1). This washing process was repeated three times. The final pellet was resuspended in 5 ml of 0.05 M phosphate buffer, and the bacterial concentration was adjusted by adding sterile 0.05 M phosphate buffer until the OD at 600 nm was 1.0 (OD_{600} = 1.0). This suspension

was retained for later use (section 5.5.5), and labeled as 'bacterial cells', a solution containing only the cells.

5.5.4 Preparation of cyanobacterial cells for growth inhibition assays

Microcystis aeruginosa strain SWCYNO4 was prepared as described in section 5.5.2 resulting in a cyanobacterial culture with OD at 750 nm of 0.1 (OD_{750}). The 14-day old cyanobacterial culture was diluted with sterile BG-11 broth until the OD_{750} was 0.1, and used as described in section 5.5.5.

5.5.5 Growth inhibition effect of the bacterium *Sphingomonas* isolate NV-3 on *M. aeruginosa* strain SWCYNO4

The growth inhibition effect of the *Sphingomonas* isolate NV-3 on *M. aeruginosa* strain SWCYNO4 was established by observing the changes of chlorophyll-*a* (Chl-*a*) content in an infected culture over a 14-day period, using a method modified from Manage et al. (2000); Choi et al. (2005); Shunyu et al. (2006) and Mu et al. (2007). Chlorophyll-*a* determination was performed as described in the standard procedure of ISO 10260 (1992).

The experiments involved six treatments which reflected the three methods of preparing the two different bacterial media (1/10 NB and PYEM broth) (treatments 1-6), plus three controls (treatment 7-9) (see Table 5.1).

Table 5.1 Treatment conditions for growth inhibition effects of *Sphingomonas* isolate NV-3 on *M. aeruginosa* strain SWCYNO4

Treatment	Components
Treatment 1	60 ml of 'bacterial culture' from 1/10 NB + 540 ml of cyanobacterial culture
Treatment 2	60 ml of 'bacterial culture' from PYEM broth + 540 ml of cyanobacterial culture
Treatment 3	60 ml of 'bacterial supernatant' from 1/10 NB + 540 ml of cyanobacterial culture
Treatment 4	60 ml of 'bacterial supernatant' from PYEM broth + 540 ml of cyanobacterial culture
Treatment 5	60 ml of 'bacterial cells' from 1/10 NB + 540 ml of cyanobacterial culture
Treatment 6	60 ml of 'bacterial cells' from PYEM broth + 540 ml of cyanobacterial culture
Control 1	60 ml of sterile BG-11 medium + 540 ml of cyanobacterial culture
Control 2	60 ml of sterile 1/10 NB + 540 ml of cyanobacterial culture
Control 3	60 ml of sterile PYEM broth + 540 ml of cyanobacterial culture

5.5.5.1 Growth inhibition effect by 'bacterial culture' (bacterial cells, extracellular products and bacterial medium) of the bacterium *Sphingomonas* isolate NV-3 (treatment 1 and 2)

Sixty ml of 'bacterial culture' prepared as in Section 5.5.3a was inoculated with 540 ml of cyanobacterium culture obtained from 5.5.4 (10% v/v) (adapted from Shunyu et al., 2006). The infected culture was incubated for 14 days in a shaking incubator at 25^{o}C and 100 rpm with fluorescent illumination under the 12L/12D cycle. An aliquot (50 ml) of the infected culture was withdrawn after 0, 1, 3, 5, 7 and 14 days of incubation. The chlorophyll-*a* content was measured in each aliquot using a Spectrophotometer 2000 at OD_{750}. Any change in chlorophyll-*a* was presumed to be a measure of growth inhibition and/or lytic effect of *Sphingomonas* isolate NV-3 on *M. aeruginosa* strain SWCYNO4. To determine whether or not the effect of *Sphingomonas* isolate NV-3 was nutrient dependent, controls 1, 2 and 3 were carried out using bacterial and cyanobacterial media. All experiments were carried out in triplicate.

5.5.5.2 Growth inhibition effect by 'bacterial supernatant' (extracellular products and bacterial medium) (treatments 3 and 4)

The procedure was the same as described in section 5.5.5.1 except that 60 ml of 'bacterial supernatant' (see 5.5.3b) was used. As previously noted this approach is an adaptation of that used by Manage et al. (2000) and Choi et al. (2005).

5.5.5.3 Growth inhibition effect by 'bacterial cells' (treatment 5 and 6)

The procedure was the same as described in Section 5.5.5.1 except that 60 ml of 'bacterial cells' (see 5.5.3c) was used. As previously noted this approach is an adaptation of that used by Manage et al. (2000) and Choi et al. (2005).

5.5.6 Effect of different 'bacterial cells' concentrations, and different volumes of 'bacterial culture' and 'bacterial supernatant' of the bacterium *Sphingomonas* isolate NV-3 on growth inhibition of *M. aeruginosa* strain SWCYNO4

An additional study of growth inhibition effect was investigated. Different 'bacterial cells' concentrations (OD_{600} = 0.1, 0.3, 0.5, 1.0 and 1.5) and different volumes of 'bacterial culture' and 'bacterial supernatants' of the *Sphingomonas* isolate NV-3 (5, 10, 15 and 20% v/v) in the infected culture were used to determine whether or not simply increasing bacterial concentration and volume of extracellular products in the culture had an effect on growth as it was suspected that the ability to inhibit *M. aeruginosa* growth may be weakened by a large volume of cyanobacterial culture. The experiment was carried out in triplicate along with controls.

5.5.6.1 Effect of different volumes of 'bacterial culture' of the bacterium *Sphingomonas* isolate NV-3 on growth inhibition

The process for the preparation of bacterial cells was similar to that described in section 5.5.3 except:

1) One tenth NB (1/10 NB) was the only medium used because PYEM medium was shown later (in Section 5.5.5) to have a direct effect on cyanobacterial growth.

2) The volume of 'bacterial culture' of *Sphingomonas* isolate NV-3 obtained from 5.5.3a, and the volume of cyanobacterial culture obtained from 5.5.4 were varied, as follows:

a) 5% v/v 'bacterial culture' (30 ml of 'bacterial culture' and 570 ml of cyanobacterium culture).
b) 10% v/v 'bacterial culture' (60 ml of 'bacterial culture' and 540 ml of cyanobacterium culture).
c) 15% v/v 'bacterial culture' (90 ml of 'bacterial culture' and 510 ml of cyanobacterium culture).
d) 20% v/v 'bacterial culture' (120 ml of 'bacterial culture' and 480 ml of cyanobacterium culture).

5.5.6.2 Effect of different volumes of 'bacterial supernatant' of *Sphingomonas* isolate NV-3 on growth inhibition

The procedure is similar to section 5.5.6.1 except that the 'bacterial supernatant' from 1/10 NB was used instead of 'bacterial culture' with 5, 10, 15 and 20% v/v of 'bacterial supernatant'.

5.5.6.3 Effect of different 'bacterial cells' concentration of *Sphingomonas* isolate NV-3 on growth inhibition

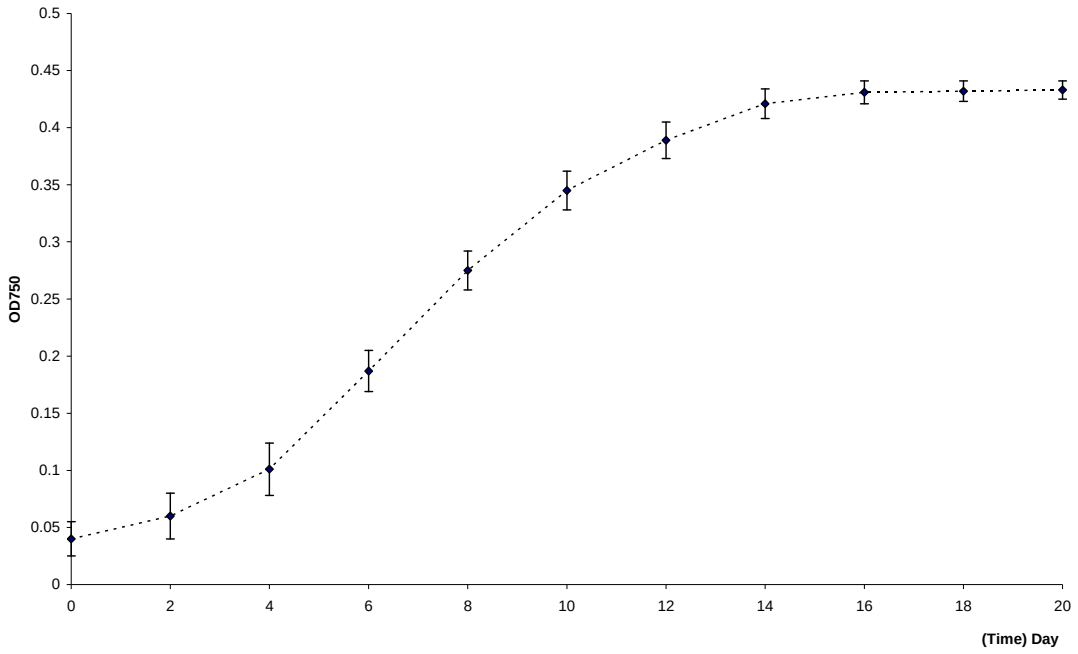
The procedure is identical to that described in section 5.5.5.3 except that the final pellet of 'bacterial cells' extracted as described in section 5.5.3c was prepared and adjusted to 5 different bacterial concentrations, by adding sterile 0.05 M phosphate buffer solution until the required ODs at 600 nm were 0.1, 0.3, 0.5, 1.0 and 1.5, respectively [OD_{600} = 0.1 (bacterial concentration approximately $7.9x10^6$ CFU/ml), OD_{600} = 0.3 ($2.5x10^7$ CFU/ ml), OD_{600} = 0.5 ($4.9x10^7$ CFU/ml), OD_{600} = 1.0 ($1.0x10^8$ CFU/ml) and OD_{600} = 1.5 ($1.45x10^8$ CFU/ml)]. Then, 60 ml aliquots from these cultures of differing bacterial concentrations were individually mixed with 540 ml of cyanobacterial culture obtained from 5.5.4 to examine growth inhibition effect.

5.6 Results

5.6.1 Cyanobacterial growth curve assays of *M. aeruginosa* strain SWCYNO4

A change in OD_{750} reflected change in biomass of bacteria in the culture. The growth represented by change in OD_{750} followed the classic s-shaped growth curve (Figure 5.1). For the first two days, growth was relatively slow, representing the lag phase. From day 4 to 14, there was a rapid change in OD_{750}, representing the log or exponential phase of cyanobacterial growth. From days 16 to 20, the OD_{750} remained relatively unchanged, representing the stationary phase.

Figure 5.1 Growth curve of *M. aeruginosa* strain SWCYNO4 using optical density of 750 nm [OD_{750}]. Each point represents ± Standard Deviation (S.D.) of the three replicates.



5.6.2 Growth inhibition effect by bacterial cells, extracellular products and bacterial media of *Sphingomonas* isolate NV-3

The bacterium *Sphingomonas* isolate NV-3 was tested against cyanobacterium *M. aeruginosa* strain SWCYNO4 using (1) the 'bacterial culture' (2) 'bacterial supernatant' and (3) the 'bacterial cells'. The changes in chlorophyll-*a* content of the algal cultures indicated that only the 'bacterial culture' and the 'bacterial supernatant'

of *Sphingomonas* isolate NV-3 cultured from PYEM broth affected the growth of *M. aeruginosa* strain SWCYNO4 (decrease of chlorophyll-*a* content) (Figure 5.2). However; control 3 containing only sterile PYEM broth also revealed a decrease of chlorophyll-*a* content (Figure 5.2), whereas control 1 (sterile BG-11 broth only) and control 2 (sterile 1/10 NB only) did not affect the growth of *M. aeruginosa* (chlorophyll-*a* content increased over the time) (Figure 5.3). Therefore, the growth inhibition effect of the 'bacterial culture' and the 'bacterial supernatant' on *Sphingomonas* isolate NV-3 cultured in PYEM broth, most likely resulted from the PYEM broth itself and thus no growth inhibition effect of *Sphingomonas* isolate NV-3 was found on the growth of *M. aeruginosa* strain SWCYNO4.

Figure 5.2 Growth inhibition effect of *Sphingomonas* isolate NV-3, as reflected in changes in the content of chlorophyll-*a*, on *M. aeruginosa* strain SWCYNO4 cultured from PYEM. Each point represents ± standard deviation (S.D.) of three replicates.

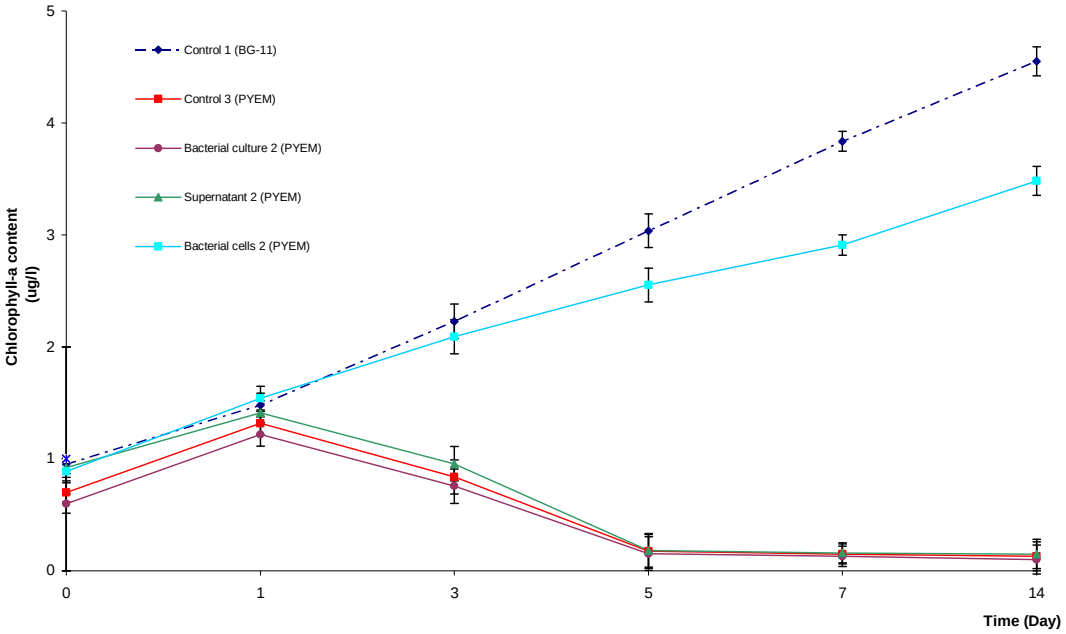
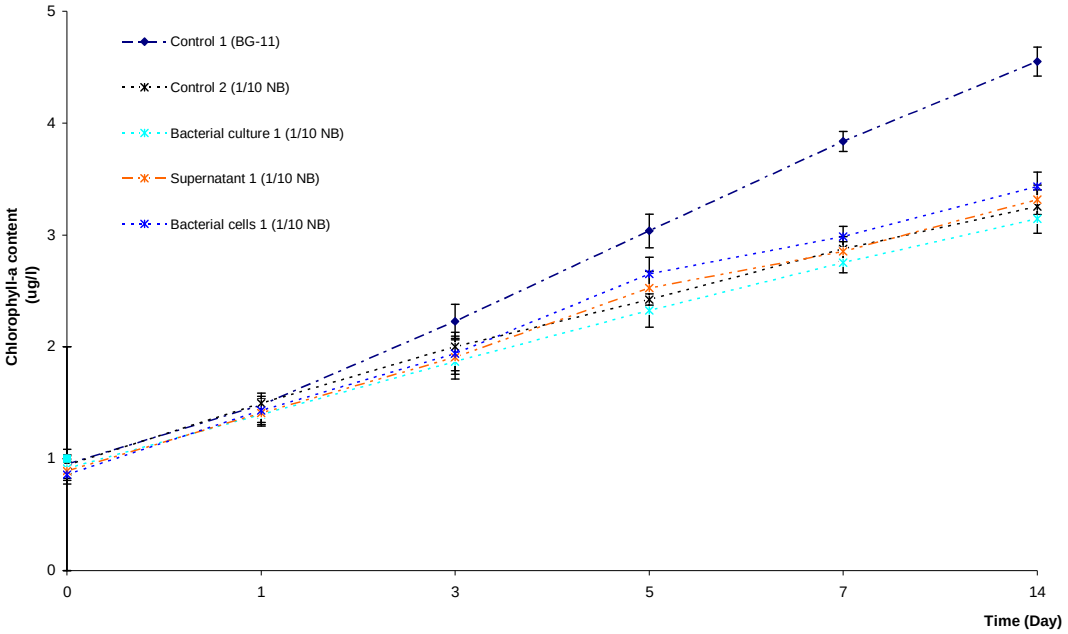


Figure 5.3 Growth inhibition effect of *Sphingomonas* isolate NV-3, as reflected in changes in the content of chlorophyll-*a*, on *M. aeruginosa* strain SWCYNO4 cultured from 1/10 NB. Each point represents ± S.D. of three replicates.



5.6.3 Effect of the volume of 'bacterial culture' and 'bacterial supernatant' of *Sphingomonas* isolate NV-3 on cyanobacterial growth

The effect of the *Sphingomonas* isolate NV-3 on the *M. aeruginosa* strain SWCYNO4 using different volumes of the culture and supernatant of *Sphingomonas* isolate NV-3, was examined. Results revealed that no inhibitory effect of *Sphingomonas* isolate NV-3 was found on the growth of *M. aeruginosa* strain SWCYNO4 (Figure 5.4 and 5.5). In addition, it was also found that more than 15% v/v of sterile 1/10 NB (used as controls) caused growth inhibition of the *M. aeruginosa* strain SWCYNO4, whereas 5 and 10 % v/v did not affect the growth of the cyanobacterium (Figure 5.4 and 5.5). That is, the medium on its own, is having an inhibitory effect on cyanobacterial cell growth, which has significant implications for similar future experiments. Therefore, 5-10 % v/v of 1/10 NB are appropriate for examining growth inhibition effects on *M. aeruginosa* strain SWCYNO4.

Figure 5.4 Effect of different volumes of 'bacterial culture' of *Sphingomonas* isolate NV-3 on growth of *M. aeruginosa* strain SWCYNO4, as reflected in changes in the content of chlorophyll-*a*. Each point represents ± S.D. of three replicates.

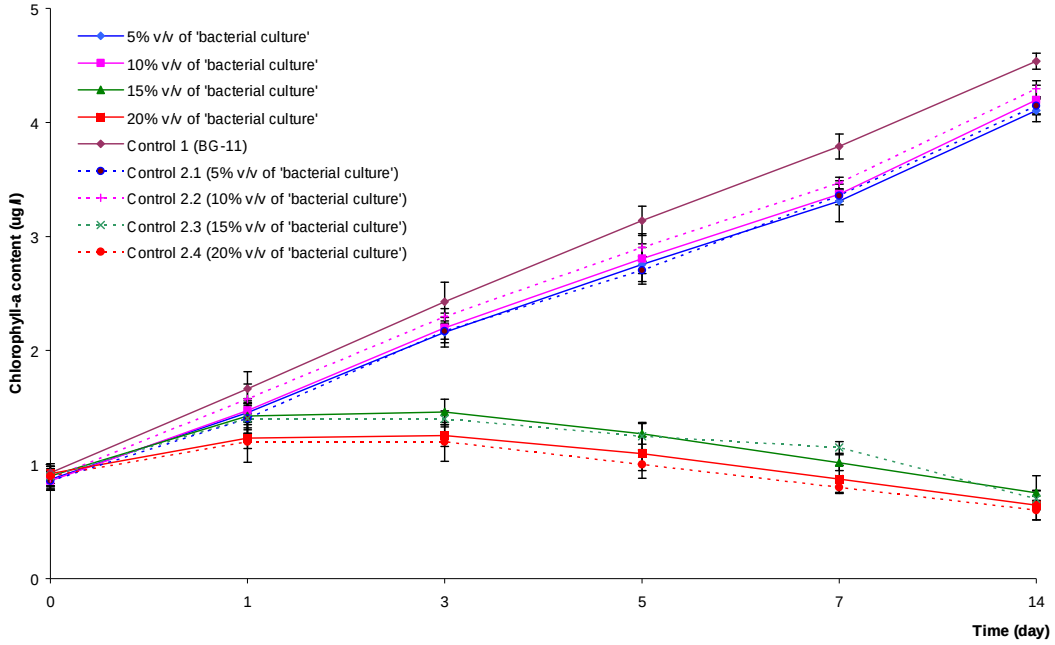
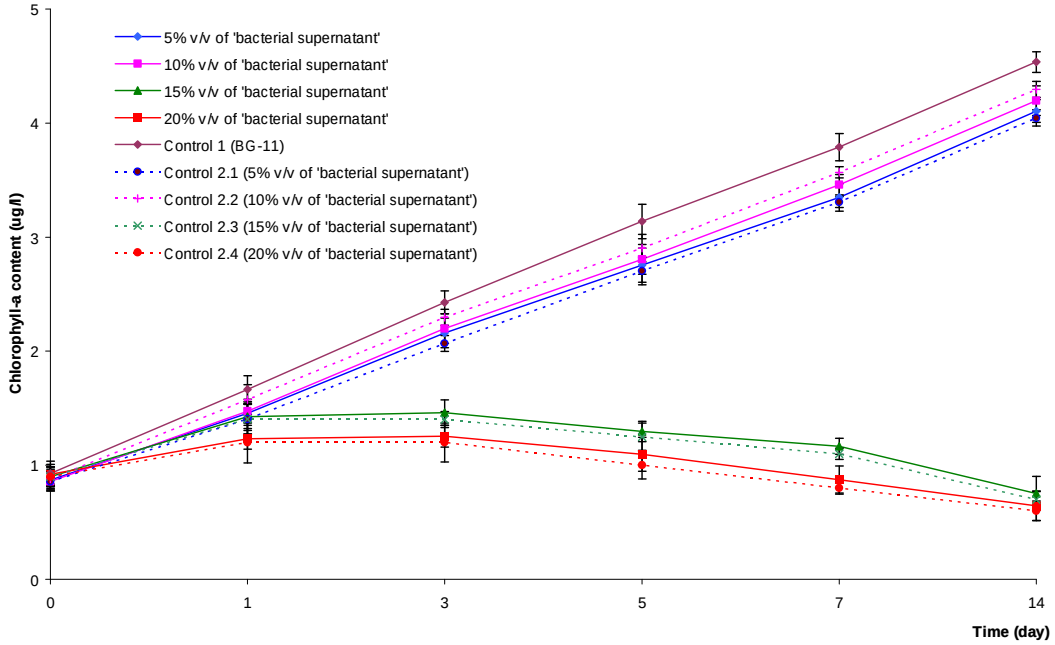


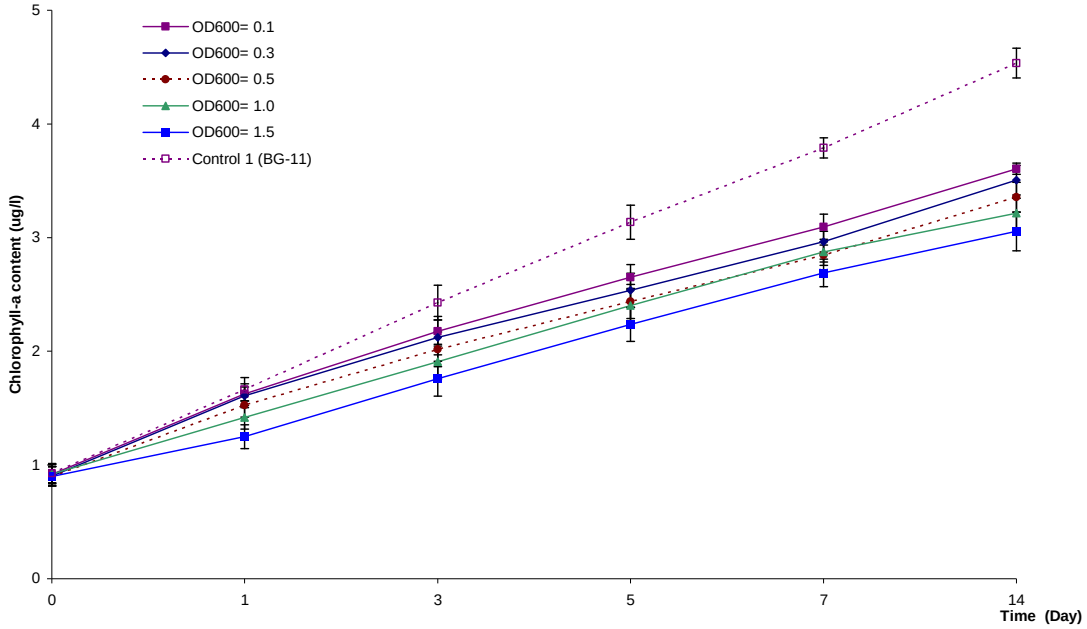
Figure 5.5 Effect of different volumes of 'bacterial supernatant' of *Sphingomonas* isolate NV-3 on growth of *M. aeruginosa* strain SWCYNO4, as reflected in the content of chlorophyll-*a*. Each point represents ± S.D. of three replicates.



5.6.4 Effect of 'bacterial cells' concentration on growth inhibition effect

Growth inhibition effect on *M. aeruginosa* strain SWCYNO4 was tested again using washed 'bacterial cells' of *Sphingomonas* isolate NV-3 at 5 different concentrations of the bacterium. Results showed that even the highest initial bacterial concentration (OD_{600} = 1.5) provided only a slight growth inhibition effect on *M. aeruginosa* strain SWCYNO4, varying very little from that of the lowest initial 'bacterial cell' concentration (OD_{600} = 0.1) (Figure 5.6). Therefore, there was no significant measured effect of differing 'bacterial cells' concentrations on the growth of *M. aeruginosa* strain SWCYNO4.

Figure 5.6 Effect of different 'bacterial cells' concentrations of *Sphingomonas i*solate NV-3 on growth of *M. aeruginosa* strain SWCYNO4, as reflected in the content of chlorophyll-*a*. Each point represents ± S.D. of three replicates.



5.7 Discussion

As part of considering the potential benefit of using MC-degrading bacteria in the management of cyanobacterial blooms, *Sphingomonas* isolate NV-3, known to be capable of degrading MCs, was also examined for its ability to inhibit growth or break down whole *Microcystis* cells. It was shown that the isolate NV-3 had no significant effect on the growth of the cyanobacterium (*M. aeruginosa* strain SWCYNO4). In general two main approaches are involved in the control (inhibition) of *Microcystis* cells as follows: (1) contact lysis which involves the activity of lysozyme-like enzymes located at the algicidal bacterial surface to degrade the peptidoglycan of *Microcystis* cell wall and (2) production of anti-cyanobacterial substances such as enzymes, antibiotics and very low molecular weight volatiles (Yamamota & Suzuki, 1970; Daft & Stewart, 1971; Caiola & Pellegrini, 1984; Sigee et al., 1999; Sigee et al., 2005; Steffensen et al., 1999; Manage et al., 2001; Choi et al., 2005; Pei, Hu, Qu, Mu, & Li, 2005; Visser et al., 2005; Mu et al., 2007). In this study, no adverse effects on the growth of *M. aeruginosa* cells by *Sphingomonas* isolate NV-3 were observed, either from cell-to-cell contact mechanisms and/or secretion of anti-cyanobacterial substances. Similarly variations in bacterial cell concentrations and volumes of bacterial culture had no noticeable effects. In broad terms there are two possible explanations for these results:

(1) Quite simply *Sphingomonas* isolate NV-3 may not actually possess the capability to inhibit or break down *M. aeruginosa* cells, or

(2) The *Sphingomonas* isolate NV-3 had the capability but:

   (i) the bacterial cells lost the ability to inhibit or break down the *Microcystis* cells because genes responsible for these abilities (cell lysis and production of anti-cyanobacterial substances) were switched off or inactive because the bacterial cells had not been exposed to *M. aeruginosa* cells for a long time (Manage et al., 2000);

   (ii) the bacterial cells had the ability to inhibit *M. aeruginosa* cells by secretion of extracellular products (algicidal/algistatic compounds), but the compounds released from the cell were diluted by relatively large volumes of bacterial culture (Imamura et al., 2001), resulting

in reduced capability of inhibiting *M. aeruginosa* cells and thus affects not being obvious.

Therefore, co-cultures between *Sphingomonas* isolate NV-3 and *M. aeruginosa* cells and condensed 'bacterial culture' and 'bacterial supernatant' could be carried out in the future, to investigate the effect of *Sphingomonas* isolate NV-3 on *M. aeruginosa* cells, following exposure to one another. This was deemed beyond the scope of the present study.

There has been a significant increase in the number of reports on the broad capabilities of strains of *Sphingomonas* species. These microorganisms have great potential for biotechnological applications in the biodegradation of water pollution (e. g. insecticides [Nagata, Miyauchi, & Takagi, 1999], herbicides [Adkins, 1999; Kohler, 1999], aromatic hydrocarbons [Bastiaens et al., 2000], MCs [Park et al., 2001; Ishii, Nishijima, & Abe, 2004; Saitou et al., 2003; Tsuji, Asakawa, Anzai, Sumino, & Harada, 2006; Valeria, Ricardo, Stephan, & Alberto, 2006]). Therefore, it would be interesting for future study to investigate whether or not the bacterium *Sphingomonas* isolate NV-3 can be used as biological control for other organisms e.g. pathogenic microorganisms or other toxic cyanobacterial strains (e.g. *Anabaena*, *Anabaenopsis*, *Planktothrix*, *Aphanizomenon*, and *Cylindrospermopsis*), as it has been shown that bacterial agents are target selective and specific for particular microorganisms (Rashidan & Bird, 2001). *Sphingomonas* strains are able to produce extracellular compounds that inhibit phytopathogenic fungi *Verticillium dahliae* (Berg & Balin, 1994), and *Sphingomonas* M-17 was shown to lyse *Microcystis* cells by producing anticyanobacterial compounds effective against *M. viridis* NIES-20 (Yamaguchi et al., 2003).

Sphingomonas sp. strain M-17 was isolated from Lake Biwa, Japan. This bacterium produced anticyanobacterial compounds, referred to as argimicin A, B and C (Imamura et al., 2000; Imamura et al., 2001; Yamaguchi, Kobayashi, Adachi, & Imamura, 2003). Argimicin A has been well studied and has stronger activity than argimicin B and C, about 2 and 10 times respectively. The argimicin A (pentapeptide) exhibited strong and highly selective activity against *M. viridis* NIES-20, and such selectivity is considered to be rare. The compound showed a unique delayed action. For example, the cyanobacterial cell division continued until at least 36 hours after

treatment even though the decrease of oxygen evolution was observed at 24 hours after treatment. It is concluded that argimicin A is a photosynthetic inhibitor which interrupts electron transport chain prior to photosystem II. The site of action was speculated to be photo energy transfer from a cyanobacterial specific complex of accessory protein pigments, phycobilisome, to photosystem II (Hibayashi & Imamura, 2003).

In this study, it was very exciting in initial pilot test runs when the results showed that the 'bacterial culture' and 'bacterial supernatant', prepared using PYEM, had a marked effect on cyanobacterial growth, with BG-11 medium (cyanobacterial medium) as the control. The BG-11 cyanobacterial medium is normally used as the control in many research studies to investigate growth inhibition effects of bacteria on cyanobacteria (Manage et al., 2000; Walker & Higginbotham, 2000; Nakamura et al., 2003; Hare et al., 2005; Shunyu et al., 2006). Initially, in this aspect of the study, PYEM was the only medium used for preparation of 'bacterial cells', 'bacterial culture' and 'bacterial supernatant' since the medium had been previously successfully used for the work described in Chapter 4.

However, when the ingredients of PYEM were compared with 1/10 NB used by Imamura et al. (2001) (1/10 NB was used for screening anti-*Microcystis* compounds from *Sphingomonas* sp. strain M-17), it was found that PYEM contained higher contents of peptone and yeast extract than 1/10 NB (PYEM containing 10 g peptone, 5 g yeast extract per liter and 1/10 NB containing 1 g peptone, 1 g yeast extract per liter). Therefore, it became evident that the growth inhibition effect of *Sphingomonas* isolate NV-3 on *Microcystis* cells was simply a result of the PYEM medium itself. For this reason, subsequent experiments were set up very carefully as follows: (1) all experiments repeated again with sterile PYEM as additional control and (2) 1/10 NB was also used as an additional medium for preparation of 'bacterial cells', 'bacterial culture' and 'bacterial supernatant' to examine possible growth inhibition effects of the bacterium on *M. aeruginosa* cells. In addition fresh 1/10 NB itself was also tested on *Microcystis* to ensure that the medium itself had no effect on the cyanobacterial cell growth. In summary, there was no direct effect of *Sphingomonas* isolate NV-3 on *M. aeruginosa* cells (in terms of noticeable growth inhibition). PYEM, on its own, had a significant negative effect on growth of *M.*

aeruginosa. Concentrations of 1/10 NB above 15% v/v also had an inhibitory effect on growth. Therefore, for future growth inhibition experiments the choice of bacterial media is crucial, and the controls for growth inhibition effects should include both cyanobacterial media and bacterial media. There may be value in further studies which establish the active ingredient of PYEM, that is capable of inhibiting cyanobacterial growth, and its potential use as a control agent.

5.8 References

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Chapter 6

Biodegradation of [Dha^7]MC-LR and MC-LR by a microcystin-degrading bacterium *Sphingomonas* isolate NV-3 in an internal airlift loop ceramic honeycomb support bioreactor

6.1 Abstract

Two types of biofilm formation assays, namely, a microtiter plate assay and coupon biofilm formation assay, were used to investigate whether or not the bacterium *Sphingomonas* isolate NV-3 was able to form a biofilm on abiotic surfaces. This was in preparation for using this bacterium in a bioreactor for biodegradation of [Dha^7]MC-LR and MC-LR. It was established that the isolate NV-3 is a moderate biofilm former. It attached most effectively to ceramic followed by PVC, polystyrene, stainless steel, and finally glass coupons. Using scanning electron microscopy (SEM), it was demonstrated that the biomass of the bacterium increased steadily over time from 24 to 72 hours on the ceramic surface. A ceramic surface was therefore chosen as the carrier support in the bioreactors.

Biodegradation of MCs by the bacterium *Sphingomonas* isolate NV-3 in an internal airlift loop ceramic honeycomb support bioreactor (IAL-CHS bioreactor) was established in batch and continuous-flow experiments. In the batch experiment, NV-3 degraded a combination of [Dha^7]MC-LR and MC-LR at an initial concentration of 25 μg/ml at 30^{o}C in 30 hours whereas in the continuous-flow experiment, NV-3 degraded the same concentration of [Dha^7]MC-LR and MC-LR in 36 hours with hydraulic retention time (HRT) of 8 hours.

6.2 Keywords: biodegradation, microcystins, IAL-CHS bioreactor

6.3 Introduction

Cyanobacterial microcystins (MCs) are increasingly becoming a threat to human health around the world (Carmichael, 1992; Skulberg, Carmichael, & Codd, 1993, Sivonen & Jones, 1999; Chorus, 2001; Svrcek & Smith, 2004; Codd, Morrison, & Metcalf, 2005). Microcystins caused the death of 56 patients during a dialysis treatment in Caruaru, Brazil (Jochimsen et al., 1998), and are considered as a possible

cause of primary liver cancer in China, where people are exposed long-term to the toxins through drinking water (Yu et al., 1995; Ueno et al., 1996). Microcystins are stable and resistant to conventional water treatment processes (Botes et al., 1984; Harada, Murata, Qiang, Suzuki, & Kondo 1996b, Lawton & Robertson, 1999). Simple water treatment, using filtration processes and an additional flocculation step, has been shown to be ineffective in removing dissolved MCs. Only complicated treatment using optimum dose ozonation, chlorination and activated carbon can reduce the concentration of MCs (Himberg, Keijola, Hiisvirta, Pyysalo, & Sivonen, 1989; Lahti & Hiisvirta, 1989; Lambert, Holmes, & Hrudey, 1996; Lawton & Robertson, 1999; Hitzfeld, Höger, & Dietrich, 2000; Grützmacher, Bottcher, Chorus, & Bartel, 2002; Hoeger, Hitzfeld, & Dietrich, 2005). However, when cyanobacteria are blooming the capacity of water treatment plants, even with the most up to date technology, may be overwhelmed (Hoffman, 1976; Keijola, Himberg, Sivonen, & Hiisvirta, 1988). Biological treatment processes, using bacterial degradation, could be an alternative option for removal of the toxins from the water.

Many studies have reported degradation of microcystin by natural bacteria (Jones, Bourne, Blakely, & Doelle, 1994; Rapala, Lahti, Sivonen, & Niemelä, 1994; Cousins, Bealing, James, & Sutton, 1996; Lahti, Rapala, Fardig, Niemela, & Sivonen 1997; Chorus & Bartram, 1999). Bacterial strains belonging to the genera *Sphingomonas*, *Pseudomonas*, *Sphingosinicella*, *Sphingopyxis*, *Paucibacter* and *Burkholderia*, have been reported to degrade MCs, and have in each case been isolated and characterized (Jones et al., 1994; Takenaka & Watanabe, 1997; Park et al., 2001; Maruyama et al., 2003; Saito et al., 2003; Harada et al., 2004; Ishii, Nishijima, & Abe, 2004; Rapala et al., 2005; Maruyama et al., 2006; Valeria, Ricardo, Stephan, & Alberto, 2006; Lemes et al., 2008). The potential for biological processes to remove MCs in water treatment plants has stimulated significant research in this area. A range of small scale experiments have been carried out using bacteria to degrade MC-contaminated water using bacterial cells immobilized on (i) polyester resin in a small scale bioreactor (Tsuji, Asakawa, Anzai, Sumino, & Harada, 2006), (ii) biologically granular activated carbon columns (Wang, Ho, Lewis, Brookes, & Newcombe, 2007) and (iii) biologically active sand filters (Ho et al., 2006; Bourne et al., 2006; Ho, Hoefel, Saint, & Newcombe, 2007). These biological degradation

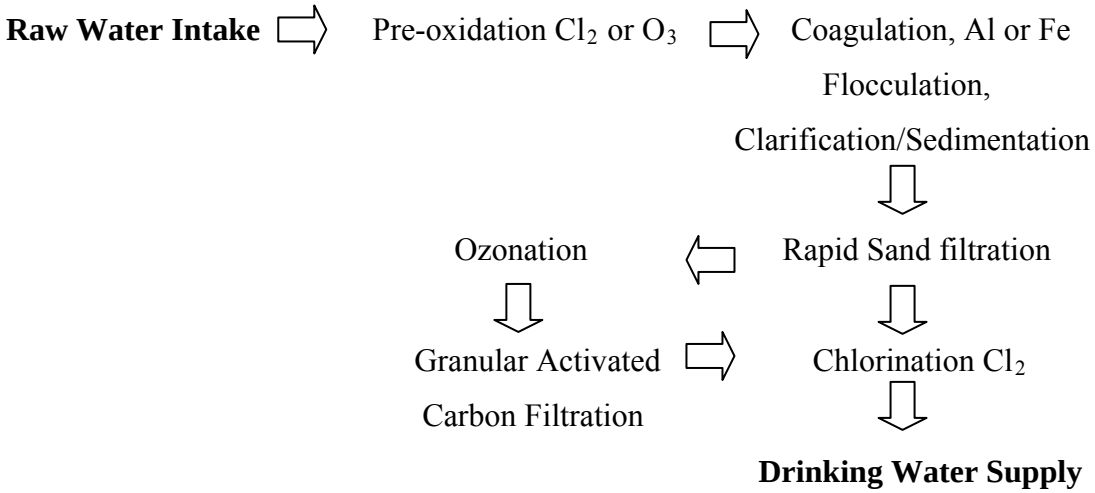
methods were successful in removing MCs completely from the contaminated water and have potential for larger scale application.

In this study an internal airlift loop ceramic honeycomb support bioreactor (IAL-CHS bioreactor), was used for removing MCs using the bacterial isolate NV-3. This type of bioreactor has been used to successfully remove 2, 4-dichlorophenol and phenol, high-carbohydrate, printing ink, and quinoline from wastewater (Zhang et al., 2002a; Zhang, Shi, & Qian, 2002b; Quan, Shi, Zhang, Wang, & Qian, 2003; Quan, Shi, Zhang, Wang, & Qian, 2004; Zhang et al., 2004; Zhang et al., 2005).

6.3.1 Water treatment processes

Water treatment is the process of removing undesirable compounds such as chemical and biological contaminants from water. The aim is to produce good quality water for a wide variety of purposes such as drinking water, household, industrial, medical and other applications. In drinking water treatment, the process generally involves physical methods (e.g. sedimentation and filtration), biological methods (e.g. slow sand filtration), chemical methods (e.g. coagulation, flocculation, and chlorination), and the application of electromagnetic radiation (e.g. ultraviolet light) (Crittenden, Trussell, Hand, Howe, & Tchobanoglous, 2005; Hendricks, 2005). Two alternative drinking water treatments are shown in Figure 6.1, one is a basic and conventional process, and the other more sophisticated, including ozone and activated carbon filtration (Falconer, 2005).

Figure 6.1 Fundamental process of a drinking water treatment plant (Falconer, 2005)



6.3.2 Water treatment processes for removal of MCs

Microcystins, intracellular toxins in healthy cyanobacterial cells, are only released into the environment when cells lyse or die (van Apeldoorn, Egmond, Speijers, & Bakker, 2007). The toxins enter water treatment plants as dissolved compounds in the raw water and within cyanobacterial cells. Therefore, water treatment systems must remove the living cyanobacterial cells as well as their toxins from the raw water (Lawton & Robertson, 1999). It is the dissolved component which is the most difficult to remove in water treatment plants. The focus of this study is the removal of MCs dissolved in water, the removal of cyanobacterial cells containing MCs will only be considered briefly.

6.3.2.1 Water treatment processes for cyanobacterial cell removal

A simple way of greatly reducing the total MCs in a water body is by removal of cyanobacterial cells at the early stages of water treatment using barriers to restrict scum movement or skimming devices to collect the cell masses. There are other methods for removal of cyanobacterial cells designed to reduce cell lysis and subsequent toxin release (Lawton & Robertson, 1999; Hitzfeld et al., 2000). Conventional water treatment using agglomeration (coagulation/flocculation) and filtration is effective in removing cyanobacterial cells, but dissolved MCs released from the cells, are not efficiently removed by these methods (Hitzfeld et al., 2000). The best chemicals for agglomeration are those which avoid cell lysis and the release of the toxins to water (Hart, Fawell, & Croll, 1997). Agglomeration involves destabilization of particles, such as cyanobacterial cells, by neutralizing their surface charge causing aggregation into larger masses. Chemicals such as iron-based and aluminum-based compounds are used (Drikas, Chow, House, & Burch, 2001). Chow et al. (1998) revealed that flocculation using ferric chloride did not cause cyanobacterial lysis, or an increase in dissolved MC concentration for *Microcystis aeruginosa* and *Anabeana circinalis*. In addition, there is an optimum concentration of these chemicals for removing cyanobacterial cells without triggering cell lysis, and subsequent increase in extracellular toxin concentration (Hoeger et al., 2005).

Filtration is an important step in removing cyanobacterial cells and particles. The most commonly used filters are granular media such as coarse sand, crushed

anthracite coal, or garnet (Lepisto, Lahti, & Niemi, 1994). In full-scale water treatment plants, rapid sand filtration, a method typically used after agglomeration, achieves only about 14% removal of cyanobacterial cells (Watanabe, 1996), whereas slow sand filtration is shown to effectively remove >85% of cyanobacterial cells as well as significant amounts of their toxins e.g. MC-LR, MC-RR and MC-YR (removal rates of 43% to 99%) (Grützmacher et al., 2002). However, in some water treatment plants, filtration is used without agglomeration, and the cyanobacterial cells, if present at high concentrations, may simply overwhelm the filtration system (Drikas et al., 2001). A filter that is coarse enough to prevent rapid clogging, will not effectively retain the cyanobacterial cells, whereas a filter fine enough to retain the cells will clog quickly resulting in short run times. Therefore, filtration alone is not recommended as the sole process for removing cyanobacterial cells (Petrusevki, Vlaski, van Breeman, & Alaerts, 1993; Drikas et al., 2001).

Flotation, an alternative water treatment method to sedimentation, is an efficient method for removal of low-density floc particles, including cyanobacterial cells. Dissolved-air flotation (DAF) is one of the most commonly used methods to remove cyanobacterial cells with 40-80% removal of *Microcystis* cells (Drikas et al., 2001). The removal is achieved by releasing air at atmospheric pressure in a flotation tank or basin, creating a stream of tiny bubbles which adhere to suspended matter or cells causing them to float or form surface scums on water bodies where they may then be removed by skimming devices (Hitzfeld et al., 2000; Wang, Hung, Lo, & Yapijakis, 2004).

6.3.2.2 Water treatment processes for dissolved MC removal

Conventional water treatment processes such as coagulation, sedimentation and filtration steps do not completely remove dissolved MCs (Hoffman, 1976; Keijola et al., 1988; Himberg et al., 1989; Lahti & Hiisvirta, 1989; Lambert et al., 1996; Grützmacher et al., 2002; Hoeger et al., 2005). Only advanced processes of water treatment such as photolysis, ozonation, activated carbon filtration and chlorination can eliminate dissolved MCs from water, and these effective water treatment processes are reviewed below.

6.3.2.2.1 Photolysis

As mentioned in Chapter 4, MCs remain relatively stable when irradiated by sunlight but are rapidly broken down by ultraviolet (UV) light around their absorption maxima of 238–254 nm (Tsuji et al., 1995). Shephard et al. (1998) introduced a photocatalytic reactor in which an oxygen purge, UV radiation and a semiconductor titanium dioxide (TiO_2) catalyst, were used for removal of MC-LR, MC-YR, and MC-YA. Experiments demonstrated that the toxins were rapidly decomposed with half-lives of less than 5 minutes. Reaction rates were strongly dependent on the amount of TiO_2 catalyst present (0–5 g/l). The use of lake water, rather than distilled water, showed that this process is feasible in natural waters, although increased levels of catalyst (up to 5 g/l) were required to achieve comparable decomposition rates. A number of researchers have successfully used photocatalytic processes for removal of MCs in water (Robertson, Lawton, Cornish, & Jaspars, 1998; Cornish, Lawton, & Robertson, 2000; Shephard, Stockenstrom, de Villiers, Engelbrecht, & Wessels, 2002; Lawton, Robertson, Cornish, Marr, & Jaspars, 2003; Lee, Kim, Kim, Chung, & Kim 2004).

6.3.2.2.2 Ozonation

Ozone, a triatomic unstable gas (O_3), is one of the most powerful oxidants and widely used in water treatment for removal of organic and inorganic contaminants (e.g. heavy metals, phenols and pesticides) and for the inactivation of microorganisms (e.g. bacteria and viruses) (Falconer, 2005). The process referred to as ozonation involves subjecting the molecule of oxygen with high electrical voltages in an aqueous medium to produce ozone as well as free radicals (mainly $^{\bullet}OH$; created from ozone decomposition) (Staehelin & Hoigne, 1985). Although, ozone is an expensive and occasionally unpredictable reagent, it is widely used in many developed countries for treating drinking water. A combination of ozonation and UV light has been shown to be very effective for removing a wide range of organic compounds, including MCs (Lawton & Robertson, 1999; Falconer, 2005).

Ozone is a highly specific reagent targeting carbon-carbon double bonds, activated aromatic systems, and neutral amines (von Gunten, 2003). It breaks down MCs by cleaving the double bonds at C-6 to C-7 of the Adda residue (Falconer,

2005). The cleavage of the Adda residue reduces overall toxicity since the toxicity of MCs has been shown to be associated with the Adda residue (Harada, Murata, Qiang, Suzuki, & Kondo, 1996a; Lawton & Robertson, 1999). Ozone was shown to be effective for the decomposition of MCs in water treatment plants, but is concentration dependent (Keijola et al., 1988; Himberg et al., 1989; Fawell, Hart, James, & Parry, 1993; Hart et al., 1997; Rositano, Newcombe, Nicholson, & Sztajnbok, 2001; Shawwa & Smith, 2001; Hoeger et al., 2005). Keijola et al. (1988) revealed that preozonation processes, at a dose of 1 mg/l of ozone was adequate to remove MCs completely (100% removal of MC at a concentration of 60 µg/l) however at this concentration, ozone residue may be inadequate to remove the toxins when cyanobacteria are blooming due to the increased soluble organic load associated with the biomass (Falconer, 2005). Therefore, intermediate ozonation with 0.5 mg/l is required to ensure the removal of toxic compounds including MCs in water (Hoeger et al., 2005). However, when there is an insufficient ozone dose, by-products of ozonation (e.g. bromate, from ozone reacting with naturally occurring bromide) may be formed and can result in adverse health effect (e.g. tumors in animals at high doses of the by-products) (Hitzfeld et al., 2000).

6.3.2.2.3 Chlorination

Chlorination is one of the most crucial steps in the water treatment process (AWWA & ASCE, 2005). Chlorine, effective and cheap, is commonly used to disinfect drinking water for human consumption, and to maintain a residual effect in the distribution system (Hendricks, 2005). Chlorine reacts with the similar moieties as ozone (double bonds, activated aromatic systems, and neutral amines) but at much lower rates than ozone (Rodriguez et al., 2007).

Works by Hoffman (1976), Keijola et al. (1988) and Himberg et al. (1989) revealed that chlorination was ineffective at removing MCs from water. This may be due to the processes occurring at a pH where the free chlorine concentrations are relatively low (Lawton & Robertson, 1999). Keijola and Himberg did not report the pH levels, but Hoffman performed his investigations at pH 8.5. The effect of pH on chlorination of MCs was consequently investigated by Nicholson and co-workers in 1994.

Nicholson, Rositano, and Burch (1994) indicated that the removal (decomposition) of MC-LR was pH dependent. Below pH 8, aqueous chlorine mainly occurs in the form of hypochlorous acid at a concentration of 15 mg/l, and is able to decompose MC-LR whereas the toxin removal was significantly reduced above pH 8. The reduction in toxin removal with increasing pH can be explained in terms of the decreasing concentrations of hypochlorous acid (79% at pH 7, 55% at pH 7.5, 28% at pH 8, 0.4% at pH 10, 20°C), which is a more powerful oxidizing agent in comparison with the hypochlorite ion (Nicholson et al., 1994). It is important to note that a chlorine residual of at least 0.5 mg/l should be present after 30-minute contact time in order to decompose the toxin (Nicholson & Rositano, 1997).

Tsuji et al. (1997) investigated the effect of chlorination on the decomposition of MC-LR and MC-RR and revealed that the toxins were easily decomposed by chlorination with sodium hypochlorite, and the decomposition depended on the free chlorine dose and pH. During the chlorination process, many reaction by-products were formed, one of which was dihydroxy-microcystin formed through the chloronium ion at the conjugated diene of Adda, followed by hydrolysis. Other products may be its stereoisomers and/or regioisomers. No toxic products were detected from the chlorination process of MC-LR (Tsuji et al., 1997). Although these results suggest that chlorination with an adequate chlorine dose is very effective for the removal of MCs, preoxidation of the cell itself with chlorine must be avoided, because it frequently causes toxin release from cyanobacteria and production of halogenated organic compounds (e.g. chloroform, other trihalomethanes (THMs), haloacetic acids (HAAs) etc.) during water treatment (Singer & Reckhow, 1999).

6.3.2.2.4 Activated carbon filtration

Activated carbon filtration is most effective in removing organic contaminants from water (Hendricks, 2005). In the United States, the application of activated carbon filtration in water treatment has been used mainly for removing undesirable contaminants such as taste and odour causing compounds, pesticides and other toxic compounds, including MCs from water (AWWA & ASCE, 2005; Crittenden et al., 2005). Activated carbon is capable of adsorbing a wide range of compounds because of its highly porous structure, with a broad variety of pore sizes (micropores < 2 nm,

mesopores 2-50 nm and macropores > 50 nm). Intermolecular attractions in the smallest pores create adsorption force, causing large and small molecules of dissolved contaminants to be condensed and precipitated from solution into the molecular-scale pores (AWWA & ASCE, 2005).

Two different forms of activated carbon, powdered activated carbon (PAC) and granular activated carbon (GAC), are used for water treatment as well as removal of MCs (Hoffman, 1976; Keijola et al., 1988; Himberg et al., 1989; Falconer, Runnegar, Buckley, Huyn, & Bradshaw, 1989; Donati, Drikas, Hayes, & Newcombe, 1994; Lambert et al., 1996; Cook & Newcombe, 2002; Newcombe, Cook, Brooke, Ho, & Slyman, 2003; Ho, 2004; Wang et al., 2007). GAC is widely used in flow-through column reactors whereas PAC can be added directly into water prior to coagulation or filtration (Lawton & Robertson, 1999). However, GAC has many other benefits over PAC such as its long life, higher adsorptive capacity, the ease of process control, more efficient use of the carbon, and the ability to regenerate the carbon for reuse (Ho, 2004).

Activated carbon as well as ozonation is reported to be the best treatment options for removing MCs in water (Rodrıguez et al., 2007). Initial work on the application of activated carbon for the removal of cyanobacterial toxins, including MCs produced by the toxic cyanobacterium *M. aeruginosa*, was carried out by Hoffmann (1976). Sequential water treatment processes of flocculation, sedimentation, filtration, and chlorination did not adequately remove the toxins, whereas water treatment processes with activated carbon were successful in removing the toxins, but removal was dependent on the dosing levels of activated carbon. Keijola et al. (1988) also confirmed that MCs were not removed by conventional flocculation treatment procedures, and even the addition of a small amount of PAC with the flocculation chemical did not improve the removal of MCs significantly. Significant removal of toxins was achieved by GAC filtration and by ozonation in both laboratory and pilot scale experiments. Himberg et al. (1989) showed that 20 mg/l of PAC was able to achieve a 90% removal of MCs combined with conventional coagulation-sedimentation treatment and pre-ozonation, and Falconer et al. (1989) reported successful removal of MCs produced by a *Microcystis* sp., as well as odour compounds from a cyanobacterial bloom by using GAC under pilot plant conditions.

Lambert et al. (1996) found that conventional coagulation-sedimentation, dual media filtration and chlorination treatment processes combined with activated carbon (GAC and PAC) removed more than 80% of MC-LR from raw water, but a residual concentration of 0.1-0.5 µg equivalents of MC-LR per liter was observed for both GAC and PAC treatment facilities.

Various sources of activated carbon have been examined for their ability to adsorb MC-LR. Drikas (1994) reported that wood-based products were the most effective MC-LR adsorbents due to their high mesopore volume. It was also found that treatment with 25 mg/l of wood-based PAC with a contact time of 30 minutes, could reduce the concentration of MC-LR from 50 to <1 µg/l. These results are supported by a similar study by Donati et al. (1994). Wood-based carbons proved the most effective adsorbents of MC-LR, followed by coal-based PACs, whereas coconut and peat moss-based carbons were the poorest MC-LR adsorbents. The extent of adsorption for each PAC was related to the volume of mesopores (diameters between 2.0 and 50 nm) which was dependent on the starting material. They also found that competitive adsorption of PAC with natural organic matter (NOM) from natural water (Murray River, Australia) significantly reduced the initial rate of MC-LR adsorption (Donati et al., 1994).

Lawton & Robertson (1999) revealed that the MC removal by PAC was relatively high in pure water (e.g. Milli-Q) and relatively low in raw water. Removal efficiency decreases because of competition for binding sites on the carbon (Lawton & Robertson, 1999). Not only competitive adsorption but also water characteristics and carbon properties affect efficiency of MC removal by activated carbon. Huang, Cheng, and Cheng (2007) demonstrated that the activated carbon with a high ratio of mesopore and macropore volume showed an increased MC-LR adsorption capacity, whereas the micropores in carbon provide only a nominal internal surface for adsorption. The adsorption capabilities of different activated carbon generally followed their pH at the zero point of charge values (pH_{zpc}). Activated carbons with higher pH_{zpc} values exhibit a neutral or positive charge under typical pH conditions, promoting MC-LR adsorption on the carbon surface. The competitive effects of natural organic matter on activated carbon showed that it caused a reduction in the

capacity of carbon and therefore decreased its adsorptive ability to remove MCs (Huang et al., 2007).

Granular activated carbon can also be used as a substrate for bacteria (biofilm formation) to mineralize (biodegrade) organic by-products produced from the ozonation (e.g. ketones, aldehydes and acids), as well as other undesirable compounds, including MCs, which may survive early water treatment processes (Lambert & Graham, 1995; Hoeger et al., 2005). Adsorption and biodegradation mechanisms are known to be the predominant factors contributing to MC removal during the GAC filtration process (Ho, 2004). Therefore, a study by Wang et al. (2008) attempted to discriminate between, and assess the respective importance of, adsorption and biodegradation mechanisms during GAC removal of MC-LR. Results showed that biodegradation is an efficient removal mechanism once it begins. However, it was found that an active biofilm, present on the surface of conventional GAC, also hindered adsorption of MC-LR. Up to 70% removal of MC-LR was still observed after 6 months of operation of the sterile GAC column, indicating that adsorption still played a vital role in the removal of MC-LR (Wang et al., 2007).

6.3.2.2.5 Slow sand filtration

Slow sand filtration has been used for treating raw water to produce drinking water, for nearly 200 years (AWWA & ASCE, 2005). Here water treatment is through biological processes (biofiltration), by the formation of a layer of microorganisms on the surface of the sand bed. The layer, called *schmutzdecke* or biofilm, acts both as a particulate filter and as an active metabolic degradation system for organic compounds (Ellis, 1995). It has been shown that some biological processes also extend deep into the sand bed, allowing uptake and metabolism of xenobiotics and natural toxins by bacteria and fungi (AWWA & ASCE, 2005; Falconer, 2005).

Biological sand filtration is becoming more attractive for the removal of undesirable compounds, including MCs, as it offers a cost-effective water treatment with low technology, maintenance, and infrastructure (Bourne et al., 2006; Ho et al., 2006). This method also provides an advantage of removing contaminants without the use of other chemicals that may result in unfavorable by-products (Ho et al., 2006). A number of studies have focused on the removal of MCs in sand filtration systems

(Lahti & Hiisvirta, 1989; Grützmacher et al., 2002; Bourne et al., 2006; Ho et al., 2006; Ho et al., 2007). Early on Lahti and Hiisvirta (1989) and Grützmacher et al. (2002) revealed that slow sand filtration was capable of removing MCs from water and also suggested that biological degradation of MCs occurred in the slow sand filters. However, these studies did not show that the removal of MCs was through biological processes (e.g. biodegradation) rather than physical processes (adsorption).

Ho et al. (2006) were among the first to successfully demonstrate the removal of MCs, within a biologically active sand filter, by biological processes (biodegradation). They examined biological sand filtration in laboratory sand columns (made of glass 30 cm long, internal diameter 2.5 cm, and a sand bed height of 15 cm) with two MC variants, MC-LR and MC-LA. Both variants, at final concentrations of 20 µg/l, were completely removed in 4 days (a lag period of 3 days). Using PCR, a biofilm extracted from one of the sand filters that had effectively removed the MCs, was shown to contain bacteria with the *mlr*A gene that encodes for an enzyme responsible for the first step in the degradation of MC-LR. Detection of this gene provided additional evidence that biological degradation of MC was the primary removal mechanism. A further study in the same area resulted in the isolation of *Sphingopyxis* isolate LH21 from a biological sand filter (Ho et al., 2007). Isolate LH21 was also shown to contain homologues to each of the four genes, *mlr*A, *mlr*B, *mlr*C and *mlr*D, previously associated with the degradation of MC-LR by *Sphingomonas* sp. ACM-3962. Isolate LH21 was able to effectively degrade MC-LR and MC-LA, in batch sand filters under environmentally relevant conditions, with complete removal observed in 4 days (initial concentration of the toxin at 20 µg/l).

Bourne et al. (2006) also investigated biologically active slow sand filtration for removal of MCs by using the MC-degrading bacterium *Sphingomonas* strain MJ-PV (ACM-3962). The dissolved MC-LR at a concentration of 50 µg/l was fed into sand filled PVC piping columns (100 mm in diameter, 1 m in length and a sand bed height of 0.5 m). Complete removal of the toxin was observed within 6 days, and sampling up to 15 days did not detect any further breakthrough of the toxin. PCR, specifically targeting amplification of 16S rDNA of MJ-PV and the *mlr*A gene, was used to monitor the presence of the bacterium in experimental trials. However, pilot scale biologically active slow sand filters demonstrated degradation of MC-LR

irrespective of MJ-PV bacterial inoculation. No amplified products, indicative of an endemic MJ-PV population, were observed in un-inoculated treatments indicating bacterial strains other than MJ-PV were active in degradation of MC-LR. Bourne's study demonstrated the effectiveness of a low-technology water treatment system like biologically active slow sand filters for removal of MCs from reticulated water supplies (Bourne et al., 2006).

6.3.3 Airlift bioreactor

In natural water, a wide variety of undesirable compounds are removed gradually by physical, chemical, and biological processes. In water treatment plants similar naturally occurring processes occur in vessels, or tanks, commonly called reactors, and when this involves biological processes, the reactor is referred to as a bioreactor (Merchuk & Asenjo, 1995; Crittenden et al., 2005).

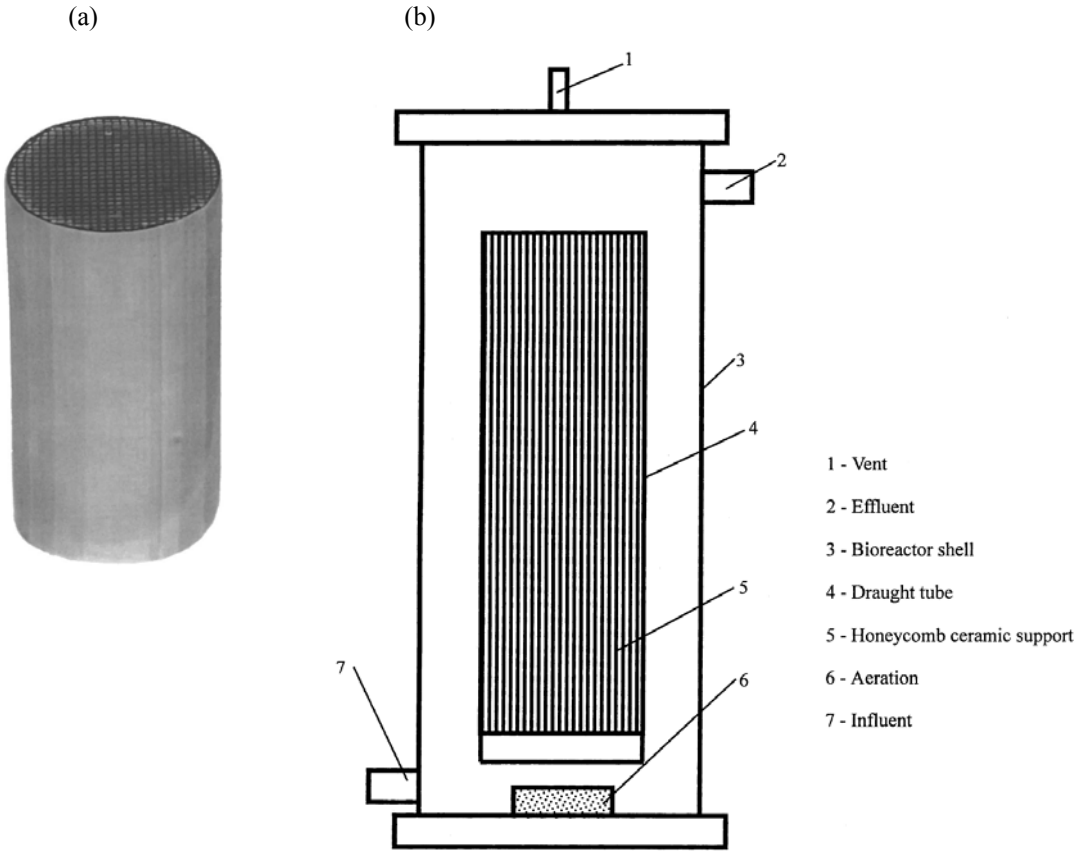
The airlift bioreactor is one of the most common small scale bioreactors, used for a wide variety of processes in bioprocess research, including the production of biochemical compounds (e.g. enzymes and antibiotics) and wastewater treatments (Merchuk, 2003). The bioreactor has significant advantages in terms of good mixing, rapid liquid flow rate, high concentration of dissolved oxygen, the absence of mechanical agitators, and relatively low cost which makes it a versatile apparatus with great potential for simulating larger scale wastewater treatment (Chisti, 1989; Loh & Liu, 2001; Merchuk, 2003; Chen, Li, Qiao, Yang, & Ding, 2005). Jin, Yan, Yu, and van Leeuwen, (2002) used a small scale airlift reactor in a comprehensive pilot plant system for starch wastewater treatment by *Aspergilius orizae*. Simultaneously, 95% chemical oxygen demand (COD), 93% biological oxygen demand (BOD), and 98% suspended solids removal was achieved, along with important production of α-amylase. Campos, Borges, Olivera, Nobrega, and Sant'Anna (2002) also used an airlift bioreactor in a combined (microfiltration and biological) oilfield wastewater treatment. They obtained satisfactory results in Total Organic Carbon (TOC) and COD reduction in a continuous process.

In this study, an internal airlift loop ceramic honeycomb support bioreactor, (IAL-CHS bioreactor) was used for removal of MCs using the bacterium *Sphingomonas* isolate NV-3. This particular design of bioreactor has demonstrated

efficient purification of water contaminated with 2, 4-dichlorophenol and phenol, high-carbohydrate, printing ink, and quinoline wastewater (Zhang et al., 2002a; Zhang et al., 2002b; Quan et al., 2003, 2004; Zhang et al., 2004, 2005).

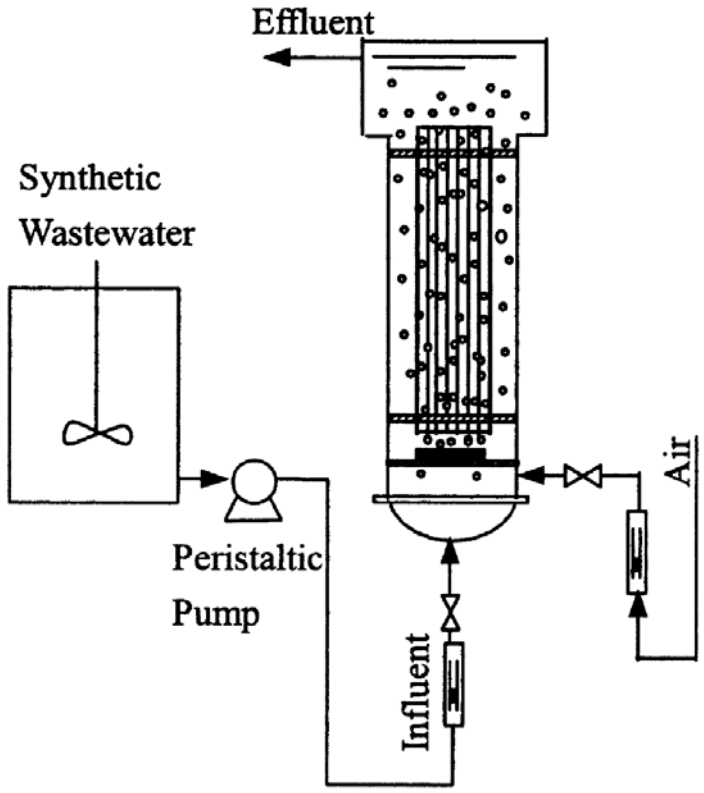
Zhang et al. (2002a) first introduced the novel internal airlift loop ceramic honeycomb support bioreactor, which they designated as the IAL-CHS bioreactor, for the removal of quinoline by biological processes. In the bioreactor, the bacterium *Burkholderia pickttii* was first immobilized onto a ceramic honeycomb support (CHS) (Figure 6.2a) as a biofilm. This was then inserted into a draught tube to make an internal airlift loop (IAL). The draught tube with honeycomb support inside was placed inside a plexiglass tube equipped with aerator stone, to make the complete IAL-CHS bioreactor (Figure 6.2b).

Figure 6.2 (a) Ceramic honeycomb support (CHS), and (b) the fully assembled internal airlift loop ceramic honeycomb support bioreactor (IAL-CHS bioreactor) (Zhang et al., 2002a)



A series of experiments were performed in a continuous mode consisting of a synthetic wastewater reservoir, a peristaltic pump, an IAL-CHS bioreactor, an outflow water flask, and an air pump to control internal water flow rate (Figure 6.3). Results indicated that more than 95% of quinoline could be removed for 4 hours of hydraulic retention time (HRT), with influent concentration of quinoline at 260 to 320 mg/l. *Burkholderia pickttii* could be easily immobilized in the micropores of the ceramic, as ceramic provided excellent absorptivity to cells, and supplied a great microenvironment for *B. pickttii* to metabolize, proliferate and degrade quinoline steadily.

Figure 6.3 Schematic diagram of IAL-CHS bioreactor (Quan et al., 2003)



The IAL-CHS bioreactor, with immobilized cells of *Bacillus* sp., was also used to treat printing ink wastewater, resulting in more than 85% reduction in chemical oxygen demand (COD) (Zhang et al., 2002b). Organic compounds and the *Bacillus* sp. were found to precipitate or absorb onto the surface of the ceramic forming an excellent microenvironment which enhanced the removal of printing ink.

The ceramic honeycomb support was reported to reduce the size of bubbles emitted from the aeration stone and thus enhance oxygen transfer, offering a large area for enrichment of cells to accelerate COD reduction in wastewater (Zhang et al., 2002b). A bacterium *Achromobacter* sp. was used for removal of 2, 4-dichlorophenol and phenol in the IAL-CHS bioreactor (Quan et al., 2003, 2004; Zhang et al., 2004). The strain of *Achromobacter* sp. used in the above three studies was not given, but it is presumed that they used the same strain of bacterium as they are members of the same research team. The results indicated that the bioreactor was useful for bacterial immobilization. The bacterium could attach to the surfaces of the ceramic carrier effectively and quickly, and maintain itself using chlorophenol and phenol as the sole carbon source, with the removal percentage ranging between 84% and 100% (Quan et al., 2003, 2004; Zhang et al., 2004).

The IAL-CHS bioreactor was also used for the treatment of high-carbohydrate wastewater and the production of a single-cell protein (SCP) simultaneously using immobilized cells of the yeast *Candida tropicali*s. It was obvious under a scanning electron microscope (SEM), that the ceramic macropores provided an excellent microenvironment for the cells to proliferate in, as *C. tropicalis* cells immobilized densely in the macropores of the ceramic support. Although biomass retention was dense in the macropores, the IAL-CHS produced a significant yield of SCP (Zhang et al., 2005).

6.4 Objectives of this chapter

The objectives of this study were to (i) assess biofilm formation of MC-degrading bacterium *Sphingomonas* isolate NV-3 on abiotic surfaces and (ii) establish whether or not the internal airlift loop ceramic honeycomb support bioreactor (previously used for biodegradation of other toxicants) was an effective mechanism for the removal of MCs under single batch and continuous mode operation.

6.5 Methods

6.5.1 Bacterial strains and culture media

The MC-degrading bacterium *Sphingomonas* isolate NV-3, characterized in Chapter 4, was used for biofilm formation characteristics, and degradation of MCs in the bioreactor. Isolate NV-3 was cultured on PYEM medium (see Appendix 5) at 30^{o}C (optimum temperature for NV-3, see Chapter 4) and stored in a manner described in Section 4.5.2.

6.5.2 Biofilm formation characteristics

Two types of biofilm formation assays were performed to assess the ability of NV-3 to form a biofilm on a range of abiotic surfaces, before immobilizing NV-3 on honeycomb ceramic in the IAL-CHS bioreactor. The assays involved quantification of dye bound to bacterial cells, thus an indirect measure of bacterial biomass. The bacteria in the biofilm were stained initially with 1% crystal violet, subsequently destained, and the amount of released crystal violet dye measured by absorbance spectrophotometry (O'Toole & Kolter, 1998).

6.5.2.1 Microtiter plate biofilm formation assay

The first biofilm assay was undertaken to determine the ability of the bacterium isolate NV-3 to form a biofilm on the surface of sterile 96-well polyvinylchloride plastic (PVC) microtiter plates, using a method modified from O'Toole and Kolter (1998); Pang, Hong, Guo, and Liu (2005); and Harvey, Keenan, and Gilmour (2007). A single colony of the isolate NV-3 was cultured in 5 ml of R2A medium broth (Appendix 13). The culture was incubated in a shaking incubator at 200 rpm and 30^{o}C for 36 h, the resultant culture was then diluted by addition of sterile

R2A broth to achieve an optical density of 0.05 at a waveledge of 600 nm (Pang et al., 2005). One hundred µl of this mixture were added to each of 8 wells of the 96-well microtiter plates (Nunc, Roskilde, Denmark) and incubated at 30°C without agitation for a total time of 96 h. Each plate included 8 control wells each with 100 µl of un-inoculated growth medium. The biomass of the biofilm formed on the walls of wells in the microtiter plates was determined by staining with a 1% crystal violet (CV) solution after 24, 48, 72 and 96 h. This dye stains the bacterial cells but not the PVC (O'Toole & Kolter, 1998). Twenty five µl of CV solution was added to each well and incubated at 30°C for 45 min. The contents of the well were discarded and the wells were then washed three times with sterile double-distilled water to remove excess dye. In order to destain the biofilm, 100 µl of 95% ethanol were added to the wells and incubated at at 30° C for 45 min. The concentration of crystal violet in the 95% ethanol wash solution was then determined using optical density at a wavelength of 600 nm (CV-OD_{600}). The average optical density from the control wells (Ac) was subtracted from the OD_{600} of all test wells. The microtiter plate biofilm assays were performed in triplicate and the averages and standard deviations (S.D.) were calculated. The ability to form biofilms was classified using the criteria of Stepanovic, Cirkovic, Ranin, and Svabic-Vlahovic (2004) as follows: $A \leq Ac$ = no biofilm producer, $Ac < A \leq (2 \times Ac)$ = weak biofilm producer, $(2 \times Ac) < A \leq (4 \times Ac)$ = moderate biofilm producer and $(4 \times Ac) < A$ = strong biofilm producer.

6.5.2.2 Coupon biofilm formation assay

To establish the best substrate for use in the bioreactor experiments, biofilm formation by *Sphingomonas* isolate NV-3, on a variety of substrates was determined by immersing coupons of different material types in the bacterial culture. Experiments were set up in triplicate. Un-inoculated growth medium was used as a control and the entire experiment was repeated on a future occasion to check reliability.

Abiotic surfaces, made of polystyrene, polyvinylchloride plastic, glass, stainless steel and ceramic were used to determine biofilm formation. Polystyrene sterile tissue culture plates (24 well plates from Corning Incorporate), PVC plastic (Corning Incorporate), stainless-steel plates (grade 304; Atlas Steels), slide cover glasses (JIA 7101 WT, Sail brand, China) and ceramic plate (Pingxiang Chemshun

Ceramics Co. Ltd.) were cut to make 1 x 2 cm coupons and prepared as abiotic surfaces. The glass, stainless steel, and ceramic coupons were cleaned by soaking in acetone for 30 min to remove grease, rinsed with sterile distilled water, soaked in alkaline detergent for a minimum of 1 h at 70°C, rinsed again in sterile distilled water and finally sterilized by autoclaving. The PVC and polystyrene coupons were cleaned by soaking in 70% ethanol for 15 min, washed aseptically with sterile double-distilled water, and placed in sterile 12 cm x 1.5 cm test tubes at 40^{o}C to dry.

Using a modified method from O'Toole and Kolter (1998) and Pang et al., (2005), 10 ml of 36 h bacterial culture diluted with R2A broth to an optical density of 0.05 (OD_{600} = 0.05), were transferred to 12 cm x 1.5 cm test tubes containing one piece of each coupon type (10 ml is adequate to completely immerse the coupons). The tubes were incubated without agitation at 30°C for 24, 48 and 72 h, allowing biofilm development. Two and a half ml of 1% crystal violet solution were then added to each tube to stain the biofilm cells on the coupon surface and incubated for 45 min at 30°C. The loosely attached cells on the surface of each coupon were removed by gently rinsing with 200 ml of sterile double-distilled water. The dye bound to the biofilm on the coupons was extracted and collected using 10 ml of 95% ethanol in a sonicator for 1 h. The concentration of crystal violet, a measure of biofilm formation, was then determined using optical density at a wavelength of 600 nm (CV-OD_{600}). The average optical density of the alcohol removed from the control coupons (Ac), for each material, was subtracted from the OD_{600} derived from each material coupon test in which *Sphingomonas* isolate NV-3 was used. Each material was tested for biofilm formation in triplicate and the averages and standard deviations (S.D.) were calculated. The ability to form biofilms was categorized as in 6.5.2.1.

6.5.3 Scanning electron microscopy

In order to mimic the actual conditions of biofilm formation in the bioreactor, *Sphingomonas* isolate NV-3 was cultured in 5 ml of R2A medium broth for 24, 48, and 72 h on ceramic coupons. The ceramic coupons were then prepared for viewing under the Scanning Electron Microscope. The preparation was carried out by the Manawatu Microscopy and Imaging Centre, within the Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand.

6.5.4 Degradation of MCs in the bioreactor

6.5.4.1 Biofilm reactor

Biodegradation of MCs was investigated using a IAL-CHS bioreactor, identical, in principle, to that used by Zhang et al. (2002). The IAL-CHS bioreactor consisted of an air pump, 0.22 μm air filter, a 10 liter 'synthetic wastewater' reservoir (plus MCs), a glass-cylinder bioreactor shell with 220 mm internal height, 110 mm internal diameter and 1500 ml of working volume (Figure 6.4a and Figure 6.4c). When operational the ceramic honeycomb support (CHS) (Figure 6.4b) was placed in the center of the IAL-CHS bioreactor, with an aerator stone beneath (Figure 6.5). In addition there was a recipient flask, and a peristaltic pump for when the bioreactor was operated in continuous mode. The CHS (Pingxiang Chemshun Ceramics), in which bacterial cells were immobilized, had a diameter of 60 mm, and a height of 150 mm. In cross-section it had numerous pores of 3 mm diameter and within the ceramic framework smaller, pores of 2-3 μm diameter, presenting significant surface area for biofilm formation. Air was continuously supplied to an aerator stone beneath the CHS via a sterile 0.22 μm filter.

Figure 6.4 IAL-CHS bioreactor (a) glass-cylinder bioreactor shell for continuous-flow mode (b) ceramic honeycomb support (CHS) and (c) glass-cylinder bioreactor shell for batch mode

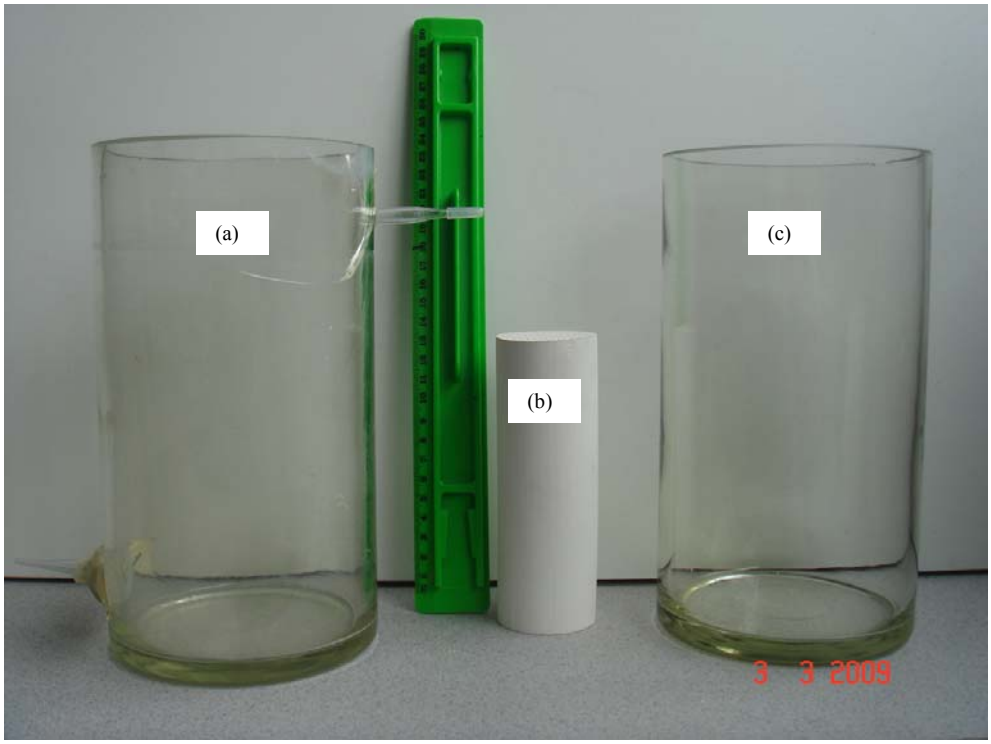


Figure 6.5 IAL-CHS bioreactor set up for batch mode experiments

6.5.4.2 Abiotic loss of the MCs through the CHS

In order to estimate the loss of MCs by mean of abiotic processes, that is other than direct degradation by bacteria, such as physical adsorption onto the CHS, an experiment was performed for 60 h using 1500 ml of 'synthetic wastewater' (Appendix 13) containing MCs at an initial concentration of 25 µg/ml. The entire IAL-CHS bioreactor was immersed in a circulating water bath, with temperature at 30^{o}C, in the dark, and arranged as for a batch mode experiment. There was no bacterial biofilm in the CHS. Every 6 h for 48 h, an aliquot (1 ml) of sample was withdrawn from the bioreactor and centrifuged at 12,000 rpm for 10 min. The MC concentration was then analyzed in the supernatant using the HPLC-UV detector as previously described (see Chapter 3, Section 3.5.6.1). The experiments were carried out in triplicate as well as using controls. The controls comprised 1500 ml of 'synthetic wastewater', containing MCs, in the absence of the CHS.

6.5.4.3 Cell immobilization on the CHS of the bioreactor

A single colony of the bacterial isolate NV-3 was grown in 30 ml PYEM broth in a shaking incubator at 200 rpm and 30°C for 36 h. The 36 h-culture was inoculated into 1500 ml (2% v/v) of fresh PYEM broth and incubated in a shaking incubator at 30^{o}C and 200 rpm for 24 h. After 24 h incubation, the bacterial culture was poured directly into and around the CHS, which was suspended in a glass-cylinder bioreactor shell (Figure 6.4c), so that the cells started to form a biofilm on the surface of the ceramic as well as in the pores. The whole unit (CHS and bacterial culture in the glass-cylinder), was then incubated at 30^{o}C for 12 h on an orbital shaker at 80 rpm, to promote sufficient bacterial biomass on the CHS. The entire bacterial culture within the CHS, had a total incubation period of 36 h, which corresponds to late exponential growth phase for NV-3 as described in Chapter 4, Section 4.5.5.

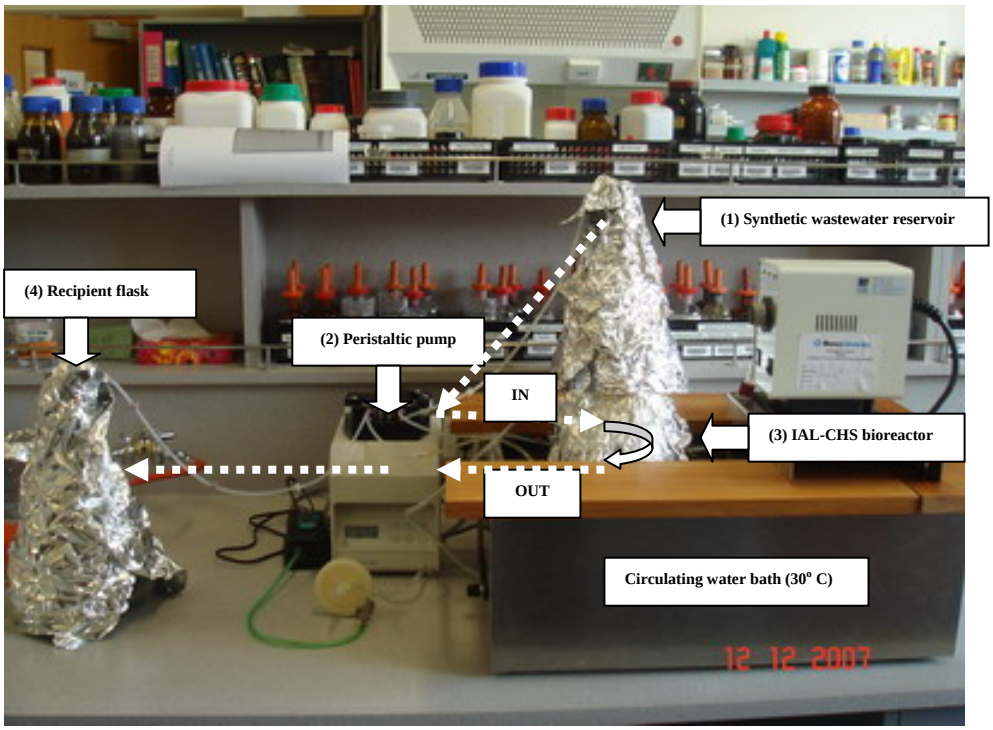
6.5.4.4 Batch experiment of MC-degradation

After the biofilm had formed, the CHS was washed with 10 liters of sterile 'synthetic wastewater' to remove loosely attached cells and excess culture medium. The primed CHS was then placed in a clean glass-cylinder bioreactor shell containing fresh synthetic wastewater plus MCs at a concentration of 32.5 µg/ml, for 6 h (see Figure 6.5 IAL-CHS bioreactor). After 6 h of incubation, the concentration of the MCs was reduced to around 25 µg/ml, due to abiotic loss of the MCs through adsorption onto the CHS as explained in Section 6.5.4.2. The IAL-CHS bioreactor was allowed to incubate, while immersed in a circulating water bath at 30^{o}C, in the dark covered in aluminium foil. Clean air was continuously fed through a 0.22 µm sterile filter to the bioreactor. Air circulation caused the 'synthetic wastewater' to flow upward in a loop over the fixed-bed reactor, making it work like a fluidized bed reactor (Quan et al., 2003). At regular intervals aliquots (1 ml) of 'synthetic wastewater' containing MCs were taken and centrifuged at 12000 rpm for 10 min (4^{o}C). The supernatants were measured for MC concentration by HPLC. The experiment was performed in triplicate as well as using controls. The controls comprised 1500 ml of 'synthetic wastewater' plus MCs (25 µg/ml) in the absence of bacteria on the CHS.

6.5.4.5 Continuous-flow experiment of MC-degradation

In water treatment plants, the treatment is generally operated in a continuous mode; therefore it is very important to determine the biodegradation of the MCs under more continuous operation. Experiments were carried out using a similar setup to that for the batch experiments. The primed IAL-CHS bioreactor (using the glass-cylinder with inlet and outlet, Figure 6.4a), with the 'synthetic wastewater' containing MCs at a concentration of 32.5 µg/ml, was incubated for 6 h, allowing time for the toxins to adsorb onto the ceramic carrier effectively reducing toxin concentration to 25 µg/ml. The bioreactor experiment then changed to flow-through mode by addition of the peristaltic pump (Figure 6.6) where the 'synthetic wastewater', containing the toxins at a concentration of 25 µg/ml, flowed from an adjacent 10 liter 'reservoir' through the bioreactor continuously, to accumulate in a recipient flask (single pass reactor). The flow rate was set at 2 ml/min, and the hydraulic retention time (HRT) was 8 h. Every 6 h for 60 h, an aliquot (1 ml) of sample was withdrawn from the outlet tube leading to the recipient flask and the MC concentration was then analyzed as previously described (Section 3.5.6.1). Triplicates as well as controls were carried out.

Figure 6.6 The IAL-CHS bioreactor in a continuous flow-through mode experiment

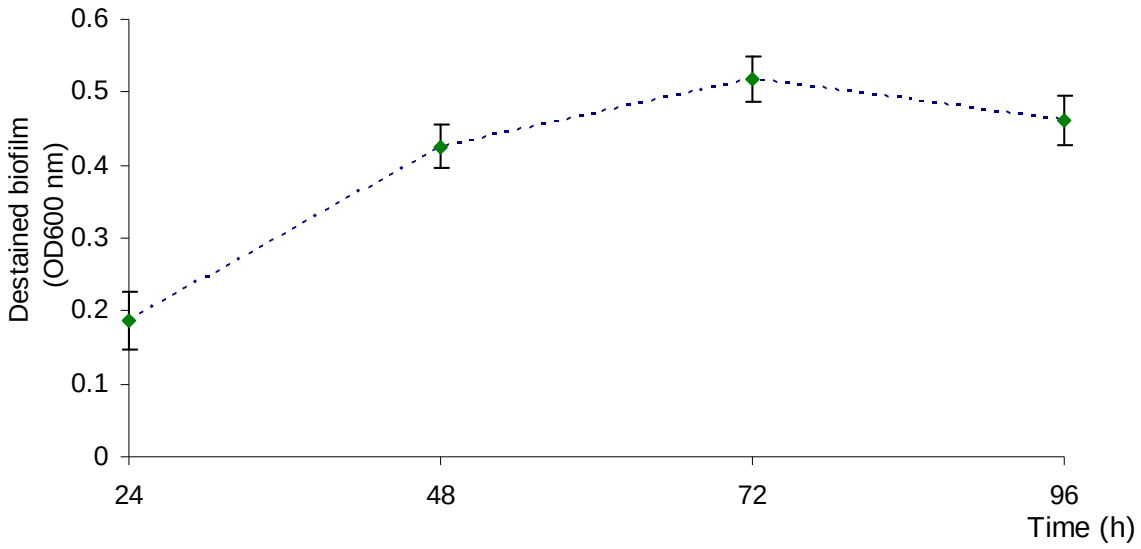


6.6 Results

6.6.1 Microtiter plate biofilm formation assay

The biofilm formation increased steadily over 24, 48, and 72 h, but declined slightly at 96 h (Figure 6.7). The isolate NV-3 proved to be a moderate biofilm former on the PVC of the microtiter plate, according to the criteria of Stepanovic et al. (2004) (see Section 6.5.2.1) using the CV-OD_{600} values (the average optical density from the control wells (Ac) was 0.16 whereas average optical density from test wells was 0.398).

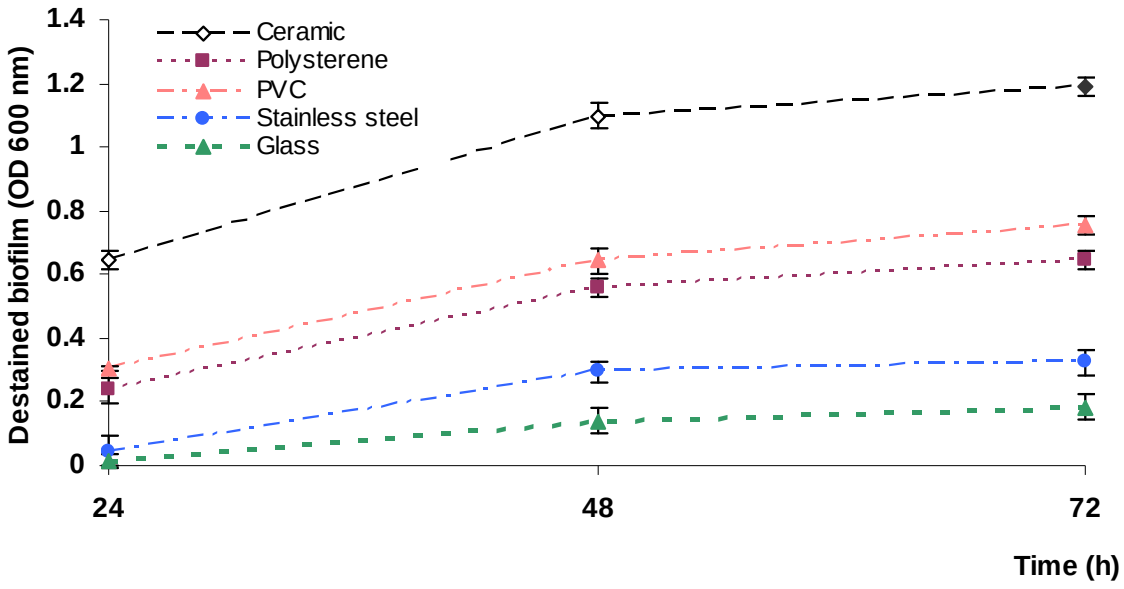
Figure 6.7 Destained biofilm (measured at OD_{600}) of *Sphingomonas* isolate NV-3 biofilm formation assay on microtiter plate at 24, 48, 72 and 96 h. Each point represents ± Standard Deviation (S.D.) of three replicates.



6.6.2 Coupon biofilm formation assay

Greater amounts of crystal violet were extracted from the ceramic than from PVC, polystyrene, stainless steel and glass surfaces, respectively (Figure 6.8). Data showed that biofilm formation increased over time on all abiotic surfaces tested. From CV-OD_{600} values at 72 h, the isolate NV-3 may be designated as a moderate biofilm producer on the ceramic, PVC and polystyrene coupon surfaces but a weak biofilm producer on stainless steel and glass coupons as defined by Stepanovic et al. (2004). The highest optical densities derived from the ceramic experiment was 1.12±0.03, while that of PVC, polystyrene, stainless steel and glass surfaces were 0.75±0.04, 0.65±0.04, 0.32±0.04 and 0.18±0.02 respectively.

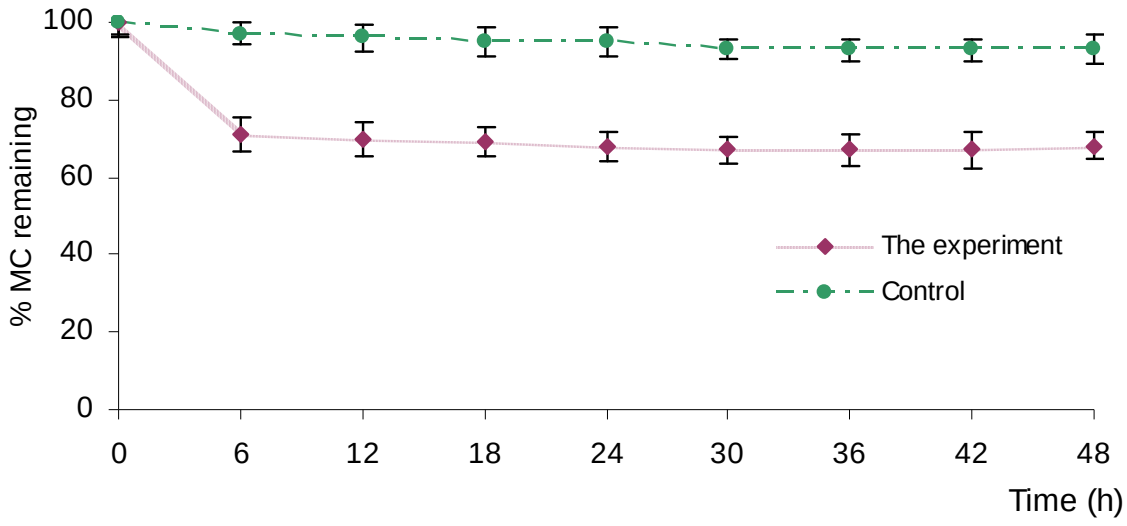
Figure 6.8 Destained biofilm (measured at OD_{600}) of *Sphingomonas* isolate NV-3 in coupon biofilm formation assays on polystyrene, polyvinylchloride plastic, glass, stainless steel and ceramic coupons (1 cm x 2 cm) at 24, 48 and 72h. Each point represents ± S.D. of three replicates.



6.6.3 Abiotic loss of the toxins adsorbed to surface of ceramic support

The percentage of MC remaining in the synthetic wastewater within the bioreactor is shown in Figure 6.9. Toxin concentration remained constant at 17.8 µg/ml after 6 h-incubation until the end of the experiment (48 h). From an initial concentration of 25 µg/ml, there was a loss of approximately 30% of the MCs in the synthetic wastewater. The results indicate that abiotic loss of the toxins through the adsorption onto the ceramic support was significant, and concentrations of synthetic wastewater, in subsequent experiments, were adjusted to allow for this. The controls, containing no CHS, showed very little change in MC concentration over the 48 hours.

Figure 6.9 Initial losses of [Dha^7]MC-LR and MC-LR by adsorption onto the ceramic core resulting in a final concentration of 17.8 µg/ml in the synthetic wastewater of the bioreactor. Each point represents ± S.D. of three replicates.

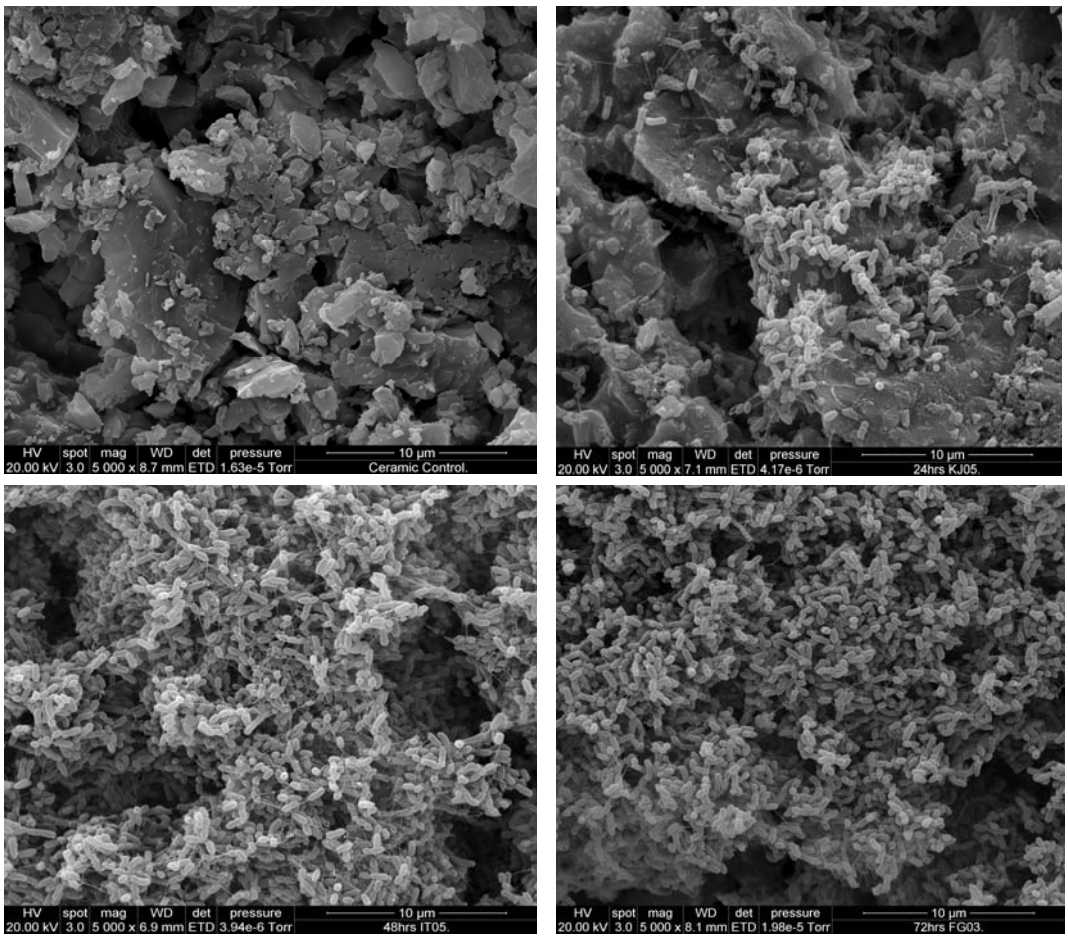


6.6.4 Biofilm formation on the ceramic coupon

The surfaces of the ceramic coupon before and after biofilm formation were examined by SEM (Figure 6.10). The images show the ceramic coupon containing a lot of pores (2-3 nm) and micropores (2-3 μm)(Figure 6.10a), a clear advantage for biofilm formation. The biomass of the bacterium increased over time 24 h (Figure 6.10b), 48 h (Figure 6.10c), and 72 h (Figure 6.10d) and this indicated that the ceramic honeycomb support (CHS) was a very good carrier for isolate NV-3 in MC-degradation.

Figure 6.10 Scanning electron micrograph of the bacterium isolate NV-3 biofilm on the ceramic coupon in (a) the control un-inoculated with culture, and (b) 24 h, (c) 48 h and (d) 72 h of inoculated culture on the ceramic.

(a) (b)



(c) (d)

6.6.5 Batch experiment of MC-degradation

Beginning at 12 h, two degradation by-products were detected in the batch experiment and the MC concentration began to decline. The degradation by-products A and B (previously characterized and described in Chapter 4, Section 4.6.7) increased over time (Figure 6.11). At the 12 h incubation time, the toxins were degraded by about 50% (Figure 6.11b and Figure 6.12) and completely degraded, including the two by-products (A and B), after 30 h of incubation (Figure 6.11d and Figure 6.12).

Figure 6.11 HPLC chromatograms from biodegradation of [Dha^7]MC-LR and MC-LR by the *Sphingomonas* isolate NV-3 at (a) time zero, (b) 12 h, (c) 24 h and (d) 30 h in an IAL-CHS bioreactor as the batch experiment. The peaks A and B represent the biodegradation by-products A and B.

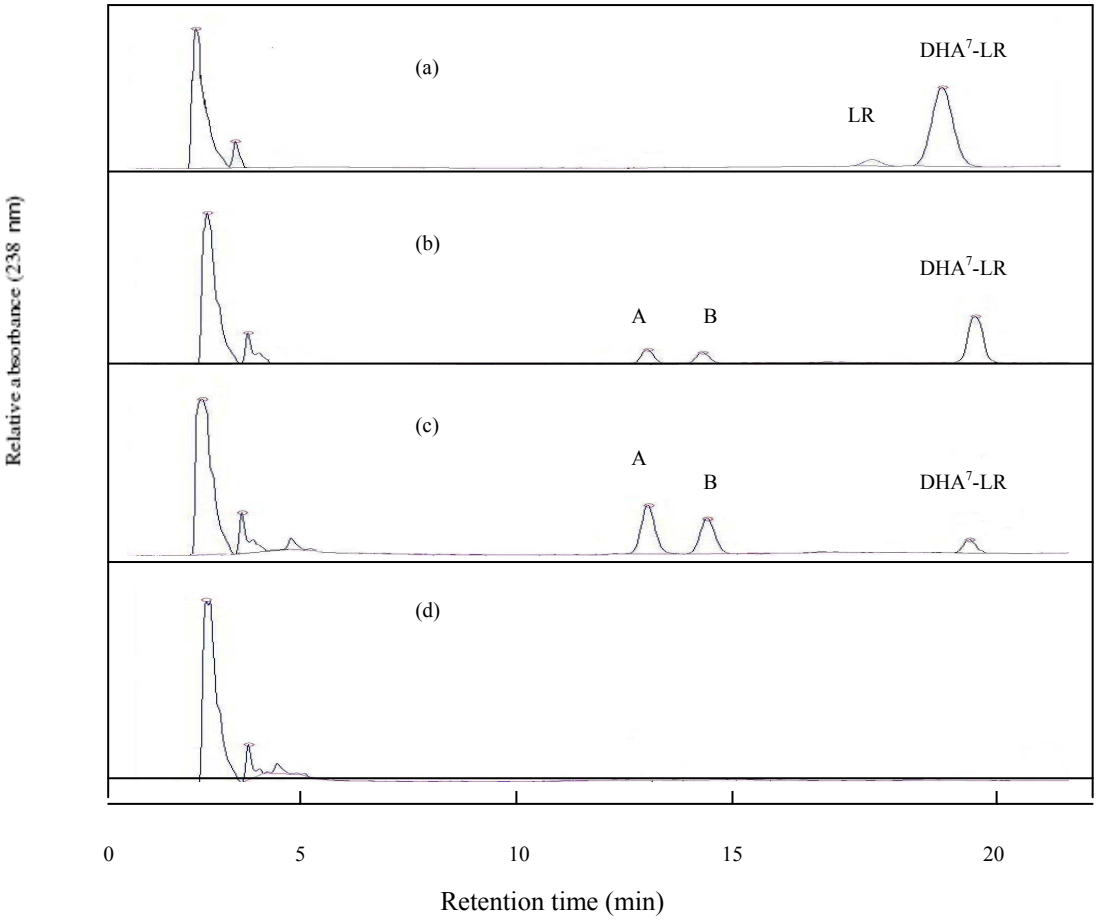
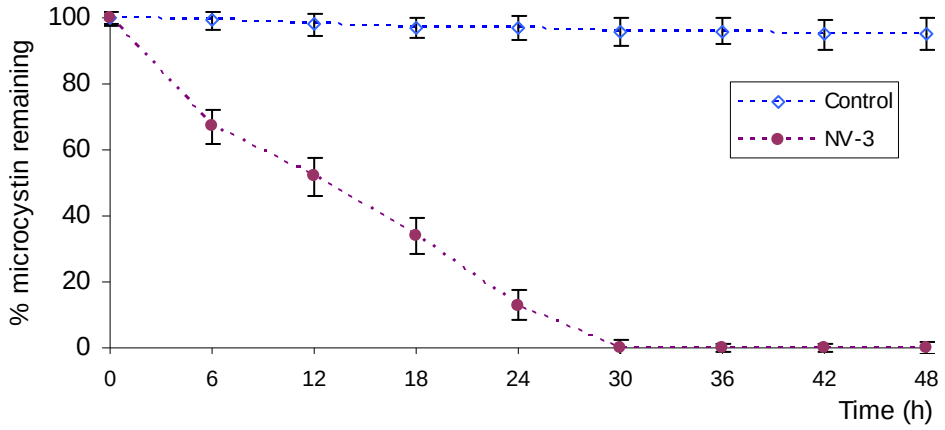


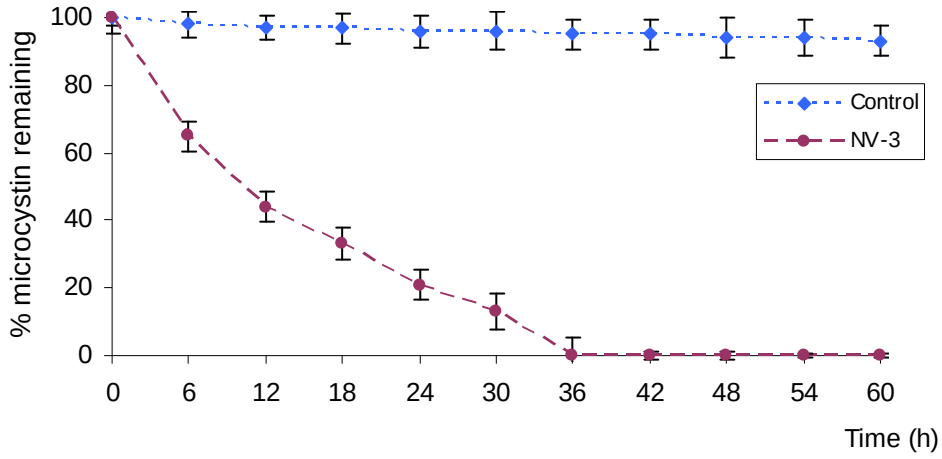
Figure 6.12 Percentage of MC remaining in the bioreactor using batch experiment mode at an initial concentration of [Dha^7]MC-LR and MC-LR of 32.5 µg/ml at 30^{o}C. Each point represents ± S.D. of three replicates.



6.6.6 Continuous-flow experiment of MC-degradation

After adsorption of the toxins onto the CHS had occurred, the 'synthetic wastewater' (with MCs at an initial concentration of 25 µg/ml) was continuously treated in the bioreactor for 8 h hydraulic residence time. The pattern of biodegradation (including appearance and subsequent removal of by-products A and B) was similar to the degradation processes in the batch experiment where the MCs were degraded by about 45% after 12 h incubation, and approximately 80% degradation at 24 h. However, in contrast, the degradation of the toxins, assessed in the effluent leaving the outlet of the bioreactor, was complete at 36 h of incubation (Figure 6.13), not 30 h as with the batch experiment.

Figure 6.13 Percentage of MC remaining in the IAL-CHS bioreactor over a period of 36 h in continuous flow-through mode at 30°C. Each point represents ± S.D. of three replicates.



6.7 Discussion

Microcystins are intracellular toxins that can be released into raw water in significant quantities, by dead and dying cyanobacterial cells, especially if water is treated by algicides and prechlorination (Falconer, 1993; Jones & Orr, 1994; Duy, Lam, Shaw, & Connell, 2000). Therefore cyanobacterial cells should be removed as soon as possible without massive cell lysis. In addition water treatment processes should be effective enough to remove remaining whole cells and the dissolved MCs within the water. As previously noted a wide range of processes have been used for removal of MCs from water, such as photolysis, ozonation, agglomeration, sedimentation, chlorination and activated carbon filtration (Staehelin & Hoigne, 1985; Keijola et al., 1988; Nicholson et al., 1994; Drikas, 1994; Shephard et al., 1998; Lawton & Robertson, 1999; Hitzfeld et al., 2000; Ho, 2004; Bourne et al., 2006). However, these processes rely on quite complicated technology and the use of significant quantities of chemicals. Biological treatment processes, using naturally occurring microorganisms, may provide a cost effective option for removing MCs from water.

Microcystin biodegradation by aquatic bacteria has been reported (Jones et al., 1994; Takenaka & Watanabe, 1997; Park et al., 2001; Maruyama et al., 2003; Saitou et al., 2003; Harada et al., 2004; Ishii et al., 2004; Rapala et al., 2005; Maruyama et

al., 2006; Valeria et al., 2006; Maruyama et al., 2007; Lemes et al., 2008). Bacteria from the genera of *Sphingomonas*, *Sphingosinicella*, *Sphingopyxis*, and *Paucibacter* have been isolated and used in small laboratory scale experiments for removal of toxins (Tsuji et al., 2006; Wang et al., 2007; Ho et al., 2006; Bourne et al., 2006). In this study, the *Sphingomonas* isolate NV-3 was used in a small scale IAL-CHS bioreactor to effectively remove MCs from synthetic wastewater, demonstrating that biodegradation has possible application in water treatment plants.

Assays were carried out to determine whether or not the isolate NV-3 could form biofilms on abiotic surfaces, a necessary first step toward use in the bioreactor. Initial biofilm formation assays on PVC microtiter plates were carried out as a rapid, simple method to assess the potential for biofilm formation by isolate NV-3 (Djordjevic et al., 2002; Harvey et al., 2007). The second stage of the biofilm study involved assays to examine biofilm formation on different abiotic surfaces. It was shown the bacterium could form biofilms on hydrophobic surfaces (polystyrene, polycarbonate, polypropylene, PVC and stainless-steel) as well as hydrophilic surfaces (glass) (O'Toole & Kolter, 1998). As a result of these biofilm experiments it was concluded that isolate NV-3 is a moderate biofilm former, based on criteria used by Stepanovic et al. (2004). Assessment of the ability of isolate NV-3 to form biofilms was achieved using crystal violet staining, ethanol extraction and optical density measurement. Crystal violet, a common dye used for staining the peptidoglycan of bacterial cell walls, was absorbed into the cell walls and released when the cell walls were washed by ethanol. In the case of the isolate NV-3, a gram-negative bacterium, the cell walls possess an external layer of lipopolysaccharide, which is disrupted by the ethanol elution, allowing the crystal violet to escape. The amount of the leaked dye, induced by ethanol extraction from the cell walls, is less than that of gram-positive bacteria, but the amount of the leaked dye can still be used to reflect the number of stained cells on the abiotic surfaces (Pawar, Rossman, & Chen, 2005).

It has been suggested that biofilm formation begins with the attachment of bacterial cells to an abiotic surface by means of flagella, pili or other materials as well as the production of exopolysaccharides to form a glycocalyx matrix entangling the bacteria (Watnick & Kolter, 1999). Harshey (2003) revealed that the bacterial motility

by swimming and swarming is dependent on flagella, whereas the motility by twitching is related to type IV pili (Harshey, 2003). As illustrated in Chapter 4, SEM and TEM results revealed that the isolate NV-3 possesses bipolar flagella and pili, and possibly exopolysaccharides around the bacterial cells. This supports the idea that flagella formation, bacterial motility and the production of exopolysaccharides play a role in the initial phases of biofilm formation (Djordjevic et al., 2002). In addition, biofilm formation by NV-3 seems influenced by cell concentration (Pang et al., 2005). Perhaps not surprisingly, bacteria used for biofilm experiments taken from older cultures, and thus more concentrated, readily formed dense biofilms. This is consistent with work carried out by Pang et al. (2005) who demonstrated that biofilm development is affected by cell density as the opportunity for cellular attachment was stochastically proportional to the number of the cells present in the bulk fluid.

It was confirmed that NV-3 isolate can form biofilms on a wide range of abiotic surfaces. It forms more effectively on hydrophobic surfaces than hydrophilic surfaces, and in descending order of biofilm formation on ceramic, PVC, polystyrene, stainless steel and glass, consistent with findings from other studies on other strains of bacteria (Fletcher & Loeb 1979; Hogt, Dankert, de Vries, & Feijen, 1983; Satou, Satou, & Shintani, 1988). It was revealed by An and Friedman (2000) that the primary force to induce biofilm formation or attachment of bacterial cells to an abiotic surface, under aqueous conditions, is the hydrophobic effect. The ceramic, plastic, metal, and glass all have different surface properties. The glass surfaces are highly electronegative, hydrophilic and possess high surface energy, whereas plastics, metals and ceramics are hydrophobic and have low surface energy (Pawar et al., 2005) - especially important with the ceramics with less surface energy than the plastics (Krol & Krol, 2006). In addition, the ceramic carriers used in these experiments contained a lot of pores (round pores of 3 mm, and 2-3 μm for the millipores), which provided additional surface area and shelter for bacterial cells. It was concluded, therefore, that the ceramic surface would be a good carrier support for use in the bioreactor for MC-degradation. This concurs with studies of Quan et al. (2003) where the ceramic honeycomb support proved to have very good adsorptive ability and promoted the attachment of bacterial cells, when used in the biodegradation of 2,4-dichlorophenol.

It was shown that the abiotic loss of the MCs within the bioreactor, within 6 hours of initial setup, was significant, being approximately 30% of total MCs. It is believed that the loss corresponds to adsorption of the toxins onto the ceramic core as well as onto the surface of the glass bioreactor similar to absorption of the toxins by activated carbon. Similar initial losses of toxins have been observed in other experiments with this type of bioreactor, using other bacterial strains such as *Pseudomonas stutzeri* strain OX1 and *Achromobacter* sp, and involving phenol biodegradation (Quan et al., 2003; Viggiani et al., 2006). It was very important that the ceramic honeycomb support became saturated with the toxins before the biodegradation experiments began. The significant loss (30%) is undoubtedly due to the inherent properties of the ceramic, with its honeycomb structure of many pores and millipores of varying sizes. However, once saturation by adsorption onto the honeycomb was complete, then it could be presumed that subsequent decreases in MC concentration in the IAL-CHS bioreactor (batch and continuous flow) was due to biodegradation of the toxins by the resident biofilm bacteria. Such biodegradation was confirmed by the appearance of degradation by-product A and B in the medium, from enzymatic breakdown of MCs.

To date, as noted earlier, biodegradation of MC-contaminated water has been applied only to small scale bioreactors and sand filtration units (Tsuji et al., 2006; Ho et al., 2006; Bourne et al., 2006; Wang et al., 2007). Tsuji et al. (2006) revealed that MC-RR, at concentrations of 200 μg/l, was entirely decomposed within 24 h (degradation rate = 0.0083 μg/ml/h) in a bioreactor using immobilized cells of the bacterium *Sphingomonas* sp. (strain B-9) on a polyester resin. The biodegradation efficiency continued for 2 months, which indicates strong potential for the practical treatment of MCs. In addition, biological sand filtration has also been tested for bioremediation application (Ho et al., 2006; Bourne et al., 2006). Ho et al. (2006) demonstrated that the removal of MC-LR and MC-LA through the use of sand filters was primarily through biological degradation, rather than any physical processes, and the filtration was able to remove MC-RR at concentrations of 20 μg/l within 4 days (degradation rate = 0.0002 μg/ml/h). Bourne et al. (2006) revealed that complete removal of MC-LR through biologically active slow sand filter columns was observed within 6 days, with initial concentrations of the toxin at 50 μg/l (degradation rate =

0.00034 μg/ml/h), and sampling up to 15 days, after which they did not detect any further breakthrough of the toxin. Therefore, the biological sand filtration is a strong potential treatment process for removal of MCs as it is generally of low technology, requiring little maintenance and infrastructure (Ho et al., 2006; Bourne et al., 2006). Wang et al. (2007) also investigated MC removal by using a column, in which the bacterial cells were immobilized on granular activated carbon (GAC). The results showed that MC-LR at a final concentration of 5 μg/l was removed due to adsorption and biodegradation in the GAC filtration process. Adsorption played a vital role in the removal of the toxins, and biodegradation was found to be an efficient method to remove the toxins once it gets underway.

In this study, the IAL-CHS bioreactor, using a ceramic honeycomb support (CHS) for biofilm formation by the bacterial isolate NV-3, was successful in the removal of [Dha^7]MC-LR and MC-LR, at an initial concentration of 25 μg/ml, within 30 hours in the batch experiment, and within 36 hours in the continuous flow experiment. The degradation rates for (a) the immobilized cells in the batch experiments, (b) the continuous flow experiments (see Sections 6.5.4.4 and 6.5.4.5 in Chapter 6) and (c) the suspended cell assays (see Section 4.5.1 in Chapter 4), were 0.833, 0.69 and 0.35 μg/ml/h, respectively. In general terms, the immobilized bacteria in the IAL-CHS bioreactor (batch and continuous flow), with the same initial concentration of 25 μg/ml of [Dha^7]MC-LR and MC-LR can decompose the toxins more rapidly than the suspended cell assays (see Section 4.6.2). That is, approximately 2 times as fast. These differences may simply reflect the level of exposure per unit time of MCs to the degrading bacteria, and bacterial biomass. High numbers of bacteria concentrated in the pores of the CHS within the IAL-CHS bioreactor were presumably more exposed to circulating MCs than those free in suspension, even though the latter in a shaking incubator. The same issue of exposure per unit time, no doubt applies to the batch and continuous flow experiments. In the batch experiments the MCs were circulated within the bioreactor by internal air lift for the 48 h duration of the experiment. During this time the biomass of bacteria within the CHS would probably have increased. In contrast with a single pass of the 'synthetic wastewater' in the continuous flow experiment, with only an 8 h residence time, it is perhaps not surprising the degradation rate was slightly less.

In addition, biodegradation of MCs by immobilized cells in the IAL-CHS bioreactor can also degrade MCs more rapidly than previous experiments reported by Tsuji et al. (2006), Ho et al. (2006), Bourne et al. (2006), and Wang et al. (2007). This may be because the ceramic honeycomb support contains a greater number of pores, and a wider variety of pore sizes, providing a better environment for bacteria to accumulate than a polyester resin (Tsuji et al., 2006) or sand (Ho et al., 2006; Bourne et al.. 2006; Wang et al., 2007).

In conclusion, therefore, the removal of MCs in the IAL-CHS bioreactor, in which the bacterium isolate NV-3 cells were immobilized onto a ceramic honeycomb support, is a promising and effective treatment process for biodegradation of MCs.

6.8 References

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Chapter 7
General discussion and conclusions

The study of the biodegradation of microcystins (MCs) using bacteria was interesting because it involved a broad range of subjects: including chemistry (analytical chemistry for purification and identification of MCs); microbiology (isolation, identification and characterization of MC-degrading bacteria); biology (cyanobacterial growth patterns, growth curves and effect of an MC-degrading bacterium on *Microcystis* sp.); molecular biology (DNA and PCR techniques for elucidation of genes coding for the MC-degrading enzymes); and environmental engineering or applied sciences (water treatment using an IAL-CHS bioreactor).

It was decided to extract MCs from cyanobacterial bloom samples collected from Lake Horowhenua because (1) it was important to look at degradation of MCs that naturally occur in New Zealand, and (2) significant quantities of MCs were required for biodegradation experiments and the toxins in pure form were expensive (i.e. standard MC-LR = $850 NZD/mg). However, extraction of these naturally occurring MCs was not without its challenges. Extraction and purification of MCs were first carried out at Cawthron Institute, Nelson, over a two week period during April, 2006. This cooperation between Cawthron Institute and the Institute of Food, Nutrition and Human Health (IFNHH) Massey University, was invaluable, as Dr Patrick Holland (senior scientist) provided expert guidance on essential techniques in the field of analytical chemistry. Extraction and purification of MCs was continued on the Wellington campus. However, some minor early problems were encountered because the biochemistry laboratory at Wellington was being upgraded, resulting in delays related to purchase of new equipment. New instruments, such as a refrigerated centrifuge, a sonicator bath, and a UV-VIS detector, were acquired. Very pure water (Milli-Q water) was obtained, and the cyanobacterial bloom material (scum) collected from Lake Horowhenua, was freeze-dried at the IFNHH Food Laboratory (Palmerston North), allowing initial purification steps to proceed, before the much needed new equipment arrived. As is common with bench work, there were decisions to make and problems to solve, such as choice of chromatography column. Initially a closed

column chromatography unit with liquid chromatography (LC) pump, previously used at Cawthron Institute, was considered, followed by an open column chromatography unit, with a positive pressure pump attached. Both proved unsuitable for a variety reasons such as inconsistent flow rate, giving variable results. A new peristaltic pump was attached to the open column chromatography unit, and this combination proved the best long term solution for MC purification. From this point on extraction and purification of MCs progressed steadily. Although a source of frustration from time to time, the sustained bench work and significant problem solving required to ensure smooth operation, was an invaluable experience.

It took about 8 months of extraction and purification to obtain enough MC, about 1.3 g of purified MCs ([Dha^7]MC-LR and MC-LR), for the main part of the study to proceed. It was revealed that every 20 g of freeze-dried material from Lake Horowhenua, New Zealand, yielded about 51 mg dry weight of mainly [Dha^7]MC-LR and small amounts of MC-LR (the major variants of MCs in the freeze-dried material). The collected scum of the cyanobacterium *Microcystis aeruginosa*, proved a rich source of these MCs.

This was the first report on MC extraction and purification from a cyanobacterial bloom in New Zealand. Most other studies simply reported the presence of MC variants and stated concentrations in cyanobacterial bloom material and/or in the water column, without further purification (Hamill, 2001; Stirling & Quilliam, 2001; Mountfort, Holland, & Sprosen, 2004; Wood, 2004; Wood et al., 2006). It proved difficult to separate [Dha^7]MC-LR and MC-LR from each other using the chromatography setup described, because they are very similar molecules. [Dha^7]MC-LR has Dha instead of Mdha (in MC-LR) at amino acid position 7, due to loss of a methyl group (Zurawell, Chen, Burke, & Prepas, 2005). This was not considered a major problem as the mixture of [Dha^7]MC-LR and MC-LR was ideal for use as a substrate for subsequent biodegradation experiments, as it reflected what is present in New Zealand lake water and what will probably be encountered in water treatment plants.

The second challenge of the study was isolation of MC-degrading bacteria. They were isolated, by acclimating water samples (bacterial sources), using purified

MCs as the only bacterial carbon and nitrogen source, following a method used by Jones, Bourne, Blakely, and Doelle (1994); Saitou, Sugiura, Itayama, Inamori, and Matsumura (2003); Valeria, Ricardo, Stephan, and Alberto (2006), and Lemes et al. (2008). Using this technique, only a few bacterial colonies grew on the agar medium. It was presumed that only bacteria which can breakdown and use MCs for their growth will survive and grow on the agar plates. However, there is another more standard microbiological technique that has been used by Takenaka and Watanabe (1997); Park et al. (2001); Tsuji, Asakawa, Anzai, Sumino, and Harada (2006); Ho, Hoefel, Saint, and Newcombe (2007), to successfully isolate MC-degrading bacteria such as *Sphingomonas* strain Y2 and B-9, and *Sphingopyxis* LH21. This involved collecting water samples (or diluted water samples) and then directly plating onto bacterial media agar, yielding a lot of bacterial isolates, but also a lot of extra work for testing authentic MC-degrading bacteria. Therefore, the enrichment technique used in this study was found to be superior to standard microbiological techniques for isolation of MC-degrading bacteria.

This is the first time MC-degrading bacteria have been isolated from New Zealand freshwater lakes, and the first time that biodegradation has been demonstrated on the [Dha^7]MC-LR variant. Three active bacterial isolates, designated NV-1, NV-2, and NV-3, capable of degrading a combination of [Dha^7]MC-LR and MC-LR, were isolated from Lake Rotoiti - a lake with a prior history of cyanobacterial blooms yielding MCs. The bacteria isolated from the water samples of Lake Rotoiti were able to breakdown MCs. In contrast, bacteria isolated from other New Zealand lakes, for example, from Lake Horowhenua where cyanobacterial blooms rich in MCs have been reported (source of MCs for biodegradation experiments), were unable to degrade the toxins. It could be that the water samples collected from the lakes other than Rotoiti, simply did not contain the MC-degrading bacteria. Therefore, more extensive sample collection for isolation of MC-degrading bacteria is recommended for future research.

It was suspected that NV-1, NV-2, and NV-3, isolated from Lake Rotoiti, belonged to the same bacterial genus based on their morphology, colony characteristics, gram staining, and a series of biochemical tests. NV-2 and NV-3 exhibited strong degradative activity with the fastest degradation rate established for

NV-3. Isolate NV-1 exhibited the slowest degradation rate. It is possible NV-1, NV-2 and NV-3 are strains of the same genus. Because NV-1 and NV-3 showed the greatest contrast in MC-degrading activity overall, they were further identified using 16S rRNA sequencing. It was shown that, in spite of differences in MC degradation ability, both isolates belong to the genus *Sphingomonas* with 100% homology of 16S rRNA sequence to the MC-degrading bacterium *Sphingomonas* strain MD-1, which has been shown to exhibit 98.5% homology with the 16S rRNA sequence of *Sphingomonas stygia* (Saitou et al., 2003). To confirm, beyond any doubt, that NV-1 and NV-3 are from the genus *Sphingomonas*, future study will require full genotypic and phenotypic (chemotaxonomic) studies. For example, this would involve DNA hybridization of NV-1 and NV-3 to a designated 'type strain' of the genus *Sphingomonas*, and confirmation of the presence of unique characteristic features of the genus *Sphingomonas*, such as sphingoglycolipids (the most distinctive lipid compound), 2-hydroxymyristic acid (major hydroxylated fatty acid), and ubiquinone Q-10 (major respiratory lipoquinone).

It has been documented that a few strains of *Sphingomonas* (i.e. *Sphingomonas paucimobilis*) can cause a wide range of human illnesses such as bacteremia (bacterial infections of the bloodstream), respiratory infections, urinary infections, and a single case of meningitis. However, infections by *S. paucimobilis* are seldom serious and life threatening (Faden et al., 1981). In this study standard microbiological safety precautions were used and therefore risks kept to a minimum. However, there are grounds for caution and before future research is carried out using *Sphingomonas* isolate NV-3, bacterial risk assessment needs to be carried out for human and environmental safety (Balkwill, Fredrickson & Romine, 2006).

According to scientific literature, world wide, cyanobacteria most commonly occur in summer or early autumn when water temperatures are between 15° and 30ºC (Carmichael, 1994; Dow & Swoboda, 2000; Haider et al., 2003; Svrcek & Smith, 2004). At these temperatures, combined with other factors (e.g. nutrients, sunlight, and calm conditions), cyanobacteria grow rapidly (bloom) and may produce significant amounts of intracellular, as well as extracellular MCs. In New Zealand cyanobacteria also commonly bloom in summer (average water temperature about 20^{o}-30^{o}C) (Pridmore & Etheredge, 1987). Therefore, there is great potential for

cyanotoxin degrading bacteria, such as NV-3, to be used for removal of MCs from water, because it can degrade MCs under a wide range of temperatures (between 10^oC and 35^oC). Significant degradation occurred at relatively high temperatures (above 20^oC) with the highest activity rate at 30^oC. In addition, the minimum number of cells required for biodegradation was established at about $7.9x10^6$ CFU/ml, but in field application the bacterial concentration may need to be above this level (probably at $1.0x10^8$ CFU/ml the same as the optimum established in this study), to ensure the biodegradation process occurs efficiently. It would be interesting, however, for future study to investigate (1) how the bacterium performs within the range of natural water temperatures, (2) what form of bacterium should be used in the actual environment (e.g. cell suspension or lyophilized cells), and (3) what bacterial concentration of NV-3 should be used in the environment.

This is the first study where (1) biodegradation by-products, and (2) genes and putative proteins, involved in MC degradation, were detected and characterized at the same time (working with the [Dha^7]MC-LR variant). Most other studies detected or characterized only the by-products, or only the genes, when working mainly with MC-LR variants (Bourne et al., 1996, 2001, 2005; Saito et al., 2003; Imanishi et al., 2005; Ho et al., 2006, 2007; Geueke et al., 2007). The detection, in this study, of two intermediate by-products (linearized peptides and tetrapeptide), and four genes (*mlr*A, *mlr*B, *mlr*C and *mlr*D) that encode four putative proteins (enzymes) involved in MC degradation, strongly indicates that (1) the biodegradation enzymes that degrade MC-LR can also degrade [Dha^7]MC-LR, and (2) the biodegradation process involved in the breakdown of [Dha^7]MC-LR by NV-3 is similar to that of MC-LR by *Sphingomonas* strain MJ-PV (ACM-3962) (Bourne et al., 1996, 2001). It is proposed that the mechanism of biodegradation of [Dha^7]MC-LR by NV-3 is as follows. The *mlr*A gene encodes a putative metalloprotease (first enzyme) that opens the aromatic ring of [Dha^7]MC-LR at the Arg-Adda peptide bond, yielding linearized peptides (biodegradation by-product A). Next, *mlr*B encodes a putative serine peptidase (second enzyme) which cleaves the linearized peptides at the Ala-Leu bond, producing a tetrapeptide (biodegradation by-product B). In addition, *mlr*C encoding another putative metalloprotease (third enzyme) cuts the peptide bonds randomly at

any peptide bond within [Dha^7]MC-LR, producing smaller peptides or undetectable peptide fragments. Finally, *mlr*D encodes a protein transporter, involved in the transport of digested peptides or small peptides across the bacterial cell. To prove the above biodegradation process, beyond doubt, in future study the enzymes will need to be purified and enzyme activity with substrate specificity examined.

A recent study by Hashimoto et al. (2009) has provided more details of MC-LR degradation processes using the *Sphingomonas* strain B-9. The process was proposed after detection of certain specific biodegradation by-products, along with the linearized peptides and tetrapeptide detected by Bourne et al. (1996). The more detailed process is as follows: MC-LR is hydrolyzed, producing a linearized MC-LR [by-product A from Bourne et al. (1996)] . The cleavage of the linearized MC-LR gives two by-products, the tetrapeptide Adda-Glu-Mdha-Ala [by-product B from Bourne et al. (1996)], and the tripeptide Leu-MeAsp-Arg. Hydrolysis of the tetrapeptide provides mainly Adda and a tripeptide (Glu-Mdha-Ala), as well as minor products such as the tripeptide (Adda-Glu-Mdha). These tripeptides are most likely hydrolyzed to dipeptides (Leu-MeAsp and MeAsp-Arg) and individual amino acids.

It would be an added advantage, in the management of cyanobacterial blooms in water sources, if bacteria such as NV-3, capable of degrading MCs, could also inhibit growth or break down whole *Microcystis* cells and not just the toxin itself. Isolate NV-3 was, therefore, investigated for its ability to inhibit the growth of *Microcystis* cells. It was established that NV-3 did not have a direct affect on the growth of the cyanobacterium by either secretion of bacterial extracellular products or cell-to-cell contact. However, in initial trials where PYEM (the bacterial medium) was involved, it seemed that NV-3 did have an effect on cyanobacterial growth, especially when the ‘bacterial culture’ and ‘bacterial supernatant’ were used. However when sterile BG-11 (a cyanobacterial medium) was used, instead of PYEM, results indicated that NV-3 cells did not, in fact, have an effect on growth of cyanobacteria. This suggested that the bacterial medium itself has an effect on *Microcystis* growth. Therefore, in order to investigate the effect of bacteria on cyanobacterial growth, it is crucial to use two controls, a cyanobacterial medium and a bacterial medium, because many previous studies have used only cyanobacterial media in the controls (Manage Kawabata, & Nakano, 2000; Walker & Higginbotham,

2000; Nakamura, Nakano, Sugiura, & Matsumura, 2003; Hare et al., 2005; Shunyu, Yongding, Yinwu, Genbao, & Dunhai, 2006). It is possible the reported effect of bacteria on intact living cyanobacterial cells was due to the medium itself, and not the bacteria. When bacterial media are used (such as PYEM) for culturing, it is vitally important that when bacterial cells alone are tested for growth inhibition, the cells be washed thoroughly in neutral solution several times, to ensure that the medium has been completely removed from the cells. Therefore, the growth inhibition effects reported in the above studies should be reexamined, to ensure that the bacterial medium used did not interfere with cyanobacterial growth, as demonstrated in this study.

Interesting future research could be carried out investigating induction of a possible algicidal capability in bacteria such as NV-3, using the acclimation technique, because certain abilities in bacterial strains can be activated by exposing bacteria to stress environments or conditions. For example, the capability to degrade p-nitrophenol (PNP) by two strains of bacteria, *Pseudomonas* sp. YTK 17 and *Rhodococcus opacus* YTK 32, was induced by pre-exposure of the bacteria to high concentrations of PNP for 1 month (Shinozaki, Kimura, & Nakahara, 2002). The acclimation process could involve three to five consecutive sub-cultures of NV-3 in fresh *Microcystis* culture, for a consecutive period of 14 days, followed by re-examination of the ability of NV-3 to impact on *Microcystis*. This presumes, of course that the ability to inhibit cyanobacterial growth or lyse cells is latent in NV-3 in the first place.

A significant question for this study is how to apply the cyanotoxin degrading capability of bacteria, such as NV-3, to the removal of the toxins from New Zealand waterbodies. Biodegradation of MCs by NV-3 in the IAL-CHS bioreactor was investigated. It was shown that NV-3 was effective in removing MCs in the IAL-CHS bioreactor. With this potential, the IAL-CHS bioreactor could be scaled up as a ready-to-use, or on-site water treatment unit, for rural areas and small communities, to manage water quality when cyanobacteria are blooming. It could minimize the costs of water treatment using low cost technology requiring relatively little infrastructure and maintenance. However, more studies are required to investigate the use of scaled-up IAL-CHS bioreactors in field application, including, (1) how IAL-CHS bioreactors

could be physically adapted in small treatment plants, (2) the efficacy, volume of water that could be treated, duration of treatment and cost of a adapted IAL-CHS design, (3) how NV-3 will perform and react in the actual environment where a wide range of substrate compounds exist, (4) whether or not the bacteria are stable in the field and perform the same biodegradation processes as noted in the laboratory, (5) difficulties associated with stimulating bacterial growth in the field, and (6) other factors that might reduce the degradation activity of NV-3 in the environment.

For applying IAL-CHS bioreactor technology to small scale water treatment, the bioreactor may be adapted as follows: (1) a flat water-flow concrete tank (around 1 m deep x 50 m long x 1 m wide) as the bioreactor, (2) crushed tiles or ceramic, as the bacterial carrier support instead of the CHS, (3) NV-3 used directly in the form of freeze-dried material (cells) (commonly used in commercial wastewater inoculants), or by suspending it in PYEM broth (or other available bacterial media). The bacterial cells would be immobilized onto the tiles by soaking them together at ambient temperature for 3-5 days, depending on how biofilm formation progresses (in controlled laboratory conditions, NV-3 was incubated at 30^{o}C for 36 hours), (4) a flow through principle used to expose water to NV-3 isolate, and (5) electronic operation of inflow-outflow of water in bioreactor (concrete tank) to maintain hydraulic retention time (HRT) in place of peristaltic pump. However, pilot studies of proposed design would need to be carried out to obtain preliminary data on performance, and improve efficiency of the bioreactor for bigger scale water treatment.

Another option for using NV-3 for small scale water treatment is the use of slow sand filtration by inoculating the bacterium into the sand layer. Slow sand filters, under experimental conditions, have proved successful for the complete removal of MCs using MC-degrading bacteria (Ho et al., 2006; Bourne, Blakeley, Riddles, & Jones, 2006). This would provide a cost-effective water treatment process, linked in with existing technology, with less operator attention, which has potential for water treatment facilities in rural areas (Logsdon, Kohne, Abel, & LaBonde, 2002; Bourne, Blakeley, Riddles, & Jones, 2006). However, laboratory scale operations, using NV-3 in slow sand filtration, need to be investigated to evaluate stability, efficacy, and potential viability of NV-3 under such conditions.

Another possible application for the use of NV-3 is by directly spraying a culture broth of the bacterium onto the surface of water where cyanobacterial blooms occur. This method might be a simple way for accelerating the removal of MCs. More research needs to be carried out to establish, for example, (1) what concentration of NV-3 should be used, and (2) potential health and environmental risks related to the use of NV-3 in the actual environment.

To apply NV-3 to existing large scale water treatment plants, the bacterium could be immobilized onto floating plastic carriers in flocculation and sedimentation tanks. It has already been shown that the bacterial isolate NV-3 is a moderate biofilm former on the ceramic, polyvinylchloride plastic (PVC) and polystyrene surfaces (see Chapter 6). According to Nakamura, Nakano, Sungira and Matsumura (2003), this technology was successful for controlling natural floating *Microcystis* blooms, by immobilizing the bacterium *Bacillus cereus* N-14 onto floating plastic carriers, resulting in 99% elimination of the cyanobacteria in 4 days.

Finally, several contributions have been made from this study to the fields of toxic cyanobacteria and hepatotoxins. Firstly, a modified method of MC extraction and purification offered a cost effective option, for obtaining significant amounts of MCs from freeze-dried samples from a cyanobacterial bloom, rather than purchase expensive commercial grade supplies. Secondly, three MC-degrading bacteria (isolates NV-1, NV-2 and NV-3) were purified from a New Zealand water body with great potential for larger scale biodegradation of MCs. Thirdly, detection of the intermediate degradation by-products and four genes responsible for MC degradation provided evidence for the mechanism of [Dha^7]MC-LR degradation by bacteria in the aquatic environment. Fourthly, the internal airlift loop ceramic honeycomb support bioreactor (IAL-CHS bioreactor), using NV-3, proved capable of degrading MCs, and thus provides an option for scaled-up operations to remove MCs from New Zealand waterbodies.

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Appendix 1

Chemicals, materials, and instruments of Chapter 3

A1.1 Chemicals, reagents and solutions

Table A1.1 Chemicals, reagent list

Chemicals and media	Company	Used for/in
Acetone (commercial grade)	BDH	General (cleaning glasses)
Absolute methanol (MeOH)	BDH	Extraction of MCs
Trifluoroacetic acid (TFA)	Sigma	Extraction of MCs
Ammonium sulfate	BDH	Protein precipitation
Absolute ethanol (EtOH)	BDH	Purification of MCs (solution A, B)
Potassium hydroxide (KOH)	BDH	Purification of MCs (solution A, B)
2-Morpholinoethanesulfonic acid (MES)	Sigma	Purification of MCs (solution A, B)
Sodium chloride (NaCl)	BDH	Purification of MCs (solution B)
Toyopearl DEAE-650M resin	Tosoh	Purification of MCs
MC-LR and MC-RR standards	Sigma	Identification of MCs
Acetonitrile (HPLC grade)	J. T. Baker	Identification of MCs (mobile phase)
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck	Identification of MCs (buffer)
Di-sodium hydrogen phosphate (Na_2HPO_4)	Merck	Identification of MCs (buffer)
Hydrochloric acid (HCl)	BDH	pH adjustment
Sodium Hydroxide (NaOH)	BDH	pH adjustment

Table A1.2 Solution and buffer preparations

70% MeOH-0.1%TFA	For 1 liter: • 700 ml of MeOH • 1.0 ml of TFA • Mix and adjust in a total volume of 1L milli-Q water
0.05 M MES-KOH(pH 5.5)-20%EtOH (solution A)	For 1 liter: • 10.66 g of MES (2-Morpholinoethane- sulfonic acid) • 200 ml of EtOH • Mix and adjust pH 5.5 using KOH with a total volume of 1L milli-Q water

Table A1.2 (continued) Solution and buffer preparations

0.05 M MES-KOH (pH 5.5)-20%EtOH- 1 M NaCl (solution B)	For 1 liter: • 10.66 g of MES (2-Morpholinoethane- sulfonic acid) • 58.5 g of NaCl • 200 ml of EtOH • Mix and adjust pH 5.5 using KOH with a total volume of 1L milli-Q water
0.05 M phosphate buffer (pH 3.0)	For 1 liter: • 7.645 g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) • 0.142 g of sodium phosphate (anhydrous) (Na_2HPO_4) • Mix and adjust pH using 0.5 M HCl with a total volume of 1L milli-Q water • Filtrate the solution with 0.22 μm membrane filter
0.5 M HCl	For 1 liter: • 41.30 ml of HCl (conc. 37.4%) • Adjust in a total volume of 1L milli-Q water
0.5 M NaOH	For 1 liter: • 20 g of NaOH • Mix and adjust in a total volume of 1L milli-Q water

Note: The water used for solution and buffer preparations was purified using Milli-Q reagent water system from Millipore (Bedford, MA, USA) with a resistance of at least 18 MΩ cm^{-1} throughout the study

A1.2 Instruments

Table A1.3 Instrument list

Instruments and materials	Company	Model / Type
Analytical balance	Mettler	AE 100 and PL 1200
pH meter	Suntex	SP-2200
Laminar flow	EcoAir	5763
Rotary evaporator	Buchi	Rotavapor R
Bench top centrifuge	Eppendorf	5810R
Micro centrifuge	Beckman	Microfuge E

Table A1.3 (continued) Instrument list

Instruments and materials	Company	Model / Type
Shaker (orbital incubator)	New Brunsick	Innova 4230
Water bath	Thermo	Haake C 10
Fraction collector	ISCO	620
Peristaltic pump	Gilson	Minipuls 3
UV-Vis spectrometer	Bausch & Lomb	Spectronic 2000
HPLC system		
- Degasser	Uniflow	DG-1210
- HPLC pump	Waters	515
- UV-VIS detector	Shimadzu	SPD-20A
- Chromatography Data Systems	PeakSimple	333
Solid phase extraction cartridge	Phenomenex	Strata-x
Anion exchange resin	Tosoh	TSK-GEL ODS-80Ts resin
Filtration system	Whatman	2 liter
Membrane filters	Whatman	GF/C and GF/F

Table A1.4 Material and miscellaneous

Miscellaneous	Company
GF/C paper filter	Whatman
Microcentrifuge tube (1.6 ml)	Neptune
PCR tubes (20 µl)	Neptune
Pipettes (0.2-2 µl, 2-20 µl, 20-200µl, 100-1000 µl)	Thermo
Pipette tips with filter (0.2-2 µl, 2-20 µl, 20-200µl, 1000 µl)	Neptune
Glassware	Schott
15 and 50 ml conical-bottom disposable plastic tubes	Nunc

Appendix 2

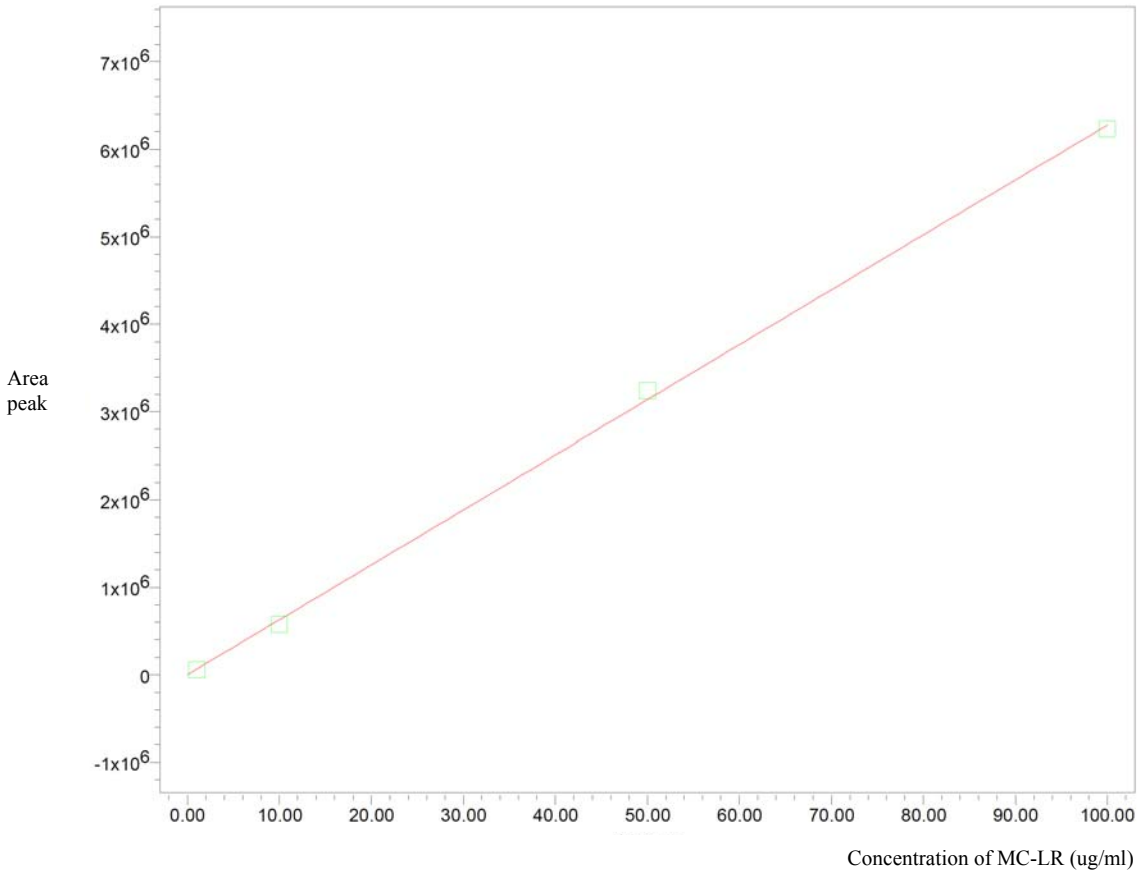
Identification and characterization of MCs with LC-MS/MS

A coupled liquid chromatography-tandem mass spectrometry system, consisting of an alliance 2790 liquid chromatography (Waters) and a Quattro Ultima (Micromass) tandem mass spectrometer, was used. A Luna C-18 column (150 mm x 2 mm i.d., 5 μm) (Phenomenex) was used for LC separation with the column oven at 30°C. The mobile phase gradient was water/acetonitrile with acidic buffer (1.7 mM ammonium formate and 24 mM formic acid at the flow rate of 0.2 ml/min. The mass spectrometer was operated in positive mode electrospray ionization (ESI^+) for parent ion spectrum, daughter ion spectrum and multiple reaction monitoring (MRM) for MS-MS channels set up. The nebulizing, desolvation and cone gas were supplied with nitrogen. During tandem mass spectrometric analysis, the parent ion and daughter ion (characteristic ion) traces monitored by tandem MS/MS and collision energy (CE) was optimized for each product-ion trace measured. The instrument was calibrated with authentic standards of MC-RR, MC-YR, and MC-LR and gave highly linear calibration curves for concentrations in the range of 5–200 ng/ml. The response factors for MC-LR were applied to the other related toxins for which no pure analytical standards were available.

Appendix 3

Calibration plot of microcystin-LR concentration

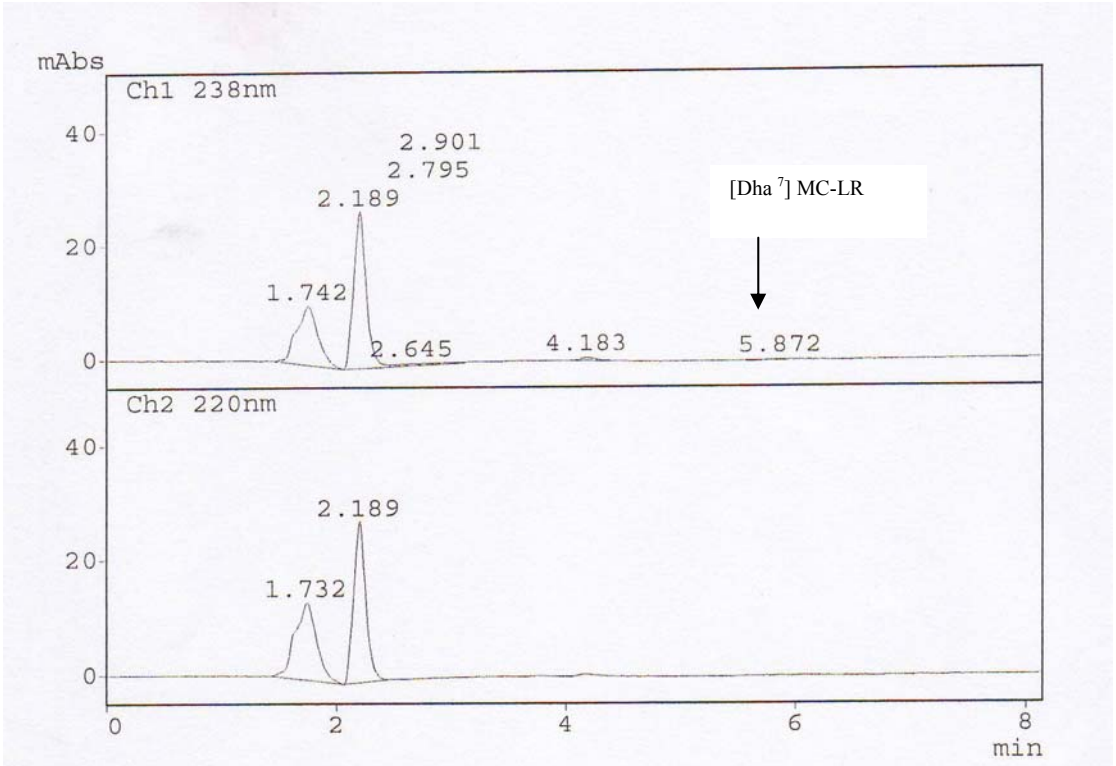
Figure A3.1 Microcystin-LR standard at 1, 10, 50 and 100 µg/ml (R^2 = 0.9994383)



Appendix 4

HPLC chromatograms of microcystin elution from the Strata-X SPE cartridges

Figure A4.1 [Dha^7]MC-LR eluted from Strata-X cartridges with 30% aqueous methanol measured by HPLC-UVD system, including channel 1 measured at 238 nm and channel 2 measured at 220 nm

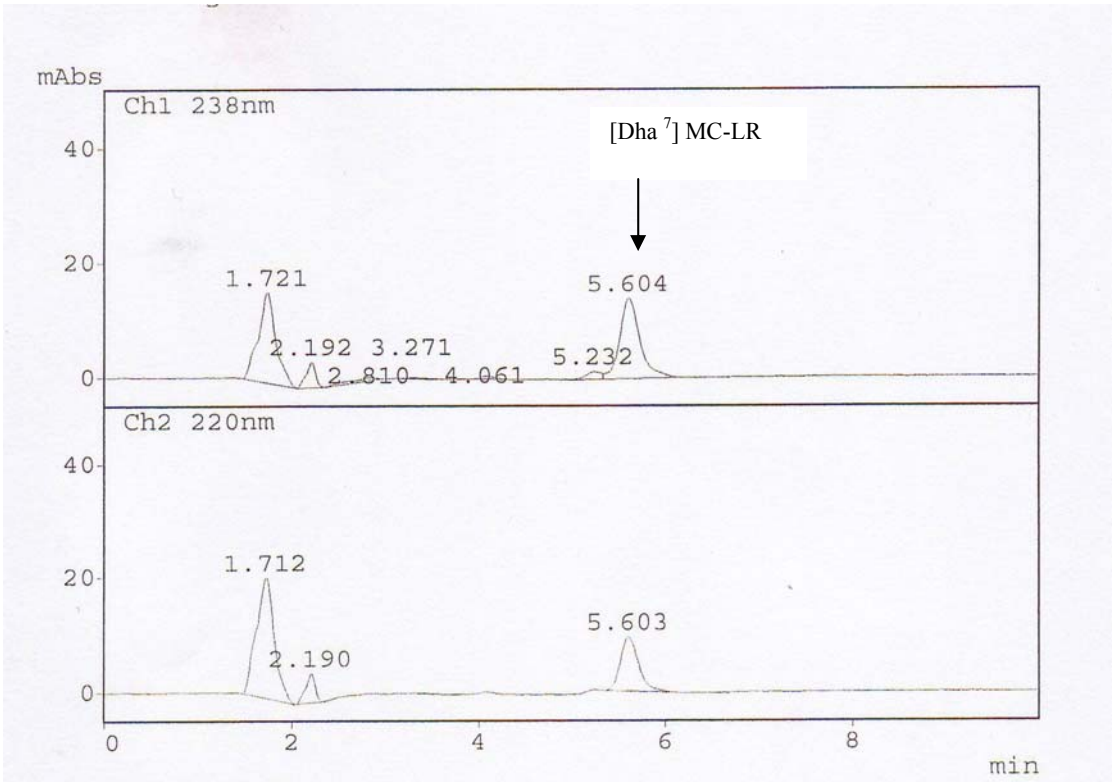


*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN
1	1	1.742	135388			
	2	1.732	177585			
2	1	2.189	210095			
	2	2.189	209213			
3	1	2.645	5328	V		
4	1	2.795	2141	V		
5	1	2.901	3396	V		
6	1	4.183	5311			
7	1	5.872	1008			
			749464			

*** [Dha 7] MC-LR **

Figure A4.2 [Dha[7]]MC-LR eluted from Strata-X cartridges with 40% aqueous methanol measured by HPLC-UVD system, including channel 1 measured at 238 nm and channel 2 measured at 220 nm

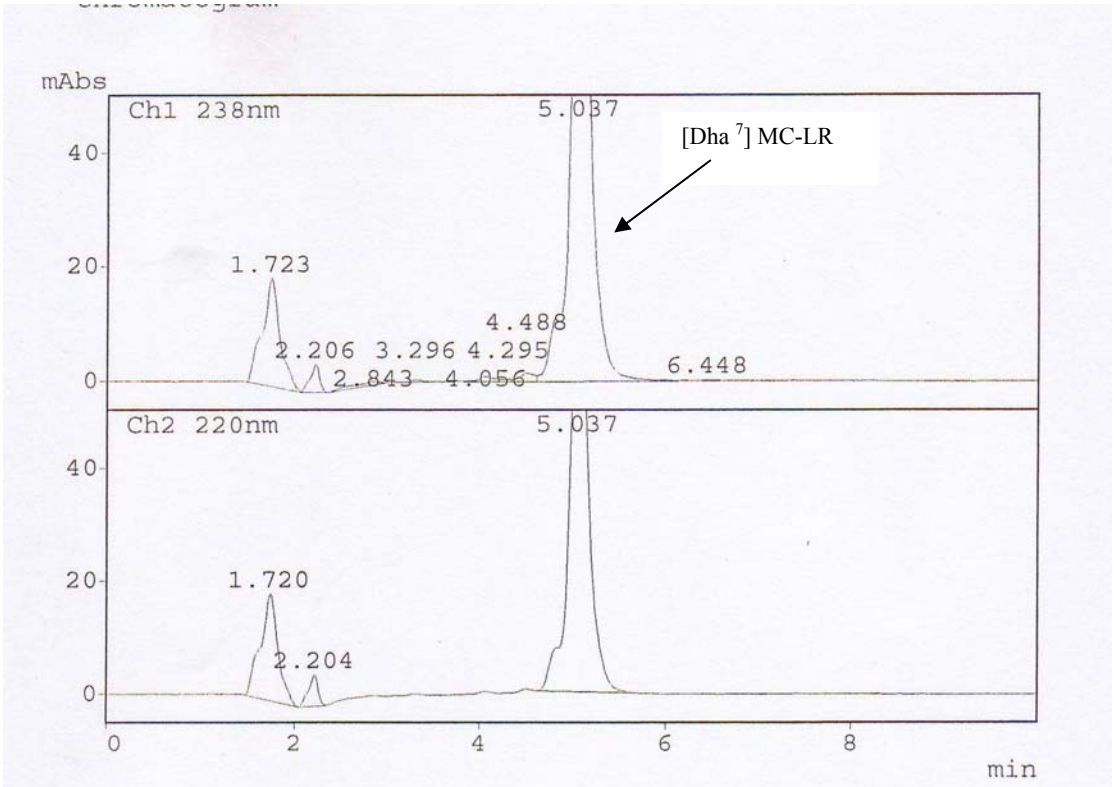


*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN
1	1	1.721	204727			
	2	1.712	280326			
2	1	2.192	31244			
	2	2.190	36486			
3	1	2.810	12836			
4	1	3.271	2320			
5	1	4.061	5767			
6	1	5.232	14668	V		
7	1	5.604	212547	V		
	2	5.603	131141			
			932062			

*** [Dha 7] MC-LR **

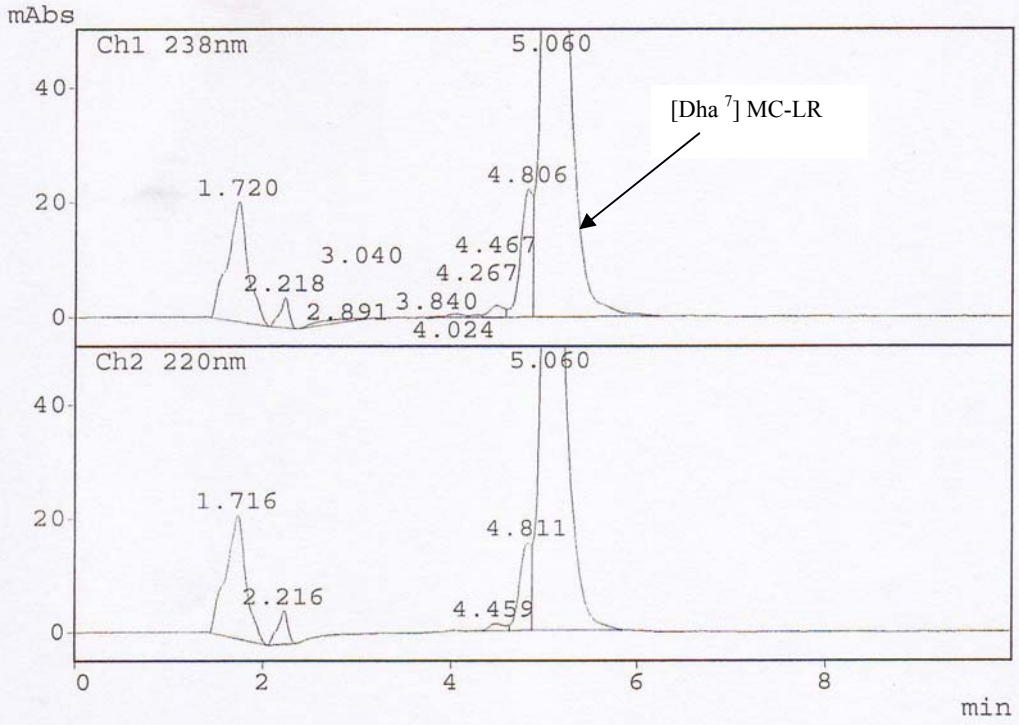
Figure A4.3 [Dha[7]]MC-LR eluted from Strata-X cartridges with 50% aqueous methanol measured by HPLC-UVD system, including channel 1 measured at 238 nm and channel 2 measured at 220 nm



*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN
1	1	1.723	250970			
	2	1.720	248079			
2	1	2.206	33879			
	2	2.204	40168			
3	1	2.843	15232			
4	1	3.296	3572			
5	1	4.056	10529	V		
6	1	4.295	3129	V		
7	1	4.488	15558	V		
8	1	5.037	1659124	SV	*** [Dha 7] MC-LR **	
	2	5.037	1153607			
9	1	6.448	1200	T		
			3435046			

Figure A4.4 [Dha^{7}]MC-LR eluted from Strata-X cartridges with 60% aqueous methanol measured by HPLC-UVD system, including channel 1 measured at 238 nm and channel 2 measured at 220 nm

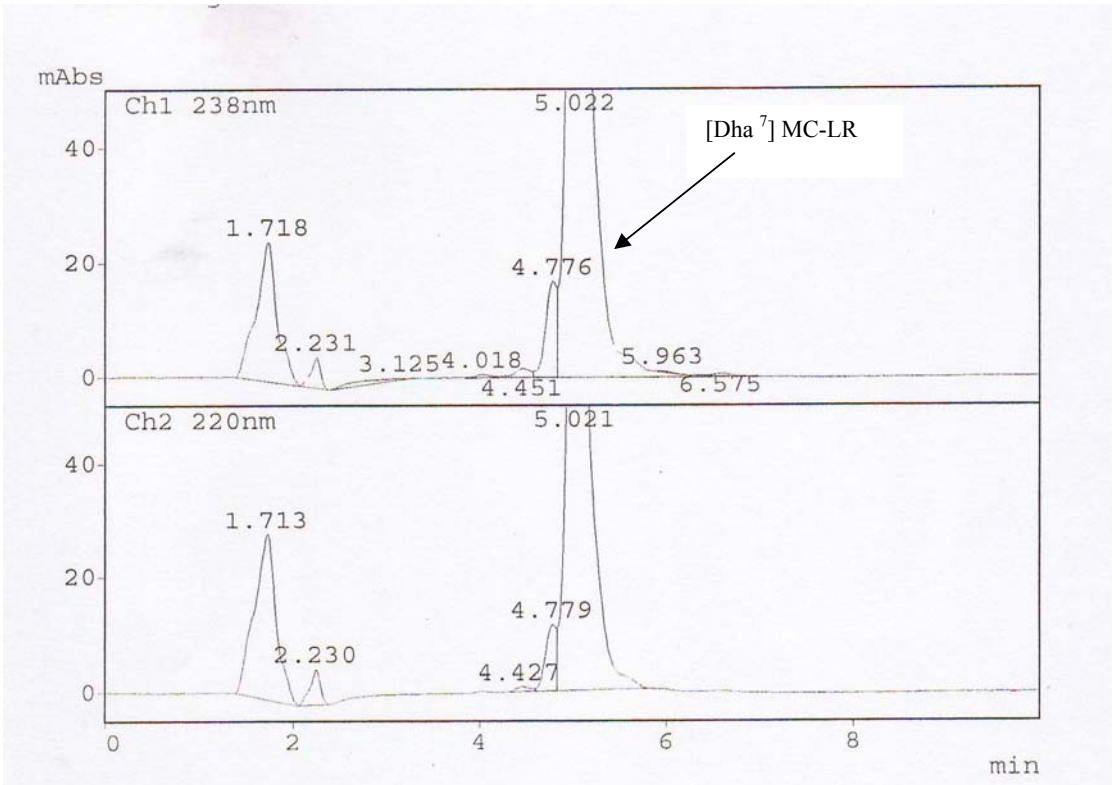


*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN
1	1	1.720	311129			
	2	1.716	325743			
2	1	2.218	35727			
	2	2.216	44958			
3	1	2.891	19036			
4	1	3.040	1234	V		
5	1	3.840	1813	V		
6	1	4.024	7139	V		
7	1	4.267	3321	V		
8	1	4.467	22010	V		
	2	4.459	14436			
9	1	4.806	203108	V		
	2	4.811	137815	V		
10	1	5.060	3261081	SV		
	2	5.060	2337332	V		
			6725882			

*** [Dha 7] MC-LR **

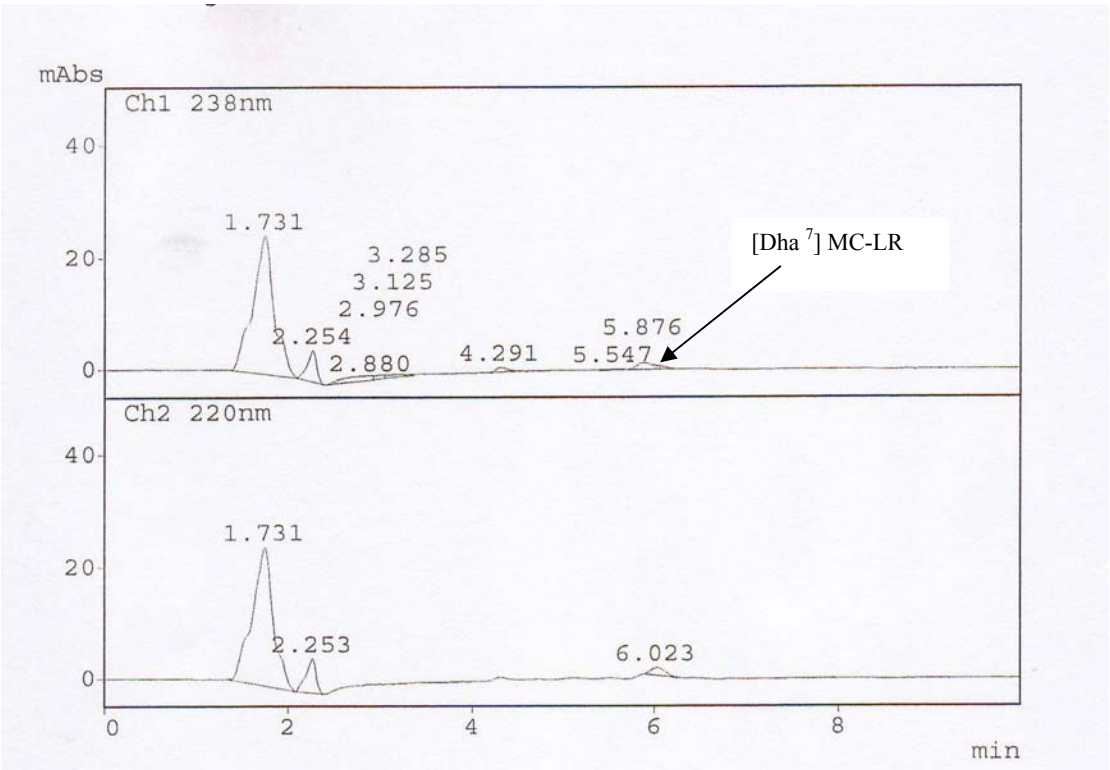
Figure A4.5 [Dha[7]]MC-LR eluted from Strata-X cartridges with 70% aqueous methanol measured by HPLC-UVD system, including channel 1 measured at 238 nm and channel 2 measured at 220 nm



*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN
1	1	1.718	376910			
	2	1.713	461541			
2	1	2.231	38709			
	2	2.230	47890			
3	1	3.125	24398			
4	1	4.018	6339	V		
5	2	4.427	8899			
6	1	4.451	19954	V		
7	1	4.776	147179	V		
	2	4.779	96573	V		
8	1	5.022	2954514	SV	*** [Dha 7] MC-LR **	
	2	5.021	2080398	V		
9	1	5.963	1492	T		
10	1	6.575	6339	T		
			6271132			

Figure A4.6 [Dha[7]]MC-LR eluted from Strata-X cartridges with 80% aqueous methanol measured by HPLC-UVD system, including channel 1 measured at 238 nm and channel 2 measured at 220 nm



*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN
1	1	1.731	401461			
	2	1.731	398602			
2	1	2.254	39368			
	2	2.253	47688			
3	1	2.880	21598			
4	1	2.976	4768	V		
5	1	3.125	6398	V		
6	1	3.285	1286	V		
7	1	4.291	7706			
8	1	5.547	1339			
9	1	5.876	17715	V		
10	2	6.023	15390			
			963318			

*** [Dha 7] MC-LR **

Appendix 5

Chemicals, materials, and instruments of Chapter 4

A5.1 Bacterial media and identification

Table A5.1 Chemicals, media and kit lists

Chemicals and media	Company	Used for/in
Peptone	Difco	Peptone-yeast extract medium
Yeast extract	Difco	Peptone-yeast extract medium
Magnesium sulphate ($MgSO_4$)	M&B	Mineral salts medium
Zine sulphate ($ZnSO_4$)	BDH	Mineral salts medium
Sodium molybdate (Na_2MoO_4)	BDH	Mineral salts medium
Potasium hydrogen phosphate (KH_2PO_4)	BDH	Mineral salts medium
Di-sodium hydrogen phosphate (Na_2HPO_4)	M&B	Mineral salts medium
Calcium chloride ($CaCl_2$)	BDH	Mineral salts medium
Ferric chloride ($FeCl_3$)	BDH	Mineral salts medium
Hydrochloric acid (HCl)	BDH	pH adjustment
Sodium Hydroxide (NaOH)	BDH	pH adjustment
API 20NE test kit	BioMerieux	Bacterial identification

Table A5.2 Bacterial media and preparations

Peptone-yeast extract medium (PYEM)	For 1 liter: • 10 g peptone • 5 g yeast extract • Dissolve in a total of 1L milli-Q water and adjust pH 7.2 using 0.1 M HCl or 0.1 M NaOH • Sterile using autoclave at 121^{o}C for 15 min • Check pH of the solution again after sterilization with pH paper
Mineral salts medium (MSM)	For 1 liter: • 112 mg $MgSO_4 \cdot H_2O$, 5 mg $ZnSO_4 \cdot H_2O$, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 340 mg KH_2PO_4, 670 mg $Na_2HPO_4 \cdot 7H_2O$, 14 mg $CaCl_2$ and 0.13 mg $FeCl_3$ • Dissolve in a total of 1L milli-Q water and adjust pH 7.2 using HCl or NaOH • Sterile using autoclave at 121^{o}C for 15 min • Check pH after sterilization with pH paper

A5.2 Molecular biology chemicals, reagents, and kits

Table A5.3 Chemicals, media and kit lists

Chemical, media and kit	Company	Used for/in
Wizard® Genomic DNA Purification kit	Promega	DNA Purification
Ethanol, for molecular biology	Sigma	DNA Purification
Isopropanol	Sigma	DNA Purification
AmpliTaq Gold DNA polymerase	Applied Biosystems	PCR
Taq DNA polymerase, recombinant	Invitrogen	PCR
10X PCR buffer	Invitrogen	PCR
50 mM Magnesium choloride	Invitrogen	PCR
10 mM dNTPs mixture	Invitrogen	PCR
UltraPure DNase/RNase-free distilled water	Invitrogen	PCR
1 Kb plus DNA ladder	Invitrogen	Gel electrophoresis
Seakem LE agarose	Cambrex	Gel electrophoresis
Glycerol, for molecular biology	Sigma	Gel electrophoresis
Tris base	Sigma	Gel electrophoresis
Ethidium bromide	Sigma	Gel electrophoresis
Bromophenol blue	M&B	Gel electrophoresis
Xylene cyano FF	M&B	Gel electrophoresis
Wizard SV gel and kit	Promega	PCR clean up

Table A5.4 Molecular buffers and solutions

Buffers and solutions	Preparation
50X TAE buffer	For 1 liter: • 242 g of Tris base • 57.1 ml of glacial acetic acid • 100 ml of 0.5M EDTA (pH 8.0) • Dissolve in a total volume of 1L milli-Q water
1X TAE buffer	For 1 liter: • Dilute 20 ml of 50X TAE buffer to a total volume of 1 L with milli-Q water
0.5M EDTA (pH 8.0)	For 1 liter: • 186.1 g EDTA • Dissolve in a total volume of 1L milli-Q water • Adjust pH with NaOH pellets
Loading dye	For 10 ml: • 0.025 g of bromophenol blue • 0.025 g of xylene cyano FF • 3 ml of 100% glycerol • Dissolve in a total of 10 ml milli-Q water

Table A5.4 (continued) Molecular buffers and solutions

Buffers and solutions	Preparation
1 Kb DNA ladder	For 100 µl: • 10 µl of 1 Kb plus DNA ladder (1 µg/µl) • 20 µl of Loading dye • 70 µl of ultraPure DNase/RNase-free distilled water
Ethidium bromide (10mg/ml)	For 100 ml: • 1 g of ethidium bromide • Dissolve in a total of 100 ml milli-Q water

Note: The water used for solution and buffer preparations was purified using Milli-Q reagent water system from Millipore (Bedford, MA, USA) with a resistance of at least 18 MΩ cm^{-1} throughout the study.

Table A5.5 Miscellaneous

Miscellaneous	**Company**
Microcentrifuge tube (1.6 ml)	Neptune
PCR tubes (20 µl)	Neptune
Pipettes (0.2-2 µl, 2-20 µl, 20-200µl, 100-1000 µl)	Thermo
Pipette tips with filter (0.2-2 µl, 2-20 µl, 20-200µl, 1000 µl)	Neptune
Glassware	Schott
15 and 50 ml conical-bottom disposable plastic tubes	Nunc

A5.3 Instruments

Table A5.6 Instrument list

Instruments and materials	**Company**	**Model**
Analytical balance	Mettler	AE 100 and PL 1200
pH meter	Suntex	SP-2200
Biological safety cabinet	ETR Environmental	4FT class II
Autoclave	Getinge	HS 6610 EC-1
Micro centrifuge	Eppendorf	5415D
Shaking incubator	New Brunsick	Innova 4250
Incubator	Contherm	Pular 1000 C
Microwave	Whirlpool	M 595
Water bath	Thermo	Haake C 10

Table A5.6 (continued) Instrument list

Instruments and materials	Company	Model
HPLC system		
- Degasser	Uniflow	DG-1210
- HPLC pump	Waters	515
- UV-VIS detector	Shimadzu	SPD-20A
- Chromatography Data Systems	PeakSimple	333
PCR machine	Thermo Hybaid	PX2
Gel electrophoresis set		
- Horizontal Mini gel	C.B.S Scientific	MGU-402T
- Power supply	C.B.S Scientific	ETS-250-II
Transilluminator	UVipro	Serial N
-80^{o} C Freezer	Thermo Forma	702
Biophotometer	Eppendorf	RS 232C

Appendix 6

Reading and interpretation of API 20 NE identification kit

1) GLU, ADH, URE, ESC, GEL and PNPG tests were recorded and interpreted according to the color without adding any reagents (see Table A6.1).

2) NO_3 test

The NO_3 cupule was added with 1 drop of NIT 1 and 1 drop of NIT 2 reagents (BioMerieux), After 5 min, a pink red color indicates a positive reaction while a negative reaction may be due to the production of nitrogen. Therefore the NO_3 cupule was added again with 2 mg of zinc dust. After 5 min, a cupule remaining colorless indicates a positive reaction.

3) TRP test

The TRP cupule was added with 1 drop of JAMES reagent (BioMerieux). The reaction takes place immediately: a pink color which develops in the whole cupule indicates a positive reaction.

Note: The reading of the two tests NO_3 and TRP should be performed while protecting the assimilation tests from airborne contamination, using the incubation box lid during the reading of the enzymatic tests.

4) Assimilation tests, [GLU], [ARA], [MNE], [MAN], [NAG], [MAL], [GNT], [CAP], [ADI], [MLT], [CIT] and [PAC], read and interpreted the results, according to bacterial growth. An opaque cupule indicated a positive reaction. Table 6.1 used for analyzing the assimilation test results.

The reactions were translated into numerical profiles and interpreted by using the API LAB software. The quality of the identification is indicated based on the values of the identification percentage (ID).

Table A6.1 Interpretation of reactions of API 20 NE. The table below was used to help analyze the results using API 20 NE.

TEST	SUBSTRATES	QTY	ENZYMZTIC ACTIVITY OR REACTION TESTED	RESULTS	
				NEGATIVE	POSITIVE
NO3	potassium nitrate	0.12 mg	reduction of nitrates to nitrites	NIT 1 (1 drop) + NIT 2 (1 drop) /up to 5 min: colorless	pink-red
			reduction of nitrates to nitrogen	Zinc dust (2-3 mg) / up to 5 min: pink-red	colorless
TRP	tryptophane	0.4 mg	indole production	JAMES (1 drop) / immediate: colorless pale green / yellow	pink
GLU	glucose	2.0 mg	acidification	blue to green	yellow
ADH	arginine	2.0 mg	arginine dihydrolase	yellow	orange / pink / red
URE	urea	0.8 mg	urease	yellow	orange / pink / red
ESC	esculin	0.56 mg	hydrolysis (β-glucosidase)	yellow	grey / brown / black
GEL	gelatin (with India ink)	0.6 mg	hydrolysis (protease)	no pigment diffusion	diffusion of black pigment
PNPG	p-nitrophenyl-β-D-galactopyranoside isopropylthiogalacto-pyranoside (IPTG)	0.2 mg 8.0 ug	β-galactosidase	colorless	yellow
GLU	glucose	1.5 mg	assimilation	transparent	opaque
ARA	arabinose	1.4 mg	assimilation	transparent	opaque
MNE	mannose	1.4 mg	assimilation	transparent	opaque
MAN	mannitol	1.4 mg	assimilation	transparent	opaque
NAG	N-acetyl-glucosamine	1.3 mg	assimilation	transparent	opaque
MAL	maltose	1.4 mg	assimilation	transparent	opaque
GNT	gluconate	1.8 mg	assimilation	transparent	opaque
CAP	caprate	0.8 mg	assimilation	transparent	opaque
ADI	adipate	1.1 mg	assimilation	transparent	opaque
MLT	malate	1.5 mg	assimilation	transparent	opaque
CIT	citrate	2.3 mg	assimilation	transparent	opaque
PAC	phenyl-acetate	0.8 mg	assimilation	transparent	opaque

Appendix 7

16s rRNA sequencing method

A7.1 PCR and sequencing primers.

All primers were obtained from Invitrogen (New Zealand).

The primer pairs used for 16s rRNA gene amplification were:

Primer name	Sequence	Corresponding *E. coli* base #
16F27	5′-AGA GTT TGA TCM TGG CTC AG-3′	8 to 27
16R1541	5′-AAG GAG GTG ATC CAG CCG CA -3′	1541to 1520

Sequencing was performed using the following primers:

Primer name	Sequence	Corresponding *E. coli* base #
16F357	5’-ACT CCT ACG GGA GGC AGC AG-3’	337 to 357
16F530	5’-GTG CCA GCM GCC GCG G-3’	514 to 530
16F945	5’-GGG CCC GCA CAA GCG GTG G-3’	926 to 945
16R518	5’-CGT ATT ACC GCG GCT GCT GG-3’	537 to 518
16R1087	5’-CTC GTT GCG GGA CTT AAC CC-3’	1107 to 1087
16R1389	5’-ACG GGC GGT GTG TAC AAG	1406 to 1389

A7.2 DNA isolation and 16S rRNA gene PCR

Genomic DNA was extracted from the purified plate-grown bacterial isolate using the Roche High Pure PCR Template Preparation Kit (USA). An initial PCR amplification was carried out using the outer primer pairs 16SF27 and 16SR1541 that amplify a ~1,500-bp fragment of the 16S rRNA that is considered to be universal for the domain *Bacteria*.

Amplification of the bacterial 16S rRNA was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems, USA) by PCR using the following reaction conditions: 35 cycles of denaturation for 30 sec at 94°C; annealing for 30 sec at 55°C; and elongation for 45 sec at 72°C. Amplicon detection was carried out by agarose gel electrophoresis of 5 µl of amplification product through horizontal 2% agarose gels in 0.5 × Tris-borate-EDTA buffers. After electrophoresis, amplicons were stained with ethidium bromide and visualised by UV light irradiation before photographing.

A7.3 Sequencing reaction

Purification of the amplicon was performed using the Roche High Pure PCR Product Purification Kit prior to sequencing. Primers internal to the outer primers (F357, F530, F, R518, R1087 & R1492) were used for amplification and sequencing of the initial PCR product to obtain overlapping products in both directions.

All resulting PCR products were then sequenced using an ABI PRISM BigDye terminator DNA sequencing kit (Applied Biosystems, USA). The sequencing products were analyzed on a model 3730XL ABI DNA sequencer (Applied Biosystems, USA), following the instructions supplied by the manufacturer.

A7.4 Sequence Analysis

Sequence analysis was performed using Bionumerics version 4. The data obtained with the six sequencing primers were aligned to produce a consensus sequence. The 16S rRNA sequence was compared with those available in the GenBank, EMBL, and DJB prokaryote databases using the default settings of the Fasta3 alignment programme through the EBI server and the similarity matrix tool from the Ribosomal Database Project II (RDP-II).

Appendix 8

Taxonomic identification reports of bacterium isolate NV-3

Bacterium name: Bacterium isolate NV-3		
Sequence Identification Number: S07/031		
Primers used:	F357, F530, F945 R518, R1087, R1389	**Length of 16S DNA sequence obtained (# of nucleotides):** 1436

Results of pairwise DNA sequence alignment:

EMBL database search (FASTA):

The sample most closely resembles the species *Sphingomonas* sp. MD-1 strain, with 100% gapped (100% ungapped) DNA sequence identity for a 1436-nucleotide overlap of the 5'-end of the 16S rRNA gene.

RDPII database search:

The sample is most closely related to the isolate *Sphingomonas* sp. strain MD-1, with 100% sequence homology for 1436 contiguous nucleotides in the 16S rRNA gene

Taxonomic Identification of sample:

Bacteria (domain); Proteobacteria (phylum); Alphaproteobacteria (class); Sphingomonadales (order); Sphingomonadaceae (family); *Spingomonas* (genus); *Sphingomonas* sp. strain MD-1 (species).

DNA Sequence

16S rRNA gene consensus sequence obtained from bacterial isolate NV-3

1-1436 bases from 5'-origin

```
   1 AACGAACGCT GGCGGCATGC CTAACACATG CAAGTCGAAC GAACCCTTCG GGGTTAGTGG   60
  61 CGCACGGGTG CGTAACGCGT GGGAATCTGC CCTTTGCTTC GGAATAACTC AGGGAAACTT  120
 121 GTGCTAATAC CGGATGATGT CTTCGGACCA AAGATTTATC GGCAAGGGAT GAGCCCGCGT  180
 181 AGGATTAGGT AGTTGGTGGG GTAAAGGCCT ACCAAGCCGA CGATCCTTAG CTGGTCTGAG  240
 241 AGGATGATCA GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTG  300
 301 GGGAATATTG GACAATGGGC GAAAGCCTGA TCCAGCAATG CCGCGTGAGT GATGAAGGCC  360
 361 CTCGGGTCGT AAAGCTCTTT TACCAGGGAT GATAATGACA GTACCTGGAG AATAAGCTCC  420
 421 GGCTAACTCC GTGCCAGCAG CCGCGGTAAT ACGGAGGGAG CTAGCGTTGT TCGGAATTAC  480
 481 TGGGCGTAAA GCGCACGTAG GCGGCTACTC AAGTCAGAGG TGAAAGCCCG GGGCTCAACC  540
 541 CCGGAACTGC CTTTGAAACT AGGTGGCTAG AATCTTGGAG AGGCGAGTGG AATTCCGAGT  600
 601 GTAGAGGTGA AATTCGTAGA TATTCGGAAG AACACCAGTG GCGAAGGCGA CTCGCTGGAC  660
 661 AAGTATTGAC GCTGAGGTGC GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT  720
 721 CCACGCCGTA AACGATGATA ACTAGCTGTC CGGGTACTTG GTACTTGGGT GGCGCAGCTA  780
 781 ACGCATTAAG TTATCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATTGAC  840
 821 GGGGGCCTGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA AGCAACGCGC AGAACCTTAC  900
 901 CAGCGTTTGA CATCCCGCGC TAACCCGAGA GATCGGGTGT TCCCTTCGGG GACGCGGTGA  960
 961 CAGGTGCTGC ATGGCTGTCG TCAGCTCGTG TCGTGAGATG TTGGGTTAAG TCCCGCAACG 1020
1021 AGCGCAACCC TCGTCCTTAG TTGCCATCAT TTAGTTGGGC ACTCTAAGGA AACCGCCGGT 1080
1081 GATAAGCCGG AGGAAGGTGG GGATGACGTC AAGTCCTCAT GGCCCTTACA CGCTGGGCTA 1140
1141 CACACGTGCT ACAATGGCGG TGACAGTGGG CAGCAAGCAC GCGAGTGTGA GCTAATCTCC 1200
1201 AAAAGCCGTC TCAGTTCGGA TTGTTCTCTG CAACTCGAGA GCATGAAGGC GGAATCGCTA 1260
1261 GTAATCGCGG ATCAGCATGC CGCGGTGAAT ACGTTCCCAG GCCTTGTACA CACCGCCCGT 1320
1321 CACACCATGG GAGTTGGTTT CACCCGAAGG CAGTGCGCTA ACCGCAAGGA GGCAGCTGAC 1380
1381 CACGGTGGGA TCAGCGACTG GGGTGAAGTC GTAACAAGGT AGCCGTAGGG GAACCT     1436
```

Fasta Summary Table

SUBMISSION PARAMETERS			
Title	S07/031 1436 nt	**Database**	em_rel_pro
Sequence length	1436	**Sequence type**	nucleotide
Program	fasta	**Version**	34.26 January 12, 2007
Expectation upper value	10.0	**Sequence range**	1-
Number of scores	20	**Number of alignments**	20
Word size	6	**Open gap penalty**	-14
Gap extension penalty	-4	**Histogram**	false

DB:ID	Source	Length	Identity%	Similar%	Overlap	E()
EM_PRO:AB110635	*Sphingomonas* sp. MD-1 gene f	1474	100.000	100.000	1436	0
EM_PRO:AJ746094	*Novosphingobium* sp. MG37 par	1448	99.930	99.930	1436	0
EM_PRO:AJ746092	*Novosphingobium* sp. MG35 par	1445	99.791	99.791	1436	0
EM_PRO:AB025013	*Sphingomonas stygia* gene for	1444	98.034	98.034	1424	0
EM_PRO:AJ009707	*Novosphingobium* sp. K39 part	1420	97.748	97.748	1421	0
EM_PRO:CP000248	*Novosphingobium aromaticivor*	3561584	97.357	97.357	1438	0
EM_PRO:AJ000920	*Novosphingobium* sp. K16 part	1438	97.147	97.147	1437	0
EM_PRO:AJ001051	*Sphingomonas* sp. strain A282	1440	96.871	96.940	1438	0
EM_PRO:AB025014	*Sphingomonas subterranea* gen	1446	97.335	97.335	1426	0
EM_PRO:AB025012	*Sphingomonas aromaticivorans*	1446	97.265	97.265	1426	0
EM_PRO:EF421434	*Novosphingobium* sp. Cep1 16S	1451	96.989	96.989	1428	0
EM_PRO:AJ746093	*Novosphingobium* sp. MG36 par	1350	99.852	99.852	1350	0
EM_PRO:AF411072	*Sphingomonas* sp. ACM-3962	1466	96.657	96.657	1436	0
EM_PRO:AJ001053	*Sphingomonas* sp. strain C282	1438	96.732	96.871	1438	0
EM_PRO:AB023290	*Sphingomonas* sp. MBIC4193	1428	96.709	96.709	1428	0
EM_PRO:EF044233	*Novosphingobium* sp. strain K	1433	96.706	96.706	1427	0
EM_PRO:D16147	*Sphingomonas capsulata* DNA	1443	96.456	96.456	1439	0
EM_PRO:AF235996	Alpha proteobacterium F06021	1444	96.489	96.489	1424	0
EM_PRO:AJ416411	*Novosphingobium hassiacum* pa	1485	96.111	96.111	1440	0
EM_PRO:DQ840049	*Sphingomonas* sp. H 16S ribos	1407	97.086	97.086	1407	0

Appendix 9

Taxonomic identification reports of bacterium isolate NV-1

Bacterium name: Bacterium isolate NV-1 **Sequence Identification Number:** S07/030		
Primers used:	F357, F530, F945 R518, R1087, R1389	**Length of 16S DNA sequence obtained (# of nucleotides):** 1437

Results of pairwise DNA sequence alignment:

EMBL database search (FASTA):

The sample most closely resembles the species *Sphingomonas* sp. MD-1 strain, with 100% gapped (100% ungapped) DNA sequence identity for a 1436-nucleotide overlap of the 5'-end of the 16S rRNA gene.

RDPII database search:

The sample is most closely related to the isolate *Sphingomonas* sp. strain MD-1, with 100% sequence homology for 1436 contiguous nucleotides in the 16S rRNA gene.

Taxonomic Identification of sample:

Bacteria (domain); Proteobacteria (phylum); Alphaproteobacteria (class); Sphingomonadales (order); Sphingomonadaceae (family); *Spingomonas* (genus); *Sphingomonas* sp. strain MD-1 (species).

DNA Sequence

16S rRNA gene consensus sequence obtained from bacterial isolate NV-1

1-1437 bases from 5'-origin

```
   1 AACGAACGCT GGCGGCATGC CTAACACATG CAAGTCGAAC GAACCCTTCG GGGTTAGTGG   60
  61 CGCACGGGTG CGTAACGCGT GGGAATCTGC CCTTTGCTTC GGAATAACTC AGGGAAACTT  120
 121 GTGCTAATAC CGGATGATGT CTTCGGACCA AAGATTTATC GGCAAGGGAT GAGCCCGCGT  180
 181 AGGATTAGGT AGTTGGTGGG GTAAAGGCCT ACCAAGCCGA CGATCCTTAG CTGGTCTGAG  240
 241 AGGATGATCA GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTG  300
 301 GGGAATATTG GACAATGGGC GAAAGCCTGA TCCAGCAATG CCGCGTGAGT GATGAAGGCC  360
 361 CTCGGGTCGT AAAGCTCTTT TACCAGGGAT GATAATGACA GTACCTGGAG AATAAGCTCC  420
 421 GGCTAACTCC GTGCCAGCAG CCGCGGTAAT ACGGAGGGAG CTAGCGTTGT TCGGAATTAC  480
 481 TGGGCGTAAA GCGCACGTAG GCGGCTACTC AAGTCAGAGG TGAAAGCCCG GGGCTCAACC  540
 541 CCGGAACTGC CTTTGAAACT AGGTGGCTAG AATCTTGGAG AGGCGAGTGG AATTCCGAGT  600
 601 GTAGAGGTGA AATTCGTAGA TATTCGGAAG AACACCAGTG GCGAAGGCGA CTCGCTGGAC  660
 661 AAGTATTGAC GCTGAGGTGC GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT  720
 721 CCACGCCGTA AACGATGATA ACTAGCTGTC CGGGTACTTG GTACTTGGGT GGCGCAGCTA  780
 781 ACGCATTAAG TTATCCGCCT GGGGAGTACG GTCGCAAGAT TAAAACTCAA AGGAATTGAC  840
 821 GGGGGCCTGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA AGCAACGCGC AGAACCTTAC  900
 901 CAGCGTTTGA CATCCCGCGC TAACCCGAGA GATCGGGTGT TCCCTTCGGG GACGCGGTGA  960
 961 CAGGTGCTGC ATGGCTGTCG TCAGCTCGTG TCGTGAGATG TTGGGTTAAG TCCCGCAACG 1020
1021 AGCGCAACCC TCGTCCTTAG TTGCCATCAT TTAGTTGGGC ACTCTAAGGA AACCGCCGGT 1080
1081 GATAAGCCGG AGGAAGGTGG GGATGACGTC AAGTCCTCAT GGCCCTTACA CGCTGGGCTA 1140
1141 CACACGTGCT ACAATGGCGG TGACAGTGGG CAGCAAGCAC GCGAGTGTGA GCTAATCTCC 1200
1201 AAAAGCCGTC TCAGTTCGGA TTGTTCTCTG CAACTCGAGA GCATGAAGGC GGAATCGCTA 1260
1261 GTAATCGCGG ATCAGCATGC CGCGGTGAAT ACGTTCCCAG GCCTTGTACA CACCGCCCGT 1320
1321 CACACCATGG GAGTTGGTTT CACCCGAAGG CAGTGCGCTA ACCGCAAGGA GGCAGCTGAC 1380
1381 CACGGTGGGA TCAGCGACTG GGGTGAAGTC GTAACAAGGT AGCCGTAGGG GAACCT     1436
```

Fasta Summary Table

SUBMISSION PARAMETERS			
Title	S07/030 1437 nt	**Database**	em_rel_pro
Sequence length	1437	**Sequence type**	nucleotide
Program	fasta	**Version**	34.26 January 12, 2007
Expectation upper value	10.0	**Sequence range**	1-
Number of scores	20	**Number of alignments**	20
Word size	6	**Open gap penalty**	-14
Gap extension penalty	-4	**Histogram**	false

DB:ID	Source	Length	Identity%	Similar%	Overlap	E()
EM_PRO:AB110635	*Sphingomonas* sp. MD-1 gene f	1474	100.000	100.000	1437	0
EM_PRO:AJ746094	*Novosphingobium* sp. MG37 par	1448	99.930	99.930	1437	0
EM_PRO:AJ746092	*Novosphingobium* sp. MG35 par	1445	99.791	99.791	1437	0
EM_PRO:AB025013	*Sphingomonas stygia* gene for	1444	98.034	98.034	1424	0
EM_PRO:AJ009707	*Novosphingobium* sp. K39 part	1420	97.748	97.748	1421	0
EM_PRO:CP000248	*Novosphingobium*	3561584	97.359	97.359	1439	0
EM_PRO:AJ000920	*Novosphingobium* sp. K16 part	1438	97.149	97.149	1438	0
EM_PRO:AJ001051	*Sphingomonas* sp. strain A282	1440	96.873	96.942	1439	0
EM_PRO:AB025014	*Sphingomonas subterranea* gen	1446	97.335	97.335	1426	0
EM_PRO:AB025012	*Sphingomonas* aromaticivorans	1446	97.265	97.265	1426	0
EM_PRO:EF421434	*Novosphingobium* sp. Cep1 16S	1451	96.989	96.989	1428	0
EM_PRO:AJ746093	*Novosphingobium* sp. MG36 par	1350	99.852	99.852	1350	0
EM_PRO:AF411072	*Sphingomonas* sp. ACM-3962 16	1466	96.660	96.660	1437	0
EM_PRO:AJ001053	*Sphingomonas* sp. strain C282	1438	96.734	96.873	1439	0
EM_PRO:AB023290	*Sphingomonas* sp. MBIC4193 ge	1428	96.709	96.709	1428	0
EM_PRO:EF044233	*Novosphingobium* sp. strain K	1433	96.706	96.706	1427	0
EM_PRO:D16147	*Sphingomonas capsulata* DNA for	1443	96.458	96.458	1440	0
EM_PRO:AJ416411	*Novosphingobium hassiacum* pa	1485	96.114	96.114	1441	0
EM_PRO:AF235996	Alpha proteobacterium F06021	1444	96.489	96.489	1424	0
EM_PRO:DQ840049	*Sphingomonas* sp. H 16S ribos	1407	97.086	97.086	1407	0

Appendix 10

Partial nucleotides of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes and predicted amino acids of putative MlrA, MlrB, MlrC and MlrD proteins of the bacterial isolate NV-1

A10.1 *mlr*A gene

a) Partial nucleotide sequences of *mlr*A gene

```
aagatactgcaggagacgcacgctcacctcaacattattaccgctgtcaggtctacgttcgagtatccg
ggagcctatacgctcttactgtttccggccgccccaatgttcgcggccctgatcgcaaccgggatcggc
tatgggcaagcaggatttcgtgaactgctcagccgctgcgccccgtggcggtcgcctgtttcctggcgt
cagggcgttactgtcatagccgtgtgtttccttgcattcttcgcgctcacaggaatcatgtgggttcag
acatacctctacgctccgcctggtacgcttgatcgtaccttcctgcgctatggggcagatccggtcgcc
atttatgtgatgctggcagcatcgctgctactcagccctggcccgctgctggaagaactgggctggcgc
ggctttgcgctgccgcagctcctcaagaagtttgacccccttaccgcagcggtgatcctcggcatcatg
tggtgggcctggcatttgccacgcgacctgccaacactgttctccggcgcccctggcgcggcctggagc
gttattgtcaaacaactcgttatcgctcctgggttcattgcgagcaccatcatcgctgtcttcgtatgc
aacaagctcggtggatcaatgtgggggggcgtgctcactcacgccatccataacgagctgggagtaaac
gtcactgccgaatgggccccaacggtcgcaggcatcgggtggcgcccatgggatctcatcgaattt
(756 bp)
```

b) Translation of *mlr*A gene

```
mlrA: AAGATACTGCAGGAGACGCACGCTCACCTCAACATTATTACCGCTGTCAGG
      K  I  L  Q  E  T  H  A  H  L  N  I  I  T  A  V  R

mlrA: TCTACGTTCGAGTATCCGGGAGCCTATACGCTCTTACTGTTTCCGGCCGCC
      S  T  F  E  Y  P  G  A  Y  T  L  L  L  F  P  A  A

mlrA: CCAATGTTCGCGGCCCTGATCGCAACCGGGATCGGCTATGGGCAAGCAGGA
      P  M  F  A  A  L  I  A  T  G  I  G  Y  G  Q  A  G

mlrA: TTTCGTGAACTGCTCAGCCGCTGCGCCCCGTGGCGGTCGCCTGTTTCCTGG
      F  R  E  L  L  S  R  C  A  P  W  R  S  P  V  S  W

mlrA: CGTCAGGGCGTTACTGTCATAGCCGTGTGTTTCCTTGCATTCTTCGCGCTC
      R  Q  G  V  T  V  I  A  V  C  F  L  A  F  F  A  L

mlrA: ACAGGAATCATGTGGGTTCAGACATACCTCTACGCTCCGCCTGGTACGCTT
      T  G  I  M  W  V  Q  T  Y  L  Y  A  P  P  G  T  L

mlrA: GATCGTACCTTCCTGCGCTATGGGGCAGATCCGGTCGCCATTTATGTGATG
      D  R  T  F  L  R  Y  G  A  D  P  V  A  I  Y  V  M

mlrA: CTGGCAGCATCGCTGCTACTCAGCCCTGGCCCGCTGCTGGAAGAACTGGGC
      L  A  A  S  L  L  L  S  P  G  P  L  L  E  E  L  G

mlrA: TGGCGCGGCTTTGCGCTGCCGCAGCTCCTCAAGAAGTTTGACCCCCTTACC
      W  R  G  F  A  L  P  Q  L  L  K  K  F  D  P  L  T

mlrA: GCAGCGGTGATCCTCGGCATCATGTGGTGGGCCTGGCATTTGCCACGCGAC
      A  A  V  I  L  G  I  M  W  W  A  W  H  L  P  R  D
```

*mlr*A: CTGCCAACACTGTTCTCCGGCGCCCCTGGCGCGGCCTGGAGCGTTATTGTC
L P T L F S G A P G A A W S V I V

*mlr*A: AAACAACTCGTTATCGCTCCTGGGTTCATTGCGAGCACCATCATCGCTGTC
K Q L V I A P G F I A S T I I A V

*mlr*A: TTCGTATGCAACAAGCTCGGTGGATCAATGTGGGGGGGCGTGCTCACTCAC
F V C N K L G G S M W G G V L T H

*mlr*A: GCCATCCATAACGAGCTGGGAGTAAACGTCACTGCCGAATGGGCCCCAACG
A I H N E L G V N V T A E W A P T

*mlr*A: GTCGCAGGCATCGGGTGGCGCCCATGGGATCTCATCGAATTT
V A G I G W R P W D L I E F

c) Predicted polypeptide sequences of *mlr*A gene (predicted MlrA protein)

KILQETHAHLNIITAVRSTFEYPGAYTLLLFPAAPMFAALIATGIGYGQAGFRELLSRCAPWRSPVSWR
QGVTVIAVCFLAFFALTGIMWVQTYLYAPPGTLDRTFLRYGADPVAIYVMLAASLLLSPGPLLEELGWR
GFALPQLLKKFDPLTAAVILGIMWWAWHLPRDLPTLFSGAPGAAWSVIVKQLVIAPGFIASTIIAVFVC
NKLGGSMWGGVLTHAIHNELGVNVTAEWAPTVAGIGWRPWDLIEF (252 aa)

A10.2 *mlr*B gene

a) Partial nucleotide sequences of *mlr*B gene

caggcgctcgaagtggcgcgcctcgcgaacgggaaactgcattctggcagacctgtcgattatgccggt
gggttgttcgtggacgatcggcaaagcgagcgtgttgtgtcgcattcgggcttggttgtgggcaatcgc
gccatggacgtactttatccggagagcgggattggtatcagcgtgatgtgtaatcgcgacgatatctcg
ccagctgagcgtgcgcgcaaaattgctttgctcgtgaagcccggggcgcccgatccagcttttgaccgc
gcaattgatcctgccgaaatgaaacgcctgggaaaaattggcgacctgcgctccgcacctgacgtctat
tat (348 bp)

b) Translation of *mlr*B gene

mlrB: CAGGCGCTCGAAGTGGCGCGCCTCGCGAACGGGAAACTGCATTCTGGCAGA
Q A L E V A R L A N G K L H S G R

mlrB: CCTGTCGATTATGCCGGTGGGTTGTTCGTGGACGATCGGCAAAGCGAGCGT
P V D Y A G G L F V D D R Q S E R

mlrB: GTTGTGTCGCATTCGGGCTTGGTTGTGGGCAATCGCGCCATGGACGTACTT
V V S H S G L V V G N R A M D V L

mlrB: TATCCGGAGAGCGGGATTGGTATCAGCGTGATGTGTAATCGCGACGATATC
Y P E S G I G I S V M C N R D D I

mlrB: TCGCCAGCTGAGCGTGCGCGCAAAATTGCTTTGCTCGTGAAGCCCGGGGCG
S P A E R A R K I A L L V K P G A

mlrB: CCCGATCCAGCTTTTGACCGCGCAATTGATCCTGCCGAAATGAAACGCCTG
P D P A F D R A I D P A E M K R L

mlrB: GGAAAAATTGGCGACCTGCGCTCCGCACCTGACGTCTATTAT
G K I G D L R S A P D V Y Y

c) Predicted polypeptide sequences of *mlr*B gene (predicted MlrB protein)

```
QALEVARLANGKLHSGRPVDYAGGLFVDDRQSERVVSHSGLVVGNRAMDVLYPESGIGISVMCNRDDIS
PAERARKIALLVKPGAPDPAFDRAIDPAEMKRLGKIGDLRSAPDVYY (116 aa)
```

A10.3 *mlr*C gene

a) Partial nucleotide sequences of *mlr*C gene

```
gcgcggatggggtcgaaagtgctgatctataccaacaacgatcagccagctgctgcctctatcgcacaa
gacttcggtcggcgctaccaagccatggcttcgatcatgaaaggcaacggccccgagcgaagctttgcg
gccgacatcgagctagccaaggcggccaccgcatacccggtaatcctggtcgatagttcggacaacccc
ggcggtggggcttcgggtgacaatatggcattggcccgagcgatgctggacaatgacctcgtcccgtcg
tgcattgggccgatatgggatcccctggcagtacaattgggctttgaagccggccttggtgccgatttt
tccctgcgcgttggcggcaaggtcggcgaggcatccgggctacctctcgacgttcgcggcaaaatcaca
gggcttgccgagaatgtcacccaaaaccttcagggctctcggccgcctctggggcgcgtcgtctgcatc
agtacaggtggtctagacatcatcgtcagcgaaattcgcgaccagtgctacggccccgatatgttccgg
gcgctcggtgttgaacctgcgaacaagcgctac (585 bp)
```

b) Translation of *mlr*C gene

```
mlrC : GCGCGGATGGGGTCGAAAGTGCTGATCTATACCAACAACGATCAGCCAGCT
    A  R  M  G  S  K  V  L  I  Y  T  N  N  D  Q  P  A

mlrC : GCTGCCTCTATCGCACAAGACTTCGGTCGGCGCTACCAAGCCATGGCTTCG
    A  A  S  I  A  Q  D  F  G  R  R  Y  Q  A  M  A  S

mlrC : ATCATGAAAGGCAACGGCCCCGAGCGAAGCTTTGCGGCCGACATCGAGCTA
    I  M  K  G  N  G  P  E  R  S  F  A  A  D  I  E  L

mlrC : GCCAAGGCGGCCACCGCATACCCGGTAATCCTGGTCGATAGTTCGGACAAC
    A  K  A  A  T  A  Y  P  V  I  L  V  D  S  S  D  N

mlrC : CCCGGCGGTGGGGCTTCGGGTGACAATATGGCATTGGCCCGAGCGATGCTG
    P  G  G  G  A  S  G  D  N  M  A  L  A  R  A  M  L

mlrC : GACAATGACCTCGTCCCGTCGTGCATTGGGCCGATATGGGATCCCCTGGCA
    D  N  D  L  V  P  S  C  I  G  P  I  W  D  P  L  A

mlrC : GTACAATTGGGCTTTGAAGCCGGCCTTGGTGCCGATTTTTCCCTGCGCGTT
    V  Q  L  G  F  E  A  G  L  G  A  D  F  S  L  R  V

mlrC : GGCGGCAAGGTCGGCGAGGCATCCGGGCTACCTCTCGACGTTCGCGGCAAA
     G  G  K  V  G  E  A  S  G  L  P  L  D  V  R  G  K

mlrC : ATCACAGGGCTTGCCGAGAATGTCACCCAAAACCTTCAGGGCTCTCGGCCG
    I  T  G  L  A  E  N  V  T  Q  N  L  Q  G  S  R  P

mlrC : CCTCTGGGGCGCGTCGTCTGCATCAGTACAGGTGGTCTAGACATCATCGTC
    P  L  G  R  V  V  C  I  S  T  G  G  L  D  I  I  V

mlrC : AGCGAAATTCGCGACCAGTGCTACGGCCCCGATATGTTCCGGGCGCTCGGT
    S  E  I  R  D  Q  C  Y  G  P  D  M  F  R  A  L  G

mlrC : GTTGAACCTGCGAACAAGCGCTAC
    V  E  P  A  N  K  R  Y
```

c) Predicted polypeptide sequences of of *mlr*C gene (predicted MlrC protein)

```
ARMGSKVLIYTNNDQPAAASIAQDFGRRYQAMASIMKGNGPERSFAADIELAKAATAYPVILVDSSDNP
GGGASGDNMALARAMLDNDLVPSCIGPIWDPLAVQLGFEAGLGADFSLRVGGKVGEASGLPLDVRGKIT
GLAENVTQNLQGSRPPLGRVVCISTGGLDIIVSEIRDQCYGPDMFRALGVEPANKRY (195 aa)
```

A10.4 *mlr*D gene

a) Partial nucleotide sequences of *mlr*D gene

```
cgtactggtctactaccttacgcaagacctgggctattcgaccgaggacgcctcacttatctatgggac
gttcctcggcgtagcctatgtaacgccaatcctgggagggtggatcgccgataggtttattggccgatc
tgcggcaattgtcggtggcgcattgctgaagatggccggttacatcggccttgtgattggcgcgaacgt
cacgggctgcctcgccgcaattgtcattggcaatggcctgtttcttcccactctgcccgctactctggg
tgcacttttttcgccgaacgcccccgatcgccagcgcagtttcagcttctactatctcgcagtgagcgc
tggtgcgctgctggcaccgctgatctgcggcacgcttggagagaatttcggctggcgctacagcttcct
cgcttccgctaccgggcttgcggctgccattgttatctttctcgccggacgccatctgctgccgccaga
ccgacctgcagcagcgtcccatccggtcgacgaagcgccggtcgcggctacgagcctgtccgtcatccc
gcttctggcaggtgtcctcgcagcagtcatcgt(585 bp)
```

b) Translation of *mlr*D gene

```
mlrD: CGTACTGGTCTACTACCTTACGCAAGACCTGGGCTATTCGACCGAGGACGC
      V  L  V  Y  Y  L  T  Q  D  L  G  Y  S  T  E  D  A
mlrD: CTCACTTATCTATGGGACGTTCCTCGGCGTAGCCTATGTAACGCCAATCCT
      S  L  I  Y  G  T  F  L  G  V  A  Y  V  T  P  I  L
mlrD: GGGAGGGTGGATCGCCGATAGGTTTATTGGCCGATCTGCGGCAATTGTCGG
      G  G  W  I  A  D  R  F  I  G  R  S  A  A  I  V  G
mlrD: TGGCGCATTGCTGAAGATGGCCGGTTACATCGGCCTTGTGATTGGCGCGAA
      G  A  L  L  K  M  A  G  Y  I  G  L  V  I  G  A  N
mlrD: CGTCACGGGCTGCCTCGCCGCAATTGTCATTGGCAATGGCCTGTTTCTTCC
      V  T  G  C  L  A  A  I  V  I  G  N  G  L  F  L  P
mlrD: CACTCTGCCCGCTACTCTGGGTGCACTTTTTTCGCCGAACGCCCCCGATCG
      T  L  P  A  T  L  G  A  L  F  S  P  N  A  P  D  R
mlrD: CCAGCGCAGTTTCAGCTTCTACTATCTCGCAGTGAGCGCTGGTGCGCTGCT
      Q  R  S  F  S  F  Y  Y  L  A  V  S  A  G  A  L  L
mlrD: GGCACCGCTGATCTGCGGCACGCTTGGAGAGAATTTCGGCTGGCGCTACAG
      A  P  L  I  C  G  T  L  G  E  N  F  G  W  R  Y  S
mlrD: CTTCCTCGCTTCCGCTACCGGGCTTGCGGCTGCCATTGTTATCTTTCTCGC
      F  L  A  S  A  T  G  L  A  A  A  I  V  I  F  L  A
mlrD: CGGACGCCATCTGCTGCCGCCAGACCGACCTGCAGCAGCGTCCCATCCGGT
      G  R  H  L  L  P  P  D  R  P  A  A  A  S  H  P  V
mlrD: CGACGAAGCGCCGGTCGCGGCTACGAGCCTGTCCGTCATCCCGCTTCTGGC
      D  E  A  P  V  A  A  T  S  L  S  V  I  P  L  L  A
mlrD: AGGTGTCCTCGCAGCAGTCATCGTG
      G  V  L  A  A  V  I  V
```

c) Predicted polypeptide sequences of *mlr*D gene (predicted MlrD protein)

```
VLVYYLTQDLGYSTEDASLIYGTFLGVAYVTPILGGWIADRFIGRSAAIVGGALLKMAGYIGLVIGANV
TGCLAAIVIGNGLFLPTLPATLGALFSPNAPDRQRSFSFYYLAVSAGALLAPLICGTLGENFGWRYSFL
ASATGLAAAIVIFLAGRHLLPPDRPAAASHPVDEAPVAATSLSVIPLLAGVLAAVIV (195 aa)
```

Appendix 11

Partial nucleotides of *mlr*A, *mlr*B, *mlr*C and *mlr*D genes and predicted amino acids of putative MlrA, MlrB, MlrC and MlrD proteins of the bacterial isolate NV-3

A11.1 *mlr*A gene

a) Partial nucleotide sequences of *mlr*A gene

```
attattaccgctgtcaggtctacgttcgagtatccgggagcctatacgctcttactgtttccggccgcc
ccaatgttcgcggccctgatcgcaaccgggatcggctatgggcaagcaggatttcgtgaactgctcagc
cgctgcgccccgtggcggtcgcctgtttcctggcgtcagggcgttactgtcatagccgtgtgtttcctt
gcattcttcgcgctcacaggaatcatgtgggttcagacatacctctacgctccgcctggtacgcttgat
cgtaccttcctgcgctatggggcagatccggtcgccatttatgtgatgctggcagcatcgctgctactc
agccctggcccgctgctggaagaactgggctggcgcggctttgcgctgccgcagctcctcaagaagttt
gacccccttaccgcagcggtgatcctcggcatcatgtggtgggcctggcatttgccacgcgacctgcca
acactgttctccggcgccccctggcgcggcctggagcgttattgtcaaacaactcgttatcgctcctggg
ttcattgcgagcaccatcatcgctgtcttcgtatgcaacaagctcggtggatcaatgtgggggggcgtg
ctcactcacgccatccataacgagctgggagtaaacgtcactgccgaatgggccccaacggtcgcaggc
atcgggtggcgcccatgggatctcatcgaat(721 bp)
```

b) Translation of *mlr*A gene

```
mlrA : ATTATTACCGCTGTCAGGTCTACGTTCGAGTATCCGGGAGCCTATACGCTC
       I  I  T  A  V  R  S  T  F  E  Y  P  G  A  Y  T  L

mlrA : TTACTGTTTCCGGCCGCCCCAATGTTCGCGGCCCTGATCGCAACCGGGATC
       L  L  F  P  A  A  P  M  F  A  A  L  I  A  T  G  I

mlrA : GGCTATGGGCAAGCAGGATTTCGTGAACTGCTCAGCCGCTGCGCCCCGTGG
       G  Y  G  Q  A  G  F  R  E  L  L  S  R  C  A  P  W

mlrA : CGGTCGCCTGTTTCCTGGCGTCAGGGCGTTACTGTCATAGCCGTGTGTTTC
       R  S  P  V  S  W  R  Q  G  V  T  V  I  A  V  C  F

mlrA : CTTGCATTCTTCGCGCTCACAGGAATCATGTGGGTTCAGACATACCTCTAC
       L  A  F  F  A  L  T  G  I  M  W  V  Q  T  Y  L  Y

mlrA : GCTCCGCCTGGTACGCTTGATCGTACCTTCCTGCGCTATGGGGCAGATCCG
       A  P  P  G  T  L  D  R  T  F  L  R  Y  G  A  D  P

mlrA : GTCGCCATTTATGTGATGCTGGCAGCATCGCTGCTACTCAGCCCTGGCCCG
       V  A  I  Y  V  M  L  A  A  S  L  L  L  S  P  G  P

mlrA : CTGCTGGAAGAACTGGGCTGGCGCGGCTTTGCGCTGCCGCAGCTCCTCAAG
       L  L  E  E  L  G  W  R  G  F  A  L  P  Q  L  L  K

mlrA : AAGTTTGACCCCCTTACCGCAGCGGTGATCCTCGGCATCATGTGGTGGGCC
       K  F  D  P  L  T  A  A  V  I  L  G  I  M  W  W  A

mlrA : TGGCATTTGCCACGCGACCTGCCAACACTGTTCTCCGGCGCCCCTGGCGCG
       W  H  L  P  R  D  L  P  T  L  F  S  G  A  P  G  A
```

```
mlrA : GCCTGGAGCGTTATTGTCAAACAACTCGTTATCGCTCCTGGGTTCATTGCG
       A  W  S  V  I  V  K  Q  L  V  I  A  P  G  F  I  A

mlrA : AGCACCATCATCGCTGTCTTCGTATGCAACAAGCTCGGTGGATCAATGTGG
       S  T  I  I  A  V  F  V  C  N  K  L  G  G  S  M  W

mlrA : GGGGGCGTGCTCACTCACGCCATCCATAACGAGCTGGGAGTAAACGTCACT
       G  G  V  L  T  H  A  I  H  N  E  L  G  V  N  V  T

mlrA : GCCGAATGGGCCCCAACGGTCGCAGGCATCGGGTGGCGCCCATGGGATCTC
       A  E  W  A  P  T  V  A  G  I  G  W  R  P  W  D  L

mlrA : ATCGAAT
       I  E
```

c) Predicted polypeptide sequences of *mlr*A gene (predicted MlrA protein)

```
IITAVRSTFEYPGAYTLLLFPAAPMFAALIATGIGYGQAGFRELLSRCAPWRSPVSWRQGVTVIAVCFL
AFFALTGIMWVQTYLYAPPGTLDRTFLRYGADPVAIYVMLAASLLLSPGPLLEELGWRGFALPQLLKKF
DPLTAAVILGIMWWAWHLPRDLPTLFSGAPGAAWSVIVKQLVIAPGFIASTIIAVFVCNKLGGSMWGGV
LTHAIHNELGVNVTAEWAPTVAGIGWRPWDLIE (240 aa)
```

A11.2 *mlr*B gene

a) Partial nucleotide sequences of *mlr*B gene

```
accggcggtcaggcgctcgaagtggcgcgcctcgcgaacgggaaactgcattctggcagacctgtcgat
tatgccggtgggttgttcgtggatgatcggcaaagcgagcgtgttgtgtcgcattcgggcttggttgtg
ggcaatcgcgccatggacgtactttatccggagagcgggattggtatcagcgtgatgtgtaatcgcgac
gatatctcgccagctgagcgtgcgcgcaaaattgctttgctcgtgaagcccggggcgcccgatccagct
tttgaccgcgcaattgatcctgccgaaatgaaacgcctgggaaaagttggcgacctgcgct (337 bp)
```

b) Translation of *mlr*B gene

```
mlrB : ACCGGCGGTCAGGCGCTCGAAGTGGCGCGCCTCGCGAACGGGAAACTGCAT
       T  G  G  Q  A  L  E  V  A  R  L  A  N  G  K  L  H

mlrB : TCTGGCAGACCTGTCGATTATGCCGGTGGGTTGTTCGTGGATGATCGGCAA
       S  G  R  P  V  D  Y  A  G  G  L  F  V  D  D  R  Q

mlrB : AGCGAGCGTGTTGTGTCGCATTCGGGCTTGGTTGTGGGCAATCGCGCCATG
       S  E  R  V  V  S  H  S  G  L  V  V  G  N  R  A  M

mlrB : GACGTACTTTATCCGGAGAGCGGGATTGGTATCAGCGTGATGTGTAATCGC
       D  V  L  Y  P  E  S  G  I  G  I  S  V  M  C  N  R

mlrB : GACGATATCTCGCCAGCTGAGCGTGCGCGCAAAATTGCTTTGCTCGTGAAG
       D  D  I  S  P  A  E  R  A  R  K  I  A  L  L  V  K

mlrB : CCCGGGGCGCCCGATCCAGCTTTTGACCGCGCAATTGATCCTGCCGAAATG
       P  G  A  P  D  P  A  F  D  R  A  I  D  P  A  E  M

mlrB : AAACGCCTGGGAAAAGTTGGCGACCTGCGCT
       K  R  L  G  K  V  G  D  L  R
```

c) Predicted polypeptide sequences of *mlr*B gene (predicted MlrB protein)

```
TGGQALEVARLANGKLHSGRPVDYAGGLFVDDRQSERVVSHSGLVVGNRAMDVLYPESGIGISVMCNRD
DISPAERARKIALLVKPGAPDPAFDRAIDPAEMKRLGKVGDLR (112 aa)
```

A11.3 *mlr*C gene

a) Partial nucleotide sequences of *mlr*C gene

```
gtagcgcggatggggtcgaaagtgctgatctataccaacaacgatcagccagctgctgcctctatcgca
caagacttcggtcggcgctaccaagccatggcttcgaccatgaaaggcaacggccccgagcgaagcttt
gcggccgacatcgagctagccaaggcggccaccgcatacccggtaatcctggtcgatagttcggacaac
cccggcggtggggcttcgggtgacaatatggcattggcccgagcgatgctggacaatgacctcgtcccg
tcgtgcattgggccgatatgggatcccctggcagtacaattgggctttgaagccggccttggtgccgat
ttttccctgcgcgttggcggcaaggtcggcgaggcatccgggctacctctcgacgttcgcggcaaaatc
acagggcttgccgagaatgtcacccaaaaccttcagggctctcggccgcctctggggcgcgtcgtctgc
atcagtacagcgggtctagacatcatcgtcagcgaaattcgcgaccagtgctacggccccgatatgttc
cgggcgctcggtgttgaacctgcgaacaagcgctac(588 bp)
```

b) Translation of *mlr*C gene

```
mlrC : GTAGCGCGGATGGGGTCGAAAGTGCTGATCTATACCAACAACGATCAGCCA
       V  A  R  M  G  S  K  V  L  I  Y  T  N  N  D  Q  P

mlrC : GCTGCTGCCTCTATCGCACAAGACTTCGGTCGGCGCTACCAAGCCATGGCT
       A  A  A  S  I  A  Q  D  F  G  R  R  Y  Q  A  M  A

mlrC : TCGACCATGAAAGGCAACGGCCCCGAGCGAAGCTTTGCGGCCGACATCGAG
       S  T  M  K  G  N  G  P  E  R  S  F  A  A  D  I  E

mlrC : CTAGCCAAGGCGGCCACCGCATACCCGGTAATCCTGGTCGATAGTTCGGAC
       L  A  K  A  A  T  A  Y  P  V  I  L  V  D  S  S  D

mlrC : AACCCCGGCGGTGGGGCTTCGGGTGACAATATGGCATTGGCCCGAGCGATG
       N  P  G  G  G  A  S  G  D  N  M  A  L  A  R  A  M

mlrC : CTGGACAATGACCTCGTCCCGTCGTGCATTGGGCCGATATGGGATCCCCTG
       L  D  N  D  L  V  P  S  C  I  G  P  I  W  D  P  L

mlrC : GCAGTACAATTGGGCTTTGAAGCCGGCCTTGGTGCCGATTTTTCCCTGCGC
       A  V  Q  L  G  F  E  A  G  L  G  A  D  F  S  L  R

mlrC : GTTGGCGGCAAGGTCGGCGAGGCATCCGGGCTACCTCTCGACGTTCGCGGC
       V  G  G  K  V  G  E  A  S  G  L  P  L  D  V  R  G

mlrC : AAAATCACAGGGCTTGCCGAGAATGTCACCCAAAACCTTCAGGGCTCTCGG
       K  I  T  G  L  A  E  N  V  T  Q  N  L  Q  G  S  R

mlrC : CCGCCTCTGGGGCGCGTCGTCTGCATCAGTACAGCGGGTCTAGACATCATC
       P  P  L  G  R  V  V  C  I  S  T  A  G  L  D  I  I

mlrC : GTCAGCGAAATTCGCGACCAGTGCTACGGCCCCGATATGTTCCGGGCGCTC
       V  S  E  I  R  D  Q  C  Y  G  P  D  M  F  R  A  L

mlrC : GGTGTTGAACCTGCGAACAAGCGCTAC
       G  V  E  P  A  N  K  R  Y
```

c) Predicted polypeptide sequences of of *mlr*C gene (predicted MlrC protein)

```
VARMGSKVLIYTNNDQPAAASIAQDFGRRYQAMASTMKGNGPERSFAADIELAKAATAYPVILVDSSDN
PGGGASGDNMALARAMLDNDLVPSCIGPIWDPLAVQLGFEAGLGADFSLRVGGKVGEASGLPLDVRGKI
TGLAENVTQNLQGSRPPLGRVVCISTAGLDIIVSEIRDQCYGPDMFRALGVEPANKR (195 aa)
```

A11.4 *mlr*D gene

a) Partial nucleotide sequences of *mlr*D gene

```
gtcgggctcagaaccgtactggtctactaccttacgcaagacctgggctattcgaccgaggacgcctca
cttatctatgggacgttcctcggcgtagcctatgtaacgccaatcctgggagggtggatcgccgatagg
tttattggccgatctgcggcaattgtcggtggcgcattgctgaagatggccggttacatcggccttgtg
attggcgcgaacgtcacgggctgcctcgccgcaattgtcattggcaatggcctgtttcttcccactctg
cccgctactctgggtgcacttttttcgccgaacgcccccgatcgccagcgcagtttcagcttctactat
ctcgcagtgagcgctggtgcgctgctggcaccgctgatctgcggcacgcttggagagaatttcggctgg
cgctacagcttcctcgcttccgctaccgggcttgcggctgccattgttatctttctcgccggacgccat
ctgctgccgccagaccgacctgcagcagcgtcccatccggtcgacgaagcgccggtcgcggctacgagc
ctgtccgtcatcccgcttctggcaggtgtcctcgcagcagtcatc (597 bp)
```

b) Translation of *mlr*D gene

```
mlrD : GTCGGGCTCAGAACCGTACTGGTCTACTACCTTACGCAAGACCTGGGCTAT
       V  G  L  R  T  V  L  V  Y  Y  L  T  Q  D  L  G  Y

mlrD : TCGACCGAGGACGCCTCACTTATCTATGGGACGTTCCTCGGCGTAGCCTAT
       S  T  E  D  A  S  L  I  Y  G  T  F  L  G  V  A  Y

mlrD : GTAACGCCAATCCTGGGAGGGTGGATCGCCGATAGGTTTATTGGCCGATCT
       V  T  P  I  L  G  G  W  I  A  D  R  F  I  G  R  S

mlrD : GCGGCAATTGTCGGTGGCGCATTGCTGAAGATGGCCGGTTACATCGGCCTT
       A  A  I  V  G  G  A  L  L  K  M  A  G  Y  I  G  L

mlrD : GTGATTGGCGCGAACGTCACGGGCTGCCTCGCCGCAATTGTCATTGGCAAT
       V  I  G  A  N  V  T  G  C  L  A  A  I  V  I  G  N

mlrD : GGCCTGTTTCTTCCCACTCTGCCCGCTACTCTGGGTGCACTTTTTTCGCCG
       G  L  F  L  P  T  L  P  A  T  L  G  A  L  F  S  P

mlrD : AACGCCCCCGATCGCCAGCGCAGTTTCAGCTTCTACTATCTCGCAGTGAGC
       N  A  P  D  R  Q  R  S  F  S  F  Y  Y  L  A  V  S

mlrD : GCTGGTGCGCTGCTGGCACCGCTGATCTGCGGCACGCTTGGAGAGAATTTC
       A  G  A  L  L  A  P  L  I  C  G  T  L  G  E  N  F

mlrD : GGCTGGCGCTACAGCTTCCTCGCTTCCGCTACCGGGCTTGCGGCTGCCATT
       G  W  R  Y  S  F  L  A  S  A  T  G  L  A  A  A  I

mlrD : GTTATCTTTCTCGCCGGACGCCATCTGCTGCCGCCAGACCGACCTGCAGCA
       V  I  F  L  A  G  R  H  L  L  P  P  D  R  P  A  A

mlrD : GCGTCCCATCCGGTCGACGAAGCGCCGGTCGCGGCTACGAGCCTGTCCGTC
       A  S  H  P  V  D  E  A  P  V  A  A  T  S  L  S  V

mlrD : ATCCCGCTTCTGGCAGGTGTCCTCGCAGCAGTCATC
       I  P  L  L  A  G  V  L  A  A  V  I
```

c) Predicted polypeptide sequences of *mlr*D gene (predicted MlrD protein)

```
VGLRTVLVYYLTQDLGYSTEDASLIYGTFLGVAYVTPILGGWIADRFIGRSAAIVGGALLKMAGYIGLV
IGANVTGCLAAIVIGNGLFLPTLPATLGALFSPNAPDRQRSFSFYYLAVSAGALLAPLICGTLGENFGW
RYSFLASATGLAAAIVIFLAGRHLLPPDRPAAASHPVDEAPVAATSLSVIPLLAGVLAAVI(199 aa)
```

Appendix 12

Chemicals, materials, and instruments of Chapter 5

A12.1. Chemicals and bacterial media

Table A12.1 Chemicals, media, and reagent lists

Chemicals and media	Company	Used for/in
Sodium carbonate ($NaCO_3$)	M&B	BG-11 medium
Di-potasium hydrogen phosphate (K_2HPO_4)	BDH	BG-11 medium
Magnesium sulphate ($MgSO_4$)	M&B	BG-11 medium
Calcium chloride ($CaCl_2$)	BDH	BG-11 medium
Citric acid ($C_6H_8O_7$)	BDH	BG-11 medium
Ethylene Diamine Tetraacetic Acid (EDTA)	BDH	BG-11 medium
Di-sodium carbonate (Na_2CO_3)	BDH	BG-11 medium
Boric acid (H_3BO_3)	M&B	BG-11 medium
Manganese chloride tetrahydrate ($MnCl_2$ $4H_2O$)	Merck	BG-11 medium
Zine sulphate ($ZnSO_4$)	BDH	BG-11 medium
Sodium Molybdate dihydrate (Na_2MoO_4 $2H_2O$)	BDH	BG-11 medium
Di-sodium hydrogen phosphate (Na_2HPO_4)	BDH	BG-11 medium
Copper sulphate pentahydrate ($CuSO_4$ $5H_2O$)	Merck	BG-11 medium
Cobalt nitrate hexahydrate ($Co(NO_3)_2$ $6H_2O$)	BDH	BG-11 medium
Peptone	Difco	1/10 nutrient broth
Yeast extract	Difco	1/10 nutrient broth
Absolute ethanol (EtOH)	BDH	Chlorophyll-a determination

Table A12.2 Material and miscellaneous

Miscellaneous	Company
GF/C paper filter	Whatman
Microcentrifuge tube (1.6 ml)	Neptune
PCR tubes (20 µl)	Neptune
Pipettes (0.2-2 µl, 2-20 µl, 20-200µl, 100-1000 µl)	Thermo
Pipette tips with filter (0.2-2 µl, 2-20 µl, 20-200µl, 1000 µl)	Neptune
Glassware	Schott
15 and 50 ml conical-bottom disposable plastic tubes	Nunc

A12.2. Bacterial media and preparations

Table A12.3 BG-11 medium preparations

Stock solution	Per Liter Distilled water	To prepare BG-11 medium Add to 983 ml sterile distilled water
$NaCO_3$	150 g	10 ml
$K_2HPO_4.3H_20$	40 g	1 ml
$MgSO_4.\ 7H_2O$	75 g	1 ml
$CaCl_2\ 2H_2O$	36 g	1 ml
Citric acid	6 g	1 ml
EDTA	1 g	1 ml
Na_2CO_3	20 g	1 ml
Trace metal solution	See below	1 ml

Table A12.4 Trace metal solution

Stock solution	Per Liter Distilled water
H_3BO_3	2.68 g
$MnCl_2\ 4H_2O$	1.81 g
$ZnSO_4\ 7H_2O$	0.222 g
$Na_2MoO_4\ 2H_2O$	0.39 g
$CuSO_4\ 5H_2O$	0.079 g
$Co(NO_3)_2\ 6H_2O$	0.0494 g

- Mix all ingredient together with 1,000 ml distilled water
- Adjust pH to approximately 7.5 using HCl or NaOH
- Sterile using autoclave at 121^{o}C for 15 min
- Check pH after sterilization with pH paper

Table A12.5 One ten (1/10) nutrient broth preparations

1/10 nutrient broth	For 1 liter: • 1 g of peptone, and • 1 g of yeast extract • Dissolve in a total of 1L milli-Q water and adjust pH 7.2 using HCl or NaOH • Sterile using autoclave at 121^{o}C for 15 min • Check pH after sterilization with pH paper

A12.3 Instruments

Table A12.6 Instrument list

Instruments and materials	Company	Model
Analytical balance	Mettler	AE 100 and PL 1200
pH meter	Suntex	SP-2200
Biological safety cabinet	ETR Environmental	4FT class II
Autoclave	Getinge	HS 6610 EC-1
Bench top centrifuge	Eppendorf	5810R
Shaking incubator	New Brunsick	Innova 4250
Incubator	Contherm	Pular 1000 C
Water bath	Thermo	Haake C 10
UV-Vis spectrometer	Bausch & Lomb	Spectronic 2000

Appendix 13

Chemicals, materials, and instruments of Chapter 6

A13.1. Chemicals, reagents and solutions

Table A13.1 Chemicals, reagent list

Chemicals and media	Company	Used for/in
Peptone	Difco	R2A medium
Yeast extract	Difco	R2A medium
Casamino acids	BDH	R2A medium
Glucose	BDH	R2A medium
Soluble starch	BDH	R2A medium
Sodium pyruvate	BDH	R2A medium
Magnesium sulphate ($MgSO_4$)	M&B	R2A medium and synthetic wastewater
Di-Potassium hydrogen phosphate (K_2HPO_4)	M&B	R2A medium and synthetic wastewater
Ammonium sulphate $(NH_4)_2SO_4$	BDH	R2A medium and synthetic wastewater
Potasium hydrogen phosphate (KH_2PO_4)	BDH	Synthetic wastewater
Di-sodium hydrogen phosphate (Na_2HPO_4)	BDH	Synthetic wastewater
Crystal violet	BDH	Biofilm formation assay
Absolute ethanol (EtOH)	BDH	Chlorophyll-a determination

Table A13.2 Bacterial media and preparations

R2A medium	For 1 liter: 0.5g of yeast extract, 0.5 g of protease peptone, 0.5 g of casamino acids, 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.05 g of $MgSO_4.7H_2O$, and 0.3 g of sodium pyruvate per liter. • Dissolve in a total of 1L milli-Q water and adjust pH 7.0 using 0.1 M HCl or 0.1 M NaOH • Sterile using autoclave at 121^oC for 15 min • Check pH of the solution again after sterilization with pH paper
Synthetic wastewater	For 1 liter: • 0.1 g L^{-1} of $(NH_4)_2SO_4$, 0.5 g L^{-1} of KH_2PO_4 , 0.5 g L^{-1} of Na_2HPO_4, 0.5 g L^{-1} of $MgSO_4.7H_2O$ and 0.02 g L^{-1} of yeast extract • Dissolve in a total of 1L milli-Q water and adjust pH 7.0 using HCl or NaOH • Sterile using autoclave at 121^oC for 15 min • Check pH after sterilization with pH paper

Table A13.3 Material and miscellaneous

Miscellaneous	Company
96-well polyvinylchloride (PVC) microtiter plate	Nunc
Polystyrene plastic (24 well plates)	Corning
PVC plastic	Corning
Stainless-steel plates (grade 304)	Atlas Steels
Slide cover glasses	Sail brand
Ceramic plate	Pingxiang Chemshun Ceramics
Honey ceramic support	Pingxiang Chemshun Ceramics
GF/C paper filter	Whatman
0.22 µm filter unit	Whatman
Microcentrifuge tube (1.6 ml)	Neptune
PCR tubes (20 µl)	Neptune
Pipettes (0.2-2 µl, 2-20 µl, 20-200µl, 100-1000 µl)	Thermo
Pipette tips with filter (0.2-2 µl, 2-20 µl, 20-200µl, 1000 µl)	Neptune
Glassware	Schott
15 and 50 ml conical-bottom disposable plastic tubes	Nunc

A13.2 Instruments

Table A13.4 Instrument list

Instruments and materials	Company	Model
Analytical balance	Mettler	AE 100 and PL 1200
pH meter	Suntex	SP-2200
Biological safety cabinet	ETR Environmental	4FT class II
Autoclave	Getinge	HS 6610 EC-1
Bench top centrifuge	Eppendorf	5810R
Shaking incubator	New Brunsick	Innova 4250
Incubator	Contherm	Pular 1000 C
Water bath	Thermo	Haake C 10
UV-Vis spectrometer	Bausch & Lomb	Spectronic 2000
Air pump	Aquarium fish air pump	100L