AN INVESTIGATION INTO THE BIODEGRADATION OF PEPTIDE CYANOTOXINS (MICROCYSTINS AND NODULARIN) BY NOVEL GRAM-POSITIVE BACTERIA

By

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A thesis submitted in partial fulfilment for the degree of

Doctor of Philosophy

Robert Gordon University

February 2012

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DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise acknowledged, and has not been submitted in any form for another degree or qualification at any other academic institution.

Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Aakash Welgamage Don

DEDICATION

FOR

MY

MUM AND DAD

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors Prof. Linda Lawton and Dr. Christine Edwards for their immense support, guidance and wonderful friendship throughout this project. I also extend my sincere thanks to Prof. Elke Dittmann (University of Potsdam, Germany) for the great support, hospitality and friendship during my research visit to Germany.

I am also very grateful to the Institute for Innovation, Design & Sustainability Research for their financial assistance.

Thank you very much Eoin, for the friendship and support through my teaching and demonstrating career. Thank you very much Heather, Mairi, John, Kirsten and Beth for your support.

Thanks also to the many people in the department who helped and advised me over the years, especially Kostas, Carlos, Vijith, Luca, Shaista, Radisti, Arif, Ania, James, Olivier and all my lab colleagues from Germany; Jan, Eva, Kathrin, Arthur, Douglas, Katarina, Sven and Chen-Lin.

I am indebted to Mum, Dad and my Brother, not only for their love but for the opportunities they have given me along the way. Thank you very much Evie for your love, courage and support; that kept me going through all the tough times. Thank you very much for everything.

ABBREVIATIONS

aa	Amino acid
ABPNB	Anabaenopeptin B
Amp	Ampicillin
ANGTN	Angiotensin III
AT	Aminotransferase
ATP	Adenosin triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair(s)
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CY	Cyclosporin A
d	Days
Da	Daltons
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	1,4-dithiothreitole
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetic acid
EMBL	European Molecular Biology Laboratory
ESI	Electrospray ionization
GRA	Gramicidin A

h	Hours
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo base pairs
kDa	kilo Dalton
LB	Lysogeny broth
LNOD	Linear nodularin
MC	Microcystin
ME	Microcystinase enzyme
min	Minutes
MPG	Microcystinase producing genes
MS	Mass spectrometry
MW	Molecular weight
NA	Nutrient agar
NB	Nurtrient broth
NCBI	The National Center for Biotechnology Information
NOD	Nodularin
OD	Optical density
ORF	Open reading frame
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCC	Pasteur Culture Collection

PCR	Polymerase chain reaction
PDA	Photodiode array
PEG	Polyethylenglycol
PLP	Pyridoxal-phosphate
PMSF	Phenyl-methyl-sulphonyl-fluoride
POLYB	Polymyxin B
RNA	Ribonucleic acid
RP	Reversed phase
rpm	Rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Seconds
TAE	Tris-acetate-EDTA buffer
TFA	Trifluoroacetic acid
Tm	Primer melting temperature
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet

Abstract

Cyanobacterial secondary metabolites, microcystins (MC) and nodularin (NOD) have become common contaminants in most aquatic ecosystems over recent years presenting a hazard to animal and human health. Unfortunately, these chemically diverse peptide hepatotoxins remain a challenge to most conventional water treatments due to their stable cyclic structures. Over recent years, bioremediation of MC and NOD has become one of the most exciting areas that holds promise for a successful and cost effective solution for water treatment process. The current work presents the biodegradation of MCs and NOD by bacterial isolates from three different bacteria genus *Arthrobacter*, *Brevibacterium* and *Rhodococcus* belonging to *Actinobacteria*. A total of five isolates representing the three genera have demonstrated an overall metabolism of MC-LR, -LF, -LY, -LW, -RR and NOD in a Biolog MT2 assay. Subsequently, these bacteria were reported to degrade the range of toxins in a separate batch experiment. The bacterial degradation rate of the above cyanobacterial peptides were found to decrease with the multiple sub-culturing of the bacteria. However, a rapid degradation was discovered when the bacteria were re-exposed to MC or other prokaryotic peptides demonstrating an inducible bacterial biodegradation. Utilising latest molecular biology techniques, the gene responsible for production of MC degrading enzymes was successfully elucidated and its activity was evaluated. Analysis of the degradation products of MC-LR revealed a glutathione conjugate detoxification mechanism involved during the degradation of MC-LR by *Rhodococcus* sp. (C1). A novel MC degradation pathway was proposed. Further studies were suggested to fully characterise the degradation pathway and to evaluate the MC detoxification mechanism in bacteria.

CHAPTER 1

INTRODUCTION

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1 Introduction

1.1 Origin and distribution of cyanobacteria

Cyanobacteria are a diverse group of prokaryotes that occupy a broad range of ecological niches. They are the Earth's oldest known oxygen-producing organisms, having first appeared billions of years ago (Adams, 1997). Cyanobacteria represent some of the most primitive cells to have appeared in the early Precambrian period, with fossils remaining dating back 3.5 billion years ago. As the progenitor of oxygenic photosynthesis, cyanobacteria are believed to have transformed the evolution of biology and geochemistry of Earth (Shi and Falkowski, 2008). In the theory of evolution, it was hypothesised that chloroplasts in plant cells, are derived from endosymbiotic cyanobacteria (Raven and Allen, 2003). The early forms of these photosynthesisers, were taken up by other microbes sometime back in the Precambrian period, and lost their ability to function independently (Carmichael, 1994). Their significant contribution to an oxygen rich environment ultimately facilitated the evolution of complex life forms with an aerobic metabolism (Falkowski *et al*., 2005). Thriving for billions of years, cyanobacteria still live today and inhabit a multiplicity of environments worldwide, contributing significantly to the global primary production (Shi and Falkowski, 2008).

Cyanobacteria are well known as "blue-green algae" due to their similar morphology to the eukaryotic algae and their ability for photosynthesis. The "blue-green" colour is denoted by their photosynthetic pigments; phycocyanins and chlorophyll *a,* giving them a blue green colour (Mur *et al*., 1999). While often referred to as eukaryotic algae, their intracellular organisation and

protein synthesis machinery are similar to those of other prokaryotes. Also, cyanobacteria lack membrane-bound organelles and nucleus, and have lipopolysaccharide layers rather than cellulose cell wall structures. Unique similarities and disparities of cyanobacteria to both the eukaryotes and prokaryotes, have made their phylogenic classification challenging. Nevertheless, cyanobacteria are now classified within eubacteria as an individual class of organisms known as cyanophyceae (Castenholz and Waterbury, 1989). Moreover, molecular phylogenetic studies have made it abundantly clear that cyanobacteria are more related to bacteria rather than eukaryotic cells (Mur *et al*., 1999).

Cyanobacteria are one of the most successful and widespread living organisms on earth. They can be found in almost all aquatic and many terrestrial environments (Knoll, 2008). They are capable of tolerating a wide range of environmental temperatures, sudden changes of temperature, salinity variations and desiccation, ensuring a successful adaptation and greater distribution (Walsby, 1987). Many species inhabit aquatic ecosystems including freshwater, marine and brackish waters as colonial or free living organisms (Ehrenreich *et al*., 2005). Cyanobacteria are commonly found in the terrestrial North and South Polar Regions as well as hot springs (Van Den Hoek *et al.*, 1995). As an adaptation for the aquatic environment, many species of cyanobacteria possess cytoplasmic inclusions, called gas vacuoles. These inclusions aid the cells to obtain buoyancy in order to move vertically within the water column towards more favourable positions for their development (Dittmann and Wiegand, 2006). Their remarkable suit of living strategies and survival mechanisms sustain their survival under extreme

habitats (Tiwari *et al*., 2005). Similarly, many of these organisms are capable of either exploiting or modifying their habitats, to make them more suitable under environmental stress conditions, i.e. by synthesis of biologically active natural products, toxins, to eliminate competitors (Ehrenreich *et al*., 2005).

Symbiotic relations of cyanobacteria have been found within various organisms, including plants, fungi, and animals (Whitton and Potts, 2000). The cyanobacterial genus *Nostoc,* presents a number of symbiotic associations with the lichenised fungi as well as with different plants, bryophytes (Adams, 2002) and cycads (Costa and Lindblad, 2002). Cyanobacteria species *Aphanocapsa* sp., *Phormidium* sp. and *Oscillatoria spongeliae*, can commonly live in a symbiotic relationship with aquatic host animals like sponges (Wilkinson, 1992; Lee *et al*., 2001). Cyanobacterial symbiotic associations are important components of the ecology as a part of nature (O'Brien *et al*., 2005).

1.2 Occurrence of cyanobacterial blooms

Blooms (mass occurrences) of cyanobacteria occur when cyanobacterial cells grow exuberantly, and reach more than 10,000 cells per millilitre (Oliver and Ganf, 2000). This can happen at any time in warmer climates, whereas in temperate countries it most often occurs in late summer or early autumn (Mur *et al*., 1999; Oliver and Ganf, 2000). The most dominant bloom forming cyanobacterial genera responsible for these mass occurrences are: *Anabaena, Aphanazomenon, Cylindrospermopsis, Gloeotrichia, Oscillatoria, Rivularia, Lyngbya, Microcystis, Nostoc* and *Planktothrix*. Surface blooms are commonly formed by *Anabaena* and *Microcystis* in freshwaters (Oliver and Ganf, 2000), *Nodularia* and *Aphanizomenon* in brackish waters, i.e. the Baltic Sea (Finni *et*

al., 2001). Also, metalimnetic populations are commonly formed by low-light-adapted *Planktothrix* species (Oliver and Ganf, 2000).

Occurrence of cyanobacterial blooms has significantly increased over the past two decades due to the dramatic climate changes (Paerl *et al*., 2011) and the anthropogenic eutrophication in aquatic systems (Vasconcelos, 2005). Increased nutrient discharge, especially nitrate and phosphate, has stimulated rapid formation of freshwater blooms of cyanobacteria; *Anabaena* and *Microcystis* (Schindler *et al*., 2008). These blooms are often toxic due to the production of a wide range of potent hepatotoxic and neurotoxic secondary metabolites, which are detrimental for several organisms including humans (Wiegand and Pflugmacher, 2005). Toxic blooms have been reported globally, including world's most resourceful water bodies such as the Baltic Sea in northern Europe (Conley *et al*., 2009), Lakes Taihu in China (Qin *et al*., 2010), Kasumiguaura in Japan; Victoria in Africa and Erie in US-Canada, Caspian Sea, West Asia (Paerl and Huisman, 2008). Thereby, occurrences of these toxic cyanobacterial blooms have been considered as a significant issue by industrial, environmental and health sectors and significant effort has been made to prevent, control and mitigate their incidence both in natural and manmade water bodies (Paerl *et al*., 2011).

1.3 Cyanotoxins

Cyanobacteria have attracted increasing interest all over the world, due to their production of a range of toxic secondary metabolites, collectively known as cyanotoxins. The ecological role of their toxin production still remains unclear (Meriluoto, 1997; Lawton and Edwards, 2001). In nature, most of the cyanotoxins are produced and remain within the cyanobacterial cells. Under the circumstances of natural or anthropogenically induced bloom collapse, high concentrations of toxins can be released into the surrounding environment (Sivonen and Jones, 1999) causing an environmental risk. Animal toxicoses of cyanotoxins have been documented over 120 years ago. It was reported that domestic animals such as dogs, sheep, pigs and horses, started dying within hours of drinking water from a lake hosting a bloom of cyanobacterium *Nodularia spumigena* (Francis, 1878). Since then, thousands of livestock fatalities and numerous poisonings in dogs have been reported worldwide, due to the ingestion of cyanotoxin contaminated water (Stewart *et al*., 2006). Cyanotoxins in marine environments have been identified as possible cause of liver disease in fish (Chorus *et al*., 2000).

To date, high numbers of economically valuable secondary metabolites have been isolated and characterised from various taxa of cyanobacteria (Welker and Dohren, 2006). Considering the vast diversity of cyanobacterial secondary metabolites and their toxicity (Burja *et al*., 2001), they fall into three different groups namely, hepatotoxins, neurotoxins and lipopolysaccharide endotoxins. Hepatotoxins (MC, NOD and Cylindrospermopsin) and neurotoxins (Anatoxins and Saxitoxins) are intracellular and are produced only by certain strains of cyanobacteria (table 1.1). Irritant toxins; lipopolysaccharide endotoxins are

found in the outer membrane of the cyanobacterial cell wall, and are commonly reported among cyanobacteria (table 1.1).

Table 1.1 Cyanotoxins in nature (adapted from Wiegand and Pflugmacher, 2005)

Toxin group	Producer	Primary target/organ	Biotransformation
Microcystins	*Microcystis*, *Anabaena*, *Planktothrix*	Liver	Glutathione *S*-transferase
Nodularin	*Nodularia*	Liver	Glutathione *S*-transferase
Cylindrospermopsin	*Cylindrospermopsis,* *Anabaena*, *Planktothirx*, *Raphidiopsis*	Liver	Cytochrome P450
Anatoxins	*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Planktothrix*	Nerve synapse	Cytochrome P450, Glutathione *S*-transferase
Anatoxins –a(s)	*Anabaena*	Nerve synapse	Cytochrome P450, Glutathione *S*-transferase
Saxitoxins	*Aphanizomenon*, *Lyngbya*, *Cylindrospermopsis*, *Anabaena*	Nerve synapse	Glutathione *S*-transferase
Lipopolysaccharide	Cyanobacteria in general	Endotoxin, potential irritant, affects any exposed tissue	Deacylation via lysosomal pathway

1.3.1 Cyclic peptide hepatotoxins - microcystins and nodularin

Cyanobacteria produce a range of cyclic peptides such as MCs, NOD, anabaenopeptin, microviridins and cyclamides (Welker and Dohren, 2006). Among them, the most commonly occurring cyanobacterial cyclic peptides found in fresh and brackish waters are MCs and NOD (Carmichael, 1994).

MCs are produced by several genera of cyanobacteria; *Microcystis*, *Anabaena*, and *Planktothrix* (*Oscillatoria*). Few strains of planktonic *Nostoc, Anabaenopsis*, and *Radiocystis* are also reported to produce MC (Vieira *et al*., 2003; Mohamed and Shehri, 2009). MCs are mono-cyclic heptapeptides consisting of seven amino acids with three D-amino acids (alanine, erythro-ß-methylaspartic acid and glutamic acid), two variable L-amino acids, and two unusual amino acids; *N*-methyldehydroalanine (Mdha), and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Botes *et al*., 1982; figure 1.1). The common structure for MC is presented as *cyclo*-(D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glu^6-Mdha^7; Edwards and Lawton, 2009). The X^2 and Z^4 positions represent variable L-amino acids helping to distinguish MCs from one another, while the other amino acids are more or less constant between variant MCs although, minor modifications such as methylation and demethylation are common. Using amino acid single letter code nomenclature, each MC is designated a name depending on the variable amino acids which complete their structure; i.e. MC-LR contains Leucine (L) at position X^2 and Arginine (R) at position Z^4. To date, more than 70 structurally different MC variants are described using their general nomenclature (Edwards and Lawton, 2009; table 1.2), however, MC-LR remains as the most common congener of MCs (Welker and Dohren, 2006).

Table 1.2 Variation of amino acid on some selected microcystins

MC variant	X- amino Acid	Z- amino acid	Molecular weight (Da)
MC- LA	Leucine	Alanine	910
MC–LF	Leucine	Phenylalanine	986
MC–LY	Leucine	Tyrosine	1002
MC–LW	Leucine	Tryptophan	1025
MC–RR	Arginine	Arginine	1038
MC–YR	Tyrosine	Arginine	1045
MC–WR	Tryptophan	Arginine	1068

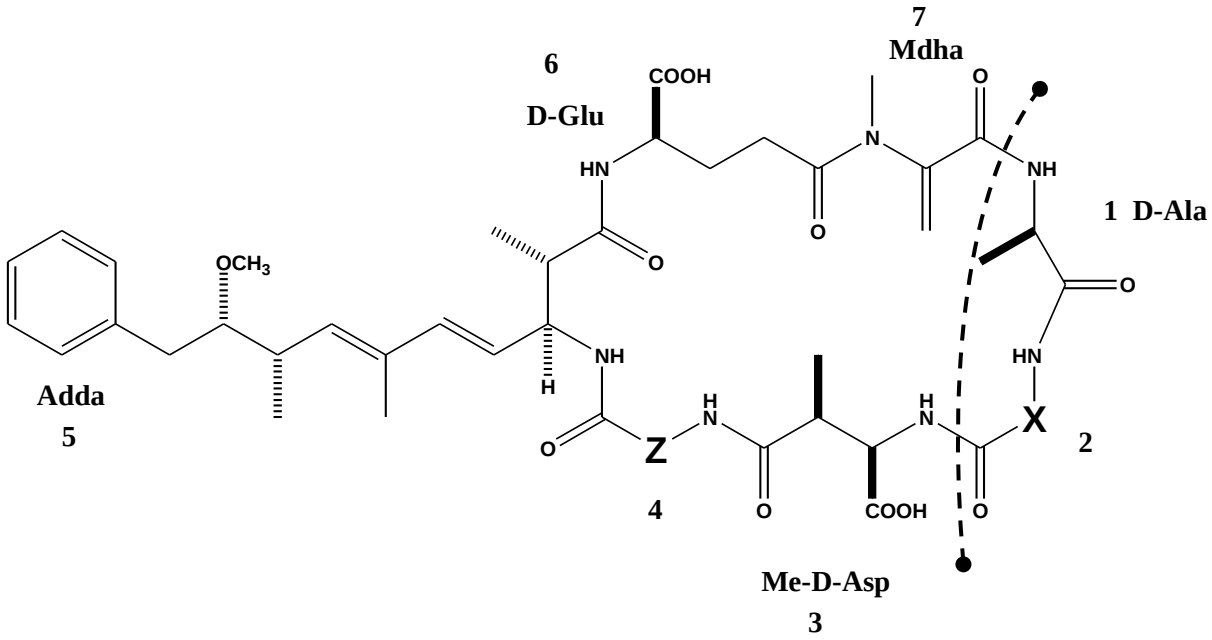

Figure 1.1 The general structure of microcystin and nodularin

MC is a cyclic peptide containing seven amino acids:

1 = D - Alanine

2 = X - Variable L- amino acid

3 = D - Methyl Aspartic acid

4 = Z - Variable L- amino acid

5 = Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

6 = D - Glutamic acid

7 = Mdha (*N*-methyldehydroalanine)

NOD, the mono cyclic pentapeptides are solely produced by planktonic *Nodularia spumigena* strains in brackish waters (Sivonen and Jones, 1999) and were first structurally identified by Rinehart *et al*., (1988). Like MC producing blooms, the formation of the blooms of *N. spumigena* has been found to be influenced by the water temperature, light intensity and nutrient status (P and N; Mazur-Marzec and Plinski, 2003). The structure and biological activity of NOD is similar to that of MCs (Mazur-Marzec *et al*., 2009). Therefore, very often, NOD is determined using the same analytical methods as those used MCs, i.e. HPLC detection methods (Edwards and Lawton, 2010).

NOD is a pentapeptide with a general structure of *cyclo*-(D-MeAsp^{1}-L-arginine^{2}-Adda^{3}-D-glutamate^{4}- Mdhb^{5}), where Mdhb stands for *N*-methyldehydrobutyrine (Sivonen and Jones, 1999). The main differences between MCs and NOD are the lack of amino acids D-Ala^{1} and X^{2} (indicated by the dashed line; figure 1.1), and the substitution of Mdha with Mdhb in NOD (Rantala *et al*., 2004). Slight structural variations of NOD have been identified; NOD-Har was produced by *Nodularia* PCC 7804 replacing homoarginine instead of arginine (Rantala *et al*., 2004).

In cyanobacteria, cyclic peptides like MCs and NOD are produced via a non-ribosomal peptide synthetase (NRPS) system (Dittmann *et al*., 1997) and their synthesis is an energy dependant process (Bickel and Lyck, 2001). Although, the ecological role of toxin production is under investigation, their significant risk for many other organisms has been widely experienced. MCs are known to affect from micro algae to mammals (Figueiredo *et al*., 2004). MC-LR has been shown to paralyse the motile green alga *Chlamydomonas reinhadtii* to provide

competition free environment for their MC producing cyanobacteria. The brine shrimp *Artemia salina* was shown to be affected by exposure to MC-LR (Delaney and Wilkins, 1995), thereby leading to a detoxification mechanism via glutathione *S*-transferase to form a MC-LR glutathione conjugate (Beattie *et al*., 2003). This enzymatic mechanism was found to be the first step of MC detoxification (Pflugmacher *et al*., 1998). Furthermore, MC-LR detoxification via glutathione conjugation has been identified and studied in plants, fish (Pflugmacher *et al*., 1998) as well as rats (Tekenaka, 2001).

However, in mammals, toxicity of MCs is selective for hepatic cells (table 1.1). Their mechanism of toxicity is by inhibiting protein phosphatases; PP1 and PP2 (Imanishi and Harada, 2004). Protein phosphatases play a key role in reversible protein dephosphorylation regulating many cellular activities such as proliferation and differentiation in the human body (Ikehara *et al*., 2008). Disruption of protein phosphatase leads to significant disintegration of the hepatocyte structure, liver necrosis and internal haemorrhage in liver ultimately leading to haemorrhagic shock and death (Dow and Swoboda, 2000). In addition to their binding with protein phosphatases, MC-LR was found to bind with ATP synthetase and deactivate its enzymatic role leading to a cell apoptosis (Mikhailov *et al*., 2003). Nonetheless, protein phosphatases are important tumour suppressing enzymes and disruption of their activity have been shown to promote liver cancer in mammals (Ito *et al*., 1997). The mechanism of the toxicity of NOD is similar to MCs (table 1.1); exceptionally NOD does not bind covalently to the protein phosphatases (Dawson, 1998). However, the toxicity occurs as a result of the interaction of the "Adda" group in MCs and NOD (figure 1.1) with the catalytic site of protein phosphatase.

Therefore, structural modifications or removal of Adda from MCs and NOD hinder their interaction with protein phosphatase and they would no longer be toxic (Abdel-Rahman *et al*., 1993). The lethal dose; LD_{50} value for MC-LR is 50 μg kg^{-1} of body weight in mice; however, it can range from 25 to 125 μg kg^{-1} (Dawson, 1998). For NOD the LD_{50} value in the mouse is 30-50 μg kg^{-1} of body weight (Chorus and Bartram, 1999).

Potent toxicity of MCs was highlighted in a recent human catastrophe that involved the death of more than 50 patients at a haemodialysis centre in Brazil in 1996. More than 100 patients experienced visual disturbances, nausea and vomiting after their routine haemodialysis treatment. It has been revealed that the water used for dialysis was contaminated with MCs (Jochimsen *et al*., 1998; Hirooka *et al*., 1999). In later studies it was estimated that about 19.5 μg l^{-1} of MC-LR was present in the water used for haemodialysis treatment (Carmichael *et al*., 2001). Moreover, human exposure to MC may occur through drinking water (Ueno *et al*., 1996), recreational activities (World Health Organization; WHO, 2003) or indirect ingestion as food (Codd *et al*., 1999). In general, therapy and prophylaxis for MCs is complicated due to the rapid damage occurring in liver (Figueiredo *et al*., 2004). Some studies have suggested the use of monoclonal antibodies for human intoxication of MCs (Nagata *et al*., 1995) while some others highlighted the use of an antibiotic rifampin and immunosuppressant drugs such as cyclosporin A (Hermansky *et al*., 1991). However, considering their potent toxicity in nature the WHO has established a guideline of 1.0 μg l^{-1} as the maximum concentration in potable waters (WHO, 1998).

1.3.2 Toxic Alkaloids

1.3.2.1 Neurotoxin alkaloids - anatoxin-a and anatoxin-a(S)

Toxic alkaloids include the neurotoxins; anatoxin-a, anatoxin-a(S), saxitoxins and hepatotoxic cylindrospermopsins (table 1.1). Anatoxin-a is a low molecular weight (MW=165 Da) secondary amine with the structural formula 2-acetyl-9-azabicyclo (4-2-1) non-2-ene (figure 1.2). Its analogue homoanatoxin-a (MW=179 Da) has a propionyl group at R position (figure 1.2) instead of the acetyl group in anatoxin-a (Chorus and Bartram, 1999). Anatoxin-a was first isolated from *Anabaena flos-aquae* by Devlin *et al*., (1977) but, also reported to produce by few genera of cyanobacteria; *Oscillatoria*, *Aphanizomenon* and *Raphidiopsis* (Edwards and Lawton, 2009). Homoanatoxin-a was isolated from *Planktothrix formosa* (Chorus and Bartram, 1999). Their mode of toxicity in birds and mammalians is to mimic the neurotransmitter acetylcholine and by binding irreversibly to the nicotinic acetylcholine receptor, thereby activating the flow of ions which induces muscle contraction (Wiegand and Pflugmacher, 2005). Unlike acetylcholine, the anatoxin stimulated nerve reception is not affected by acetylcholinesterase; hence, continues to act on muscle cells causing them to become over-stimulated (Carmichael, 1997). Disrupting the neuromuscular junctions blocking further electrical transmission can lead to paralysis and in the case of respiratory arrest may lead to death by asphyxiation (Van Apeldoorn *et al*., 2007).

Anatoxin-a(S) is a unique guanidinium methyl phosphate ester (figure 1.2b). Anatoxin-a(S) is also produced by *Anabaena flos-aquae* and acts as a potent cholinesterase inhibitor. Anatoxin-a(s) permits the binding of acetylcholine to

its receptors. However, it prevents the neurotransmitter from being degraded by acetylcholinesterase, causing muscle cells to become over-stimulated (Carmichael, 1997). The intraperitoneal LD_{50} value for mice and rats is 31 and 20 μg kg^{-1} of body weight respectively (Van Apeldoorn *et al*., 2007).

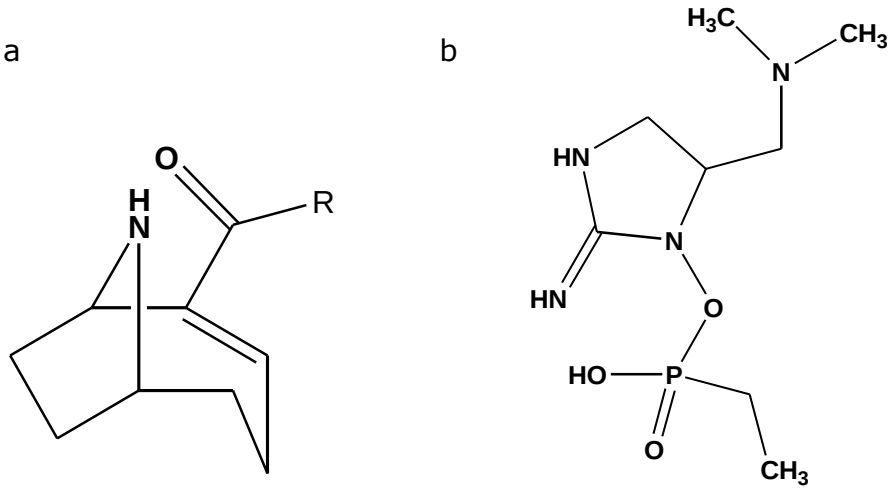

Figure 1.2 General structure of alkaloid toxins (a) anatoxin-a (R = CH_3); homoanatoxin-a (R = CH_2CH_3) and (b) anatoxin-a(S).

1.3.2.2 Saxitoxins

Saxitoxins (STX) are group of alkaloid neurotoxins produced by marine dinoflagellates such as *Protogonyaulax* sp., *Alexandrium catenella*, *Alexandrium minutum*, *Alexandrium ostenfeldii*, *Alexandrium tamarensis* and *Gymnodinium catenatum* (Carmichael, 1997; Chorus and Bartram, 1999). Moreover, the production of STX has been related to several cyanobacterial genera; *Aphanizomenon* sp., *Anabaena* sp., *Lyngbya* sp., and *Cylindrospermopsis raciborskii* (table 1.1). STX is composed of a unique triclcylic structure with hydropurine rings (figure 1.3). In nature the STX structure may vary depending on addition of sulphated (gonyautoxins – GTX) or doubly sulphated (C-toxins) groups (figure and table 1.3).

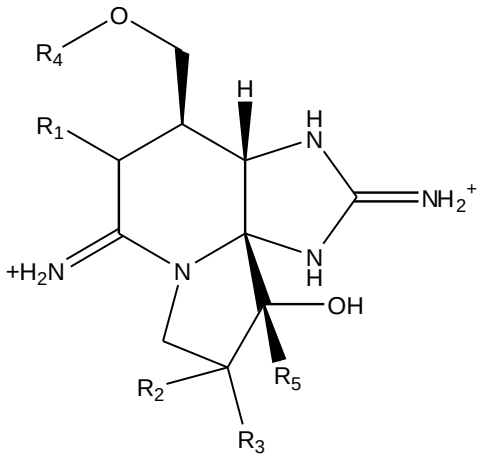

Figure 1.3 General structure of saxitoxins

Table 1.3 Structures of the saxitoxin (adapted from Van Apeldoorn *et al*., 2007)

Toxin variant	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**
STX	H	H	H	$CONH_2$	OH
NEOSTX	OH	H	H	$CONH_2$	OH
GTX1	OH	H	OSO_3^-	$CONH_2$	OH
GTX2	H	H	OSO_3^-	$CONH_2$	OH
GTX3	H	OSO_3^-	H	$CONH_2$	OH
GTX4	OH	OSO_3^-	H	$CONH_2$	OH
GTX5	H	H	H	$CONHSO_3^-$	OH
GTX6	OH	H	H	$CONHSO_3^-$	OH
C1	H	H	OSO_3^-	$CONHSO_3^-$	OH
C2	H	OSO_3^-	H	$CONHSO_3^-$	OH
dcSTX	H	H	H	H	OH
dcGTX2	H	H	OSO_3^-	H	OH
dcGTX3	H	OSO_3^-	H	H	OH

STX – Saxitoxins **NEOSTX – Neosaxitoxin** **GTX - Gonyautoxins**

C - C-toxins **dc - decarbamoyl**

STX are also known as paralytic shellfish poisons (PSPs) since the toxin has been found to accumulate in bivalve shellfish such as mussels and clams due to their indiscriminate ingestion of STX producing dinoflagellates. The transport of STX via the food chain and bioaccumulation has been reported as the main source of human toxicoses (consumption of STX contaminated shellfish).

In mammals, the STX toxicity mechanism occurs due to the interruption of neuronal communication as a result of their binding to the voltage-gated Na^+ channels in nerve cells (Wiegand and Pflugmacher, 2005). This causes muscle cells to receive no stimulation and become paralysed leading to subsequent death by respiratory arrest (Su *et al*., 2004).

1.3.2.3 Hepatotoxic cylindrospermopsin

Cylindrospermopsin (CYN) is a tricyclic sulphated alkaloid (figure 1.4) produced by different fresh water cyanobacteria such as *Cylindrospermopsis, Anabaena*, *Planktothirx* and *Raphidiopsis* (table 1.1). CYN is referred to as a potent hepatotoxin due to the severe liver necrosis it causes. However, unlike the MCs and NOD, CYN does not pose any inhibitory action for PP1 and PP2 (Chong *et al*., 2002). However, it was reported to irreversibly inhibit the protein synthesis (Froscio *et al*., 2003). Consequently, CYN appears to have a progressive effect on a number of other vital organs in addition to the liver (Chorus and Bartram, 1999). By oral ingestion, CYN can cause damage to the gastrointestinal route; liver necrosis and kidney malfunction from cell injury (Van Apeldoorn *et al*., 2007). The pyrimidine of CYN (figure 1.4) was found to be responsible for the toxic mechanisms of the compound. Unlike other

hepatotoxic cyanobacterial metabolites, the toxicity of CYN in animals is caused by the interaction with cytochrome P450 system (Froscio *et al*., 2003).

The toxic effect of CYN was highlighted by the "Palm island disease" in Australia, 1979. It has been reported that severe gastrointestinal diseases have been developed after the consumption of water from a reservoir in Palm Island, Queensland, Australia containing a collapsed bloom of *Cylindrospermopsis raciborskii* (Griffiths and Saker, 2003). Despite the potent toxicity of CYN, to date, relatively little work has been done on the toxicology of CYN, thereby further studies would enhance the elucidation of possible biotransformation pathways; e.g. glutathione *S*-transferase mechanism.

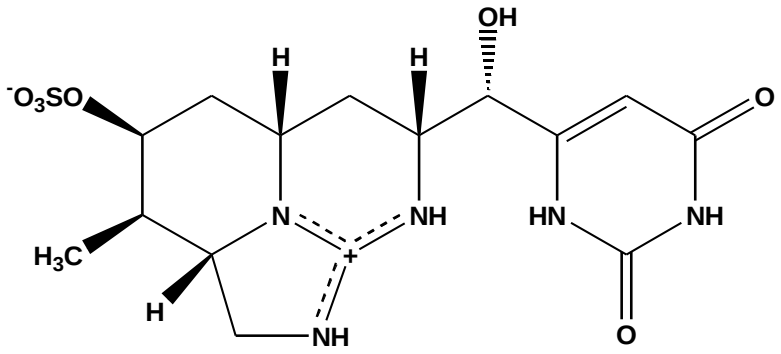

Figure 1.4 General structure of cylindrospermopsin

1.4 Persistence and degradation of microcystins

In nature, microcystins remain within the cells of exponentially growing cyanobacteria, and are released into water after viral infection, photodamage or chemically induced lysis (Welker and Steinberg, 2000). The cell-bound concentrations of MC-LR have been found to range from 50-10000 µg l^{-1} in German surface waters between 1995 and 1996(Chorus *et al*., 2000). Upon the collapse of a cyanobacterial bloom, large quantities of MCs can be released into the water posing a threat to many living organisms. The amount of the toxin released by a bloom collapse is unpredictable. However, once released, they are naturally removed by the dilution and chemical or biodegradation in natural environments (Edwards and Lawton, 2009). Dissolved toxins are of special concern due to their greater stability and the persistence of the toxicity to many other users of the aquatic system (Tsuji *et al*., 1994).

Degradation of MCs in the environment can be assumed to be part of the natural balance in between the production and elimination. In nature, bioremediation of the toxins is subjected to different environmental conditions such as concentration of the toxin, sunlight, temperature and pH of the water. To investigate the effect of sunlight on MCs degradation, Tsuji *et al*., (1994) exposed MC-LR (0.007-14 mg l^{-1}) to sunlight for 26 days and found 86% of the compound still available after the exposure period. However a clear decomposition of MC-LR was observed in the presence of photosynthetic pigments and sunlight together (Tsuji *et al*., 1994). This scenario was further confirmed by Welker and Steinberg, (2000), whereas more rapid degradation was caused by sunlight in the presence of fulvic acids and natural dissolved organic matter compared to that of sunlight alone. Jones and Orr, (1994)

investigated the persistence of MC-LR in Lake Centenary after an algicide treatment of a toxic bloom of *M. aeruginosa*. Surprisingly, MC-LR was still detectable after a month. Harada *et al*., (1996) reported that MCs, being cyclic peptides are extremely stable and resistant to chemical hydrolysis or oxidation near neutral pH. Authors further reported that MC was persistent under temperatures higher than the usual environmental temperatures (40 °C) as well as high and low pH values. About 90% of MC-LR was decomposed at highly acidic solutions (pH=1) after 10 weeks where it took more than 12 weeks when the pH was adjusted to pH 9 (Harada *et al*., 1996).

Meanwhile, many researchers identified the importance of natural bacterial consortia in lake water and sediments for the degradation of MCs (Jones *et al*., 1994). Interestingly, the majority of studies have followed a similar procedure to explore the bacteria biodegradation of MCs. In general, water samples or sediment samples collected from different lakes were exposed to MCs over a period of time and the remaining amount of the compound was monitored by HPLC (high performance liquid chromatography) methods. The results were compared to that of autoclaved water or sediment samples to ensure loss of MC is solely by a biological activity. Jones *et al*., (1994) demonstrated the degradation of MC-LR (1 mg l^{-1}) by the indigenous microflora from Murrumbidgee River, Australia. Interestingly, the degradation was commenced with an initial lag period of 3 days (d) and rapidly degraded by 5 d. However, in the same study, the lag phase was not observed following a re-addition of MC-LR into the pre-conditioned water sample indicating presence of bacteria and enzymes for rapid degradation of MCs (Edwards and Lawton, 2009). In later studies, Cousins *et al*., (1996) and Edwards *et al*., (2008) have reported

similar lag phases and half-lives to those presented by Jones *et al*., (1994). The scenario of the lag phase was explained as the time required for the induction of specific bacterial enzymes responsible for the degradation of MCs (Jones *et al*., 1994). However, in contrast to this, Lam *et al*., (1995) suggested that lag phases were associated with the number of bacteria in the water sample rather than an induction period. Meanwhile, few studies have indicated the importance of benthic microorganisms for the degradation of MCs. Holst *et al*., (2003) studied the degradation of ^{14}C labelled MC-LR in the presence of lake sediment samples. The mineralisation of radio-labelled MC-LR was observed by the evolution of $^{14}CO_2$ and ~ 50 µg l^{-1} of MC-LR was mineralised within 1-2 weeks. In another study, Torunska *et al*., (2008) demonstrated that NOD was rapidly degraded in sea water with added sediment samples indicating the importance of benthic microflora for the degradation of MCs and NOD in nature.

1.5 Removal of microcystin from drinking water

Drinking water can come from surface water and/or ground water. Ground water is filtered through different soil particles and collected beneath the ground surface in soil pore spaces. However, compared to the ground water sources, surface drinking water (lakes, rivers, man-made reservoirs) has been found to be significantly vulnerable for the MCs contamination (Lawton and Robertson, 1999) due to the increase in bloom occurrence. MC producing blooms may break out not only in large water bodies, but also in small reservoirs, ponds and pools (Ishii *et al*., 2004). Their persistence in water may last from weeks to months and their natural removal is regulated by environmental factors such as pH, sunlight, temperature and microorganisms

(Harada *et al*., 1996). However, due to the increasing threat of contamination of drinking water sources by MCs combined with the increasing water scarcity, natural water remediation is inadequate. Consequently, the emerging risk of MC contamination in drinking water has prompted many countries to establish regulations for the limitation of cyanobacterial toxins in drinking water supplies. United Kingdom, New Zealand and Brazil have followed the lead of WHO (1998) provisional guidelines value of 1 μg l^{-1} for total MC-LR (intra and extracellular toxin concentration). In Canada and Australia the drinking water guideline for total MC (free and cell bound) is extended to 1.5 μg l^{-1} of MC-LR (Van Apeldoorn *et al*., 2007). Furthermore, United States Environmental Protection Agency (USEPA) has listed MC as a drinking water contaminant and reviewed along with chemical warfare agents (Reemtsma 2003; Richardson and Ternes, 2005).

Although many guidelines and regulations have been presented, the removal of MCs still remains challenging for many traditional water treatment methods (Edwards *et al*., 2008). If MCs remained within the cells, removal through conventional methods such as coagulation, flocculation and filtration would likely to be efficient as a water treatment. However, once the cyanotoxins are released, their removal may require advanced water treatment (Himberg *et al*., 1989). Lawton and Robertson, (1999) reviewed different water treatment methods for their effectiveness against MCs. According to the review, well established methods such as chlorination is likely to require relatively high dosing rates and considerably long time may be required for an effective removal of MCs. The fate of the MC degradation and the toxicity of the degradation products caused by chlorination are barely known. Although,

ozonation was acknowledged as an effective method for removal of MCs, the cost of treatments has been considered as unaffordable for many parts of the world. Potential of the photocatalytic degradation of MCs using titanium dioxide (TiO_2) has been identified as potential low cost and clean water treatment (Robertson *et al*., 1997; Lawton *et al*., 1999). However, furhter evaluation of photocatalytic MC degradation is required before it can be applied for water treatment.

Whilst many water treatment methods are either ineffective or expensive to implement, biodegradation of MCs has been acknowledged as an emerging, potentially reliable and cost effective method for water treatment (Babica *et al*., 2005; Bourne *et al*., 2006; Ho *et al*., 2006). As previously discussed, lake water and sediments might serve as rich resources of the natural MC degrading consortia (Jones *et al*., 1994). Thereby, bacterial biodegradation of MCs can be implemented by exploiting natural MC degrading bacteria. Accordingly, many studies have focused on harnessing MC degrading bacteria from water sources exposed to MC-producing blooms which resulted in low diversity of bacteria. For instance, many of the MC degraders are reported as *Sphingomonas* or *Sphingomonas*-like (table 1.4). Until Manage *et al*., (2009), identified MC degrading Gram-positive *Actinobacteria*, all of the MC degraders had belonged to Gram-negative *Proteobacteria* isolated in classical microbiology procedures (table 1.1).

In the quest of exploring the potential of bacterial biodegradation of MCs, Bourne *et al*., (1996) identified and characterised a degradation pathway for MC-LR by *Sphingomonas* sp. (figure 1.5). This bacterium was previously

identified by Jones *et al*., (1994) and shown to harbour specific MC degrading enzyme producing genes, namely, *mlrA*, *mlrB* and *mlrC* along with *mlrD* (oligo peptide transporter) in *mlr* gene cluster.

Table 1.4 Microcystin and nodularin degrading bacteria, adapted from Ho *et al*., (2007) and Shimizu *et al*., (2011).

Bacteria	Degradable analogous	Gene/gene cluster	Grams identification	Reference
Sphingomonas sp. ACM-3962	MC-LR and -RR	*mlr*[a]	-ve	Bourne *et al*., (2001)
Novosphingobium sp. MD-1	MC-LR, -YR, and -RR	*mlrA*	-ve	Saitou *et al*., (2003)
Sphingosinicella microcystinivorans Y2	MC-LR, -RR, -YR, 6(Z)-Adda-LR	*mlrA*	-ve	Saitou *et al*., (2003)
Sphingomonas sp. B9	MC-LR, -RR, dh-LR, LR-Cys, NOD	*mlrA*	-ve	Harada *et al*., (2004)
Sphingomonas sp. 7CY	MC-LR, -RR, -LY, -LW, -LF	NI[b]	-ve	Ishii *et al*., (2004)
Paucibacter toxinivorans	MC-LR, MC-YR, NOD	NI	-ve	Rapala *et al*., (2005)
Sphingosinicella microcystinivorans B9	MC-LR, -RR	NI	-ve	Tsuji *et al*., (2006)
Sphingomonas sp. CBA4	MC-RR	NI	-ve	Valeria *et al*., (2006)
Sphingopyxis sp. MG-15 and MG-22		NI	-ve	Fujimoto *et al*., (2007)
Sphingopyxis witflariensis LH21	MC-LA, MC-LR	*mlr*	-ve	Ho *et al*., (2007)
Burkholderia sp.	MC-LR, [D-leu1]LR		-ve	Lemes *et al*., (2008)
Sphingopyxis sp. C-1	MC-LR	*mlr*	-ve	Okano *et al*., (2009)
Methylobacillus sp. J10	MC-LR, -RR	NI	-ve	Hu *et al*., (2009)
Arthrobacter sp., *Brevibacterium* sp. and *Rhodococcus* sp.	MC-LR	NA[c]	+ve	Manage *et al*., (2009)
Stenotrophomonas sp. EMS	MC-LR, -RR	*mlrA*	-ve	Chen *et al*., (2010)
Sphingopyxris sp. USTB-05	MC-RR	NI	-ve	Zhang *et al*., (2010)

a – *mlr* gene cluster with *mlrA, mlrB, mlrC, mlrD* **b – Not investigated** **c – Not available**

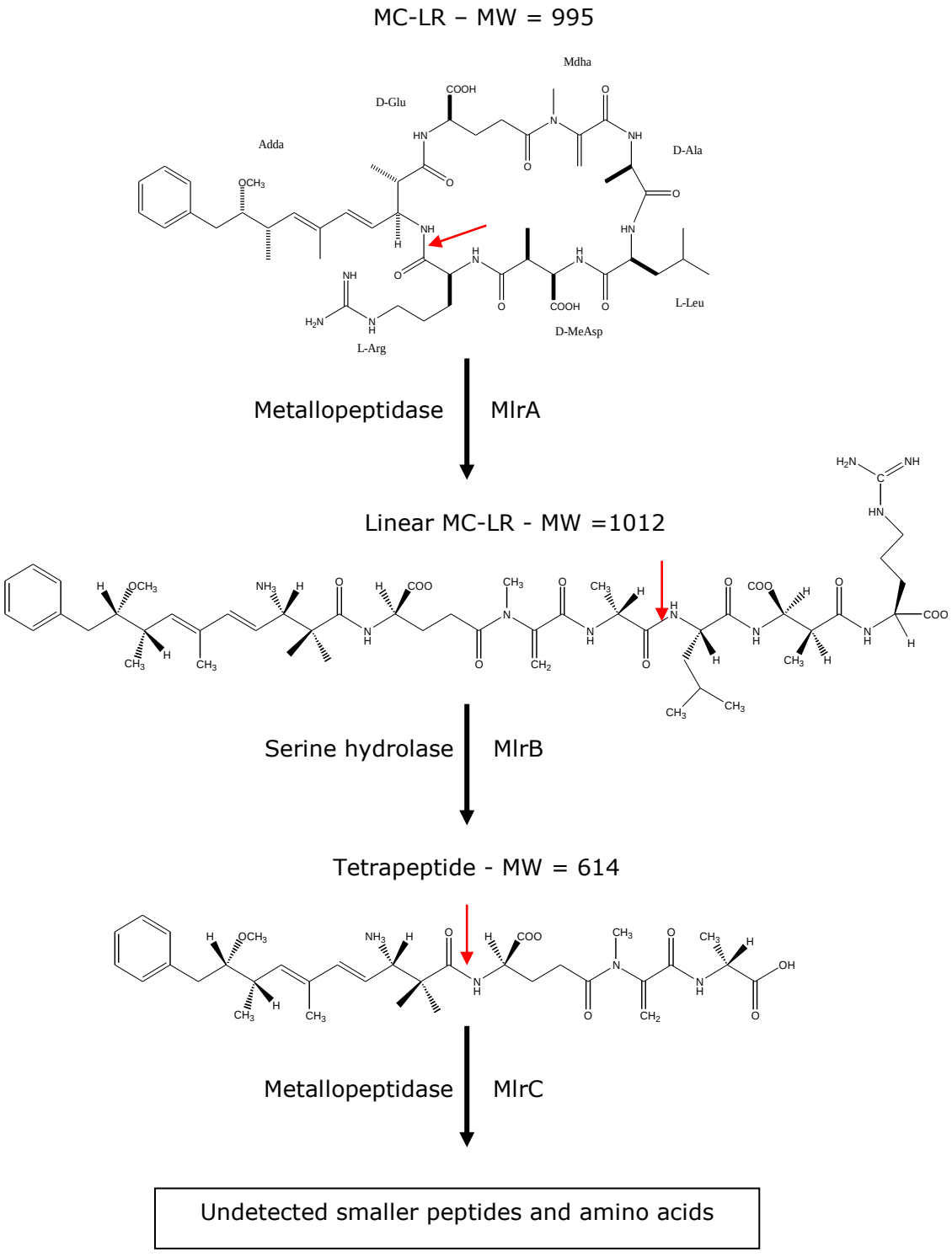

Figure 1.5 Proposed MC-LR degradation pathway (Bourne *et al*., 1996, 2001)

A metallopeptidase enzyme (MlrA) produced by *mlrA* gene initiated the degradation by the cleavage of Adda-Arginine (Arg) bond resulting in a 160 fold reduction of toxicity compared to the natural cyclic structure (Bourne *et al*., 1996). Subsequently, linear MC-LR was further hydrolysed into a tetrapeptide by MlrB which was a serine hydrolase. MlrC performed further degradation of the tetrapeptide into smaller peptides and amino acids. In later studies, Saito *et al*., (2003) and Harada *et al*., (2004) have identified the prevalence of microcystinase enzyme producing genes (*mlr*) previously reported by Bourne *et al*., (1996, 2001). Similarly, Ho *et al*., (2007) isolated MC degrading bacterium; *Sphingopyxis witflariensis* from a biologically active sand filter in Australia. In that study, authors used the DNA sequence of *mlrA* gene in a PCR assay to screen 32 bacterial isolates for the presence of MC degrading genes, and only one bacterium was found to contain the *mlr* gene cluster. Furthermore, in recent studies, Okano *et al*., (2009) and Chen *et al*., (2010) isolated MC degrading bacteria containing *mlrA* gene. Utilisation of these elegant molecular techniques may facilitate the future of the bacterial biodegradation of MCs. However, on the other hand, significant concern about using *mlr* gene sequences for PCR screening was the limitation of the diversity of MC degraders since, these methods will detect only particular bacteria while many other MC degraders may be available in the environment.

The main objective of harnessing MC degrading bacteria is to allow water remediation. Potential MC degrading strains can be immobilised in water treatment systems. This was demonstrated by the use of *Sphingomonas* sp. B9 (table 1.4) in a bioreactor (figure 1.6) where MC-LR and –RR were completely degraded within 24 h (Tsuji *et al*., 2006). When the bioreactor was

further implemented using several different substrates (cellulose and different polyester types), more than 90% of MC-RR was degraded within 24 h period. Their removal efficiency was found to be consistent while MC-RR was continuously added in regular intervals for at least 2 months. This work has demonstrated the use of MC degraders in a water purification system and their greater potential for the removal of MC from potable water. However, considering the possible existence of greater diversity of MC degrading bacteria, the harnessing of novel MC degraders may facilitate the implementing of more efficient and reliable methods of water purification.

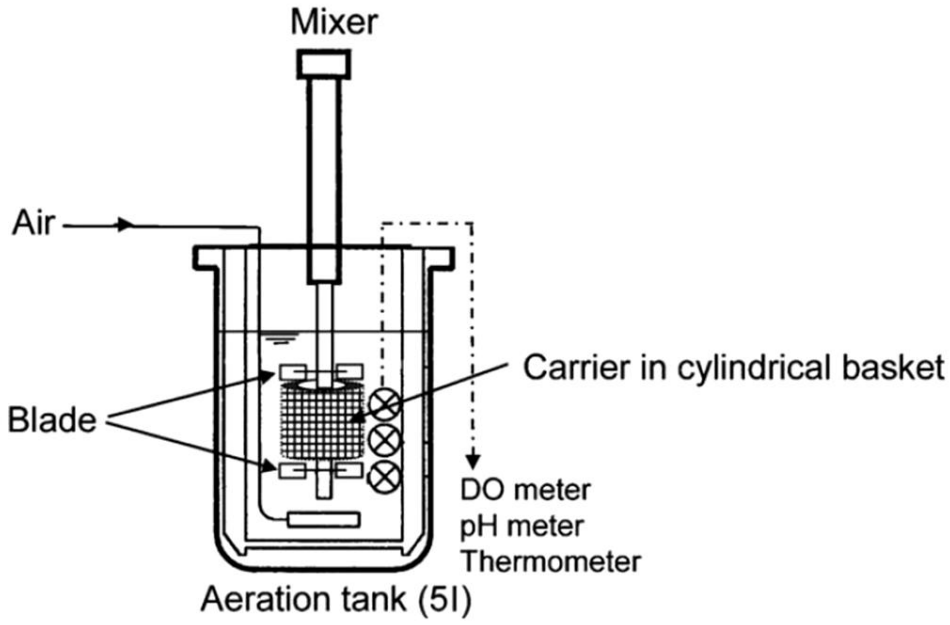

Figure 1.6 Bioreactor diagram using immobilised *Sphingomonas* sp. B9 for removal of MC from lake water (adapted from Tsuji *et al*., 2006)

1.6 Aims and objectives

While conventional water treatments remain ineffective for the removal of MC and NOD from potable water, bioremediation, utilisation of bacteria to remove MC and NOD has received greater attention as a reliable and cost effective approach for water treatment. However, the number and diversity of reported MC degraders are still extremely low. Recent work in our laboratory, has resulted in successful isolation and characterisation of 10 novel bacteria (*Arthrobacter* sp*., Brevibacterium* sp. and *Rhodococcus* sp.), capable of degrading MC-LR. Following the previous work, the current research aimed to investigate the biodegradation potential of these novel bacteria towards a cost effective and reliable method for removal of MCs and NOD from drinking water.

Major objectives of this study were to:

1. **Evaluate the bacterial metabolism and biodegradation on array of chemically diverse MCs and NOD**

Manage *et al*., (2009) demonstrated the metabolism and degradation of MC-LR by novel *Actinobacteria* isolates. However, their potential to degrade other MCs has not been evaluated. Conversely, cyanobacteria produce multiple classes of toxins and their chemical diversity, toxicity and stability are more diverse in nature. To date, most of the studies have focused on one or two MC variants (table 1.4); hence, their degradation capacity on different MC variants and NOD is unknown. The first objective of this study was to evaluate biodegradation potential of novel *Actinobacteria* on an array of chemically diverse MCs and NOD. The 96 well Biolog MT2 plates (contain no carbon

source in the wells but a tetrazolium dye) have been implemented as a rapid and cost effective tool for screening the bacterial isolates against a wide range of MCs (MC-LR, -LF, -LW, -LY and -RR) and NOD as sole carbon source. The utilisation/oxidisation of these carbon sources would transform tetrazolium dye in the Biolog plate wells to a formazan dye which can be spectroscopically measured. To confirm the occurrence of bacterial metabolism in MT2 plates, biodegradation of above toxins were HPLC-monitored in a traditional batch experiment.

2. Investigate the effects of water chemistry on the biodegradation of MCs

Bacterial biodegradation can be affected by the nutrient status of the water. In nature, bacteria undergo different nutrient stress conditions such as no, low or high amounts of different nutrients (C, N, P, metals). During a water treatment, MC degrading community may experience different nutrient compositions depending on different periods of a year (summer, winter), rainfall and nutrient discharges. Some studies have evaluated the effect of carbon on the degradation of MC-LR, while many other biodegradation studies addressed neither the effect of carbon nor other nutrients such as N and P. To date, there is very limited information available regarding the nutrients essential to optimise MC degradation to be utilised in water treatment. However, understanding the nutrient status which enhances bacterial degradation might play an important role utilising the bacteria in a water treatment. Furthermore, this will undoubtedly facilitate determining the location the bacteria should be employed in a water purification pathway.

3. Evaluate the bacterial degradation of different prokaryotic and eukaryotic peptides

It is highly unlikely that MC degrading enzymes are produced only for the degradation of MCs. However, many studies have hypothesised that MC degrading genes are solely for degradation of MCs. On the other hand, biodegradation studies are extremely limited to MCs and only very little information is available regarding their degradation of different peptides in nature. Conversely, bacteria like *Arthrobacter* sp. and *Rhodococcus* sp. are well acknowledged for their remarkable ability to degrade many recalcitrant environmental pollutants. Therefore, it is important to understand their role in biodegradation of naturally occurring peptides other than MCs. This may also facilitate understanding of the natural peptide degradation mechanisms as well as some aspects of antibiotic resistance of bacteria. Furthermore, to facilitate understanding whether peptide degradation is influence of by their chemical structure or origin; cyclic, linear, prokaryotic and eukaryotic peptides were evaluated in a batch degradation assay.

4. Elucidate gene(s) responsible for degradation of MCs

As a result of the limited diversity of MC degrading bacteria, to date, only one degradation pathway has been characterised using a Gram-negative *Sphingomonas* sp. from *Proteobacteria*. However, since *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp. belong to Gram-positive *Actinobacteria*; their degradation pathway of MC may be different. In previous studies, Manage *et al*., (2009) reported that *Arthrobacter* sp., *Brevibacterium* sp. and *Rhodococcus* sp. do not harbour *mlr* genes, but proposed that *mlr* homologues may exist. Thus, the main objective here was to investigate the

existence of *mlr* homologous genes in *Actinobacteria* isolates. Under the circumstances that *mlr* homologues genes are not present, the gene(s) responsible for degradation of MC has been elucidated.

CHAPTER 2

CULTURING AND PROCESSING OF CYANOBACTERIA

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2 Culturing and processing of cyanobacteria

2.1 Introduction

Cyanobacteria produce a diverse group of secondary metabolites (Moore, 1996; Burja *et al*., 2001) for which the natural function has not been clearly revealed yet (Lawton and Edwards, 2001; Wiedner *et al*., 2002). Among them, MC and NOD have widely been studied due to their diversity of different analogues, acute and chronic toxicity on human and animals, worldwide distribution and long persistence in the environment. Moreover, both MCs and NOD are well known to inhibit protein phosphatase; hence, disrupts the cell regulatory processes (Mackintosh *et al*., 1990) which in turn increase the risk of liver cancer (Falconer, 1994; Ueno *et al*., 1996). Thus, indicating the importance of rigorous environmental monitoring and toxicological studies of MCs and NOD. Emerging interest in cyanobacterial secondary metabolites in pharmacology (biochemical assays, i.e. protein phosphatase inhibition) and toxicology (environmental standards, toxicity measurements) has increased the demand for purified cyanobacterial secondary metabolites (Lawton and Edwards, 2001). To satisfy this, harvesting of cyanobacterial secondary metabolites has gained interest amongst the researchers in this field of study.

Cyanobacteria are extracted either from field collected blooms or laboratory grown cultures. Harvesting cyanotoxins from naturally available blooms may be cost effective due to no extra investment in culturing and maintenance. This can be achieved by collecting large quantities of cyanobacterial cells from surface waters along the shoreline. However, in most cases cyanobacteria can remain in the water column rather than being accumulated in the shoreline

where additional resources are required for collection (sample collection gear e.g. plankton net, boat; Lawton and Edwards, 2001). Hence, collection and concentration of environmental bloom material can become a time consuming process. In addition, samples collected from the field usually contain more than a single strain of cyanobacteria. This makes further characterisation necessary to determine which organism is responsible for the production of the cyanotoxins needed (Lawton and Edwards, 2001). However, culturing and processing of laboratory cultures of cyanobacteria has been shown to be an efficient and reliable alternative to field collection of cyanobacterial blooms. Laboratory cultures, often well characterised, have already been established and maintained as single strain cultures. This, in turn facilitates a rapid monitoring of toxin production and a reliable analysis of the quantity of toxin produced at a particular time period. As described by Lawton and Edwards, (2001), laboratory cultured cyanobacteria remain highly reproducible, less complex for harvesting and provide rapid purification of cyanotoxins (Lawton *et al*., 1999).

Cyanotoxins are generally extracted as a mixture of chemically different compounds. However, to enable their use either as an analytical standard or in a biochemical assay, high quality individual compounds are required. This will be typically achieved by extracting cyanotoxins from cyanobacterial cells followed by multiple purification steps (Lawton and Edwards, 2001; Edwards and Lawton, 2010). In the quest for the purification of cyanotoxins a number of different methods have been proposed to purify MCs and NOD. Botes *et al*., (1982) purified MCs using diethylaminoethyl cellulose column chromatography

with a Sephadex G50 column obtaining the final purity by high-voltage paper electrophoresis. Subsequently, Poon *et al*., (1987), adapted the high performance thin layer chromatography along with a concentration step through Sep-Pack C18 cartridge with resulting purification of microgram quantities. However, these methods have been found to involve a number of time consuming steps leading to a poor yield. Some advances in purification of MCs included methanol extraction, followed by concentration through a C18 solid phase extraction cartridge with an elution step gradient of methanol and water (Lawton *et al*., 1995). Furthermore, this technique was developed using reversed-phase (RP) flash chromatography and HPLC, scaling-up the final yield from milligram to gram quantities of pure compounds (Edwards *et al*., 1996). The purity of MCs and NOD is generally determined by an ultra violet (UV) chromatogram extracted at 238 nm (λ_{max} of the majority of MCs) using reversed-phase HPLC with photo diode array (PDA) detection (Lawton and Edwards, 2001). However, impurities which do not absorb at UV 200-300 nm remains a challenge for obtaining high purity MC and NOD standards. Therefore, the choice of mass spectrometry (MS) to evaluate purified compounds has been widely acknowledged due to the high sensitivity and broad detection range along with information in mass spectra for identification and confirmation of MCs and NOD (Edwards and Lawton, 2010). The reliability combined with the sensitivity and the accuracy of both UV and MS methods together has established them as reference methods for the quality control of MCs and NOD. In contrast, recent advances to improve the quality of the purified material revealed the advantages of using charge aerosol detector (CAD) in the assessment of the purity of MCs (Edwards and Lawton, 2010).

Interestingly, whilst the study confirmed the reliability of UV and MS methods when they are integrated, the authors (Edwards and Lawton, 2010) also demonstrated the novel application of CAD as an integrated detector in the purification process for a greater range of high quality purified compounds.

In the current study, a wide range of purified cyanobacterial secondary metabolites such as MCs (MC-LR, -LF, -LW, -LY and -RR), NOD, Linear NOD (LNOD) and anabaenopeptin B (ABPNB) were utilised to evaluate their bacterial biodegradation. Due to the high demand and cost of these compounds, purchasing pure material and standards was not an affordable choice. On the other hand, cyanotoxins such as MC-LF, -LW were only available at considerably low amounts whereas ABPNB and LNOD were commercially unavailable. However, some elegant studies (Edwards *et al*., 1996; Barco *et al*., 2005; Edwards and Lawton, 2010), have previously described efficient HPLC based methods for the purification of different cyanotoxins which are consistently proven to obtain high yields of pure material. This research, therefore, aimed to adapt the HPLC based toxin purification methods previously described by Edwards *et al*., (1996), and Edwards and Lawton, (2010), in order to extract and purify the required cyanotoxins. RP flash chromatography was employed to provide semi-purified samples for the preparative HPLC separations (Lawton *et al*., 1995; Edwards and Lawton, 2010). The purity of the separated compounds was assessed by analytical HPLC equipped with UV-PDA and electrospray ionisation-mass spectrometry (ESI-MS) detector (PDA-LC-MS). In summary, this chapter describes the culturing and processing of cyanobacteria as well as the HPLC

based methods to obtain high quality cyanotoxins to underpin accuracy and reliability of the biodegradation studies described in chapter 3, 4 and 5.

2.2 Materials and methods

2.2.1 Chemicals

Chemicals were of analytical-reagent grade unless stated and obtained from Fisher Scientific, Leicestershire, UK. HPLC-grade methanol and acetonitrile were purchased from Rathburn, Walkersburn, UK. Trifluoroacetic acid (TFA) for HPLC systems was purchased from Fisher Scientific, Leicestershire, UK. Pure water was obtained from a Milli-Q system (purified to 18.2 MΩ, Millipore, Watford, UK). MC (-LR, -LY, -LW, -LF and -RR), ABPNB standards were purified from batch cultures of *Microcystis aeruginosa* (table 2.1) as previously described (Edwards *et al*., 1996; Edwards and Lawton, 2010). NOD and LNOD standards were obtained from the cultures of *Nodularia spumigena* KAC66 (table 2.1).

2.2.2 Cyanobacterial strains

Strains of cyanobacteria used in the following study are listed in table 2.1.

Table 2.1 Cyanobacterial strains used for lab scale culturing and the secondary metabolite (cyanotoxins) harvesting

Cyanobacterial strain	Culture collection	Secondary metabolites produced	Reference
Microcystis aeruginosa PCC 7820	Pasteur culture collection, Paris, France	MC-LR, -LY, -LW, and -LF	Lawton *et al*., (1995), Lawton and Edwards, (2001)
Microcystis aeruginosa UTEX B2666	University of Texas, Texas, USA	MC-LR, -LA, -LL and -LF	Barco *et al*., (2005) Diehnelt *et al*., (2006)
Microcystis aeruginosa Sciento	Sciento Education service, Manchester, UK	MC-LR, -WR, -RR, and ABPNB	Barco *et al*., (2005)
Nodularia spumigena KAC66	Kalmar culture collection, Kalmar, Sweden	NOD, LNOD	Edwards *et al*., (2008)

2.2.3 Laboratory scale culturing of cyanobacteria

All cyanobacteria cultures (table 2.1) were maintained on a regular basis and sub-cultured every 6 weeks in 10 l Pyrex glass containers using BG-II medium (Stanier *et al*., 1971; table 2.2). In order to avoid precipitation of the chemicals, BG-II culture medium was prepared from stock solutions of macronutrients and trace metals (table 2.2) and autoclaved (121 °C, 50 min; Astell Scientific, UK). Exceptionally, *Nodularia spumigena* KAC66 was grown in BG-II with 20% instant ocean water (Mazur-Marzec *et al*., 2006).

Table 2.2 Composition of the BG-II media (Stanier *et al*., 1971)

Component	**BG-II (g l^{-1})**
$NaNO_3$	0.750
K_2HPO_4	0.040
$MgSO_4\ 7H_2O$	0.075
$CaCl_2\ 2H_2O$	0.036
Na_2CO_3	0.020
Citric acid	0.006
$FeSO_4\ 7H_2O$	0.006
EDTA (disodium)	0.001
Trace element solution	1 ml l^{-1}
Trace element solution	
H_3BO_3	2.680
$MnCl_2\ 4H_2O$	1.810
$NaMoO_4\ 2H_2O$	0.390
$ZnSO_4\ 7H_2O$	0.222
$CuSO_4\ 5H_2O$	0.079
$Co(NO3)_2\ 6H_2O$	0.049

Figure 2.1 *Microcystis aeruginosa* PCC 7820 grown in 10 l Pyrex flasks under continuous illumination and sparging with sterile air.

A volume of 1 l cyanobacterial culture in stationary phase was inoculated into 9 l sterile freshly prepared BG-II, incubated under continuous flow of sterile air (0.22 µm filter, Millipore, UK). Cultures were exposed to continuous illumination generated by cool white Osram fluorescent tubes (58 W) 20 µmol m^{-2} s^{-1} (Li-Cor intelligent light meter Li-250) in a temperature controlled (22 °C ± 2) room.

2.2.4 Harvesting and extraction of the cultures

Cyanobacterial cultures growing in aerated 10 l glass pots were harvested by centrifugation (1500 g, 30 min) and stored at -20 °C prior to being thawed for extraction. All cyanotoxins were extracted in methanol (Lawton *et al*., 1995; Barco *et al*., 2005). Freeze-thawed wet cyanobacteria cell pellets were centrifuged (1500 g, 30 min), supernatants were decanted and the pellets were re-extracted a further two times in methanol. Extracted material in methanol was diluted 20% (v/v methanol) and loaded into C18 cartridge for RP flash chromatography (Edwards *et al*., 1996).

2.2.5 Concentration and cleanup - reverse phase flash chromatography

HPLC based methods described by Edwards *et al*., (1996) and Lawton and Edwards, (2010) were adapted for the purification of all cyanotoxin variants (figure 2.2). The concentration and clean up steps were performed using a pre-conditioned (methanol and water) C18 cartridge (40 mm I.D. x 75 mm long; 40-63 µm particle size) attached to Biotage Horizon flash chromatography system (Biotage, Cardiff, UK). Aqueous extracts of cyanobacteria were loaded at 40 ml min^{-1} and eluted with an increasing gradient of methanol from 0% to 100%, in 10% increments with 240 ml volume per fraction. The quality and quantity of the eluted cyanotoxins in each fraction was determined by analysing a sample (10 µl) by PDA-LC-MS (section 2.2.8). Fractions selected through PDA-LC-MS containing cyanotoxins were pooled and cleaned up through a second flash chromatography step for further

concentration of the samples. Instrumental parameters were maintained as previously described by Edwards and Lawton, (2010).

2.2.6 Purification of cyanotoxins – preparative HPLC

Further purification of the RP flash samples (section 2.2.5) were carried out using a Phenomenex (Macclesfield, UK) Luna C18 column (21 mm I.D. x 250 mm long; 10 µm particle size) attached to a preparative HPLC system (Biotage, Parallex Flex, Cardiff, UK) with Milli-Q water (A) and acetonitrile (B) both containing 0.05% TFA as the mobile phase. Cyanotoxins were eluted as fractions over a gradient that increased from 20% to 80% B over 60 min at a flow rate of 20 ml min^{-1}. The gradient of the organic mobile phase was adjusted to elute compounds with different polarity using the parameters described by Edwards and Lawton, (2010). Separation of the fractions was performed by the UV absorption threshold as defined by the chromatography system (Flex V3 software) and collected into deep well microtiter plates (58 x 5 ml). The quality control of the separated samples in the microtiter plates was performed by PDA-LC-MS as described in section 2.2.8 (figure 2.2). The relative purity of each sample was calculated as the proportion of the corresponding MC peak over all other peaks in the chromatogram.

2.2.7 Desalting and drying of the purified cyanotoxins

Preparative HPLC fractions of acceptable purity (≥ 95%) were pooled and diluted (1:5) with Milli-Q water. The diluted sample was then loaded into preparative HPLC cartridge with a subsequent Milli-Q washing step. Trapped

cyanotoxins in the cartridge were eluted with 100% methanol (Edwards *et al*., 1996). Eluted cyanotoxins were aliquot into weight determined dry vials, dried under gaseous nitrogen and stored at -20 °C.

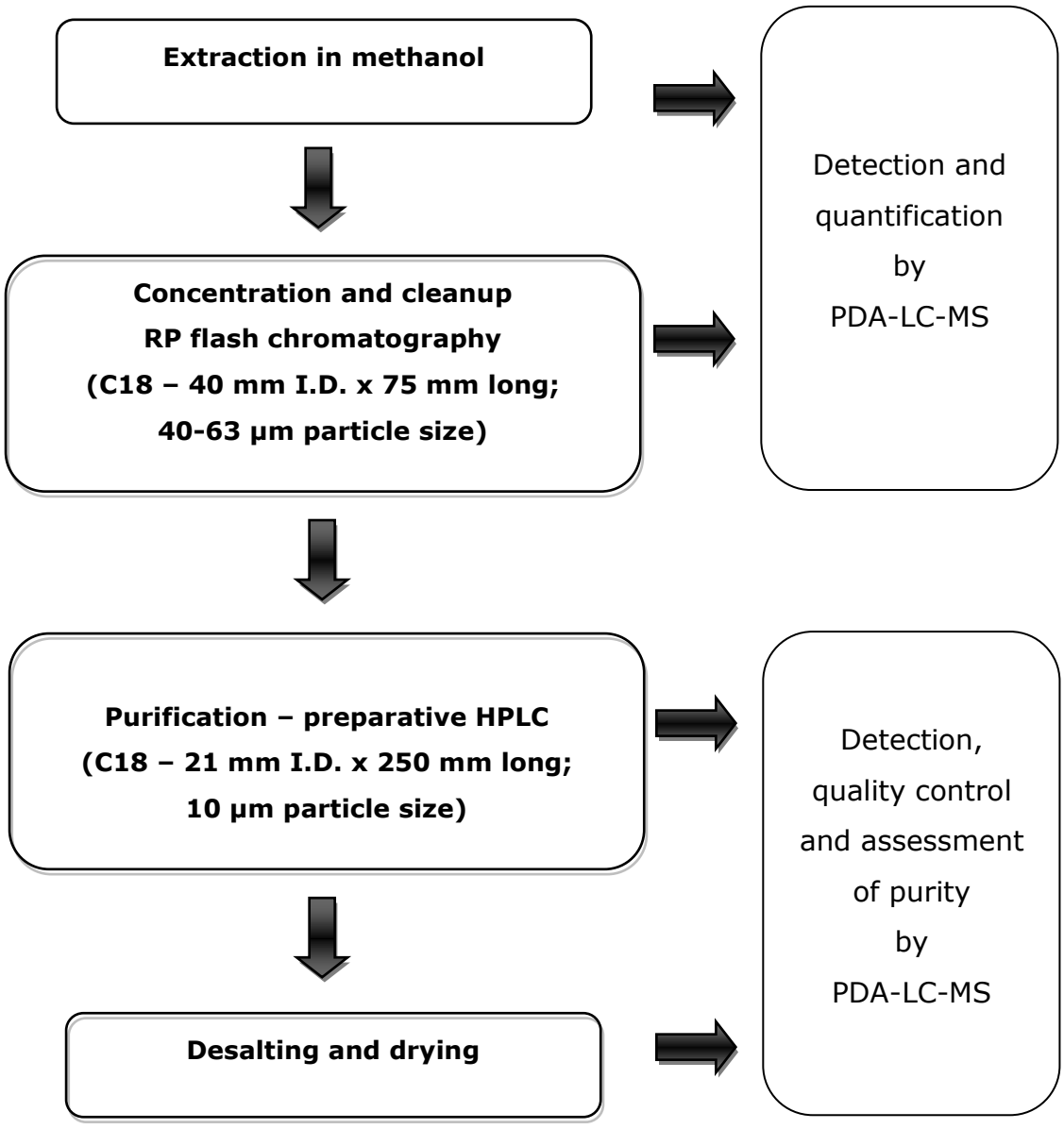

Figure 2.2 Schematic of the HPLC based purification of cyanotoxins (Edwards *et al*., 1996)

2.2.8 Analysis of cyanotoxins – analytical HPLC (PDA-LC-MS)

Analysis of cyanotoxins was performed based on the HPLC methods described by Edwards and Lawton, (2010) using LC-MS (Waters Alliance 2695 solvent delivery system) with photodiode array (2996 PDA) equipped with a mass detector (ZQ 2000 MS) in series (Waters, Elstree, UK). The separation was effected on a Sunfire C18 column (2.1 mm I.D. x 150 mm long; 5 µm particle size) and maintained at 40 °C. Milli-Q water (A) and acetonitrile (B) both containing 0.05% TFA constituted the mobile phase. Samples were separated using a gradient increasing from 15% to 60% B over 25 min at a flow rate of 0.3 ml min^{-1} followed by ramp up to 100% B and re-equilibration over the next 10 min. Eluent was monitored by UV absorption from 200-300 nm with a resolution of 1.2 nm. Analysis by mass spectrometry was performed in positive ion electro-spray mode, scanning from *m/z* 100 to 1200 Da with a scan time of 2 s and inter-scan delay of 0.1 s. Ion source parameters; sprayer voltage, 3.07 kV; cone voltage, 80 V; desolvation temperature, 300 °C; and source temperature, 100 °C. Instrumental control, data acquisition and processing were achieved using Masslynx v4.0. Purified MCs were identified on the basis of both their retention time and characteristic UV spectra using previously purified validated MC-LR standards.

2.2.9 Preparation of calibration curves for purified cyanotoxins

Standard stock solutions of purified MCs were prepared gravimetrically by re-dissolving known amounts of the dry compound in 80% methanol (Edwards and Lawton, 2010). Working solutions for the biodegradation studies (chapter 3, 4 and 5) were always made by the dilution of stock solutions in Milli-Q

water. Calibration curves were prepared using serial dilutions of the cyanotoxins that represented the typical working range; 1 µg to 5 ng on PDA-LC-MS column (100-5 µg ml^{-1}). Triplicates of the samples were analysed by PDA-LC-MS as described in section 2.2.8. Regression curve and correlation coefficient was obtained from the plotted graphs of concentration against peak area of the UV and ESI-MS ion mass chromatograms.

2.2.10 Purification and analysis of other cyanotoxins

MC-RR, NOD, LNOD and ABPNB (table 2.1) were harvested and extracted in methanol as previously described in section 2.2.4. Extracted compounds were concentrated using RP flash chromatography (section 2.2.5) and purified by preparative HPLC (section 2.2.6). Sample identification and quality control was performed by PDA-LC-MS (section 2.2.8). Purified MC-RR, NOD, LNOD and ABPNB were desalted (section 2.2.7) and calibration curves were prepared (section 2.2.9) before being used for biodegradation studies in chapter 3 and 4.

2.3 Results

2.3.1 Extraction of cyanobacterial secondary metabolites

PDA-LC-MS analysis of the aqueous methanolic cell extract of *Microcystis aeruginosa* PCC 7820, revealed four main variants of MCs. The UV chromatogram extracted at 238 nm (figure 2.3) indicated the presence of MC-LR, -LY, -LW and -LF. Extracted MCs were identified on the basis of both their retention time and characteristic UV spectra using previously purified validated MC-LR standards (appendix C).

The initial extraction successfully provided 130 mg (76%) of MC-LR and 11 mg (92%) of MC-LY with two other MC variants, MC-LW (20 mg; 77%) and MC-LF (15 mg; 79%; table 2.3). Re-extraction of the cell pellets resulted in comparatively low yield of all four MC strains (table 2.3). MC-LR was found to be the most predominant variant in the second extraction (35 mg; 20%). MC-LW has indicated a slightly higher amount of 6 mg compared to the 4 mg of MC-LF during the second methanol extraction. MC-LY resulted in a comparatively low yield (1 mg) compared to other MC variants. Third methanolic extraction contained 5 mg of MC-LR whilst other variants remained below the limit of detection. The three extraction steps provided 170 mg of MC-LR in total (table 2.3). In contrast, MC-LY demonstrated the least amount (12 mg) of MCs compared to other variants. The final yield of the extracted MC-LW (26 mg) indicated slightly higher than the closely eluting MC-LF (19 mg; table 2.3).

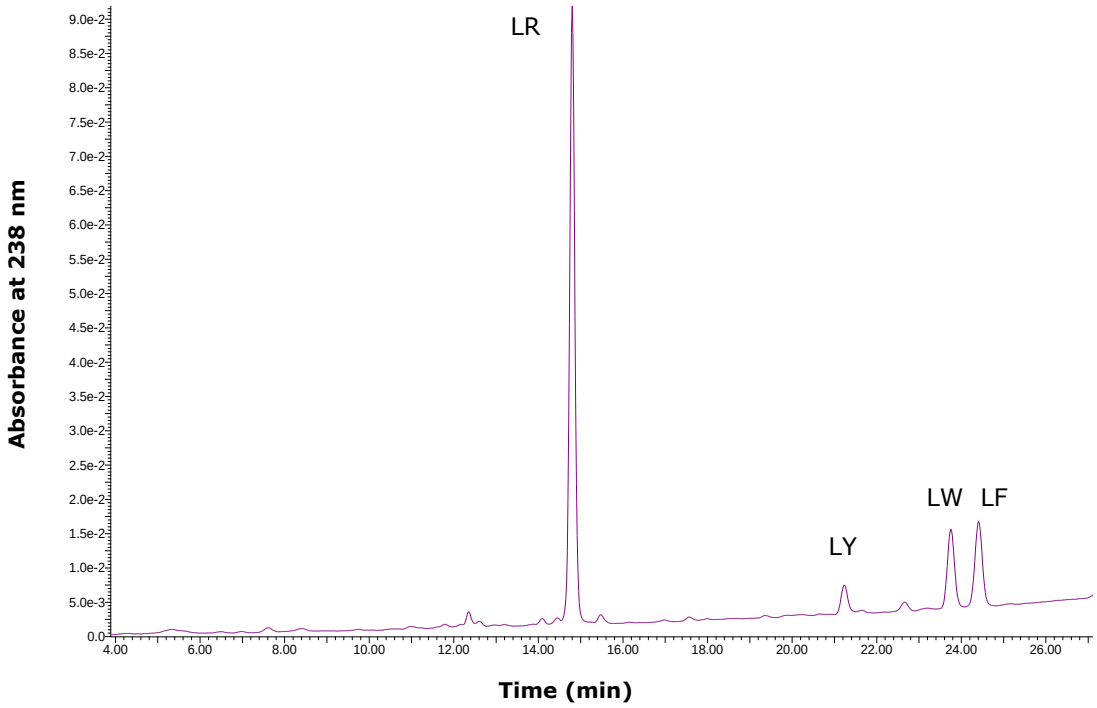

Figure 2.3 Aqueous methanolic extract of *Microcystis aeruginosa* PCC 7820.

Table 2.3 Recovery of MC variants during methanol extraction of *Microcystis aeruginosa* PCC 7820.

MC variant	1st extraction (mg), % recovery*	2nd extraction (mg), % recovery*	3rd extraction (mg), % recovery*	Total amount extracted (mg)
MC-LR	130 (76)	35 (21)	5 (3)	170
MC-LY	11 (92)	1 (8)	0	12
MC-LW	20 (77)	6 (23)	0	26
MC-LF	15 (79)	4 (21)	0	19

* Percentage recovery is shown in brackets

2.3.2 Sample concentration and clean up – RP flash chromatography

Methanolic extraction of *Microcystis aeruginosa* PCC 7820 revealed four major MC variants, MC-LR, -LY, -LW and –LF (figure 2.3). Prior to the flash chromatography, the extracted material was diluted with Milli-Q water to facilitate retention of cyanotoxins in the C18 cartridge (section 2.2.5). Preconditioning of the cartridge with methanol and water resulted in activating the packing material to retain the cyanobacterial secondary metabolites in the cartridge. Eluent waste passed through the C18 cartridge was analysed by PDA-LC-MS and revealed no breakthrough of MCs following sample loading. Compounds retained in the C18 cartridge were eluted in a gradient of methanol according to the conditions described in section 2.2.5. Fractions eluted from 10% to 20% methanol contained no MCs (table 2.4). MC-LR, generally the most abundant MC variant in *Microcystis aeruginosa* PCC 7820, was eluted in a range of different methanol concentrations (30-60%; figure 2.4). The fraction eluted with 30% of methanol contained 6 mg MC-LR of 47% purity (table 2.4). Fractions with 40% to 50% methanol exclusively contained MC-LR with a purity ≥ 90%. A quantity of 37 mg of MC-LR was eluted in 40% methanol. The fractions eluted with 50% methanol (table 2.4) contained 92 mg of MC-LR at a purity of 90%. This was recorded as the highest amount of MC to elute in a fraction during the RP flash chromatography. MC-LY was found in 30% (3 mg) and 60% (4 mg) methanol fractions. However, the purity of the both fractions indicated comparatively low values (13% and 5% respectively; table 2.4). Mixture of the MCs was collected in 60% methanol fraction. MC-LW was found to be the predominant compound (15 mg) with 10 mg of MC-LF. However, the purity of the two compounds, MC-LW and -LF was

relatively low (21% and 18% respectively; table 2.4). Interestingly, MC-LR was still eluting at 60 % which finally provided an amount of 30 mg, but indicated a slightly low purity (72%). MC-LW (7 mg) and –LF (3 mg) continued to elute up to 70% solvent concentration due to their high hydrophobicity compared to other variants (figure 2.4; table 2.4). The 70% fraction also eluted uncharacterised cyanobacterial peptides. As expected, fractions eluted with 80-100% of methanol contained no MC compounds (table 2.4). Fractions of similar composition were pooled to give two major fractions prior to the preparative HPLC injection. Flash fractions containing mainly MC-LR (30-50%) were pooled to give LR pool whilst, more hydrophobic MCs (MC-LW and -LF) contained in the LW-LF pool (fractions 60-70%).

Table 2.4 Quantity and purity of MCs resulted in RP flash chromatography

Methanol fraction (%)	**MC variant**	**MC quantity (mg)**	**% Purity**
10-20	-	-	-
30	MC-LR	6	47
	MC-LY	3	13
40	MC-LR	37	92
50	MC-LR	92	90
60	MC-LR	30	27
	MC-LY	4	5
	MC-LW	15	21
	MC-LF	10	18
70	MC-LW	7	12
	MC-LF	3	5
80-100	-	-	-

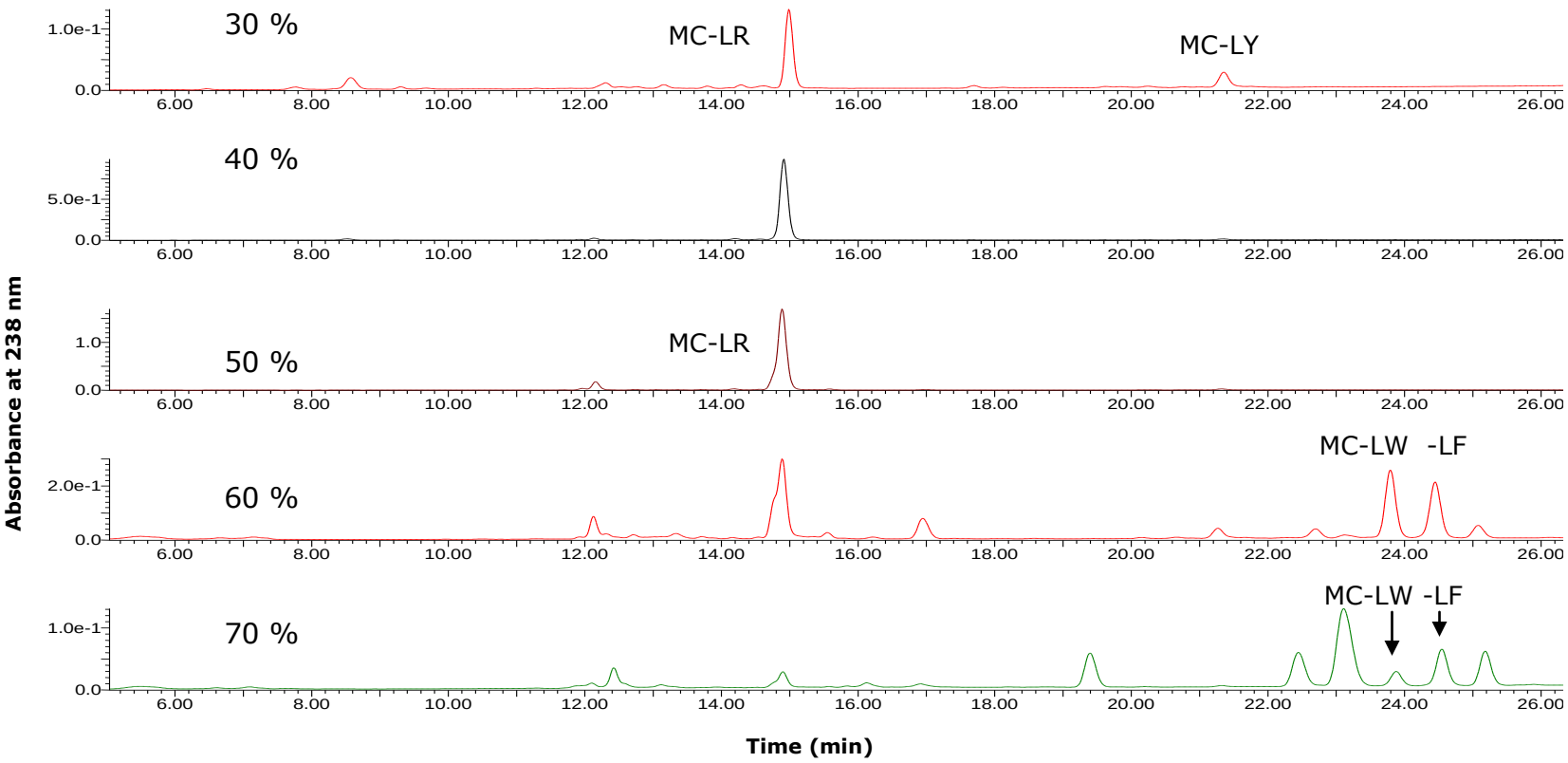

Figure 2.4 PDA-LC-MS chromatogram of different variants of MC from 10% gradient elution.

2.3.3 Purification of cyanotoxins - preparative HPLC

Previous step gradient of RP flash chromatography resulted in 240 ml methanol fractions containing MC-LR, -LY, -LW and -LF of which a further purification was required. Further separation and purification of MCs were achieved by preparative HPLC. Four MC variants were separated into single compounds from both LR pool (30-50%) and LW-LF pool (60-70%) to obtain a purity exceeding 95% (table 2.5). LR pool contained two MC variants, MC-LR and –LY which was finally separated into their single compounds. Purification of both variants, MC-LR and -LY resulted in 99% and 98% pure compounds respectively (table 2.5; figure 2.5). PDA-LC-MS indicated 133 mg of MC-LR and 3 mg of MC-LY separated from LR pool. Separation of the LW-LF pool (appendix A) finally resulted in preparative HPLC fractions containing 97% pure MC-LW (21 mg) and -LF (12 mg) (table 2.5; figure 2.5). MC-LR and -LY which had eluted in 60-70% fractions in the RP flash chromatography was separated as 28 mg of MC-LR and 3.5 mg of MC-LY (table 2.5; figure 2.5).

Table 2.5 Different variants of MCs separated and purified by preparative HPLC

Fractions from RP flash	MC variant	Quantity (mg)	Purity (%)
LR pool (30-50%)	MC-LR	133	99
	MC-LY	3	98
LW-LF pool (60-70%)	MC-LR	28	99
	MC-LY	3.5	98
	MC-LW	21	97
	MC-LF	12	97

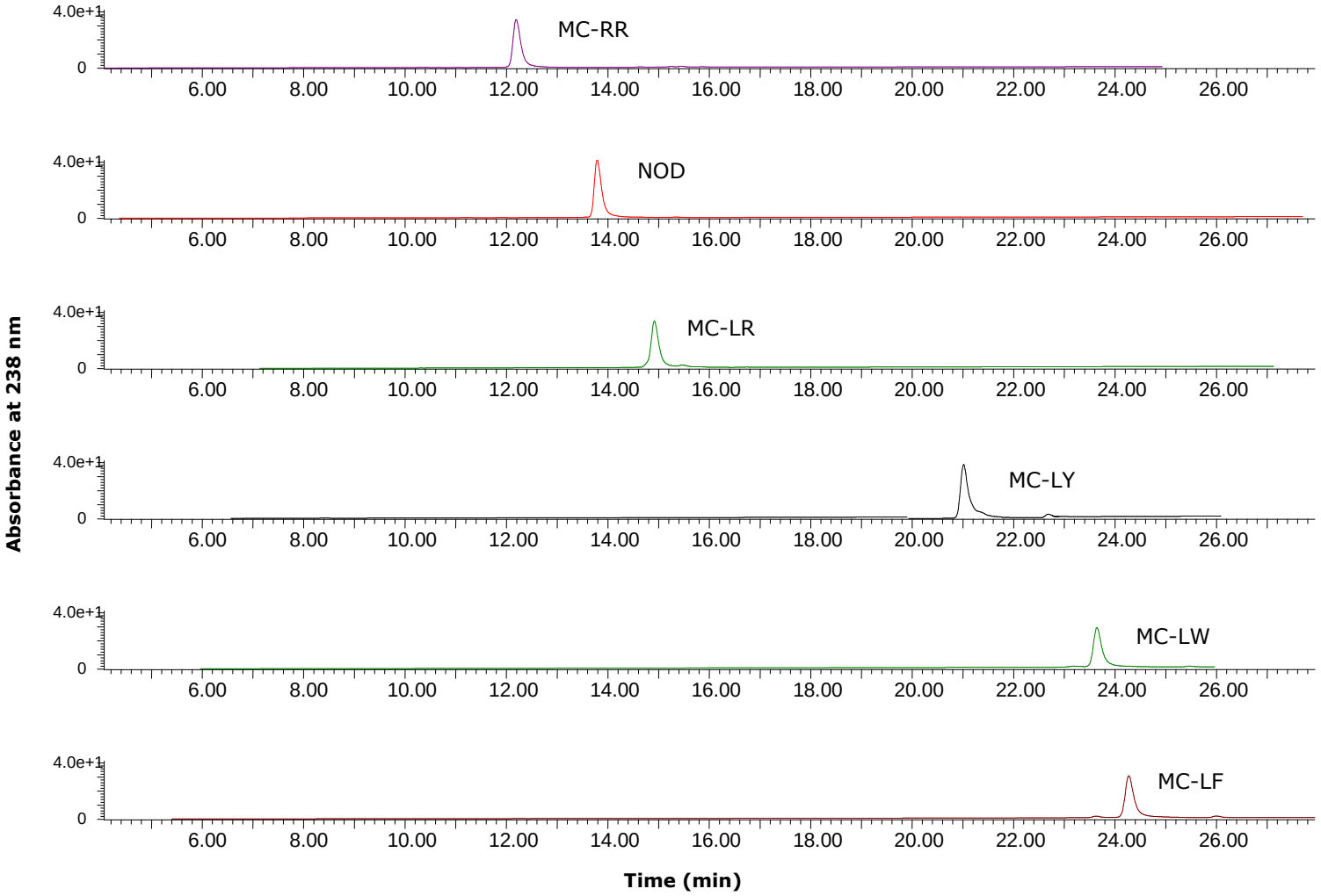

Figure 2.5 PDA-LC-MC chromatograms of different MCs purified by preparative HPLC with relative purity > 95%

2.3.4 Desalting and drying of cyanotoxins

Preparative HPLC fractions containing MCs with a percentage purity of 95% or higher were pooled, desalted and dried. Final purity of all MCs was determined to be ≥ 95% (figure 2.6; appendix C). When the samples were dried under gaseous flow of nitrogen, a white powder remained, confirming that the cyanotoxins were of high purity with no pigmented impurities. The yield at this purity for all purified material was determined by gravimetric analysis. As observed in previous steps of purification, MC-LR predominantly resulted in 125 mg of pure dried powder (table 2.6). MC-LY, having a comparatively low quantity during the extraction and purification, gave a final yield of 4.5 mg (table 2.6; appendix B). The two hydrophobic, closely eluting MC variants, MC-LW and –LF were dried as two single compounds (appendix B) with a quantity of 14 mg and 5.5 mg respectively (table 2.6).

Table 2.6 Table of summary of the purification process

Compound	Initial amount extracted (mg)	Quantity purified from prep. HPLC (mg)	Desalted amount (mg)	% yield
MC-LR	170	161	125	74
MC-LY	12	6.5	4.5	38
MC-LF	26	21	14	54
MC-LW	19	12	5.5	29

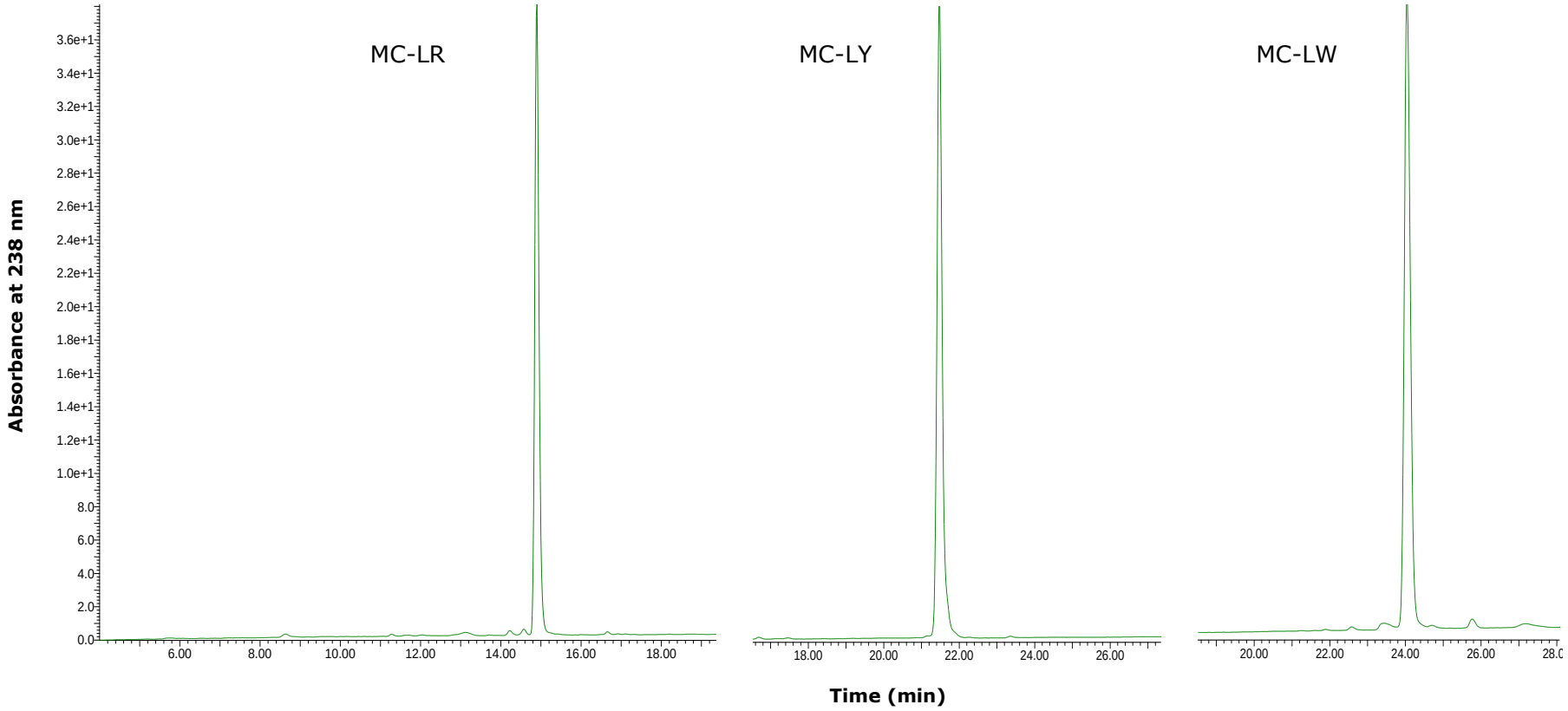

Figure 2.6 UV chromatograms extracted at 238 nm of desalted MC-LR, -LY and -LF with relative purity > 95%.

2.3.5 Preparation of calibration curves for purified cyanotoxins

Calibration curves of purified MCs were prepared as the concentration versus peak area response by PDA-LC-MS. UV-PDA chromatograms were extracted at their highest absorption wave lengths (238 nm). ESI-MS detector range was set from 100 to 1600 Da and the chromatograms were extracted using mass of the parent ion (*m/z*; table 2.7). All purified MCs demonstrated a linear response at UV-238 nm (figure 2.7; appendix C) and maintained a correlation coefficient (R^2) greater than 0.99 on their linear regression (table 2.8). However, compared to the purity determined by UV, all MC variants indicated slightly low percentage purity at ESI-MS (table 2.7). Consequently, their linear response was comparatively low to that of UV. The highest correlation coefficient R^2 = 0.988 at ESI-MS was reported from MC-LF while MC-LY gave the lowest at R^2 = 0.975 (table 2.8).

Table 2.7 Percentage purity determined for purified MCs by UV-PDA and ESI-MS

Compound	Assessed purity by UV-PDA (%)	Assessed purity by ESI-MS (100-1600 Da) (%)	Structure of the fragment	Observed *m/z*
MC-LR	99	96	$[M+H]^+$	996
MC-LY	98	94	$[M+Na]^+$	1024
MC-LF	97	95	$[M+Na]^+$	1009
MC-LW	97	95	$[M+Na]^+$	1048

Table 2.8 Correlation coefficient (R^2) of different peptides

Compound	R^2 at UV-PDA	R^2 at ESI-MS
MC-LR	0.999	0.980
MC-LY	0.999	0.975
MC-LF	0.998	0.988
MC-LW	0.999	0.978

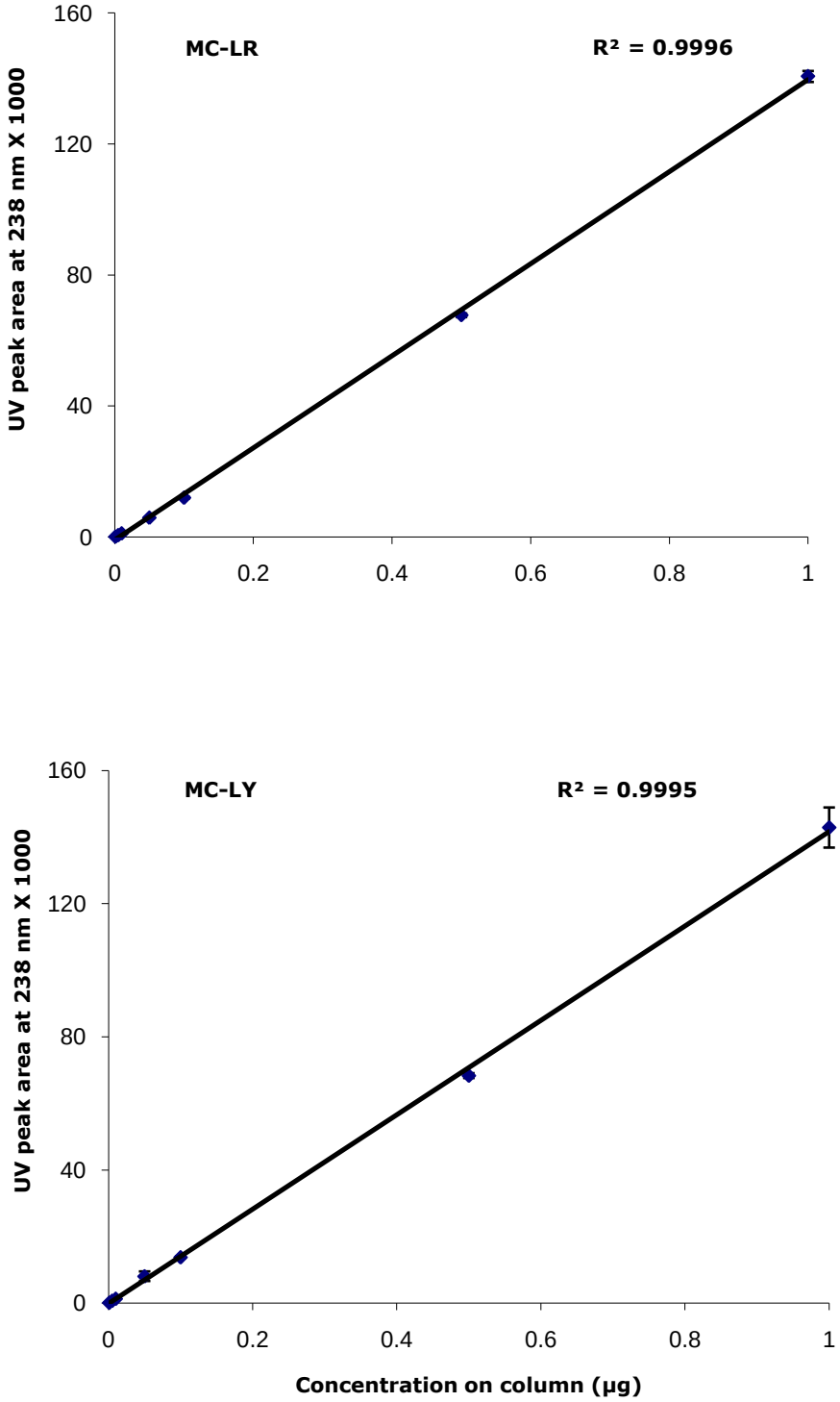

Figure 2.7 Calibration curves of purified MC-LR and MC-LY by UV-PDA (238 nm). Error bars represent one standard deviation (n=3).

2.3.6 Analysis of other cyanotoxins

MC-RR, NOD, LNOD and ABPNB (table 2.1) were purified as previously described in sections 2.2.4-2.2.6 and their analysis was performed by PDA-LC-MS (appendices A, B and C). Calibration curves prepared for MC-RR, NOD, LNOD and ABPNB demonstrated a linear response at UV-PDA and maintained a correlation coefficient (R^2) greater than 0.99 on their linear regression (appendix E).

2.4 Discussion

This chapter has described an effective purification of a wide range of cyanotoxins using RP flash chromatography and HPLC based cyanotoxin purification methods presented by Edwards *et al*., (1996) and Edwards and Lawton, (2010). Purification of the cyanotoxins from the laboratory-grown cyanobacterial cultures contained; extraction of the toxins, concentration and clean up of the extracts and purification and the quality control of the cyanotoxins have been explained.

Extraction of intracellularly produced cyanobacterial secondary metabolites is a crucial step in cyanotoxin purification. Quality and quantity of the isolated cyanobacterial secondary metabolites rely on the mode of their extraction. It is therefore important to employ a reliable and efficient procedure to extract cyanotoxins and provide sufficient material for further purification. To achieve this, various studies have developed different methods for extraction of cyanotoxins. However, the majority of the procedures failed not only to recover a sufficient yield but also to extract hydrophobic MC variants such as MC-LW and -LF (Botes *et al*., 1982; Grabow *et al*., 1982; Harada *et al*., 1988). In contrast, aqueous methanolic extract of *Microcystis aeruginosa* PCC 7820 (Lawton *et al*., 1995; Edwards *et al*., 1996) prepared for this study has successfully resulted in MC-LR, -LY and the hydrophobic MC-LW and –LF (figure 2.3). Using large volumes of methanol and water by the dilution and re-extraction of the cyanobacterial cells resulted in low concentration of cyanotoxins in the large volume of medium (~ 8 l). To overcome this, one of the commonly used approaches was rotary evaporation (Lawton *et al*., 1994).

However, rotary evaporation of large volumes of media is time consuming and impractical. The current study required purifying eight different cyanotoxins in high quantities (> 3 mg); thus, a rapid and reliable concentration and cleaning up procedure was desirable.

Concentration of the samples on C18 flash cartridge followed step gradient (10%) elution in 0-100% aqueous methanol (v/v) facilitated a simple and effective method for the separation and partial purification of these cyanotoxins (Edwards *et al*., 1996). Fractions eluted with 10–20% of methanol contained no MCs indicating a higher percentage of solvent concentration required for the elution of MCs (table 2.4). MC-LR, generally the most available MC variant in *Microcystis aeruginosa* PCC 7820, was eluted in a range of different methanol concentrations from 30% to 60% and contained 165 mg. The two closely eluting MC variants, MC-LW and –LF typically retains long due to their affinities to the sorbent (figure 2.4). Hence, the elution starts only at higher concentrations of methanol (60%; figure 2.4; table 2.4). Uncharacterized peaks detected in 70% MeOH fraction could be implied to different unidentified cyanobacterial peptides or peptide degradation products formed during the extraction and purification procedure. It was interesting to note a loss of MC-LR during the sample concentration and gradient elution of reverse phase chromatography. HPLC analysis of methanol extraction provided a total quantity of 170 mg of MC-LR (table 2.3). However, the RP flash chromatography has resulted in 165 mg of MC-LR (table 2.4) indicating a loss of 5 mg during the process. It is probable that some of the MC-LR remained irreversibly bound in the C18 column, due to the presence of free silanol groups which provide extremely strong sorbent sites (Thurman and Mills,

1998). Furthermore, cyanotoxins may be subjected to degradation as a result of reactions with wash solvents, i.e. MeOH.

The purity of MC in the methanol fraction was shown to be affected by the presence of multiple MC variants and uncharacterised cyanobacterial intracellular material. Fractions of 40 % and 50% exclusively contained MC-LR indicating almost no further purification needed. However, fractions eluted with 30%, 60% and 70% MeOH contained multiple MCs and few uncharacterised impurities. Thus, step gradient eluted fractions were further purified and separated in to single cyanotoxins using semi-preparative HPLC (Edwards *et al*., 1996; Edwards and Lawton, 2010). MC-LR was easily purified at the preparative scale and contained 99% pure fractions sufficient (161 mg) for the degradation studies described in chapters 3, 4 and 5. Purification of MC-LW and -LF can be associated with difficulties in separation of the two compounds due to its closely eluting behaviour (Lawton *et al*., 1994). However, in this study, using the step gradient elution (Edwards and Lawton, 2010), an initial separation of MC-LW and -LF was achieved during the sample concentration step (figure 2.4). Subsequently, it was successfully separated as single MC variants over 95% relative purity using the polarity guided intelligent separation available through preparative HPLC (figure 2.5; table 2.5).

There are a few points worthy of comment about the purification of LNOD and ABPNB. To date, purification of MCs and NOD has been widely studied and considerable development of their identification and purification has been established (Botes *et al*., 1982; Poon *et al*., 1987; Harada *et al*., 1988; Lawton *et al*., 1995; Edwards *et al*., 1996; Diehnelt *et al*., 2006). However, it was

interesting to note only a little interest on the purification of anabaenopeptins (ABPN) and LNOD so far. Since Harada *et al*., (1995) reported the isolation and structural determination of anabaenopeptin A (ABPNA) and ABPNB, many researchers have reported its detection and identification to date (Fujii *et al*., 1997; Murakami *et al*., 1997; Shin *et al*., 1997; Fastner *et al*., 2001; Welker *et al*., 2004). In addition to that, Morrison *et al*., (2006) reported the methanolic extraction and preparative HPLC purification of ABPNA and ABPNB using *Anabaena* NRC 525-17. However, in this study, the HPLC methods described by Edwards *et al*., (1996) and Edwards and Lawton, (2010) were successfully adapted to extract and purify ABPNB from the cells of *Microcystis aeruginosa* Sciento (appendix A).

The other compound of interest was LNOD, the precursor of cyclic NOD (Rinehart *et al*., 1994). To date, there is very little information available regarding the extraction and purification procedure for LNOD. Mazur-Marzec *et al*., (2006) presented the characterisation of different variants of NOD, which also described the characterisation of LNOD. However, it was noted that LNOD was extracted according to Lawton *et al*., (1994); in similar manner as described in the current study (sections 2.2.4-2.2.6). It is probable that less availability of LNOD in cyanobacteria has hindered studies related to its purification. Furthermore, LNOD may be less stable for purification due to its linear structure compared to NOD. However, the purification methods described here enables effective purification of LNOD, ABPNB as well as a wide range of chemically different MCs and NOD (appendices A, B and C).

2.5 Conclusion

This chapter has described an inclusive methodology for laboratory scale culturing and maintenance of several strains of cyanobacteria. Extraction was required to isolate the cyanotoxins since they were produced intracellularly. Since HPLC methods described by Edwards *et al*., (1996) with flash chromatography, preparative HPLC, UV and MS for the determination of MCs and NOD are accurate, sensitive and provide highly reliable quantitative results, these techniques were used as reference methods.

The difficulties associated with the separation of closely eluting MC-LW and –LF was overcome successfully using the RP flash chromatography and the preparative HPLC methods (Edwards and Lawton, 2010). Although MCs and NOD have been widely purified, little information was available for the purification of ABPNB and LNOD. However, it was successfully demonstrated that not only MCs and NOD, but LNOD and ABPNB were also successfully purified by the HPLC methods described by Edwards *et al*., (1996). Finally, all purified cyanotoxins demonstrated a relative purity exceeding ≥ 95% and a linear response in the calibration curves. Thus, all of the purified cyanotoxins were satisfactory for future use in biodegradation studies.

CHAPTER 3

BACTERIAL METABOLISM AND BIODEGRADATION OF MCS AND NOD

TABLE OF CONTENTS

3 Bacterial metabolism and biodegradation of MCs and NOD

3.1 Introduction

The occurrence and the persistence of MCs and NOD, in both natural and man-made water bodies have been recorded in many regions all over the world (Falconer and Humpage, 2005). As a result, the cases of deaths and illnesses of wild animals, livestock and humans due to the ingestion of cyanotoxin (MCs and NOD) contaminated water, have significantly increased over the past few decades (Bell and Codd, 1994; Cousins *et al*., 1996; Jochimsen *et al*., 1998; Hirooka *et al*., 1999; Baldia *et al*., 2003; Ishii *et al*., 2004). One of the most highlighted incidents of human poisoning of MCs was the death of 53 patients at a haemodialysis centre in Brazil, in 1996. It was later revealed, that the water used for dialysis had been contaminated with MC-LR (Jochimsen *et al*., 1998; Hirooka *et al*., 1999). Furthermore, the potential chronic toxicity associated with MCs, has led the WHO to establish a provisional guideline value for the concentration in drinking water of MC-LR (1 μg l^{-1}; WHO, 1998).

Effective removal of MCs from drinking water is a major goal for all water utilities (Ho *et al*., 2007). However, due to the chemically stable cyclic structures of MCs and NOD (Watanabe *et al*., 1992), many conventional water treatment methods such as coagulation, flocculation and filtration have shown limited effect on their removal from potable water (Himberg *et al*., 1989). For instance, Keijola *et al*., (1988), reported poor or no removal of MCs when flocculation filtration-chlorination and flocculation-filtration were used. On the other hand, water treatment technologies such as granular activated carbon, powdered activated carbon, and membrane filtering approaches have shown promising results for the removal of MCs (Lawton and Robertson, 1999;

Schmidt *et al*., 2002). However, these technologies require expensive facilities, making them difficult to implement and maintain (Eleuterio and Batista, 2010). Moreover, there are also concerns about their efficiency under high carbon loads, in cases where highly eutrophic water is used (Edwards *et al*., 2008). Overall, unaffordable water treatment costs, water scarcity and the world's increasing population have set a global challenge: The need for a simple, reliable and cost effective approach to remove these contaminants ensuring safe drinking water.

One of the most exciting and promising areas is the biodegradation of MCs by heterotrophic bacteria (Berg *et al*., 1987; Watanabe *et al*., 1992; Kenefick *et al*., 1993; Jones and Orr, 1994; Jones *et al*., 1994; Cousins *et al*., 1996; Holst *et al*., 2003). Further advances in this approach include the use of MC degraders in slow sand filters and biofilms to remove MCs from potable water (Babica *et al*., 2005; Bourne *et al*., 2006; Ho *et al*., 2006; Tsuji *et al*., 2006). However, it is evident, that a successful bioremediative water treatment against MCs relies highly on the potency of MC degrading bacteria to degrade a wide range of chemically different MCs and NOD. To date, many studies have been carried out exploiting naturally occurring heterotrophic bacteria capable of degrading MCs into non-toxic compounds (Bourne *et al*., 1996, 2001; Lathi *et al.,* 1998; Harada *et al*., 2004; Ishii *et al*., 2004). However, only a few studies, so far, have successfully isolated bacteria capable of degrading several variants of MCs. In the majority of the studies, the MC degrading bacteria were isolated only from the water sources exposed to MCs, restricting the diversity of the MC degraders (Edwards *et al*., 2008). Further concerns regarding the low number of bacteria include the use of traditional

microbiological techniques where the majority of the aquatic bacteria including MC degraders may be presented as non-culturable under laboratory conditions. This was clearly revealed by the study of Lathi *et al*., (1998) where a total of 100 bacterial strains were isolated from lake water and sediments, 17 were capable of degrading MC and only three were able to degrade NOD. Not surprisingly, only a single biodegradation pathway has been characterised to date (Bourne *et al*., 1996, 2001) while many may yet remain to be identified (Edwards *et al*., 2008).

In the quest of elucidating novel MC degraders, more than 15 bacterial strains have been isolated and characterised so far. Jones *et al*., (1994) successfully isolated the first MC degrading bacterium, which was later characterised by Bourne *et al*., (2001) as *Sphingomonas* sp. ACM 3962. Saitou *et al*., (2003) have isolated two bacterial strains; *Novosphingobium* sp. MD-1 and *Sphingosinicella microcystinivorans* Y2 capable of degrading several variants of MCs such as MC-LR, -YR, and -RR. In later studies, Harada *et al*., (2004) and Ishii *et al*., (2004) identified and characterised two bacterial strains from the genus of *Sphingomonas*. Until Rapala *et al*., (2005) isolated and characterised the MC degrading bacterium *Paucibacter toxinivorans*; the majority of the isolated bacteria were either *Sphingomonas*-like or belonged to the genus *Sphingomonas.* Furthermore, recent studies (Fujimoto *et al*., 2007; Ho *et al*., 2007; Okano *et al*., 2009; Zhang *et al*., 2010) have described five more different bacteria from the same genus of *Sphingopyxis,* whilst Chen *et al*., (2010) isolated *Stenotrophomonas* sp. degrading MC-LR and -RR. It has also been proven that probiotic bacteria such as *Lactobacillus rhamnosus* can be effective in removal of MCs (Nybom *et al*., 2008). Shimizu *et al*., (2011), in a

recent review reported that the MC degrading bacteria present great similarities in their phylogeny, implying that all the MCs degraders evolved from *Proteobacteria* and share the common physio-chemical features as Gram-negative bacteria. Also, the degradation of MCs has been solely limited to Gram-negative *Proteobacteria* until a recent study in our laboratory resulted in isolation and characterisation of novel MC degraders belonging to *Actinobacteria* (Manage *et al*., 2009). Unlike the *Proteobacteria*, all the *Actinobacteria* isolates namely, *Arthrobacter*, *Brevibacterium* and *Rhodococcus* were Gram-positive (Manage *et al*., 2009). These bacteria have been isolated from three Scottish water bodies, previously shown to contain a rich microflora of MCs and NOD degraders (Edwards *et al*., 2008). Unlike the traditional screening methods, the researchers rapidly screened 31 fresh water bacterial isolates for utilisation of MC-LR, by use of the Biolog MT2 plates. After screening, 10 bacterial isolates were found to metabolise MC-LR and had proven to degrade MC-LR in a subsequent batch study where MC-LR was completely degraded by all isolates after 72 h (Manage *et al*., 2009). Amongst the 10 bacterial isolates shown to degrade MC-LR, four *Arthrobacter* sp. were isolated from Loch Rescobie, which is known to produce annual cyanobacterial blooms with MCs (Richard *et al*., 1983). Two *Arthrobacter* sp. and one *Brevibacterium* sp. have been isolated (Manage *et al*., 2009) from Forfar Loch, a eutrophic water body which has no record of MCs (Edwards *et al*., 2008). Two *Rhodococcus* sp. and one *Arthrobacter* sp. were isolated from fast flowing Carron river. Surprisingly, the isolates indicated a rapid degradation of MC-LR (Manage *et al*., 2009), despite no previous exposure to any blooms or MCs (Edwards *et al*., 2008). This fact implies a more widespread existence of MC degraders.

Due to MCs chemically diverse occurrence and abundance in nature, it is important that a MC degrading candidate is capable of degrading a wide range of multiple MCs and NOD. Consequently, this study has been carried out to further evaluate the biodegradation potential of novel MC degraders (Manage *et al*., 2009) against a wide range of MCs and NOD. To achieve this, the Biolog MT2 assay (Rapala *et al*., 2005; Manage *et al*., 2009) was implemented as a rapid tool for examining bacterial strains with a range of MC variants and NOD along with different toxin concentrations. To support the bacteria metabolism in Biolog MT2 assay, actual degradation potency of the bacteria were assessed and confirmed in a typical batch study. Further evaluations were carried out in order to unravel the bacterial MC degradation dynamics under different environmental challenges (different nutrient composition, different synthetic media, different carbon supplements) with a view to establishing the suitability of these organisms for the removal of MCs from drinking water.

3.2 Materials and methods

3.2.1 Chemicals

Chemicals were of analytical-reagent grade unless stated and obtained from Fisher Scientific, Leicestershire, UK. HPLC-grade methanol and acetonitrile were purchased from Rathburn, Walkersburn, UK. Trifluoroacetic acid (TFA) for HPLC systems was purchased from Fisher Scientific, Leicestershire, UK. Pure water was obtained from a Milli-Q system (purified to 18.2 MΩ, Millipore, Watford, UK). MC (-LR, -LY, -LW, -LF and -RR) and NOD standards were purified from batch cultures of *Microcystis aeruginosa* and *Nodularia spumigena* KAC66 respectively (Edwards *et al*., 1996) as described in chapter 2. Cyanotoxins were dissolved in Milli-Q and sterilised in a passage through 0.22 µm filters (Dynaguard filter, Fisher, UK).

3.2.2 Bacterial strains

Bacterial strains used in the following study are listed in table 2.1 (Manage *et al*., 2009). *Paucibacter toxinivorans* (DSMZ-16998) from Braunschweig Germany was isolated and identified by Rapala *et al*., (2005).

Table 3.1 Identification and origin of bacterial isolates screened for MC-LR metabolism and degradation (Manage *et al*., 2009).

Bacterial isolate	Identification	Origin of isolation	Reference
C1	*Rhodococcus* sp.	River Carron (NO 877857)	Manage *et al*., (2009), Lawton *et al*., (2011)
C3	*Rhodococcus* sp.		
C6	*Arthrobacter* sp.		
F3	*Brevibacterium* sp.	Forfar Loch (NO 293458)	Manage *et al*., (2009), Lawton *et al*., (2011)
F7	*Arthrobacter* sp.		
F10	*Arthrobacter* sp.		
R1	*Arthrobacter* sp.	Loch Rescobie (NO 52505159)	Manage *et al*., (2009), Lawton *et al*., (2011)
R4	*Arthrobacter* sp.		
R6	*Arthrobacter* sp.		
R9	*Arthrobacter* sp.		

3.2.3 Sub-culturing and long-term storage of bacterial isolates

3.2.3.1 Regular processing and sub-culturing of bacteria

All of the bacterial strains (table 3.1) underwent routine culturing for the maintenance of the bacteria for current research work. Bacteria were cultured by inoculating a loop-full of each isolate in 5 ml sterile nutrient broth (NB) (Oxoid, Ltd., Hants, UK) with overnight incubation (25 ºC, 150 rpm). The exponentially growing cultures were streaked on sterile nutrient agar (NA) (Oxoid, Ltd., Hants, UK) slopes under aseptic conditions prior to the incubation at 25 ºC.

3.2.3.2 Long-term storage of the bacteria

For the long-term storage of the isolated bacterial strains, 1 ml of overnight grown bacterial culture (log phase) was centrifuged (10000 g, 25 ºC) and 900 µl supernatant was removed. Bacterial pellets (100 µl) were introduced into the screw capped cryoprotection vials according to the manufacturer's instructions (Technical Service Consultants Ltd, Lancashire UK) with immediate storage (-80 ºC). Depending on the requirements, bacteria were re-grown in NB under aseptic conditions.

3.2.4 Gram stain of bacteria

Bacterial isolates (table 3.1) were stained by the basic Gram method described by Valle *et al*., (1999). A thin smear of bacteria was created by placing a drop of sterile Ringer's solution and spreading the bacterial culture in thin film over slide. The slide was pre-cleaned by washing in 95% ethanol to remove residual particles. The bacterial smear was air dried for 3 min and heat fixed passing over a Bunsen flame. The smear was stained by flooding the surface with crystal violet solution for 1 min, washing quickly with Milli-Q water, flooding with iodine solution for 1 min. The slide was washed with running Milli-Q water over the bacterial smear and decolourised with 95% ethanol for 30 sec (ethanol was added drop wise to avoid over decolourisation). The smear was washed with Milli-Q water to remove remaining ethanol over the side and counterstained with safranin for 1 min.

Finally, the slide was washed with Milli-Q water and blotted dry for examination. A single slide was prepared for each different individual bacterial

isolate (table 3.1) and analysed using an optical microscope with a Zeiss 100/1.25 oil 160-objective lens (Carl Zeiss, Germany) under a total magnification of ×1000 on immersed oil.

3.2.5 Evaluation of MC-LR utilisation by isolated bacteria

3.2.5.1 Preparation of bacterial inocula

Bacterial isolates (loop-full; table 3.1) were transferred into 8 ml of liquid NB and incubated in a shaker (25 °C, 24 h, 150 rpm; Stuart scientific- Orbital incubator SI 50). *Paucibacter toxinivorans* DSMZ-16998 (Braunschweig, Germany) was used as the positive control bacterial strain as it has been found to degrade MC-LR, -YR and NOD (Rapala *et al*., 2005). The exponentially growing bacterial cultures were washed three times (x3) with an equal volume of 0.01 M Phosphate buffered Saline (PBS) by centrifugation at 3000 g for 15 min with re-suspension of the pellet in sterile 0.01 M PBS. All the bacterial cultures were incubated for a further 24 h at 25 °C to deplete residual carbon. Optical density (OD) of the cell suspensions were adjusted to $OD_{590\ nm}$ of 0.35 using spectrophotometer (Pharmacia biotech Nova Spec II) by adding overnight grown bacterial cultures or by diluting with sterile 0.01 M PBS.

3.2.5.2 Bacterial metabolism of MC-LR

Sterile aqueous MC-LR was added to the Biolog MT2 plates (Technopath, Limerick, Ireland) in triplicates (10 µl) at final concentrations of 0.1, 1.0, 10 µg ml^{-1}. Figure 3.1 shows the MT2 plate layout containing different concentrations of MC-LR. An inoculum of 150 µl OD adjusted bacterial

suspension was inoculated in MT2 plates. The control wells in the Biolog MT2 plate contained bacterial isolates and sterile PBS in triplicates. The colour development in the plate was measured by a Dynex microplate reader (Jencons, Leighton Buzzard, UK) at a wavelength of 595 nm immediately after the inoculation of bacteria (0 h) followed by 3, 6, 12, 18, 24, 36 and 48 h. The oxidisation of the analyte (MC-LR) would result in a colour reaction taking place in the well by the reduction of tetrazolium dye which can be measured and quantified spectroscopically (Garland and Mills, 1991).

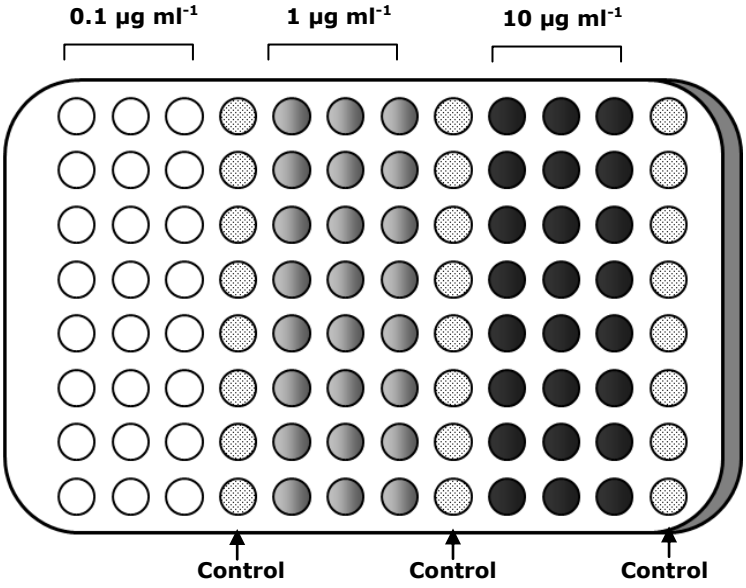

Figure 3.1 Biolog MT2 plate layout for the evaluation of metabolism of MC-LR by *Actinobacteria* isolates.

3.2.6 Evaluation of the bacterial metabolism of multiple MCs and NOD

Previous study (section 3.2.5) has confirmed that all of the bacterial isolates were still capable of utilising MC-LR as a sole carbon source. To allow the evaluation of the metabolism of a range of MCs (MC-RR, -LR, -LY, -LF and -LW) and NOD, five bacterial isolates (*Rhodococcus* sp. C1, *Arthrobacter* sp. C6, F7 and R4, *Brevibacterium* sp. F3) representing the three different genera, different sources of origin and with high utilisation of MC-LR (section 3.2.5) were selected. Bacteria were cultured and processed as previously described in section 3.2.5.1. The Biolog MT2 assay was carried out and the bacterial metabolism of MCs and NOD was evaluated as described in section 3.2.5.2. *P. toxinivorans* was used as the positive control (Rapala *et al*., 2005). According the previous data (section 3.2.5.2), little or no notable increase in absorption levels (bacterial metabolism) had been observed after 24 h of incubation in MT2 plates. Therefore, in this study, the absorption difference was considered representing the difference of the degree absorption at 24 h incubation period to that of 0 h.

3.2.7 Biodegradation of multiple MCs and NOD

Rhodococcus sp. (C1), *Arthrobacter* sp. (C6, F7 and R4), *Brevibacterium* sp. (F3) and *P. toxinivorans* were grown overnight (25 °C, 150 rpm) in liquid NB. Bacterial inocula were prepared as previously described (section 3.2.5.1). Aliquots of 0.5 ml bacterial suspension were added to sterile glass universal bottles containing 9 ml sterile water (0.22 µm filter, Millipore, UK) from their original location (table 3.1; collected in February 2009). Aqueous, 0.5 ml MC-RR, -LR, -LY, -LF, -LW and NOD were added to each bottle under aseptic condition at a final concentration of 10 µg ml^{-1}. Triplicate samples were

prepared for each isolate and incubated at 25 °C and shaken at 150 rpm. *P. toxinivorans* was processed similarly and inoculated in Loch Rescobie water. At 24 h intervals, aliquots of 0.5 ml were removed into microcentrifuge tubes (1.5 ml) under sterile conditions, frozen (-20 °C) immediately and freeze dried. Samples for PDA-LC-MS analysis were prepared from freeze dried samples, reconstituted in 200 µl of 50% (v/v) aqueous methanol and centrifuged at 15000 g for 10 min, room temperature (RT) and the supernatant (100 µl) was removed for PDA-LC-MS analysis (chapter 2; section 2.2.8). The half-life ($D_{1/2}$) of all tested MCs was calculated as the length of time taken for removal of 50% of the compound. Controls were performed containing the five MCs and NOD in sterile water from the three sources but in the absence of bacteria and sampled as described to confirm the loss of toxin is a result of microbial activity. Prior to the experiments all of the water samples collected from three different locations were analysed by PDA-LC-MS to ensure that no MCs or NOD were present in the original water samples. Thus, confirming the toxins in the sample bottles were only those added for experimental purposes.

3.2.8 Seasonal dynamics of nutrients in Carron river and biodegradation of MC-LR

3.2.8.1 Degradation of MC-LR in river water

Based on the results from the batch experiment, *Rhodococcus* sp. (C1) was selected to perform a comparative study of biodegradation of MC-LR in Carron river water collected at the end of winter period (February 2009) and mid-summer (July 2009). This was to determine whether different seasonal

nutrient status of the water influences degradation rates of MC-LR. Bacteria were cultured in liquid NB (25 °C, 24 h, 150 rpm) and the inocula were prepared as described above (section 3.2.5.1). An aliquot of 0.5 ml bacterial suspension ($OD_{595\ nm}$) was inoculated in sterile universal bottles (x3) containing 9 ml of 0.2 µm filter sterilised Carron river water collected in February or July 2009. MC-LR (0.5 ml) was added to all replicates at a final concentration of 10 µg ml^{-1}. All sample bottles were incubated and shaken for 5 d (25 °C, 150 rpm). From each bottle, an aliquot of 0.5 ml was removed at T = 0 d and 5 d and processed (section 3.2.7) for PDA-LC-MS analysis (chapter 2; section 2.2.8). A control (x3) was prepared using sterile Carron river water and MC-LR with no bacteria and sampled as described to confirm the loss of toxin is a result of microbial activity.

3.2.8.2 Metabolism of MC-LR in Biolog MT2 assay

To assess the influence of pre-exposure of the bacteria to winter and summer water on the subsequent metabolism of MC-LR, Biolog MT2 assay was carried out. After the 5 d incubation period, the bacterial cultures in both February (x3) and July water (x3) were centrifuged at 3000 g for 15 min. The bacterial suspensions ($A_{590\ nm}$ = 0.35) were prepared as previously described (section 3.2.5.1) and added (150 µl) into Biolog MT2 plates. Sterile MC-LR (10 µl) was added to the plates in triplicates to give final concentrations of 0.1, 1 and 10 µg ml^{-1}. The plates were incubated at 25 °C. The colour development was measured at $A_{595\ nm}$ using the microplate reader immediately after inoculation (0 h) followed by 1, 2, 4, 6, 9, 12, 22, 28, 36, 48 and 72 h.

To determine whether MC-LR degradation had taken place in the Biolog MT2 plates, the remaining samples in the wells were transferred into microcentrifuge tubes (1.5 ml), frozen immediately (-20 °C) and freeze dried after 24 h. Samples were prepared (section 3.2.7) and PDA-LC-MS analysis was carried out as described in section 2.2.8.

3.2.9 Biodegradation of MC-LR in synthetic waters

3.2.9.1 Preparation of synthetic waters

To facilitate the investigation of nutrient statues on MC degradation rates, it was necessary to select suitable defined synthetic water that provides maximum bacterial degradation. Three different synthetic fresh waters were prepared. Synthetic fresh water recipes described by Smith *et al*., (2002) and Deleebeeck *et al*., (2007) has been adapted for the current study. Preparation of soft water (S1), hard water (S2) (Smith *et al*., 2002) and S3 synthetic water (Deleebeeck *et al*., 2007) described in table 3.2 was carried out using several composite stock solutions made up in glass volumetric flasks at up to 1000 times the final concentration. To allow efficient dissolution of $CaCO_3$, the pCO_2 in the water was increased by vigorously sparging of CO_2 for 10 min before finely powdered $CaCO_3$ is added to the stock solution. The stock solution was subjected to subsequent sparging of 4 h with CO_2 to achieve maximum dissolution of added $CaCO_3$. Once all the stock solutions were prepared, synthetic soft and hard water was prepared using the different volumes of stock solutions as described in table 3.3. During the preparation procedure, all stock solutions and synthetic waters were stirred continuously to avoid localised supersaturation of salts and possible precipitation. The water

used during the entire procedure was obtained through a deionised filter system (Milli-RO; Millipore, Watford, UK).

Table 3.2 Preparation of stock solutions required for synthetic fresh water S1, S2 (Smith *et al*., 2002) and S3 (Deleebeeck *et al*., 2007).

Stock solution	**S1**	**S2**	**S3**
Stock solution 1	**Amount (g l^{-1})**	**Amount (g l^{-1})**	**Amount (g l^{-1})**
$MgCl_2$ $6H_2O$	12.168	-	7.55
$CaCl_2$ $6H_2O$	17.5	7.491	13.3
$Ca(NO_3)_2$ $4H_2O$	3.542	1.181	-
KCl	-	-	5.81
Stock solution 2			
$CaCO_3$	0.01872	0.0916	-
Stock solution 3			
Na_2SO_4	16.334	2.805	-
$KHCO_3$	2.502	0.751	-
$NaHCO_3$	1.678	2.268	49.98
KH_2PO_4	-	0.408	-
Stock solution 4			
$MgSO_4$ $7H_2O$	-	10.044	7.22
Trace elements			
$FeCl_3$	-	-	0.04866
H_3BO_3	-	-	0.185
$MnCl_2$	-	-	0.265
$ZnCl_2$	-	-	0.00299
Na_2MoO_4	-	-	0.00597
$CoCl_2$	-	-	0.00078
$CuSO_4$	-	-	0.00159
EDTA	-	-	0.001
pH	7.83	8.49	7.56

Table 3.3 Preparation of synthetic water S1 and S2 using the stock solutions

Stock solution	S1 (ml)	S2 (ml)	S3 (ml)
Stock solution 1	1	10	1
Stock solution 2	909	909	-
Stock solution 3	1	10	1
Stock solution 4	-	10	1
Trace elements	-	-	1
Water	89	61	996
Total volume	1000	1000	1000

In addition to the above synthetic fresh waters, BG-II media (chapter 2; section 2.2.3) which is typically used for cyanobacterial cultures has been exploited as synthetic minimal media, typical of a eutrophic lake. All synthetic media were sterilised by autoclaving (121 °C, 15 min).

3.2.9.2 Biodegradation of MC-LR in synthetic media

The biodegradation assay in different synthetic media was carried out as previously described in section 3.2.7. Bacterial suspensions were prepared (section 3.2.5.1) and inoculated (0.5 ml) in sterile universal bottles (x3) containing 9 ml sterile synthetic media S1, S2, S3 and BG-II. MC-LR (0.5 ml) was added to all replicates at a final concentration of 10 µg ml^{-1}. Sampling was carried out at T=0 d, T=5 d and T=10 d and the samples for PDA-LC-MS analysis were prepared (section 3.2.7). PDA-LC-MS analysis was performed as explained in section 2.2.8. Control (x3) experiment contained sterile 0.01 M PBS instead of synthetic media described above and sampled as described to confirm the loss of toxin is a result of microbial activity.

3.2.10 Media optimisation for the biodegradation of MC-LR by *Rhodococcus* sp. (C1)

3.2.10.1 Degradation of MC-LR in BG-II

BG-II was selected as a potential candidate for an enrichment media for MC-LR biodegradation by *Rhodococcus* sp. (C1). BG-II was prepared using the stock solutions as previously described in section 2.2.3. *Rhodococcus* sp. (C1) suspension was carried out as previously described (section 3.2.5.1) and added (0.5 ml) into sterile universal bottles (x3) containing 9 ml sterile (121 °C, 15 min) BG-II media. MC-LR (0.5 ml) was added to all replicates at a final concentration of 10 µg ml^{-1}. Sampled (T=0 d, T=5 d and T=10 d) and PDA-LC-MS analysis was performed as explained in section 3.2.7. Control (x3) experiment contained sterile 0.01 M PBS instead of BG-II and sampled as described to confirm the loss of toxin is a result of microbial activity.

3.2.10.2 Effect of an additional carbon source

To assess the effect of an additional carbon source in the media, BG-II was enriched with 5 mM glucose. Batch study was conducted using the same experimental procedures described above (section 3.2.10.13.2.10.1).

3.2.10.3 Effect of trace metal in BG-II for the degradation of MC-LR

To determine the effect of trace metals in the BG-II, one batch of BG-II media was prepared with no trace metals. Batch study was conducted using the same experimental procedures described above (section 3.2.10.1)

3.3 Results

3.3.1 Gram stain of bacteria

The bacterial isolates (table 3.1) were previously identified as Gram-positive bacteria by Manage *et al*., (2009). However, due to the long term storage and multiple sub-culturing it is important to confirm their identification using Gram staining procedure. Bacterial isolates stained by the basic Gram method described by Valle *et al*., (1999) were stained in blue-purple colours by retaining crystal violet-iodine complex. Gram identification and their specific cellular characteristics are listed in table 3.5.

Table 3.4 Gram identification of the *Actinobacteria* isolates using an optical microscope.

Bacterial isolate	**Gram identification**	**Cellular shape under microscope**
Rhodococcus sp. (C1)	Gram-positive	Clustered rods
Rhodococcus sp. (C3)	Gram-positive	Clustered rods
Arthrobacter sp. (C6)	Gram-positive	Rod shape cells
Brevibacterium sp. (F3)	Gram-positive	Short, thick rods
Arthrobacter sp. (F7)	Gram-positive	Rod shape cells
Arthrobacter sp. (F10)	Gram-positive	Rod shape cells
Arthrobacter sp. (R1)	Gram-positive	Rod shape cells
Arthrobacter sp. (R4)	Gram-positive	Rod shape cells
Arthrobacter sp. (R6)	Gram-positive	Rod shape cells
Arthrobacter sp. (R9)	Gram-positive	Rod shape cells

3.3.2 Culturing of *Actinobacteria* isolates

All of the ten bacterial isolates indicated abundant growth in NB at 25 °C. Isolates *Brevibacterium* sp. (F3) and *Arthrobacter* sp. (R1) indicated a slow growth compared to other bacterial isolates (results not shown). All of the *Arthrobacter*, *Rhodococcus* and *Brevibacterium* isolates formed smooth, circular shaped, raised colonies with entire margins on NA Petri plates. No pigmentation was observed on *Arthrobacter* sp. or *Rhodococcus* sp. isolates while *Brevibacterium* sp. (F3) formed pigmented colonies of yellow-orange on NA plates.

3.3.3 Evaluation of MC-LR utilisation by isolated bacteria

Eight out of 10 MC degrading bacterial isolates still retained the capacity to metabolise MC-LR as a sole carbon source (figures 3.2 and 3.3). Carron isolates, C1 and C3 belonging to *Rhodococcus* sp. indicated a clear metabolism of MC-LR (figure 3.2) as evident by the higher absorbance compared to the control during the entire incubation period. However, *Arthrobacter* sp. (C6) also isolated from Carron, demonstrated comparatively low absorbance, i.e. lower metabolic response (figure 3.2) to that of isolates C1 and C3. The two *Arthrobacter* isolates from Forfar (F7 and F10) demonstrated the highest absorption levels (>1.6) after 18 h incubation period, compared to the other bacterial isolates (figures 3.2 and 3.3). In contrast, MC-LR utilisation by *Brevibacterium* sp. (F3) and *Arthrobacter* sp. (R1) was limited attaining only a maximum absorption level of 0.4 and 0.3 respectively. Interestingly, in *Arthrobacter* sp. (R1), the absorption levels seemed to drop below its control when it was inoculated with higher concentrations of MC-LR (1 µg ml^{-1} and 10

μg ml^{-1}). It could be due to a possible inhibitory response to high concentration of MC-LR.

The Rescobie isolates *Arthrobacter* sp. R4, R6 and R9 indicated a pronounced metabolic response compared to the control experiment (figure 3.3). Likewise, the positive control *P. toxinivorans* achieved a rapid utilisation of MC-LR demonstrating an average absorption level exceeding 1.3 for the entire toxin concentrations tested (figure 3.3). In conclusion, these bacterial isolates (eight out of 10) are still capable of utilising MC-LR as a sole carbon source.

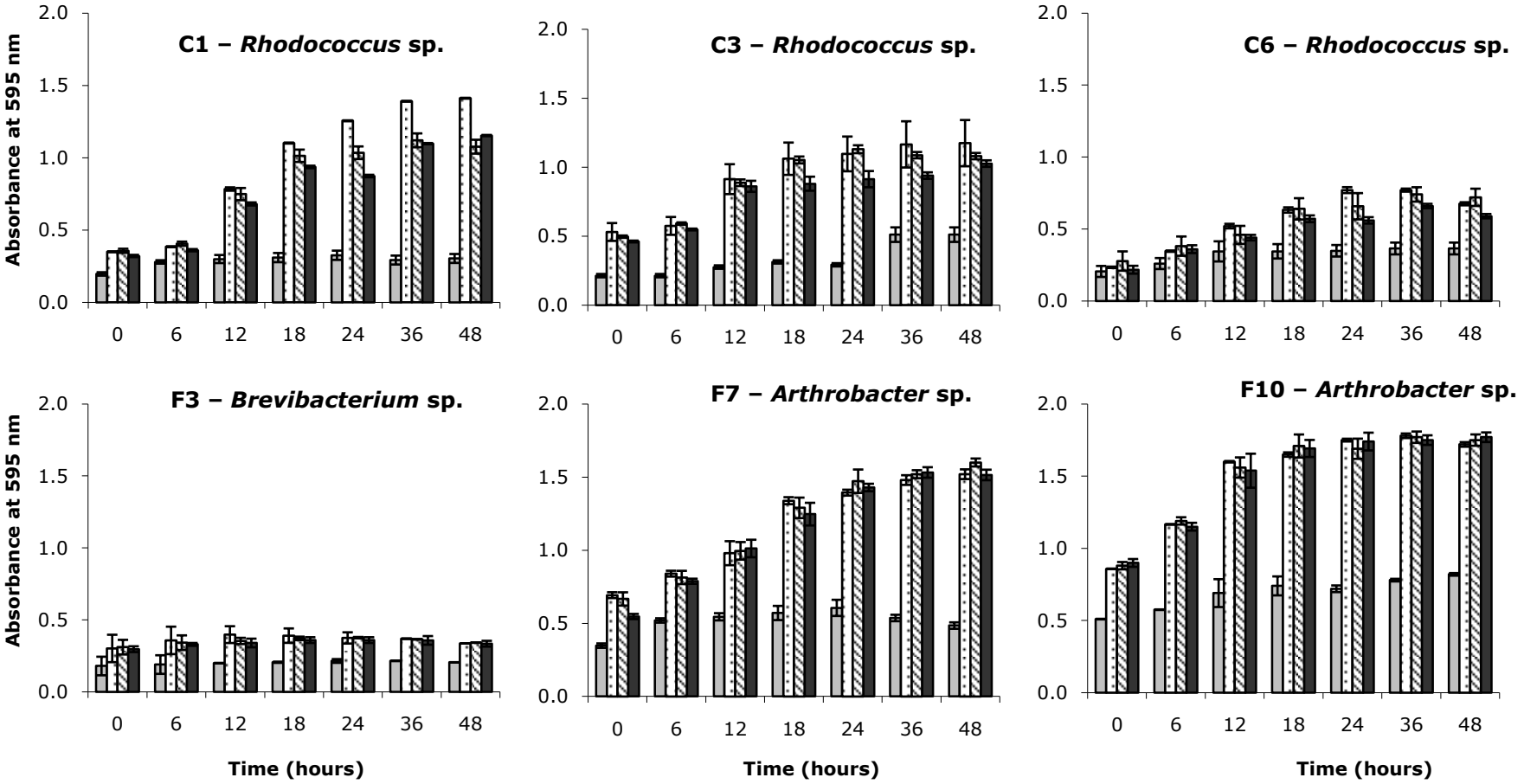

Figure 3.2 Biolog screen of MC-LR metabolism by the isolated bacteria (Carron river and Forfar Loch) during 48 h. Control (▭) contains no added carbon. Final MC-LR concentration in wells were 0.1 µg ml^{-1} (⬚), 1 µg ml^{-1} (▨) and 10 µg ml^{-1} (■). Error bars represent one standard deviation (n=3).

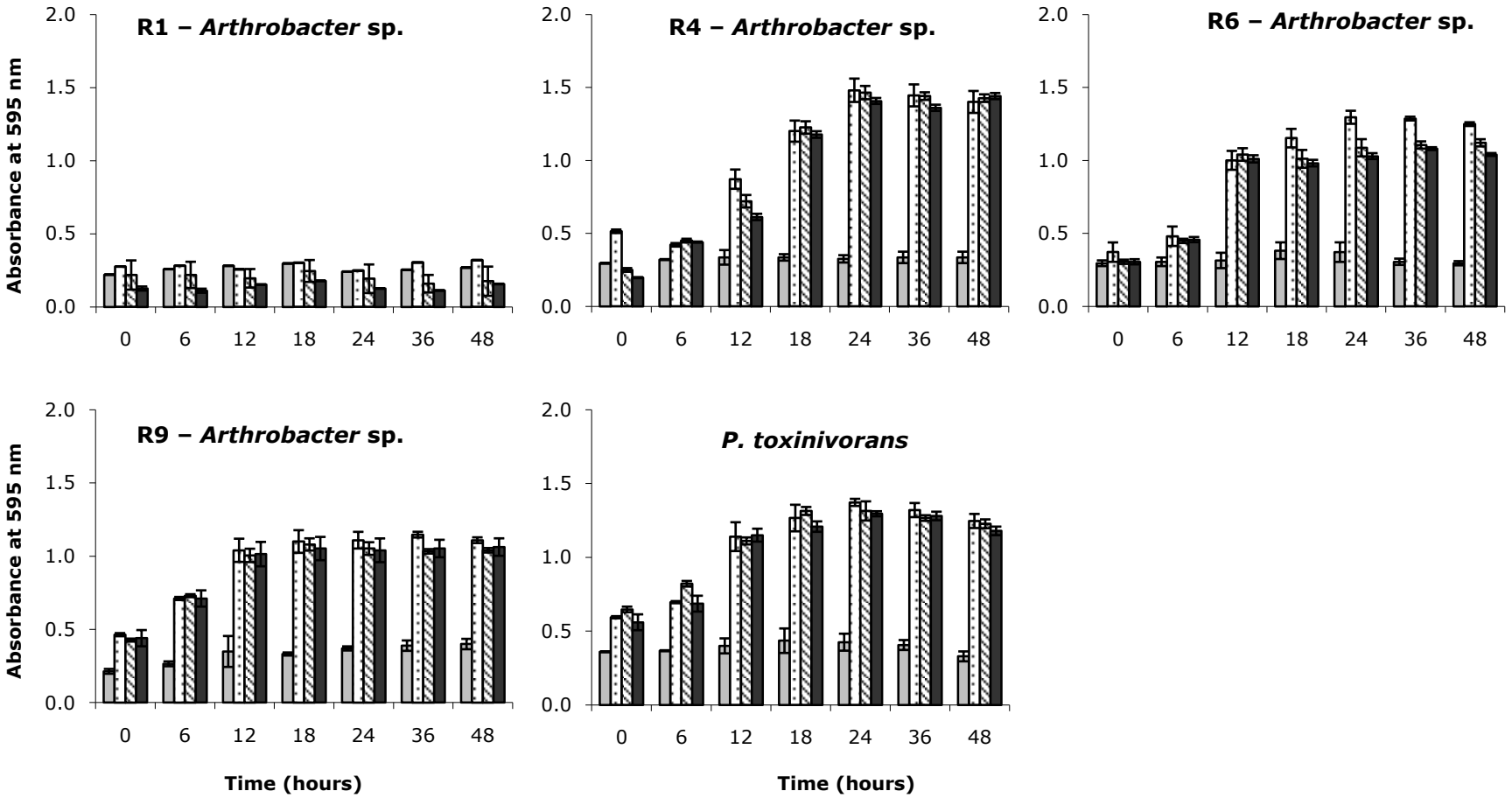

Figure 3.3 Biolog screen of MC-LR metabolism by the isolated bacteria (Loch Rescobie) during 48 h. Control (▭) contains no added carbon. Final MC-LR concentration in wells were 0.1 µg ml^{-1} (▭), 1 µg ml^{-1} (▨) and 10 µg ml^{-1} (■). Error bars represent one standard deviation (n=3).

3.3.4 Bacterial metabolism of multiple MCs and NOD

All of the five isolates selected based on the results of the previous Biolog screening and the positive control, successfully metabolised the range of MCs and NOD. However, the level of cyanotoxin utilisation was notably different depending on bacterial isolate and different toxin variants (figure 3.4). Isolates C1 (*Rhodococcus* sp.) and C6 (*Arthrobacter* sp.) were shown to utilise most MCs to a similar degree. *Arthrobacter* sp. (F7) has shown highest overall metabolism of all the toxin variants, a typical absorption of over 0.7 (figure 3.4). *Brevibacterium* sp. (F3) which indicated low utilisation of MC-LR, demonstrated an overall metabolism on all MCs and NOD. The positive control, *P. toxinivorans* has indicated a higher metabolism on MC-LY (≥ 0.7 of absorption difference) compared to other MCs (figure 3.4). Metabolism of NOD by *P. toxinivorans* was distinctly lower (~ 0.3 absorbance) compared to its metabolism on MC variants, i.e. absorption difference for MC-RR (0.6) and –LY (0.8; figure 3.4). *Rhodococcus* sp. (C1) and *Arthrobacter* sp. (C6) performed highest metabolism under the lowest toxin concentration (metabolism of MC-RR and –LF and -LY). Nevertheless, considering the metabolic response for MC-LR and -LW, the highest metabolism was evident when they were exposed to high toxin concentrations (figure 3.4). In contrast, metabolism of MC-LY by two of the Forfar isolates *Brevibacterium* sp. (F3) and *Arthrobacter* sp. (F7) had indicated metabolic preference on 1 μg ml^{-1} of MC-LY (figure 3.4).

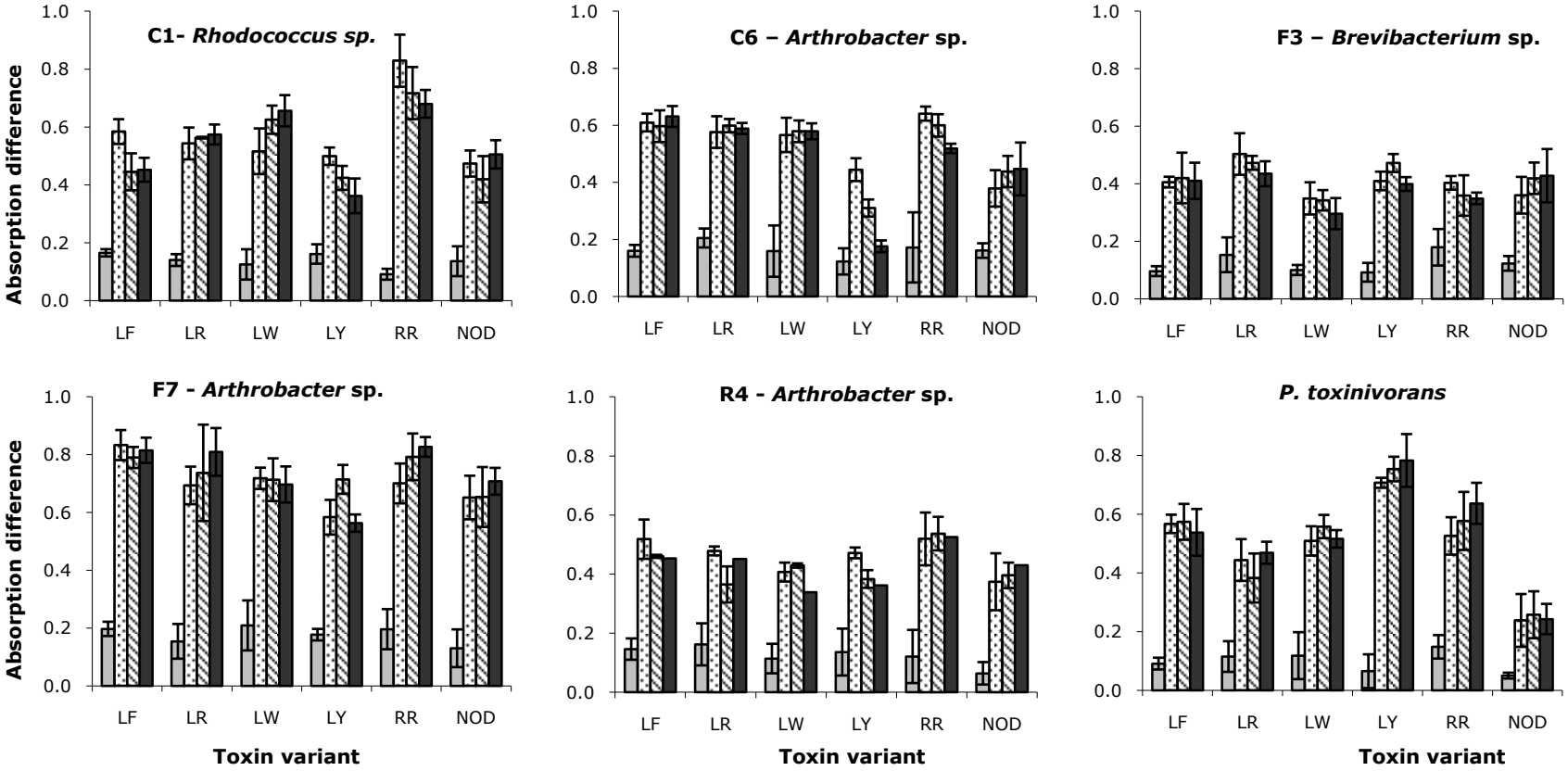

Figure 3.4 Biolog screen of MC-LF, -LR, -LW, -LY, -RR and NOD metabolism by the six bacterial isolates at 24 h of incubation. Control (□) contains no added carbon. MC-LR concentration in wells were 0.1 µg ml^{-1} (⬚), 1 µg ml^{-1} (▨) and 10 µg ml^{-1} (■). Error bars represent one standard deviation (n=3).

3.3.5 Biodegradation of multiple MCs and NOD

All of the six bacterial isolates were shown to degrade five different MC variants and NOD in Carron, Forfar and Rescobie water (figures 3.5, 3.6 and 3.7). Degradation of MCs and NOD commenced immediately and no lag phases were observed. However, there were considerable differences in the rate of degradation MCs and NOD (table 3.5). About 50% of MC-LR, -LY, -LF and -LW were degraded by the *Actinobacteria* isolates during the 10 d course of incubation (figures 3.5, 3.6, 3.7; table 3.5). Regardless of the bacterial isolate and the water in which they were incubated, the half life of the MC-LR was 9 d (table 3.5). All of the bacterial isolates were capable of degrading MC-LY most rapidly showing an average of 30% of the toxin remaining on 10 d. In contrast, MC-RR and NOD have shown relatively slow degradation having ~ 60% of the toxins remaining at 10 d (figures 3.5, 3.6 and 3.7). Moreover, none of the bacteria inoculated in the batch cultures achieved complete degradation of MCs and NOD. No considerable losses were evident in the sterile control confirming the toxin degradation occurred exclusively as a result of the bacterial activity.

Table 3.5 Degradation half-life for five MC variants and NOD, incubated with individual toxins and monitored daily.

	Time taken to degrade 50% of MC or NOD (days)					
Bacterial isolate	**MC-LR**	**-LY**	**-LF**	**-LW**	**-RR**	**NOD**
Carron water						
Rhodococcus sp. (C1)	9	5	8	6	10	>10
Arthrobacter sp. (C6)	9	5	8	8	>10	>10
Forfar water						
Brevibacterium sp. (F3)	9	6	7	9	>10	10
Arthrobacter sp. (F7)	9	6	6	7	>10	>10
Rescobie water						
Arthrobacter sp. (R4)	9	7	7	6	>10	10
P. toxinivorans	9	6	6	7	>10	>10

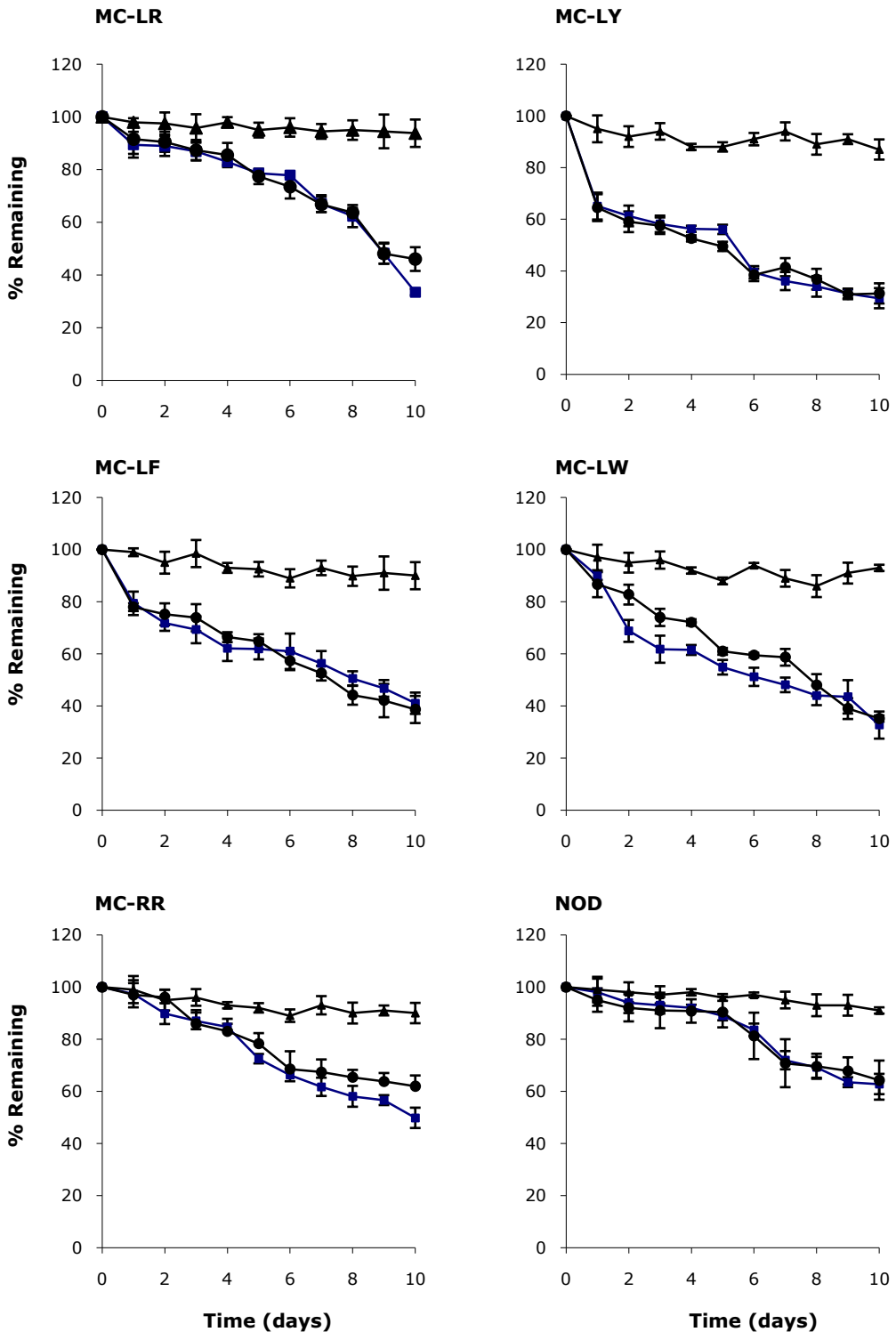

Figure 3.5 Degradation of multiple MCs and NOD (10 µg ml^{-1}) in sterile Carron river water by *Rhodococcus* sp. (C1; ■) and (C6; ●) isolates. The control (▲) contains no bacteria. Error bars represent one standard deviation (n=3).

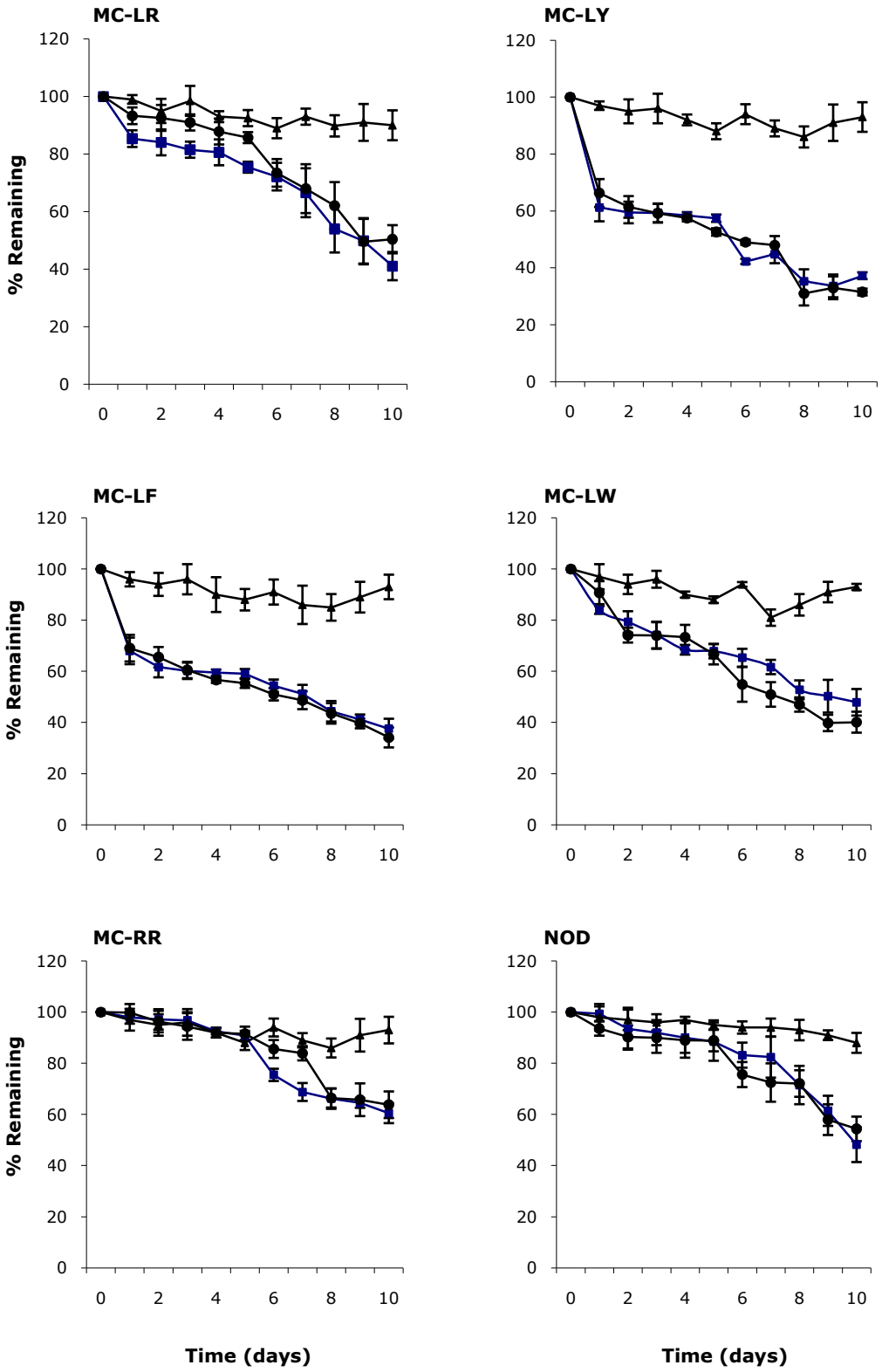

Figure 3.6 Degradation of multiple MCs and NOD (10 µg ml^{-1}) in sterile Forfar Loch water by *Brevibacterium* sp. (F3; ■) and *Arthrobacter* sp. (F7; ●) isolates. The control (▲) contains no bacteria. Error bars represent one standard deviation (n=3).

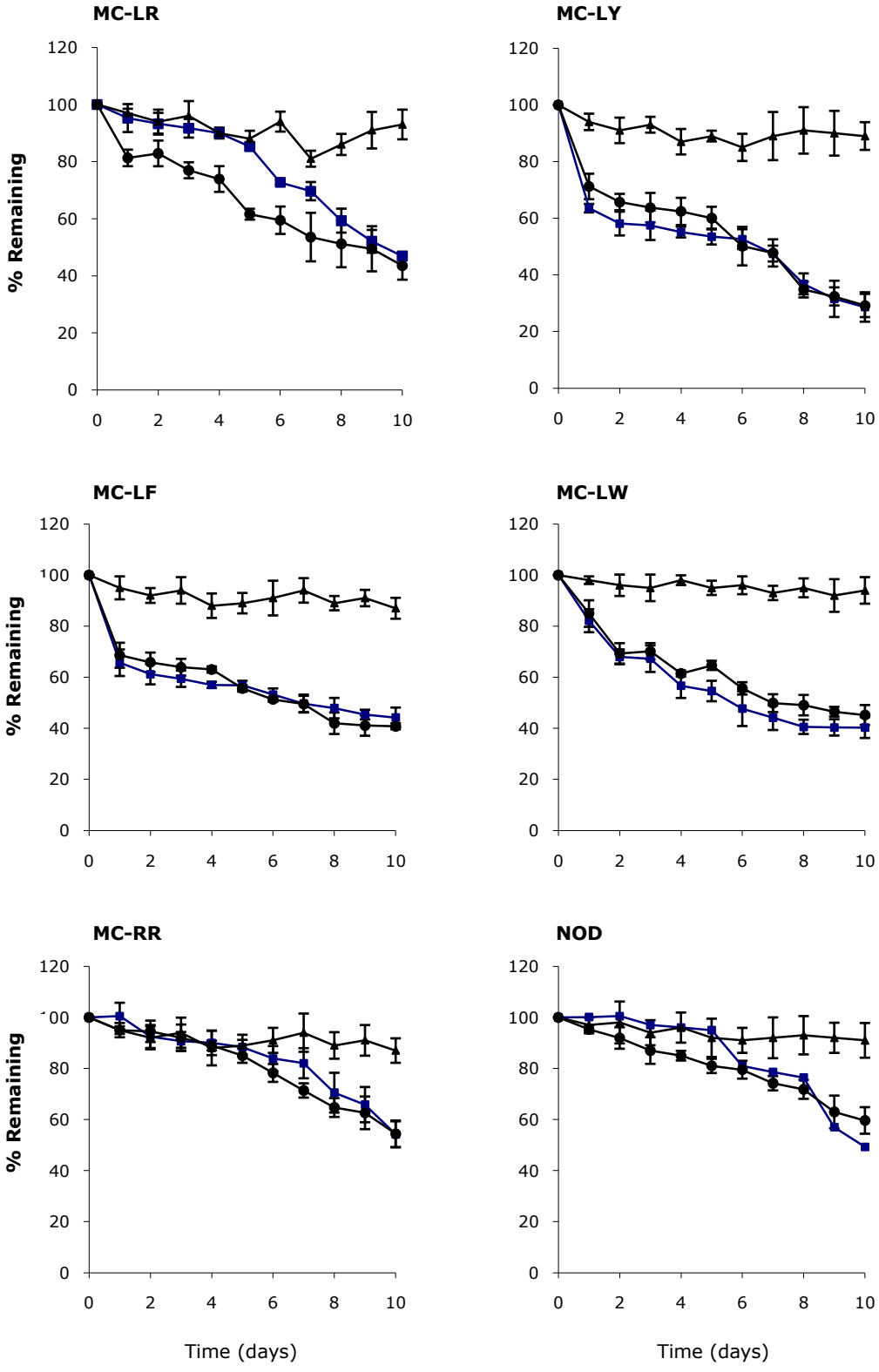

Figure 3.7 Degradation of multiple MCs and NOD (10 µg ml^{-1}) in sterile Loch Rescobie water by *Arthrobacter* sp. (R4; ■) and *P. toxinivorans* (●). The control (▲) contains no bacteria. Error bars represent one standard deviation (n=3).

3.3.6 Seasonal dynamics of nutrients in Carron river and biodegradation MC-LR

Despite different period of the year (winter or summer) in which, the water samples were collected, a clear degradation of MC-LR was observed by *Rhodococcus* sp. (C1). However, the degradation rate was shown to differ between February water and the July water. After 5 d of incubation, the amount of MC-LR remaining in February and July water samples were 70% and 55% respectively (figure 3.8). As previously observed in batch studies, *Rhodococcus* sp. (C1) was not capable of achieving the half life degradation of MC-LR during the 5 d course incubation. However, compared to the degradation rate of the previous study (figure 3.5) more rapid degradation was observed here. Interestingly, it should be noted that when bacteria were incubated in the summer water (July), a faster MC-LR degradation was evident whereas 45% of the toxin was degraded. No losses of MC-LR was evident in the parallel universal bottles containing sterile Carron river water and sterile MC-LR, confirming that the degradation in the subsequent universal bottles containing MC-LR and *Rhodococcus* sp. (C1) were through bacterial biodegradation process.

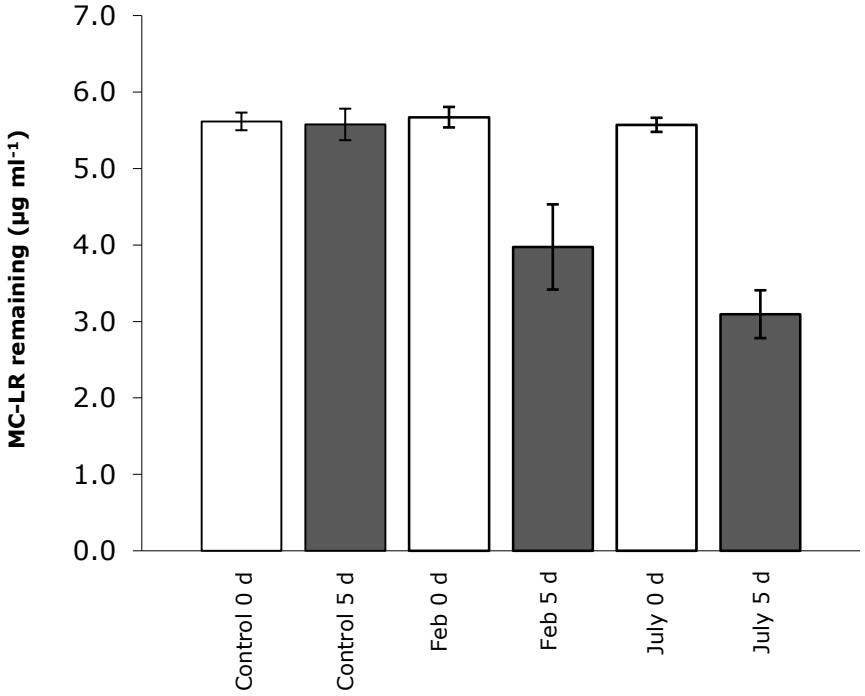

Figure 3.8 The degradation of MC-LR by *Rhodococcus* sp. (C1) in Carron river water collected on February 2009 and July after 5 d course of incubation. Error bars represent one standard deviation (n=3).

To further understand if seasonal nutrient status influences the ability of a bacterium to degrade MC-LR cells were pre-incubated in water collected at different times of the year. Figure 3.9 illustrates the dynamics of subsequent utilisation of MC-LR by *Rhodococcus* sp. (C1) after a 5 d pre-incubation period in Carron river water collected at two different times of the year (February and July). Despite pre-incubation of the bacteria in two different water types, *Rhodococcus* sp. (C1) was able to effectively metabolise MC-LR in the Biolog MT2 assay (figure 3.9). However, the bacterial responses to MC-LR after the pre-incubation have indicated considerable differences. *Rhodococcus* sp. (C1) pre-incubated in July water have demonstrated considerably low utilisation of MC-LR during the first 12 h (maximum absorption difference 0.12) followed by a pronounced utilisation of MC-LR during the next 48 h. During this period, the maximum absorption difference noted was 1.27 (figure 3.9). Nonetheless, the bacteria inoculated in February water indicated comparatively low metabolic response in the Biolog assay. The bacterial utilisation has gradually increased with the time of incubation and achieved a maximum 0.54 absorption difference after 72 h. Moreover, another observation of note was the similar metabolic response of *Rhodococcus* sp. (C1) for different MC-LR concentrations regardless of the different water in which they were pre-incubated. Figure 3.9 illustrates that bacteria from both February and July had comparatively high utilisation at the highest concentration of MC-LR.

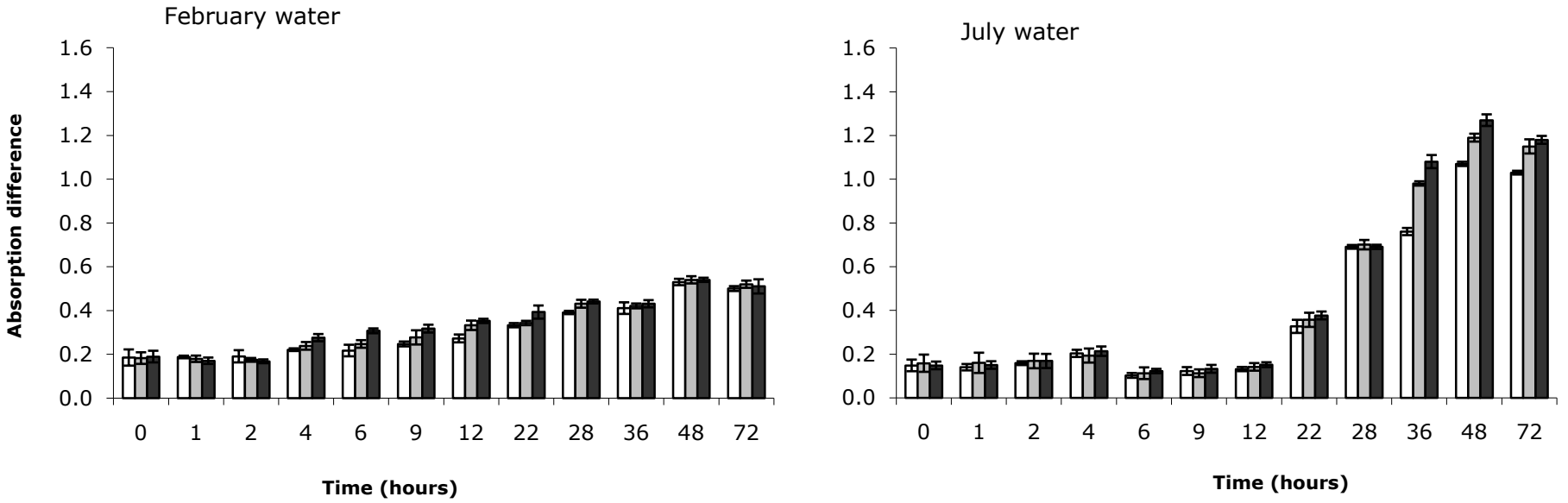

Figure 3.9 Biolog screen of MC-LR metabolism by *Rhodococcus* sp. (C1) inoculated in February and July waters. MC-LR concentration in wells were 0.1 µg ml^{-1} (□), 1 µg ml^{-1} (▒) and 10 µg ml^{-1} (■). Control wells contained no added carbon. The absorption difference was calculated as the difference between the absorption in the sample well and the corresponding control well. Error bars represent one standard deviation (n=3).

Confirmation that metabolism occurred in the Biolog MT2 plates was performed by PDA-LC-MS, analysing the remaining samples in the wells of the Biolog plate (after 72 h). It revealed that the degradation of MC-LR had taken place in the wells of Biolog MT2 plates in which the *Rhodococcus* sp. (C1) bacteria were incubated with water collected in July. During the bacterial metabolism of MC-LR in the Biolog MT2 assay, new peaks were evident compared to that of the control experiment (data not shown). Investigation of the PDA-LC-MS chromatograms and MS spectra revealed the presence of characteristic degradation products of MC-LR (table 3.6). Thus, revealed possible hydrolysis of MC-LR taken place as explained by Bourne *et al*., (1996). In contrast, there was no evidence of degradation products of MC-LR in the Biolog assay contained bacteria pre-incubated with February water.

Table 3.6 PDA-LC-MS analysis of MC-LR related degradation products identified in Biolog MT2 assay.

m/z	**Ion/Degradation product**
1013	M + H (Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH+H)
879	M + H – $PhCH_2CHOMe$
726	CO-Glu-Mdha-Ala-Leu-Masp-Arg-OH+2H
571	Mdha-Ala-Leu-Masp-Arg-OH+2H
486	Ala-Leu-Masp-Arg-OH
304	MeAsp-Arg-OH+2H
286	MeAsp-Arg+H
213	Glu-Mdha+H
175	Arg-OH+2H
155	Mdha-Ala+H
135	$PhCH_2CHOMe$

3.3.7 Biodegradation of MC-LR in synthetic fresh waters

The results of the biodegradation of MC-LR in synthetic fresh waters, PBS and BG-II media for 10 d indicated MC-LR was degraded in all treatments (figure 3.10). Although, the amount of MC-LR degraded did vary considerably depending on the composition of the media (figure 3.10). The highest percentage degradation of MC-LR was indicated on the BG-II media whereas 67% of the toxin was removed after 10 d. The degradation occurred in S1 and S2 indicated similar degradation rates; an average of 29% of MC-LR was removed during the first 5 d and 45-48% of the compound was removed after 10 d (figure 3.10). The degradation which occurred in S3 was considerably lower with only 30% of the toxin being degraded by the bacterial strain.

Degradation of MC-LR in PBS (12%) was the lowest clearly indicating that the lack of some important nutrients resulted in reduced rates of MC-LR degradation.

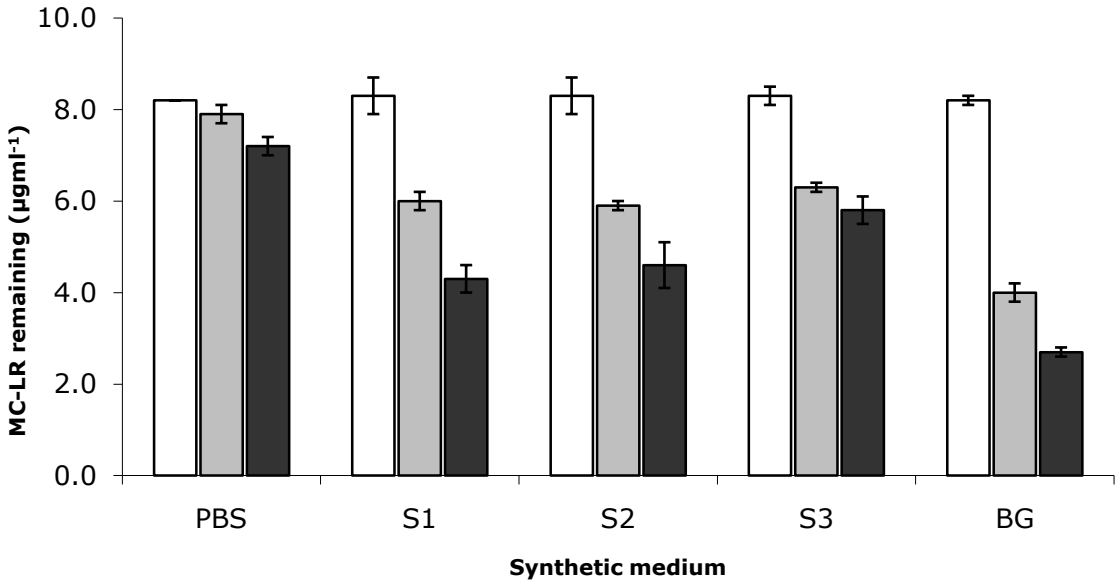

Figure 3.10 Results of the degradation of MC-LR in different synthetic media at 0 d (□), 5 d (▭) and 10 d (■). Synthetic media S1 and S2 represent soft and hard lake water described by Smith *et al*., (2002) respectively. Synthetic medium S3 was prepared according Deleebeeck *et al*., (2007). BG-II (BG), typical cyanobacteria growth media (Stanier *et al*., 1971) prepared as previously described in chapter 2; section 2.2.3. Error bars represent one standard deviation (n=3).

3.3.8 Media optimisation for the biodegradation of MC-LR by *Rhodococcus* sp. (C1)

The *Rhodococcus* sp. (C1) has shown a pronounced MC-LR degradation in BG-II regardless of the nutrient modification in the media; however different degradation rates were evident (figure 3.11). With the presence of a supplementary carbon source in the media, a clear difference in bacterial degradation was observed during 10 d of incubation. As previously seen in batch experiments (section 3.3.5), the *Rhodococcus* sp. (C1) degradation of MC-LR has indicated an average removal of 37% during the first five days of incubation. However, more rapid degradation of MC-LR was evident when the degradation media was inoculated with glucose (carbon source) whereas the half life degradation of MC-LR was achieved before 5 d. About 68% of degradation was achieved by *Rhodococcus* sp. (C1) within first 5 d, while 75% of the MC-LR was degraded within 10 d in the presence of additional carbon source. In contrast, in the absence of an additional carbon source, *Rhodococcus* sp. (C1) was not capable of achieving 50% MC-LR removal by 5 d; however, half life degradation of MC-LR had occurred within 10 d of incubation in BG-II with no glucose (72% MC-LR degraded; figure 3.11).

An interesting observation to be noticed was the similar degradation rates in the BG-II; despite the omitted trace metals from the media. During the first 5 d, *Rhodococcus* sp. (C1) has achieved an average degradation of 38% and 72% after 10 d in both BG-II and BG-II with no trace metals. This observation suggests that there is no considerable effect of trace metals in the media on the bacterial biodegradation of MC-LR. Another point worth mentioning is the complete removal of MC-LR before 5 d in one of the sample bottles

supplemented with glucose resulting in higher recorded error bars between replicates. Only minor degradation (10%) was detected by the bacteria growing in sterile 0.01 M PBS compared to both BG-II and BG-II glucose media confirming a relation between bacterial degradation and the available nutrients in the media (figure 3.11).

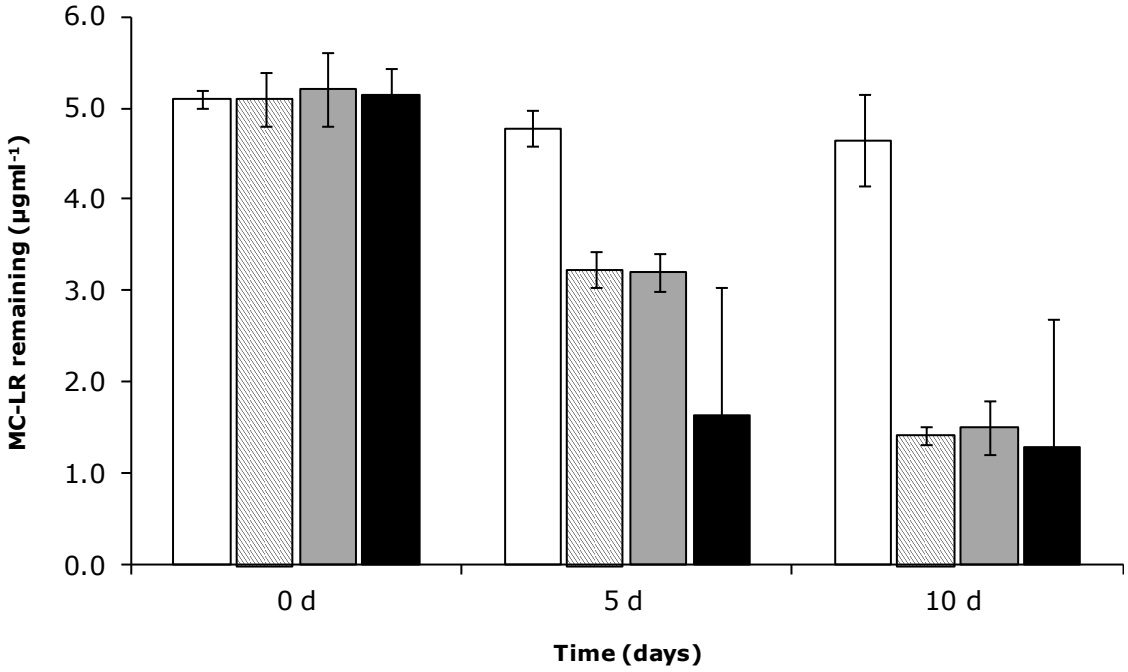

Figure 3.11 The degradation of MC-LR by *Rhodococcus* sp. (C1) in sterile 0.01 M PBS (□) BG-II (▨), BG-II with no trace metals (▩) BG-II enriched with 5 mM glucose (■) during a 10 d incubation period. Error bars represent one standard deviation (n=3).

3.4 Discussion

3.4.1 Bacterial metabolism of MCs and NOD

Manage *et al*., (2009) successfully isolated 10 novel bacterial strains from three different Scottish waters capable of utilising and degrading MC-LR. The cultures from the initial identification and Biolog MT2 screening selection had been stored for a number of months (> 8 months) and subjected to multiple sub-culturing for routine maintenance of the bacteria. Prior to a full evaluation and in-depth studies it is important to confirm that they could still actively metabolise MC-LR. Hence, in the present study, the bacterial metabolism of MC-LR was evaluated using the Biolog MT2 assay. The Biolog MT2 plates had been successfully exploited in the previous study as a rapid screening tool to evaluate the bacterial metabolism of MC-LR; (Manage *et al*., 2009) hence, extending its initial application to test multiple carbon sources against any set of bacteria (Garland and Mills, 1991).

Eight out of 10 bacterial isolates tested in Biolog MT2 assay were found to metabolise MC-LR, confirming that they still retained the ability to utilise MC-LR as a sole carbon source (figures 3.2 and 3.3). Three different concentrations of MC-LR was tested (0.1, 1, 10 μg ml^{-1}) with 10 isolated bacterial strains using the Biolog screening to investigate the bacterial response against a wide range of toxin concentrations. It was found that higher concentration of toxin did not appear to enhance the metabolism. In fact, high concentration of MC-LR was produced an inhibitory action in some bacterial strains (*Rhodococcus* sp. C1, C6) where they demonstrated slightly lower metabolism at higher concentration of the toxin (figure 3.2).

Rhodococcus sp. (C3), *Brevibacterium* sp. (F3) and *Arthrobacter* sp. (R1 and R9) have demonstrated comparatively low response to that of other isolates. This could be explained as the effects of long term storage and the continuous multiple sub-culturing before they were exploited for the current study. It is probable that these isolates might have suppressed their ability to metabolise MC-LR since they were not exposed to MC-like peptides during the routine maintenance (Lawton *et al*., 2011). Furthermore, it was not surprising to observe a slow utilisation of MC-LR by *Brevibacterium* sp. (F3) since it was found to demonstrate comparatively slow growth rate compared to the other bacterial isolates (section 3.3.2). Slow bacterial growth; i.e. slow metabolic activity of *Brevibacterium* sp. (F3) might have hindered the metabolic interaction with MC-LR which finally resulted in low absorption levels during the incubation. Similarly, *Arthrobacter* sp. (R1), which demonstrated a pronounced utilisation of MC-LR in the initial screening process (Manage *et al*., 2009), has shown no metabolic activity against MC-LR in the following study. Most of the absorption values were recorded below its control levels showing either no utilisation of MC-LR or a complete metabolic inhibition in the presence of MC-LR. The poorest response being at the highest MC-LR concentration (10 μg ml^{-1}) suggests a potential effect of MC-LR in the bacterial strain. It is probable that inoculation of low levels of MC-LR may reverse the inhibitory response.

However, when these bacteria (*Rhodococcus* sp. C1, *Arthrobacter* sp. C6, F7, R4 and *Brevibacterium* sp. F3) were evaluated with a wide range of MCs and NOD, an overall metabolism was observed (figure 3.4). This bacterial behaviour might justify the importance of using these bacteria in future

experiments to explore their capability of removing a mixture of MCs and NOD from cyanotoxin contaminated waters. Moreover, *Rhodococcus* sp. (C1 and C6) and *Arthrobacter* sp. (R4) has predominantly utilised MC-RR which could be more stable (Takenaka and Tanaka, 1995) to bacterial attack with its two arginine groups. This clearly demonstrates that these bacteria might harbour different metabolic pathways for different peptide (MCs and NOD) degradation (Lawton *et al*., 2011). Furthermore, it was interesting to note that positive control *P. toxinivorans*, which was previously reported to degrade only MC-LR, MC-YR and NOD (Rapala *et al*., 2005) clearly showed metabolism in the presence of MC-LF, -LW, -LY and -RR but comparatively low metabolism on NOD.

This study has confirmed that Biolog MT2 plates are an excellent cost effective rapid screening tool for the evaluation of bacterial metabolism with highly diverse different respiratory substrates (Manage *et al*., 2009; Lawton *et al*., 2011). In addition, without an excessive sample preparation and analysis involved in traditional protocols it has been shown to give accurate results at relatively low concentrations of toxins (Manage *et al*., 2009). Although, Biolog studies have clearly revealed the bacterial metabolism in the presence of different MCs and NOD; it could not confirm the actual degradation that occurred in Biolog MT2 plates. Therefore, further investigation is needed to confirm the actual degradation of MCs and NOD by these bacterial isolates.

3.4.2 Biodegradation of multiple MCs and NOD

The bacterial isolates which had been shown to metabolise a wide range of different MC variants and NOD in the Biolog MT2 assay were further evaluated in a typical batch shake study. However, prior to further development of isolates for use in water remediation it is important to confirm that the microbes could degrade the compounds.

The main purpose of the traditional batch experiment was to investigate the bacterial biodegradation of typically available different MCs and NOD in the original waters where they were isolated from. Interestingly, all of the five bacterial isolates namely, *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6, R4 and F7) *Brevibacterium* sp. (F3) were found to degrade all the tested MC variants (MC-LR, -LY, -LF, -LW and –RR) and NOD in the batch study. The degradation observed in the current study has supported the Biolog MT assay confirming an active metabolism occurred in the Biolog MT2 plates. However, the relative degree of metabolism observed in the Biolog assay did not reflect the rate of degradation (sections 3.3.3 and 3.3.4) whereas the actual biodegradation of MCs was much slower than expected. It is clear from the results that the most significant factor influencing degradation is the chemistry of the compound, not the bacterial isolate itself (Lawton *et al*., 2011).

Amongst the MC variants, MC-LY was found to be degraded more rapidly compared to MC-LR, -LF, -LW whereas 50% of the toxin was removed in an average of 6 d. It is also probable that the specific peptide arrangement or peptide bond(s) specific for MC-LY facilitated the degradation process. Furthermore, it could be implied that, due to the substitution of tyrosine at the

Z position of MCs ring structure (chapter 1; figure 1.1) has contributed to relatively low bond energy in MC-LY (Lawton *et al*., 2011). Interestingly, hydrophobic MC-LW and MC-LF were more rapidly degraded compared to MC-LR, which is possibly due to their high cell permeability (Goodman *et al*., 1999). These MCs could, therefore, be readily available for the degradation with its feasible uptake and transportation into the bacteria (Nybom *et al*., 2008). Furthermore, Imanishi *et al*., (2005) also observed differences in the degradation of different MC variants using a *Sphingomonas* sp. B9. In the study, the authors suggested that strain B9 was only able to degrade analogues which contained the Adda-arginine peptide bond. However, this behaviour does not appear to be universal for all MC and NOD degrading bacteria. Ishii *et al*., (2004), Rapala *et al*., (2005) and Ho *et al*., (2007) have clearly demonstrated the degradation of MC variants which did not contain this specific bond, particularly, MC-LW, -LF, -LY and LA. In the current study, all the different *Rhodococcus* sp., *Arthrobacter* sp. and *Brevibacterium* sp. were able to degrade cyanotoxins composed of Adda-arginine bond (MC-LR, -RR and NOD) as well as MC-LW, -LF and -LY which does not contain the particular peptide bond. This suggests that the bacterial degradation mechanism involved in the non-specific degradation of these different cyanotoxins could be completely different to that described before (Cousins *et al*., 1996; Bourne *et al*., 1996; Imanishi *et al*., 2005). Degradation diversity of the *Actinobacteria* isolates was also supported by comparatively slow degradation of the two cyanotoxins, MC-RR and NOD. A possible explanation for this could be that they are structurally most different to the other four, all of which contain Leucine. MC-RR contains two arginines in the variable positions which increases its polarity and may cause steric hindrance, reducing the rate of an

enzymic attack (Lawton *et al*., 2011). In addition to that, as previously described, MC-RR and NOD being more hydrophilic, could have demonstrated considerably low cell permeability (Goodman *et al*., 1999) resulting in low cellular uptake (Nybom *et al*., 2008). On the other hand, NOD is a much smaller and tighter ring structure containing only five amino acids compared to seven in the MCs and it has been suggested that this makes it harder to open the ring structure to facilitate degradation (Lawton *et al*., 2011). A study carried out by Edwards *et al*., (2008) has also reported the stability of NOD, compared to MCs, in a mixed population of bacteria naturally present in water from the Carron river and Forfar Loch. For example, the report indicated a much slower rate of NOD degradation in Loch Rescobie water where they found a complete degradation after 18 d compared to 11 d for MC-LR. However, this becomes more interesting since, many of the MC degrading *Sphingomonas* bacteria were unable to degrade NOD. For example, *Sphingomonas* sp. ACM-3962 (Jones *et al*., 1994; Bourne *et al*., 1996) from an Australian river, *Novosphingobium* sp. MD-1 (Saitou *et al*., 2003) and *Sphingomonas* sp. 7CY (Ishii *et al*., 2004) from Japanese lakes were not capable of degrading NOD or NOD-Har. In contrast, Harada *et al*., (2004) presented another *Sphingomonas* sp. B9 which could degrade NOD and few other MC variants. Comparing the previously described degradation characteristics, it is highly likely; these bacterial isolates harbour a universal cyanopeptide degrading mechanism.

Another interesting degradation pattern to observe in the current study was the lack of characteristic lag phases. Batch degradation studies carried out by Jones *et al*., (1994), Cousins *et al*., (1996), Ho *et al*., (2007) and Edwards *et*

al., (2008) using natural bacterial populations had shown to maintain relatively long lag periods. However, subsequently, Jones *et al*., (1994), observed no lag phase was on the re-addition of MC-LR to the pre-incubated bacteria in river water with MC-LR, suggesting an inducible enzymatic degradation involved in the MC degradation. Since, all of the bacteria were incubated in NB and the inocula were adjusted before being inoculated, it is also probable that a notable lag period is not visible prior to the degradation of peptide toxins (Lam *et al*., 1995). Furthermore, instead of a lag period, it was interesting to note a rapid breakdown of MC-LR, -LY, -LF and -LW during the first 24 h. This degradation behaviour could be explained as a result of the pre-starvation of the bacteria in PBS for 24 h before they are inoculated with MCs. Under the stress of the carbon starvation, the bacteria could induce expression of catabolic enzymes (Leung *et al*., 2005) which could enhance the degradation of cyanobacterial peptides.

It is also worth noting that the degradation rates of the isolates was significantly slower than when first reported (Manage *et al*., 2009), whereas the half-life for MC-LR was typically below 2 d with most toxin undetected at 3 d. The cultures used in the study have been maintained in lab culture for over a year and it may be that their ability to biodegrade is diminished over time (Lawton *et al*., 2011). It is essential that this aspect of their behaviour be investigated fully to ensure optimum performance (chapter 4). Furthermore, it is also probable that the MC-LR degradation (Manage *et al*., 2009) was enhanced by the water chemistry (water collected in early October 2008). In contrast, the water used in the current batch study was collected during the winter period (early February 2009) of the year where it is considerably lower

in nutrients compared to that of summer/autumn (Huang *et al*., 2008); hence, it was not surprising to observe slow rate of MC degradation. However, the effect of available nutrients and bacterial degradation could not be confirmed by this study due the limited data available regarding the water chemistry. Therefore, another aspect that needs to be explored in future studies are the availability of nutrients necessary to support bacterial growth and activity, ultimately promoting a rapid degradation.

No notable degradation products were observed in the PDA-LC-MS analysis, although the compounds were reasonably degraded by the bacterial isolates. It is possible that the intermediate degradation products were readily utilised by the bacteria or no longer detectable by the PDA-LC-MS methods used to monitor the degradation of MCs and NOD, i.e. a lack of chromophore in the degradation products. Moreover, the degradation products of MCs and NOD may be less stable to be processed and the concentration of the compounds were insufficient to observe degradation products. These results seem to be consistent with those of Manage *et al*., (2009), where the authors did not observe any intermediate product of MC-LR in a batch study.

Furthermore, Rapala *et al*., (2005), Tsuji *et al*., (2006), Lames *et al*., (2008), Hu *et al*., (2009) and Zang *et al*., (2010) described the degradation of MCs; however degradation products were either not detected or not described. These results can be attributed to their greater diversity of degradation pathways which are yet to be elucidated. In order to understand the biodegradation diversity, it is important to elucidate the degradation mechanism, hydrolysing enzyme and the genes involved in the whole process.

As previously described by Dittmann *et al*., (1997), the cyanotoxins like MCs, NOD, ABPN are synthesised by non-ribosomal peptide synthesise system (NRPS) similar to the production of most cyclic antibiotics (Marahiel *et al*., 1997). Thus, it is interesting to speculate the possibility of these bacteria degrading several different cyanobacterial and non-cyanobacterial peptides in nature (chapter 4).

3.4.3 Seasonal dynamics of nutrients in Carron river and biodegradation MC-LR

To evaluate the possible influence of seasonal dynamics of the nutrients in water affecting the bacterial biodegradation; *Rhodococcus* sp. (C1) was inoculated in the Carron river water which had been collected in the summer and the winter time of the year. MC-LR was added to the samples representing the MCs, and the degradation was monitored by PDA-LC-MS at regular intervals.

The PDA-LC-MS analysis of the remaining amount of MC-LR has revealed a rapid degradation performed by *Rhodococcus* sp. (C1). However, the MC-LR introduced into the July water was degraded more rapidly compared to that of the February water suggesting, the degradation of MC-LR was influenced by the nutrient composition in the July water. Generally, the nutrient composition in the water during the summer period of the year is considered to be rich with different nutrients (Huang *et al*., 2008); specially carbon, nitrate and phosphate. Moreover, the studies carried out by Zaccone *et al*., (2002) have clearly described the effect of seasonal variance of nutrients in the water that

increases metabolic activities in heterotrophic bacteria. Furthermore, another interesting point worth considering regarding the influence of water chemistry is the presence of different peptides or amino acids which could possibly induce the bacterial metabolism towards MCs and NOD. This was clearly evidenced by the study of Ishii *et al*., (2004) where, *Sphingomonas* sp. 7CY could degrade NOD-Har only when MC-RR is present in the media. The authors suggested that NOD-Har degradation is induced either by MC-RR or its degradation products. It is probable that the bacteria are frequently exposed to different amino acids, peptides in nature, which could subsequently enhance rapid degradation of cyanotoxins.

Furthermore, the present study was extended with a Biolog MT2 assay as a rapid tool to confirm the variation of bacterial metabolism in the two different types of river water. Since the bacteria were pre-incubated (5 d) in two different waters (winter and summer), it was not surprising to observe diverse metabolic response in the Biolog MT2 assay. The bacterial degradation in the July water has shown a slight metabolic inhibition with MC-LR during the 12 h of inoculation compared to the February water. This disparity could have occurred due to an inhibitory substances present in the river water causing a metabolic hindrance in bacteria. Amor *et al*., (2001) determined that the presence of some heavy metals such as nickel, cadmium and zinc could inhibit the bacterial degradation process. Likewise in this study, it is also probable that some inhibitory substances present in the Carron river water is the result of an extended lag period on bacterial metabolism. Nevertheless, once the metabolism commenced approximately after 12 h, a rapid utilisation of MC-LR was evident. Once again, it is not surprising that the bacterial metabolism

occurred in the February water was considerably low suggesting the relationship between the water chemistry and bacterial metabolism.

Further analysis of Biolog samples through the PDA-LC-MS revealed the presence of MC-LR degradation products from the samples of the July water, confirming that MC-LR degradation had taken place in the Biolog MT2 assay. The characteristic degradation products of MC-LR representing Adda at *m/z* 135 ($PhCH_2CHOMe$) and linear MC-LR at *m/z* 1013 (Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH+H) were shown to be present in PDA-LC-MS analysis. These typical MC-LR degradation products (table 3.6) were well characterised by Bourne *et al*., (1996), Imanishi *et al*., (2005) and Kato *et al*., (2007). Edwards *et al*., (2008) reported the presence of linear MC-LR, characteristic degradation products of MC-LF and NOD by the indigenous bacterial community from Loch Rescobie where *Arthrobacter* sp. (R1, R4, R6 and R9) were isolated from. Surprisingly, the previous studies carried out by Manage *et al*., (2009) have revealed that specific *mlrA, mlrB* and *mlrC* genes were not found in these *Actinobacteria* isolates. Bourne *et al*., (1996) have described that the above characteristic degradation products were exclusively produced by hydrolysing enzymes encoded by *mlrA, mlrB* and *mlrC* genes of *Sphingomonas* sp. (Bourne *et al*., 2001). From the similarity of the characteristic MC-LR degradation products shown here, it is fascinating to speculate the possible prevalence of a similar gene cluster producing MC-LR hydrolysing enzymes from novel *Actinobacteria*. It is also possible that present *Actinobacteria* isolates may harbour entirely new genes which could produce similar MC degrading enzymes to that described by Bourne *et al*., (2001), Ho *et al*., (2007) and Okano *et al*., (2009). However, due to the low concentration

of the degradation products and the quantity of the Biolog assay samples, further investigation of the degradation products were not feasible at this point.

3.4.4 Biodegradation of MC-LR in synthetic fresh waters and optimisation of the enrichment media

According to the previous studies, it was evident that *Rhodococcus* sp. (C1) is capable of degrading MC-LR regardless of the synthetic media. However, compared to the synthetic media they have been exposed, MC-LR was rapidly degraded in BG-II medium where 67% of the compound was degraded by *Rhodococcus* sp. (C1). In contrast to that, the bacteria in PBS has shown comparatively low MC-LR degradation rate indicating a clear influence of the nutrient composition in BG-II. It was interesting to note that BG-II contained 750 µg l^{-1} of nitrate and 40 µg l^{-1} phosphate (table 2.2) within the range of a eutrophic lake where nitrate and phosphate rates between 500-1100 µg l^{-1} and 30-100 µg l^{-1} respectively (Yang *et al*., 2008). MC-LR degradation occurred in synthetic medium S3 has shown considerably slow degradation rate compared to S2, S3 and BG-II. This could be explained with reference to the nutrient composition of the S3 medium. Despite the slight similarity of the nutrient content compared to that of BG-II, major nutrients, such as nitrate and phosphate were not available in the S3 media (table 3.2). Hence, it is evident that essential nutrients, such as nitrate and phosphate had enhanced the bacterial biodegradation in the current study. Furthermore, this argument could be supported by observing the subsequent biodegradation rate in the S2 medium. Synthetic medium S2 had contained both nitrate and phosphate

sources as in BG-II; however, the final concentration of each single nutrient in the media was reasonably low compared to that of BG-II (table 3.2). Hence, it's not surprising to observe comparatively slow rates of degradation in S3 medium compared to BG-II. Huang *et al*., (2008) has reported that, under no essential nutrients in the media, hydro-carbon degrading bacteria are not able to efficiently metabolise the oil components despite abundant carbon sources. Moreover, Leahy and Colwell, (1990) and Alexander, (1991) has clearly described that the scarcity of necessary nutrients (C, N, P) limits the growth and degradation abilities of the bacteria. To overcome this, Huang *et al*., (2008) has reported the use of fertilisers as rich sources of nitrate and phosphate. In a recent study, Li *et al*., (2011) clearly demonstrated the effect of nitrate on the biodegradation of MC-LR using biofilm forming bacteria. The degree and rate of degradation of MC-LR was significantly stimulated with the addition of nitrate (sodium nitrate). Interestingly, this literature supports the diversity of the MC-LR biodegradation which related to their abundance of essential nutrients required for maximum rate of biodegradation. Subsequently, upon the removal of the BG-II's trace metal elements, no alteration of MC-LR degradation has been observed. It is probable that the vital nutrient component which enhances biodegradation is not in the trace metal elements itself. However, it is also important to note that the trace metals in BG-II do not pose any inhibitory effect on the bacterial degradation, possibly due to the low concentration of the compounds. As previously described, BG-II is a designed synthetic media for culturing of the cyanobacteria; typical of a eutrophic lake. In general, MC producing cyanobacteria, such as *Mycrocystis* sp. are highly abundant in fresh water lakes of rich contents such as nitrate and phosphate. Furthermore,

eutrophication and the consequential bloom production have been well described in the literature elsewhere (Paerl *et al*., 2011). Hence, it is highly likely that the eutrophic surface water which requires a bacterial remediation against MC is possibly similar to nutrient rich media like BG-II. This consequently makes BG-II a potential candidate for a biodegradation media to evaluate and optimise MC degradation dynamics. Further evaluation of the MC degradation in synthetic media has been extended to determine the effect of an additional carbon source in the media. Most studies on MC degradation have been investigated in the presence of an additional carbon source in the media (Jones *et al*., 1994; Jones and Orr, 1994; Rapala *et al*., 1994; Cousins *et al*., 1996; Park *et al*., 2001; Christofferson *et al*., 2002; Ishii *et al*., 2004; Eleuterio and Batista, 2010; Shimizu *et al*., 2011). However, it should be noted that only Christofferson *et al*., (2002) and Eleuterio and Batista, (2010) have compared the degradation of MCs in the presence and the absence of an additional carbon source in the media. Christofferson *et al*., (2002) has demonstrated that MC-LR and the additional source of carbon degradation commenced at the same time, whilst Eleuterio and Batista, (2010) had observed that the degradation of MC-LR had been delayed until the concentration of the carbon source (acetate) was significantly diminished. This behaviour of bacteria suggests that the degradation process is based on an enzymatic activity rather than a nutrient inducible mechanism. However, having an additional carbon source in media could possibly lead to an ultimate substrate competition that potentially reduces the effectiveness of biodegradation of MCs. Thus, it is important to study the effect of an additional carbon source on current *Actinobacteria* isolates before they are employed in the water remediation process. However, in contrast to the findings of

Eleuterio and Batista, (2010), results of the current study indicate a rapid degradation of MC-LR in the presence of a carbon source (glucose). Surono *et al*., (2008) suggested that glucose supplies energy to microbial cells at stationary phase which also produces additional ATP for cell maintenance and survival during the MC-LR degradation by *Lactobacillus plantarum*. The authors have studied the cell metabolism of MC-LR under different concentrations of glucose and described low cell viability with an increasing dose of glucose besides the high removal of MC-LR. However, the current study did not evaluate the effect of high doses of a carbon source with regards to the bacterial metabolism of MC-LR. This could possibly be important to implement prior to these isolates being utilised in the water purification since all surface waters contain a variable amount of carbon derived from natural organic matter.

Considering the degradation potential of these bacteria (*Rhodococcus* sp., *Brevibacterium* sp. and *Arthrobacter* sp.) it is possible to propose a technical set up for drinking water purification. MC contaminated water could be pumped and filtered through MC degrading biofilms to allow the degradation of MCs in the water. The set up could be optimized using a batch reactor to achieve maximum degradation of MCs by adjusting the filtration time and the surface area of the biofilms. To further enhance the aeration of the biofilms and the water passing through them the implementation of several slowly rotating plates would be useful. Filtered water would be analyzed using HPLC-MS to determine the MC concentration and could be diverted to the reactor depending on the concentration of the toxins. Purified water with no MCs can be pumped out for the next step of water purification. However, further

studies are necessary to fully understand the optimum nutrient condition that provides the maximum biodegradation of MCs and NOD as this could play a key role to determine the major steps of the bacterial water remediation system. For example, water which has undergone full treatment steps will be much lower in many nutrients (i.e. nitrogen, phosphorus and carbon) compared to the water in early steps of the treatment process. Thus, it may be important to employ the bacteria in the early stages of water purification to achieve maximum removal of MCs.

3.5 Conclusions

This study reveals a number of novel findings on bacterial bioremediation of MCs and NOD. This is the first report on the multiple degradation of a wide range of MCs and NOD by a number of bacterial isolates belonging to *Actinobacteria*. Isolates of *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6, F7 and R4) and *Brevibacterium* sp. (F3) were capable of performing an overall degradation on MCs and NOD. From the results shown in the current studies, the following hypothesis could clearly be drawn, of which the novel *Actinobacteria* isolates harbour non-specific cyanopeptide (MC and NOD) degradation mechanism. It is probable that these bacteria might harbour gene(s) that produces universal MC degrading enzyme(s) which could be potential of elucidating in further studies. One of the most fascinating observations in this study was the disparity of the bacterial metabolism and the actual degradation of MCs and NOD. Conversely, it was interesting to note that the most significant factor which influences the biodegradation of MCs and NOD is the chemistry of the compound, not the bacterial isolate itself.

The influence of essential nutrients such as carbon, nitrogen and phosphorus for the degradation of MC-LR was confirmed by the versatile degradation dynamics in different waters (summer and winter) and different synthetic media. Additionally, the previously isolated and characterised *P. toxinivorans* degrading MC-LR, -LY and NOD was found to degrade MC-LF and -LW in the present study. Further studies are required to unravel the new degradation mechanisms, pathways and novel genes involved in the *Actinobacteria* degradation of diverse cyanotoxins.

CHAPTER 4

UNRAVELLING THE INDUCIBLE PEPTIDE DEGRADATION MECHANISMS OF NOVEL MC DEGRADERS

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4 Unravelling the inducible peptide degradation mechanisms of novel MC degraders

4.1 Introduction

In this work it has been shown that the novel *Actinobacteria* (*Rhodococcus* sp., *Brevibacterium* sp. and *Arthrobacter* sp.) are capable of degrading a wide range of MCs and NOD. In chapter 3, commonly occurring MCs such as MC-LR, -LY, -LF, -LW, -RR and NOD underwent rapid biodegradation from these MC degraders. In addition to the non-specific biodegradation of MCs and NOD, a number of observations needing further evaluation were noted. The relative degree of metabolism observed in the Biolog MT2 assay (chapter 3; figure 3.4) did not reflect the rate of degradation in the subsequent batch study (chapter 3; table 3.5). The degradation products of cyanotoxins were no longer detectable in the media suggesting that, they are further degraded or biotransformed into undetectable compounds (Manage *et al*., 2009). Furthermore, it was demonstrated that the most significant factor influencing degradation was the chemistry of the compound, not the bacterial isolate itself (Lawton *et al*., 2011); hence the rates and the patterns of degradation were notably different. Consequently, taking all these observations into consideration it implied a possible enzyme mediated degradation.

Enzymatic degradation of MC-LR had been investigated and characterised by a number of authors. Bourne *et al*., (1996, 2001) described an enzymatic degradation pathway for MC-LR by MlrA, MlrB and MlrC enzymes using a

Sphingomonas sp. Further studies (Imanishi *et al*., 2005; Kato *et al*., 2007) using similar bacterial strains have confirmed the previous findings as the only known degradation pathway. Initial enzyme cleavage of the Adda-arg bond forms linear MC-LR which subsequently degraded into a tetrapeptide and finally produced Adda or smaller amino acids (chapter 1; figure 1.5). However, little information is available regarding how microorganisms trigger this activity and how *mlr* gene cluster (MC degrading gene cluster; Bourne *et al*., 2001) is expressed to produce MC degrading enzymes MlrA, MlrB and MlrC. To investigate this, Shimizu *et al*., (2011) studied the influence of MC pre-exposure on its subsequent degradation, which interestingly indicated that MC induce the activity of MC degrading enzymes. Moreover, authors also reported elevated MC degradation rates when MC was added twice to the media, and observed that degradation products (tetrapeptide and Adda) consequently influence *mlrA* and *mlrB* (MC degrading genes) expression towards the production of MC degrading enzymes. Ho *et al*., (2010) studied the biological removal of MC-LR in waste water treatment plants using a real-time PCR (polymerase chain reaction) targeting *mlrA* gene abundance during the degradation process. It was reported that the MC-LR removal was directly proportional to the number of *mlrA* copies detected in the real-time PCR. In contrast to that, in the absence of MC-LR, the *mlrA* gene did not indicate any increasing number of copies.

These bacterial isolates (*Rhodococcus* sp., *Arthrobacter* sp. and *Brevibacterium* sp.) are Gram-positive *Actinobacteria* and do not harbour *mlr* gene cluster (Manage *et al*., 2009) reported from the Gram-negative *Proteobacteria* isolates.

However, when they were isolated through a MC enrichment step (14 d exposure to MC-LR, -LF and –LW), all of the isolates performed complete degradation of MC-LR within 3 d suggesting that different MC degradation pathways may exist. A point worth considering for the present studies here is, whether MC enrichment had induced the expression of the genes responsible for the rapid degradation of MC-LR. Sub-culturing of bacteria under no exposure for MC like peptides had clearly been affected to reduce the rate of MC-LR degradation in later studies (chapter 3 and Lawton *et al*., 2011) which implies an inducible degradation mechanism. If these bacteria are to be employed in water remediation against MCs, it is essential that this aspect of their behaviour be fully investigated to ensure optimum biodegradation performance.

This chapter aims to investigate the possible existence of an inducible degradation mechanism in novel MC degraders. To ensure the maximum performance, bacteria stored in - 80 °C after the initial screening (Manage *et al*., 2009) were retrieved, revived and their biodegradation potential was evaluated in a traditional batch study. The optimal bacterial density and carbon starvation period was assessed in a batch study to determine the maximum biodegradation efficacy that can be obtained from the revived bacteria.

Prior to the in-depth studies to evaluate the bacterial inducible mechanisms, *Rhodococcus* sp. (C1) was exposed to MC-LR for 7, 10, 14 and 21 d then subsequent degradation rates of MC-LR were assessed. This has revealed that, the rate of MC-LR degradation was increased with the length of pre-exposure. To

understand the influence of pre-exposure to different peptides other than MC-LR on the expression of bacterial degradation mechanisms, *Rhodococcus* sp. (C1) was inoculated with different peptides. The peptides were selected depending on their origin (cyanobacterial, non-cyanobacterial, eukaryotic or prokaryotic) and the structure (cyclic or linear). In addition to that, to assess the possible degradation of different peptides by the *Rhodococcus* sp. (C1) during the pre-exposure time, the level of peptide added to the media was monitored periodically using PDA-LC-MS.

4.2 Materials and methods

4.2.1 Chemicals

Chemicals were of analytical-reagent grade unless stated and obtained from Fisher Scientific, Leicestershire, UK. HPLC-grade methanol and acetonitrile were purchased from Rathburn, Walkersburn, UK. Trifluoroacetic acid (TFA) and formic acid for HPLC systems were purchased from Fisher Scientific, Leicestershire, UK and Sigma-Aldrich, Dorset, UK, respectively. Pure water was obtained from a Milli-Q system (purified to 18.2 MΩ, Millipore, Watford, UK). MC-LR and ABPNB were purified from batch cultures of *Microcystis aeruginosa* (table 2.1) as previously described (Edwards *et al*., 1996; Edwards and Lawton, 2010). LNOD was obtained from the cultures of *Nodularia spumigena* KAC66 (table 2.1). Angiotensin III (ANGTN), Cyclosporin A (CY), Gramicidin A (GRA) and Polymyxin B (POLYB) were purchased from Sigma-Aldrich, Dorset, UK.

4.2.2 Water samples

Water samples were collected (July, 2009) from Carron river (NO 876864) which was the source of the isolate *Rhodococcus* sp. (C1; table 3.1). The collection and storage was made using sterile Pyrex glass bottles (1 l) and kept refrigerated (4 °C) until required. All water samples were first filtered through Whatman GF/C filters to remove suspended particles and sterilised by passage through 0.22 µm filters (Millipore, UK). Prior to use in experiments, water samples were analysed by PDA-LC-MS to determine the presence of any MCs, ABPN, NOD or any other naturally occurring peptides as described in chapter 2, section 2.2.8.

4.2.3 Preparation and the analysis of peptides

4.2.3.1 Preparation of peptide stock solutions

Dry material of MC-LR, ABPNB, NOD and LNOD were re-suspended in 500 µl of 80% methanol and diluted to 200 µg ml^{-1} with Milli-Q to prepare stock solutions. ANGTN and POLYB were directly diluted with Milli-Q as they were readily soluble in water to make up stock solutions (200 µg ml^{-1}). CY and GRA were first re-suspended in 500 µl of dimethyl sulfoxide (DMSO) and diluted to 200 µg ml^{-1} with Milli-Q. The solvent concentrations of MeOH and DMSO were 0.02%. All of the peptide stock solutions were sterilised by passage through 0.22 µm filter (Dynaguard, Fisher, UK).

4.2.3.2 Peptide calibration curves

Calibration curves for ANGTN, POLYB, CY and GRA were prepared using serial dilutions as previously described in section 2.2.9. Triplicates of the samples were analysed by PDA-LC-MS; section 2.2.8. Analysis of GRA was performed by ultra performance liquid chromatography (UPLC; Waters, Elstree, UK) as described in section 4.2.3.3. Regression curves and correlation coefficient was obtained from the plotted graphs of concentration against peak area of the ESI-MS ion mass chromatograms.

4.2.3.3 Analysis of peptides

Analysis of MC-LR, ABPNB, LNOD, ANGTN, CY, GRA and POLYB were based on the HPLC methods described previously in chapter 2, section 2.2.8. Analysis of GRA was performed by UPLC system (ACQUITY UPLC System) with photodiode array (ACQUITY UPLC PDA) equipped with Tandem Quadruple Mass Spectrometer (Xevo TQD) in series (Waters, Elstree, UK). The separation was affected with an ACQUITY UPLC BEH C_{18} column (2.1 mm; 100 mm long; 1.7 µm particle size; Waters, UK) maintained at 40 °C. Mobile phase was Milli-Q water (A) and acetonitrile (B) both containing 0.1% formic acid constituted the mobile phase. Samples were separated using a gradient increasing from 50% to 100% B over 10 min at a flow rate of 0.2 ml min^{-1} followed by ramp up to 100% B and re-equilibration over the next 5 min. Eluent was monitored from 200-400 nm with a resolution 1.2 nm and peptides were quantified by external standard at its highest concentration. Mass spectrometry analysis were all performed in positive ion electro-spray mode (ES+), scanning from *m/z* 50 to 2000 Da with a scan time of 0.25 s and inter-scan delay of 0.025 s. The capillary voltage was set at 3.0 kV and cone voltage at 25.0 V. The source and desolvation temperatures were set to 80 °C and 300 °C respectively. Flow rate for cone gas and desolvation gas were arranged at 50 and 40 l h^{-1} respectively. Instrumental control, data acquisition and processing were achieved using Masslynx v4.1.

4.2.4 Recovery of bacteria stored at -80 °C

Actinobacteria isolates previously stored at -80 °C were recovered to evaluate the MC-LR degradation difference between initially isolated bacteria which had been stored since isolation and the same isolates that had repeatedly undergone sub-culturing. Cryopreserved vials were recovered from the -80 °C freezer and immediately revived by streaking on a solidified NA plate to obtain single colonies. NA plates were incubated at 25 °C for 24 h; single colonies were transferred aseptically into sterile universal bottles containing 5 ml of NB and subjected to a subsequent incubation at 25 °C for 24 h with shaking (150 rpm).

To differentiate the recovered isolates from regularly sub-cultured bacteria, recovered isolates were named with an additional letter of "N". For example, *Rhodococcus* sp. (C1) recovered from -80 °C was named as *Rhodococcus* sp. (NC1).

To determine the bacterial growth, *Rhodococcus* sp. (NC1) was grown in liquid NB media (100 ml culture volume in 500 ml flask) with incubating (25 °C, 150 rpm) and the OD ($A_{590\ nm}$) of the culture was monitored spectroscopically at regular intervals (3 h).

The bacterial suspensions required for the biodegradation studies were prepared from the exponentially growing cultures by centrifuge (3000 g, 15 min, 25 °C), re-suspended and washed with sterile 0.01 M PBS.

4.2.5 Degradation of MC-LR by cryopreserved (-80 °C) *Actinobacteria* isolates

Cell suspensions of *Rhodococcus* sp. (NC1), *Arthrobacter* sp. (NC6, NF7 and NR4) and *Brevibacterium* sp. (NF3) were prepared (section 4.2.4) and bacterial inocula ($A_{590\ nm}$ = 0.35) were prepared after depleting the carbon overnight in sterile 0.01 M PBS. To evaluate the MC-LR degradation by the bacteria immediately recovered from -80 °C storage, a batch study was performed. A volume of 50 µl, OD adjusted bacterial cell suspension were added into sterile Bijou bottles (x3) containing sterile Carron water (900 µl) spiked with 50 µl aqueous sterile MC-LR (final concentration 10 µg ml^{-1}) to give a final volume of 1 ml in the bottle. Regularly sub-cultured *Rhodococcus* sp. (C1) was prepared as the experimental control to the recovered isolates (-80 °C). Bottles were incubated (25 °C, 150 rpm), sampled (0, 3 and 5 d) by placing the Bijou plus contents in the freezer then freeze drying. Samples for PDA-LC-MS analysis were prepared from freeze dried samples, reconstituted in 500 µl of 50% aqueous methanol, vortexed thoroughly, transferred into microcentrifuge tubes (1.5 ml) and centrifuged at 10000 g for 10 min and the supernatant (100 µl) was withdrawn for PDA-LC-MS analysis (section 2.2.8).

4.2.6 The effect of bacterial carbon starvation on MC-LR degradation

To evaluate the effect of carbon starvation history on subsequent degradation of MC-LR, *Rhodococcus* sp. (C1) isolate was prepared as described in section 4.2.4 and cell suspensions were incubated (25 °C, 150 rpm) in sterile 0.01 M PBS and

carbon starved for 24, 36 and 48 h. To obtain non-starved bacteria, in a parallel batch, *Rhodococcus* sp. (C1) were grown for 24 h in NB and harvested by centrifuge (3000 g, 15 min) and immediately used as the experimental control. Carbon starved and non-starved bacterial suspensions were prepared ($A_{590\ nm}$ = 0.35), inoculated (50 µl) in sterile Bijou bottles (x3) containing sterile Carron water (900 µl) spiked with 50 µl of MC-LR (final concentration 10 µg ml^{-1}). Bottles were incubated (25 °C, 150 rpm), sampled (0, 3 and 5 d), freeze dried and prepared for PDA-LC-MS analysis as described in section 4.2.5.

4.2.7 Effect of bacterial cell density on MC-LR degradation

Rhodococcus sp. (C1) cell suspensions were prepared from the exponentially growing cultures as described earlier (section 4.2.4) and carbon depleted for 24 h (section 4.2.6). Their cell densities were adjusted to $OD_{590\ nm}$ values of 0.05, 0.1 and 0.35 to determine the effect of cell density on MC-LR degradation. Batch study was conducted using the same experimental procedures described above (section 4.2.5).

4.2.8 MC-LR degradation by MC-LR induced *Rhodococcus* sp. (C1)

4.2.8.1 Preparation of MC-LR induced *Rhodococcus* sp. (C1)

Rhodococcus sp. (C1) cultures prepared (section 4.2.4) and cell suspensions ($A_{590\ nm}$ = 0.35) were made in 0.01 M PBS after overnight carbon depletion (section 4.2.5). Immediately, an aliquot of 0.5 ml of the equalised bacterial suspension

was inoculated into sterile 50 ml BD Falcon conical tubes (Fisher, UK) (x3) containing 9 ml of 0.22 µm sterile Carron river water. Cultures were spiked with sterile aqueous MC-LR solutions (0.5 ml) at a final concentration of 10 µg ml^{-1}. The control experiment contained sterile river water inoculated with C1 bacteria in the absence of MC-LR to determine the influence of MC pre-exposure for their response of subsequent MC-LR degradation. The sample bottles were incubated for 21 d at 25 °C with shaking at 150 rpm. To maintain the concentration of MC-LR available to induce the bacteria, identical re-spikes (0.5 ml) of MC-LR were made from sterile stock solutions (section 4.2.3.1), at 7 d and 14 d.

4.2.8.2 Degradation of MC-LR by induced *Rhodococcus* sp. (C1)

After the pre-exposure of bacteria to MC-LR and the sterile Carron river water for 21 d, the bacteria were harvested by centrifuging at 3000 g for 15 min, 25 °C. The bacterial pellets were washed (x1) as described (section 4.2.4) and the MC-LR remaining in the suspension was removed. The final turbidity of all bacterial suspensions was $A_{590\ nm}$ = 0.25. As previously described (section 4.2.5), 900 µl of sterile Carron water was spiked with 50 µl of MC-LR (10 µg ml^{-1}) and 50 µl of bacterial cell suspension to give a final volume of 1 ml in the Bijou bottle (x3). Bottles were incubated at constant temperature (25 °C, 150 rpm) and sampled at 0, 3 and 5 d. Samples for PDA-LC-MS were prepared as previously described in section 4.2.5. Experimental control contained regularly sub-cultured *Rhodococcus* sp. (C1) which had not been exposed to MC-LR (> 8 months) during this study (NEX). NEX bacteria were grown in NB (24 h, 25 °C, 150 rpm), cell suspensions

adjusted to $A_{590\ nm}$ = 0.25, inoculated in Bijou bottles with MC-LR (10 µg ml^{-1}), incubated in the same batch (25 °C, 150 rpm), sampled (0, 3, 5 d) and analysed by PDA-LC-MS as previously described.

4.2.9 MC-LR degradation by cyanobacterial and non-cyanobacterial peptide induced *Rhodococcus* sp. (C1)

4.2.9.1 Preparation of peptide induced *Rhodococcus* sp. (C1)

Rhodococcus sp. (C1) cell suspensions ($A_{590\ nm}$ = 0.35) were prepared as previously described in section 4.2.4. Batch culture protocol was carried out (section 4.2.8.1) inoculating an aliquot of 0.5 ml optical density adjusted bacterial suspension into sterile 50 ml BD Falcon™ conical tubes (x3) containing 9 ml of 0.22 µm filter sterilised Carron river water. Culture media were spiked with sterile, aqueous peptides solutions (MC-LR, ABPNB, LNOD, ANGTN and CY; figures 4.1 and 4.2) at a final concentration of 10 µg ml^{-1}. The sample bottles were incubated for 21 d at 25 °C and shaken at 150 rpm. To maintain the concentration of peptides available to induce the bacteria, identical re-spikes (0.5 ml) were made from sterile peptides stock solutions (section 4.2.3.1), at 7 d and 14 d. To determine whether Carron river water (RW) has any influence to induce bacteria for MC degradation, C1 bacteria were inoculated in sterile Carron river water in the absence of peptides and incubated in the same batch (25 °C, 150 rpm, 21 d).

4.2.9.2 Biodegradation of cyanobacterial and non-cyanobacterial peptides by *Rhodococcus* sp. (C1)

To evaluate the possible biodegradation occurring during the peptide pre-exposure period, an aliquot of 500 µl was removed aseptically from the peptide induction media at regular intervals (0, 7, 14 and 21 d) and transferred into microcentrifuge tubes (1.5 ml), frozen immediately and freeze dried. Samples for PDA-LC-MS analysis were prepared as previously described in section 4.2.5.
To investigate the stability of the peptides in sterile Carron river water, all peptides were spiked in sterile river water at 10 µg ml^{-1} and incubated in the same batch, sampled and analysed as previously described.

4.2.9.3 Biodegradation of MC-LR by peptide induced *Rhodococcus* sp. (C1)

After the pre-exposure to MC-LR, ABPN, LNOD, ANGTN, CY and sterile Carron river water for 21 d, the bacteria were harvested (3000 g, 15 min, 25 °C), cell suspensions ($A_{590\ nm}$ = 0.25) were adjusted and the batch degradation study was prepared as previously described in section 4.2.8.2. Bijou bottles containing 50 µl of MC-LR, 900 µl sterile Carron water and 50 µl bacterial inocula were incubated at constant temperature (25 °C, 150 rpm) and sampled at regular intervals (0, 3 and 5 d). A control study contained non-induced bacteria (section 4.2.8.2) and processed in the same batch. Samples for PDA-LC-MS analysis were prepared as previously described in section 4.2.5.

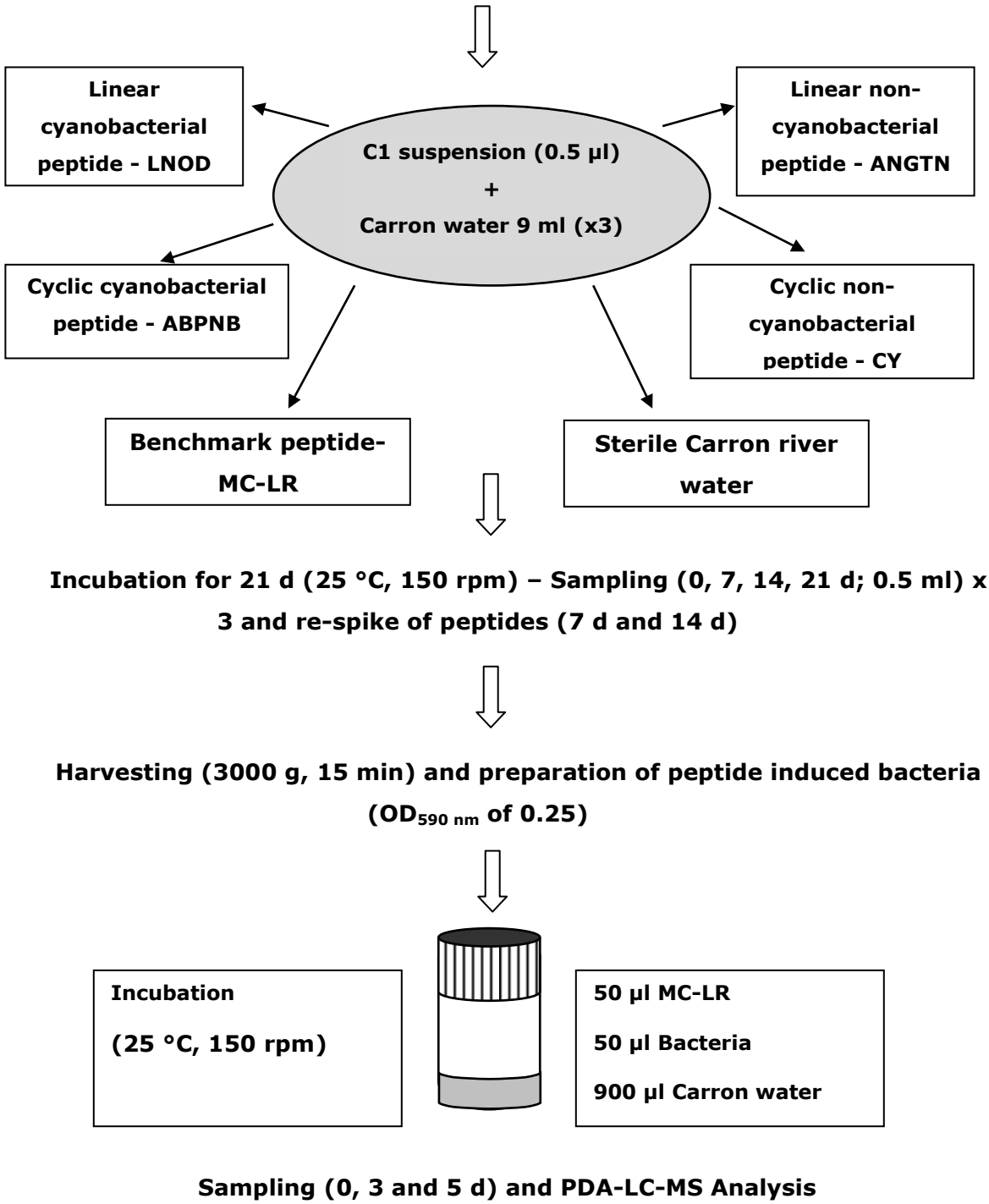

Figure 4.1 Schematic diagram of induced MC-LR degradation by *Rhodococcus* sp. (C1) in sterile Carron water. Experimental control contained bacteria exposed to sterile river water with no peptides inoculated.

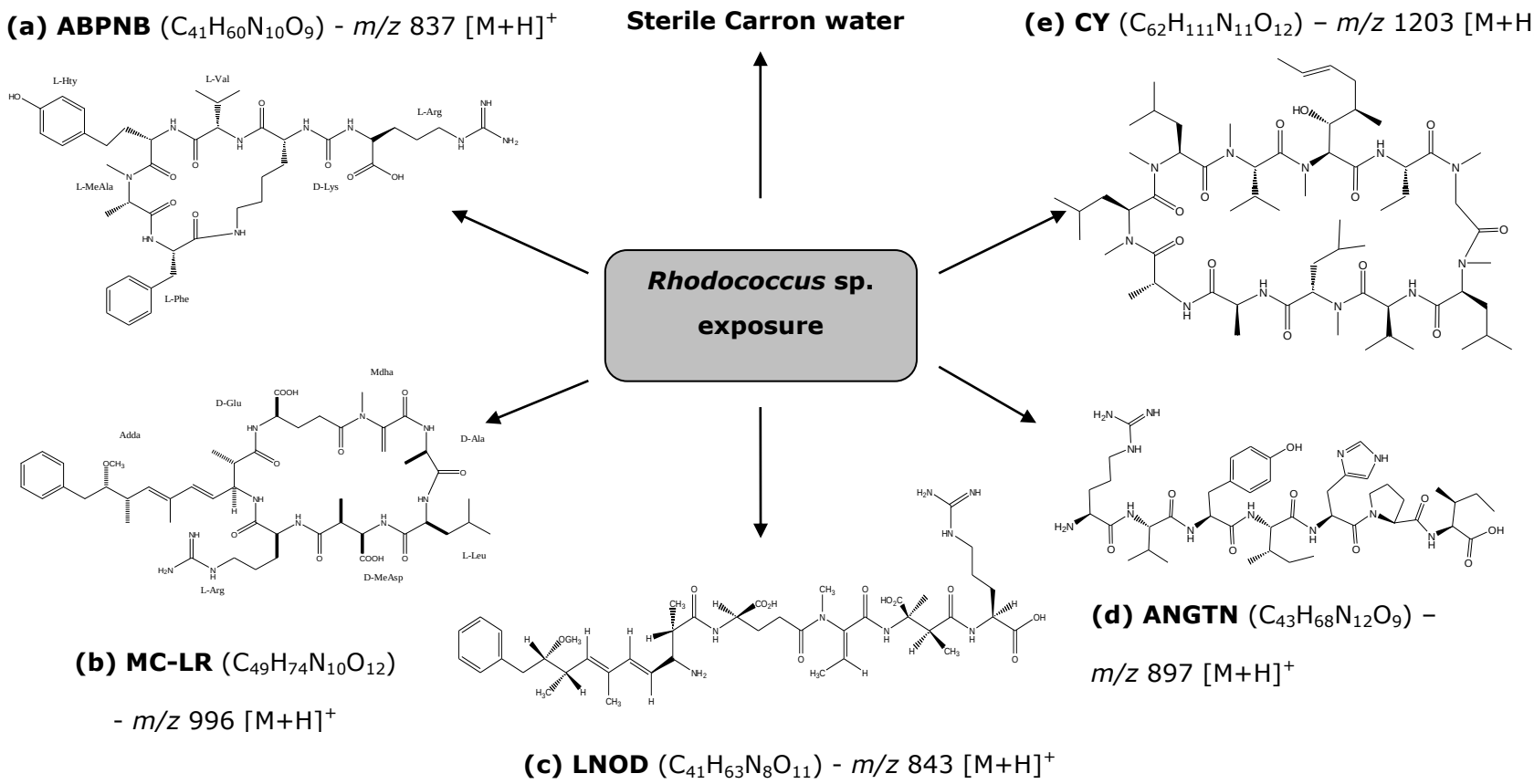

Figure 4.2 The structures of cyanobacterial cyclic (a and b) linear (c), non-cyanobacterial linear (d) and cyclic (e) peptides used for exposure and induction of *Rhodococcus* sp. (C1) in river water.

4.2.10 MC-LR degradation by prokaryotic and eukaryotic peptide induced *Rhodococcus* sp. (C1)

4.2.10.1 Induction and the degradation of peptides by *Rhodococcus* sp. (C1)

Rhodococcus sp. (C1) was induced by MC-LR, ANGTN, CY, GRA and POLYB as previously described in section 4.2.9.1. To maintain the concentration of peptides available to induce the bacteria, identical re-spikes (0.5 ml) were made from sterile peptides stock solutions (section 4.2.3.1), at 7 d and 14 d. Analysis of the degradation of the peptides during the induction period was carried out as previously described (section 4.2.9.2). To determine whether Carron river water (RW) has any influence to induce bacteria for MC degradation, C1 bacteria were inoculated in sterile Carron river water in the absence of peptides and incubated in the same batch (25 °C, 150 rpm, 21 d). To investigate the stability of the peptides in sterile Carron river water, all peptides were spiked in sterile river water at 10 µg ml^{-1} and incubated in the same batch, sampled and analysed with similar conditions as previously described. GRA was analysed by UPLC-MS (section 4.2.3.3).

4.2.10.2 Degradation of MC-LR by *Rhodococcus* sp. (C1) after the exposure to prokaryotic and eukaryotic peptides

After the pre-exposure of bacteria to MC-LR, ANGTN, CY, GRA, POLYB and sterile Carron river water for 21 d, effects on subsequent MC-LR degradation were evaluated according to the previous MC-LR degradation assay by peptides induced bacteria (section 4.2.9.3). Bijou bottles (x3) contained 50 µl of OD adjusted ($A_{590\ nm}$ = 0.25) bacterial suspension and 50 µl of MC-LR (final concentration of 10 $\mu g ml^{-1}$) spiked in 900 µl sterile Carron river water. Experimental control contained non-induced *Rhodococcus* sp. (C1; section 4.2.8.2).

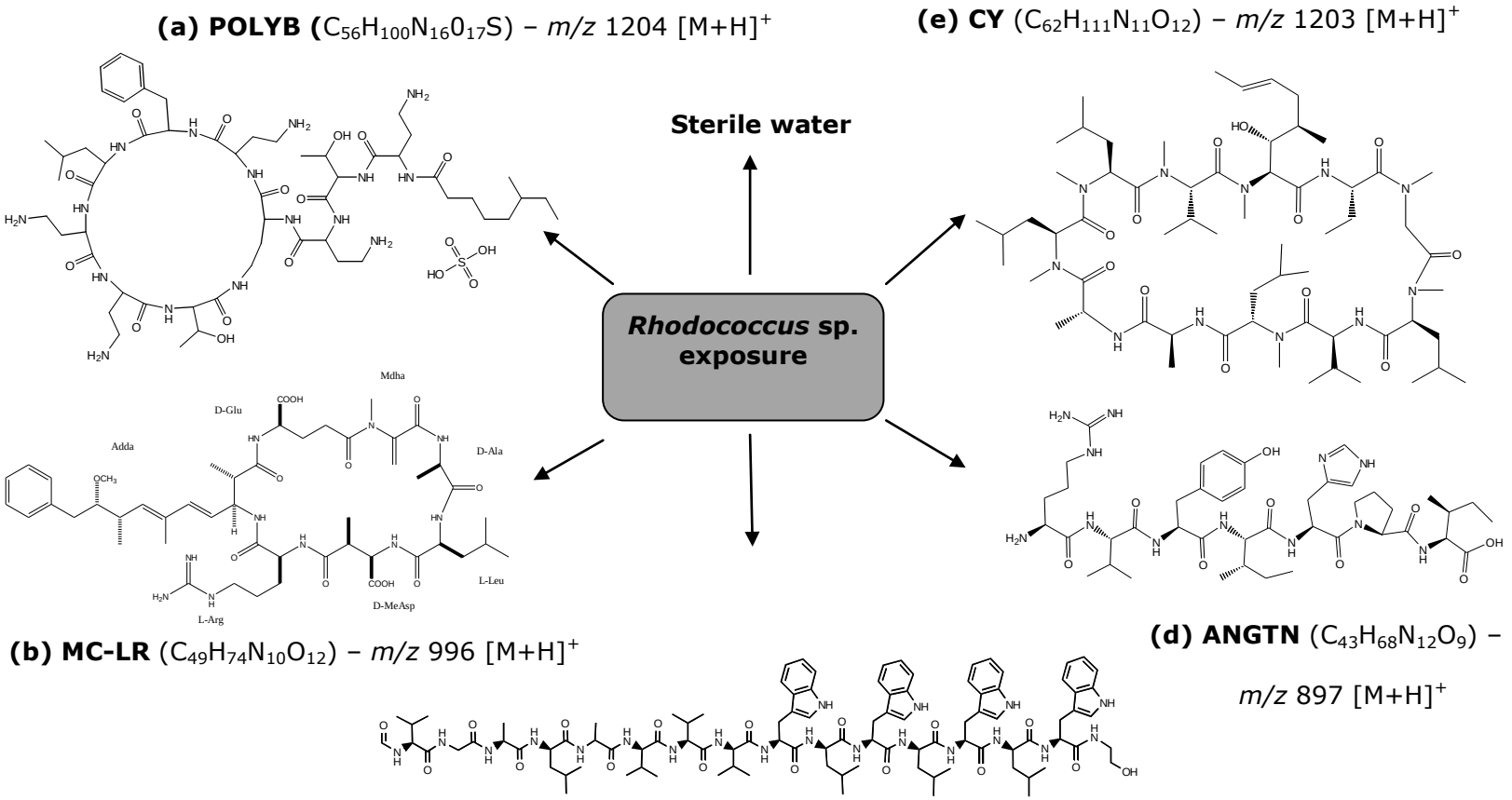

Figure 4.3 The structures of prokaryotic cyclic (a and b) linear (c), eukaryotic linear (d) and cyclic (e) peptides used for exposure and induction of *Rhodococcus* sp. (C1) in river water.

4.2.11 Degradation of different peptides by MC-LR induced *Rhodococcus* sp. (C1)

As previously observed (section 4.2.10.1), MC degradation mechanisms of *Rhodococcus* sp. (C1) can be successfully induced by prokaryotic peptides. Hence, this study was carried out to investigate whether *Rhodococcus* sp. (C1) can be successfully induced by MC-LR towards a rapid degradation of prokaryotic peptides (figure 4.4).

To determine this, MC-LR induced *Rhodococcus* sp. (C1) cells were prepared as previously described in section 4.2.8.1. After 21 d of exposure to MC-LR, bacteria were harvested (3000 g, 15 min, 25 °C), cell suspensions ($A_{590\ nm}$ = 0.25) were prepared (section 4.2.8.1.) and added (50 µl) into separate Bijou bottles (x3) containing 900 µl of sterile Carron river water spiked with MC-LR, LNOD, ABPNB, POLYB and GRA (figure 4.4). Final concentration of the peptides in Bijou bottles was 10 µg ml^{-1}. Sample bottles were incubated (25 °C, 150 rpm), sampled (0, 3 and 5 d) and analysed by PDA-LC-MS as previously described (section 4.2.8.2). GRA was analysed by UPLC-MS (section 4.2.3.3). The control experiment contained *Rhodococcus* sp. (C1) with no history of pre-exposure to MC-LR, LNOD, ABPNB, POLYB or GRA.

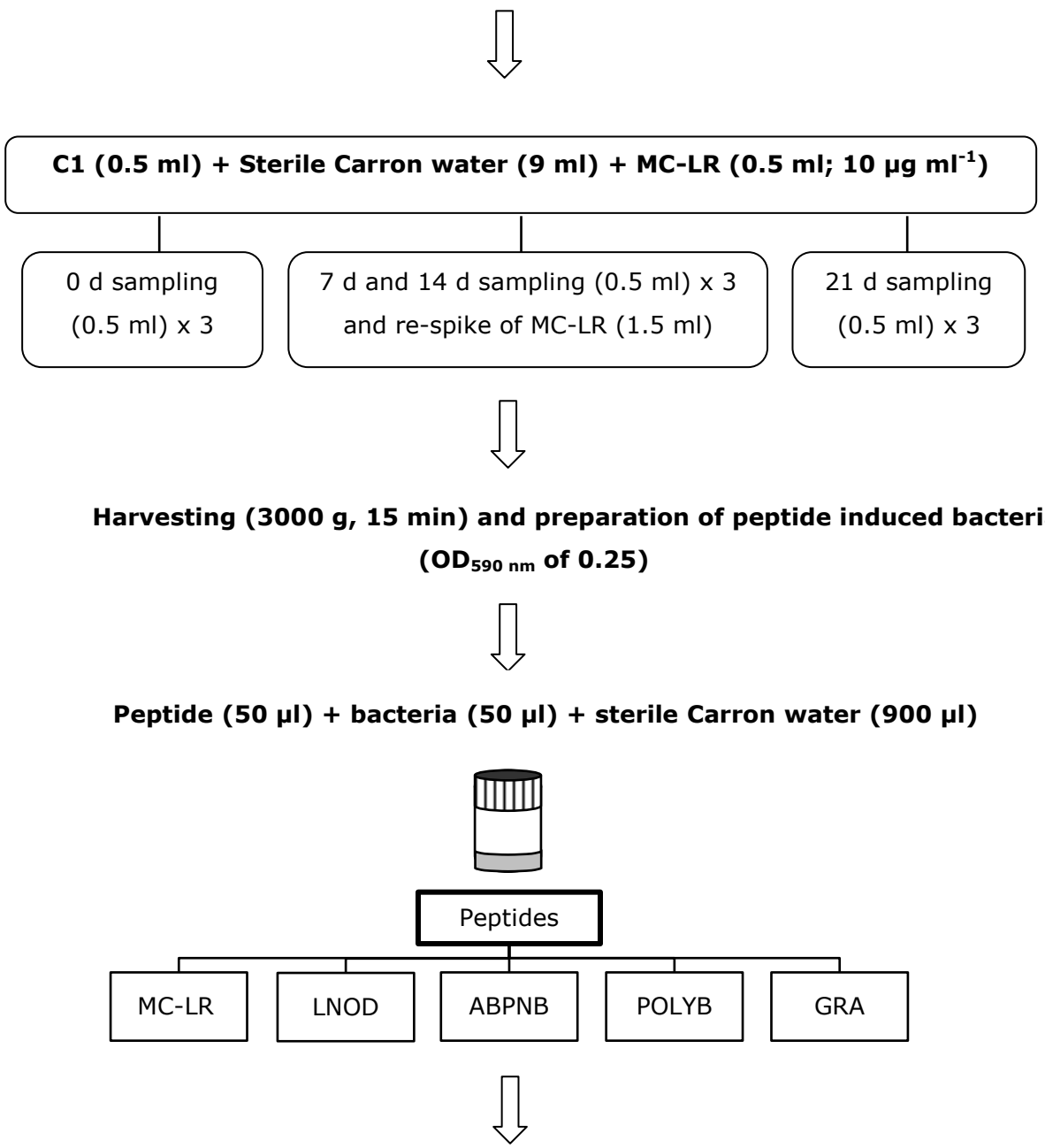

Figure 4.4 Schematic diagram of degradation of prokaryotic peptides (MC-LR, LNOD, ABPNB, POLYB and GRA) by MC-LR induced *Rhodococcus* sp. (C1). Experimental control contained non-induced bacteria.

4.3 Results

4.3.1 Water samples

PDA-LC-MS analysis of the water samples collected in July 2009 from the Carron river revealed that it contained neither MCs nor any other peptides used in the experiment (data not included). The peptides contained in the sample bottles were therefore confirmed as solely added for the experimental purpose. The water contained no other bacterial strains other than *Rhodococcus* sp. as it was sterilised through 0.22 µm filters prior to the bacterial inoculation and therefore any bacterial interaction with the peptides in the river water was solely by *Rhodococcus* sp. (C1).

4.3.2 Calibration curves for peptides

Peptide calibration curves for commercially acquired peptides (ANGTN, CY and POLYB) were prepared using the concentration versus peak area response by PDA-LC-MS. The identification of the peptides was based on their MS spectra and the parent ion described in appendix D. GRA was analysed using the UPLC-MS (appendix D). ESI-MS detection on both HPLC and UPLC-MS was made using the mass of the parent ion (*m/z*) and is described in table 4.1. All of the peptides demonstrated a linear response at ESI-MS (figure 4.2) and maintained a correlation coefficient (R^2) greater than 0.98 on their linear regression (table 4.1). This confirmed the aptness of utilising these peptides in biodegradation studies. In addition to the ESI-MS detection, ANGTN has also demonstrated a

linearity greater than 0.99 in UV absorbance as the maximum absorption wave length of 210 nm (calibration curve is not shown here). The linearity evaluated by $UV_{210\ nm}$ for ANGTN was slightly higher compared to that of linearity by MS. UPLC-MS has revealed comparatively high linearity for GRA ($R^2 > 0.99$) to that of POLYB and CY. In brief, ANGTN has revealed the highest linear response while POLYB gave the lowest linear response within the batch of assessed peptides.

Table 4.1 Evaluation of correlation coefficient (R^2) of different peptides by PDA-LC-MS (ANGTN, CY and POLYB) and UPLC-MS (GRA).

Compound	**R^2 at MS-TIC**	**Structure of the fragment**	**Observed *m/z***
ANGTN	0.995	$[M+H]^+$	897
CY	0.985	$[M+H]^+$	1203
POLYB	0.970	$[M+H]^+$	1204
GRA	0.994	$[M+2H]^{2+}$	942

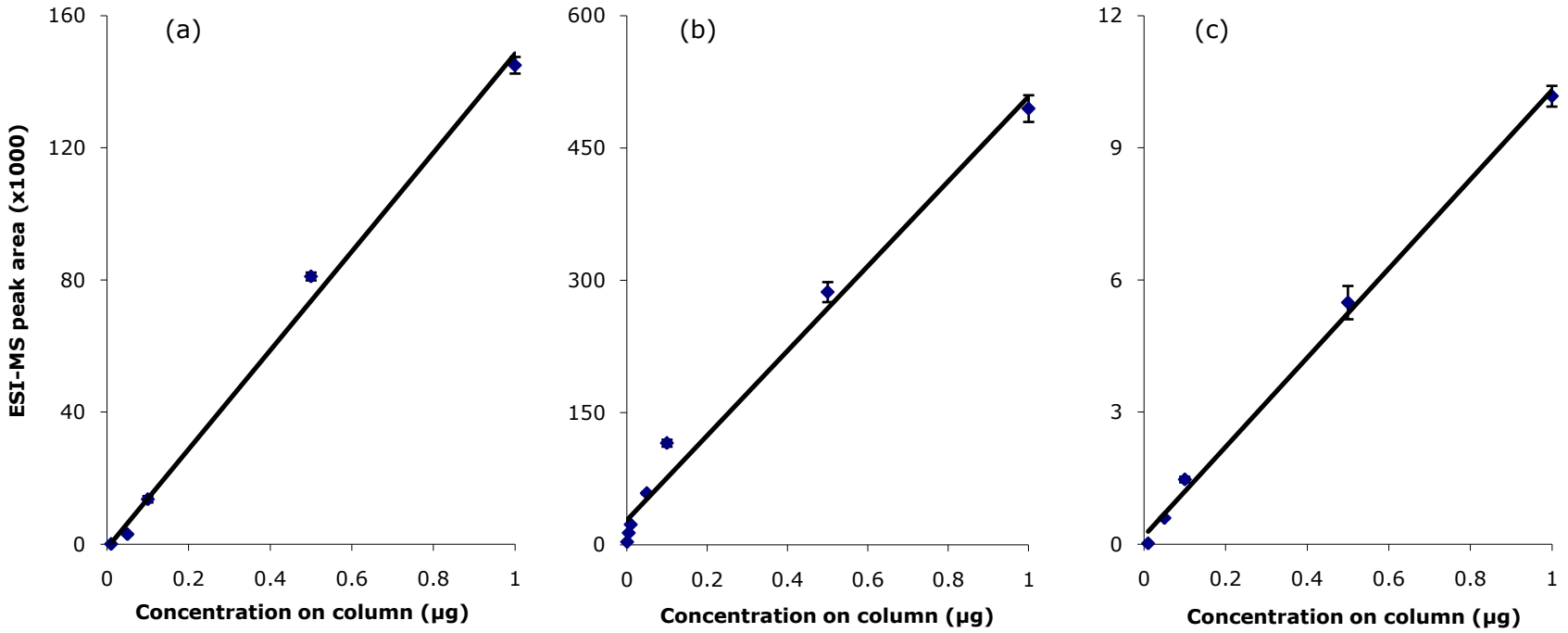

Figure 4.5 Calibration curves for commercially acquired ANGTN, CY and GRA using PDA-LC-MS/UPLC-MS at (a) ANGTN at *m/z* 897, (b) CY at *m/z* 1203 and (c) GRA at *m/z* 942. Error bars represent one standard deviation (n=3).

4.3.3 Recovery of bacteria stored at -80 °C

Long-term (-80 °C) stored bacteria, re-cultured in NB indicated an abundant growth comparatively similar (visual observation) to the regularly maintained *Actinobacteria* isolates (Table 3.1). The time dependant measurement of growth rate of *Rhodococcus* sp. (NC1) has revealed a lag period of ~ 3 h (results not shown) and subsequently proceeded to the exponential state where it reached the maximum cell density of $OD_{590\ nm}$ of 1.55 after 15 h indicating a good cell viability. In addition to that, cell viability was further confirmed by the colonies growing in NA plates. For further maintenance of the freshly recovered bacteria from long-term storage, they were streaked on NA slopes and a parallel batch was transferred into NA stab cultures.

4.3.4 Evaluation of the biodegradation of MC-LR by pre-stored (-80 ºC) bacteria

From the batch of cryopreserved *Actinobacteria* MC degraders, *Rhodococcus* sp. (NC1), *Arthrobacter* sp. (NC6, NF7 and NR4) and *Brevibacterium* sp. (NF3) have been selected to evaluate their biodegradation potential against MC-LR. All of the five isolates demonstrated a pronounced degradation of MC-LR; however, as expected, a slight difference in the degradation rate was evident. In contrast, it was clearly noticed that regularly sub-cultured *Rhodococcus* sp. (C1) -RC1 showed considerably slow degradation of MC-LR (figure 4.6). *Rhodococcus* sp. (NC1), *Arthrobacter* sp. (NR4 and NC6) indicated similar degradation pattern whereas they all removed 88-90% of the compound after 5 d. Meanwhile,

Rhodococcus sp. (NC1) attained the highest degradation of MC-LR whereas 80% and 90% of the toxin was removed respectively after 3 d and 5 d; indicating its potential to be utilised in further biodegradation studies. As observed previously, the degradation of NF3 was low compared to that of other isolates which can be once again attributed to its slow growth rate, i.e. metabolic responses. Despite the rapid removal of MC-LR, none of the bacterial isolates has achieved complete removal of the compound within the test period.

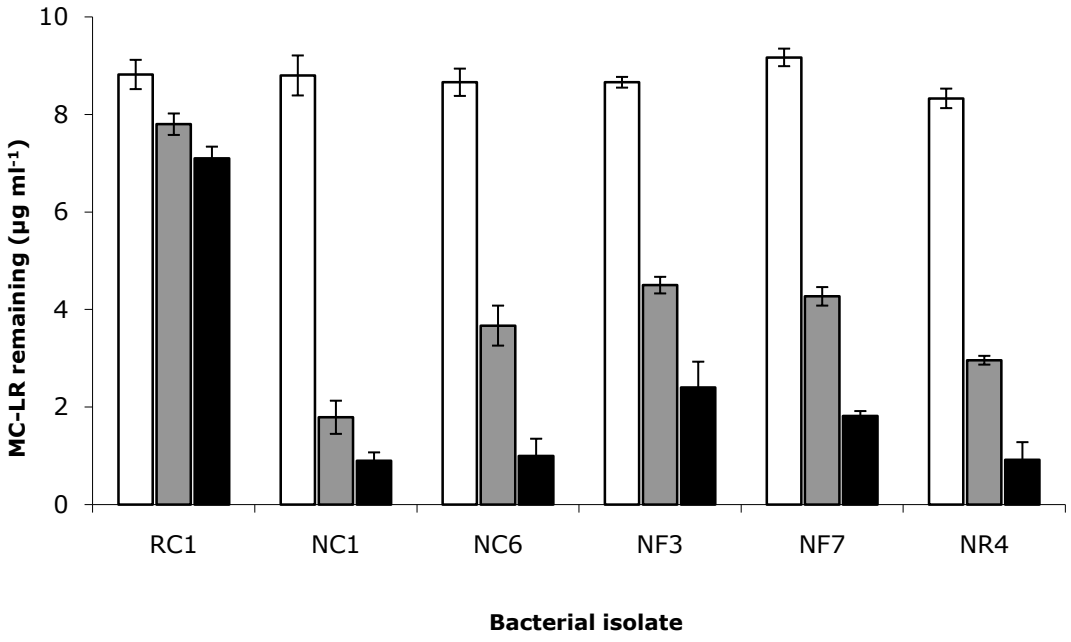

Figure 4.6 Degradation efficiency of MC-LR by pre-stored (-80 ºC) *Actinobacteria* isolates at 0 d (□), 3 d (▩) and 5 d (■) in Carron river water spiked with 10 µg ml^{-1}. RC1 represents the control bacterial strain which was subjected to regular multiple sub-culturing. Error bars represent one standard deviation (n=3).

4.3.5 The effect of bacterial carbon starvation on MC-LR degradation

Degradation of MC-LR has been monitored under carbon starved and non-starved bacteria. Both of the carbon starved and non-starved bacteria were able to degrade MC-LR; although degradation rates were different depending on the length of the carbon starved period. *Rhodococcus* sp. (NC1) starved for 24 h showed a more rapid degradation of MC-LR than the non-starved culture. This is well illustrated by figure 4.7, as 79% of the initial MC-LR has been removed from the media whereas the non-starved bacteria achieved only 35% of toxin removal after 5 d of the study. However, the degradation rate of MC-LR was reduced comparatively when the length of starvation extended beyond 24 h. As shown in figure 4.7, the removal of the compound has been reduced to 64% and 32% when the starvation was extended up to 36 h and 48 h respectively. After 3 d of the incubation, 36 h carbon starved bacteria has shown considerably similar MC-LR degradation rate to the non-starved cells. For example, remaining amount of MC-LR after 5 d was reported as 7 μg ml^{-1} for both 36 h carbon starved and non-starved bacteria (figure 4.7). In summary, the results show a clear influence of carbon starvation of the bacteria on the subsequent degradation of MC-LR.

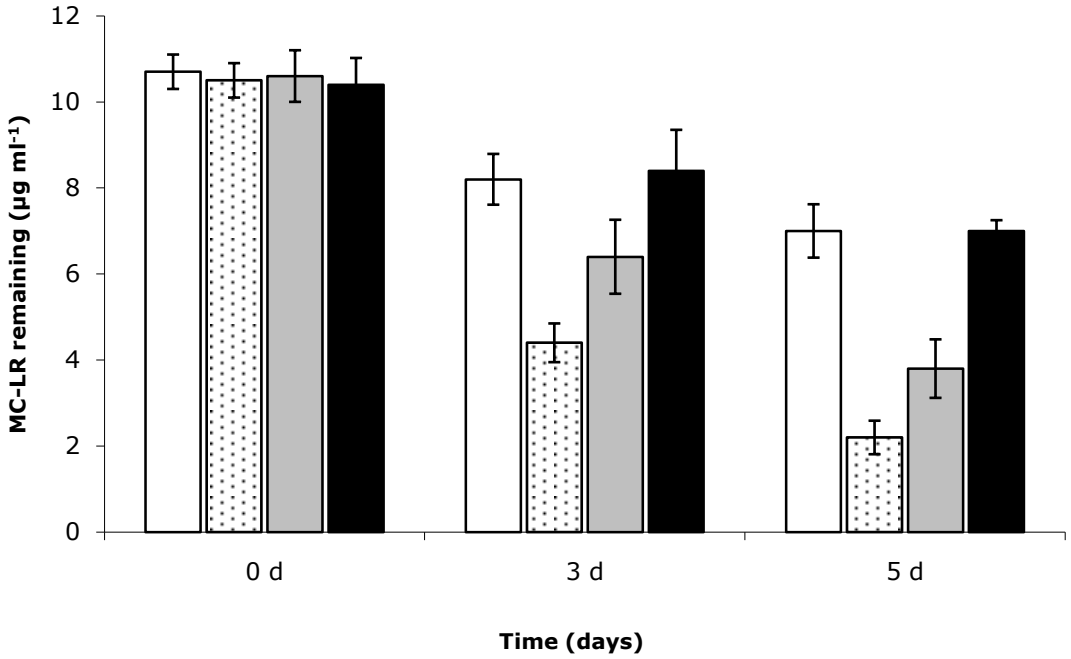

Figure 4.7 Degradation of MC-LR by 0 d (□), 24 h (⊡), 36 h (■) and 48 h (▩) carbon starved *Rhodococcus* sp. (NC1) in Carron river water spiked with 10 µg ml^{-1}. Error bars represent one standard deviation (n=3).

4.3.5.1 Effect of bacterial cell density on MC-LR degradation

The effect of the bacterial density on the degradation rate of MC-LR was examined to determine optimum cell density that gave a maximum toxin removal (figure 4.8). Regardless of the cell density, *Rhodococcus* sp. (NC1) has indicated a notable degradation of MC-LR during the 5 d study period. However, it should be noted that, the degradation of the compound was faster with the increasing number of cells inoculated in the media (figure 4.8). The maximum cell density adjusted in the current study was $OD_{590\ nm}$ of 0.35 to allow the comparison with previous studies (chapter 2). An average of 86% of MC-LR was removed by the *Rhodococcus* sp. (NC1) when the bacterial inoculum was adjusted to $OD_{590\ nm}$ of

0.35. Moreover, figure 4.8 indicates reasonably rapid degradation of MC-LR although the cell density is below ($OD_{590\ nm}$) 0.35; the percentage removal of MC-LR was 67% and 77% for $OD_{590\ nm}$ of 0.05 and 0.1 respectively.

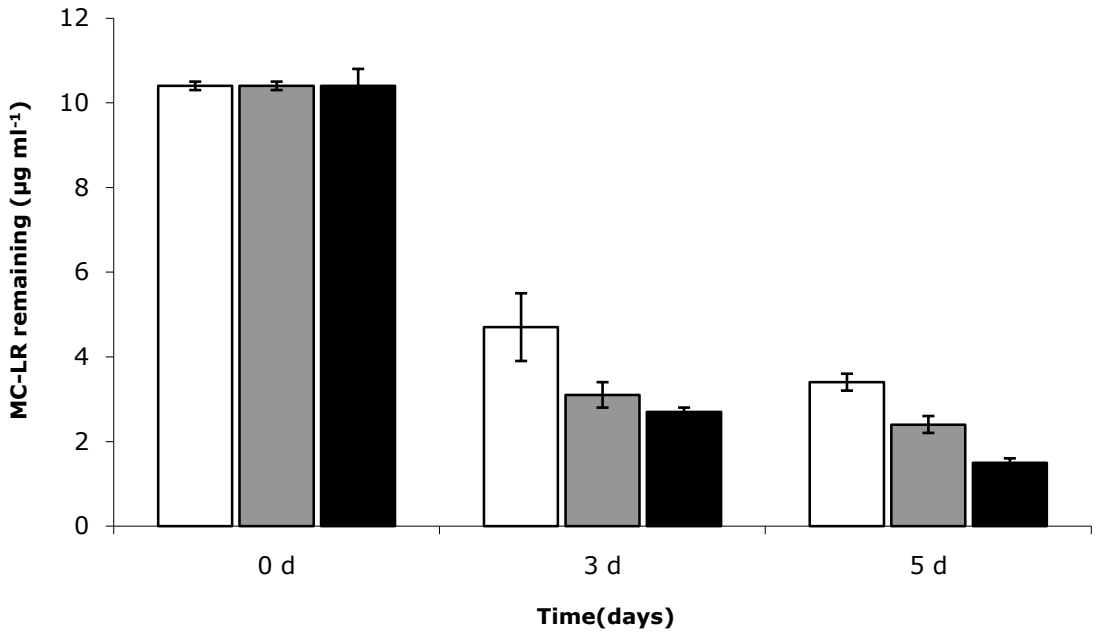

Figure 4.8 Degradation of MC-LR under different cell densities of *Rhodococcus* sp. (NC1) at $OD_{590\ nm}$ of 0.05 (□), 0.1 (▩) and 0.35 (■). Error bars represent one standard deviation (n=3).

4.3.6 MC-LR degradation by MC-LR induced *Rhodococcus* sp. (C1)

Rhodococcus sp. (C1) induced by MC-LR was found to rapidly degrade MC-LR in the subsequent batch degradation assay (figure 4.9). Thus, it reveals an inducible mechanism of the *Actinobacteria* involved in the degradation of MC-LR. This is indicated by the disparity of the degradation rates of the non-exposed (non-induced; NEX) bacteria and the MC-LR induced bacteria. To determine the minimum length of induction required for a rapid removal of MC-LR, bacteria were exposed for different lengths of time up to 21 d in river water spiked with MC-LR. The degradation of MC-LR has increased rapidly with the increasing length of the pre-exposure (figure 4.9). When bacteria were MC-LR induced for 7 d, they subsequently removed 87% of the MC-LR within 5 d. However, compared to that, 21 d induced cells required only 3 d to remove 90% of the toxin with only 5 % remaining after 5 d (figure 4.9). For all MC-LR induced bacteria, the half life of MC-LR can be estimated below 3 d. It is now clear that the bacteria attain reasonably high biodegradation efficiency (90% and 95% after 3 d and 5 d respectively) when they are pre-exposed for at least 21 d. In contrast to the MC-LR induced biodegradation of MC-LR, the non-exposed *Rhodococcus* sp. (C1) removed maximum 20% of the compound during the course of 5 d experiment (figure 4.9), confirming the theory of an inducible mechanism occurring during the pre-exposure.

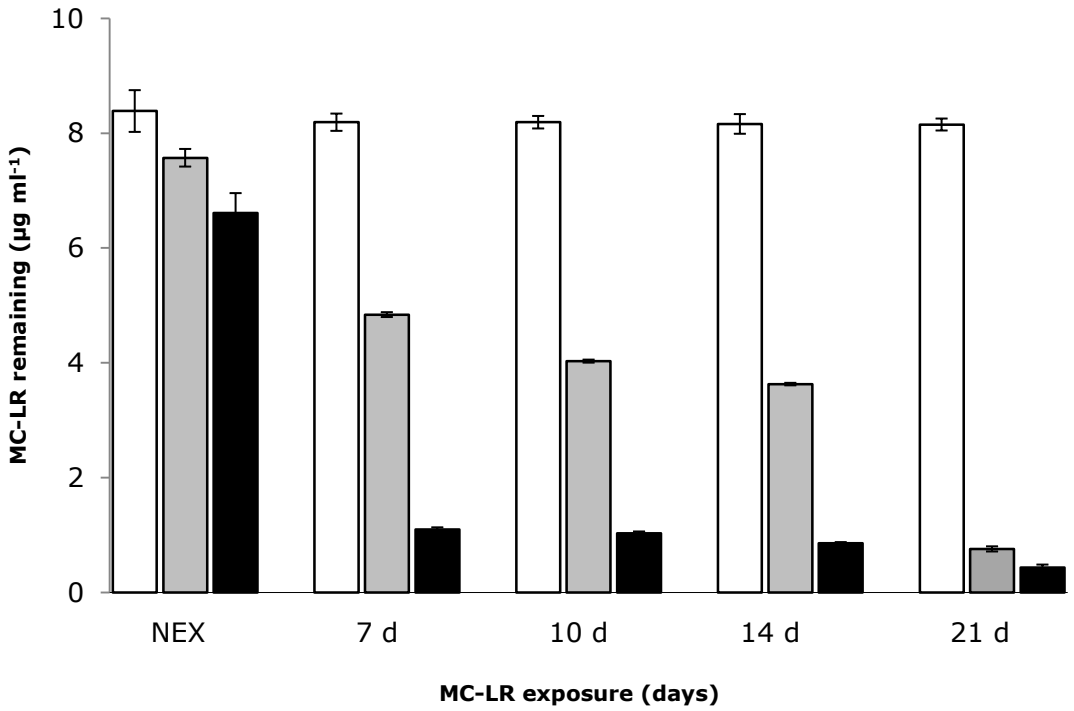

Figure 4.9 Degradation of MC-LR by MC-LR induced *Rhodococcus* sp. (C1). The length of the bacterial exposure time to MC-LR was 7, 10, 14 and 21 d. NEX stands for the bacteria which were not previously exposed to MC-LR. The subsequent MC-LR degradation was monitored after 0 d (□), 3 d (▩) and 5 d (■). Error bars represent one standard deviation (n=3).

4.3.7 MC-LR degradation by cyanobacterial and non-cyanobacterial peptide induced *Rhodococcus* sp. (C1)

4.3.7.1 Pre-exposure and the degradation of the peptides

Previous study has revealed an inducible degradation of MC-LR by *Rhodococcus* sp. (C1). Thus, the present study was further implemented to understand whether this strain could only be induced by MC-LR or other cyanobacterial and non-cyanobacterial peptides as well. The first step of this study, the pre-exposure of different peptides was carried out for 21 d. To determine the possible degradation occurring during the induction period, samples were removed periodically, analysed in PDA-LC-MS and illustrated in figure 4.10. Interestingly, it revealed that *Rhodococcus* sp. (C1) is capable of degrading not only MCs, but also other different cyanobacterial and non-cyanobacterial peptides (figures 4.10 and 4.11). Despite the slight differences, *Rhodococcus* sp. (C1) has shown a clear degradation of MC-LR, ABPNB, LNOD, ANGTN and CY before they were subjected to a fresh re-spike of an additional dose of the peptides (figure 4.10). For example, when *Rhodococcus* sp. (C1) was initially exposed to 88 µg of MC-LR, after 7 d of incubation, only 18 µg of the compound remained in the media. This indicates an average 80% degradation of MC-LR before the media was re-spiked. Nonetheless, since it was anticipated that degradation of the peptides may have occurred, an identical peptide re-spike was made at 7 d intervals in order to maintain a sufficient amount of peptides to induce the bacteria. For example, after the re-spike of MC-LR at 7 d, the total amount of MC-LR remaining in the media was estimated to be 106 µg (figure 4.10).

About 70% of ABPNB and 61% of LNOD was degraded during the first 7 d of the peptide exposure (figures 4.10 and 4.11). Figure 4.11 shows the UV chromatograms (UV_{max} 210 nm for ABPNB and 235 nm for LNOD) of the remaining amount of ABPNB and LNOD after 0, 7, 14 and 21 d. It clearly indicates the disappearance of LNOD after 14 d regardless of the peptide re-spike at 7 d. In addition to the UV peak of the compound, several other peaks were visible (figure 4.11) in the PDA-LC-MS analysis. These peaks were attributed to the possible degradation products of the peptides.

It was interesting to note a complete removal of ANGTN after 7 d, indicating potential degradation mechanisms of *Rhodococcus* sp. (C1). In contrast, during the first 7 d of the induction, CY was degraded comparatively slowly attaining 42% of the compound removed by *Rhodococcus* sp. (C1).

Once the media was re-spiked with identical doses of peptides, the rate of the peptide degradation has clearly increased. Degradation of MC-LR has reached 97% over the following days, leaving only trace amounts of the compound (2.7 µg). Meanwhile, *Rhodococcus* sp. (C1) has completely removed LNOD before it was subjected to a subsequent re-spike after 14 d. However, after the 2^{nd} re-spike of the peptides at 14 d, the degradation efficiency decreased noticeably compared to that of previous degradation occurred from 7 d to 14 d. This could be attributed to the rapid changes occurring in the media such as pH variation due to bacterial by products as well as the possible cause of inhibitory products

(some degradation products might pose inhibitory actions). The percentage removal of MC-LR was decreased to 54%. Although a complete removal of LNOD has been noticed previously, the bacterial strain has not degraded LNOD completely after the second identical re-spike of the peptides after 14 d (figure 4.11). However, once again, the bacteria were still capable of performing a complete removal of ANGTN. The degradation efficacy of CY remained with no observable difference until 21 d regardless of the peptide re-spike.

No losses of any of the peptide were evident in a parallel batch containing peptide inoculated sterile river water confirming that the removals of subsequent peptides in the sample bottles were through biological degradation process.

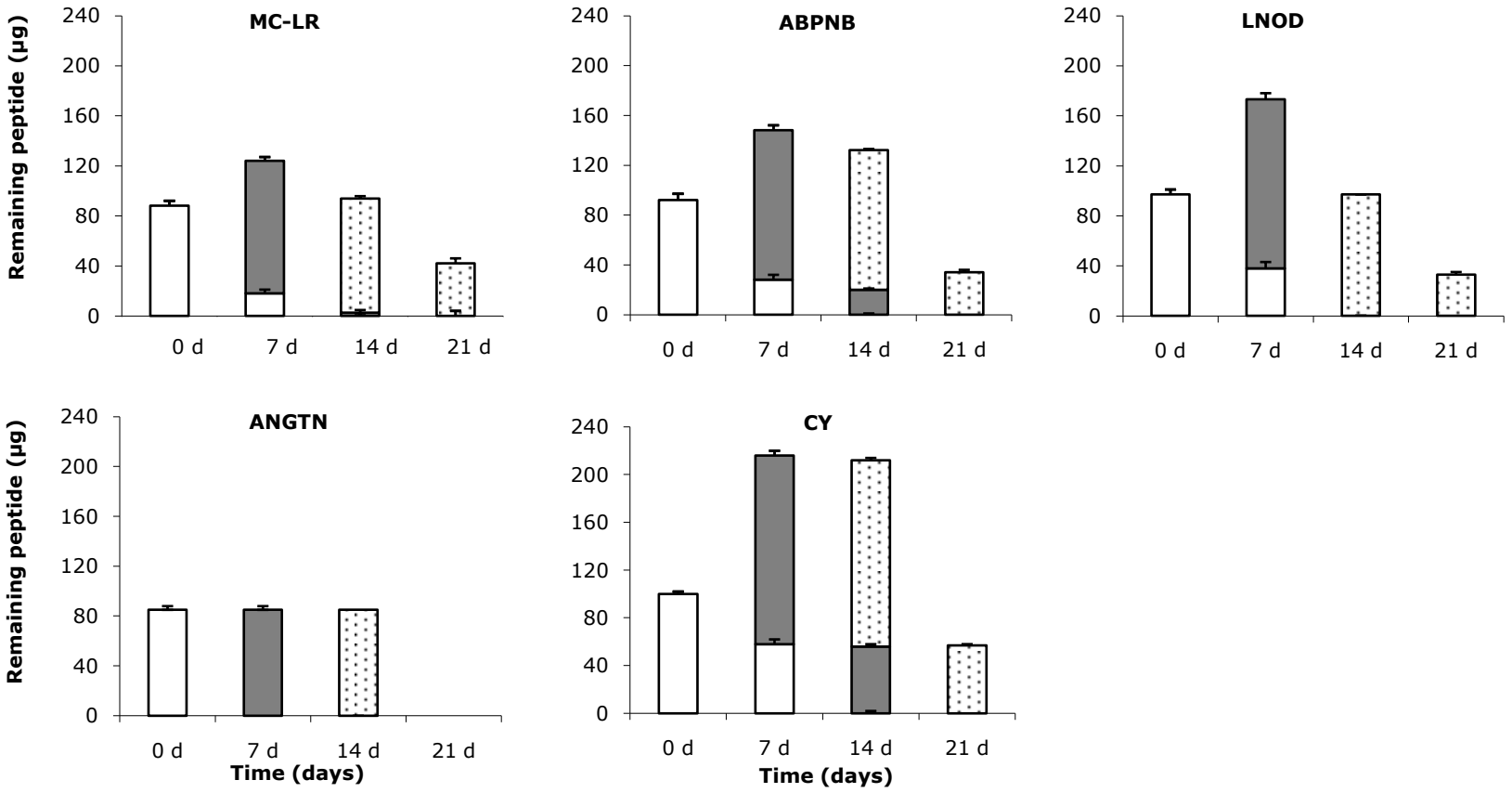

Figure 4.10 Degradation of different cyanobacterial and non-cyanobacterial peptides by *Rhodococcus* sp. (C1) spiked in sterile Carron water. Final peptide concentration in the media at 0 d (□), re-spike of peptides from stock peptide solutions at 7 d (■), and 14 d (⊡). Error bars represent one standard deviation (n=3).

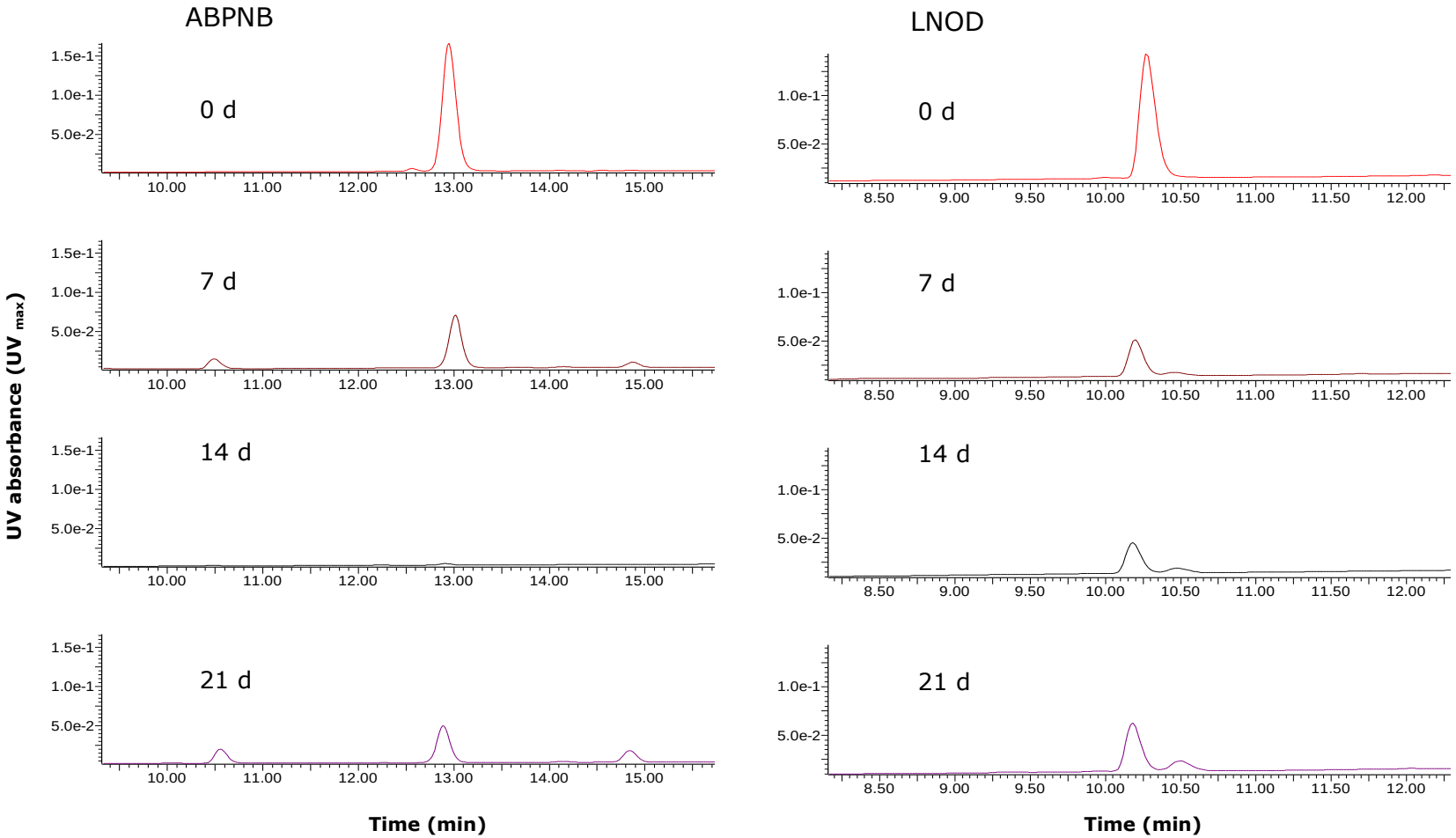

Figure 4.11 PDA-LC-MS analysis of the degradation process of ABPNB and LNOD by *Rhodococcus* sp. (C1). UV chromatograms show the remaining amount of ABPNB and LNOD at 0, 7, 14 and 21 d in the media. Identical peptide re-spikes were made at 7 d and 14 d. UV_{max} for ABPNB was 210 nm and 235 nm for LNOD.

4.3.7.2 Biodegradation of MC-LR by *Rhodococcus* sp. (C1) after the pre-exposure of cyanobacterial and non-cyanobacterial peptides

Results of this study confirmed, MC-LR degradation mechanisms of *Rhodococcus* sp. (C1) can be successfully induced by exposure to different cyanobacterial peptides (figure 4.12). *Rhodococcus* sp. (C1) exposed to MC-LR, ABPNB and LNOD have attained complete removal of MC-LR (~10 µg ml^{-1}) during the course of 5 d incubation in the present batch study (figure 4.12). However, considering the amount of MC-LR remaining after 3 d, most rapid degradation has occurred when the bacteria were induced by LNOD. Approximately 70% of MC-LR remaining in the media during the first 3 d was no longer available after 5 d due to rapid degradation occurring after third day (figure 4.12). In contrast to this, ANGTN, CY and RW did not induce the bacteria towards the subsequent MC-LR degradation (figure 4.12). There were no observable differences discerned between the bacteria pre-exposed to peptides (ANGTN and CY) and RW with the bacteria that had no previous exposure to any of the peptides. Interestingly, the degradation of peptides exposed (ANGTN, CY and RW) and the non-exposed bacteria were similar. This indicates that regardless of their ability to degrade different cyanobacterial and non-cyanobacterial peptides, the induction of MC-LR degradation is specific to the origin or the composition of the peptide they are pre-exposed.

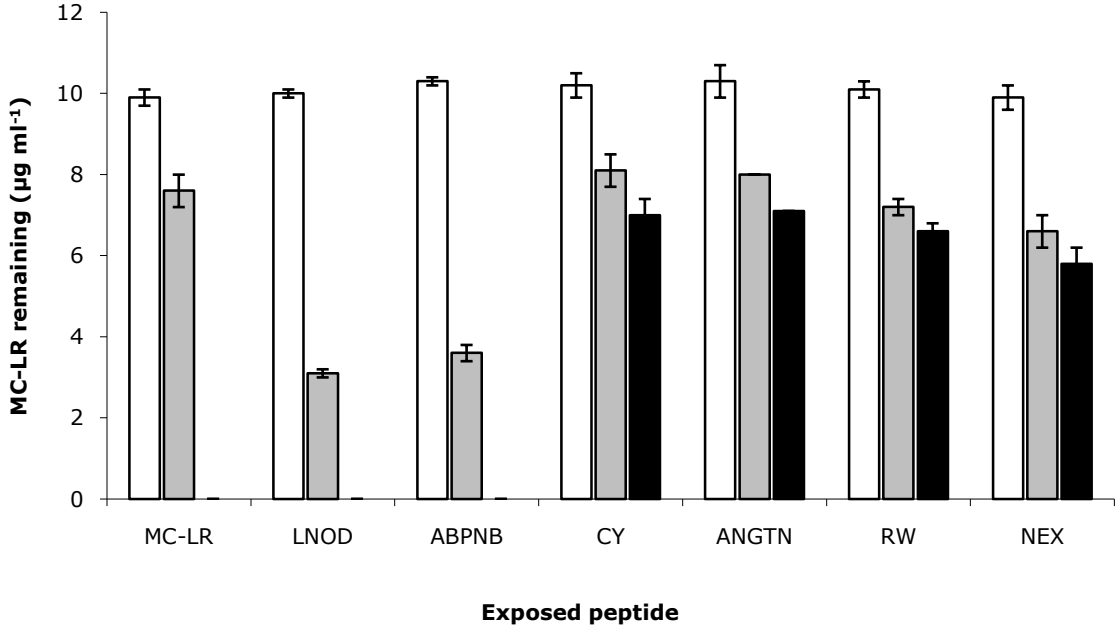

Figure 4.12 Degradation of MC-LR by peptide induced *Rhodococcus* sp. (C1) after 0 d (□), 3 d (▩) and 5 d (■). C1 isolate was continuously exposed to MC-LR, LNOD, ABPNB, CY or ANGTN for 21 d spiked in sterile Carron river water. Parallel set of bacteria were continuously exposed to sterile river water with no other peptides (RW). The control experiment contained bacteria with no exposure to any peptides (NEX). Error bars represent one standard deviation (n=3).

4.3.8 MC-LR degradation by prokaryotic and eukaryotic peptide induced *Rhodococcus* sp. (C1)

4.3.8.1 Peptide degradation and pre-exposure

Initial results suggested *Rhodococcus* sp. (C1) was an excellent universal peptide degrader harbouring an inducible MC-LR degradation mechanism (figures 4.11 and 4.12). An observation worth noticing was the disparity of the induction between cyanobacterial peptides and non-cyanobacterial peptides such as ANGTN and CY, although they were successfully degraded. Thus, the main objective of the following study was to determine whether bacterial induction is dependent on the origin of the peptide or the structural difference. To allow this, *Rhodococcus* sp. (C1) was exposed and induced by different eukaryotic (ANGTN and CY) and prokaryotic peptides such as POLYB and GRA. Interestingly, the bacterial strain is capable of degrading all of the peptides regardless of the structural difference and the different source origin (figure 4.13). The degradation of MC-LR has been consistent with the previous study (figure 4.11); whereas 81% of the compound was removed during the first 7 d, subsequently attaining a rapid degradation after the re-spike of (88 μg ml^{-1}) MC-LR, reducing to an average removal of 45% of the peptide after the second re-spike at 14 d. Furthermore, it was interesting to note a complete removal of MC-LR at the onset of the course of 21 d peptide exposure (figure 4.13). Cyclic prokaryotic peptide, PLOYB was easily degraded by *Rhodococcus* sp. (C1). Complete removal of the peptide was indicated by the PDA-LC-MS analysis of the samples retrieved at 7, 14 and 21 d (figure 4.13). This degradation pattern was clearly noticed in the previous study when the bacteria

were incubated with ANGTN (figure 4.11). However, in the present study, the degradation of ANGTN has shown to be slightly different to that previously discussed because; 25% (21 µg ml^{-1}) of the compound was still remaining after the 7 d of the incubation (figure 4.13). Nonetheless, once the degradation commenced, the peptide was completely decomposed after 14 d and 21 d regardless of the additional dose of ANGTN on 14 d (figure 4.13). In contrast to the comparatively slow response of bacteria against the cyclic eukaryotic peptide CY in the previous study (figure 4.11), a rapid degradation has occurred in the present study. During the first 7 d, CY was completely degraded by *Rhodococcus* sp. (C1); however, the degradation efficacy seems to be affected by the re-spike of CY after 7 d whereas the bacterial strain could attain only 8% removal of the cyclic peptide (figure 4.13) from 7 d to 14 d. Once again, regardless of the considerably high amount of CY remaining in the media (154 µg ml^{-1}); a rapid removal of over 80% was observed after 21 d. GRA is a hydrophobic linear polypeptide composed of 15 amino acids which was rapidly degraded by *Rhodococcus* sp. (C1). Despite, GRA being an antibiotic against Gram-positive bacteria like *Rhodococcus* sp. (C1), 73% of the peptide was rapidly degraded within the first 7 d during the peptide exposure. Similar to the degradation pattern of the other peptides, the percentage degradation was slightly reduced to 67% after the first re-spike and subsequently achieved 84% degradation after the second re-spike of GRA (figures 4.13 and 4.14).

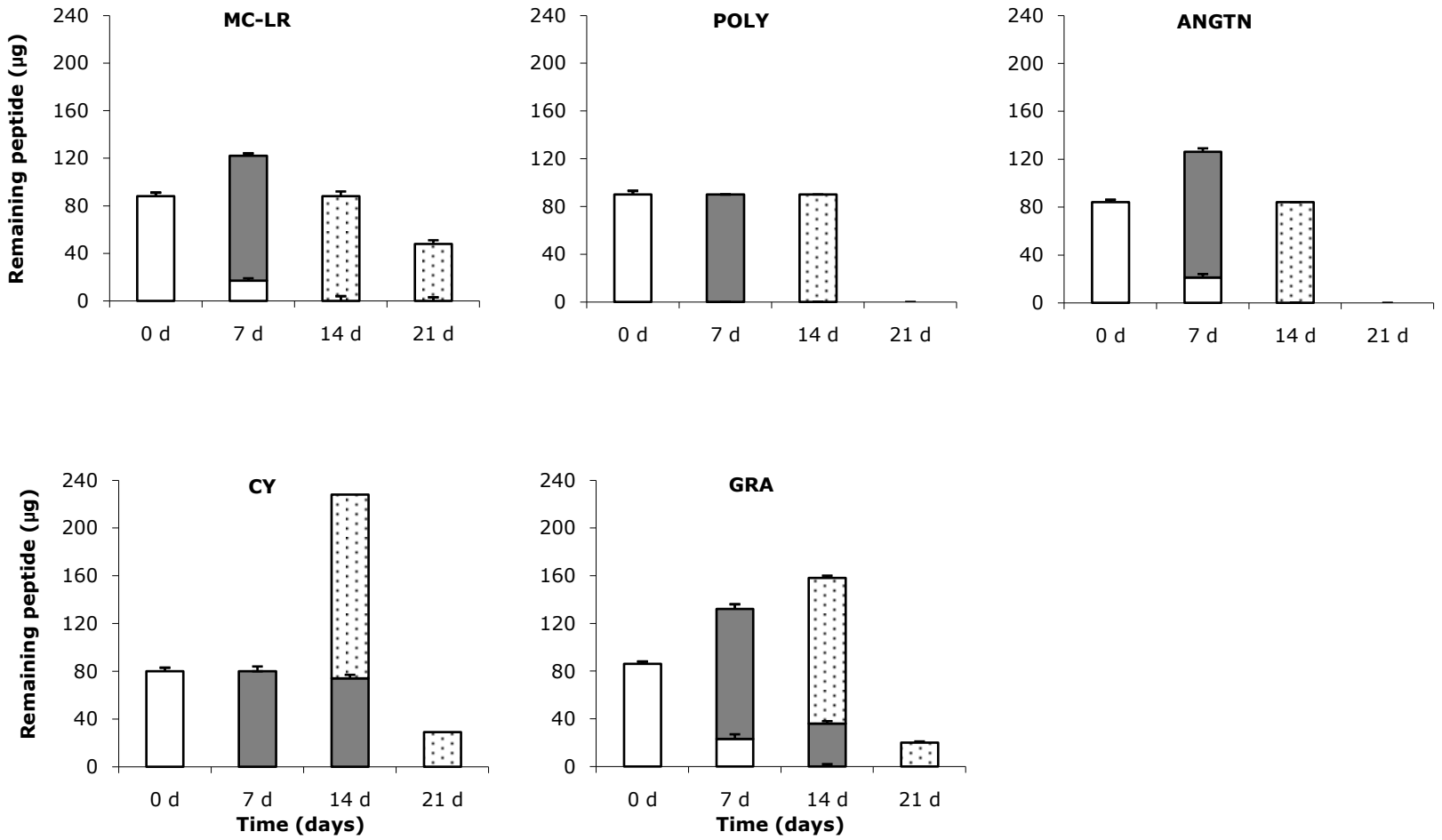

Figure 4.13 Degradation of different eukaryotic and prokaryotic peptides by *Rhodococcus* sp. (C1) in sterile Carron water. Final peptide concentration in the media at 0 d (□), re-spike of peptides from stock peptide solutions at 7 d (■) and 14 d (⊡). Error bars represent one standard deviation (n=3).

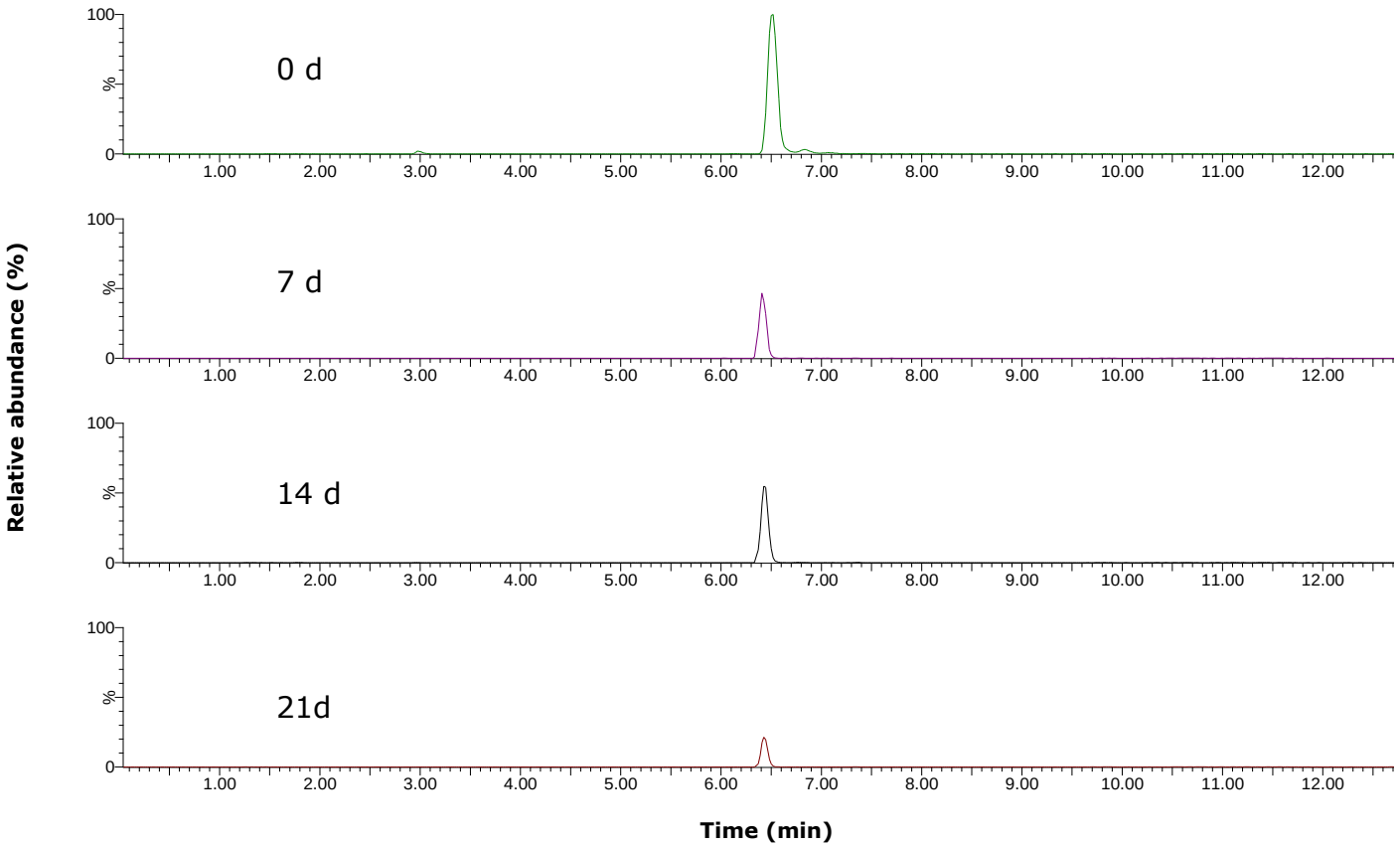

Figure 4.14 UPLC-MS analysis of the degradation of GRA by *Rhodococcus* sp. (C1) during the 21 d of pre-exposure. MS chromatograms extracted at *m/z* 942 of $[M+2H]^{2+}$ shows the remaining amount of GRA at 0, 7, 14 and 21 d. Identical doses of GRA were re-spiked at 7 d and 14 d.

4.3.8.2 Degradation of MC-LR by *Rhodococcus* sp. (C1) after the pre-exposure to prokaryotic and eukaryotic peptides

After the exposure of the prokaryotic peptides (MC-LR, POLYB and GRA), the bacteria were successfully induced to degrade MC-LR in the present study (figure 4.15). MC-LR introduced in the present study was rapidly removed before 5 d. MC-LR and POLYB induced bacteria were capable of completely removing MC-LR after 5 d whereas GRA induced bacteria removed 90% the toxin spiked in the media. Considering the MC-LR degradation by MC-LR and GRA induced bacteria, more rapid degradation occurred within the first 3 d compared to the degradation that occurred after 3 d. In contrast, the highest MC-LR degradation was commenced after 3 d when the bacteria were induced by POLYB.

Compared to the rapid degradation performed by MC-LR, POLYB and GRA induced bacteria, ANGTN and CY did not induce the bacteria towards the subsequent MC-LR degradation (figure 4.15). These results are consistent with the previous study (figure 4.12) where ANGTN and CY showed no inducible effect on *Rhodococcu*s sp. (C1).

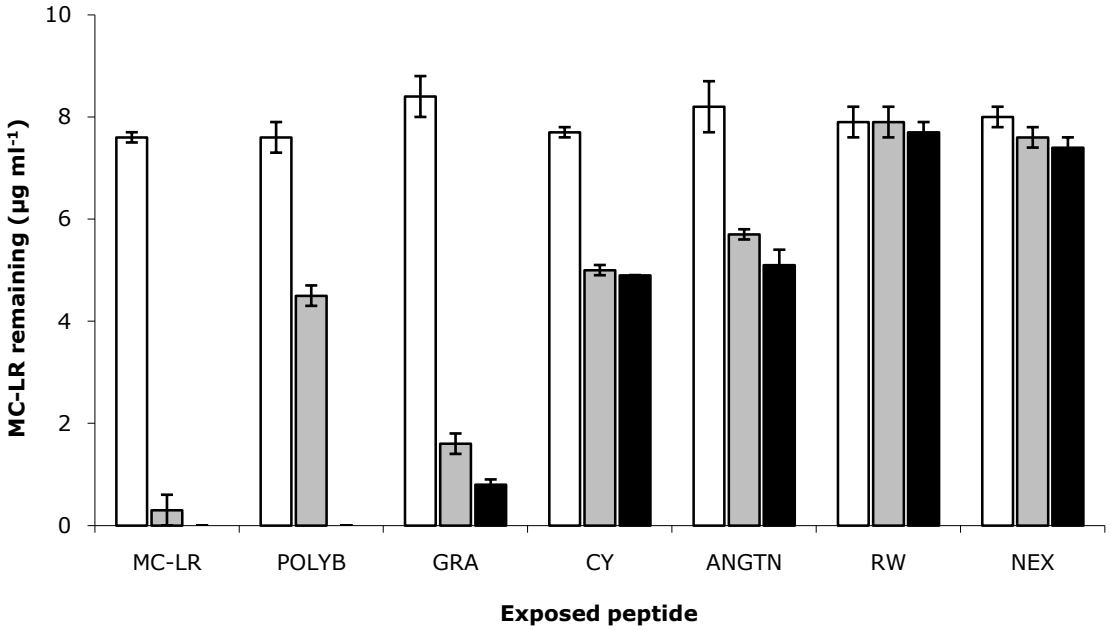

Figure 4.15 Degradation of MC-LR by peptide induced *Rhodococcus* sp. (C1) after 0 d (□), 3 d (▨) and 5 d (■). C1 isolate was continuously exposed to MC-LR, POLYB, GRA, CY, ANGTN and RW for 21 d spiked in sterile Carron river water. The control experiment contained bacteria with no exposure to any peptides (NEX). Error bars represent one standard deviation (n=3).

4.3.9 Degradation of different peptides by MC-LR induced *Rhodococcus* sp. (C1)

In previous studies, *Rhodococcus* sp. (C1) induced by different prokaryotic peptides (MC-LR, LNOD, ABPNB and POLYB) were found to degrade ~ 10 µg of MC-LR in less than 5 d. However, the current study was carried out to evaluate their efficiency in decomposing other prokaryotic peptides after they are induced by MC-LR. *Rhodococcus* sp. (C1) induced by MC-LR has successfully degraded all

tested prokaryotic peptides spiked in river water (figure 4.16). Approximately 95% of MC-LR, LNOD, ABPNB and POLYB were degraded after 5 d while 85% of GRA was degraded by *Rhodococcus* sp. (C1). Compared to the induced bacteria, non-induced bacteria attained comparatively low degradation (figure 4.16). For example, non-induced bacteria removed ~ 50% of LNOD, ABPNB, GRA and 40% MC-LR after 5 d of the study. However, it should be noted that, C1 has degraded 81% of POLYB within the same period (5 d; figure 4.16). It is clear that *Rhodococcus* sp. (C1) is capable of rapidly degrading POLYB regardless of pre-exposure. This result is consistent with the complete degradation observed for POLYB in previous studies (figure 4.13). However, none of the *Rhodococcus* sp. (C1) was capable of completely removing any of the peptides unless induced by MC-LR. Thus, pre-exposure to peptides plays an important role in the consequent expression of the peptide degradation mechanisms in *Rhodococcus* sp. (C1). Consistent results from consecutive studies (figures 4.10, 4.11, 4.13 and 4.14) have revealed a universal peptide degradation mechanism(s) of *Rhodococcus* sp. (C1). Interestingly, this mechanism(s) can be successfully induced upon the demands of the degradation of different peptides in nature. Further studies are required to unravel the genes responsible for the inducible mechanisms and to exploit the universal peptide hydrolysing enzymes from *Actinobacteria*.

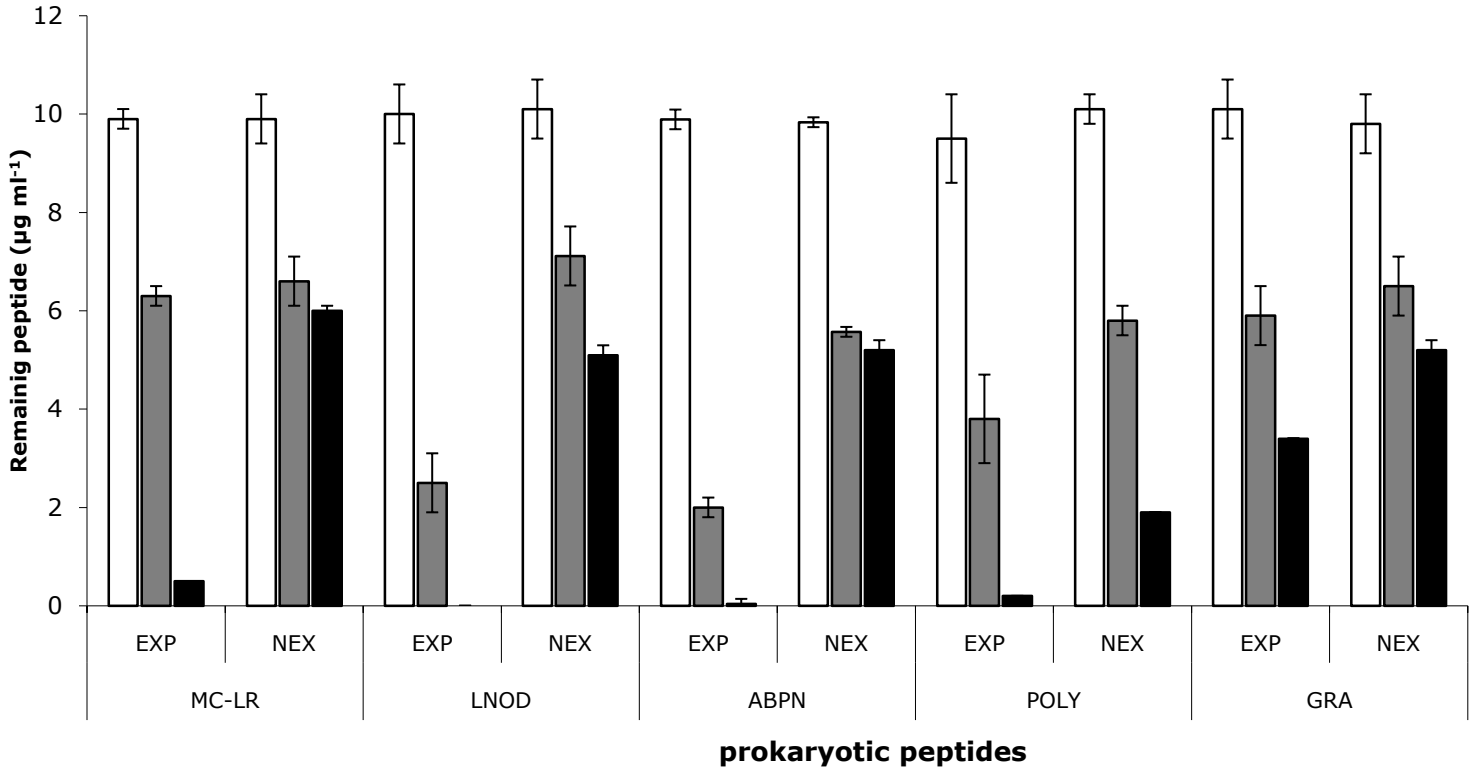

Figure 4.16 Degradation efficacy of MC-LR, LNOD, ABPNB, POLYB and GRA by MC-LR-induced (EXP) *Rhodococcus* sp. (C1) at 0 d (□), 3 d (▨) and 5 d (■). The control experiment contained non-induced *Rhodococcus* sp. (C1; NEX). Error bars represent one standard deviation (n=3).

4.4 Discussion

4.4.1 Evaluation of MC-LR degradation by -80 ºC stored bacteria

The biodegradation studies described in chapter 3 have indicated a clear degradation of MC variants and NOD. However, the rate of degradation indicated was comparatively slow to that of Manage *et al*., (2009) and none of the bacteria achieved complete removal of MC-LR despite the optimisation of batch studies examined here. These observations have been previously justified as the possible effects of the repeated sub-culturing and lack of exposure to MC-like peptides (Lawton *et al*., 2011). This could be further justified by the studies of Li *et al*., (2011), who evaluated the effect of different concentrations of peptone in biodegradation of MC-LR using a bacterial biofilm. It was observed that, peptone (1 mg l^{-1}) has imposed a significant inhibitory effect on MC-LR degradation. Thus, if this scenario occurred during the bacterial sub-culturing and storage in NB and NA, which generally contains 5 mg l^{-1} of peptone, it is possible the degradation of MC-LR was considerably affected. However, if these bacteria isolates are to be employed in a water remediation process, they should be capable of a rapid removal of MCs. Hence, it is important to investigate the possible routes to attain an efficient biodegradation performance as previously reported by Manage *et al*., (2009).

To achieve this, the cryopreserved bacterial isolates (*Rhodococcus* sp. NC1, *Arthrobacter* sp. NC6, NF7, NR4 and *Brevibacterium* sp. NF3) were successfully revived and their biodegradation efficacy of MC-LR has been evaluated in

traditional batch studies. Consequently, a rapid degradation occurred with ~ 90% MC-LR removed after 5 d (figure 4.6). Hence, it revealed the biodegradation ability was retained during the cryopreservation. However, in contrast to the rapid degradation, regularly cultured *Rhodococcus* sp. C1 (RC1) could only remove 20% of MC-LR during the 5 d course of incubation (figure 4.6). These degradation rates of MC-LR are consistent with the results of the batch study (figure 3.5) previously described in chapter 3. Therefore, it is now clear that long-term bacterial sub-culturing had affected the biodegradation efficiency of the *Actinobacteria* isolates. Despite the rapid degradation of the bacteria revived from the long-term storage, a complete degradation of MC-LR was still not achieved (figure 4.6). Once Manage *et al*., (2009) had isolated and screened these bacteria using the Biolog MT2 assay, they were maintained in NA slopes for at least 3 months before they were subjected to the cryopreservation. As previously described, it is possible that bacterial culturing in NA and NB during this period has affected their optimum biodegradation activity (Li *et al*., 2011). In contrast to this explanation, Bourne *et al*., (1996) has reported a constitutive MC degrading activity of MC degrading bacterium *Sphingomonas* sp. ACM 3962 maintained on peptone yeast extract agar. This suggests the degradation mechanism and the genes involved in Gram-positive *Rhodococcus* sp. (C1) are different from the MC-LR degradation mechanisms in Gram-negative *Proteobacteria* like *Sphingomonas* sp. (Bourne *et al*., 1996, 2001; Saitou *et al*., 2003; Ho *et al*., 2007; Okano *et al*., 2009; Chen *et al*., 2010).

Before further evaluation of these bacteria to understand the degradation mechanisms involved in novel *Actinobacteria*, it is essential to revive their optimal biodegradation (Manage *et al*., 2009) against MCs. To achieve this, the biodegradation studies were optimised with different numbers of bacteria and the length of carbon starvation required for maximum removal of MC-LR. It was clearly noted that carbon-starved bacteria were capable of rapidly degrading MC-LR compared to that of non-starved bacteria (figure 4.7). This implied that MC-LR degradation mechanisms are expressed due to the starvation. Groat *et al*., (1986) showed that *E. coli* had induced more than 50 starvation proteins under carbon starvation. It was also reported that bacterial catabolic pathways are induced to produce common catabolic enzymes such as hexokinase under carbon starvation conditions (Martin, 1996). Thus, it is possible *Rhodococcus* sp. (C1) is readily induced to utilise the carbon sources (MC-LR) available in the environment (Manage *et al*., 2009; Lawton *et al*., 2011). This could possibly be the cause of onset degradation of MCs with no lag periods in the previous studies (chapter 3, figures 3.5, 3.6 and 3.7). However, the degradation rates were seen to decrease with the length of carbon starvation (figure 4.7). These results are similar to the degradation patterns of *p*-nitrophenol reported by Leung *et al*., (2005) using a *Moraxella* strain. In the study, authors have reported a rapid removal of the compound by 24 h carbon starved bacteria whereas the degradation rates rapidly decreased when the bacteria were carbon-starved for more than 24 h. This observation can be attributed to the fact that elongated carbon starvation might have shut down some of the metabolic systems (Leung *et al*., 2005) related to MC uptake and/or degradation temporally. Due to the

energy starvation, many bacteria convert to a viable but non-culturable condition rather than die and lyse (Surono *et al*., 2008); hence they are unable to proliferate (Kell *et al*., 1998; Colwell, 2000; Pruzzo *et al*., 2003) when they are inoculated in river water with MC-LR. Conversely, this could have hindered the degradation of MC-LR by 48 h starved bacteria compared to that of 24 h starved.

The effects of bacterial carbon starvation on the degradation of MCs have not being investigated previously. Given that all of the *Actinobacteria* MC degraders are heterotrophic it is probable that they could frequently experience carbon stress conditions during the water purification process. Thus, it is essential to monitor their MC degradation under carbon stressed conditions in order to maintain the optimum degradation levels during water treatment. This would undoubtedly facilitate the use of novel *Actinobacteria* isolates in future water remediation, i.e. to determine in which stage of water purification the bacteria should be employed. Compared to the range of measured concentrations of dissolved MCs (0.1–100 µg l^{-1}) in water (Van Apeldoorn *et al*., 2007), the results here seems to be promising. However, it is important to investigate the rate of degradation of MCs under low concentrations such as 1-10 µg l^{-1} since the low concentrations of MCs may not be sufficient to induce the bacterial degradation.

To further optimise the biodegradation studies, the number of bacteria required for rapid degradation of MC-LR was evaluated. Optimisation of the bacterial population required for the rapid removal of MCs indicates an important aspect of the bioremediation process. Accordingly, the degradation of MC-LR was shown be

more rapid with the increasing OD of the bacterial inocula; i.e. number of bacteria inoculated in the media (figure 4.8). Thus, it confirmed that the degradation of MC-LR was dependent upon bacterial numbers. In similar batch degradation study, Ho *et al*., (2007) have reported that degradation of MC-LR was increased with the number of *Sphingomonas* sp. LH21 inoculated in the biodegradation assay. In fact, the enzymatic interaction between bacteria and MC-LR (enzyme units per molecule of MC-LR) increases with the number of viable bacteria available in the media. Nevertheless, the cellular uptake of MC-LR might have increased with the number of bacteria posing the ultimate result of rapid removal of MC-LR. This could be also be explained in relation to the characterised enzymatic degradation pathway of MC-LR (Bourne *et al*., 1996, 2001), where an enzyme called MlrD transports MC-LR into the bacteria cell and with subsequently hydrolysed by MlrA, MlrB and MlrC.

In terms of bioremediation efficiency, it is probable that the biodegradation rates here still do not reflect the maximum values that can be obtained from *Rhodococcus* sp. (C1). Although, *Actinobacteria* isolates were freshly revived from the initial storage (-80 ºC), the biodegradation rates were shown to be affected by the multiple sub-culturing before they were subjected to the cryopreservation; hence, the degradation efficiency could be under determined. The rate and the extent of MC-LR degradation can be influenced by the history of energy (carbon) starvation and the number of bacteria available in the media. These findings will advance the feasibility of any future experiments to obtain the optimum biodegradation behaviour of *Rhodococcus* sp. (C1).

4.4.2 Degradation of prokaryotic and eukaryotic peptides by *Rhodococcus* sp. (C1)

This study has clearly shown that MC degrading *Rhodococcus* sp. (C1) is an excellent universal peptide degrader. Moreover, the findings suggested that MC and other peptide degradation mechanisms can be induced towards a rapid degradation of both MC and other prokaryotic peptides. However, the degradation was expressed only by the pre-exposure to the prokaryotic peptides such as MC-LR, ABPNB, LNOD, POLYB and GRA.

Interestingly, *Rhodococcus* sp. (C1) has demonstrated a rapid removal of the peptides regardless of the peptide re-spikes during their exposure period of 21 d. This specific behaviour can be explained using observations of a similar batch study of degradation of MC-LR and MC-LA using a *Sphingomonas* sp. (Ho *et al*., 2007). The bacterial strain degrading MC-LR and MC-LA had shown more rapid degradation after the identical re-spikes of the compound. Thus, it was explained that once MC degrading genes are induced, efficient production of MC degrading enzymes can proceed during the re-exposure of the toxins irrespective of the initial concentration of MCs (Ho *et al*., 2007). This could be the possible scenario involved in the continuous degradation of all peptides regardless of any subsequent addition of further peptides.

Further explanations can be implied in relation to the structural specificity, for example, LNOD which was subjected to a rapid degradation also induced the bacteria to rapid degradation of other peptides (figures 4.10, 4.12 and 4.16). Few

studies have reported the degradation of NOD (Imanishi *et al*., 2005; Rapala *et al*., 2005; Kato *et al*., 2007) using single strain of bacteria while many others reported the degradation of NOD by a community of aquatic bacteria (Cousins *et al*., 1996; Heresztyn and Nicholson 1997; Lathi *et al*., 1998; Christoffersen *et al*., 2002; Holst *et al*., 2003; Bourne *et al*., 2006; Chen *et al*., 2008; Edwards *et al*., 2008). Furthermore, some authors have identified the degradation products of NOD (Imanishi *et al*., 2005; Kato *et al*., 2007; Edwards *et al*., 2008; Mazur-Marzec *et al*., 2009) and acknowledged LNOD as a prominent degradation product of pentapeptide cyclic NOD. Conversely, Jones *et al*., (1994) and Saitou *et al*., (2003) reported NOD cannot be degraded by two bacterial strains from the genus *Sphingomonas* which was reported to degrade several analogous of MCs. In addition to that, Ishii *et al*., (2004) observed that NOD-Har cannot be degraded alone but can be degraded in the presence of MC-RR and suggested that degradation activity was induced by a factor(s) produced in the degradation process of MC-RR. Hence, it is possible that *Rhodococcus* sp. was induced to degrade LNOD during the pre-exposure (figure 4.16) and undoubtedly enhanced the degradation by its open-ring structure of the compound (figure 4.10) which may be expected to be more easily degraded (Mazur-Marzec *et al*., 2009).

Degradation of hexapeptide ABPNB was not reported elsewhere. However, Kato *et al*., (2007) have studied the degradation of anabaenopeptin A (ABPNA), where L-Arg in ABPNB was replaced by L-Tyrosine. In this investigation, *Sphingomonas* sp. B9 (cell extract) has successfully degraded few cyanobacterial cyclic peptides such MC-LR, NOD, Microviridin I, Nostophycin whereas ABPBA was only slightly

degraded. However, in the current study, *Rhodococcus* sp. (C1) has successfully degraded ABPNB while it had induced the bacteria to degrade MC-LR suggesting the degradation mechanisms are different from the Gram-negative *Sphingomonas* like bacteria.

ANGTN and CY are eukaryotic peptides composed of seven (Wright *et al*., 1996) and 11 amino acids (Survase *et al*., 2011) respectively. The degradation of ANGTN was more rapid compared to CY possibly due to the structural difference of CY being a cyclic peptide. Although, *Rhodococcus* sp. (C1) has degraded both of the compounds, this did not induce the degradation of MC-LR. In contrast to that, *Rhodococcus* sp. has degraded POLYB and GRA and the peptides have successfully induced the cells to degrade MC-LR subsequently. This clearly indicates that *Rhodococcus* sp. harbour a universal peptide degradation mechanism; although their peptide degradation genes are induced when exposed to prokaryotic peptides.

GRA and POLYB are peptide antibiotics, where GRA is a 15 amino acid linear hydrophobic polypeptide with a bactericidal activity against Gram-positive bacteria (Bano *et al*., 1989; Salom *et al*., 1997). In contrast, cyclic, heptapeptide POLYB acts as an antibiotic against Gram-negative bacteria in general (Cao *et al*., 2008). However, it is interesting to note that *Rhodococcus* sp. (C1) degrade the peptides regardless of their bactericidal activity. Bacterial responses to different peptide antibiotics is well described in literature (Lambert, 2002; Fajardo and Martinez, 2008; Linares *et al*., 2006). Gram-positive bacteria like *Rhodococcus*

sp. generally posses a permeable cell wall compared to the Gram-negative bacteria that usually does not restrict the penetration of antimicrobials (Lambert, 2002). Thus, it is possible the peptide uptake is facilitated in *Rhodococcus* sp. (C1) compared to the Gram-negative bacteria ultimately enhancing the degradation process. In addition to that, the peptide antibiotics at low concentrations, plays a key role in bacterial cell signalling towards bacterial metabolic activity (Fajardo and Martinez, 2008). Consequently, this clearly supports the bacterial induction of various metabolic mechanisms during the peptide pre-exposure resulting in subsequent rapid degradation of MC-LR. As previously mentioned in chapter 3, unlike eukaryotic peptides such as ANGTN, prokaryotic peptides (MC-LR, ABPNB, NOD, GRA and POLYB) are synthesised through NRPS (Dittmann *et al*., 1997; Nishizawa *et al*., 1999). Although, CY is produced in a similar pathway, the bacteria were not successfully induced. Thus, bacterial induction could be possibly caused by the composition or arrangements of the amino acids specific to prokaryotes and requires further studies to understand the function and mechanisms of emerging bacterial resistance to antibiotics.

Rhodococcus bacteria are regarded as one of the most promising groups of organisms suitable for the biodegradation of compounds that are not easily transformed by any other organisms (Warhurst and Fewson, 1994). Their capability to degrade aliphatic and aromatic hydrocarbons, halogenated compounds, heterocyclic compounds, various pesticides and the diversity of degradation pathways are reported elsewhere (Bell *et al*., 1998; Banerjee *et al*.,

2002; Martinkova and Kren, 2002; Singh *et al*., 2006; Larkin *et al*., 2006). Considering their biodegradation diversity, it is highly desirable that *Rhodococcus* sp. (C1) contains such a universal degradation mechanism for any different peptide which has not previously been explored. However, further studies are required to understand the bacterial induction specific to prokaryotic peptides, to unravel the degradation mechanisms and to elucidate the genes responsible for degradation of MCs as well as other prokaryotic and eukaryotic peptides.

4.5 Conclusion

Rhodococcus sp. (C1) being isolated from a source of no previous exposure to MCs (River Carron), posses an important role by its universal peptide degradation ability regardless of the origin (eukaryotic and prokaryotic) or the chemistry of the peptide (cyclic or linear). Although, bacteria like *Rhodococcus* sp. (C1) have been involved in the self purification of nature's own contaminants (MCs and NOD) and many of the man made compounds (aromatic and halogenated compounds), their ability to mineralise a wide range of different peptides have never been reported elsewhere. Hence, this is the first report of *Actinobacteria* degrading a wide range of peptides such as ABPNB, POLYB, GRA, ANGTN and CY.

Another interesting finding of this study was the bacterial inducible mechanism. The bacterial degradation rate of cyanobacterial peptides were found to decrease with the multiple culturing of bacteria which was successfully reversed when they were exposed to prokaryotic peptides for 21 d. Some of the previous studies have stated that previous exposure or degradation products of MCs may induce the degradation genes (Ishii *et al*., 2004; Ho *et al*., 2007). However, this is the first detailed study to report and confirm the inducible mechanism involved in the degradation of MC-LR and a range of different peptides.

It can be further highlighted; that degradation mechanisms are expressed only by the exposure to prokaryotic peptides and eukaryotic peptides have little or no effect towards the expression of the subsequent MC-LR or different peptides. Further studies are essential to expose and evaluate biochemistry and molecular

biological aspects of the inducible biodegradation pathway of MCs and other peptides. This would not only enhance the use of these bacteria in future water remediation strategies but also unravel important evidence on emerging bacterial antibiotic resistance mechanisms.

CHAPTER 5

ELUCIDATING THE MICROCYSTINASE GENE(S) INVOLVED IN THE BACTERIAL DEGRADATION OF MC-LR

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5 Elucidating the microcystinase gene(s) involved in the bacterial degradation of MC-LR

5.1 Introduction

Microorganisms play an important role in the purification of the environment by eliminating a wide range of natural and man-made pollutants. Bacteria such as *Rhodococcus* and *Arthrobacter* have been well acknowledged for their astonishing catabolic versatility to degrade recalcitrant xenobiotics (Singh *et al*., 2006; Larkin *et al*., 2006; Ferreira *et al*., 2008). Research into the biotechnological use of the members of *Rhodococcus* has significantly increased over the past two decades (Martankova *et al*., 2009). Interestingly, many of these bacteria were widely exploited for the environmental remediation industry (Bell *et al*., 1998). Moreover, previous studies have successfully demonstrated that bacteria from the genus *Rhodococcus* and *Arthrobacter,* not only eliminate the recalcitrant xenobiotics, but also are excellent degraders of MCs, NOD, prokaryotic and eukaryotic peptides (Manage *et al*., 2009; Lawton *et al*., 2011; chapters 3 and 4 in this thesis).

MCs and NOD are cyanobacterial secondary metabolites which are structurally stable against physicochemical and biological stresses (pH, temperature, sunlight; Jones and Orr, 1994; Harada *et al*., 1996). Common bacterial proteases, such as trypsin, elastase, thrombin, papain and pepsin, are not capable of degrading MCs (Saito *et al*., 2003; Okano *et al*., 2006); hence specific MC degrading enzymes are required (Shimizu *et al*., 2011). Jones *et al*., (1994) isolated the first MC degrading bacterium, *Sphingomonas* sp. which

was later found to contain MC hydrolysing enzymes, namely, MlrA, MlrB, MlrC produced by the *mlr* gene cluster. Since then, more than 10 bacterial isolates from different environments were found to degrade MCs. Nevertheless, genotype studies on MC degrading bacteria exclusively focused on the *Spingomonadaceae* as the bacteria from this group was proven to harbour specific MC degrading genes previously described (Ho *et al*., 2010; table 5.1). Consequently, the *mlr* gene cluster involved in MC hydrolysis remains as the only characterised MC degradation pathway to date (Edwards and Lawton, 2009; Chen *et al*., 2010).

Table 5.1 MC degrading bacteria and their genes involved in the degradation of MCs (adapted from Shimizu *et al*., 2011)

Bacteria	**Gene**	**Gram identification**	**Reference**
Sphingomonas sp. ACM-3962	*mlrA*, *mlrB*, *mlrC* and *mlrD*	-ve	Bourne *et al*., (2001)
Novosphingobium sp. MD-1	*mlrA*	-ve	Saitou *et al*., (2003)
Sphingosinicella microcystinivorans Y2	*mlrA*	-ve	Saitou *et al*., (2003)
Sphingomonas sp. B9	*mlrA*	-ve	Harada *et al*., (2004)
Sphingopyxis witflariensis LH21	*mlrA*, *mlrB*, *mlrC* and *mlrD*	-ve	Ho *et al*., (2007)
Sphingopyxis sp. C-l	*mlrA*, *mlrB*, *mlrC* and *mlrD*	-ve	Okano *et al*., (2009)
Stenotrophomonas sp. EMS	*mlrA*	-ve	Chen *et al*., (2010)

Given that the *mlr* gene cluster has been successfully characterised (Bourne *et al*., 2001), it can be speculated that subsequent studies have intended to investigate the existence of the *mlr* genes in environmental bacteria (table

5.1). Saito *et al*., (2003), successfully designed a PCR assay for the detection of *mlrA* and reported that two of the MC-degrading bacteria *Novosphingobium* sp. and *Sphingosinicella* sp. contained gene homologues of *mlrA*. Utilising a similar method, Ho *et al*., (2006) investigated the existence of MC-degrading bacteria in a biological sand filter. In later studies (Ho *et al*., 2007), authors isolated and characterised the *Sphingopyxis* sp. LH21 harbouring *mlr* gene cluster that had been previously characterised by Bourne *et al*., (2001). Recent implementations of biodegradation studies have used the *mlrA* gene as a tool to identify the MC-degrading bacteria (Hoefel *et al*., 2009; Ho *et al*., 2010) from biological sand filters and waste water systems. On the contrary, MC degradation studies by Ishii *et al*., (2004), Rapala *et al*., (2005), Tsuji *et al*., (2006), Lemes *et al*., (2008), Hu *et al*., (2009) and Zhang *et al*., (2010) have not attained to describe the existence of either *mlr* or different MC degrading genes. Considering this scenario of MC bioremediation, it is not surprising that little information is available regarding the diversity of bacterial degradation mechanisms that underlies the natural removal of MCs in the environment.

In contrast, Manage *et al*., (2009) reported that *Rhodococcus* sp., *Arthrobacter* sp. and *Brevibacterium* sp. do not contain any of the *mlr* genes. Nevertheless, these bacteria achieved a rapid degradation of MC-LR, suggesting that the MC degradation is more diverse than so far has been understood. As previously mentioned here, *Rhodococcus* and *Arthrobacter* genera are widely known to degrade diverse recalcitrant contaminants in the environment. This has been proven by the rapid degradation of a wide range of different peptides (MCs, NOD, ABPNB, GRA, POLYB and ANGTN) by

Rhodococcus sp. (C1) described in chapter 4. Hence, it is highly unlikely that MC degrading enzymes exist not only to degrade MCs but also for the general metabolism for the existence of these bacteria (Kato *et al*., 2007).

Therefore, this study aims to elucidate this universal peptide degrading gene(s) and enzyme(s) as well as their degradation pathway(s). Primarily, the existence of *mlrA* or *mlrA* homologous genes was investigated by a PCR assay to confirm the findings of Manage *et al*., (2009). Elucidation of the novel MC/peptide-degrading gene, commenced with the construction of the fosmid library of *Rhodococcus* sp. (C1). The active recombinants were screened for their MC-LR degradation activity, a screening that ultimately led to the identification of a single gene producing MC degrading enzymes. Microcystinase (MC hydrolysis) activity was then expressed in an expression vector, and the MC degrading protein was purified. Finally, the degradation products and their toxicity were assessed by a protein phosphatase inhibition assay.

5.2 Materials and methods

5.2.1 Chemicals

Chemicals were of analytical-reagent grade unless stated and obtained from Roth, Karlsruhe, Germany. HPLC-grade methanol and acetonitrile were purchased from Rathburn, Walkersburn, UK and Roth, Karlsruhe, Germany. TFA for HPLC systems were purchased from Fisher Scientific, Leicestershire, UK and Roth, Karlsruhe, Germany. Formic acid was obtained from Sigma-Aldrich, Poole, Dorset, UK. Enzymes were purchased from Fermentas, St. Leon-Rot, Germany. Antibiotics were purchased from Roth, Karlsruhe, Germany. Pure water was obtained from a Milli-Q system (purified to 18.2 MΩ, Millipore, Watford, UK). MC-LR was purified from batch cultures of *Microcystis aeruginosa* (table 2.1) as previously described (Edwards *et al*., 1996; Edwards and Lawton, 2010).

5.2.2 Analysis of MC-LR

Analysis of MC-LR was conducted using two separate HPLC systems. HPLC analysis using Waters Alliance 2695 solvent delivery system with PDA and mass detector was carried out as previously described in chapter 2, section 2.2.8. For the rest of the analysis, sub-samples (20 µl) were injected in to Shimadzu HPLC system (Kyoto, Japan) comprising a LC-20AD solvent delivery module and system controller CBM-20A, SPD-M20A UV/VIS PDA detector and CTO-10AS$_{vp}$ column oven, on-line degasser DG-20A5, controlled by the CLASS-VP 6.21 SP5 software. The analysis was performed with a Sunfire C_{18} column (2.1 mm i.d. X 150 mm long; 5 µm particle size) maintained at 40 °C. The mobile phase was (A) Milli-Q water / (B) acetonitrile, both containing

0.05% (v/v) TFA with a linear gradient increasing from 20% to 60% B over 10 min at a flow rate of 1 ml min^{-1} followed by ramp up to 100% B and re-equilibration over the next 5 min. Eluent was monitored at 238 nm wave length and calculated against a standard curve with MC-LR.

5.2.3 Cultivation and maintenance of bacterial strains

5.2.3.1 Preparation of *Actinobacteria* isolates

Bacterial isolates, *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) and *Brevibacterium* sp. (F3) were grown in sterile liquid LB (Lysogeny broth) and they were maintained by regular streaking for every 21 d on fresh, solid LB agar slopes with no antibiotics added in the media. Liquid cultures of above isolates were always incubated in LB media with no antibiotics at 25 °C for 24 h (220 rpm) before they were harvested.

5.2.3.2 Preparation of *Escherichia coli*

E. coli strains were used for the PCR cloning and preparing the fosmid libraries. For PCR cloning, *E. coli* was cultivated in sterile liquid LB medium as well as on LB agar Petri dishes. *E. coli* (Epi 300, Epicentre, Madison, USA) for the fosmid library was incubated in LB media with added ingredients (2% maltose, 0.5 mM $MgSO_4$). Selective growth of transgenic *E. coli was* performed according to their resistance markers by adding antibiotics to the growth medium in the following final concentrations: ampicillin 100 µg ml^{-1}; chloramphenicol 12.5 µg ml^{-1}. *E. coli* cultures were always maintained at 37 °C with constant shaking (220 rpm) of liquid media.

5.2.4 Evaluation of the enzymatic degradation of MC-LR

5.2.4.1 Preparation of the cell extract of *Rhodococcus* sp. (C1)

Rhodococcus sp. (C1) was grown in liquid LB media (250 ml culture volume in 1 l flask) as previously described in section 5.2.3.1 with no antibiotics (25 °C, 24 h, 220 rpm). Exponentially growing bacteria were harvested by centrifugation 3000 g at 25 °C (Biofuge fresco Heraeus, Hanau, Germany) for 15 min and the pellet was washed twice with 50 mM Tris-HCl containing 0.5 mM 1,4-dithiothreitole (DTT; Kato *et al*., 2007). To obtain the bacterial enzyme extract, the cell suspension was sonicated (60 W output) using Sonopuls HD 60 sonicator (Bandelin electronic, Berlin, Germany) on ice for 7 min (4 bursts per min). Homogenised bacterial cell debris was pelleted by centrifugation at 11000 g for 20 min in a Sartorius SIGMA, Germany model 6-16K centrifuge at 4 °C, and the cell extract was decanted, filtered (0.22 µm filter, Millipore, UK) and used for the enzyme activity.

5.2.4.2 Degradation of MC-LR

Fifty microliters of sterile aqueous MC-LR (0.22 µm) was added to 450 µl of the cell extracts (section 5.2.4.1) of *Rhodococcus* sp. (C1) at a final concentration of 10 µg ml^{-1} and incubated at 25 °C for 0, 1, 3, 5, 10, 15 and 24 h respectively. Enzyme assay samples were produced in triplicates in sterile single microcentrifuge tubes (1.5 ml). After the incubation period, 500 µl of methanol containing 0.2% (v/v) of formic acid was added to microcentrifuge tubes containing MC-LR and cell extracts and vortexed gently. The microcentrifuge tubes were centrifuged (11000 g, 10 min, RT) and the

supernatant was analysed in PDA-LC-MS as described in chapter 2, section 2.2.8.

5.2.5 Investigation of the existence of *mlrA* gene in *Actinobacteria* strains

Previous studies (Manage *et al*., 2009) had confirmed that the isolated *Actinobacteria* strains do not harbor *mlr* gene cluster previously reported in *Sphingopyxis* sp. LH21 (Ho *et al*., 2007). To investigate the existence of the *mlrA* or *mlrA* homologues genes in the *Actinobacteria* isolates, two sets of primers were designed to amplify the *mlrA* (National centre for biotechnology and information (NCBI) accession number DQ112243) conserved sequences in the genomes of the isolated bacteria. Forward and reverse primers (table 5.2) were designed using the web-based freely publicly available online software program "Primer3" (http://frodo.wi.mit.edu/primer3/).

Table 5.2 PCR primers designed to investigate the *mlrA* gene in *Actinobacteria* isolates

Name	5'-3' Sequence	Tm (°C)	Target
mlrA FW1	AAAGCCCCGCCAGCCCAG	72	*mlrA*
mlrA RV1	GCAACGTCGTTCCTACCAAT	60	*mlrA*
mlrA FW2	CGAAGAACTGGGCTGGCG	69	*mlrA*
mlrA RV2	CAGGACCACGTAAGACTGCC	66	*mlrA*

5.2.5.1 Preparation of total genomic DNA from *Actinobacteria*

Rhodococcus sp. (C1), *Arthrobacter* sp. (C6) and *Brevibacterium* sp. (F3) were cultured (100 ml) in 500 ml flasks in an incubator (37 °C, 220 rpm) for 24 h in sterile liquid LB medium. Cells were harvested by centrifugation at 4700 rpm

(25 °C) for 10 min and the supernatant was discarded. Bacterial pellets were washed three times with 5 ml of 10 mM TE buffer (10 mM Tris-Hcl, 1 mM EDTA and pH 8.0) by re-suspending the bacterial pellet followed by centrifuging and removing the supernatant. The final pellet was re-suspended in 1 ml of 10 mM Tris-HCl (pH 7.0). Cell lysis was performed by adding freshly prepared lysozyme solution (Serva, Germany) at a final concentration of 2 mg ml^{-1} followed by incubation for 1 h at 37 °C. Sodium dodecyl sulphate (SDS) was added to the solution (final concentration 2%), mixed by inverting the tubes, proteinase K was added (Boehringer, Germany) at a final concentration of 50 µg ml^{-1} as the DNase inhibitor and to degrade cellular proteins followed by an incubation (50 °C) for 2-3 h until the solution becomes clear. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding 1/10 x volume of 3 M Na-acetate and 2 x volume of ice cold 95% ethanol. DNA pellet was washed with 70% ice cold ethanol, air-dried, re-suspended in 100 µl of sterile water. RNase treatment was performed to obtain high quality DNA by adding 1 µl of an RNase (MBI Fermentas, Germany) followed by incubation for 1 h at 37 °C and stored at -20 °C.

5.2.5.2 Determination of nucleic acid concentrations

Concentrations of the purified nucleic acids (DNA) were determined spectrophotometrically with Nanodrop 2000 Spectrophotometer (PEQLAB Biotechnologie GmbH, Germany) by the absorption of the DNA solution (1.5 µl) at a wavelength of 260 nm, which proportionally correlates with the content of nucleic acids.

5.2.5.3 Polymerase Chain Reactions

PCR (Peltier Thermal Cycler PTC-200 Biozym, Oldenburg, Germany) was carried out to amplify the specific DNA reflecting the cDNA (section 5.2.5.1) template, using Qiagen Taq DNA polymerase system (Qiagen, Düsseldorf, Germany). PCR was performed in 20 µl reaction volumes composed of 2 µl of 10 x Taq-buffer, 0.5 µM of each primer, 0.15 mM dNTPs, 1 U of Taq polymerase and 100 ng of genomic DNA or 10 ng of plasmid DNA in 1 µl of TE buffer or water. PCR thermal cyclers were programmed (table 5.3) to alter temperature within sample tubes time-dependently to allow several cycles of DNA synthesis.

Table 5.3 Thermal cyclers for PCR amplification of the template DNA

PCR Step	Temperature	Time span	Effect on DNA
1	95 °C	3 min	Primary denaturation of double stranded (ds) DNA
2	95 °C	30 sec	Denaturation of ds DNA
3	55 °C	30 sec	Specific annealing of primers to amplification site
4	72 °C	1 min/1000 base pair (bp) of amplicon	DNA synthesis according to template
		Repetition of steps from 2 to 4 in 35 cycles	
5	72 °C	10 min	Final elongation step
6	12 °C	∞	Cooling

5.2.5.4 Agarose gel electrophoresis of DNA samples

Agarose gel electrophoresis was used to investigate the quality, nature of the DNA (section 5.2.5.3) and to resolve the DNA fragments according to their size for further cloning purposes. Depending on the size of nucleic acid fragments to be separated, agarose concentrations were adjusted to 0.8 – 1.2% (w/v) where low concentrations of agarose were used for larger DNA i.e. genomic DNA (section 5.2.5.1) and plasmid DNA, and vice versa. Agarose was added to TAE buffer (table 5.4) and the gel was prepared by heating the agarose TAE mixture in a microwave oven. Melted agarose was cooled down to approximately 50 °C and ethidium bromide (Sigma, St. Louis, USA) was added (0.05 µg ml^{-1}) and poured into an electrophoresis chamber (BioRad Laboratories, München, Germany). DNA loading dye (1 µl; table 5.4) was added to the DNA samples (5-7 µl) and loaded to the solidified agarose gel and run at a constant voltage of 100 V (Electroporation unit Gene Pulser II, BioRad Laboratories, München, Germany). DNA was visualised by the Molecular Imager® ChemiDoc™ XRS+ using UV-light emissions (BioRad Laboratories, München, Germany). Parallel ran PstI digested λ-phage DNA was utilised as the size marker.

Table 5.4 Compositions of TAE buffer and DNA loading dye

Component	composition
TAE running buffer	40 mM Tris-HCl and 1 mM EDTA
DNA loading dye	50% (w/v) Glycerol, 1 mM EDTA, 0.05% (w/v) Bromophenol blue and 0.05% (w/v) Xylene cyanol

5.2.5.5 Elution of DNA fragments from agarose gels

Resolved DNA fragments (section 5.2.5.4) of PCR were either eluted from agarose gels or purified as described in section 5.2.5.6 before they were ligated into a vector. Separated DNA fragments in the agarose gel electrophoresis (section 5.2.5.4) were recovered from the gels using the JETSORB Gel Extraction Kit (Genomed, Löhne, Germany). DNA was extracted as recommended by the manufacturer and stored at -20 °C.

5.2.5.6 Purification of PCR products and other DNA

The PCR products obtained were purified before being used in any enzymatic reactions. QIAquick PCR purification kit (Qiagen, Düsseldorf, Germany) was utilised according to the manufacturer's instructions to remove unwanted primers and oligonucleotides, salts and other ingredients from DNA solutions.

5.2.5.7 Ligation of linear DNA fragments into plasmid vectors

DNA fragments synthesised by a PCR using Taq DNA polymerase were ligated into Qiagen pDrive cloning vector (Qiagen, Düsseldorf, Germany) according to the instructions provided by the manufacturer.

5.2.5.8 Transformation of *E. coli*

Once the ligation was completed, recombinant plasmid vectors (pDrive) were transformed into chemically competent ($CaCl_2$-induced; Sambrook*, et al.*, 1989) Top10 *E. coli* cells. An aliquot of 200 µl chemically competent *E. coli* culture was mixed gently and incubated with the respective ligated plasmid

vector on ice for 30 min. The mixture was then subjected to a heat shock in a temperature maintained (42 °C) water bath for 1 min. Recreation of the heat-shocked *E. coli* cells were performed by the incubation (37 °C, 220 rpm, 30 min) in SOC medium (table 5.5; Hanahan, 1983). Transformed *E. coli* cells were plated on LB-agar Petri dishes containing 100 $\mu g ml^{-1}$ ampicillin. Based on a *lacZ* knockout (Jacob *et al.*, 1960) in the vector plasmid, positive ligation of a cloning fragment into pDrive was determined by addition of 40 $\mu g\ ml^{-1}$ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) made blue/white screening. For further analysis, single white coloured colonies were picked and transferred into 5 ml of liquid LB medium containing 100 $\mu g ml^{-1}$ ampicillin.

Table 5.5 Composition of the SOC medium (Hanahan, 1983)

Medium	**Composition**
SOC-medium	2% (w/v) trypton and 0.5% (w/v) yeast extract, 20 mM glucose, 8.6 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$ and pH 7.0

5.2.5.9 Colony PCR

In order to determine the successful cloning of amplified PCR products, a colony PCR was adapted using the basic techniques mentioned above (section 5.2.5.3). The colony PCR was performed using bacterial cellular material directly from the cloned bacteria. Colonies were re-suspended in 10 µl of sterile Milli-Q water, incubated for 10 min at room temperature and 1 µl was used as the bacterial DNA template for the colony PCR. The primary denaturation step was extended for 10 min in order to disrupt the bacterial cell wall and obtain cellular DNA as the template. Other parameters were maintained as described above (section 5.2.5.3).

5.2.5.10 Preparation of plasmid DNA from *E. coli*

In order to sequence the positive recombinants revealed from colony PCR (section 5.2.5.9) and further restriction endonucleases analysis, plasmid DNA of positive clones were extracted using alkaline lysis standard procedure (Sambrook *et al*., 1989). Bacteria were grown in liquid LB media (4 ml) containing 100 $\mu g ml^{-1}$ ampicillin, and harvested by centrifugation at 3000 g for 5 min. The pellet was re-suspended in 300 µl of buffer P1 (table 5.6). Subsequently, 300 µl buffer P2 (table 5.6) was added and the suspension was mixed gently for 5 min at room temperature. Buffer P3 (300 µl; table 5.6) was added to the mixture and incubated in ice for further 5 min period. No vortex mixing was involved during the plasmid DNA preparation to avoid dissociation of genomic DNA from membrane fraction. To precipitate the cellular debris from the DNA, the suspension was centrifuged at 15000 g (4 °C) for 10 min and the supernatant was transferred into clean microcentrifuge tube (1.5 ml).

Nucleic acids in the clear supernatant was precipitated with 0.7 volumes of isopropanol, centrifuged as previously described (15000 g, 4 °C, 10 min) and the supernatant was removed. The remaining pellet was washed with 1 ml of 70% ethanol, re-pelleted and the supernatant was removed. The precipitated plasmid DNA was air-dried and re-suspended in 100 µl of TE buffer.

Table 5.6 Compositions of Buffers P1, P2 and P3

Buffer	Composition
Buffer P1	50 mM Tris-Hcl, 10 mM EDTA and pH 8.0
Buffer P2	200 mM NaOH and 1% SDS
Buffer P3	3 M Potassium acetate and pH 5.0

5.2.5.11 Restriction digests of DNA

Endonucleolytic cleavage of DNA was used as a tool to investigate the successful cloning of an insert DNA as well as to quantify the size to the insert DNA during the cloning of an unknown insert DNA. The restriction digestion of DNA was performed using endonucleases and compatible buffers supplied by either Fermentas or New England Biolabs (NEB) according to the reaction conditions suggested in manufacturer's recommendations. The amount of DNA for the digestion was fixed at 0.1– 0.5 µg in 20 µl reaction volumes. The digestion took place at 37 °C (or else otherwise according to the demands of particular enzyme provided by the manufacturer) for 1 h and the reaction was stopped by heat inactivation at recommended temperature supplied by the manufacturer.

5.2.5.12 Sequencing of DNA fragments

Successfully cloned positive recombinants identified through colony PCR (section 5.2.5.9) and restriction digestion analyses (section 5.2.5.11) were sequenced to identify the PCR amplified insert DNA from *Actinobacteria* genomes. DNA sequencing was performed through commercially available service by GATC Biotech, Germany. Sequencing prerequisites and preparations were arranged according to the company demands provided (30-100 ng ml^{-1} purified plasmid DNA). DNA sequences were complied and aligned with freely publicly available software program "Chromas Lite 2.01" (Technelysium Pty Ltd, Brisbane, Australia). NCBI BLAST online searches were used to identify similar sequences of DNA (http://blast.ncbi.nlm.nih.gov) from the NCBI public database (http://www.ncbi.nlm.nih.gov/Genbank).

5.2.6 Elucidating microcystinase producing genes in *Actinobacteria*

Previous studies (Manage *et al*., 2009 and section 5.2.5) indicated that none of the *Actinobacteria* isolates contained *mlrA* or *mlrA* homologous genes. This clearly suggested that, genes responsible for the degradation of MC-LR in the *Actinobacteria* were completely different to the *mlr* genes of *Sphingomonas* sp. Therefore, to identify and characterise the gene(s) involved in the degradation mechanism, the following molecular biology techniques and standard procedures were adapted and utilised according to the requirements of the study.

5.2.6.1 Selection of the *Actinobacteria* candidate for the fosmid library

To construct the fosmid library, one bacterial strain was selected to represent the *Actinobacteria* MC degraders. To allow this, MC-LR degradation efficiency of *Rhodococcus* sp. (C1), *Arthrobacter sp*. (C6) and *Brevibacterium* sp. (F3) was evaluated in a MC-LR degradation assay to select the most efficient MC degrading strain.

Cell extracts of *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) and *Brevibacterium* sp. (F3) were prepared as previously described in section 5.2.4.1 and the degradation of MC-LR was evaluated (section 5.2.4.2). Samples for HPLC analysis were removed at 0, 1, 3, 5, 10, 15 h and analysed using Shimadzu HPLC system as described in section 5.2.2.

5.2.6.2 Construction of the fosmid genomic library

MC-LR degradation assay (section 5.2.6.1) has enabled the selection of *Rhodococcus* sp. (C1) as the potential candidate for construction of the fosmid genomic library. *Rhodococcus* sp. (C1) genomic DNA fosmid library was generated with the Copy Control Fosmid Library Production kit (Epicentre, Madison, USA) using the fosmid vector pCC2FOS. The library construction was performed according to the manufacturer's instructions provided with the fosmid kit (figure 5.1). Isolated *Rhodococcus* sp. (C1) genomic DNA (section 5.2.5.1) was randomly sheared by passing through a 200 µl small-bore pipette tip to obtain ~ 40 kb (kilo bases) DNA fragments (figure 5.1). The size of the sheared genomic DNA was examined by running on an agarose gel using the fosmid control DNA as a size marker. The ligation of the genomic DNA was

performed with Fast-Link 10X ligation buffer, Fast-Link DNA ligase, ATP, pCC2FOS cloning-ready vector (figure 5.1). The ligated DNA was packed into the lambda phage, infected EPI300-T1^{R} *E. coli* (section 5.2.3.2) and the fosmid library was plated on LB agar Petri plates containing 12.5 µg ml^{-1} chloramphenicol. For screening purposes of the library, 600 fosmid clones were picked and stored in grid number labelled LB agar Petri plates containing chloramphenicol (five plates, each containing 120 individual clones; figures 5.1 and 5.2).

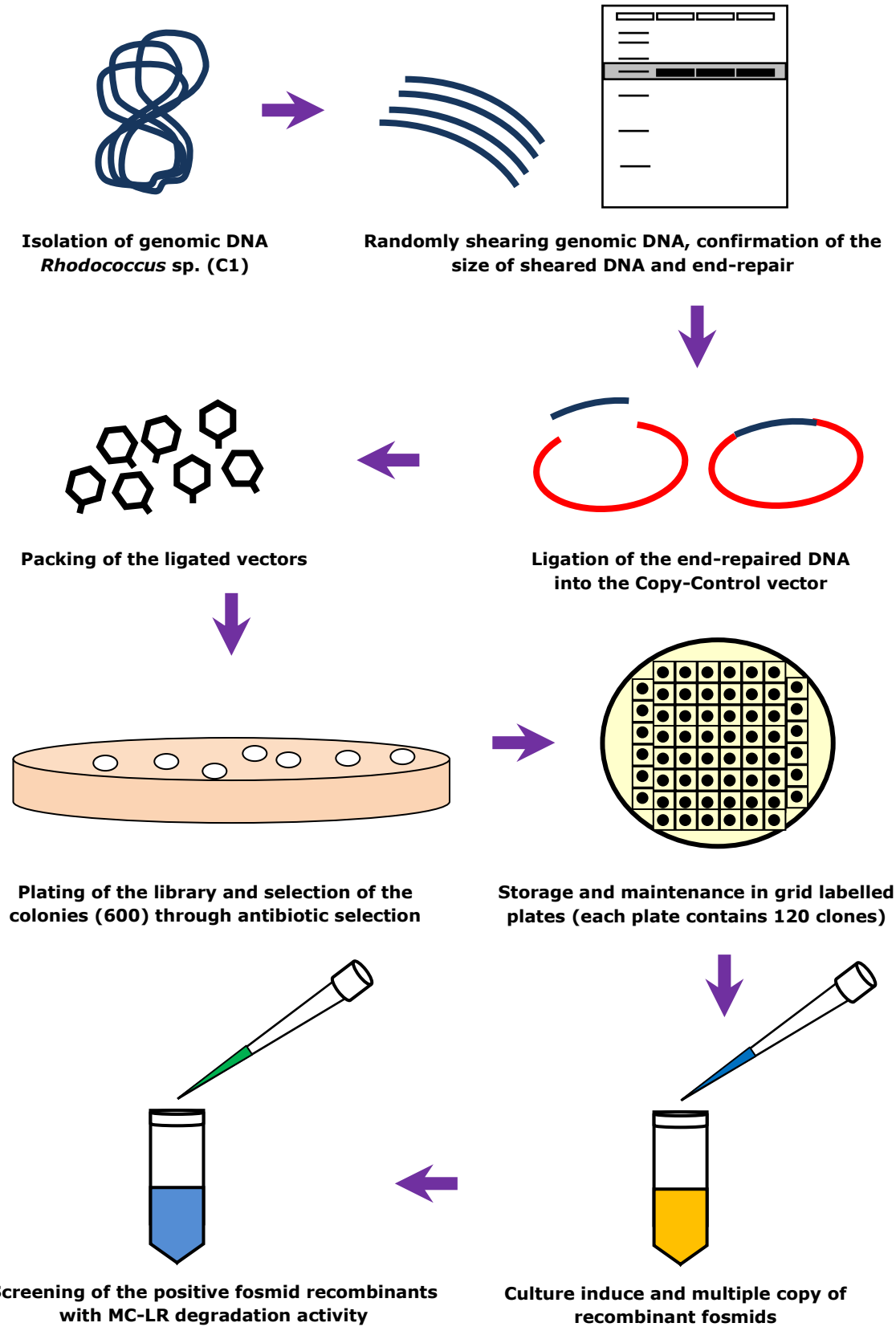

Figure 5.1 Schematic of the construction of the *Rhodococcus* sp. (C1) fosmid library and screening for active fosmid recombinants.

5.2.6.3 Library screening for the microcystinase activity

Screening of the fosmid library was carried out using the pools of individual bacteria. Each pool for screening contained 25 individual fosmid clones. Liquid cultures of fosmid clones were prepared in liquid LB medium supplemented with 12.5 µg ml^{-1} chloramphenicol (37 °C, 250 rpm). According to manufacturer's instructions (Epicentre, Madison, USA) the clones were amplified into a higher fosmid number by adding the Copy-Control fosmid auto-induction solution provided with the kit (figure 5.1).

Exponentially growing bacteria was harvested; cell suspensions were prepared in Tris-HCl; sonicated and the cell extract was prepared for the enzyme activity (section 5.2.4.1). MC-LR degradation assay was performed as described in 5.2.4.2. Samples for HPLC analysis were removed at 0, 1, 5, 10, 15 h and analysed using Shimadzu HPLC system as described in section 5.2.2. Once a positive pool of clones were found with MC-LR degradation activity, the enzyme extracts of the 25 individual clones were pooled again into smaller groups of 5 individual clones and the screening was carried out to obtain each single positive fosmid clones (figure 5.2). Finally, the screening resulted in four active fosmid recombinants (F105, F112, F113 and F114).

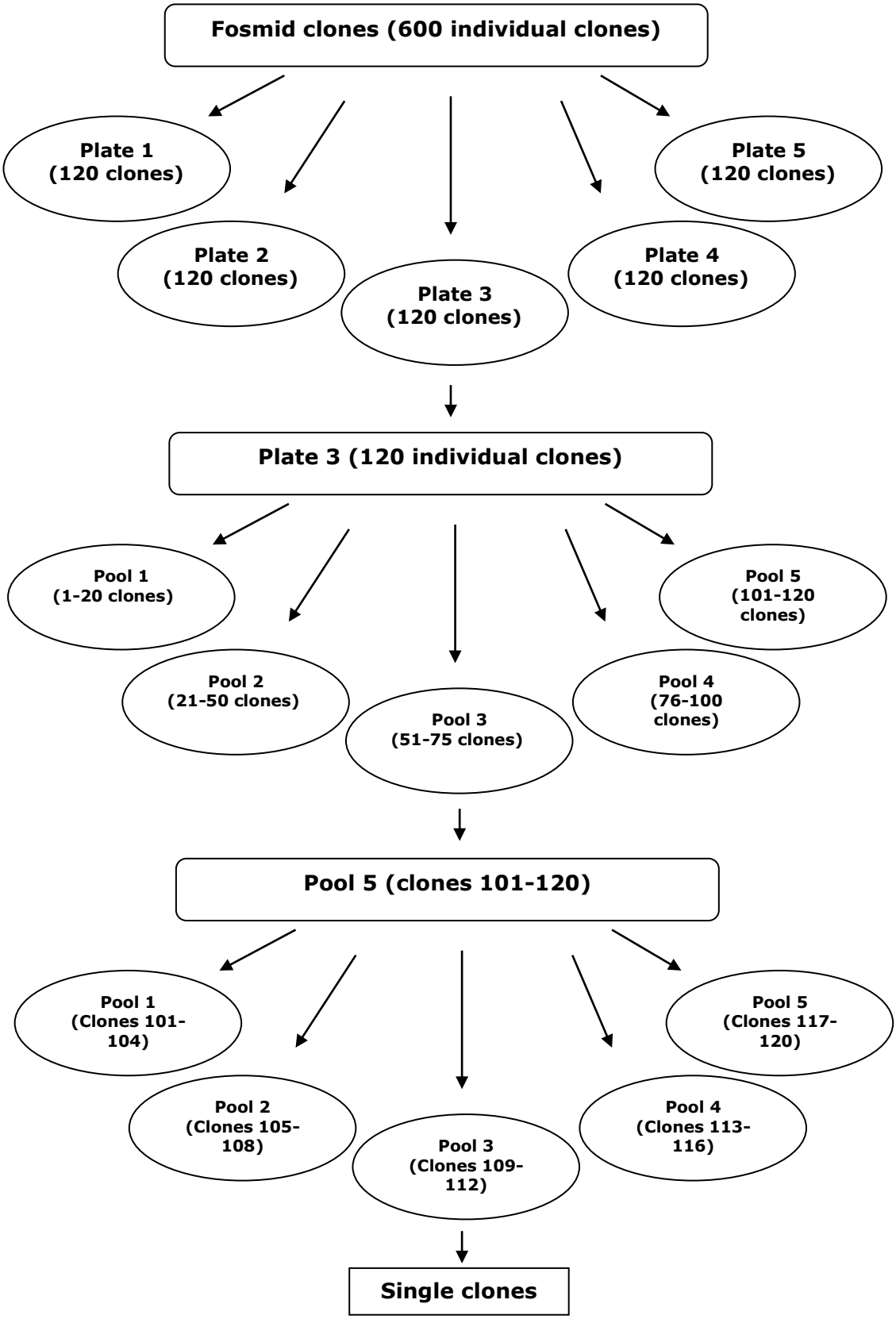

Figure 5.2 Schematic of the screening procedure (section 5.2.6.3) for the positive clones with MC-LR degradation activity

5.2.6.4 Sub-cloning of the active recombinant fosmids

Active recombinant fosmids contained ~ 40 kb insert DNA, which may contain a number of different genes from the *Rhodococcus* sp. (C1) in a single clone. Thus, to facilitate successful isolation of single genes, the insert DNA was further digested into smaller fragments and sub-cloned as follows (figure 5.2).

5.2.6.4.1 Preparation of insert DNA

Active recombinant fosmids (F105, F112, F 113 and F114) were isolated and purified using the alkaline lysis standard procedure (Sambrook *et al*., 1989) described in section 5.2.5.10. Sequencing of the positive fosmids was performed at University of Humboldt, Berlin, Germany. Purified DNA was digested using the restriction endonucleases (EcoRV, ScaI and HindIII) recommended by the manufacturer (Epicentre, Madison, USA; figure 5.2). To determine the eligibility of the digested DNA for sub-cloning, digested DNA fragments were resolved in 0.8% (w/v) agarose in TAE buffer (section 5.2.5.4).

5.2.6.4.2 Preparation of the vector DNA and ligation

Plasmid vector DNA - pUC 19 (Amersham Pharmacia, Germany) was prepared through the processes of restriction digestion, dephosphorylation and purification of the vector DNA. Circular pUC 19 vector was linearised by a blunt end restriction digestion (section 5.2.5.11) using HincII enzyme. The vector DNA went through a dephosphorylation step to avoid the natural re-ligation. The dephosphorylation was performed by adding alkaline phosphatase

(Fermentas FastAP) into ~1 µg of the purified linear vector DNA (through Qiagen PCR cleaning kit), alkaline phosphatase buffer provided by the manufacturer and nuclease free water up to a volume of 20 µl. The reaction was incubated at 37 °C for 30 min before inactivating the enzymatic reaction by heating to 75 °C for 15 min.

HincII, created blunt end ligation, performed with 20-100 ng of leaner vector DNA with a molar ratio up to 1:5 of the insert DNA (section 5.2.6.4.1). T4 DNA ligase (Fermentas, St. Leon-Rot, Germany) was added into a 20 µl reaction mixture containing T4 DNA ligase buffer, 50% polyethylene glycol (PEG) 4000 solution and nuclease free water (Fermentas, St. Leon-Rot, Germany). The ligation reaction was incubated for 1 h at 22 °C, directly transformed into *E. coli* cells and plated on LB-agar Petri dishes containing ampicillin (section 5.2.5.8).

5.2.6.5 Screening of the sub-cloned library

Individual sub-clones selected by ampicillin and X-Gal, IPTG made blue/white selection were grown (section 5.2.3.2) and harvested; the cell suspensions were prepared in Tris-HCl; sonicated and the cell extract was obtained for the enzyme activity (section 5.2.4.1). MC-LR degradation assay was performed as described in 5.2.4.2. Samples for HPLC analysis were removed at 0, 1, 5, 10, 15 h and analysed using Shimadzu HPLC system as described in section 5.2.2. Once a sub-clone was found with MC-LR degradation activity, their plasmid DNA was isolated (section 5.2.5.2), sequenced (section 5.2.5.12) and the gene sequence responsible for MC degradation was elucidated. The size and

the orientation of the insert DNA was determined by the restriction digestion (HindIII and EcoRI) of the plasmid DNA of the positive sub-clones (section 5.2.5.11).

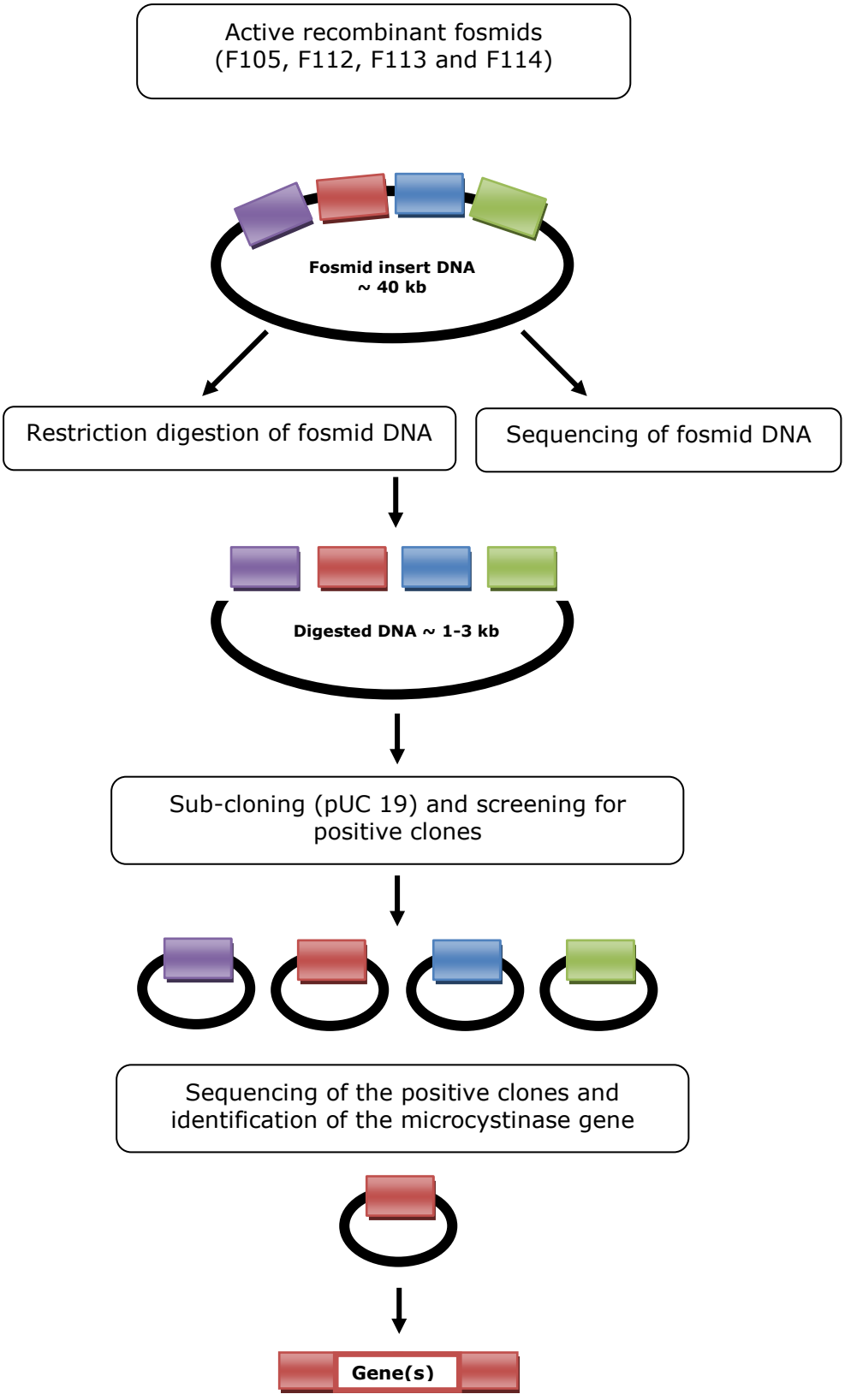

Figure 5.3 Schematic of the isolation and identification of the microcystinase enzyme producing gene(s) using the constructed fosmid library.

5.2.6.6 PCR gene cloning of the microcystinase producing gene (MPG)

Elucidated DNA sequence has revealed MPG belongs to the class of aminotransferase (AT) enzyme producing genes. To further evaluate the expression of the gene and the production of microcystinase enzymes (ME), MPG was amplified using a PCR and cloned into an expression vector. PCR primers designed (table 5.7) matched the start and stop codons of the elucidated DNA sequence of MPG to enable open reading frame (ORF) for the production of the ME(s). The primer pairs AT FW and AT RV were used in PCR reactions to amplify the MPG of *Rhodococcus* sp. (C1). To allow accurate amplification of the gene, proofreading Pfu polymerase enzyme (Fermentas, St. Leon-Rot, Germany) was used according to the manufacturer's instruction; a short incubation with Taq polymerase (72 °C, 10 min,) followed to add A-overhangs for TA-cloning. Restriction sites, NdeI and BamHI were deliberately added to each amplicon's 5' and 3' end, respectively. PCR amplified MPG was then cloned into pDrive vector, re-digested with NdeI and BamHI, and ligated into the pET 15b expression vector (Novagen/Merck, Darmstadt, Germany) previously linearised with the same restriction enzymes. Following the ligation, pET 15b expression vectors were transformed into *E.coli* BL21 (DE3) cells (Novagen/Merck, Darmstadt, Germany) for the co-expression of the recombinant protein.

Table 5.7 Primers designed for the amplification of the microcystinase producing genes

Name	5'-3' Sequence	Tm (°C)	Target
AT FW	CATATGAGCCCGCTGCAAGTGC	72	MPG
AT RV	GGTTCTCAGTTCGCGCAGGGCCTTC	78	MPG

5.2.6.7 Preparation of proteins from bacterial cells

The pET-15b vector carries an N-terminal 6xHis-Tag sequence which allows purification of the recombinant protein. To investigate whether pET 15b had expressed the production of 6xHis-Tag recombinant protein (6xHis-Tagged AT) and if expressed, to determine the target protein solubility, a pilot protein extraction was carried out. Bacterial cells of logarithmic phase were harvested by centrifugation at 3000 g (4 °C) for 10 min. Pellets were re-suspended in native extraction buffer (table 5.8; 1 ml per 100 ml cell culture) and sonicated on ice for 5 min (4 bursts per min). Sonication mixture was centrifuged (11000 g, 6 min, 4 °C) and cell debris was pelleted to recover the soluble protein fraction (supernatant). Insoluble protein fraction was recovered from the cell pellets remaining after native extraction. Pellet was re-suspended in denaturing extraction buffer (table 5.8), sonicated up to 5 bursts (5 sec each) and supernatant with insoluble proteins was recovered. Expression of the protein was analysed by SDS-Polyacrylamide gel electrophores (PAGE) analysis.

Table 5.8 Compositions of the native and denaturing extraction buffers

Buffer	**Composition**
Native extraction buffer	500 mM Tris-HCl, 50 mM EDTA, 0.5 mM phenyl-methyl-sulphonyl-fluoride (PMSF) and pH 8.0
Denaturating extraction buffer	100 mM Na_2HPO_4, 10 mM Tris-HCl, 8 M urea and pH 8.0

5.2.6.8 SDS – Polyacrylamide gel electrophoresis

SDS – PAGE has been set up to assess the total and recombinant proteins produced by pET 15b BL21 cells. Protein samples were electrophoretically separated under the denaturing conditions, according to the discontinuous method as described by Laemmli, (1970). Protein gels were prepared using Mini-PROTEANR Tetra system by BioRad (BioRad Laboratories, München, Germany) and the electrophoresis was conducted through Tris-glycin buffer system based SDS polyacrylamide gels (PAA). Separating gel was composed of 12.5% acrylamide while stacking gel was made up with 4% acrylamide (table 5.9). Protein concentration of a sample was adjusted (5-20 µg ml^{-1}), added reducing SDS-5x loading (table 5.9) dye and denatured at 95 °C for 10 min. Each gel was completely immersed in SDS-PAGE running buffer (table 5.9) and ran at constant currents of 30 mA in a BioRad Mini Protean electrophoresis chamber (BioRad Laboratories, München, Germany) loaded with Fermentas PageRuler Pre-stained Protein Ladder (Fermentas, St. Leon-Rot, Germany). Gels were floated in "Roti-Blue" Coomassie staining for 3 h on a platform shaker. Stained backgrounds were gently removed by floating and rotate shaking in distilled water for an hour.

Table 5.9 Compositions of buffers and gels prepared for SDS-PAGE

Component	Composition
Separating gel	12.5% (v/v) acrylamide/bisacrylamide 37.5:1, 375 mM Tris-HCl,pH 8.8 and 0.1% (w/v) SDS
Stacking gel	4% (v/v) acrylamide/bisacrylamide 37.5:1, 125 mM Tris-HCl, pH 6.8 and 0.1% (w/v) SDS
5x loading dye	0.3125 M Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 25% - mercaptoethanol and 0.005% (w/v) bromophenol blue.
Running buffer	192 mM Glycin, 25 mM Tris and 0.1% (w/v) SDS

5.2.6.9 Western blot transfer of SDS–PAGE

To confirm the expression of the recombinant 6xHis-Tagged proteins in section 5.2.6.7, PAA gels were transferred into nitrocellulose membranes for immunodetection of the SDS-PAGE separated proteins. The proteins were immobilised on Hybond C-extra Nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany) via electro-blotting. Blotting was performed using the BioRad Mini TransBlot Cell system (BioRad Laboratories, München, Germany). Membranes were prepared in equal size to the PAA Gel, equilibrated for 10 min in Western blot transfer buffer (15.6 mM Tris and 120 mM Glycine) before blotting "sandwich" was prepared according to manufacturer's instructions. Blots were run at a constant voltage of 100 V for 1 h.

5.2.6.10 Immunodetection on Western blot membranes

Followed by the Western blot, an immunodetection was performed to determine the 6xHis-Tagged recombinant proteins produced by the expressed vector. Membranes were blocked with 5% (w/v) skimmed milk powder in TBS-T buffer for 2 h. Primary antibody, anti-mouse, poly histadine (Pineda-Antikorper-Service, Germany) was added at 1:50000 concentration and the incubation was continued for at least 1 h by gently rocking the membranes at 4 °C. Membranes were rinsed twice for 10 min with TBS-T, added secondary antibodies (anti-mouse; Sigma-Aldrich, Buchs, Switzerland) conjugated with horseradish peroxidase in a dilution of 1:10000. Membranes were incubated in 20 ml of secondary antibody solution for 1 h at 4 °C. Finally, membranes were washed 4 times with fresh TBS-T (10 mM Tris-HCl, 150 mM NaCl and 0.1%

(v/v) Tween 20) for 30 min. Visualisation of band signals was performed using the Pierce SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA) according to manufacturer's instructions. Image detection and development was performed with ChemiDoc XRS+ imaging system under standard photographic conditions.

5.2.6.11 Heterologous expression and purification of recombinant proteins

Recombinant BL21 cells containing MPG were grown in liquid LB media (200 ml x 4) up to $OD_{600\ nm}$ of 1.0, and were induced with 0.5 mM IPTG for the heterologous expression. After the induction, cells were further incubated (150 rpm, 15 °C) overnight in a cold room. Cells were harvested, disrupted by sonication and the soluble protein fraction was recovered as previously described (section 5.2.6.7). Protein containing native extraction buffer was supplemented with 20 mM imidazole and 0.5 mM PMSF to facilitate binding of the proteins to the Ni-NTAagarose matrix added. The matrix was gently mixed using a rotate mixer for 1 h prior to the washing steps (x2) with 40 mM imidazole containing native buffer. Proteins attached to the Ni-NTAagarose matrix were eluted with 250 mM imidazole containing native extraction buffer (6 fractions). Purity of eluted protein was determined by SDS-PAGE analysis (section 5.2.6.8).

5.2.6.12 Concentration and desalting of protein solutions

Purified protein fractions were concentrated with Amicon Ultra centrifugal filters (Millipore, Billerica, USA) with a molecular weight cut-off of 30000 Da following the manufacturer's instructions. Desalting and buffer exchange (100 mM Tris-HCl, 1mM DDT, pH 8.0) of the purified proteins was performed using Sephadex G-25M pre-packed column according to manufacturer's instructions (Sigma-Aldrich, Buchs, Switzerland).

5.2.6.13 Quantification of extracted proteins – Bradford assay

Bradford assay (Bradford, 1976) has been used to scale the concentrations of total bacterial protein of the purified fractions. Volume of 795 µl of double-distilled water was mixed with 200 µl of the Bradford reagent for a 5 µl of protein solution to be analysed. After an incubation time of 10 min, change of the Bradford reagent was measured spectrophotometrically at 595 nm. A standard calibration curve prepared with a serial dilution of bovine serum albumin (BSA) was used to determine individual concentrations of the protein samples.

5.2.7 Disruption of microcystinase producing gene expression

To confirm the essential function of MPG for degradation of MC-LR, the degradation profiles of SC105 and SC105 with partially digested MPG (SC105D) were evaluated. To create SC105D, the SC105 plasmid DNA was digested by XhoI and EcoRI (section 5.2.5.11; figure 5.4), 888 bp of the MPG was removed and re-ligated (section 5.2.5.7) into pUC 19 vector. MC-LR degradation assay (section 5.2.4.2) was performed using the cell extracts of SC105 and SC105D. Degradation of MC-LR was analysed by HPLC methods described previously (section 5.2.2).

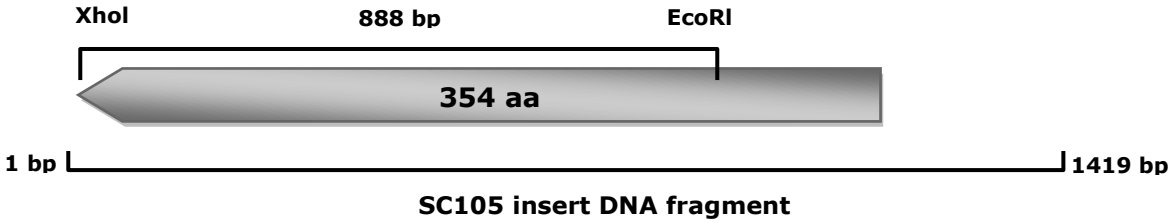

Figure 5.4 Digested sequence of SC105 to disrupt the activity of MPG.

5.2.8 Optimisation of MC-LR degradation assay with microcystinase enzyme

Protein sequence searches using EMBL-EBI database (European Bioinformatics Institute; http://www.ebi.ac.uk/Databases/) revealed that ME is a pyridoxal phosphate (PLP) dependent aminotransferase enzyme (accession number IPR000192). Therefore, to optimise the MC-LR degradation assay, 0.5 mM PLP, aspartate and PMSF were added into pure enzyme fractions. MC-LR degradation assay was performed (section 5.2.4.2) using purified enzyme instead of the cell extracts. Final concentration of the ME was adjusted to 100 µg ml^{-1}.

5.2.9 Analysis of the MC-LR degradation products

Induced pET 15 b BL21 *E. coli* cell extracts were prepared and the enzymes were extracted in 50 mM Tris-HCl containing 0.5 mM DTT (section 5.2.4.1). MC-LR degradation assay was performed (section 5.2.4.2) and the samples were removed hourly. In addition to the cell extracts, purified and optimised ME was utilised to enhance the hydrolysis of MC-LR into its degradation products. MC-LR and its degradation products were analysed by the UPLC-MS (chapter 4; section 4.2.3.3).

5.2.10 Evaluation of the toxicity of the degradation products

The toxicity of the degradation products were evaluated using a protein phosphatase inhibition assay (PPIA) described by Liu *et al*., (2005). Buffer solutions required for the PPIA were freshly prepared (table 5.10). Degradation products of MC-LR (10 µl; section 5.2.8) and equal volume of buffer A was added to the 96 well microtiter plates (Fisher Scientific, Leicestershire, UK) in triplicates. The microtiter plates were shaken gently (5 min) to allow adequate mixing of the enzyme and the degradation products. To start the enzyme reaction, 200 µl of buffer b (substrate) was added and the plates were immediately incubated for 1 h at 37 °C. The colour development in the plate was measured by a Dynex microplate reader (Jencons, Leighton Buzzard, UK) at a wavelength of 405 nm. Production of p-nitrophenol would result in an enzyme substrate reaction taking place in the well which can be measured and quantified spectroscopically. The positive control contained 10 µg ml^{-1} of MC-LR where negative control contained Milli-Q instead of the degradation products. Care was taken to avoid buffers and the microtiter

plates being exposed to direct light as p-nitrophenyl phosphate is light sensitive.

Table 5.10 Compositions of buffers used for protein phosphatase assay

Buffer	**Composition**
Buffer A	50 mM Tris-HCl, 1.0 g l^{-1} BSA, 1.0 mM $MnCl_2$, 2.0 mM DTT, 5 µg ml^{-1} protein phosphatase 1 (PP1) and pH 7.4
Buffer B	50 mM Tris-HCl, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 5 mM p-nitrophenyl phosphate and pH 8.0

5.3 Results

5.3.1 Evaluation of the enzymatic degradation of MC-LR

Degradation of MC-LR was analysed using the cell extract of *Rhodococcus* sp. (C1) to evaluate the efficiency of the MC-LR degradation by intracellular enzymes. HPLC analysis of the assay samples revealed a rapid degradation of MC-LR during the 24 h course of incubation (figure 5.3). During the first 1 h, almost 95% of the MC-LR was degraded. However, the degradation decreased considerably after 1 h, suggesting that critical enzymatic degradation occurred during the first hour of the degradation assay (figure 5.3). Total percentage degradation of MC-LR achieved during the 24 h period was 97%. Some other peaks were detected in the HPLC chromatograms at 1 h and 24 h while MC-LR decreased rapidly (figure 5.3). These peaks could be attributed to the degradation products of MC-LR.

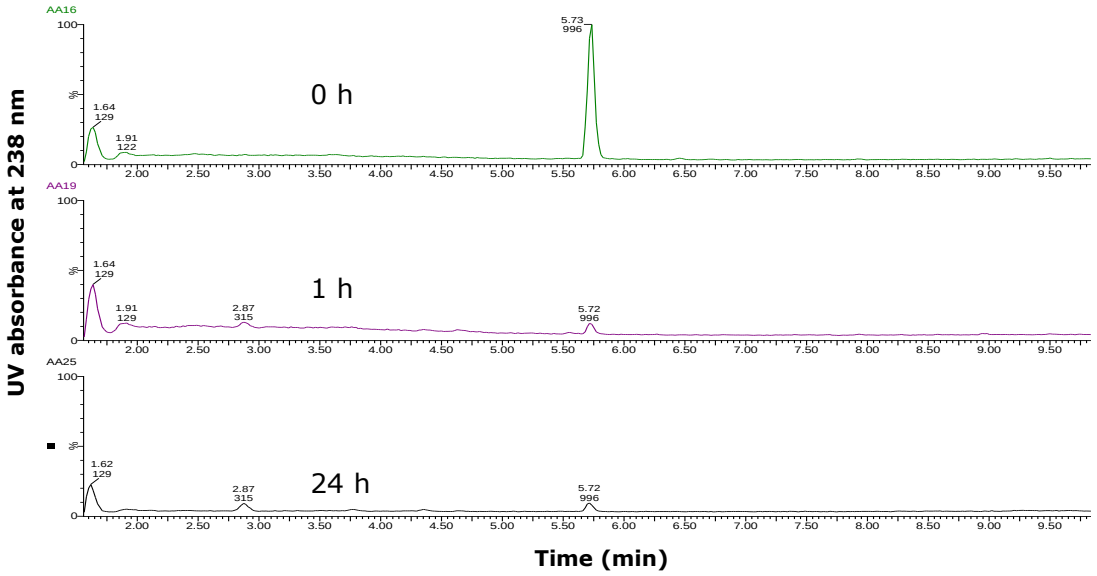

Figure 5.5 HPLC chromatograms of the degradation of MC-LR in the presence of *Rhodococcus* sp. (C1) cell extract at 0 h, 1 h and 24 h.

5.3.2 Investigation of the existence of *mlrA* in *Actinobacteria* strains

Investigation of the existence of the *mlrA* gene in the *Actinobacteria* isolates was mainly based on the PCR amplifications using the genomic DNA from *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) and *Brevibacterium* sp. (F3) (figure 5.6).

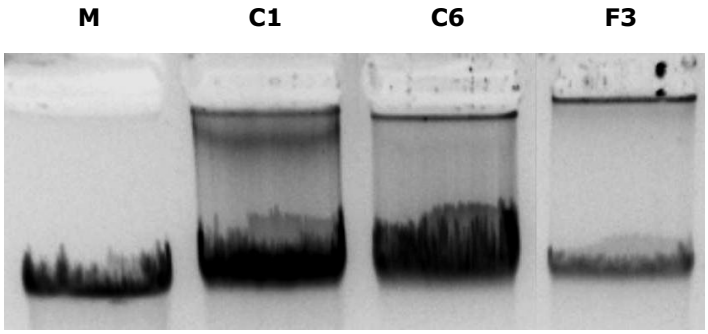

Figure 5.6 Total genomic DNA isolated from *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) and *Brevibacterium* sp. (F3). Fosmid control DNA (M) was used as the size selection marker (~ 40 kb).

Two different pairs of primers were designed to ensure the maximum amplification of the conserved sequence of the *Actinobacteria* genomes to *mlrA*. PCR contained four different polymerase reactions using the combinations of the four different primers, i.e. FW1 RV1, FW2 RV1, FW2 RV1 and FW2 RV2 (figure 5.7). Using the primer set FW1 RV1; PCR amplified two different sizes of DNA (bands at ~ 900 bp and ~ 700 bp) with *Rhodococcus* sp. (C1) genomic DNA as a template. *Arthrobacter* sp. (C6) genomic DNA was utilised to amplify two different sizes of PCR products indicating the bands at ~ 700 bp and ~ 450 bp, while they amplified only one size of DNA fragment for *Brevibacterium* sp. (F3; figure 5.7). Similar banding patterns to C6 and F3 were found in C1 and C6 when they were amplified using the primers FW1 RV2. Single DNA fragment was amplified for C1 (~ 1700 bp) and F3 (~ 1500 bp) by FW2 RV1. No bands were visible in the negative control (N) showing no DNA contamination in the PCR.

For PCR cloning, three different sizes of amplified DNA were selected representing the three different bacteria. PCR product of ~ 700 bp was identical for *Rhodococcus* sp. (C1) and *Arthrobacter* sp. (C6) genomic DNA. The ~ 500 bp DNA fragment was common to all three bacteria using FW1 RV1 and FW2 RV2 primers. Third candidate PCR product had size of ~ 1500 bp from *Brevibacterium* sp. (F3; figure 5.7). Colony PCR analysis has confirmed the accuracy of the pDrive cloning of the selected PCR products (figure 5.8). Clear bands were visible from the positive clones selected for colony PCR at ~ 700 bp, ~ 500 bp and ~ 1500 bp, confirming their potency for sequencing (figure 5.8).

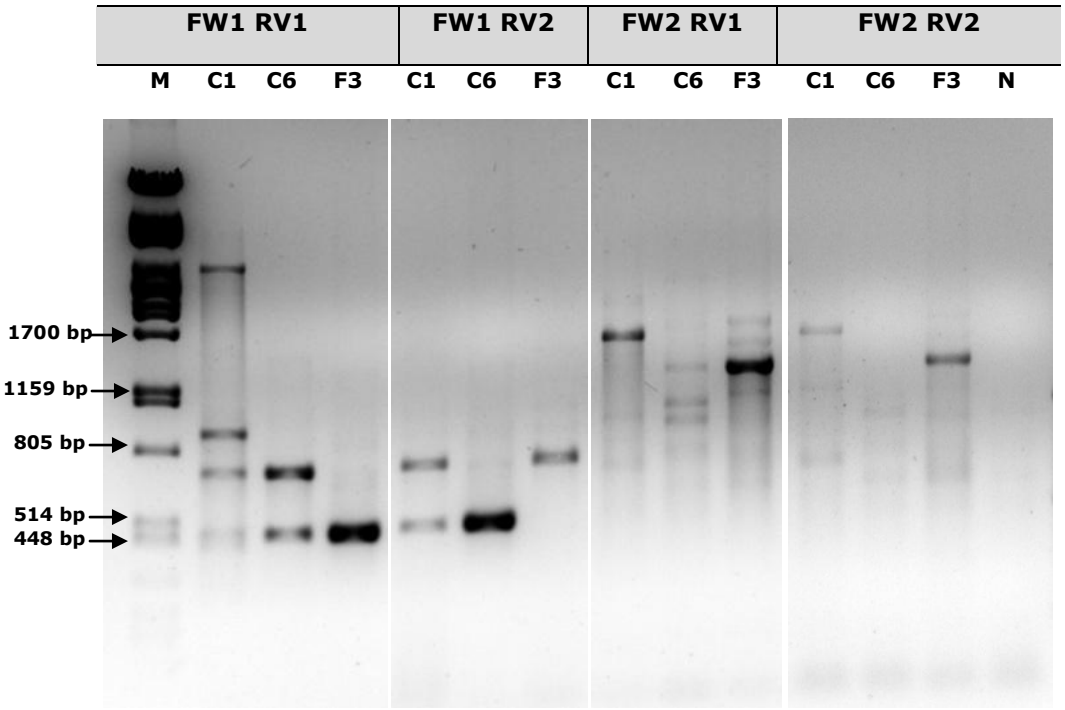

Figure 5.7 PCR screen of *Rhodococcus* sp. (C1), *Arthrobacter sp.* (C6) and *Brevibacterium* sp. (F3) for the presence of the *mlrA* gene. PCR performed with the primers *mlrA* FW1, RV1, FW2 and RV2 as described in section 5.2.5. DNA size marker (M) was Lambda (λ) PstI and labelled at 1700 bp, 1159 bp, 805 bp, 514 bp and 448 bp. N stands for the negative control which contained sterile water instead of the template DNA.

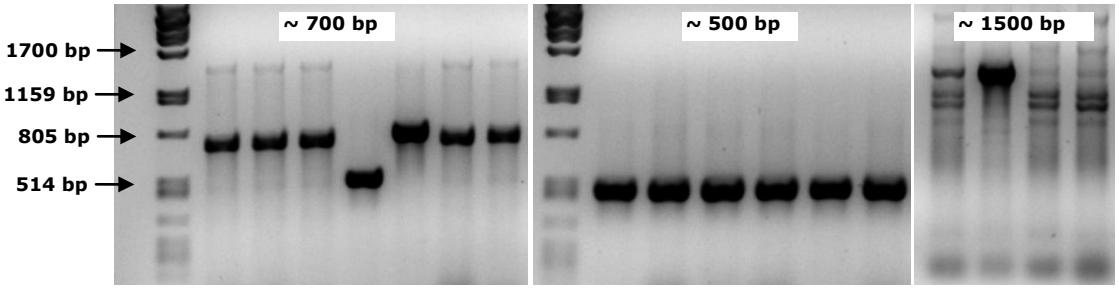

Figure 5.8 Investigation of the pDrive cloning of the amplified PCR products of ~ 700 bp, ~500 bp and ~ 1500 bp. λ PstI was used as the DNA size marker and labelled at 1700 bp, 1159 bp, 805 bp and 514 bp.

Search results from GenBank revealed that none of the PCR amplified DNA sequences were similar to that of *mlrA* sequences (GeneBank accession numbers DQ112243, AF411068, AB114203, AB161685 and AB114202) reported by Bourne *et al*., (2001), Saito *et al*., (2003) and Ho *et al*., (2007). The sequence of ~ 500 bp PCR product was similar (100% identity) to the transposition helper protein in *Rhodococcus erythropolis* while, ~ 700 bp and ~ 1500 bp sized PCR products showed 99% identity for hypothetical protein RER18230 and RER18240 respectively in the same bacterial strain. Thus, it is possible that *Actinobacteria* MC degraders harbour entirely new genes for MC degradation pathways. This indicated the importance of further studies to elucidate the novel genes involved in the degradation of MC by *Actinobacteria*.

5.3.3 Elucidating microcystinase producing genes in *Actinobacteria*

5.3.3.1 Selection of the *Actinobacteria* candidate for the fosmid library

MC-LR was exclusively degraded by the cell extracts of *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) and *Brevibacterium* sp. (F3). *Arthrobacter* sp. (C6) achieved 70% of the MC-LR degraded whereas *Brevibacterium* sp. (F3) removed ~ 50% of MC-LR after 15 h incubation of the assay (figure 5.9). A notable degradation occurred in the presence of the *Rhodococcus* sp. (C1) cell extract. Only trace amounts of MC-LR remained after removing 97% of the compound within 15 h (figure 5.9). This result was consistent with the previously observed degradation of MC-LR by the cell extract of *Rhodococcus* sp. (C1) in section 5.2.4 (figure 5.5). In conclusion, this study clearly indicated *Rhodococcus* sp. (C1) as a potential MC degrading candidate for construction of the fosmid library.

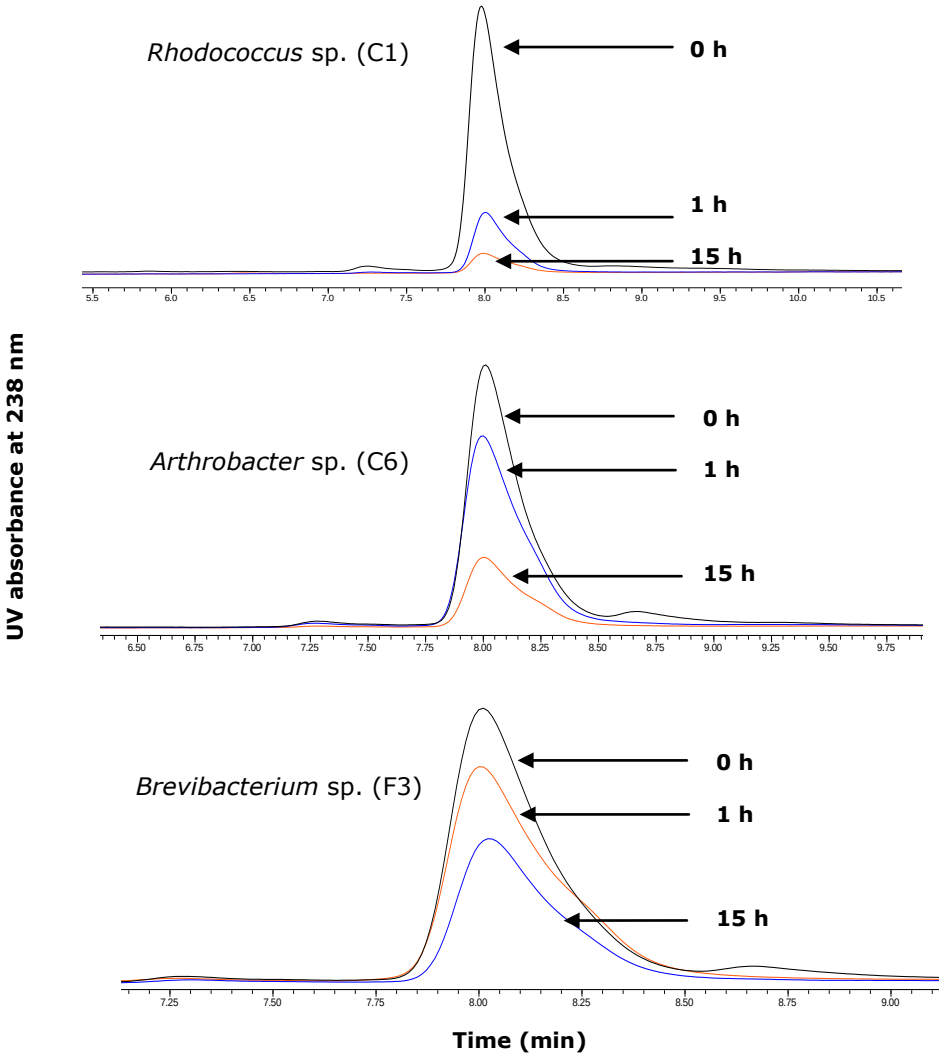

Figure 5.9 HPLC chromatograms of the degradation of MC-LR in the presence of *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) and *Brevibacterium* sp. (F3) cell extracts after 0 h, 1 h and 24 h.

5.3.3.2 Construction of the fosmid library and screening

Fosmid library was constructed using the total genomic DNA of *Rhodococcus* sp. (C1) and has resulted in 684 recombinant *E. coli* selected through their antibiotic resistance. For screening and maintenance, 600 clones were randomly selected and plated in five different grid marked plates each containing 120 recombinant fosmid clones (figure 5.2). Initial screening of the

fosmid library has revealed the microcystinase activity in a pool of 25 recombinants. For further screening, the cell extracts of the positive pool (25 clones) were separated in to five different pools each containing cell extracts of five different clones (figure 5.2). In addition to the rapid degradation, another dominant peak eluting just after MC-LR appeared on the HPLC chromatogram after 15 h incubation (figure 5.10). Although degradation products were not analysed at this stage of study, it could be attributed to a degradation product derived from MC-LR. Individual screening of the fosmid clones indicated four active fosmid recombinants with microcystinase activity (figure 5.10) and designated as F105, F112, F 113 and F114. The active fosmid recombinants were sequenced and their DNA sequences were compared (NCBI BLAST) with published sequences of related species of the genus *Rhodococcus* and several representative species of *Actinobacteria*. Sequencing of the fosmid DNA (F105, F112, F 113 and F114) confirmed their origin of the insert DNA is solely from *Rhodococcus* sp. (C1); hence, no DNA contamination occurred during the construction of the fosmid library. Accordingly, F105 and F112 sequences indicated over 90% similarity to the genomic DNA from *Rhodococcus opacus* B4 and *Rhodococcus jostii* RHA1.

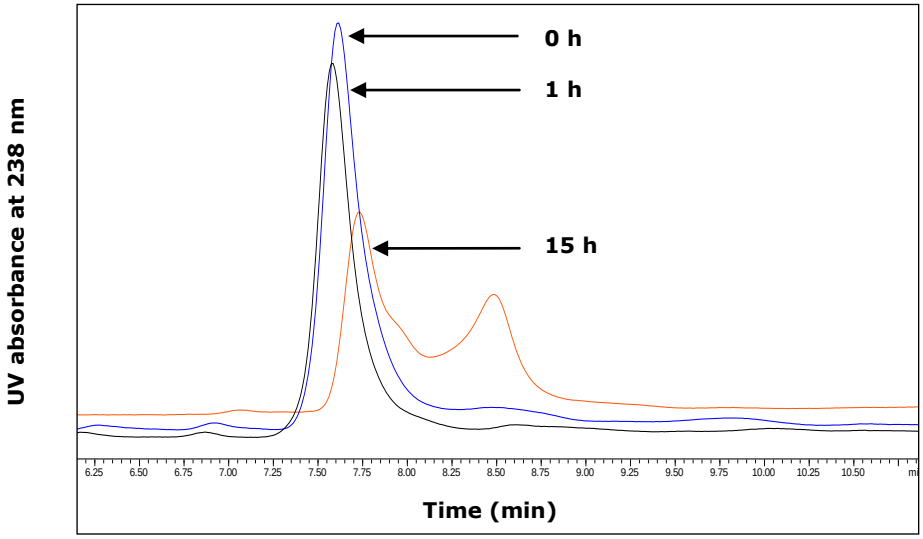

Figure 5.10 Degradation of MC-LR in the presence of the pooled cell extracts of five fosmid recombinants at 0 h, 1 h and 15 h.

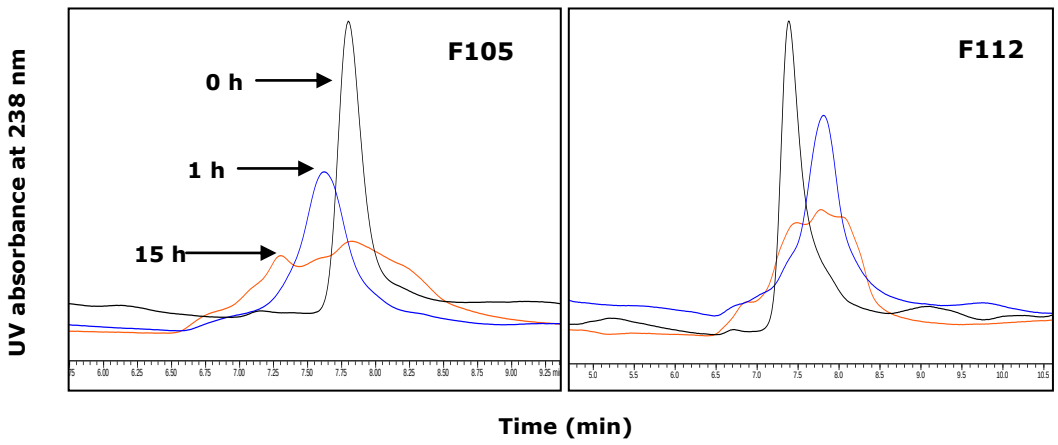

Figure 5.11 Degradation of MC-LR in the presence of the cell extracts of fosmid F105 and F112 after 0 h, 1 h and 15 h.

5.3.3.3 Microcystinase producing gene sub-cloning and screening

Fosmid clones F105, F112, F113 and F 114 were partially digested using EcoRV, ScaI and HindIII. The main objective of the restriction digestion of fosmid was to obtain appropriate DNA fragments (insert DNA ~ 2-3 kb) for the sub-cloning. Endonuclease digestion by ScaI and HindIII exclusively resulted in large DNA fragments > 5 kb (figure 5.12). Hence, they were not further considered as candidates for sub-cloning into pUC 19 vector. In contrast, EcoRV has successfully digested the fosmid DNA into < 3 kb fragments (figure 5.12), which may possibly contain 1-3 genes located in the DNA fragment. Thus, EcoRV digested fosmid DNA fragments from F105 and F112 were sub-cloned into the pUC 19 vector where they were pooled together and the microcystinase activity was monitored. HPLC screening for the degradation of MC-LR revealed a rapid degradation from sub-clones derived from both F105 and F112 (figure 5.13). Adjacent peaks deriving at 1 h and 15 h supported the fact of rapid degradation of MC-LR and the occurrence of its degradation products. This pattern of degradation was consistent with the previously identified F105 and F112 (figure 5.11). Thus, restriction digestion of the fosmid clones has not affected the activity of the MPG. The two positive sub-clones were designated as SC105 and SC112 for further evaluation. The size of the insert DNA (*Rhodococcus* sp. (C1) DNA) containing MPG was determined by a restriction digestion of the pUC 19 vector at the multiple cloning sites (figure 5.14). Results indicated that SC105 and SC112 contained ~ 1.5 kb and ~ 1.8 kb insert DNA in their pUC 19 plasmids respectively (figure 5.14). Considering the size of the insert DNA, it can be concluded that the complete microcystinase activity is expressed most likely by a single gene.

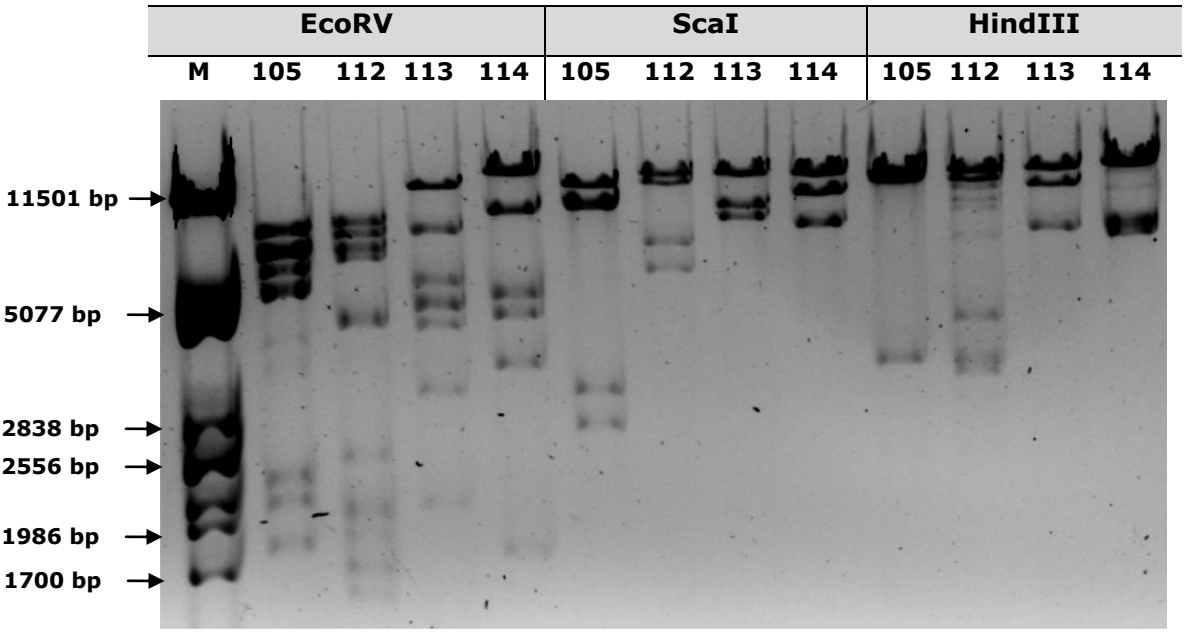

Figure 5.12 Restriction digestion of the F105, F112, F113 and F114 DNA by EcoRV, ScaI and HindIII. λ Pst I was used as the DNA size marker (M).

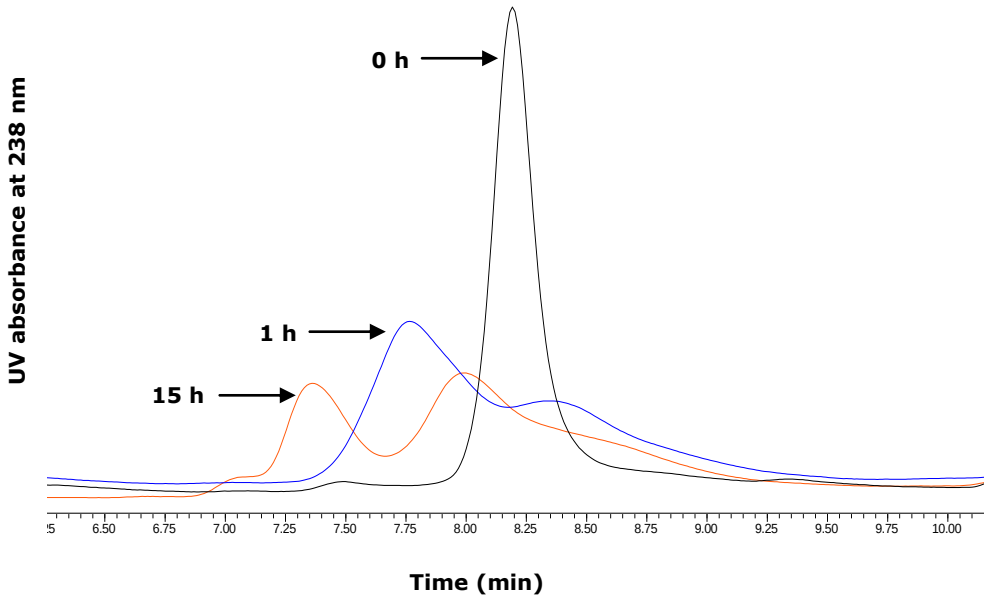

Figure 5.13 Degradation of MC-LR by SC105 cell extracts at 0 h, 1 h and 15 h.

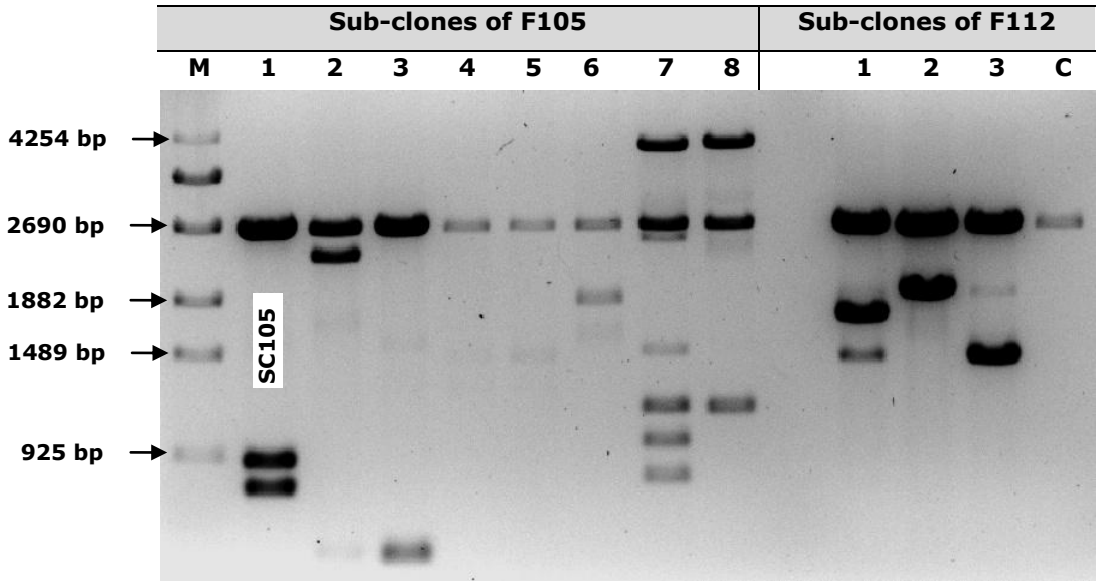

Figure 5.14 Restriction digestion analysis of the sub-clones of F105 and F112 using EcoRI and HindIII. Partially digested SC105 is labelled in the figure itself. λ sty was served as the DNA size marker (M). Experimental control (C) contained the empty linear pUC 19 vector to differentiate the location of the leaner vector from the insert DNA in the sub-clones.

Once the microcystinase activity and the size of the insert DNA in SC105 and SC112 had been confirmed, their plasmid DNA was sent for sequencing. Comparison (Blastx) of the DNA sequences of SC105 and SC112 with available protein databases in the NCBI GenBank revealed both SC105 and SC112 contained a greater similarity to putative aminotransferase producing gene from *Pseudomonas fluorescens* (accession number YP002871003). Moreover, the sequence was 78% similar to aminotransferase producing genes from the bacteria of *Rhodococcus* and *Arthrobacter* genus. Results of Blastx analysis of the elucidated nucleotide sequence are described in table 5.5.

Table 5.11 Selection of protein sequences showing percentage (%) identity and similarity to the elucidated sequences of SC105 and SC112 (NCBI, Blastx).

Matching sequence	**Database accession number**	**% Identity**	**% Similarity**
Putative aminotransferase *Pseudomonas fluorescens* SBW25	YP002871003	85	79
Aromatic amino acid beta-eliminating lyase/threonine aldolase *Pseudomonas fluorescens* Pf0-1	YP346989	67	80
Aminotransferase class V *Achromobacter xylosoxidans* A8	YP003976236	59	78
Aminotransferase class V *Burkholderia* sp. H160	ZP03268682	58	78
Aminotransferase *Rhodococcus pyridinivorans* AK37	ZP09310937	38	75
Aminotransferase *Rhodococcus opacus* B4	YP002782960	37	78
Cysteine desulfurase *Rhodococcus opacus* PD630	EHI45878	37	78
Putative aminotransferase *Arthrobacter globiformis* NBRC 12137	ZP09280303	37	78
Aminotransferase class V *Rhodococcus equi* ATCC 33707	ZP08156298	37	78
Aminotransferase *Rhodococcus erythropolis* PR4	YP002767769	36	79

5.3.3.4 PCR cloning of microcystinase producing gene

MPG of *Rhodococcus* sp. (C1) is similar to the aminotransferase and aromatic amino acid beta-eliminating lyase/threonine aldolase. To evaluate the expression and the activity of the MC degrading enzyme, the MPG was amplified using a PCR and cloned into an expression vector (pET 15b). PCR amplification was carried out using SC105 plasmid DNA as a template. PCR revealed that the actual length of the MPG nucleotide sequence is ~ 1.2 kb (figure 5.15). Using genomic DNA of *Rhodococcus* sp. (C1) and F105 plasmid DNA as positive controls it confirmed that newly amplified DNA is identical with MPG of *Rhodococcus* sp. (C1) and F105. Thus, no contamination or alteration occurred during cloning and handling up to this stage of the study (figure 5.15).

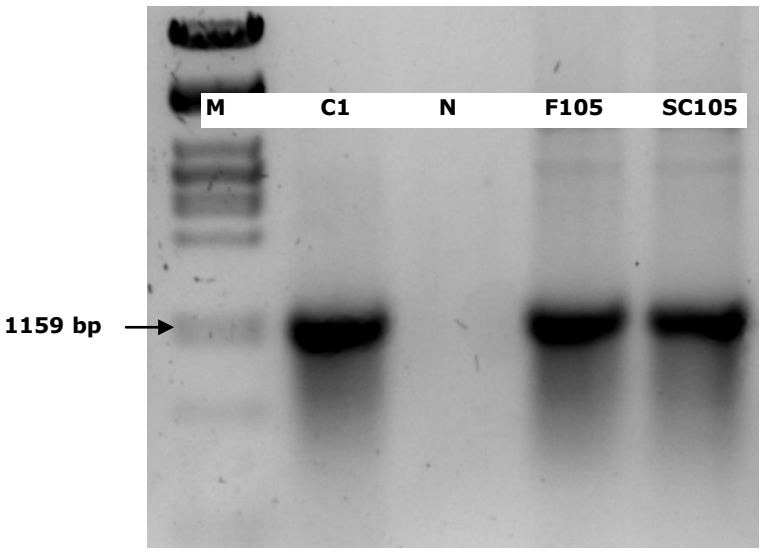

Figure 5.15 PCR amplification of the MPG using SC105 DNA as a template. Positive controls C1 and F105 contained template DNA from *Rhodococcus* sp. (C1) and F105 respectively. Negative control (N) contained sterile Milli-Q water instead of DNA. λ PstI was used as the DNA size marker.

To obtain high purity protein samples to evaluate the microcystinase activity, MPG was heterologously expressed in the *E.coli* (BL21) using the pET 15b vector optimised for T7 RNA polymerase mediated heterologous expression of proteins. Figure 5.16 shows the constructed pET 15b vector ligated with MPG for expression of the recombinant protein. Specific N-terminal 6xHis-Tag sequence has allowed MPG to produce 6xHis-Tagged recombinant protein to facilitate immunodetection and purification.

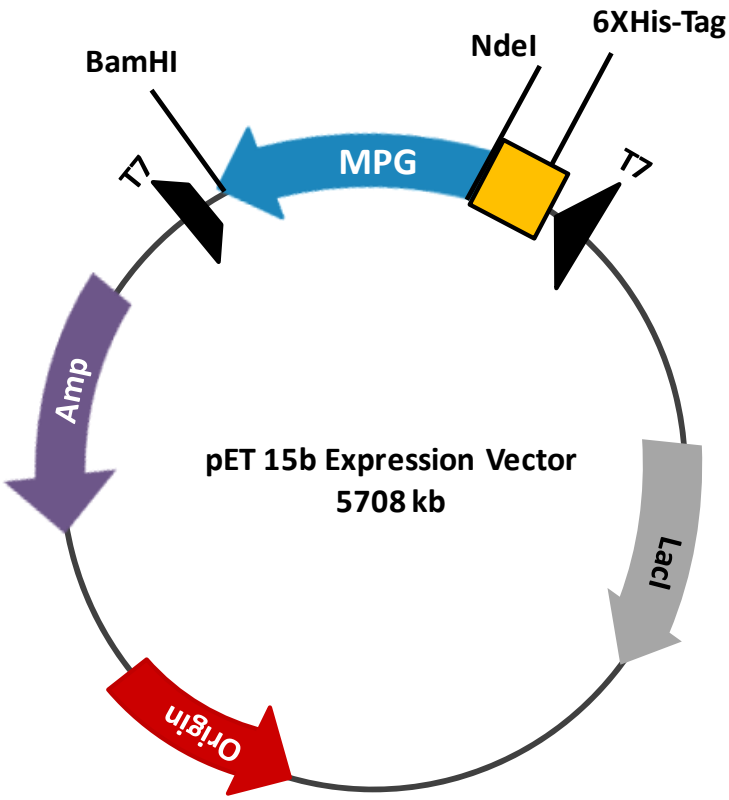

Figure 5.16 Vector map of pET 15b generated for heterologous expression of MPG. The MPG (blue), the ampicillin resistance gene ("Amp"), the lacinhibitor ("LacI"), the origin of replication ("ori"), and the location of the 6xHis-Tag are shown. The map does not show all restriction sites. T7 promoter and terminator regions are indicated "T7" at the N- and the C-terminus of the inserted gene, respectively.

Prior to the purification of the recombinant proteins from pET 15b *E.coli* cells, aptness of the pET 15b cloning was confirmed by sequencing, colony PCR (~ 1.2 kb PCR product, figure 5.17) and restriction analysis (data not shown).

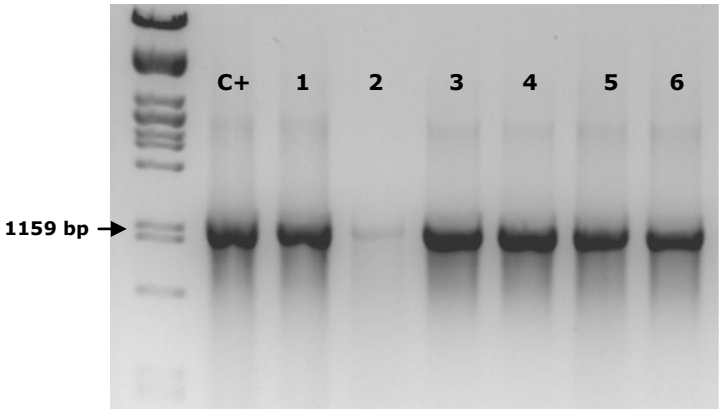

Figure 5.17 Colony PCR confirmation of the cloning of the PCR amplified MPG into the pET 15b vector. Positive control (C+) contained SC105 DNA as the template for the PCR. λ PstI was used as the DNA size marker.

5.3.3.5 Heterologous expression and purification of recombinant proteins

To investigate expression of the recombinant proteins (ME) in BL21 cells, cell extracts were prepared and the proteins were extracted under native conditions (soluble fraction; SF). Insoluble protein fraction (IS) was extracted using the denaturing buffer. SDS-PAGE analysis of the extracted protein revealed that ME was exclusively extracted under denaturing conditions. Hence, the purification procedure has undergone slight modification to obtain required amount of ME for the degradation assays. ME was tentatively identified as the thicker protein bands visible in-between 43 kDa-55 kDa (figure 5.18).

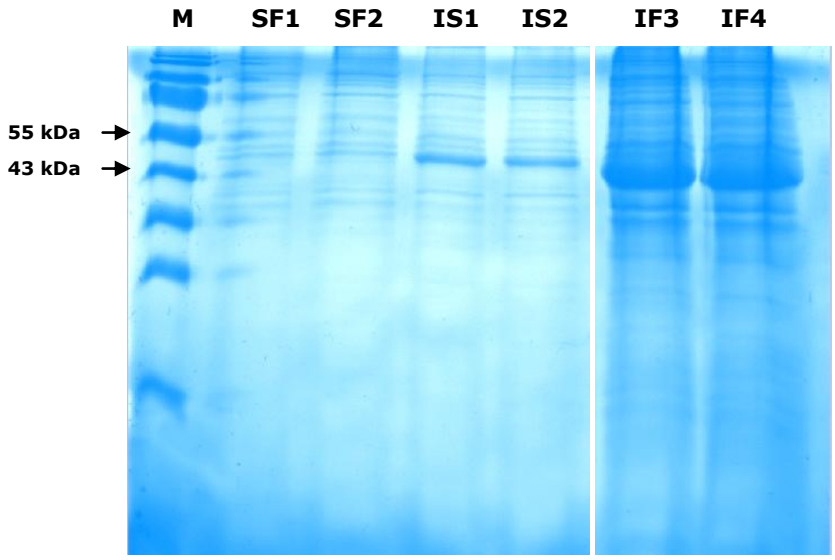

Figure 5.18 SDS-PAGE analysis of the initial extraction of the total proteins from pET 15b BL21 cells using native (SF1 and SF2) and denaturing buffers (IS1-IS4). PageRuler pre-stained protein ladder was used as the size maker (M).

Expression of the recombinant 6xHis-Tagged protein was further confirmed by Western blot immunodetection. SDS-PAGE containing the soluble protein fraction showed no visible bands in the Western blot immunodetection whereas all the insoluble fractions (IS1, IS2, IS3 and IS4) were clearly visible in figure 5.19. This confirmed that MCD protein had remained in the insoluble fraction. The bands in between 43 kDa-55 kDa seen on SDS-PAGE gels can be detected at ~ 47 kDa with an antibody interaction in Western immunoblots of heterologously expressed ME. In conclusion, this confirmed successful heterologous expression of the recombinant MPG and ME in *E. coli* suitable for large scale batch purification for further studies.

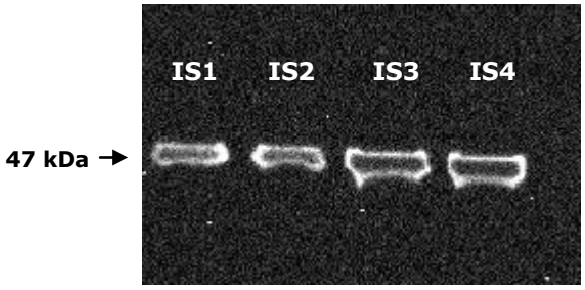

Figure 5.19 Western immunoblot analyses of microcystinase enzyme (6xHis-Tagged).

ME was expressed in large volume culture (800 ml) in BL21 cells and the pure recombinant protein was obtained from a batch purification. Slight modifications made (overnight incubation at 15 ºC) sufficiently enhanced production of the protein. ME was successfully purified and eluted with 250 mM imidazole-containing native extraction buffer and resulted in 6 fractions (0.5 ml; figure 5.20). Fractions F1-F4 eluted ~ 1.5 mg of ME while fractions F5 and F6 eluted with trace amounts of the pure protein.

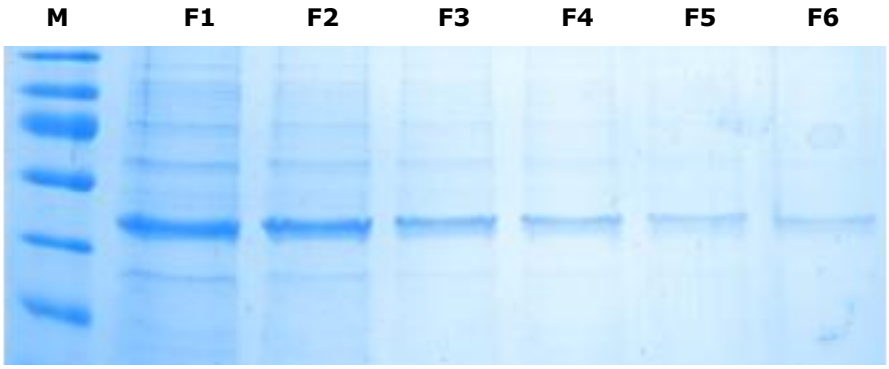

Figure 5.20 SDS-PAGE analysis of microcystinase enzyme purifications. Purified protein was eluted in six fractions (0.5 ml). PageRuler pre-stained protein ladder was used as the size maker (M).

5.3.4 Disruption of microcystinase producing gene expression

Degradation of MC-LR was evaluated using the cell extracts of SC105 and SC105D. Analysis of the assay samples confirmed the degradation of MC-LR was exclusively by the cloned MPG (figure 5.21). Rapid degradation of MC-LR was evident in the presence of the cell extracts of SC105 while no noticeable degradation occurred in SC105D (figure 5.21). Previous studies using restriction digestion, DNA sequencing and colony PCR revealed that MPG was located in ~ 1.2 kb insert DNA in pUC19 vector. Thus, partial digestion (removal of ~ 900 bp) of the MPG has undoubtedly hindered the production of the enzyme which degrades MC.

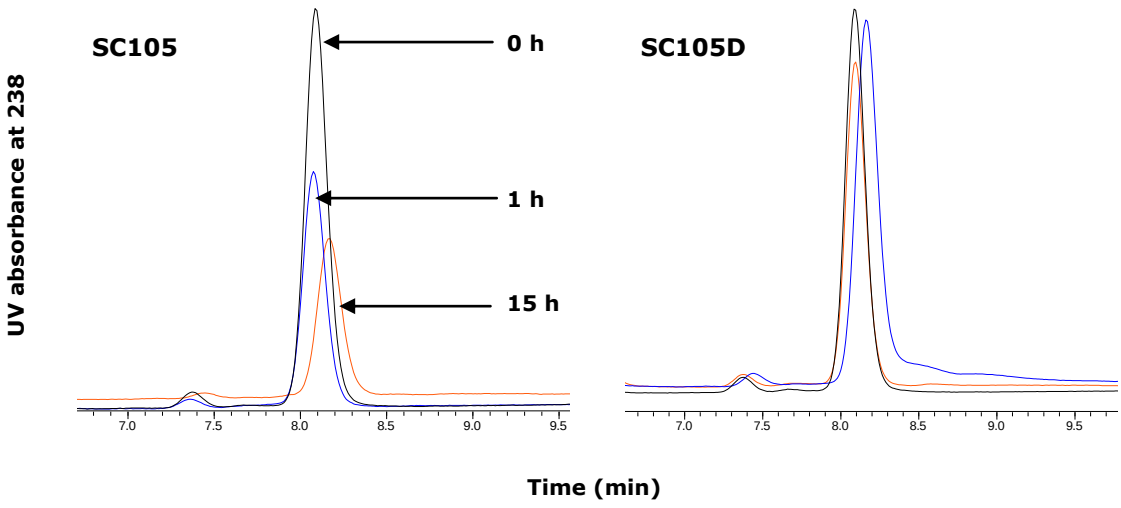

Figure 5.21 HPLC analysis of the degradation of MC-LR by SC105 and SC105D. The degradation was monitored at 0h, 1 h and 15 h.

5.3.5 Optimisation of MC-LR degradation assay with microcystinase enzyme

Degradation of MC-LR was more rapid when the assay was optimised using the PLP enzyme cofactor, aspartate and PMSF (figure 5.22). It was evident that 90% of MC-LR was removed after 4 h achieving complete degradation of MC-LR after 15 h (figure 5.22a) when the assay was optimised. In contrast, ME without PLP achieved only 10% and 40% degradation after 1 h and 15 h respectively (figure 5.22b). This rate of MC-LR degradation (figure 5.22b) is considerably lower that that previously observed using bacterial cell extracts of SC105 (figure 5.21). It can be explained that bacterial cell extracts contained naturally produced enzyme cofactors which enhanced the performance of ME in figure 5.21. This characteristic of the enzyme; PLP dependant enzyme activity is consistent with the AT description in the EMBL protein database (accession number IPR000192).

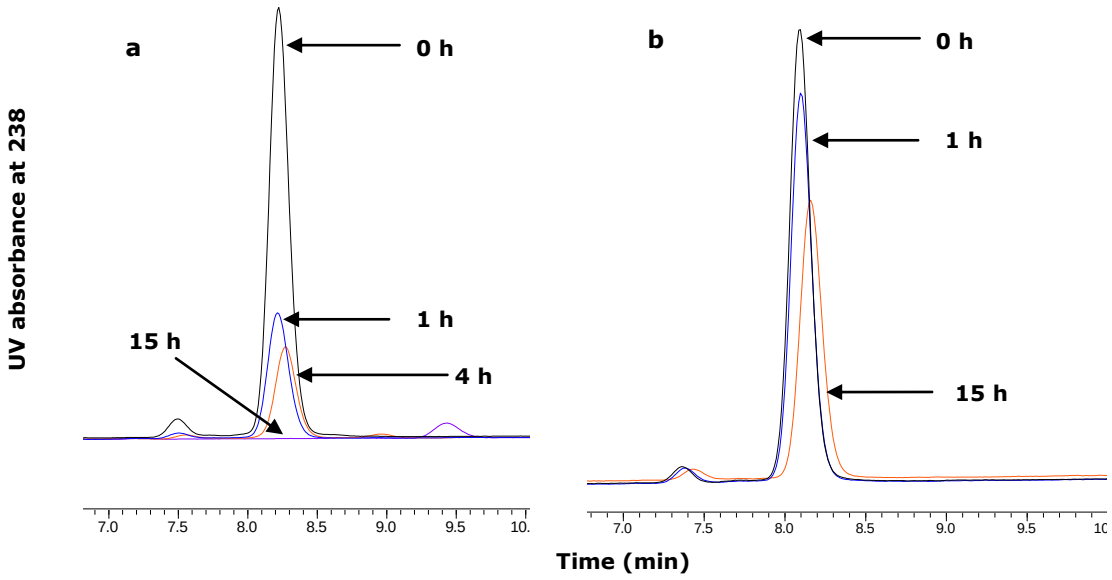

Figure 5.22 HPLC analysis of the degradation of MC-LR by (A) purified ME without optimisation and (B) optimised with PLP, aspartate and PMSF. Degradation was monitored for (A) 0 h, 1 h, 15 h and (B) 0 h, 1 h, 4 h and 15 h.

5.3.6 Analysis of MC-LR degradation products

UPLC-MS analysis revealed a rapid degradation of MC-LR with 80% of the compound removed from the degradation assays after 5 h. Peak A representing a degradation product with a retention time 5.5 - 6 min was visible after 2 h incubation of the assay (figure 5.23). Along with the disappearance of the MC-LR peak, the peak of the tentatively identified degradation product increased in size and separated into an adjacent peak (peak B; figure 5.23). However, peak A appeared to diminish with degradation process; meanwhile peak B increased gradually. It could be possible that the degradation product represented by peak A was further degraded into a more polar compound which was indicated by peak B. ESI-MS analysis of the peak A indicated at *m/z* 652 (figure 5.24) was identified as the doubly protonated ion $[M+ 2H]^{2+}$ of MC-LR glutathione conjugate (MC-LR-GSH). ESI-MS spectrum of peak A (MC-LR-GSH) revealed identical fragment ions of MC-LR-GSH (figure 5.25) previously identified in literature (Pflugmacher *et al*., 1998; Dai *et al*., 2008). Major fragment ions of MC-LR-GSH indicating *m/z* 1302 was identified as $[M+H]^{+}$ and *m/z* 1168 was identified as $[M+H\text{-Adda}]^{+}$ formed by the removal of Adda from MC-LR-GSH. Identified characteristic MC-LR-GSH fragments are described in the ESI-MS spectrum (figure 5.25) of the peak A.

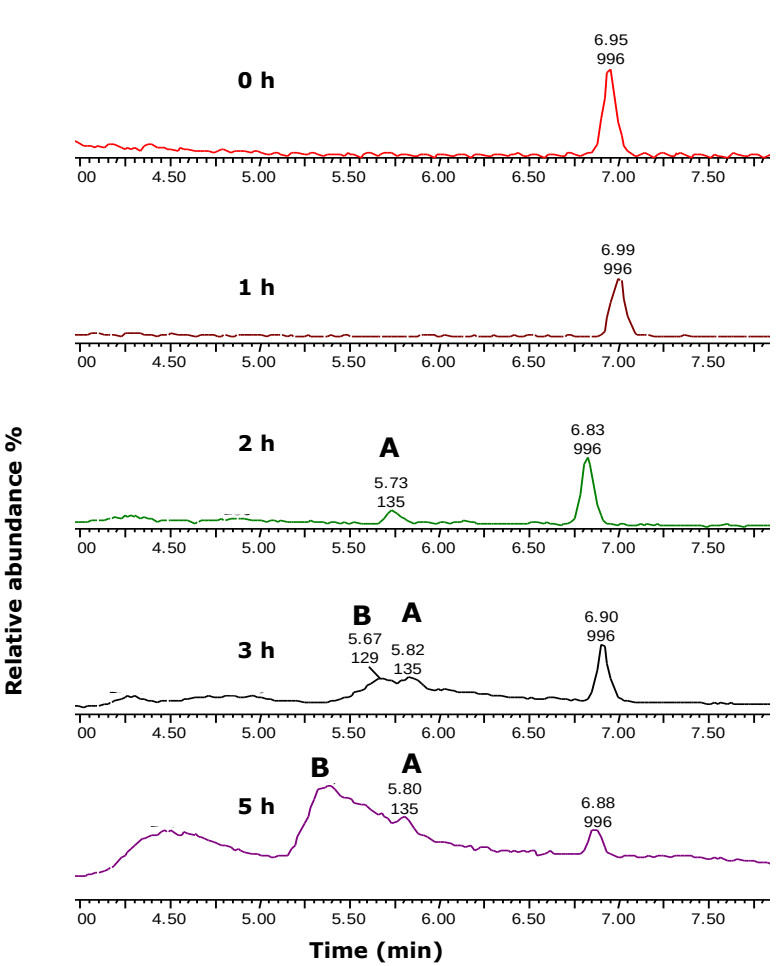

Figure 5.23 UPLC-MS analysis of the degradation of MC-LR (*m/z* 996) by recombinant BL21 cell with MPG. Peak A and B represent the degradation products of MC-LR.

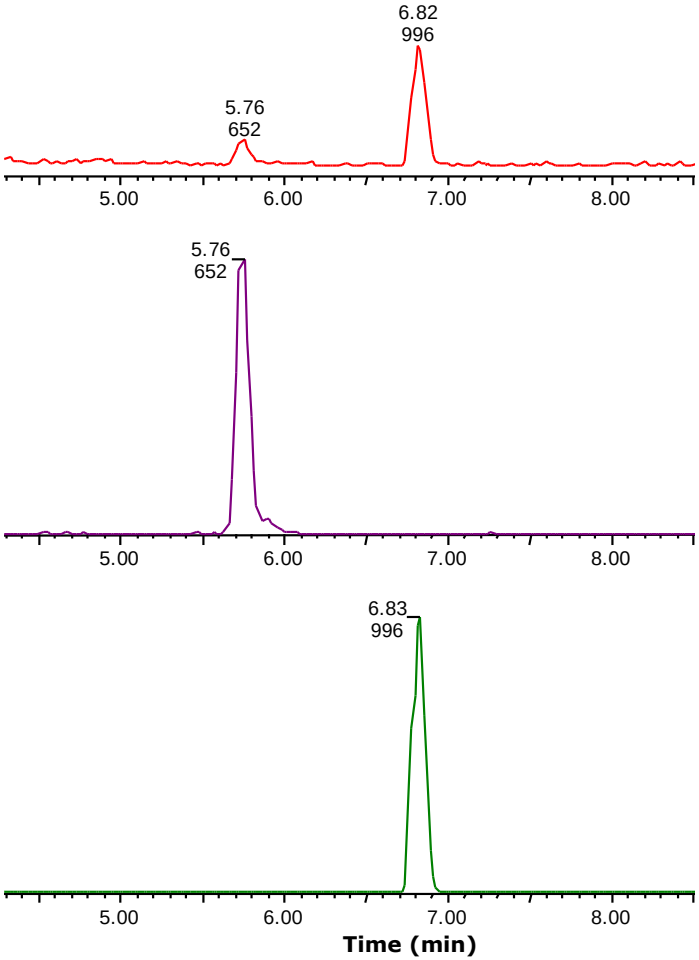

Figure 5.24 MS chromatogram of MC-LR and its glutathione conjugate (peak A). Chromatogram of MC-LR-GSH (middle) extracted at *m/z* 652 and MC-LR at *m/z* 996.

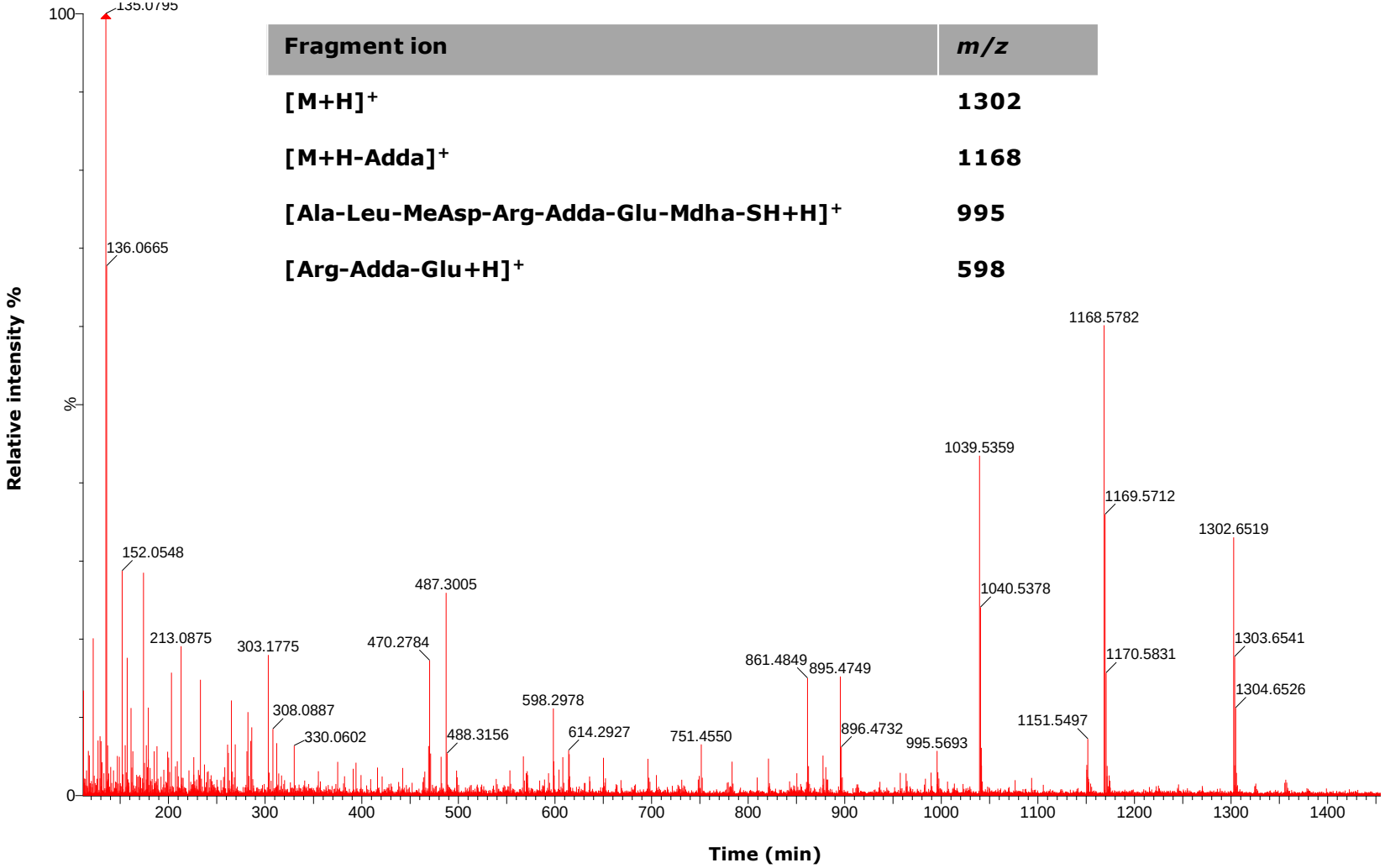

Figure 5.25 ESI-MS spectrum of MC-LR-GSH (peak A at *m/z* 652).

To determine whether glutathione conjugation was formed by the enzymes of host *E. coli* cell or by the interaction with the ME, samples that remained from the MC-LR degradation assay (performed with purified ME) were analysed by UPLC-MS. Figure 5.26 illustrates the degradation of MC-LR using the purified ME. The degradation pattern of the enzyme was similar to that of the cell extracts, although more rapid degradation was evident using the purified enzyme. In the previous study, no degradation product was visible up to 2 h (figure 5.23). However, in the current study, degradation products were clearly detected during the first hour (figure 5.26). Peak A indicated the formation of MC-LR-GSH, confirmed by its ESI-MS spectrum compared to previously identified product ions (figure 5.25). The fragment ions were identical with previous study and consistent to that available in literature. It was interesting to note the formation of peak B during the first hour. This peak was only visible after 3 h when MC-LR was degraded by the cell extracts (figure 5.23). Thus, it is evident rapid biochemical reactions occurred when purified and optimised enzyme was utilised. The MS chromatogram shows the doubly protonated ion $[M+ 2H]^{2+}$ of the degradation product of peak B yielding a mass at *m/z* 532 (figure 5.26). Peak B is further illustrated by the ESI-MS spectra (figure 5.27) with its precursor ion $[M+H]^{+}$ at *m/z* 1064. MC-LR-GSH was degraded along with MC-LR (figure 5.26). Meanwhile, peak B was gradually increased and an additional peak (peak C) appeared at *m/z* 460 in the ESI-MS chromatograms (figure 5.26). Further characterisation of peak B and C was challenging due to their low concentration. However, after 15 h, almost all MC-LR and the degradation products were degraded by ME (figure 5.26). Evaluation of the toxicity of the degradation products of MC-LR using

protein phosphatase inhibition assay (section 5.2.10) confirmed that there was no inhibition of the PP1 activity after 15 h.

In addition to the bacterial degradation of MC-LR, these results revealed the glutathione conjugation detoxification mechanism in bacteria. It is likely that the glutathione conjugation is different from the degradation mechanism of MCD enzymes. The sequence analysis using the NCBI Blastx revealed MCD enzyme belongs to the AT protein family which converts amino acids into α-keto acids. A lack of sufficient amounts of purified proteins prevented further characterisation of the degradation products at this stage of study.

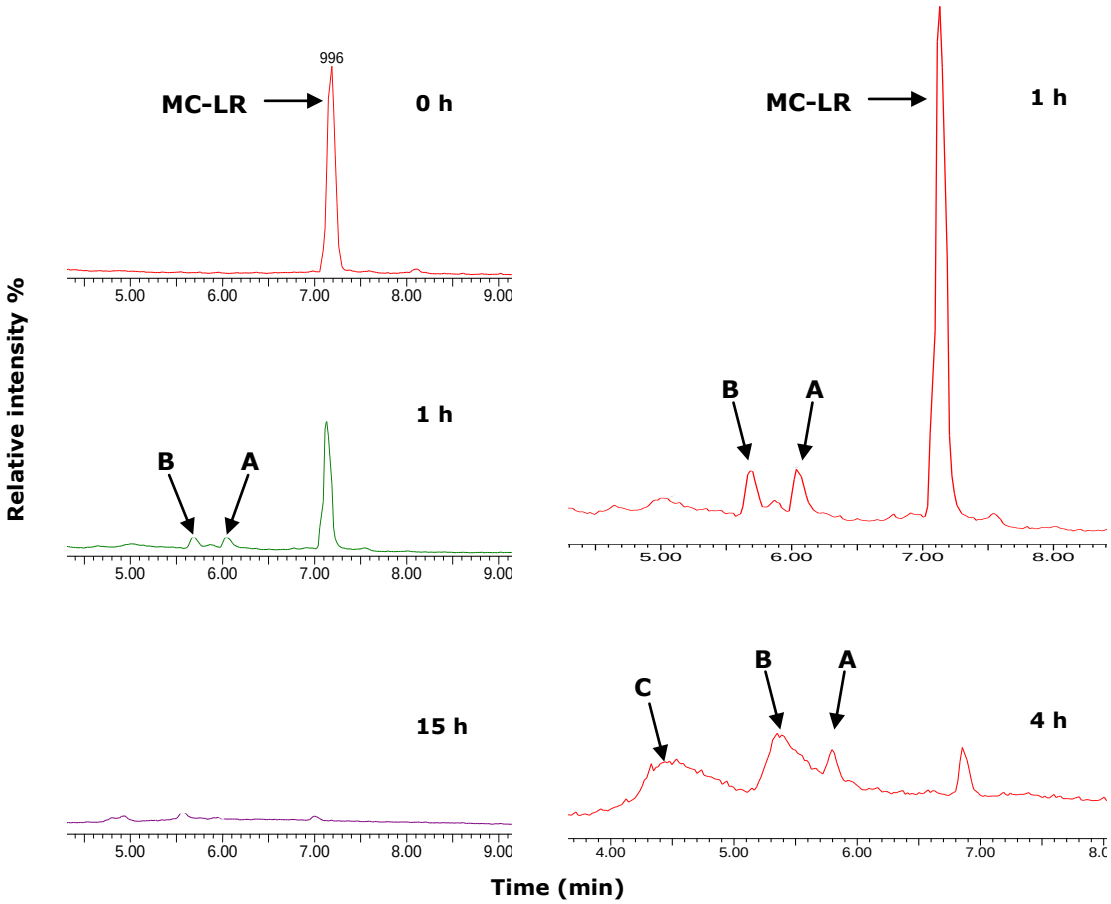

Figure 5.26 UPLC-MS analysis of the degradation of MC-LR by the purified ME. Peak A is $[M+ 2H]^{2+}$ of MC-LR-GSH at *m/z* 652, $[M+ 2H]^{2+}$ of peak B at *m/z* 532 and peak C at *m/z* 460.

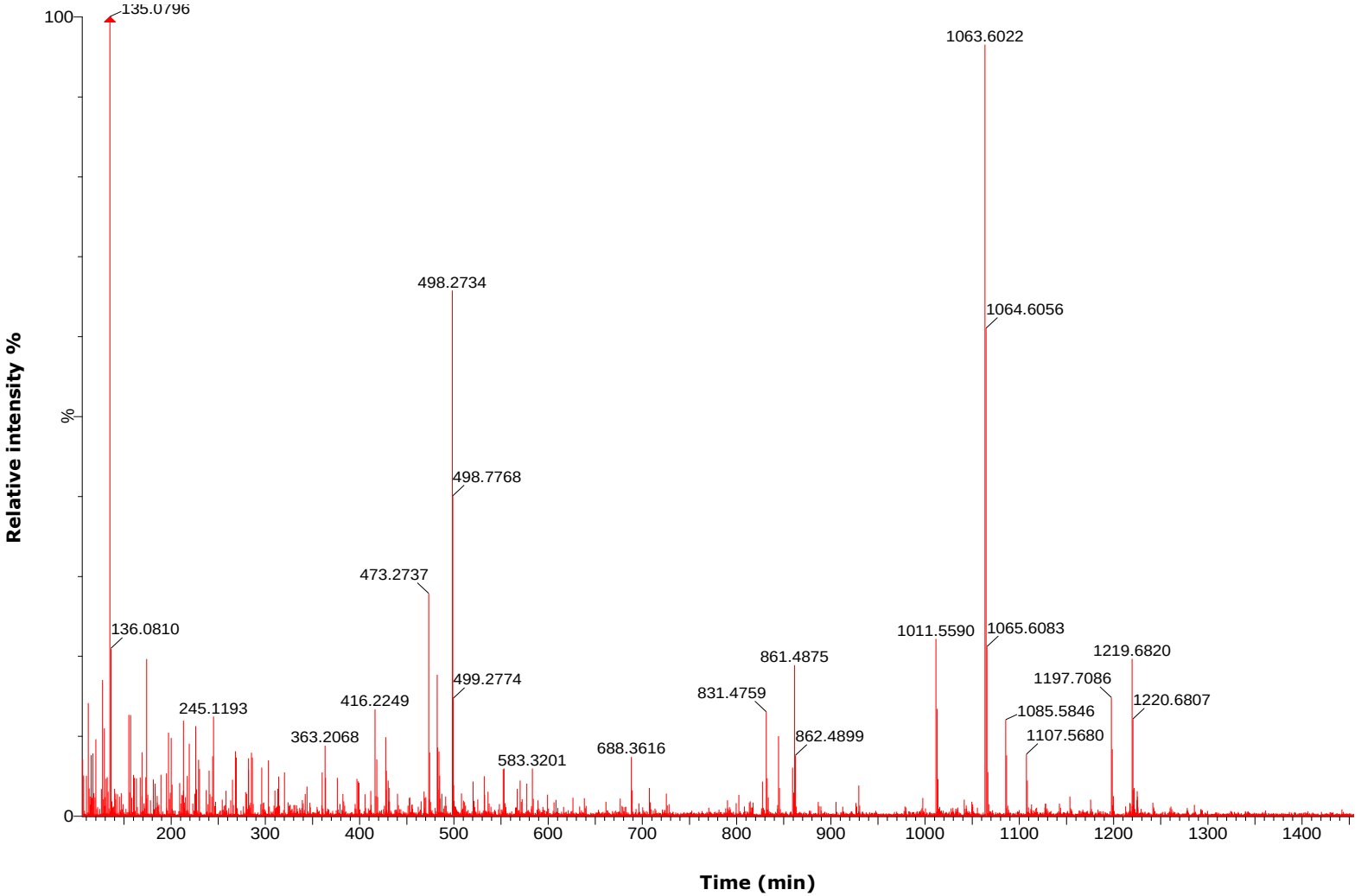

Figure 5.27 ESI-MS spectrum of the degradation product represented by $[M+ 2H]^{2+}$ of peak B at *m/z* 532 in figure 5.26.

5.4 Discussion

5.4.1 Investigation of the existence of *mlrA* gene in isolated bacterial strains

The main aim of the work presented here was to investigate the existence of the *mlrA* or *mlrA* homologous genes in the MC degrading *Actinobacteria*. With the understanding that *mlrA* or *mlrA* homologous genes do not exist in these bacterial strains, the gene(s) responsible for the degradation of MC has been elucidated.

Prior to these studies, the enzymatic degradation of MC-LR has been investigated to confirm the existence of MCD enzymes; thus, genes producing MCD enzymes. Up to this stage of the study, degradation of MCs has been evaluated using the bacterial cells themselves. In the presence of the cell extracts of *Rhodococcus* sp. (C1) a rapid degradation occurred. This indicated the intracellular degradation of MC-LR by *Rhodococcus* sp. (C1) suggesting the occurrence of MCD enzyme producing genes.

Investigation of the existence of the *mlrA* gene was achieved using a PCR assay. However, a point worth considering prior to this assay was the previous PCR analysis conducted by Manage *et al*., (2009) using the genomic DNA from the *Actinobacteria* MC degraders. The primers have been designed to meet the requirements of the previously identified *mlrA*, *mlrB*, *mlrC* and *mlrD* of *Sphingopyxis* sp. LH21. Subsequently, the PCR was performed in similar conditions to that reported previously (Ho *et al*., 2007). However, no PCR products were detected apart from the positive control *Sphingopyxis* sp. LH21

(Manage *et al*., 2009). One obvious explanation was that none of the novel MC degraders contained *mlr* gene cluster; hence, different genes are responsible for the degradation of MC. Nonetheless, the question that arose here was whether these bacteria harbour gene(s) homologues for *mlr*. Accordingly, the present study was undertaken to fill this knowledge gap. To achieve this, firstly, *mlrA* gene sequence (NCBI accession number DQ112243) was selected representing the four genes of *mlr* gene cluster from *Sphingopyxis* sp. LH21 (Ho *et al*., 2007). MlrA protein produced by *mlrA* gene is responsible for the initial enzymatic cleavage of cyclic MCs (Bourne *et al*., 2001; Saito *et al*., 2003); hence, conserved in many of the MC degrading bacteria (table 5.1). Considering these key facts, *mlrA* (~ 800 bp, Saito *et al*., 2003) gene sequence was chosen as a potential candidate for the current study. NCBI BLAST analysis revealed that *mlrA* conserved regions were rare in the *Actinobacteria* isolates. Hence, design of the primers for *Actinobacteria* was challenging. However, design of two primer sets was to exploit maximum amplification of the *mlrA* conserved regions in *Actinobacteria* MC degraders. Sequencing of the PCR amplified products revealed that neither *mlrA* nor *mlrA* homologues occur in *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) or *Brevibacterium* sp. (F3). In consequence, degradation of MC and other peptides were by a different pathway mediated by novel MC degrading enzymes.

5.4.2 Elucidating MC-LR degrading genes in *Actinobacteria*

Given that *mlr* was not detected in the MC degraders, the study was extended to elucidate the genes responsible for the degradation of MC. Elucidation of the MCD genes was performed by constructing a genomic library of a MC degrading strain. In a previous study, Bourne *et al*., (2001) has successfully characterised the *mlr* gene cluster in *Sphingomonas* sp. ACM-3962 using a cosmid library. However, for the current study, a fosmid library was selected over a cosmid library due to its novelty and proven efficiency as a genomic library. Compared to the other vector controlled libraries, fosmid library facilitates insertion of small DNA fragments (~ 40 kb) which enables rapid detection, deletions, and rearrangements of the insert DNA (Ammiraju *et al*., 2011). Moreover, their inducible (50 copies per cell) vector amplification greatly increased the DNA/protein yields during the screening of active recombinants in this study.

Rhodococcus sp. (C1) was selected for the construction of the fosmid library for its proven potential as a MC degrader (figures 5.4 and 5.8). Moreover, it was interesting to discover the occurrence of MC hydrolysing enzymes in *Rhodococcus* sp. (C1) since this bacterium was isolated from a MC-free environment (Edwards *et al*., 2008; Manage *et al*., 2009). Construction of the fosmid library successfully resulted in two active sub-clones ligated with ~ 1.5 kb insert DNA. Considering their rapid microcystinase activity, it revealed that MC degrading enzyme production is most likely by one gene. However, to date, the only enzymatic degradation pathway characterised was by Bourne *et al*., (2001). As described, the degradation of MC was mediated by a gene cluster (*mlr*) collectively by four genes (*mlrA*, *mlrB*, *mlrC* and *mlrD*). Three

enzymes namely MlrA, MlrB and MrlC have enabled the aminolysis of the MCs cyclic structure whereas MlrD facilitated the uptake of the peptides (Bourne *et al*., 2001). In contrast to gene cluster mediated MC degradation, in the present study, for the first time, MC degradation was successfully demonstrated by a single enzyme. Hence, the biochemical pathway of MC-LR degradation is highly likely to deviate from that characterised by Bourne *et al*., (2001). On this basis, the cellular uptake of the peptides must have been facilitated by their gram positive cell wall permeability (Lambart, 2002). Unlike other MC degraders, bacteria of *Rhodococcus* contain long aliphatic chains in the cell envelope enhancing cellular uptake of hydrophobic substrates such as polychlorinated biphenyls (PCBs; Martankova *et al*., 2009). The hydrophobic properties of MCs, (MC-LR, -LY, -LF and -LW; Edwards *et al*., 1994) have undoubtedly facilitated their cellular uptake, enhancing their degradation (Nybom *et al*., 2008). However, further studies are required to support this mechanism of MC and peptide uptake in *Rhodococcus* sp. (C1).

5.4.3 Microcystin glutathione conjugation

With the emerging risk of MCs, a considerable amount of research has been devoted to finding its detoxification mechanisms. Consequently, it has been reported that glutathione conjugation of MC is the fist step of detoxification (Pflugmacher *et al*., 1998). To date, MC glutathione conjugation has been reported in human (Buratti *et al*., 2011), mice (Takenaka, 2001), fish (Best *et al*., 2002) and plants (Pflugmacher *et al*., 1998). Although, glutathione conjugation has been reported in bacteria (Zablotowicz *et al*., 1995), MC glutathione conjugation, related to bacteria, has not been reported previously.

Nevertheless, for the first time, this study has described the occurrence of MC-LR-GSH in bacteria.

MC-LR-GSH formation is an enzyme mediated reaction of aquatic animals and plants catalysed by *S*-transferases (Pflugmacher *et al*., 1998). Glutathione conjugates of MC-LR have been shown to be less toxic in comparison to their respective toxin (Kondo *et al*., 1992; Metcalf *et al*., 2000). The glutathione is conjugated with Mdha residue of MC-LR (figure 5.28) yielding a mass of *m/z* 1302 at ESI-MS chromatograms (figure 5.24). The doubly protonated ion ([M+ 2H]$^{2+}$, *m/z* 652) was also found in the ESI-MS chromatogram in the UPLC-MS analysis (figure 5.23). The distribution of parent and product ions of MC-LR-GSH in the ESI-MS chromatograms and spectrums were identical to the literature available so far (Pflugmacher *et al*., 1998; Dai *et al*., 2008; Buratti *et al*., 2011).

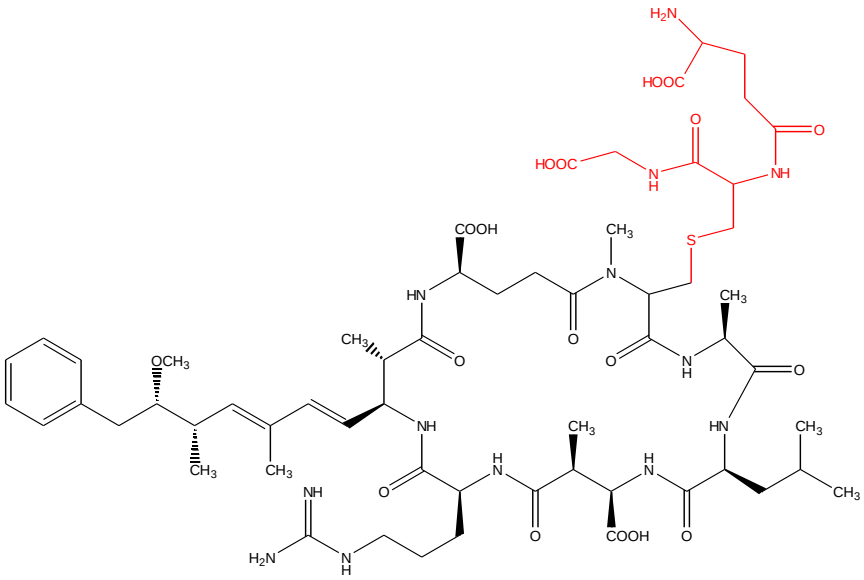

Figure 5.28 Chemical structure of MC-LR-GSH

It is likely that the degradation of MC-LR by MCD enzymes is completely different from the MC-LR-GSH formation. However, it was interesting to note that MC-LR-GSH was not stable in the assay samples and rapidly disappeared. Therefore, in future studies, it is important to reduce the time interval of the UPLC-MS sampling to investigate the MC-LR-GSH formation. Furthermore, it can also be suggested to increase the concentration of MC-LR in the degradation assay to unravel the MC-LR-GSH formation. Further studies would facilitate the discrimination of the detoxification and degradation mechanisms in bacteria.

5.4.4 Possible degradation pathway of MC-LR by aminotransferase

Successful heterologous expression of the recombinant *Rhodococcus* sp. (C1) gene has enabled the purification of its microcystinase enzymes. Sequencing of the recombinant gene revealed a greater similarity to the AT protein family in *Rhodococcus* bacteria (table 5.5). AT is a common enzyme found in both prokaryotes (Pedraza *et al*., 2004) and eukaryotes (Shull *et al*., 1995). Their general function is to convert amino acids into α-keto acids and *vice versa* (figure 5.29; Pedraza *et al*., 2004). Many studies have reported the involvement of the AT enzymes in the bio-catalytic pathways of amino acid production and transformation into α-keto acids (Curtin and McSweeney, 2003; Pedraza *et al*., 2004; Miyazaki *et al*., 2004; Liu *et al*., 2004; Yang and Lu, 2007). In the human body, ATs serves as an important metabolic pathway for the elimination of amino acids by the deamination to produce urea (Osei and Churchich, 1995). However, in the present knowledge, there has been no literature to support the degradation of peptides by AT enzymes.

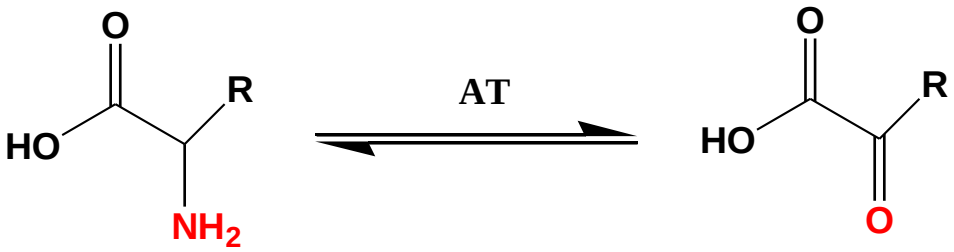

Figure 5.29 Aminotransferase deamination of amino acids

Given that MC is a cyclic peptide composed of seven amino acids, it can be hypothesised that the most abundant primary target for AT is the amino groups (-NH-) in the structure (figure 5.30). Hence, the amino groups transformed into α-keto acids. For instance, transformation of tertiary amino

groups of the Adda-Arg bond and D-MeAsp-Arg bond by AT would isolate Arg from the cyclic structure of MC. Consequently, hydrolysis of Arg would ultimately result in degradation products urea and ornithine. Ornithine can be subjected to a further deamination process which would result in an ornithine ketoglutarate (figure 5.30; Meister, 1954). As evidence, Lu, (2006) reported that deamination of the L-Arg is catalysed by an Arg inducible AT enzyme in bacteria. Further studies confirmed the involvement of AT in bacterial metabolic pathways. Yang and Lu, (2007) reported the utilisation of Arg in pseudomonads by AT mediated pathway whereas Watanabe *et al*., (2008) and Karsten *et al*., (2011) reported the transamination of lysine. Hence, the degradation of MCs is likely to commence with a modification of the amino acid structures of the main compound. Modifications of the chemical structures are commonly found in the antibiotic resistance mechanisms in bacteria (Yeaman and Yount, 2003). This mechanism is performed by the largest family of resistance enzymes; transferases, covalently modifying the structure (Wright, 2005). In these respects, it can be postulated that degradation of the peptides (NOD, ABPNB, GRA, POLYB and ANGTN) in previous studies possibly occurred by the modification of the amino group in their chemical structure, making it more susceptible to further hydrolysis by bacterial catabolic pathways.

However, with no available literature related to the degradation of peptides, suggestion of a detailed pathway of MC degradation is not possible at this stage of study. Hence, the complete characterisation of the MC degradation pathway of *Rhodococcus* sp. (C1) will be achieved in future studies.

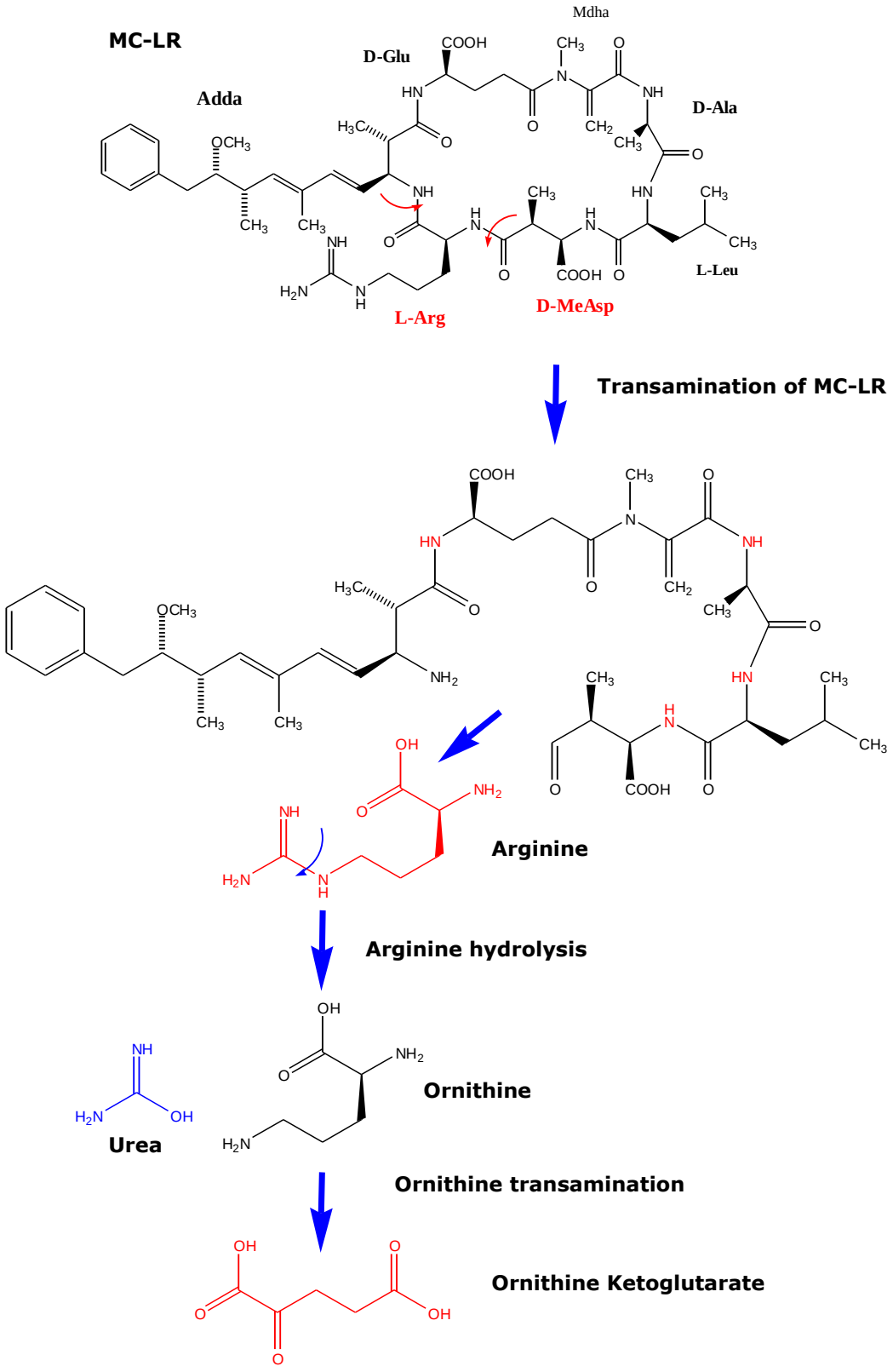

Figure 5.30 Proposed general degradation pathway of MC-LR by AT

5.5 Conclusion

Biodegradation of MC is more diverse than previously understood so far. *Rhodococcus* sp. (C1), *Arthrobacter* sp. (C6) or *Brevibacterium* sp. (F3) did not harbour either *mlrA* gene or *mlrA* homologous genes. Hence, *mlr* gene cluster is not essential for degradation of MCs and different degradation pathways exist. The fosmid library was an excellent tool for the analysis of the metagenomic library of *Rhodococcus* sp. (C1). Successful construction of the genomic library enabled the elucidation of the MPG, heterologous expression and the purification of the target protein. Knowledge of MC-degrading enzymes is extremely limited. This is the first study to purify and perform the MC degradation assays using a MC degrading enzyme.

Degradation of MC-LR is exclusively by a single gene which was identified belonging to AT protein family. Although AT has been reported to catabolise different amino acids in human, animals and bacteria, this is the first report of an AT enzyme degrading a wide range of different peptides. It is probable; the degradation of the peptides (NOD, ABPNB, POLYB, GRA and ANGTN) was performed by the same pathway as described (figure 5.30). Thus, degradation of the peptides is a universal degradation mechanism of *Rhodococcus* sp. (C1). It is now confirmed that MC degrading enzymes exist not only to degrade MCs but for the general metabolism for the existence of the bacteria in diverse environments. An interesting point worth mentioning is the first appearance of the MC-LR-GSH in bacteria. Although, MC-LR-GSH is well documented, no published data is available for this detoxification mechanism in bacteria. Further studies are essential to characterise the degradation pathway and to further evaluate the MC-LR-GSH formation in *Rhodococcus* sp. (C1).

CHAPTER 6

DISCUSSION

6 Discussion

"An investigation into the biodegradation of peptide cyanotoxins (MCs and NOD) by novel Gram-positive bacteria"

Bacterial bioremediation is an essential component maintaining the balance of nature. Their promising bioremediative ability helps self purification of the environment while it is widely employed in the industry. This study was carried out exploiting this remarkable ability of bacteria to purify nature's own contaminants, MCs and NOD, preventing their potential risk for drinking water systems. In nature, degradation of MC is determined by environmental factors such as sunlight, pH, temperature and mainly the aquatic bacterial consortia decomposing MCs. However, the chemical stability of MCs facilitates their long persistence in posing a threat to the users of the aquatic system. Under the circumstances of contamination of drinking water, the removal of MCs by conventional water treatment is unreliable and may cause serious health damage or death in case of ingestion. Thus, there is high demand for a reliable and cost effective approach to remove MCs from drinking water due to the potential health risk and prevailing water scarcity. Bacterial bioremediation of MCs is known to be promising for treatment of contaminated waters. However, many of the studies into MC biodegradation, have resulted in harnessing limited diversity of bacteria for potential employment in water treatment. In contrast, the current study presented efficient removal of MCs by *Rhodococcus* sp., *Arthrobacter* sp. and *Brevibacterium* sp. which have the potential of exploiting water treatment. One of the most important findings of this study is the "universal" degradation of different MCs and NOD. This unspecific

degradation of chemically different MCs and NOD has led to the understanding that, these bacteria may contain a general degradation mechanism for many other environmental peptides. Interestingly, *Rhodococcus* sp. (C1) was capable of degrading a wide range of different prokaryotic and eukaryotic peptides such as ABPNB, LNOD, GRA, POLYB, ANGTN and CY. Although, GRA and POLYB are antibiotics, MC degrading *Rhodococcus* sp. (C1) has shown an effective breakdown of these peptide antibiotics. This became more interesting with the finding of an inducible peptide degradation mechanism of *Rhodococcus* sp. (C1). This strain can be induced to rapidly degrade MC-LR when it is pre-exposed to either MC-LR or prokaryotic peptides such as GRA, POLYB, ABPNB, LNOD and vice versa. However, at this stage of the study, further evaluation could not be achieved to understand the cause of this inducible behaviour, specific to prokaryotic peptides. On the other hand, bacterial degradation of peptides was limited in literature. However, it can be suggested that future studies into exploring this specific bacterial response may facilitate understanding of bacterial peptide degradation mechanisms in nature. It may be possible some prokaryotic peptides are acting as signalling molecules to induce bacterial genes networking the metabolic pathways. On the other hand, it may be the case of substrate specificity on different peptides or peptide bond(s) that induce the peptide degrading enzyme producing genes. Furthermore, there is emerging evidence of antibiotics as intermicrobial signalling agents instead of bacterial inhibitory compounds (Linares *et al*., 2006; Fajardo and Martinez, 2008). For example, at high concentrations, antibiotics are inhibitory while they produce specific changes that help bacterial growth and activity, i.e. biofilm formation. Therefore, it is possible that these results might help future research into understanding the

bacterial interaction with different peptides, antibiotic sub-inhibitory concentrations and resistance mechanisms.

Although a number of biodegradation studies achieved isolation of MC-degrading bacteria, so far, only one degradation pathway has been characterised (Bourne *et al*., 1996; 2001). In contrast, in the current study, a novel microcystinase producing gene has been successfully identified, and the microcystinase activity was successfully demonstrated. The enzyme responsible for the degradation of MC was identified as an aminotransferase (AT) enzyme. Based on the function of AT enzyme, a possible MC degradation pathway was proposed. However, further studies to evaluate the function of this enzyme in peptide degradation will be exciting. Rhodococci typically harbour large linear and circular plasmids, which possibly contain large number of catabolic genes (Martankova *et al*., 2009) which makes it promising for bioremediation of recalcitrant xenobiotics. Accordingly, an interest for further studies is to investigate the possibility of peptide degrading gene islands in *Rhodococcus* sp. (C1). Noteworthy, the isolated MC degrading gene sequence of *Rhodococcus* sp. (C1) showed greater similarity (79%) to *Pseudomonas fluorescens* SBW25 (table 5.11). This may suggest a possible horizontal gene transfer in between the two bacteria, since rhodococci were reported to acquire some genes by this process (McLeod *et al*., 2006).

Another interesting point for further studies is the MC-LR glutathione conjugation by *Rhodococcus* sp. (C1). It is thought that this study has been the first to report MC-LR-GSH in bacteria. Moreover, MC-LR-GSH was found in the biodegradation assay performed with purified enzyme. This may not

directly link to the activity of the AT enzyme; however, this could be resolved by characterising the degradation products in detail and using mass spectrometry and stable labelled isotopic (MC-LR) investigation in future studies.

Although, *Rhodococcus* sp. (C1) was widely investigated for its degradation ability on MCs and different peptides in here, *Arthrobacter* sp. and *Brevibacterium* sp. still remain to be evaluated. Bacteria of *Arthrobacter* are well known to degrade many environmental pollutants (Ferreira *et al*., 2008) while *Brevibacterium* is widely exploited as an important component of the microbial flora of smear-ripened cheeses, such as Tilsit, Romadur, Danbo, Limburger and Appenzeller (Noordman *et al*., 2006). However, their capability to degrade different variants of MC is exciting and has potential for further studies unravelling the degradation mechanism(s).

In conclusion, this study has provided valuable information about a wide diversity of novel Gram-positive MC and NOD degrading bacteria and their potential to be exploited for treatment of contaminated waters. Further studies would facilitate their practical use in future water treatment for cyanotoxins such as MCs and NOD.

CHAPTER 7

REFERENCES

7 References

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APPENDICES

Appendix A. Purification and identification of ABPNB

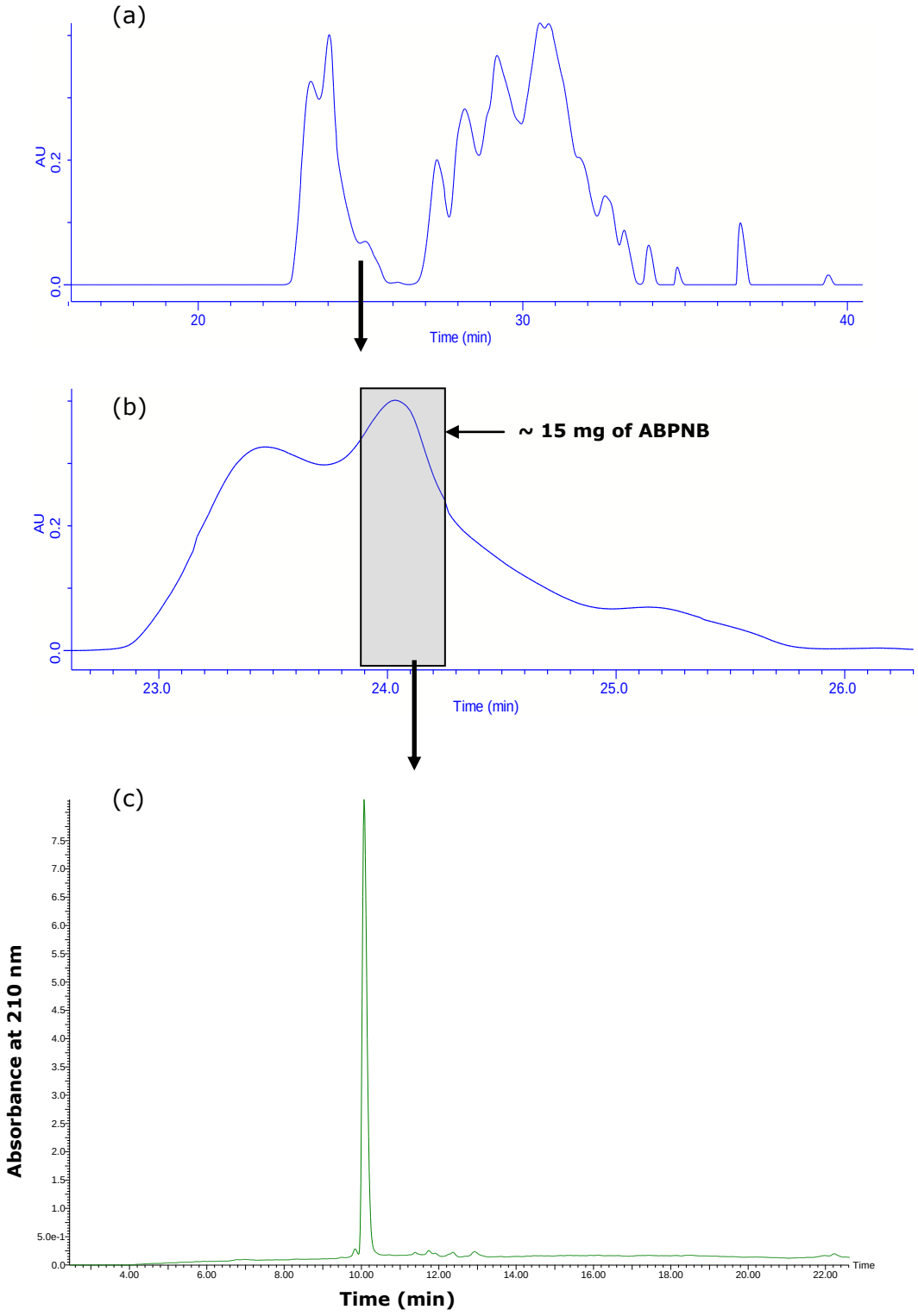


Figure 1 (a) Preparative HPLC chromatogram of ABPNB with (b) fractions containing relative purity > 95% highlighted and (c) UV chromatogram of ABPNB extracted at 210 nm with relative purity > 95%.

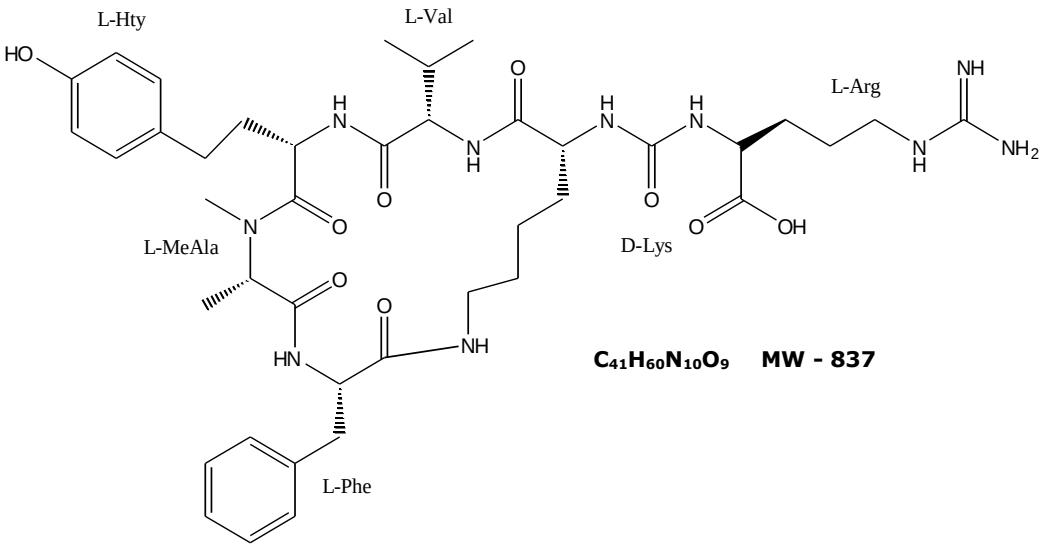


Figure 2 General structure of ABPNB

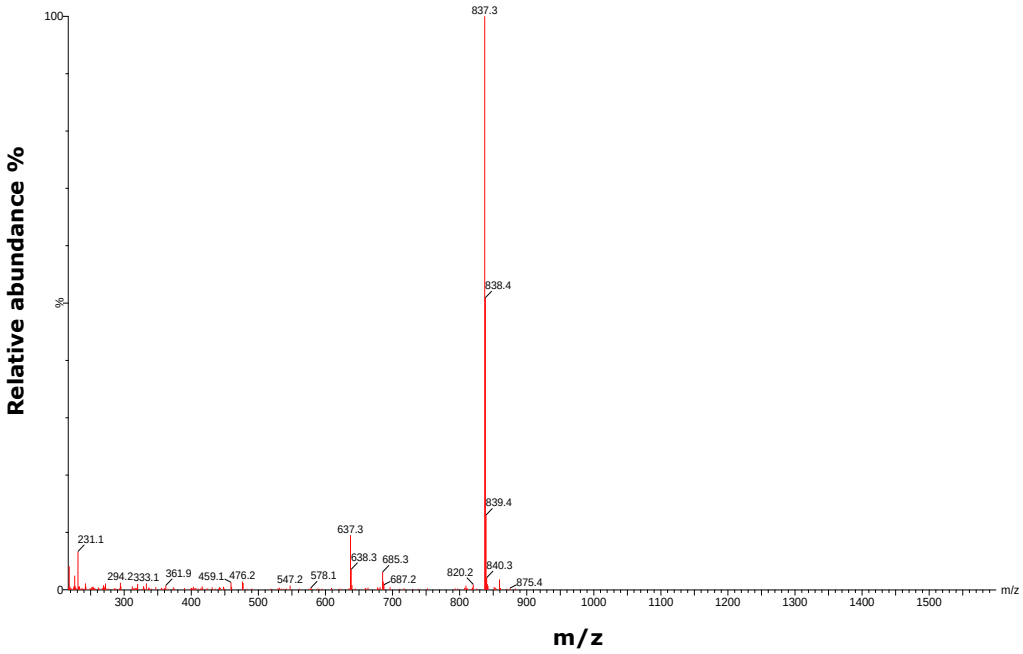


Figure 3 ESI-MS spectrum of ABPNB; *m/z* 837 $[M+H]^+$

Appendix B. Purification and identification of LNOD

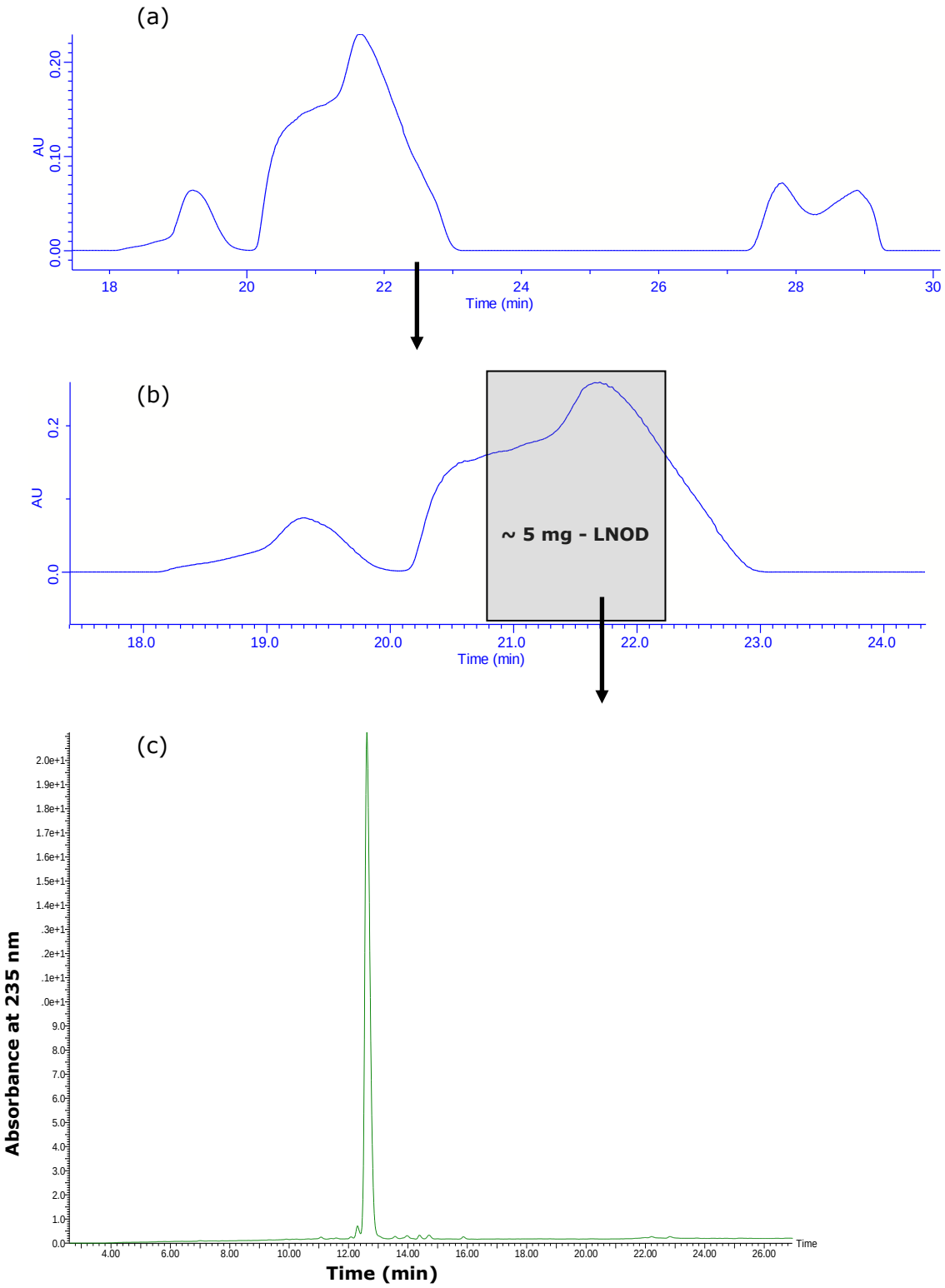


Figure 4 (a) Preparative HPLC chromatogram of LNOD with (b) fractions containing relative purity > 95% highlighted and (c) UV chromatogram of LNOD extracted at 235 nm with relative purity > 95%.

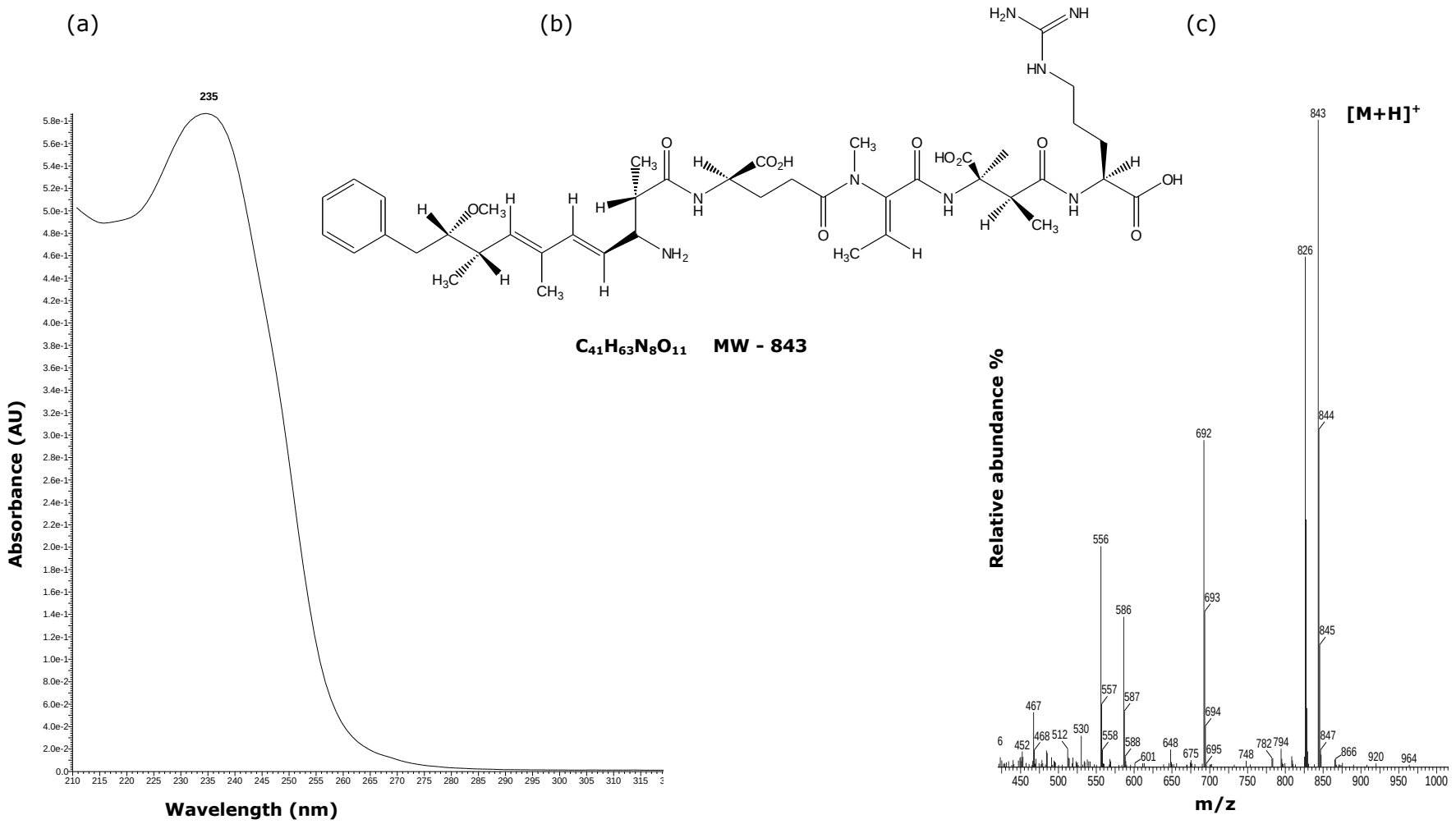


Figure 5 (a) UV spectrum (200-300 nm), (b) general structure and (c) MS spectrum of LNOD.

Appendix C. Analysis and identification of MCs and NOD

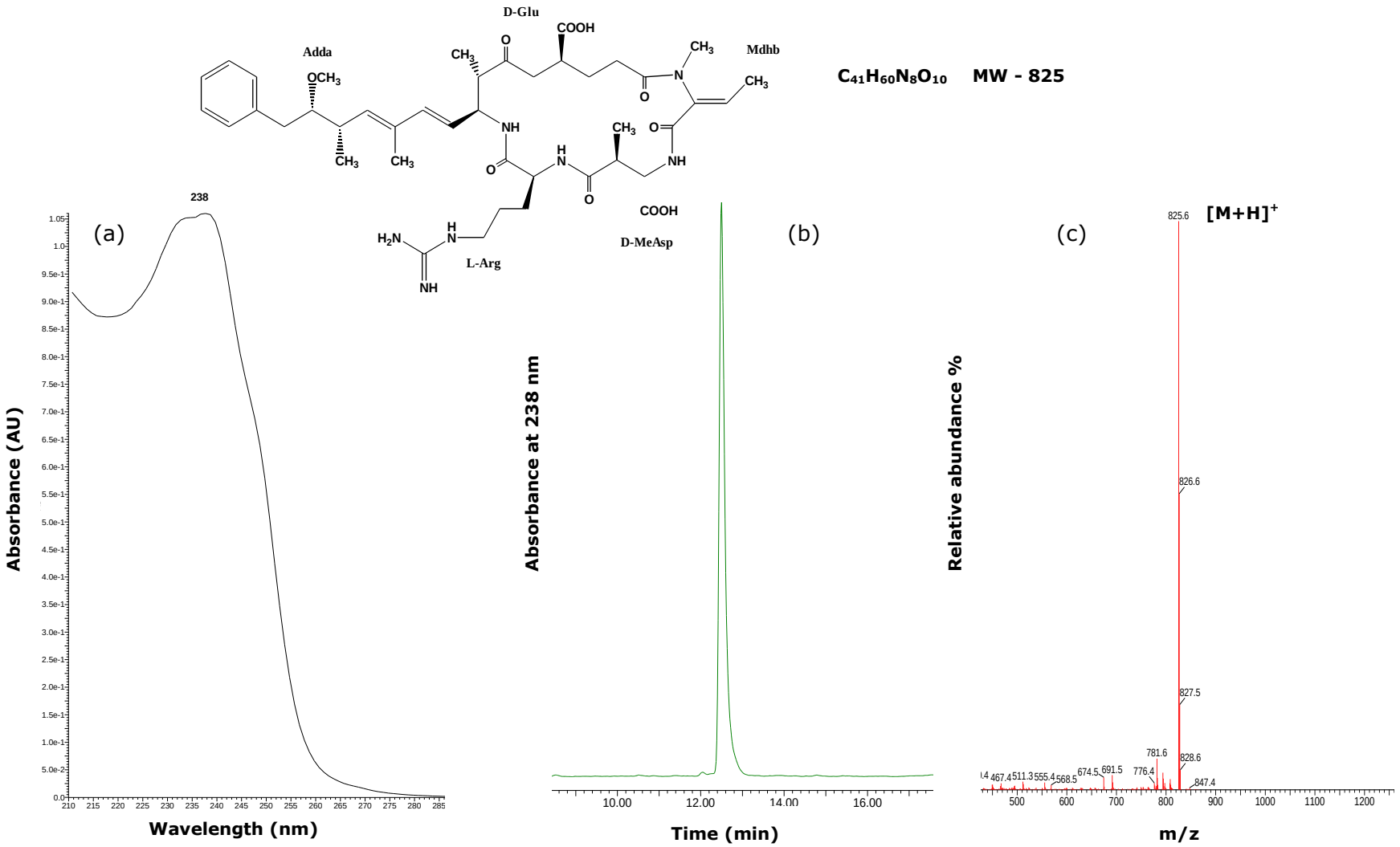


Figure 6 (a) UV spectrum (200-300 nm), (b) UV chromatogram at 238 nm with relative purity > 95% and (c) MS spectrum of NOD.

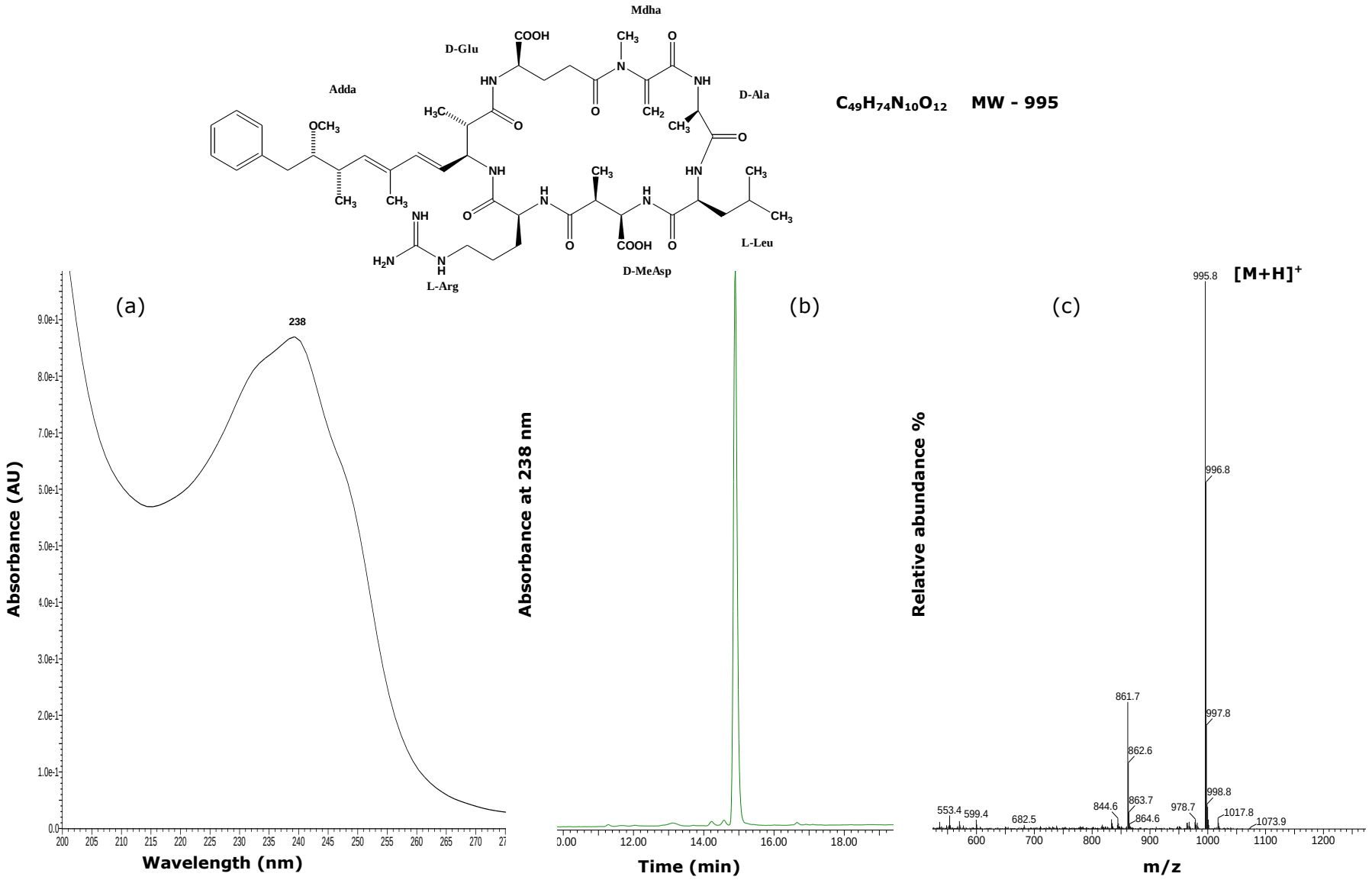


Figure 7 (a) UV spectrum (200-300 nm), (b) UV chromatogram at 238 nm with relative purity > 95% and (c) MS spectrum of MC-LR.

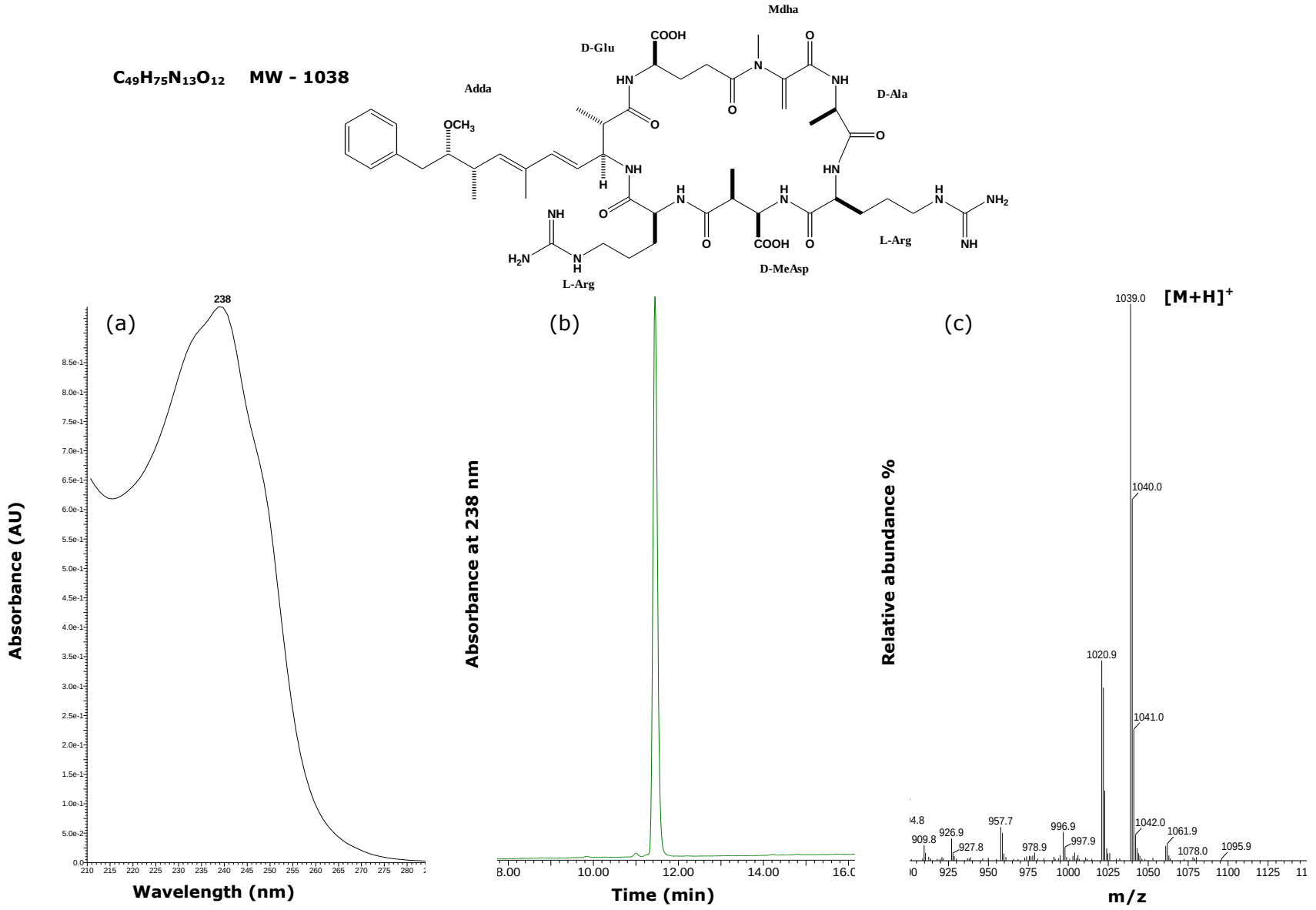


Figure 8 (a) UV spectrum (200-300 nm), (b) UV chromatogram at 238 nm with relative purity > 95% and (c) MS spectrum of MC-RR.

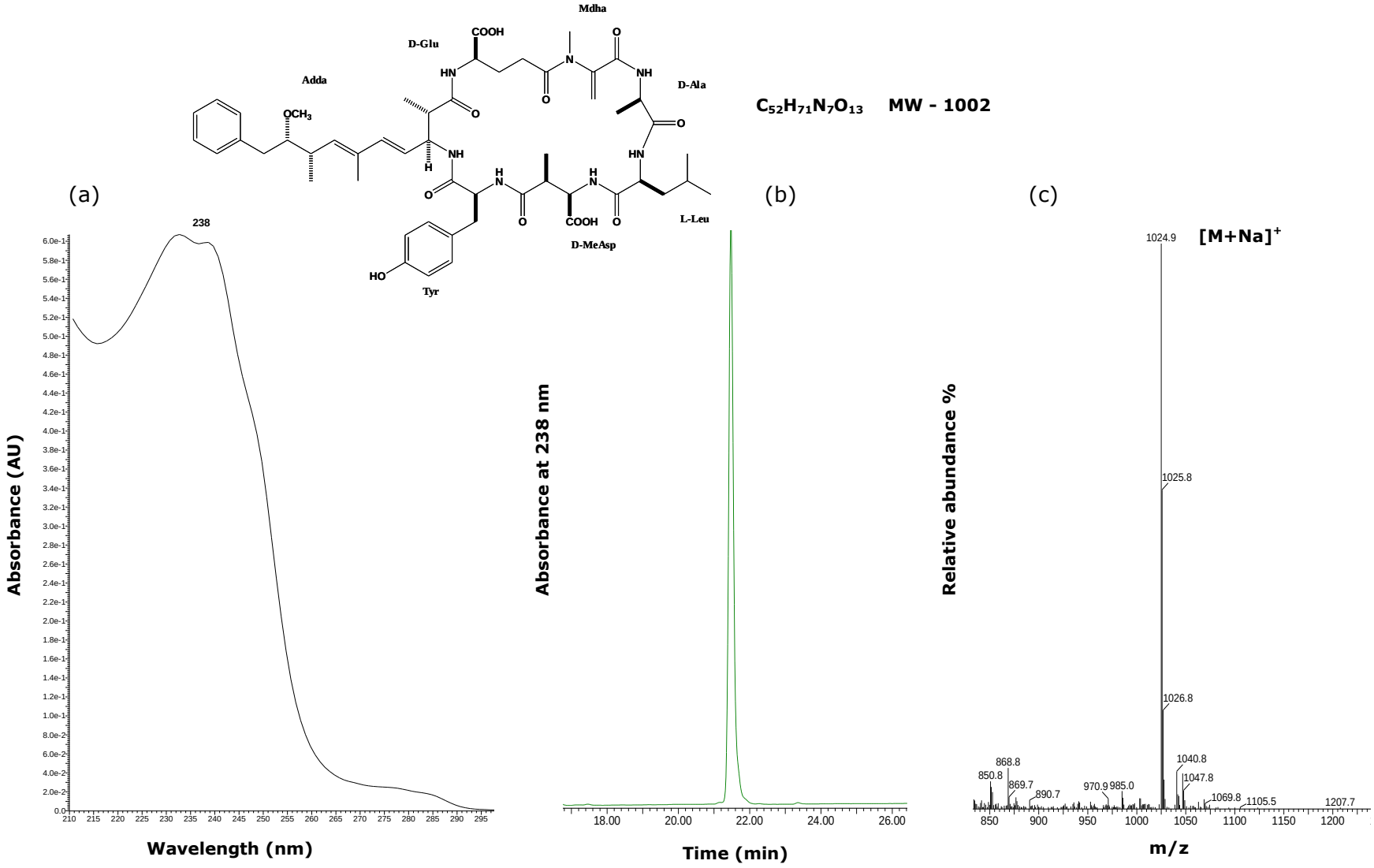


Figure 9 (a) UV spectrum (200-300 nm), (b) UV chromatogram at 238 nm with relative purity > 95% and (c) MS spectrum of MC-LY.

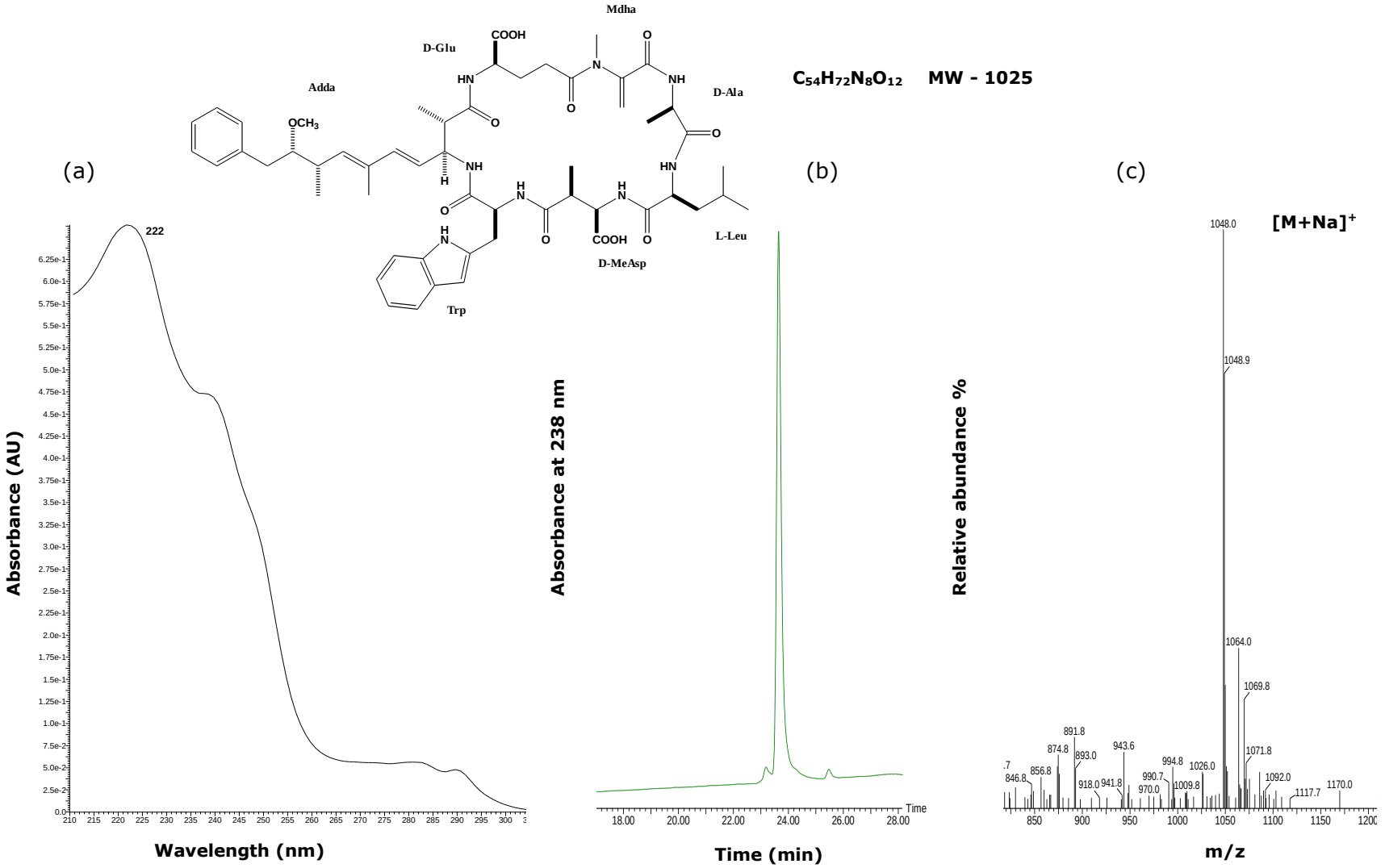


Figure 10 UV spectrum (200-300 nm), (b) UV chromatogram at 238 nm with relative purity > 95% and (c) MS spectrum of MC-LW.

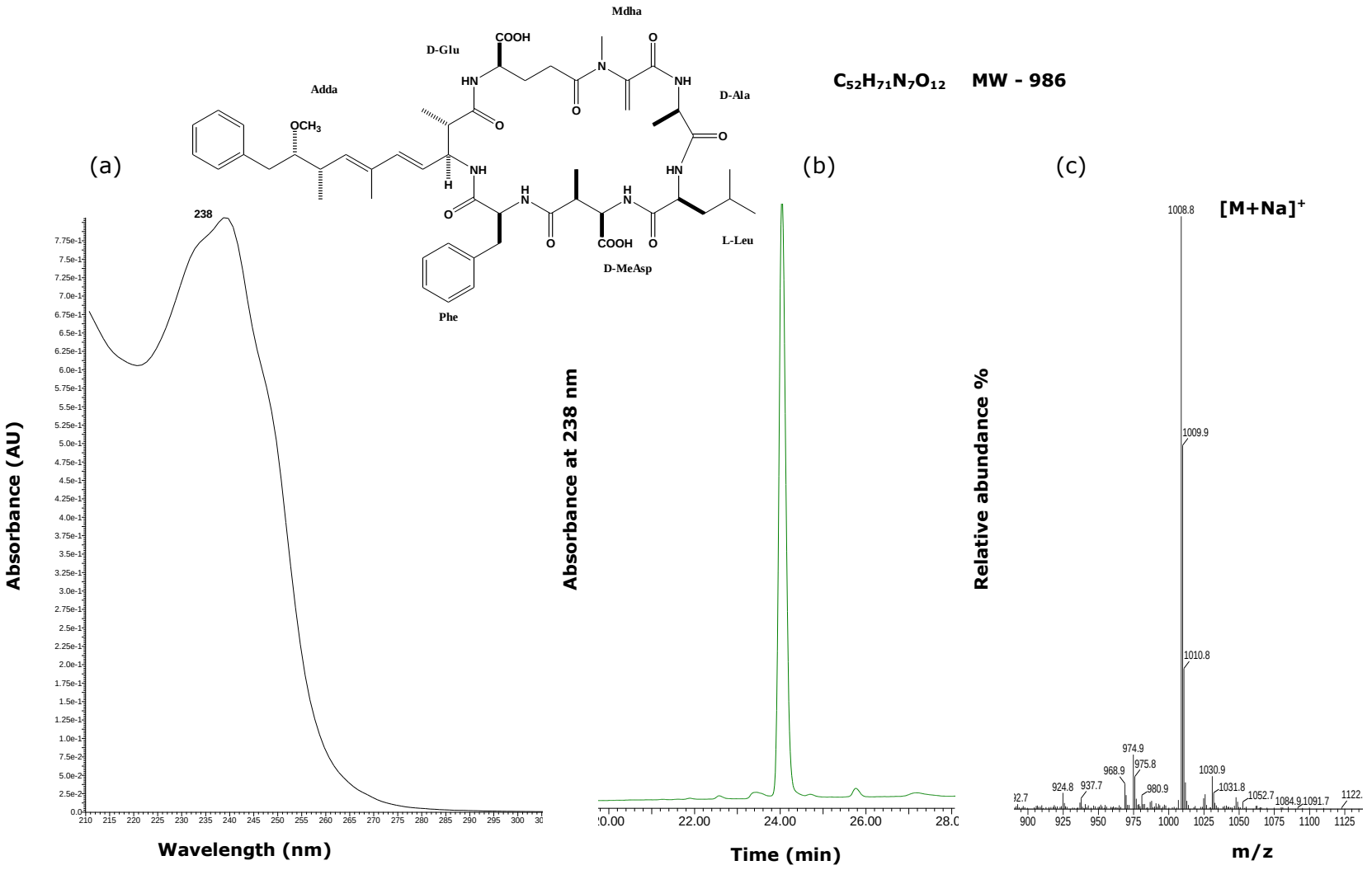
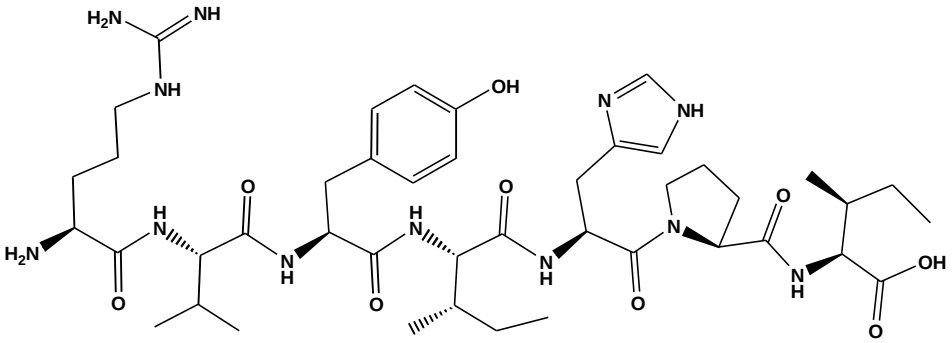


Figure 11 UV spectrum (200-300 nm), (b) UV chromatogram at 238 nm with relative purity > 95% and (c) MS spectrum of MC-LF.

Appendix D. Analysis of commercially acquired peptides



$C_{43}H_{68}N_{12}O_9$

MW: 897

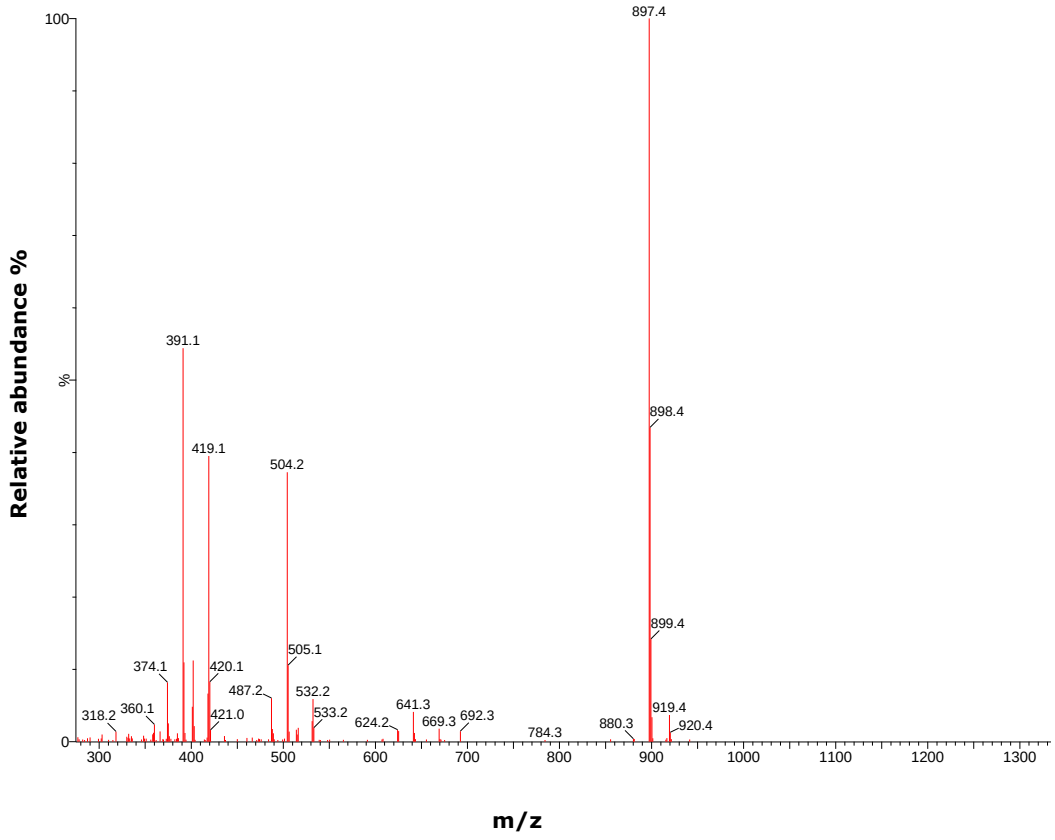
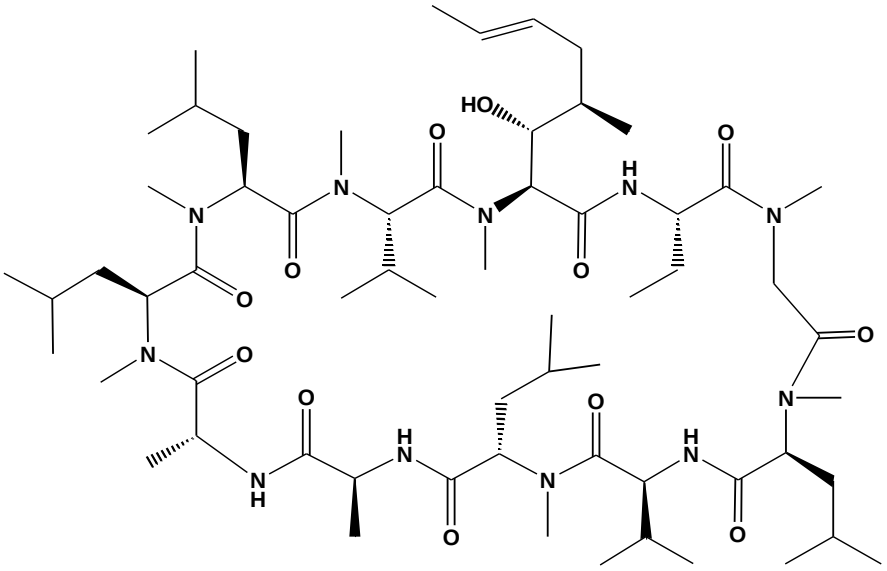


Figure 12 ESI-MS spectrum of ANGTN (Sigma-Aldrich, Dorset, UK); *m/z* 897 $[M+H]^+$



$C_{62}H_{111}N_{11}O_{12}$

MW: 1203

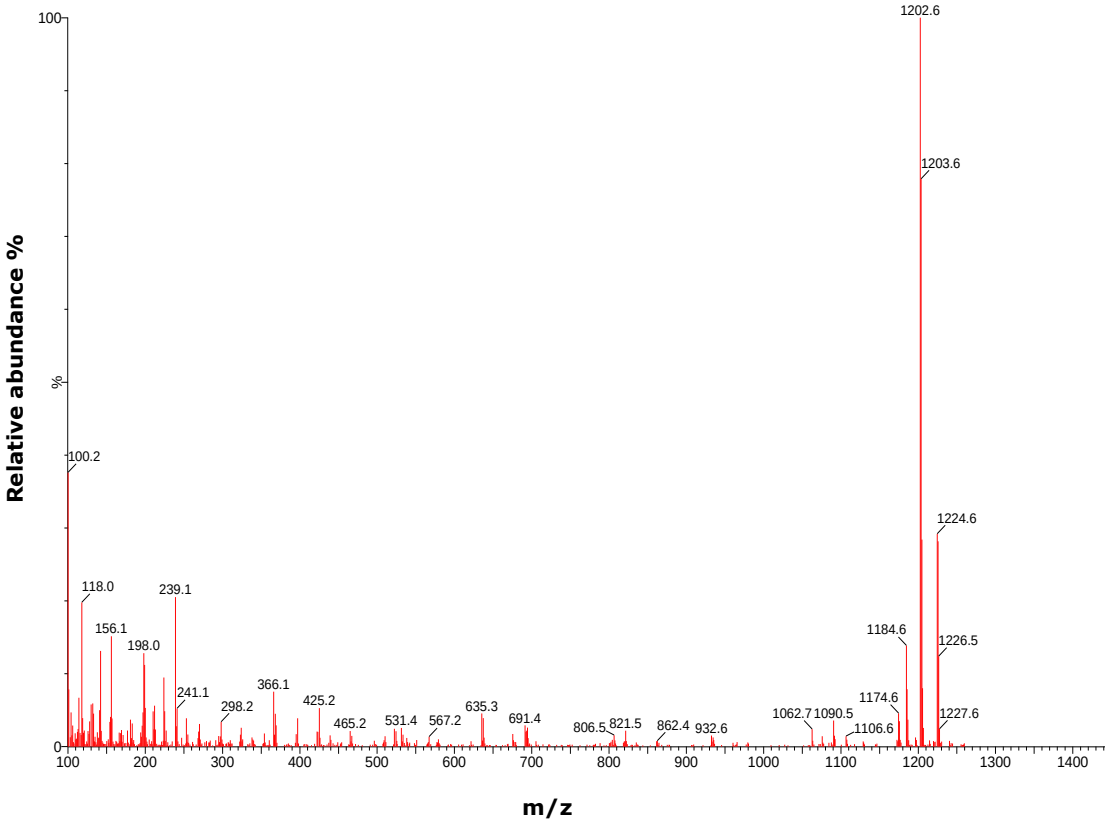
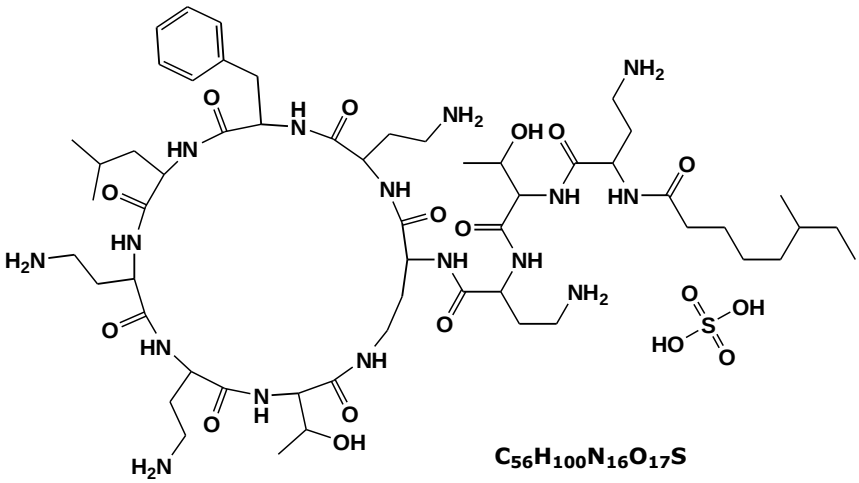


Figure 13 ESI-MS spectrum of CY (Sigma-Aldrich, Dorset, UK); *m/z* 1203 $[M+H]^+$



$C_{56}H_{100}N_{16}O_{17}S$

MW: 1302

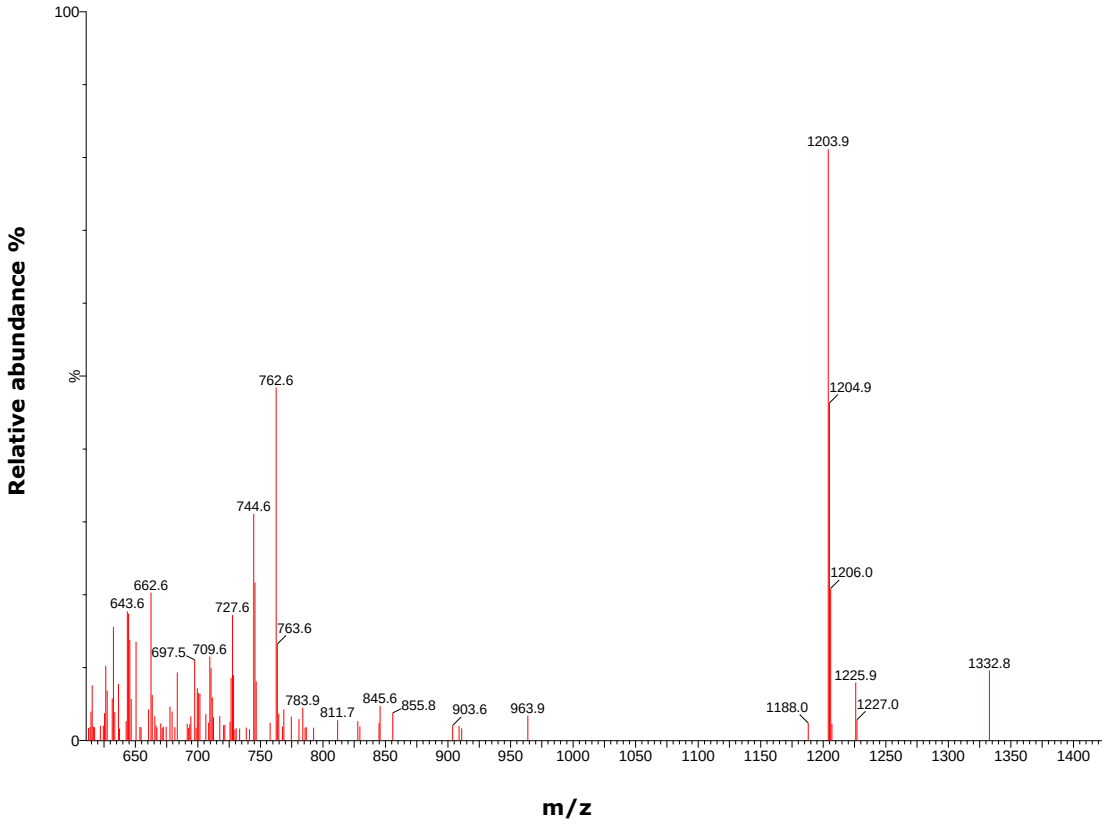
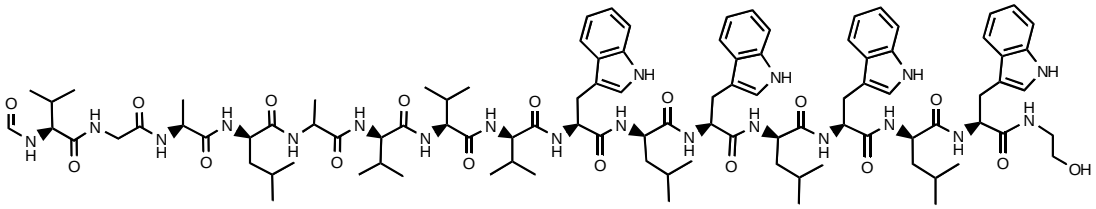


Figure 14 ESI-MS spectrum of POLYB (Sigma-Aldrich, Dorset, UK); *m/z* 1204 $[M+H]^+$



$C_{99}H_{140}N_{20}O_{17}$ MW - 1882

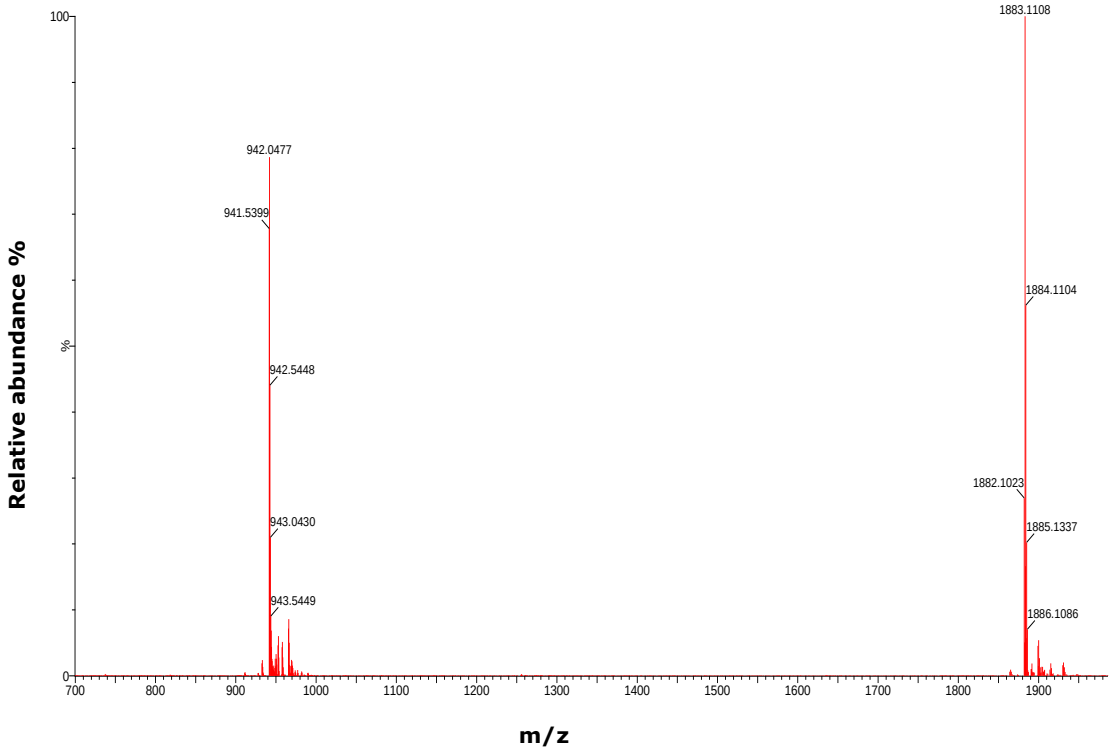


Figure 15 ESI-MS spectrum of GRA (Sigma-Aldrich, Dorset, UK); *m/z* 942 $[M+2H]^{2+}$ and *m/z* 1883 $[M+H]^{+}$

Appendix E. Calibration curves of purified cyanotoxins

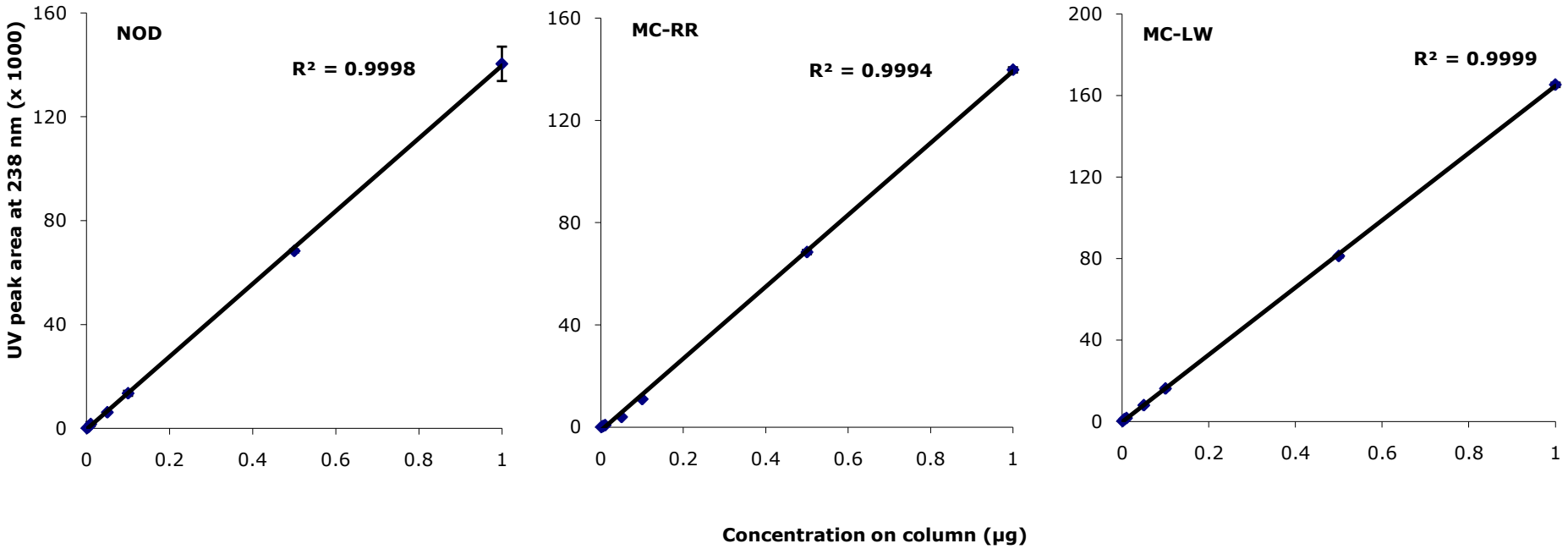


Figure 16 Calibration curves of purified NOD, MC-RR and -LW by UV at 238 nm. Error bars represent one standard deviation (n=3).

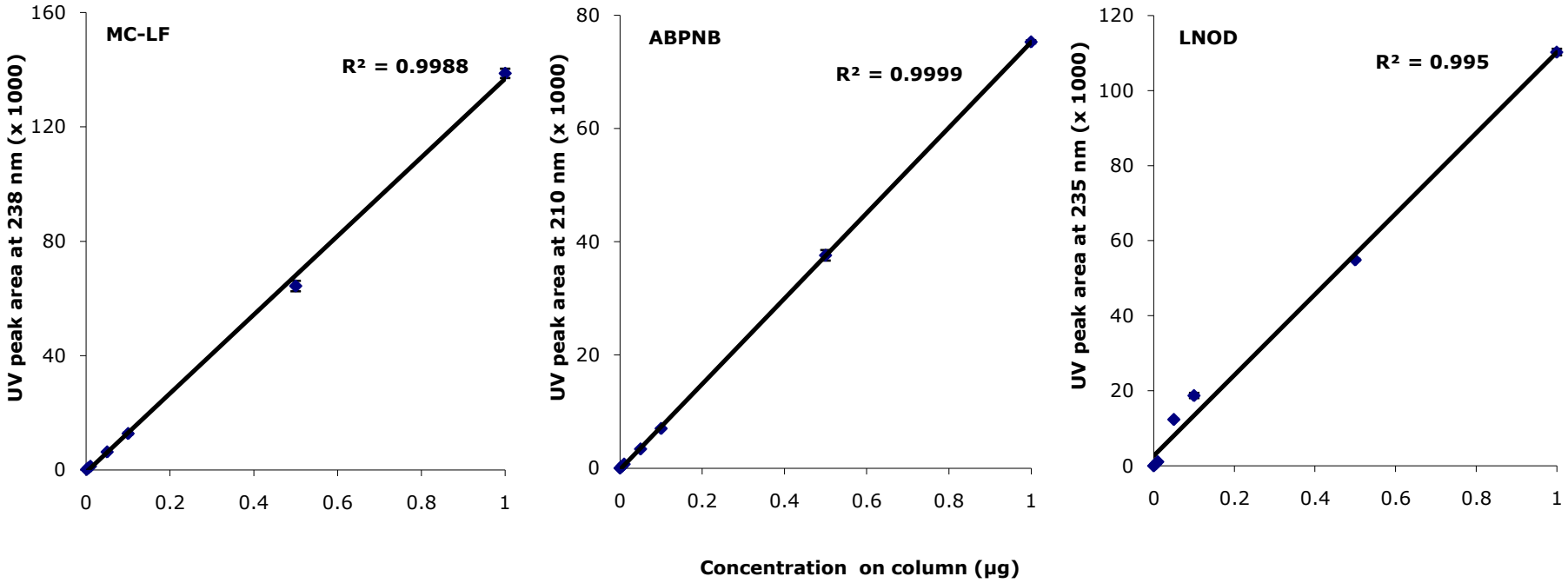


Figure 17 UV Calibration curves of purified MC-LF, ABPNB and LNOD. Error bars represent one standard deviation (n=3).

CONFERENCE PRESENTATIONS AND PUBLICATIONS

Conference presentations

WELGAMA, A., EDWARDS, C. and LAWTON, L. A. (2011) *Biodegradation of nature's own contaminants, microcystins and nodularin. 15th International Symposium on Biodeterioration and Biodegradation.* 19th-21st September 2011. Vienna, Austria.

WELGAMA, A., EDWARDS, C., LAWTON, L. A. and MANAGE, P. (2010) *Novel bacteria to remove cyanotoxins from drinking water. 8th International Conference on Toxic Cyanobacteria.* 2nd-9th September 2010. Istanbul, Turkey.

LAWTON, L.A., EDWARDS, C., **WELGAMA, A**., MANAGE, P. and GRAHAM, D. (2008) *Evaluation and Exploitation of Microbes in the Elimination of Cyanotoxins From Water. AWWA Water Quality Technology Conference.* 18th November 2008. Cincinnati, USA.

LAWTON, L.A., EDWARDS, C., **WELGAMA, A.**, MANAGE, P. and GRAHAM, D. (2008) *Evaluation and Exploitation of Microbes in the Elimination of Cyanotoxins from Water. USEPA one-day symposium.*19th November 2008. Cincinnati, USA.

WELGAMA, A., MANAGE, P. M., CHRISTINE, E. and LAWTON, L. A. (2009) *Putting microbes to work: Bioremedial microcystin removal strategies for water purification. Society for general Microbiology, autumn meeting.* 7th-12th September 2009. Edinburgh, UK.

WELGAMA. A., MANAGE, P. M., CHRISTINE, E. and LAWTON, L. A. (2008) *Harnessing novel microbial strains for bioremediation of cyanotoxins. Research student symposium.* Robert Gordon University. 10th October 2008. Aberdeen, UK.

WELGAMA, A., MANAGE, P. M., CHRISTINE, E. and LAWTON, L. A. (2008) *Novel Microbial strains for bioremediation of microcystins and nodularin. Proceedings of Scottish microbiological society.* 7th August 2008. University of Glasgow, Scotland.

WELGAMA, A. and PATHMALAL, M. M. (2007) *A microbiological study of some well water quality parameters with reference to the garbage dumping site at Navinna area. Proceedings of the Sri Lanka association for the advancement of Science*. 14th August 2007. Colombo, Sri Lanka.

WELGAMA, A., PATHMALAL, M. M. and LIYANAGE, J. A. (2007) *Heavy metal contamination in well water around a dumping site at Navinna-Maharagama*. *International Forestry and Environment Symposium*. 10th October 2007. Colombo, Sri Lanka.

Novel bacterial strains for the removal of microcystins from drinking water

L. A. Lawton, A. Welgamage, P. M. Manage and C. Edwards

ABSTRACT

Microcystins (MC) and nodularin (NOD) are common contaminants of drinking water around the world and due to their significant health impact it is important to explore suitable approaches for their removal. Unfortunately, these toxins are not always removed by conventional water treatments. One of the most exciting areas that hold promise for a successful and cost effective solution is bioremediation of microcystins. Recent work resulted in successful isolation and characterisation of 10 novel bacterial strains (*Rhodococcus* sp., *Arthrobacter* spp. and *Brevibacterium* sp.) capable of metabolizing microcystin-LR (MC-LR) in a Biolog MT2 assay. The work presented here aims to further investigate and evaluate the metabolism and the degradation of multiple microcystins (MC-LR, MC-LF, MC-LY, MC-LW and MC-RR) and nodularin by the bacterial isolates. A total of five bacterial isolates representing the three genera were evaluated using Biolog MT2 assay with a range of MCs where they all demonstrated an overall metabolism on all MCs and NOD. Subsequently, the results were confirmed by observing the degradation of the range of toxins in a separate batch experiment.

Key words | abstract, algal toxins, blue-green algae, cyanobacteria, cyanotoxins

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INTRODUCTION

Cyanobacteria are one of the most successful and widespread living organisms on earth. They can be found in almost all aquatic environments and many terrestrial environments. Blooms of cyanobacteria occur in late summer or early autumn in temperate countries, but in warmer climates, they can grow year round (Mur *et al.* 1999; Oliver & Ganf 2000). Blooms of cyanobacteria are often toxic, producing a wide range of potent hepatotoxic and neurotoxic secondary metabolites (Sivonen & Jones 1999). Toxic cyanobacteria have been reported from every continent of the world. The most commonly occurring cyanotoxins, microcystins, are potent liver toxins and can increase the risk of cancer following chronic exposure. Microcystins are cyclic peptides (Carmichael 1994) making them extremely stable during typical water treatment conditions. This has prompted the search for alternative removal methods which has included isolation of microcystin-degrading bacteria. Many studies have focused on isolating bacteria from water sources exposed to microcystin producing blooms (Edwards *et al.* 2008). These studies have resulted in the isolation of a small number of bacteria (especially *Sphingomonas* sp.) capable of metabolising the microcystins.

A recent study carried out by Manage *et al.* (2009) successfully isolated a group of novel bacteria capable of rapidly degrading microcystin-LR. A novel method of rapid screening for bacterial biodegradation activity was applied. Biolog MT2 plates, a 96 well format containing a tetrazolium redox dye provide a rapid indication of metabolic activity, indicating if a bacterial isolate can use microcystin. The novel bacterial isolates have so far only been shown to degrade microcystin-LR however many microcystin variants occur. Therefore it is essential to explore a number of chemical variants. The Biolog MT2 method was implemented as a rapid tool for examining multiple bacterial strains with multiple microcystin variants along with different toxin concentrations with a view to establishing the suitability of these organisms for the removal of microcystins from drinking water.

doi: 10.2166/wst.2011.352