EVIDENCE FOR FUNCTIONAL DRIFT OF BACTERIAL ISOLATES IN RESPONSE TO CYANOBACTERIAL MICROCYSTIN-LR AND MULTIPLE PEPTIDE DEGRADATION IN *PAUCIBACTER TOXINIVORANS*

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Evidence for functional drift of bacterial isolates in response to cyanobacterial microcystin-LR and multiple peptide degradation in *Paucibacter toxinivorans*

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DEDICATION

To

MY GRANDPARENTS

ACKNOWLEDGEMENTS

Dear Linda and Christine, thank you for having accompanied me on my first journey in the world of research and gotten me prepared for the ones still to come.

Dear Aakash, thank you for having been a great friend from the very first day and all throughout.

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Dear Kostas, thank you for all the laughter you have caused! ☺

Dear Tina, many thanks that you were always there to help.

Dear Hanna and Andrew, thank you for your helpful comments on this work.

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Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Johanna Kreitz

Abstract

Bacterial bioremediation has been proposed as an efficient, low cost and ecologically safe method to clean vital water reservoirs from cyanobacterial peptide toxins microcystins (MCs). In previous work carried out in 2008 several bacteria were isolated from Scottish freshwaters that effectively degraded MCs. *Rhodococcus* sp. C1, among the biocatalytic isolates, exhibited particular catabolic capacities as it degraded a range of chemically and structurally diverse prokaryotic and eukaryotic peptides.

The work presented here aimed to unravel the universal peptide degradation mechanisms in *Rhodococcus* sp. C1. However, current biodegradation studies indicated repeated sub-culturing and long-term cryopreservation to have caused changes in the cellular mechanisms involved in MC-LR degradation as MC-LR degradation activity was no longer observed. Therefore, the focus of the study was shifted towards other isolates of the freshwater samples as well as a MC-LR degrading organism of unknown origin. Based on 16S rRNA gene analysis the isolates were identified as *Rhodococcus* sp., *Arthrobacter* sp. and *Pseudomonas* sp., respectively. The different bacterial genera were subjected to MC-LR biodegradation studies including *Paucibacter toxinivorans* (2007), a MC degrading bacterium from Finnish water previously used as positive control organism. However, it was shown that the three isolates and *P. toxinivorans* (2007) also lost their MC-LR degradation activity over long-term maintenance under laboratory conditions.

This led to the belief that routine maintenance of the bacterial isolates in nutrient rich media such as Luria-Bertani (LB) broth had caused a functional drift that impeded the isolates' ability to degrade MC-LR. To assess whether

nutrient availability has an impact on the bacterial MC-LR degradation activity a simple and rapid 96-well plate based method was developed for testing MC-LR biodegradation in growth media of different nutrient concentration and composition. In addition to the long-term maintained and repeatedly sub-cultured strain of *P. toxinivorans* (2007) a new *P. toxinivorans* strain (2015) from the German Collection of Microorganisms and Cell Cultures was included in the nutrient assay. Comparison studies between the two strains supported the occurrence of a physiological drift in the repeatedly sub-cultured strain as cell morphology, oxidase activity and media tolerance of the strains were found to be different. The nutrient assay showed that the use of different growth media had little effect on MC-LR degradation activity of the long-term preserved bacteria. However, the newly obtained *P. toxinivorans* (2015) effectively removed MC-LR from all media except LB broth. Furthermore, UPLC-PDA-MS analysis revealed MC-LR intermediates in samples exposed to *P. toxinivorans* (2015). Two of the degradation products were identified as linearised (acyclo-) MC-LR and one as the side chain Adda. Broader investigation of the organism's catabolic abilities demonstrated *P. toxinivorans* (2015) is capable of degrading multiple MC variants, nodularin (NOD), anabaenopeptin-type peptides and human peptides. MC variants and NOD were found to be cleaved by hydrolysis indicating a single mechanism to be involved in their degradation. This is the first study to report partial elucidation of the MC and NOD degradation pathway in *P. toxinivorans*. Further research could include a complete elucidation of the enzymatic degradation pathway in *P. toxinivorans* (2015) along with studies to determine the genes encoding the enzymes involved.

Abbreviations

AP	Anabaenopeptin
AOTs	Advanced oxidation technologies
BLAST	Basic Local Alignment Search Tool
BMAA	β-methyl-amino-L-alanine
CE	Cell extract
CYN	Cylindrospermopsin
d	Days
DMC-LR	Desmethyl MC-LR
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FIB	[Glu^1]-Fibrinopeptide B
GRA	Gramicidin A
h	Hours
HPLC	High performance liquid chromatography
LB	Luria-Bertani
LEU	Leucine Enkephalin acetate salt hydrate
LTEE	Lenski Long-Term Evolution Experiment
MC	Microcystin
min	Minutes
MSM	Mineral salt medium
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
NOD	Nodularin
OXY	Oxytocin acetate salt hydrate

PBS	Phosphate buffered saline
qTOF	Quadrupole Time-of-flight
R2A	Reasoner's 2A broth
STX	Saxitoxin
TBE	Tris/Borate/EDTA
TDI	Tolerable daily intake
TE	Tris-EDTA
TFA	Trifluoroacetic acid
Tris-HCl	Tris-hydrochloride
UPLC-PDA-MS	Ultra-high performance liquid chromatography photodiode array mass spectrometry

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1 General Introduction

1.1 History and Worldwide Distribution of Cyanobacteria

Cyanobacteria are photosynthetic, nitrogen fixing prokaryotes commonly referred to as blue-green algae that have a high ability to adapt to a variety of different environments. As one of the most successful organisms on earth, they played an important role in developing the oxygenated atmosphere of our planet (Whitton and Potts, 2012). Blooms of cyanobacteria have been recorded in aquatic ecosystems all over the world where, as major primary producers, they supply organic carbon to higher organisms (Hamasaki *et al.*, 1999; Quiblier *et al.*, 2008).

In freshwater the prevalent bloom forming genera are *Dolichospermum*, *Microcystis* and *Planktothrix*. Furthermore, estuarine and brackish waters such as the Baltic Sea are commonly occupied by cyanobacterial blooms of *Nodularia*, *Aphanizomenom* and *Dolichospermum*. In marine environments filamentous members of the genera *Lyngbya*, *Trichodesmium* and the coccoid *Synechococcus* are the predominant cyanobacterial bloom formers (Zhang *et al.*, 2012).

Rapid expansion of mass cyanobacterial populations occurs globally and has been linked to climate change and eutrophication (Grossmann *et al.*, 1994; Paerl *et al.*, 2009). The rise in atmospheric carbon dioxide caused by the burning of fossil fuels increased the global temperature which is expected to further rise by 1.5 to 5 °C in the 21st century (O'Neil *et al.*, 2012). Evidence for the positive effects of temperature on cyanobacterial expansion has been given by a number of laboratories (Reynolds, 2006; Domis *et al.*, 2007) and in field observations (Jeppesen *et al.*, 2009; Wagner *et al.*, 2009).

A study exploring the relationship between temperature and phytoplankton growth took samples from over 140 lakes along the latitudinal gradient from northern Europe to South America (Kosten *et al.*, 2012). It demonstrated the percentage of cyanobacteria in the phytoplankton community to rise steeply with temperature elevation.

Moreover, the increase of nutrient sources caused by anthropogenic activities is a strong environmental driver for cyanobacterial development (Quiblier *et al.*, 2008). Urbanisation, agriculture and industrial development have led to an increased influx of phosphorus and nitrogen into lakes and rivers (Paerl *et al.*, 2008). Especially in temperate countries, phosphate is a limiting factor for the cells and in high concentration enhances the development of the blooms.

The enormous bloom proliferation has received considerable attention not only due to their severe impacts on light conditions, oxygen concentrations and biodiversity but also due to their production of a wide range of odours, noxious compounds and toxic secondary metabolites (Codd *et al.,* 2005).

1.2 Toxicity and Health Effects of Cyanobacterial Toxins

Among a wide array of secondary metabolites produced by cyanobacteria, some have been identified as potent toxins (cyanotoxins). Based on their toxicological target and mode of action cyanobacterial toxins fall into several main groups: hepatotoxins, neurotoxins, cytotoxins and dermatotoxins (Van Apeldoorn *et al.*, 2007; Table 1.1).

Hepatotoxins, known to cause liver damage, include MCs, NODs and cylindrospermopsins (CYNs) (Table 1.1). Neurotoxins are named after their mode of action on the neurological system and the most commonly produced types are anatoxin-a, anatoxin-a (S) and saxitoxins (STX) (Codd *et al.,* 2005;

Table 1.1 Groups of cyanotoxins, their toxicities and the cyanobacterial genera that produce them. (Compiled from Codd *et al.* (2005), Edwards and Lawton (2009) and O'Neil *et al.* (2012).)

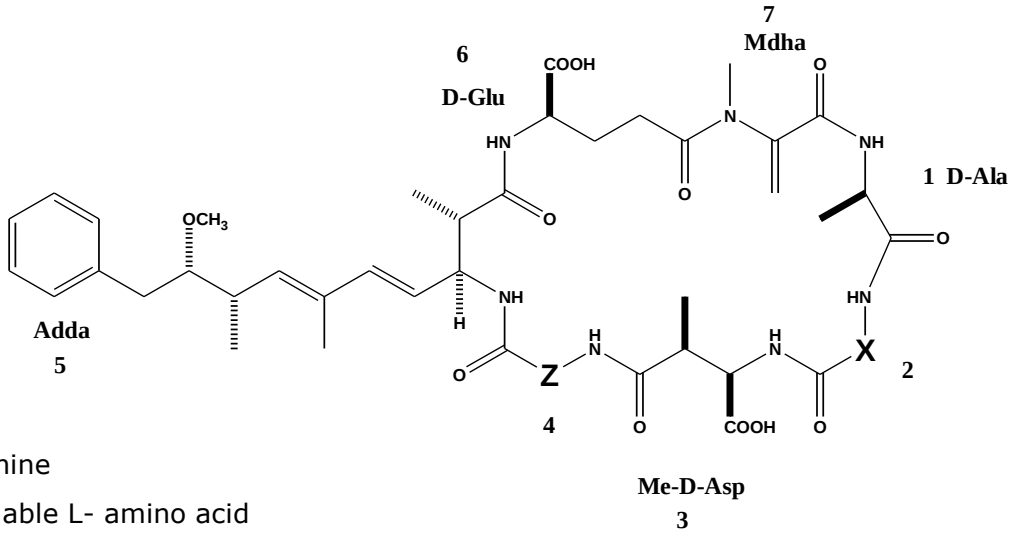
Toxins (LD_{50})*	**Primary target organ in mammals and activity**	**Cyanobacterial genera**
Hepatotoxins		
Microcystins (25 - ~ 1000)	Liver; inhibitors of protein phosphatases and membrane integrity, tumour promoters, class B carcinogens	*Microcystis, Dolichospermum, Planktothrix, Nostoc, Hapalosiphon, Anabaenopsis, Trichodesmium, Synechococcus, Snowella*
Nodularin (30 - 50)	Liver; inhibitors of protein phosphatases and membrane integrity, tumour promoters, carcinogenic	*Nodularia*
Cylindrospermopsin (200 – 2100)	Liver (also kidneys, spleen, lungs, intestine); inhibitors of protein synthesis, genotoxic	*Cylindrospermopsis, Umezakia, Aphanizomenon, Lyngbya, Raphidiopsis, Dolichospermum*
Neurotoxins		
Anatoxin-a (250)	Nerve synapse; depolarising neuromuscular blockers	*Dolichospermum, Planktothrix, Oscillatoria, Aphanizomenon, Phormidium, Rhaphidiopsis*
Anatoxin-a (S) (40)	Nerve synapse; inhibitor of acetylcholinesterase	*Dolichospermum*
Saxitoxins (10 – 30)	Nerve axons; sodium channel blockers	*Dolichospermum, Planktothrix, Aphanizomenon, Lyngbya, Cylindrospermopsis, Scytonema*
BMAA	Nerve synapse; excitotoxin on various receptors like glutamate receptors	Many (for example *Microcystis* and *Nostoc*)
Dermatotoxins and cytotoxins		
Lyngbyatoxin-a	Skin, gastro-intestinal tract; inflammatory agent, protein kinase C activator	*Lyngbya*
Aplysiatoxin	Skin; inflammatory agent, protein kinase C aktivators	*Lyngbya, Schizothrix, Planktothrix, Oscillatoria*

* Acute toxicity in mouse bioassay (LD_{50} - µg/kg body weight)

Table 1.1). The neurotoxic amino acid variant β-methyl-amino-L-alanine (BMAA) is involved in overactivation of neuroexcitatory glutamate receptors and found in many cyanobacteria (Vyas and Weiss, 2009; Table 1.1).

These major cyanobacterial bloom toxins are produced by a wide range of cyanobacterial genera including *Microcystis, Dolichospermum, Nodularia, Cylindrospermopsis* and *Aphanizomenon* (O'Neil *et al.*, 2012; Table 1.1). Among all cyanotoxins, MCs have been studied most extensively as they are most prevalent in the environment and show most acute hepatotoxicity and tumour promoting activity in humans and animals (Zurawell *et al.*, 2005). MCs are a group of chemically related heptapeptides produced by the cyanobacterial genera *Microcystis, Dolichospermum, Nostoc* and others. They are potent inhibitors of eukaryotic serine/threonine-protein phosphatases 1 and 2A and class B carcinogens (Grosse *et al.*, 2006). The extremely stable cyclic peptides are composed of seven amino acids including the unusual ß-amio acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). Adda plays a main part in the toxicity mechanism of MCs as it irreversibly binds and subsequently interacts with the catalytic site of protein phosphatase (Campos and Vasconcelos, 2010). 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} (Figure 1.1). The X2 and Z4 positions represent variable L-amino acids that distinguish MCs from one another (Zurawell *et al.*, 2005). Amino acid variations in these positions account for many of the over 70 MC variants that have been identified. The most common MC variants are MC-LR, MC-RR and MC-YR which differ in the combination of leucine (L), arginine (R) or tyrosine (Y) (Kim *et al.*, 2015). MC-LR has gained most attention due to its toxicity and increasing occurrence (Campos and Vasconcelos, 2010).

Upon rising concern about the chronic toxicity of MCs, the World Health Organisation (WHO) established a guideline of 1.0 µg/l as the permissible level of MC-LR in drinking water (WHO, 1998). The guideline was based on a liver



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)

Figure 1.1 The general structure of MC.

pathology study in mice (which determined a tolerable daily intake (TDI) level of 0.04 µg/kg body weight per day), an average adult body weight of 60 kg, an average adult water intake of 2 litres per day and the proportion of the TDI allocated to drinking water (assumed to be 0.8; guideline level = 0.04 µg/kg x 60 kg x 0.8 / 2 l = 0.96 µg/l ≈ 1 µg/l). Many countries in the world (including Australia, New Zealand, Canada, Brazil and several nations in Europe) have since adopted the guideline value in their legislation (Jurczak *et al.*, 2005). For recreational waters the WHO established MC-LR guideline values dependent on cyanobacterial intensities and defined the probability of adverse effects at three levels: low risk (< 20,000 cyanobacterial cells/ml corresponding to < 10 µg/l of MC-LR), moderate risk (20,000 – 100,000 cyanobacterial cells/ml

corresponding to 10 – 20 µg/l of MC-LR) and high risk (> 100,000 cyanobacterial cells/ml corresponding to > 20 µg/l of MC-LR) (WHO, 1998). Over recent decades, toxic cyanobacterial blooms have been frequently linked to many incidents of animal and human poisonings. Fatal poisoning has affected animals including cattle, sheep, chickens, pigs, horses, dogs and wild birds. Moreover, a wide range of aquatic organisms are exposed to the toxins and especially filter-feeding animals such as mussels have a high potential to act as toxin vectors for higher predators including humans (de Magalhães *et al.*, 2001).

Human exposure is most frequently through the consumption of contaminated drinking water or trough water swallowed during recreational activities (Grosse *et al*., 2006). Many countries including USA, Canada, Australia, UK and Sweden reported cyanotoxin (mostly MC) associated health (WHO, 2003). Colorectal and liver cancer have been associated with drinking MC contaminated water in epidemiological studies in China (Lun *et al.*, 2002). Recreational or occupational water contact has been linked to gastroenteritis, rashes, fever, eye irritation, vomiting and other health effects caused by cyanotoxins including MC and STX (Codd *et al.,* 2005). The most significant incidence of human toxicoses by MC leading to the death of 76 dialysis patients through liver failure occurred in Caruaru, Brazil in 1996 and was caused by the use of MC contaminated water in renal dialysis treatment (Carmichael *et al.*, 2001).

1.3 Different Approaches for the Removal of MCs from Water Reservoirs

The presence of cyanotoxins in drinking water is a serious concern worldwide and has prompted investigations into feasible and effective technologies that remove cyanotoxins and/or cyanobacterial cells from vital water sources. However, two factors make the search for an appropriate technology challenging. Firstly, owing to their cyclic structure MCs are resistant to many biological and biochemical factors including enzymes and temperature (Kato *et al.*, 2007). Also, sunlight decomposes and isomerises MCs only to some degree (Tsuji *et al.*, 1994). Secondly, cyanotoxins occur intracellular and dissolved following cell lysis (Schmidt *et al.*, 2002).

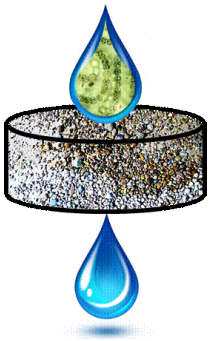
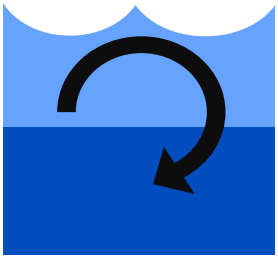
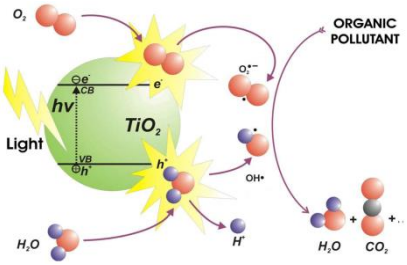
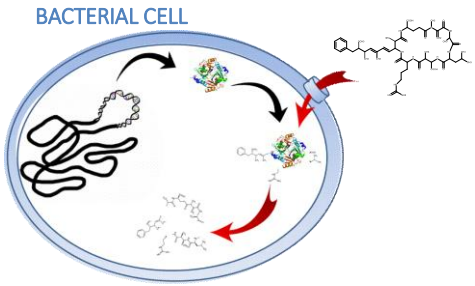
Researchers have explored conventional as well as chemical and biological processes for the treatment of contaminated water (Table 1.2).

Efforts to remove the toxins via domestic water filters (packed with activated carbon and an ion exchange resin; Lawton *et al.*, 1998), sand filters, mixing of surface water with ground water (Jurczak *et al.*, 2005) or other approaches have resulted in a reduction of toxin concentration. Yet, none of the conventional water treatment method was successful in removing these toxins rapidly and effectively from potable water (Liu *et al.*, 2009).

Coagulation, ozonation and chlorination, water treatments that make use of chemicals and are used on a commercial scale, have shown efficient elimination of MCs (Jurczak *et al.*, 2005). Though, such methods are chemically intensive and the formation of undesirable by-products and high operational costs diminish their potential (Gamage and Zisheng, 2010).

Advanced oxidation technologies (AOTs) have been considered for the treatment of soluble toxins.

Table 1.2 Different approaches for the removal of MCs from water.

TREATMENTS		SUITABILITY
Conventional water treatments		
 Sand or activated carbon filters	 Mixing of surface water with ground water	⊖ Do not remove MCs effectively
Chemical water treatments		
 Addition of chemicals like chlorine or ozone	 Advanced oxidation technology using TiO_2	⊕ Remove MCs effectively ⊖ High operational costs; concern of environmental safety
Biological water treatment		
 Bioremediation		⊕ Environmentally safe, efficient and cheap ⊖ Only remove extracellular MCs

One of the most common AOTs is titanium dioxide (TiO_2) photocatalysis (Antoniou *et al.*, 2009). A number of studies proved TiO_2 photocatalysis to be successful in the rapid removal of even high concentrations of toxins in aqueous solutions (Robertson and Lawton, 1997; Antoniou *et al.*, 2009; Liu *et*

al., 2009). However, the application of TiO_2 has not been applied at a commercial scale so far.

Biodegradation of MCs is an emerging method that has the potential to allow efficient, ecologically safe and cost effective removal of the toxins (Edwards and Lawton, 2009). In field studies Grützmacher *et al.* (2002) and Chorus *et al.* (2003) attributed high elimination rates of dissolved MCs by slow sand filters to biodegradation taking place inside the filter. Natural bacterial consortia in many various aquatic environments including rivers, reservoirs and sediments contain microorganisms capable of degrading MCs (Jones *et al.*, 1994). Numerous studies have been exploiting such microbial consortia with an emphasis on isolating an organism with strong MC degrading capability. Jones *et al.* (1994) isolated a MC degrading bacterium, *Sphingomonas* sp. strain ACM-3962 (MJ-PV), from Australian river water and identified its MC-LR degradation pathway (Bourne *et al.*, 1996 and 2001). Three hydrolytic enzymes encoded by *mlrA, mlrB* and *mlrC* were shown to mediate MC-LR breakdown. Microcystinase (MlrA) was the first enzyme in the degradation pathway cleaving the cyclic peptide at the Arg-Adda bond (Figure 1.2). The linear product was further degraded by a serine hydrolase (MlrB) and the resulting tetrapeptide was reduced to the Adda side chain by a metallopeptidase (MlrC) (Figure 1.2).

Another *Sphingomonas* strain, strain B-9 from the Lake Tsukui in Japan, was found to possess the same MC degrading enzymes (Kato *et al.*, 2007). MC degrading bacteria were discovered in many other surface waters all over the world (Park *et al.*, 2001; Maruyama *et al.*, 2006; Ho *et al.*, 2007; Eleuterio and Batista, 2009; Mohamed and Alamri, 2012). However, in order to develop a safe and effective biological treatment system, it is essential to characterise

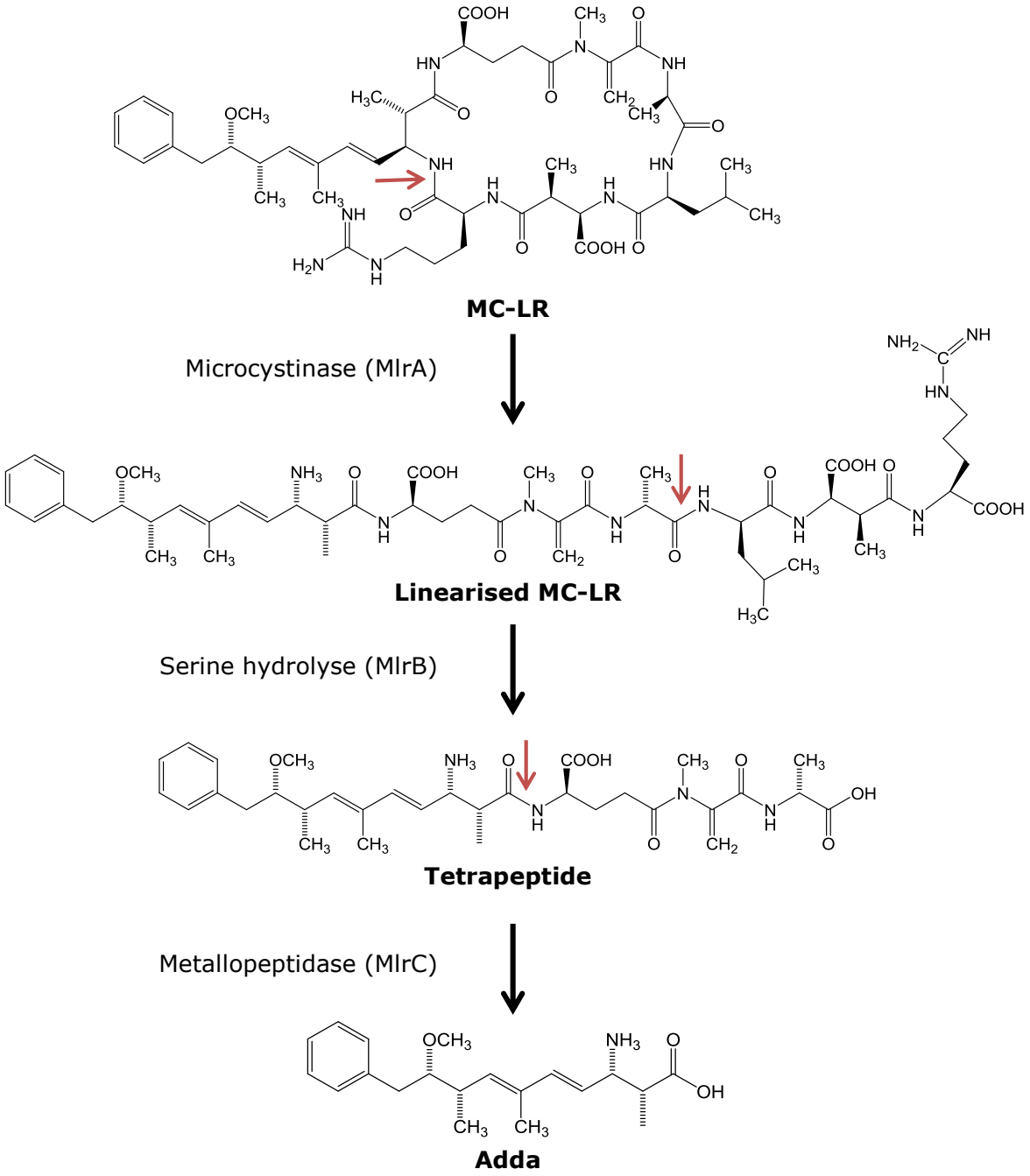


Figure 1.2 The degradation pathway of MC-LR in *Sphingomonas* sp. strain ACM-3962 proposed by Bourne *et al.* (1996). Bourne *et al.* (2001) later elucidated the corresponding *mlrABCD* gene cluster that encodes the hydrolytic MC-LR degrading enzymes. Red arrows indicate the sites of hydrolytic cleavage.

target bacteria, their mechanisms of degradation in association with gene expression and the toxicity of by-products. It is necessary to gain an extensive knowledge about the impact of ecological factors such as nutrients and temperature on bacterial MC degradation to predict the success of MC bioremediation under different environmental conditions.

The current study lays the groundwork for characterising novel bacterial isolates capable of degrading MCs. The following chapter will outline the origin of these isolates and their relevance among the community of MC degrading bacteria.

1.4 Novel Bacterial Isolates Capable of MC Biodegradation

In a study of 2008, Edwards *et al.* demonstrated the capacity of microbial consortia from six different Scottish water bodies to successfully degrade cyanobacterial toxins such as MC-LR. Since then, several bacteria with MC degrading activity have been isolated from the water samples using enrichment cultures. *Rhodococcus* sp. C1, amongst the biocatalytic bacteria, was reported to show the most promising MC-LR degradation activity (Manage *et al.*, 2009). In the current study, *Rhodococcus* sp. C1 and two yet unidentified isolates originating from the Scottish River Carron were selected for characterisation and further assessment of their MC-LR degradation ability. Unlike many other bacteria that have been studied for their MC degradation activity these isolates originate from waters without previous MC history. This suggests the ability to degrade MCs to be widespread among bacteria and not dependent on prior exposure (Edwards *et al.,* 2008). How efficient MC degrading bacteria from different habitats with different toxin histories remove

the harmful cyanobacterial metabolites is one of the main interests in this field of research.

Elucidation of the mechanisms involved in MC degradation, including genes, their regulation and the MC degrading enzymes they express is further important in understanding bacterial removal of MCs from the environment. Beside the growing number of isolates identified with MC biodegrading abilities, these mechanisms are unknown for most of the degraders (Dziga *et al.*, 2013). This also accounts for the *ß*-proteobacterial strain *P. toxinivorans* (DSMZ-16998) which was isolated from the sediment of a eutrophic lake in southern Finland and was shown to degrade MC variants –LR and –YR as well as NOD (Rapala *et al.*, 2005). Therefore, *P. toxinivorans* was included in the current study.

To further expand the range of microorganisms, biodegradation studies were also carried out on a bacterium of unknown origin which was found to rapidly degrade MC-LR during previous research on the River Carron isolates. Consequently, the current study was based on a total number of five bacteria that showed MC degrading activity.

2 Preliminary Work on *Rhodococcus* sp. C1

2.1 Introduction

Subsequent to identification and initial biodegradation studies on *Rhodococcus* sp. C1 (Manage *et al.*, 2009), Welgamage (2012) further investigated peptide degradation of the novel organism and revealed its ability to degrade a range of prokaryotic and eukaryotic peptides. These included amongst MC variants and NOD common antibiotics like gramicidin A, the immunosuppressant drug cyclosporine A and fibrinopeptide B of human origin. In the quest to understanding the universal peptide degradation of *Rhodococcus* sp. C1, the current project aimed to investigate the mechanisms involved in the degradation of the chemically and structurally diverse peptides.

Several members of the catabolically versatile genus *Rhodococcus* have been reported to degrade recalcitrant organic compounds (McLeod et *al.*, 2006). Amongst these, *Rhodococcus* sp. RHA1 has been described as a catabolic powerhouse and sequence analysis of its complete genome predicted the 9,145 protein-encoding genes to be exceptionally rich in aromatic compound degrading oxygenases and ligases.

If well characterised rhodococci such as *Rhodococcus* sp. RHA1 were to show a similar peptide degradation capacity as *Rhodococcus* sp. C1, they could be used as model organism to characterise the multiple peptide degradation mechanism. Therefore, MC-LR degradation activity was assessed in *Rhodococcus* sp. RHA1 with *Rhodococcus* sp. C1 as positive control organism. In addition, the *Rhodococcus* strains were compared in regard to their Gram stain, morphology and oxidase and catalase activity.

2.2 Materials and Methods

2.2.1 Chemicals

Chemicals were of analytical-reagent grade unless stated otherwise and obtained from Fisher Scientific, Leicestershire, UK. For PDA-LC-MS analysis, methanol and acetonitrile were purchased from Rathburn, Walkersburn, UK. Formic acid and trifluoroacetic acid (TFA) were acquired from Sigma Aldrich, Dorset, UK and Fisher Scientific, Leicestershire, UK, respectively. Water used for the studies was purified to 18.2 MΩ, through the ELGA laboratory water purification system (UK). MC-LR standards were purified from batch cultures of *Microcystis aeruginosa* PCC 7813.

2.2.2 High Performance Liquid Chromatography for the Detection of MC-LR

High Performance Liquid Chromatography (HPLC) analysis was performed using Waters Alliance 2695 solvent delivery system with photodiode array detector (Waters, Elstree, UK). Samples were separated on Symmetry C18 Column (2.1 mm i.d. x 150 mm; 5 µm particle size) maintained at 40°C. Eluent was monitored by UV absorption between 200 - 400 nm with detector resolution of 1.2 nm. The mobile phase constituted of ELGA water (A) and acetonitrile (B) both contained 0.05 % TFA. Samples were separated using a gradient increasing from 15 % to 65 % B over 25 min at a flow rate of 0.3 ml/min, followed by ramp up to 100 % B then re-equilibration at 15 % over the next 10 min. Data acquisition and processing was performed using Empower software (Version 2.0). MC-LR was quantified by external calibration using the range of 0.1 - 50 µg/ml.

2.2.3 MC-LR Calibration Curve

A linear calibration curve for MC-LR concentration (µg/ml) versus integrated intensity was obtained using the Waters Alliance HPLC system according to section 2.2.2. The calibration curve was generated by running samples with known MC-LR concentrations ranging from 0.1 to 50 µg/ml prepared from MC-LR dissolved in 80 % methanol. The curve was constructed by plotting the peak area at 238 nm (UV absorption maximum of MC-LR) to the respective MC-LR concentration forming the regression curve with corresponding correlation coefficient.

2.2.4 Culturing and Maintainance of the *Rhodococcus* Strains

Rhodococcus sp. C1 was isolated from the River Carron, Scotland, in 2007 and has since been cultured in River Carron water, LB broth and nutrient broth (NB). For long-term preservation it has been stored on Technical Service Consultants cryobeads immersed in a specially designed cryo-solution at – 80 °C. Experiments on *Rhodococcus* sp. C1 for the current study started in 2015 and were initiated by recovering the organism from a 2 year preservation period. Since then, *Rhodococcus* sp. C1 was routinely maintained on LB agar and grown in 10 ml LB broth at 25 °C, 150 rpm for 2 days prior to biodegradation studies. The cultivation temperature of 25 °C was chosen according to previous studies on *Rhodococcus* sp. C1 which showed a temperature of 25 °C to allow efficient cell growth (Manage *et al*., 2009; Lawton *et al.,* 2011; Welgamage, 2012).

Rhodococcus sp. RHA1 was kindly provided by the University of British Columbia, Vancouver, Canada. Like *Rhodococcus* sp. C1 it was routinely

maintained on LB agar and grown in 10 ml LB broth shaken at 150 rpm at 25 °C for 2 days prior to biodegradation studies.

2.2.5 Gram Staining and Testing for Catalase and Oxidase Activity

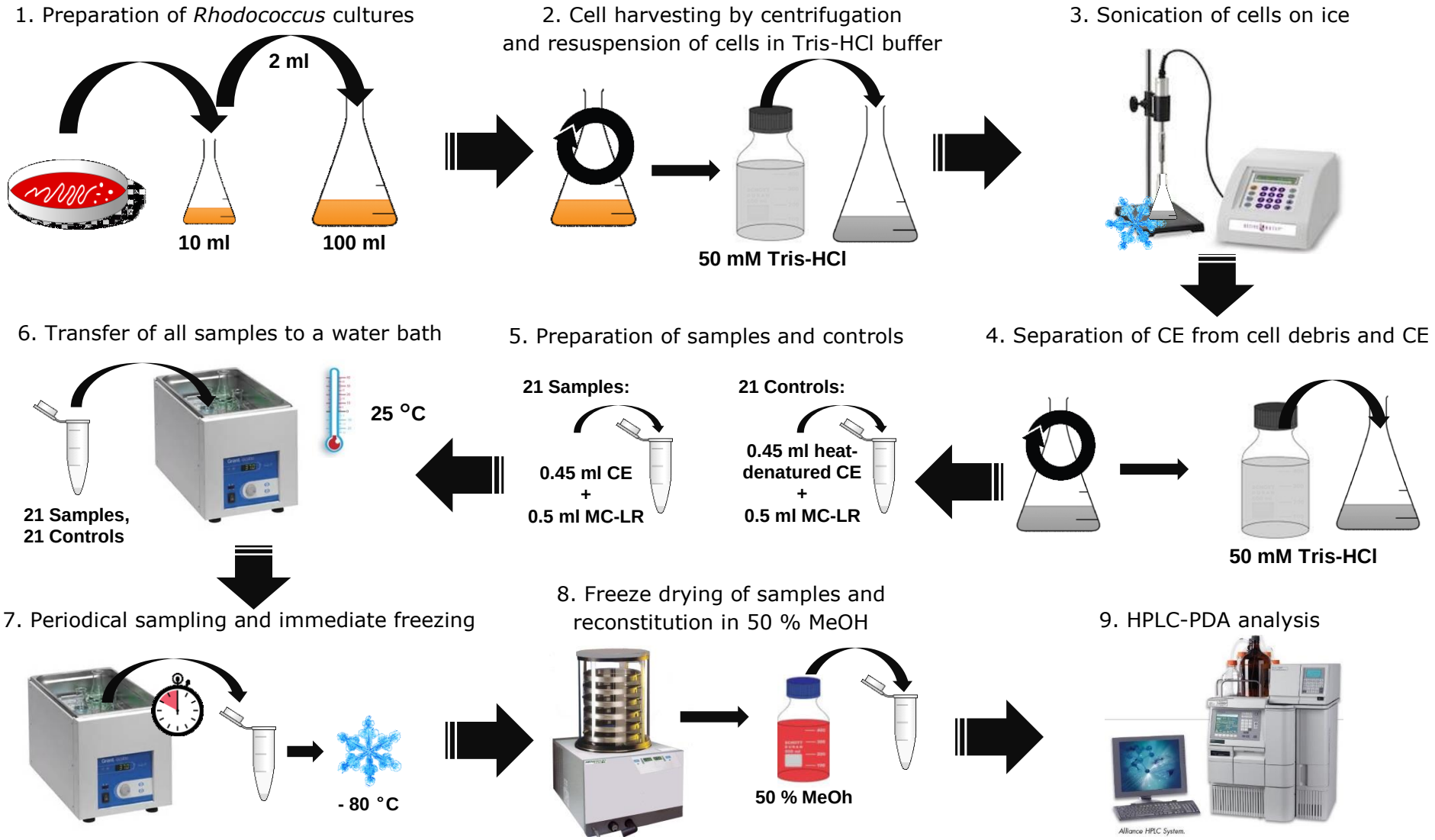
Gram staining. A colony of *Rhodococcus* sp. C1 and *Rhodococcus* sp. RHA1 was transferred to a film of sterile water on a glass slide. After evaporation of the water the bacterial cells were heat-fixed by passing the slide through a flame of a Bunsen burner. The staining process was initiated by covering the cells with crystal violet for 60 seconds. Excess of crystal violet was rinsed off with water and the stain was fixed with iodine for 45 seconds and decolourised with ethanol. The cells were then incubated with the second stain, safranin, for an additional 60 seconds before they were rinsed with water for the final time. When the slides had dried the bacterial smears were examined under oil immersion magnification at x1000 (Ex20 Series Biological Microscope) and captured photographically at x400 magnification (Olympus IX50 Inverted System Microscope connected to Lumenera's Infinity 2-2 digital CCD camera).

Catalase test. For each sample a drop of hydrogen peroxide (35 % concentration) was placed on a glass slide and bacterial colonies of *Rhodococcus* sp. C1 and *Rhodococcus* sp. RHA1, respectively, were mixed into the drop. A positive result was recorded when heavy bubbling occurred.

Oxidase test. Colonies of *Rhodococcus* sp. C1 and *Rhodococcus* sp. RHA1, respectively, were applied on the paper zone of a diagnostic strip (FLUKA Oxidase Strips). The results were read after one minute. A positive result was recorded when the colour of the paper zone turned blue.

2.2.6 Bacterial Cell Extract Based MC-LR Degradation Assay

The complete methodology is depicted in schemata 2.1. From a 10 ml culture of *Rhodococcus* sp. C1 and *Rhodococcus* sp. RHA1 (prepared according to section 2.2.4) 2 ml were transferred to 100 ml LB broth and incubated at 25 °C at 150 rpm for two nights. Cells were harvested by centrifugation (20 min, 3500 rpm, 4 °C; ALC 4237R Refrigerated Centrifuge), resuspended in 5 ml 50 mM Tris-hydrochloride (Tris-HCl) buffer (pH 7.6) and sonicated on ice for 28 cycles (5 s pulse, 10 s rest per cycle) with a MSE (UK) LTD Soniprep 150 (100 W, 23 kHz, max. amplitude, probe ∅ 3 mm). Cell debris were pelleted (10 min, 4000 rpm, 4 °C) and the cell extracts (CEs) were decanted, diluted with 50 mM Tris-HCl buffer (pH 7.6) to reach a volume of 25 ml and stored on ice. Enzyme assays were performed by using 0.45 ml CE spiked with 0.5 ml MC-LR to a final concentration of 10 µg/ml. All assay samples were prepared in 1.5 ml Eppendorf tubes, vortexed and incubated in a water bath at a temperature of 25 °C which was previously shown to allow successful MC-LR degradation (Manage *et al*., 2009; Lawton *et al.,* 2011; Welgamage, 2012). In triplicates, samples were removed periodically at 10 minute intervals for 60 minutes and immediately transferred to - 80 °C. A time frame of 60 minutes was chosen as previous CE based degradation studies on *Rhodococcus* sp. C1 showed that the MC degrading enzymes contained in the CE react rapidly with MC-LR (Welgamage, 2012). Samples were then freeze dried (Edwards Modulyo Freeze Dryer), reconstituted in 1 ml 50 % aqueous methanol, centrifuged (15 min, 13000 x g) and the supernatant was analysed using the Waters Alliance HPLC-PDA System according to section 2.2.2. Controls (triplicates) contained heat-denatured CEs (denatured in a 100 °C heating block for 10 min).



Schemata 2.1 Complete process of bacterial cell extract based MC-LR degradation assay carried out on *Rhodococcus* sp. C1 and RHA1.

2.3 Results

2.3.1 MC-LR Calibration Curve

By plotting the integrated intensity measured by HPLC-PDA at 238 nm to the respective MC-LR concentrations the calibration curve and the corresponding line function was obtained (Figure 2.1). The different MC-LR concentrations demonstrated a linear response of PDA detector. The highly linear regression was indicated by a correlation coefficient (R^2) of 1. By using the line function, the MC-LR concentration of biodegradation samples was estimated.

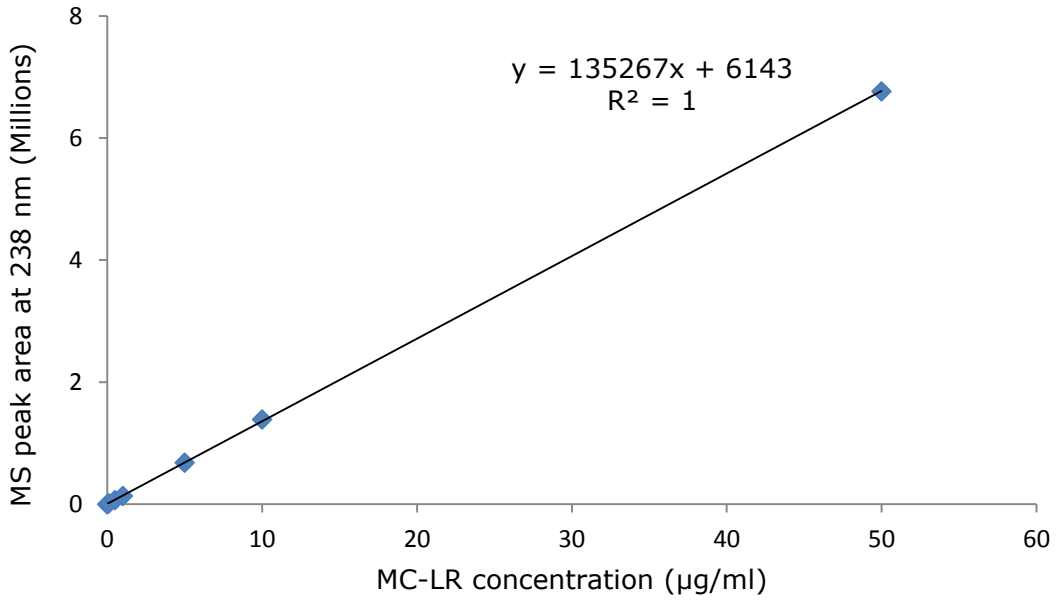
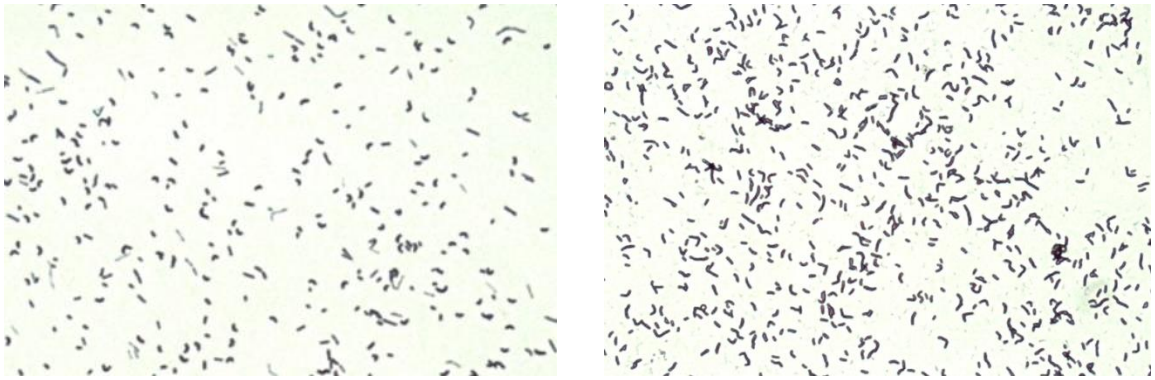


Figure 2.1 MC-LR calibration curve obtained from HPLC-PDA analysis at 238 nm.

2.3.2 Gram Stain and Catalase and Oxidase Activity of the *Rhodococcus* Strains

Gram staining of *Rhodococcus* sp. C1 showed a microscopic morphology consisting of Gram-positive short rods (Figure 2.2 (A)). Moreover, cells of this organism were catalase-positive and oxidase-negative.

Cells of *Rhodococcus* sp. RHA1 were also Gram-positive and oxidase-negative but catalase-negative. Similar to *Rhodococcus* sp. C1, *Rhodococcus* sp. RHA1 formed rod-shaped cells, however, slightly larger in comparison (Figure 2.2 (B)).



A **B**

Figure 2.2 Gram stained cells of (A) *Rhodococcus* sp. C1 and (B) *Rhodococcus* sp. RHA1 photographically captured at x400 magnification using the Olympus IX50 Inverted System Microscope connected to Lumenera's INFINITY2-2 digital CCD camera.

2.3.3 Bacterial Cell Extract Based MC-LR Degradation Assay

Incubation of CE of *Rhodococcus* sp. C1 and *Rhodococcus* sp. RHA1 with MC-LR did not result in removal of MC-LR (Figure 2.3). To confirm the outcome presented here, the biodegradation assay was repeated several times (data not shown) including efforts to increase the CE concentration and the conduction of a whole cell MC-LR biodegradation assay (methodology found in section 3.2.6). However, the former ability of *Rhodococcus* sp. C1 to degrade MC-LR remained unobserved.

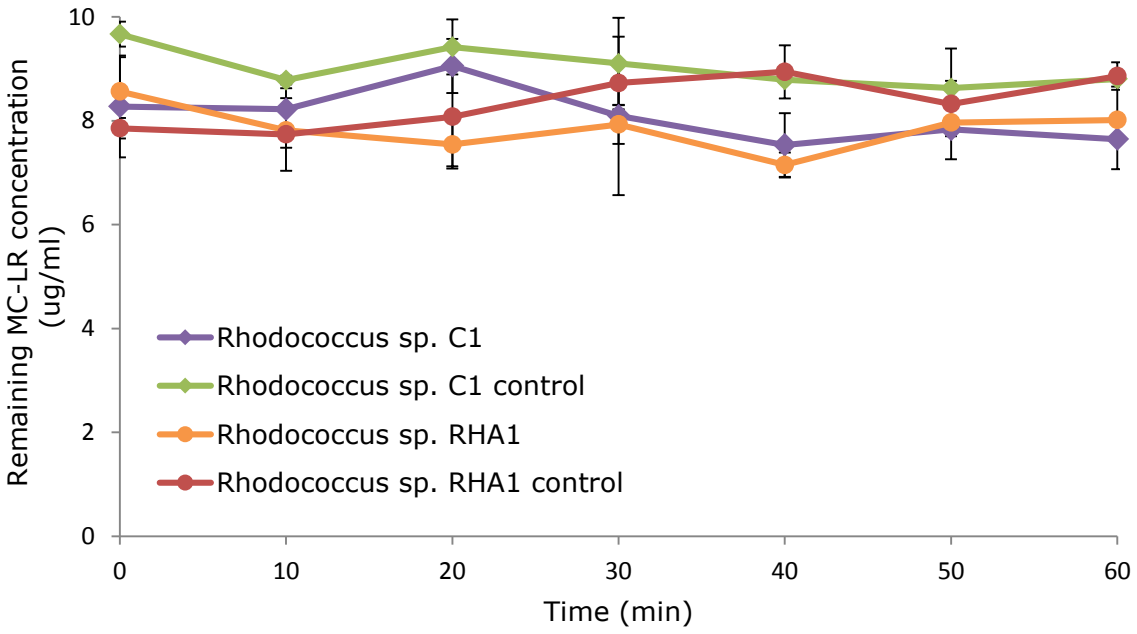


Figure 2.3 Remaining MC-LR concentrations monitored by HPLC-PDA (238 nm) in MC-LR degradation assay samples of CEs of *Rhodococcus* sp. C1 (◆) and *Rhodococcus* sp. RHA1 (●) incubated with the toxin at 25 °C over time. Control samples of *Rhodococcus* sp. C1 (◆) and *Rhodococcus* sp. RHA1 (●) contained heat-denatured CEs. Standard errors are displayed (n = 3).

2.4 Discussion

The study described above was conducted to unveil whether the ability to degrade cyanobacterial MC-LR was common among members of the catabolically versatile genus *Rhodococcus*. The metabolically diverse *Rhodococcus* sp. RHA1 has one of the largest bacterial genomes (9.7 mbp) sequenced up to date. It was chosen for the study as catabolic activity of the organism towards MC-LR would have provided advances in elucidating the enzymes and genes involved in MC-LR degradation by for example inhibiting known protease families possibly involved in MC degradation. However, despite its ability to catabolise a wide range of chemically stable compounds, enzymes from *Rhodococcus* sp. RHA1 were found catabolically inactive towards MC-LR.

Unlike *Rhodococcus* sp. C1, *Rhodococcus* sp. RHA1 is a soil bacterium isolated from agricultural contaminated ground. Its requirement and regulation of catabolic genes might differ largely from the freshwater bacterium *Rhodococcus* sp. C1.

Also *Rhodococcus* sp. C1 was incapable of removing MC-LR which was verified in repeated MC-LR biodegradation assays. These results were in contrast to previous findings of Welgamage (2012) where 95 % MC-LR removal was observed in 60 minutes. To exclude that a lack of bacterial cell extract based degradation activity was due to inefficient sonication or heat-denaturation of bacterial enzymes, cell viability and heat generation was evaluated over the length of sonication. The applicability of the 7 minute sonication treatment was confirmed as cell viability decreased by 95 % while the temperature remained far below a threshold value that would have caused enzyme denaturation (< 24 °C).

The inability of the organism to degrade the cyanobacterial toxin was assumed to be the result of frequent sub-culturing and long storage periods to which the organism was subjected over the past eight years since its isolation. Transfer of *Rhodococcus* sp. C1 from its original environment (River Carron water; nutrient poor, > 20 °C) to the laboratory environment (nutrient rich, 25 °C) will have affected its phenotype and the transfer most likely also induced changes in its genotype.

Several papers have reported the often undesirable effects of sub-culturing on the expression of genes involved in the biosynthesis of bacterial proteins. Koga *et al.* (1989) for example demonstrated a great decrease of the expression of a cell-surface protein antigen by *Streptococcus mutans* important for the organisms' binding to tooth surfaces after subculturing the strain 60 times.

Another work extensively reviewed studies on replicate *E. coli* populations that were passaged over 50,000 generations in a simple environment with only glucose for carbon and energy as part of the Lenski Long-Term Evolution Experiment (LTEE) (Cooper, 2014). Long-term passaging allowed examination of potential natural selection and mutational erosion of unused traits in the LTEE *E. coli* populations. A study testing cellular respiration across a range of different environments over the first 2,000 generations observed specialisation in the use of glucose by the *E. coli* lines and few losses in substrate respiration (Flynn *et al*., 2013). However, between 2,000 and 20,000 generations, fitness losses in foreign conditions were obvious and over 20,000 generations, LTEE *E. coli* lines performed worst in multiple environments (Cooper *et al*., 2001; Cooper, 2002). These observations supported the occurrence of antagonistic

pleitropy, a process of natural selection favouring traits that simultaneously enhance fitness in one environment but compromise fitness elsewhere.

It is difficult to estimate the number of generations experienced by *Rhodococcus* sp. C1. However, the findings of Cooper *et al.* (2001) support that long-term passaging of *Rhodococcus* sp. C1 caused a genetic drift leading to the loss of the organism's MC-LR degradation ability.

Also other papers reported genetic drifts in microbial cultures under laboratory conditions and stressed the importance for appropriate protocols to process and maintain bacterial strains of functional interest (Katouli *et al.*, 1990; Cross *et al.,* 2011).

Although unlikely, it has to be taken into consideration that a lack of MC-LR degradation activity could have been due to a contamination of the *Rhodococcus* sp. C1 culture that was introduced during sub-culturing of the organism. Contaminations occur as a result of inadequate sterile techniques and unless a bacterial culture is phylogenetically profiled or tested for a known characteristic (such as the production of specific toxins in case of cyanobacterial species) a contamination cannot be fully excluded.

Moreover, *Rhodococcus* sp. C1 was identified on the genus level and it might have been the case that the culture of *Rhodococcus* sp. C1 contained clonal strains incapable of MC-LR degradation. Since however cells of *Rhodococcus* sp. C1 successfully degraded MC-LR in several biodegradation assays over a period of at least four years (Manage *et al.*, 2009; Lawton *et al.* 2011; Welgamage, 2012) the possibility of a non-active clonal strain causing the lack of MC-LR degradation activity was assumed to be low.

If *Rhodococcus* sp. C1 had been still active towards MC-LR it would have been of interest to also test the supernatant of the bacterial cells towards MC-LR

degradation activity. This would have provided conclusions whether cellular uptake of MC-LR prior to intracellular breakdown is essential for degradation by *Rhodococcus* sp. C1 or whether exoproteases (contained in the supernatant) degrade MC-LR outside the cell.

3 MC-LR Degradation Studies on Bacterial Isolates of Different Origin

3.1 Introduction

The MC-LR degradation activity previously demonstrated by Manage *et al.* (2009) and Welgamage (2012) was no longer observed in biodegradation studies on *Rhodococcus* sp. C1. Therefore, the study was re-focused towards two other MC degrading bacteria isolated in the course of earlier work on the biodegrading water samples and yet another MC degrading organism of unknown origin. Since isolates were of unknown identity, phylogenetic analysis based on 16S rRNA sequence similarity were carried out prior to further investigations. In addition, the bacterial strains were assessed in regard to their Gram stain, morphology and oxidase and catalase activity.
Subsequently, a whole cell based MC-LR degradation assay was carried out on the phylogenetically profiled isolates. Included in the assay was the long-term (since 2007) laboratory maintained *P. toxinivorans* strain that was successfully used as positive control organism for MC-LR degradation in previous studies by Manage *et al.* (2009) and Welgamage (2012).
When isolates failed to remove MC-LR in the degradation assay they were exposed to MC-LR for 21 days in an approach to re-induce their MC-LR degradation activity.

3.2 Materials and Methods

3.2.1 Chemicals

See section 2.2.1.

3.2.2 Ultra Performance Liquid Chromatography for the Detection of MC-LR

Ultra Performance Liquid Chromatography (UPLC) analysis was conducted using the ACQUITY UPLC system with photodiode array (ACQUITY UPLC PDA) and Quadruple time of flight (Xevo QToF) mass spectrometer (Waters, Elstree, UK). For all the analysis, the Waters ACQUITY UPLC BEH C18 column (2.1 mm x 100 mm in size; 1.7 µm particle size) was maintained at 40 °C. The mobile phase was composed of ELGA water (A) and acetonitrile (B) both provided with 0.1 % formic acid. At a constant flow rate of 0.2 ml/min acetonitrile was increased from 20 % to 70 % over 10 min and kept at 100 % for an additional two minutes. Re-equilibration of the column to starting conditions was set for a period of 2.5 min. MC-LR contained in the eluent was analysed by ultra violet (UV) absorption from 200 to 400 nm with a resolution of 1.2 nm. For mass spectrometry (positive ion electro-spray mode), the eluent was then scanned from *m/z* 100 to 1200 Da for 2 s followed by a 0.1 s delay. Ion source parameters and sprayer voltage and cone voltage at 3.07 kV and 80 V, respectively, were applied together with a source temperature of 100 °C and desolvation temperature of 300 °C.

3.2.3 Culturing and Maintainance of the Bacterial Strains

P. toxinivorans (2007) obtained by the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ) in 2007, the isolates from the River Carron (later identified as *Rhodococcus* sp. and *Arthrobacter* sp.) and the isolate of unknown origin (later identified as *Pseudomonas* sp.) were preserved at – 80 °C on Technical Service Consultants cryobeads immersed in

a specially designed cryo-solution. The bacteria were recovered and routinely maintained on LB agar plates.

Prior to degradation studies new agar plates were inoculated with the respective organisms and incubated over 2 nights at 25 °C. A single colony was then transferred to 10 ml liquid LB media and incubated at 25 °C, 150 rpm until culture turbidity indicated bacterial growth (20 to 44 hours).

3.2.4 Gram Staining and Testing for Catalase and Oxidase Activity

Colonies of the two isolates from the River Carron and the isolate of unknown origin were Gram stained and tested for their catalase and oxidase activity according to section 2.2.5.

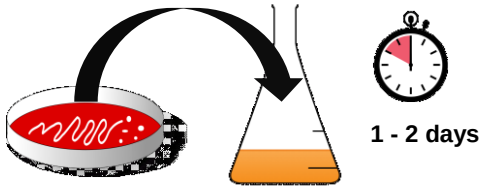
3.2.5 16S rRNA Based Profiling

The complete methodology is depicted in schemata 3.1.

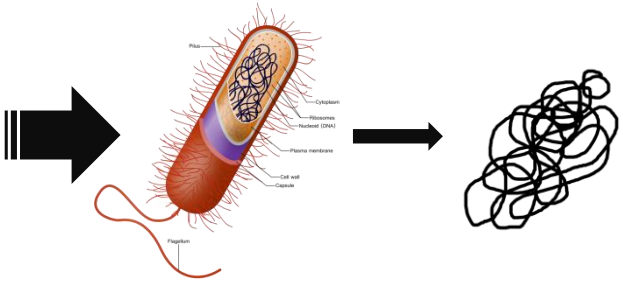
Preparation of cultures. Cultures of the two isolates from the River Carron and the isolate of unknown origin were prepared according to section 3.2.3.

DNA extraction. A 1 ml culture aliquot of each isolate was pipetted into sterile Eppendorf tubes and centrifuged at 13500 rpm for 5 minutes with a Sigma 1-15 Microfuge. Cell pellets were resuspended in 100 µl 0.1 M Tris-EDTA (TE) buffer supplemented with 10 mg/ml lysozyme and incubated at 37 °C for 45 minutes. After addition of 1 µl protinease K and 1 µl 10 % SDS the cells were incubated at 37 °C for additional 30 minutes. 30 % Chelex (100 µl) were mixed with the cells and the tubes were transferred to a 56 °C heating block for 30 minutes.

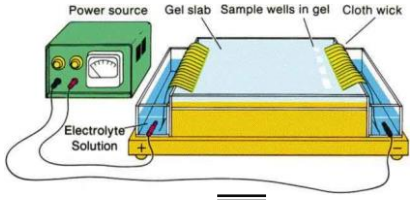
1. Preparation of cultures



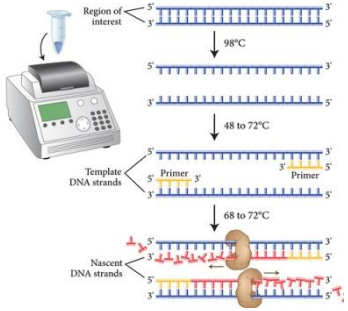
2. DNA extraction



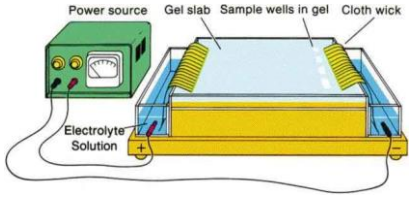
3. Gel electrophoresis of extracted DNA



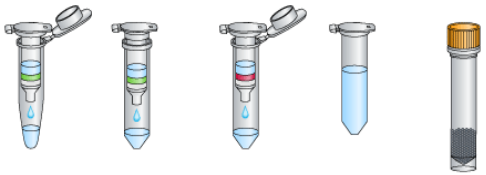
4. 16S rRNA specific PCR amplification



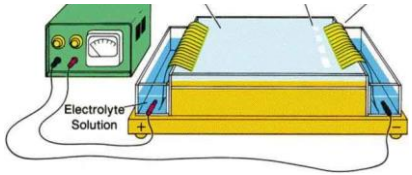
5. Gel electrophoresis of PCR products



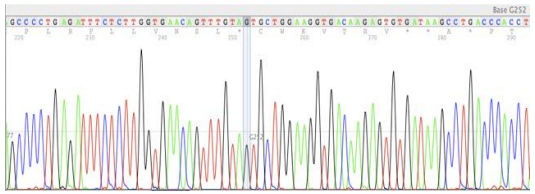
6. PCR product purification



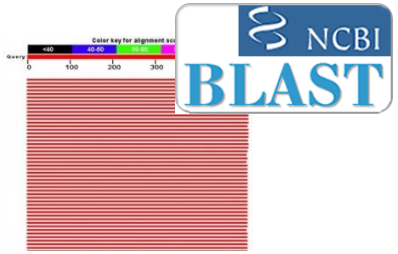
7. Gel electrophoresis of purified PCR products



8. Sequencing of PCR products (by MRC PPU DNA and Sequencing Services, Dundee)



9. Sequence analysis using BLAST



Schemata 3.1 Procedure for the 16S rRNA profiling of isolates previously shown MC-LR degradation activity.

Subsequently, the tubes were kept in 100 °C boiling water for 10 minutes as this was found to be essential for lysing the majority of the bacterial cells and for obtaining good quality DNA. Successful DNA extraction was confirmed by gel electrophoresis on a 1.5 % agarose gel supplemented with 2 % GelRed™ (Nucleic Acid Gel Stain, 10000 x in water by BIOTIUM). The gel was immersed in 1 x Tris/Borate/EDTA (TBE) buffer. Thereafter, 8 µl of DNA sample was mixed with 2 µl 5 x DNA Loading Buffer, Blue (BIOLINE) and loaded onto the gel together with 4 µl HyperLadder 1 kb, 100 lanes (BIOLINE). The gel was documented using the UVItec Gel Documentation System (UVITEC, Cambridge, UK).

16S rRNA specific PCR amplification. PCR was performed for each isolate to be identified using 45 µl PCR mastermix (containing a 16S rRNA primer set; Table 3.1), 5 µl extracted DNA and 1 µl GoTaq Flexi DNA polymerase (Promega). Including a negative control lacking bacterial DNA PCR amplification was performed with a Techne® Endurance TC-412 Thermal Cycler according to the program described in Table 3.2. Successful 16S rRNA specific PCR amplification was confirmed by gel electrophoresis in the same manner as described in the previous section.

Table 3.1 Specifics on preparation of the PCR mastermix.

Reagent	Amount per sample	Amount for twelve samples
5 x Colorless GoTaq Flexi buffer (Promega)	10 µl	120 µl
25 mM Magnesium Chloride (Promega)	3 µl	36 µl
10 mM dNTP Mix (Promega)	1 µl	12 µl
16S Forward (Sigma, Tm = 62.3 °C, 5'-GCTCAGATTGAACGCTGG, 10 µM)	1 µl	12 µl
16S Reverse (Sigma, Tm = 60.6 °C, 5'-TACTGCTGCCTCCCGTA, 10 µM)	1 µl	12 µl
0.22 µm filtered ELGA water	34 µl	408 µl

Table 3.2 PCR program of the 16S rRNA specific gene amplification.

Condition	Temperature	Duration	Cycle number
Denaturation	94 °C	5 min	1
	94 °C	1 min	30
Annealing	63 °C	1 min	
Extension	72 °C	2 min	
Final extension	72 °C	5 min	1

PCR product purification. For the purification of the 16S rRNA specific PCR products a QIAquick PCR Purification Kit (QIAGEN) was utilised according to the manufacturer's protocol and its success was confirmed by gel electrophoresis as described before.

Sequencing and sequence analysis. The 16S rRNA specific PCR products of the different isolates were sequenced by MRC PPU DNA and Sequencing Services (Dundee, Scotland). The identity of the isolates was determined by Basic Local Alignment Search Tool (BLAST) using the 16S ribosomal RNA sequences (Bacteria and Archaea) database of the National Center for Biotechnology Information (NCBI).

3.2.6 Whole Cell MC-LR Degradation Assay

Six times 100 µl cultures of *Arthrobacter* sp., *Rhodococcus* sp., *Pseudomonas* sp. and *P. toxinivorans* (2007) (prepared according to section 3.2.3) were separately transferred to 5 ml filter-sterilised (Millipore, Stericup 500 ml Duropore 0.22 µm PVDF) River Carron water contained in universal glass bottles. The cultures were spiked with filter-sterilised (IWAKI Disposable Sterile Syringe Filter, 3 mm, 0.20 Micron) MC-LR to a final concentration of 10 µg/ml. Controls contained sterile River Carron water with MC-LR which were monitored along with the sample bottles to confirm that degradation was due to microbial activity alone. All bottles were incubated at 150 rpm at 25 °C for

10 days. (Personal observations on other isolates showed degradation optima to be at 25 °C.) A prolonged time period of 10 days (in contrast to 3 days used in initial whole cell biodegradation studies; Manage *et al.*, 2009) was chosen as previous investigations showed that MC-LR degradation time for the in 2008 isolated bacteria was significantly slower (50 % MC-LR degradation after 9 days; Lawton *et al.*, 2011) than when first reported (> 90 % MC-LR degradation after 3 days; Manage *et al.*, 2009).

Three sample bottles of each organism and the control were frozen (– 80 °C) immediately (Day 0). The remaining three samples of each organism and the control were frozen after their 10 day incubation period. Samples were freeze dried (Edwards Modulyo Freeze Dryer), reconstituted in 1 ml 50 % aqueous methanol, centrifuged (15 min, 14000 x g) and 100 µl of the supernatant were analysed by Waters Aquity UPLC-PDA-MS System according to section 3.2.2.

3.2.7 21 Day MC-LR Induction of Bacterial Isolates

Cultures of *Rhodococcus* sp., *Arthrobacter* sp. and *Pseudomonas* (prepared according to section 3.2.3) were pelleted (20 min, 4000 x g; Thermo Scientific™ Heraeus™ Megafuge™ 40 Centrifuge), washed three times with 0.01 M phosphate buffered saline (PBS) and kept in 0.01 M PBS overnight (25 °C, 150 rpm) for carbon depletion. The bacterial suspensions were adjusted to a turbidity of A_{590} = 0.24 and 0.5 ml was added to glass universal bottles (triplicates) containing 0.22 µm filter sterilised River Carron water spiked with 0.5 ml MC-LR to reach a final concentration of 10 µg/ml. Controls contained sterile river Carron water plus MC-LR without bacteria. The sample bottles were incubated for 21 days at 25 °C and shaken at 150 rpm. The time period was chosen according to initial MC-LR degradation studies on River Carron

water samples containing the MC-LR degrading bacteria which revealed a lag phase of 7 to 11 days prior to successful MC-LR degradation which resulted in ~ 80 % MC-LR removal after 19 days (Edwards *et al.*, 2008). To maintain a high MC-LR concentration samples were re-spiked with 0.5 ml of the same MC-LR stock at day 7 and 14. Aliquots of 0.5 ml were removed into Eppendorf tubes periodically at seven day intervals for 21 days and immediately frozen (– 80 °C). On day 7 and 14 aliquots were taken before and after the re-spike. The samples were processed for UPLC-PDA-MS analysis as described above (section 3.2.6).

3.3 Results

3.3.1 Gram Stain and Catalase and Oxidase activity

Cells of the two isolates from the River Carron were Gram-positive, catalase-positive, oxidase-negative and rod-shaped.

Cells of the isolate of unknown origin were also catalase-positive and rod-shaped but Gram-negative and oxidase-positive.

3.3.2 16S rRNA Based Profiling

Gel electrophoresis confirmed the success of DNA extraction (Figure 3.1 (A)), 16S rRNA primer specific PCR amplification (Figure 3.1 (B)) and PCR product purification (Figure 3.1 (C)).

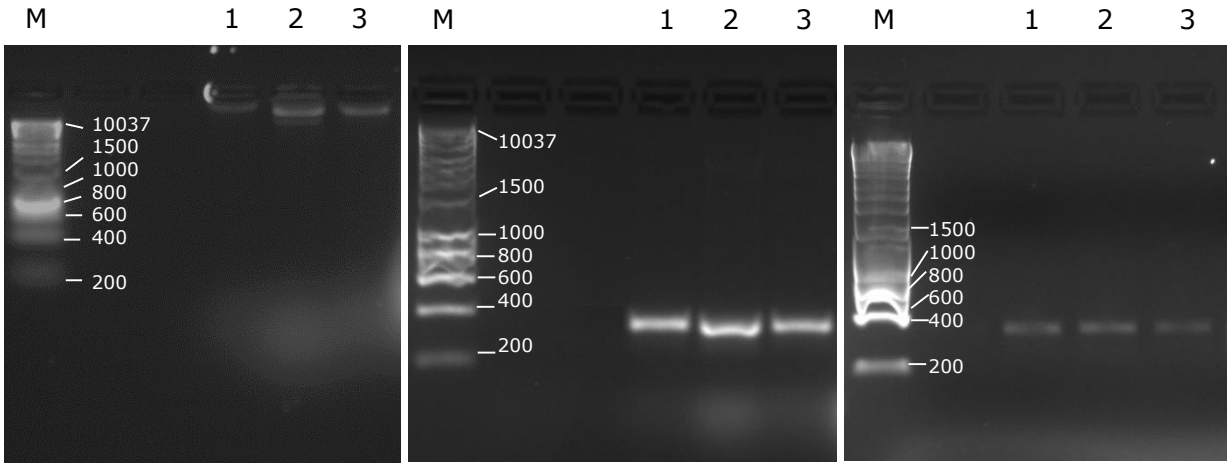


Figure 3.1 Gel electrophoresis of (A) extracted DNA, (B) 16S rRNA primer specific PCR products and (C) purified 16S rRNA primer specific PCR products of River Carron bacterial isolates (1, 2) and the isolate of unknown origin (3). DNA and PCR samples were run on a 1.5 % agarose gel for 60 min at 80 volt together with a marker (M) (HyperLadder 1 kb 100 lanes by Bioline).

The phylogenetic profiles were obtained from matches of the partial 16S rRNA sequences to sequences of the 16S ribosomal RNA sequences (Bacteria and Archaea) database which were ranked according to sequence identity, query cover and similarity score (describes the overall quality of sequence alignment which is the better the higher the score). Based on the top ten sequence identity matches the two isolates from the River Carron were classified as *Arthrobacter* sp. (sequence identities: 96 - 99 %, query covers: 98 %, similarity scores: 486 - 540) and *Rhodococcus* sp. (sequence identities: 96 - 98 %, query covers: 87 - 99 %, similarity scores: 494 - 562), respectively. The organism of unknown origin was identified as *Pseudomonas* sp. (sequence identities: 99 - 100 %, query covers: 75 %, similarity scores: 1196 - 1207).

3.3.3 Whole Cell MC-LR Degradation Assay

Despite incubation of MC-LR with the isolates previously proven to rapidly degrade MC-LR, no removal of the toxin was observed in any of the assay samples. The initial MC-LR concentration (Day 0) in all samples was 8.7 µg/ml (Figure 3.2). The negative control - sterile MC-LR spiked Carron water - showed a remaining MC-LR concentration of 8.3 µg/ml after 10 days of incubation confirming that MC-LR was not removed by the impact of physico-chemical factors (Figure 3.2). In all other samples inoculated with different bacterial cultures remaining MC-LR concentration was also found to be around 8.3 µg/ml with maximal values of 8.4 µg/ml and minimal values of 8.0 µg/ml (Figure 3.2). From these findings it was concluded that MC-LR biodegradation did not occur demonstrating the inability of the different isolates to degrade MC-LR under the applied conditions.

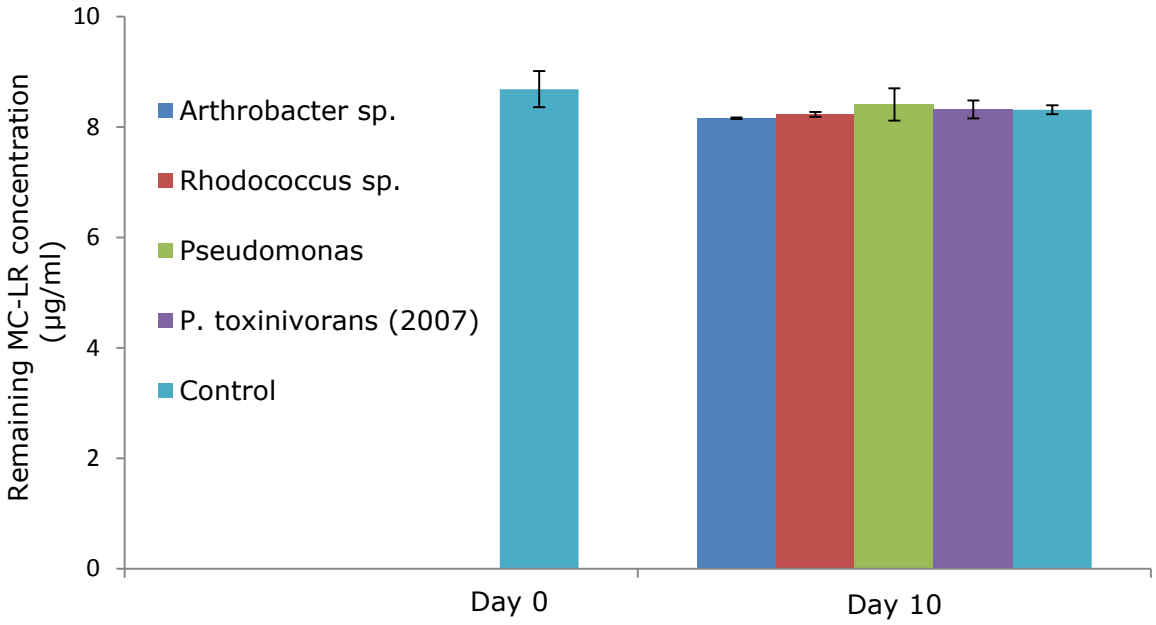


Figure 3.2 Remaining MC-LR concentrations monitored by UPLC-PDA (238 nm) after 10 days of incubation of toxin spiked River Carron water with the different isolates. Control samples contained toxin spiked River Carron water without bacteria. Standard errors are displayed (n = 3).

3.3.4 21 day MC-LR Induction of Bacterial Isolates

Incubation of *Rhodococcus* sp., *Arthrobacter* sp. and *Pseudomonas* sp. with increasing concentrations of MC-LR in a prolonged degradation assay did not induce the bacterial biodegradation activity. This was evident from the steady concentration of MC-LR in-between the seven day intervals over which the toxin concentration was increased from ~ 8 µg/ml (day 0) to ~ 17 µg/ml (day 7) to ~ 28 µg/ml (day 21) (Figure 3.3).

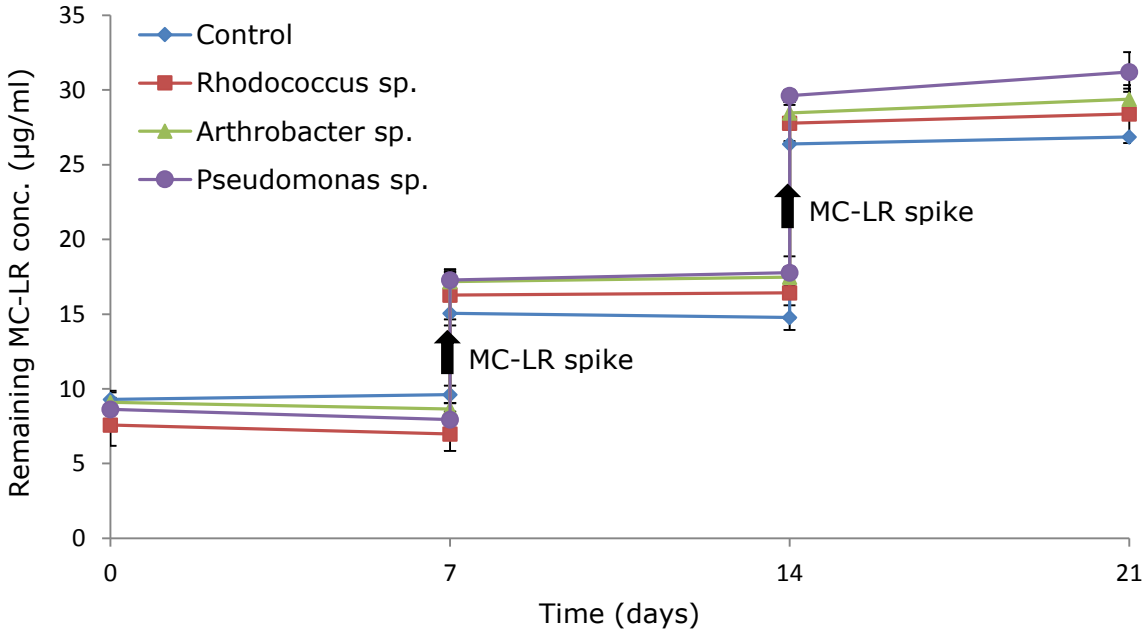


Figure 3.3 Remaining MC-LR concentrations over 21 day MC-LR induction of *Rhodococcus* sp., *Arthrobacter* sp. and *Pseudomonas* sp.. Bacterial cultures were re-spiked with MC-LR on day 7 and 14. Sterile River Carron water plus MC-LR served as control. Standard errors are displayed (n = 3).

3.4 Discussion

Phylogenetic analysis based on 16S rRNA sequence similarity identified the two organisms isolated from the River Carron as *Arthrobacter* sp. and *Rhodococcus* sp.. The two genera belong to the phylum *Actinobacteria*. *Actinobacteria* represent Gram-positive bacteria with diverse metabolic and physiological properties that include primary antibiotic producing species used in the pharmaceutical industry like *Streptomyces* sp. as well as pathogens such as *Mycobacterium* spp. (Ventura *et al.*, 2007). Many actinobacterial strains have been found to recycle refractory compounds by decomposition including hydrocarbons and soil herbicides (Prince *et al.*, 2010; Veselá *et al.*, 2010).

The organism previously found to degrade MC-LR was subjected to 16S rRNA analysis and was classified as *Pseudomonas* sp.. Like *Sphingomonas* sp. and *P. toxinivorans, Pseudomonas* sp. belongs to the phylum *Proteobacteria* which also comprises many organisms capable of biodegrading a range of natural and man-made compounds (Parales, 2010). A MC-LR degrading *Pseudomonas* sp. was reported before by Takenaka and Watanabe (1997). The strain was isolated from surface water of a Japanese lake and identified as *Pseudomonas aeruginosa.*

In this study *Arthrobacter* sp., *Rhodococcus* sp., *Pseudomonas* sp. and *P. toxinivorans* (2007) were found to be unable to degrade MC-LR, although former studies (following work published by Edwards *et al.* (2008)) had demonstrated their MC-LR degradation ability. Also long-term culturing (21 days) of the bacteria in the presence of increasing concentrations of MC-LR as dominant carbon source did not induce the bacterial MC-LR degradation

activity. The findings suggested that like *Rhodococcus* sp. C1 the organisms lost their ability to degrade MC-LR in the course of repeated sub-culturing and long storage periods.

As discussed in the previous chapter a number of studies investigated the occurrence of functional drifts in bacterial cultures subjected to long-term sub-culturing. Several demonstrated the influence of sub-culturing on functional diversification of nutrient utilisation. Scortichini and Rossi (1995) isolated phytopathogenic *Pseudomonas* spp. and compared their capability of carbohydrate utilisation with that shown by the same isolates when they were sub-cultured for one week and one month on nutrient agar and King's medium B (a *Pseudomonas* isolation agar). The authors reported a different carbohydrate utilisation for all isolates sub-cultured on laboratory culture media in comparison to freshly isolated bacteria. Bacterial culture media, very different in chemical composition to the plant tissue from which the isolates originated, simultaneously induced the capability to utilise different carbohydrates and disabled the utilisation of others.

Alterations of genes or gene expression in response to changing nutrients or nutrient concentrations have also largely been studied in biofilm forming bacteria (Stewart and Franklin, 2008). Cells within biofilms have to adapt to local chemical conditions. Consequently, variant sub-populations from a homogenous inoculum emerge within a few days and can constitute 10 % or more of a biofilm population (Boles *et al.*, 2004; Kirisits *et al.*, 2005). These variants arise through mutation and genetic rearrangements as well as through regulation of gene expression (Stewart and Franklin, 2008). Stover *et al.* (2000) reported proportions of regulatory genes in some bacterial genomes to be as much as 10 %.

It is likely that the bacterial isolates assessed in this study adapted to nutrient rich media routinely used for their maintenance through genetic or regulatory variations that simultaneously impeded their capability to degrade MC-LR.

4 A Simple and Rapid 96-Well Plate Based Method to Test the Effect of Different Growth Media on MC-LR Biodegradation and Partial Elucidation of the MC-LR Degradation Pathway in *P. toxinivorans*

4.1 Introduction

Adaptations to fluctuating carbon source availability are of particular importance for bacterial survival. Adjustments to a change of carbon source involves enzymatic and genetic regulations that are up to date only understood for a few metabolic regulatory systems such as the adaptation to lactose in *E. coli* (Ozbudak *et al.*, 2004).

This study observed a loss of activity in MC-LR degradation in a number of phylogenetically different bacteria that had been repeatedly sub-cultured and long-term cryopreserved since their MC degradation activity was demonstrated in initial degradation studies. It was proposed that the impediment of MC-LR degradation was the result of bacterial adaptation to rich culture media. Nutrients are readily available in carbon rich media such as LB and enzymes involved in MC-LR degradation may no longer have been essential for the survival of the organisms.

To test whether nutrient availability affects MC-LR degradation activity of the metabolically altered bacteria MC-LR degradation was examined in different growth media. For this purpose, a simple and rapid 96-well plate method was developed which tests the effect of five media differing in carbon composition and concentration on bacterial MC-LR degradation. A newly obtained strain of *P. toxinivorans* (2015) was included in the nutrient assay. Its availability allowed direct comparison to the long-term sub-cultured and preserved *P. toxinivorans* (2007).

4.2 Materials and Methods

4.2.1 Chemicals

See section 2.2.1.

4.2.2 Ultra Performance Liquid Chromatography for the Detection of MC-LR

See section 3.2.2.

4.2.3 Culturing of the Bacterial Strains

A new culture of *P. toxinivorans,* from here on called *P. toxinivorans* (2015), was purchased by DSMZ. The freeze-dried organism was revived according to the protocol provided with the culture. *Rhodococcus* sp., *Pseudomonas* sp. and both existing and newly obtained *P. toxinivorans* strains were streaked onto LB and R2A agar plates and incubated at 29 °C for 40 hours. The temperature of 29 °C was chosen according to the optimal growth temperature of the new *P. toxinivorans* strain and used for all organisms to allow growth comparison (and later on comparison of MC-LR degradation activity).

4.2.4 Gram Staining and Testing for Catalase and Oxidase Activity

Colonies of both *P. toxinivorans* strains were Gram stained and tested for their catalase and oxidase activity according to section 2.2.5.

4.2.5 Testing the Effect of Growth Media on Bacterial MC-LR Biodegradation Activity

The complete methodology is depicted in Schemata 4.1. Cells of each organism grown on R2A agar were transferred to 3 ml PBS and adjusted to a turbidity of A_{595} = 0.37 ± 0.01. Under sterile conditions 140 µl of 100 % and 10 % mineral salt medium (MSM; composed according to Yang *et al.,* 2014), River Carron water, R2A broth, NB and LB broth (diluted beforehand by addition of Milli-Q water) were transferred to 96-well plates (Thermo Scientific™ Nunc™ MicroWell™ 96-Well Microplates) (Figure 4.1). (See appendix for composition of media (Table 9.1)). The different growth media were spiked with 5 µl MC-LR to reach a final concentration of 10 µg/ml and inoculated with 5 µl OD adjusted cultures of *Rhodococcus* sp., *Pseudomonas* sp. and the *P. toxinivorans* strains, respectively (Figure 4.1). Controls contained 150 µl of the different media (100 % concentration) spiked with MC-LR but lacking bacteria to confirm loss of MC-LR to be due to bacterial degradation (Figure 4.1). The plates were covered with the provided lids, placed in a closed sandwich box layered with wet paper towels to prevent the samples from evaporating and incubated at 29 °C, 150 rpm for 7 days. After the incubation period 150 µl of 50 % methanol supplemented with 2 % formic acid were added to each sample contained in the 96-well plates to precipitate proteins and kill the bacterial cells. The 96-well plates were centrifuged on a microplate carrier (15 min, 2272 x g) using the Thermo Scientific™ Heraeus™ Megafuge™ 40 Centrifuge and 100 µl of each supernatant were analysed in high recovery vials (Waters LCGC Certified Clear Glass 12 x 32mm Screw Neck Max Recovery Vials) by Waters Aquity UPLC-PDA-MS System according to section 3.2.2.

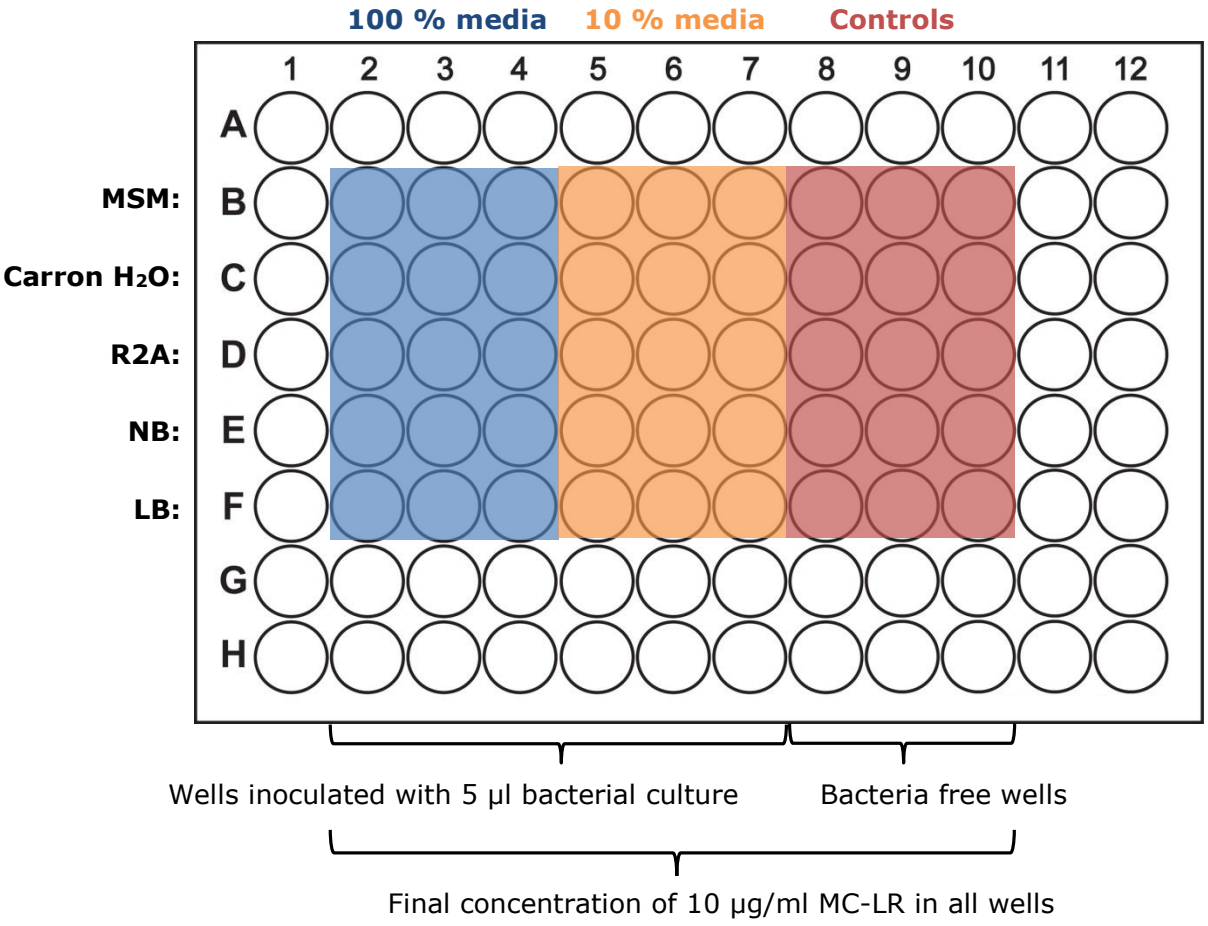
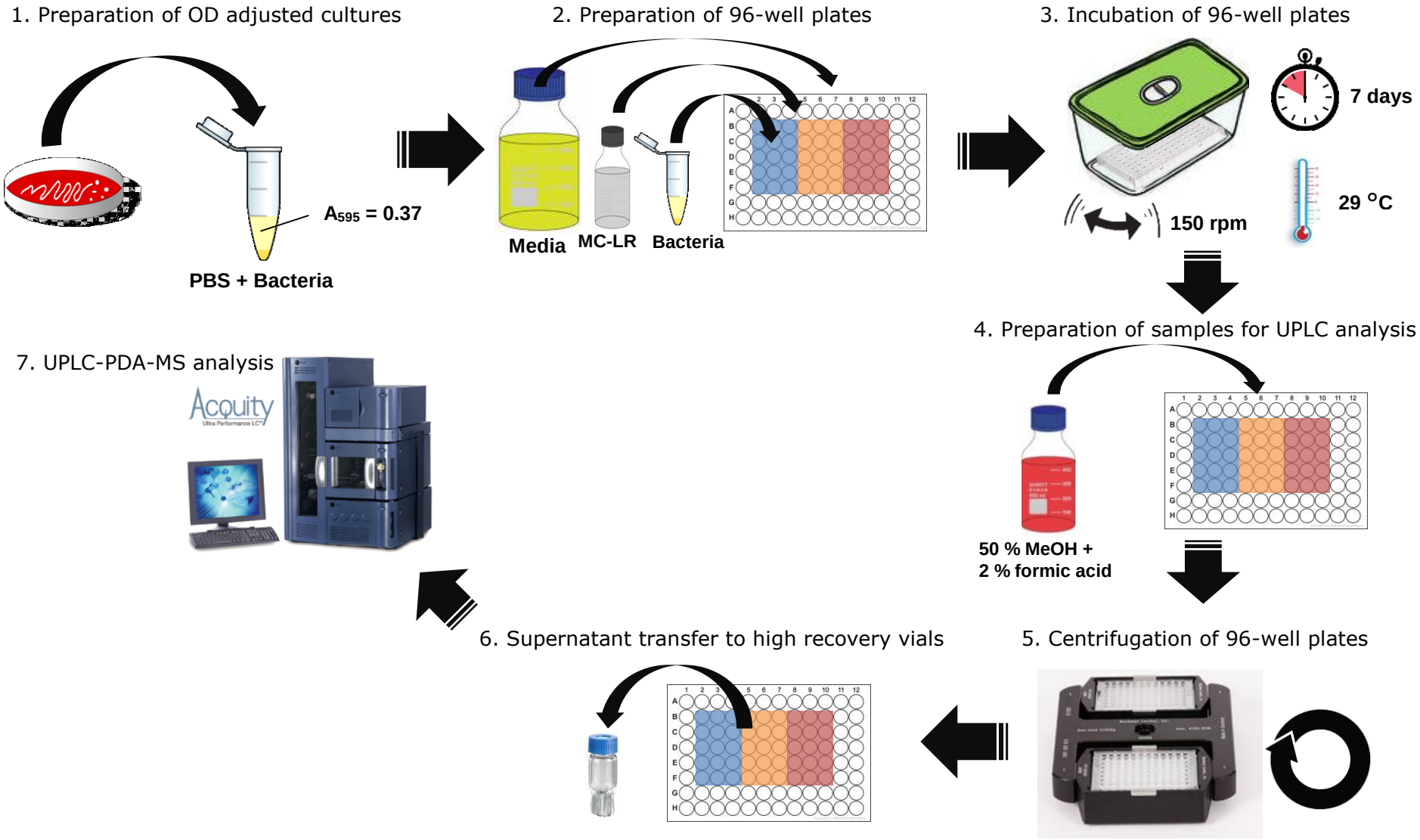


Figure 4.1 Composition of the MC-LR degradation assay carried out in four 96 well plates each containing a different culture: (1) *P. toxinivorans* (2007), (2) *P. toxinivorans* (2015) (3) *Rhodococcus* sp. and (4) *Pseudomonas* sp..



Schemata 4.1 96-well plate based method testing the effect of different growth media on bacterial MC-LR biodegradation.

4.2.6 Detection of *mlrABCD* Genes Involved in MC-LR Degradation

DNA of *P. toxinivorans* (2015) was extracted under conditions documented in section 3.2.5. The DNA and specific oligonucleotide primer sets (Saito *et al.*, 2003; Ho *et al.,* 2007; Table 4.1) were then used in PCR to screen *P. toxinivorans* (2015) for the *mlrA, mlrB, mlrC* and *mlrD* genes. DNA of *Sphingomonas* sp. strain ACM-3962 (kindly provided by the Australian Water Quality Centre, South Australia) was used as positive control since primers were initially designed from its *mlrABCD* gene cluster. Amplifications were performed in 51 µl reaction volume containing 45 µl PCR mastermix (Table 4.2), 5 µl DNA extract and 1 µl GoTaq Flexi DNA polymerase (Promega). The PCR was conducted on a Techne® Endurance TC-412 Thermal Cycler under conditions previously reported by Ho *et al.* (2007) (Table 4.3). PCR products were separated by gel electrophoresis and visualised as described before (section 3.2.5).

Table 4.1 Specific oligonucleotide primer sequences used in PCR to target regions in the *mlrA, mlrB, mlrC* and *mlrD* genes previously shown to be involved in MC-LR degradation (Bourne *et al*., 2001).

Gene	Primer	Sequence (5′ – 3′)	Length (bp)	Tm (°C)	Position*
mlrA	mlrAFw	GACCCGATGTTCAAGATACT	20	58	103-123
	mlrARv	CTCCTCCCACAAATCAGGAC	20	63	891-911
mlrB	mlrBFw	CGACGATGAGATACTGTCC	19	58	99-117
	mlrBRv	CGTGCGGACTACTGTTGG	18	63	530-547
mlrC	mlrCFw	TCCCCGAAACCGATTCTCCA	20	71	98-117
	mlrCRv	CCGGCTCACTGATCCAAGGCT	21	72	744-764
mlrD	mlrDFw	GCTGGCTGCGACGGAAATG	19	71	51-69
	mlrDRv	ACAGTGTTGCCGAGCTGCTCA	21	71	702-722

* On respective gene of *Sphingomonas* sp. strain ACM-3962

Table 4.2 Specifics on preparation of the PCR mastermix.

Reagent	Amount per sample	Amount for nine samples
5 x Colorless GoTaq Flexi buffer (Promega)	10 µl	90 µl
25 mM Magnesium Chloride (Promega)	3 µl	27 µl
10 mM dNTP Mix (Promega)	1 µl	9 µl
mlr Forward Primer	1 µl	9 µl
mlr Reverse Primer	1 µl	9 µl
0.22 µm filtered ELGA water	34 µl	306 µl

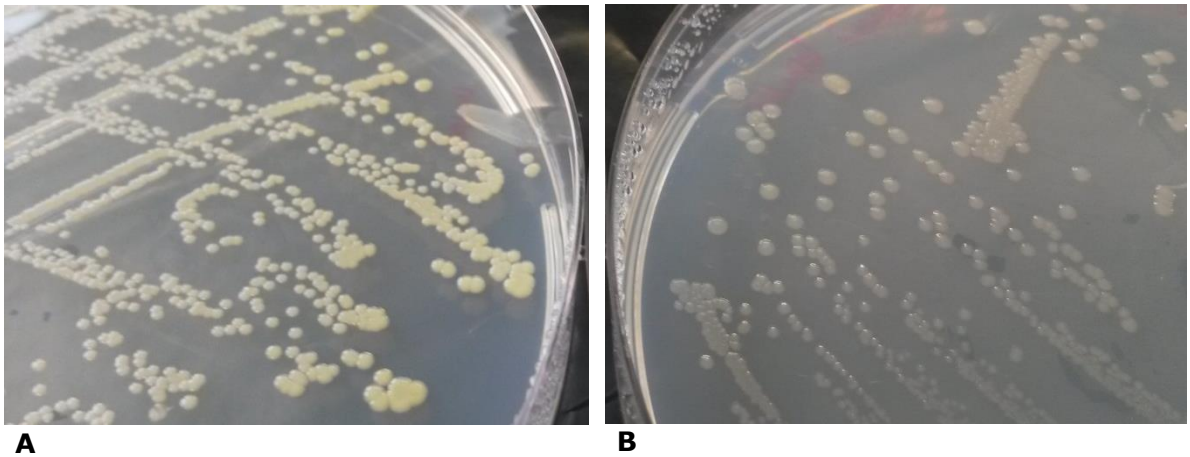
Table 4.3 PCR program of the *mlrABCD* specific gene amplification (Ho *et al.*, 2007).

Condition	Temperature	Duration	Cycle number
Denaturation	94 °C	10 min	1
	94 °C	20 s	35
Annealing	60 °C	10 s	
Extension	72 °C	30 s	
Final extension	72 °C	10 min	1

4.3 Results

4.3.1 Gram Stain, Catalase and Oxidase Activity and Colony Morphology of the *P. toxinivorans* Strains

Cells of *P. toxinivorans* (2007) were Gram-negative, catalase-positive, oxidase-negative and cocci-shaped. Cells of *P. toxinivorans* (2015) were also Gram-negative but, contrary to *P. toxinivorans* (2007), they were catalase-negative, oxidase-positive and rod-shaped. Despite the catalase activity, findings corresponding to *P. toxinivorans* (2015) were in agreement with findings of Rapala *et al.* (2005) who first described the novel genus and species *P. toxinivorans*, but reported the organism to be weakly catalase-positive. Colony shape, margin and texture of *P. toxinivorans* (2007) and *P. toxinivorans* (2015) were congruent. However, two distinctive colonies were noted concerning the appearance, pigmentation and optical property.
While convex, yellow-pigmented, opaque colonies were characteristic for *P. toxinivorans* (2007) (Figure 4.3 (A)), *P. toxinivorans* (2007) colonies were umbonate, non-pigmented and translucent (Figure 4.3 (B)).



A **B**

Figure 4.2 Appearance of (A) *P. toxinivorans* (2007) and (B) *P. toxinivorans* (2015) colonies on R2A agar plates after several days of incubation at 29 °C.

4.3.2 Testing the Effect of Growth Media on Bacterial MC-LR Biodegradation Activity

To obtain single colonies of the *P. toxinivorans* strains, *Rhodococcus* sp. and *Pseudomonas* sp. bacterial cultures were streaked onto R2A as well as LB agar plates. All organisms except the newly obtained strain of *P. toxinivorans* grew on both media. The new *P. toxinivorans* strain (2015) only grew on the minimal R2A agar medium.

P. toxinivorans (2015) degraded 100 % of MC-LR in all the different media regardless of the concentration of the media (Figure 4.4 (A)). The LB broth of 100 % concentration was the only exception as it did not promote MC-LR degradation at all. All the controls which were composed of the respective media (100 % concentration) spiked with MC-LR but lacking bacteria showed remaining MC-LR concentrations of close to 10 µl/ml confirming MC-LR removal to be due to bacterial activity.

In contrast to the *P. toxinivorans* strain of 2015 the *P. toxinivorans* strain obtained in 2007 showed a much weaker degradation activity (Figure 4.4 (B)). *P. toxinivorans* (2007) showed the highest degradation activity in 100 % NB broth but degraded considerably less (32 % compared to 100 %) than *P. toxinivorans* (2015). Incubation of *P. toxinivorans* (2007) in MC-LR spiked MSM, Carron water, R2A and LB broth also supported MC-LR degradation. While 100 % concentrated MSM and Carron water showed a higher reduction in MC-LR concentration compared to the 10 % concentrated compositions it was 10 % concentrated LB broth (23% reduction) that promoted more removal compared to the 100 % concentrated LB broth (10 % reduction). These findings were consistent with MC-LR removal by *P. toxinivorans* (2015)

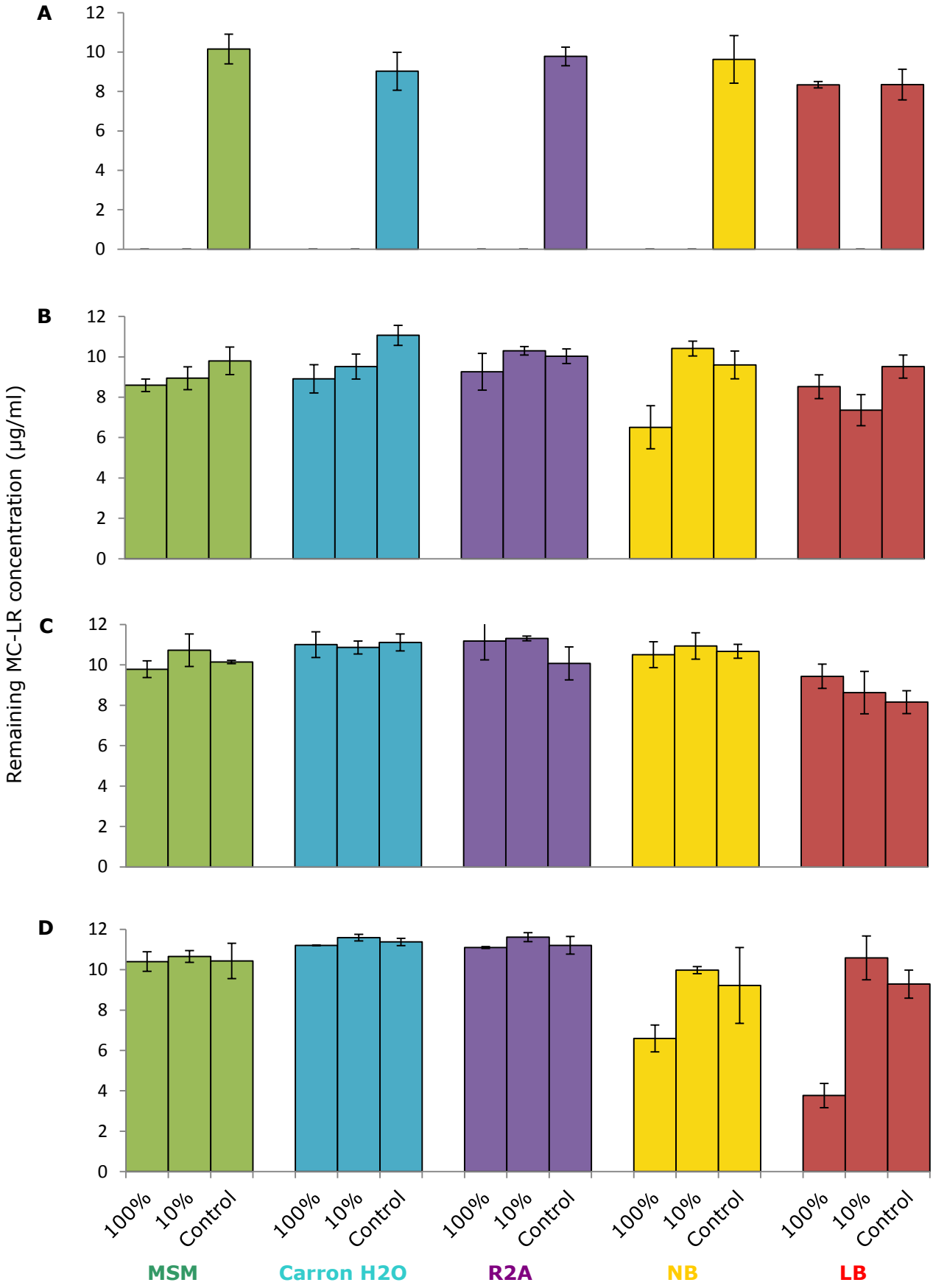


Figure 4.3 Remaining MC-LR concentrations after 7 days in samples containing different media of varying concentrations spiked with MC-LR and inoculated with (A) *P. toxinivorans* (2015), (B) *P. toxinivorans* (2007), (C) *Rhodococcus* sp. and (D) *Pseudomonas* sp.. Controls contained 100 % media spiked with MC-LR but lacking bacterial cultures. Standard errors are displayed (n = 3).

which also showed higher MC-LR degradation activity in less concentrated LB broth. The same degradation assay was carried out on *P. toxinivorans* (2007) before to test the feasibility of the assay (data not shown). The results of the current assay were in agreement with those of the previous assay in that degradation by *P. toxinivorans* (2007) was promoted the most in 100 % concentrated NB broth (32 % reduction) and the least in R2A media (0 % reduction). In the previous assay high MC-LR reduction (33 %) was also achieved in 100 % concentrated LB broth (yet, only 10 % in the current assay). MC-LR reduction in 100 % concentrated MSM and Carron water was in both assays between 10 % and 20 %. At this point it is important to mention that the previous assay was incubated at 25 °C while the current assay was incubated at 29 °C which was chosen according to the optimal growth temperature of *P. toxinivorans* (2015).

In contrast, *Rhodococcus* sp. did not show MC-LR degradation activity regardless of the type of media (Figure 4.4 (C)). Similar to *Rhodococcus* sp. *Pseudomonas* sp. did not degrade MC-LR in MSM, Carron water and R2A media (Figure 4.4 (D)). However, the organism achieved 28 % MC-LR reduction in 100 % concentrated NB broth and an even higher reduction of 59 % in 100 % concentrated LB broth. Yet, no removal of the toxin occurred in the 10 % concentrated equivalents of the two media.

UPLC chromatograms of *P. toxinivorans* (2007) and *Pseudomonas* sp. samples in which MC-LR degradation had occurred did not reveal MC-LR breakdown products. However, UPLC profiles of *P. toxinivorans* (2015) which had completely removed MC-LR in all assay samples but 100 % LB broth showed a consistent appearance of three degradation products (Figure 4.5).

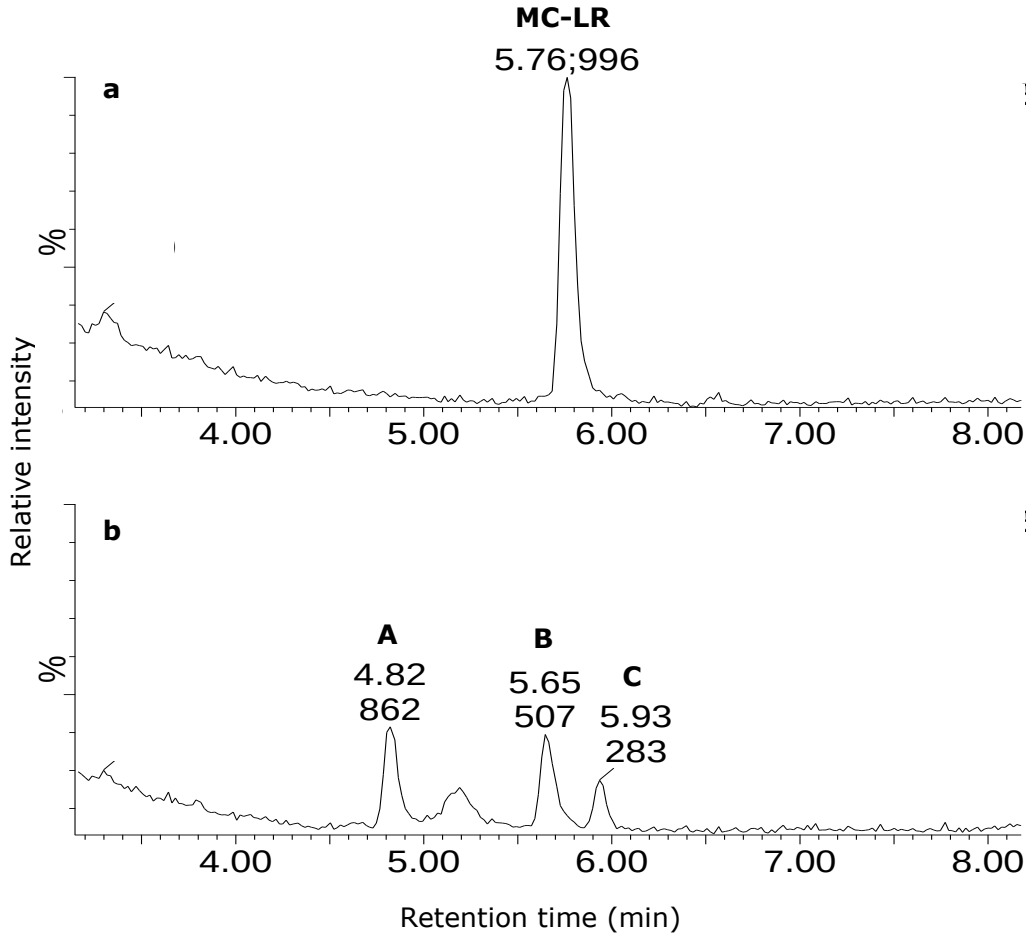


Figure 4.4 UPLC profiles at (a) day 0 and (b) day 7 for the biodegradation of MC-LR by *P. toxinivorans* (2015), illustrating the accumulation of the three degradation products A, B and C.

The peaks of the degradation products at retention time 4.82, 5.65 and 5.93 minutes were indicated as product A, B and C, respectively (Figure 4.5 (b)). Yet a forth peak was observed at retention time 5.20 minutes, however, the peak most likely corresponds to a bacterial product or column material as mass spectral analysis showed no relation to MC-LR. The MS/MS spectral analysis of the degradation product A revealed a major ion at *m/z* 862.5 ($[M+H-NH_2-PhCH_2CHOMe]^+$), corresponding to linear MC-LR that lost the

terminal phenylethylmethoxy group (MW: 135) and the amino NH_2 group (MW: 16) from Adda during fragmentation of the parent ion *m/z* 1013.6 (Figure 4.6 (b)). The protonated molecular ion at *m/z* 1013.6 is typical of linearised MC-LR ($[M+H_2O+H]^+$) and its appearance strongly suggested that the degradation product A was the result of MC-LR hydrolysis. A series of fragment ions were identified from product A which included, among others, ions at *m/z* 135 which correspond to terminal Adda (Figure 4.6 (b), Table 4.4). Like the degradation product A the degradation product B also exhibited the protonated molecular ion at *m/z* 1013.6 (Figure 4.6 (c)) but the base peak was at *m/z* 507 (Figure 4.5 (b)) which represents the dimeric ion of linearised MC-LR ($[M+H_20+2H]^{2+}$). The fragmentation pattern of product B revealed with ions at *m/z* 488, 304, 175 and 135 similar daughter ions compared to that of product A (Table 4.4). However, daughter ions of higher mass-to-charge ratios (862, 726, 571) observed in the mass spectrum of product A were not observed for product B (Figure 4.6 (c)). Instead, an ion at *m/z* 879 was present that was identified as linear MC-LR that lost the terminal Adda group ($[M+H-PhCH_2CHOMe]^+$) (Figure 4.6 (c), Table 4.4). Based on the different retention time and the different mass spectrum compared to product A it was suggested that product B represented linear MC-LR hydrolysed at a different peptide bond than product A. Alternatively, based on the high similarity of fragmentation patterns product B could have been a stereoisomer of product A. Further analysis is required to characterise product B as either a distinct degradation product or as a stereoisomer of product A.

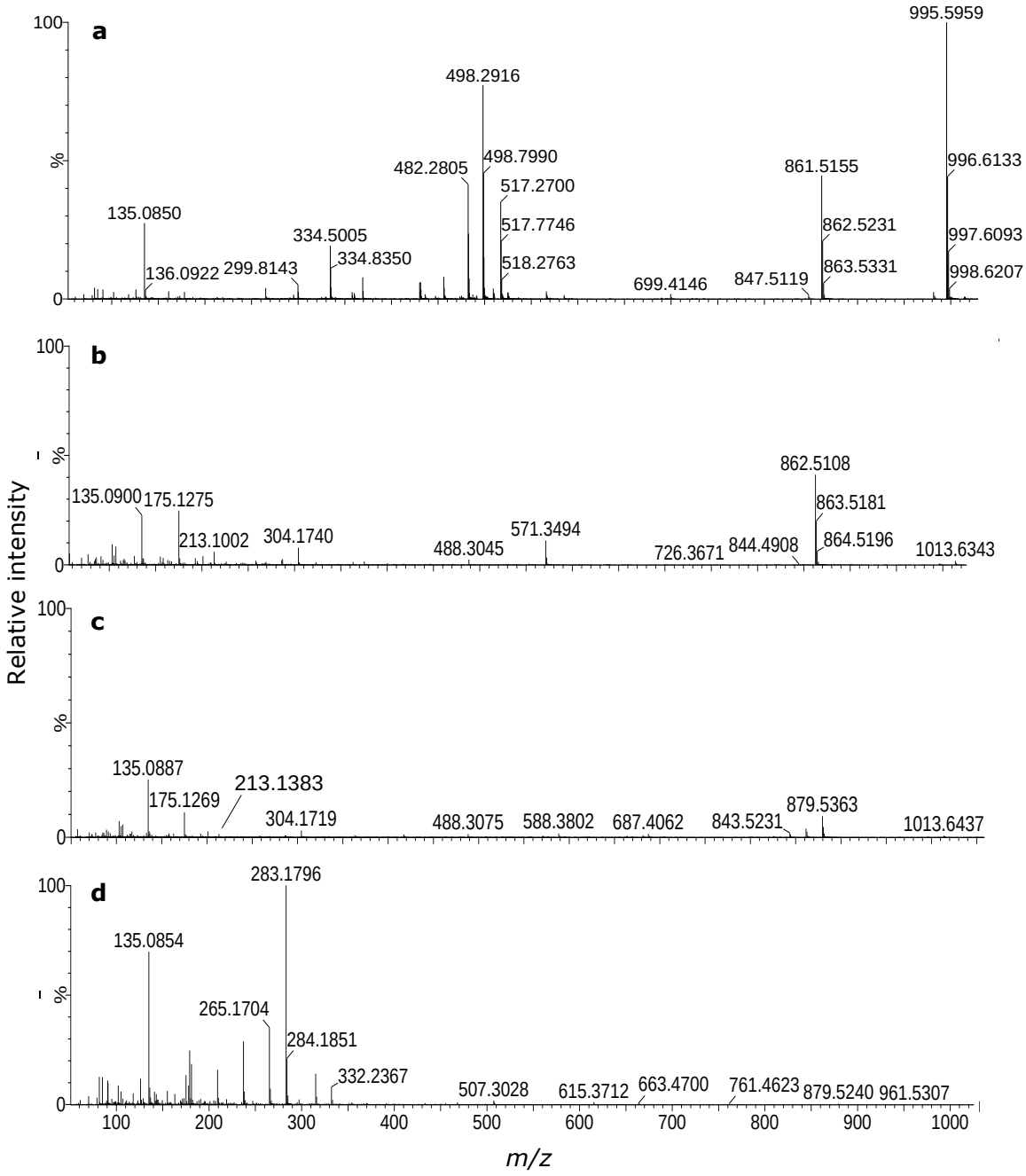


Figure 4.5 Mass spectra at high collision energy (20 – 55 V) of (a) cyclic MC-LR, MC-LR degradation products (b) A and (c) B and mass spectrum at low collision energy (6 V) of MC-LR degradation product (d) C.

As shown in the mass spectrum (Figure 4.6 (d)), degradation product C exhibited a protonated molecular ion at *m/z* 332 with a major peak at *m/z* 283. The product ions, including the precursor ion ($[M+H]^+$ *m/z* 332), a

Table 4.4 MS parent ion and daughter ions of linear MC-LR.

m/z	Identity
1013*^	M + H (Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH + H)
879^	M + H - $PhCH_2CHOMe$
862*	M + H - NH_2 - $PhCH_2CHOMe$
726*	Co-Glu-Mdha-Ala-Leu-Masp-Arg-OH - 2H
571*	Mdha-Ala-Leu-Masp-Arg-OH + 2H
507^[1]	M + H_20 + 2H
488*^	Ala-Leu-Masp-Arg-OH + 2H
304*^	Masp-Arg-OH + 2H
213*^	Glu-Mdha + H
175*^	Arg-OH + 2H
135*^	$PhCH_2CHOMe$

* identified in degradation product A ^ identified in degradation product B

[1] only visible in mass spectrum of product B at low collision energy (6 V)

dimeric ion ($[2M+H]^{2+}$ *m/z* 663) and other daughter ions at *m/z* 283 ($[M+H-NH_3-MeOH]^+$), 265 ($[M+H-NH_3-MeOH-H_2O]^+$) and 135 (terminal Adda) were consistent with those reported by Harada *et al.* (2004) for the mass spectral characteristics of the Adda amino acid (Table 4.5). Therefore, it was strongly believed that beside the potentially two different linear MC-LR compounds the complete Adda amino acid was a third breakdown product generated in the enzymatic MC-LR degradation of *P. toxinivorans* (2015).

Table 4.5 MS parent ion and daughter ions of the MC-LR Adda amino acid.

m/z	Identity
663	2M + H
332	M + H
283	M + H - NH_3 - MeOH
265	M + H - NH_3 - MeOH - H_2O
135	$PhCH_2CHOMe$

4.3.3 Detection of *mlrABCD* Genes Involved in MC-LR Degradation

As MC-LR breakdown products of *P. toxinivorans* (2015) were similar to those reported for *Sphingomonas* sp. strain ACM-3962 (Bourne *et al.,* 1996) it was tested for the presence of the *mlrABCD* genes associated with MC degradation in the *Sphingomonas* strain. Partial *mlrA, mlrB, mlrC* and *mlrD* gene sequences were successfully amplified from the DNA of *Sphingomonas* sp. strain ACM-3962 confirming the applicability of primers and PCR conditions. The band of *mlrA* was surprisingly weak in appearance and detected in the range of 1250 bp although the *mlrA* primer target sequence is of approximately 800 bp (Figure 4.7). Bands of approximately 400 to 600 bp products (Figure 4.7) indicated the presence of the partial gene sequences of *mlrB* (446 bp)*, mlrC* (666 bp) and *mlrD* (671 bp). The respective bands were not detected in PCR products of *P. toxinivorans* (2015) revealing the absence of *mlrA,B,C,D* homologues (Figure 4.7).

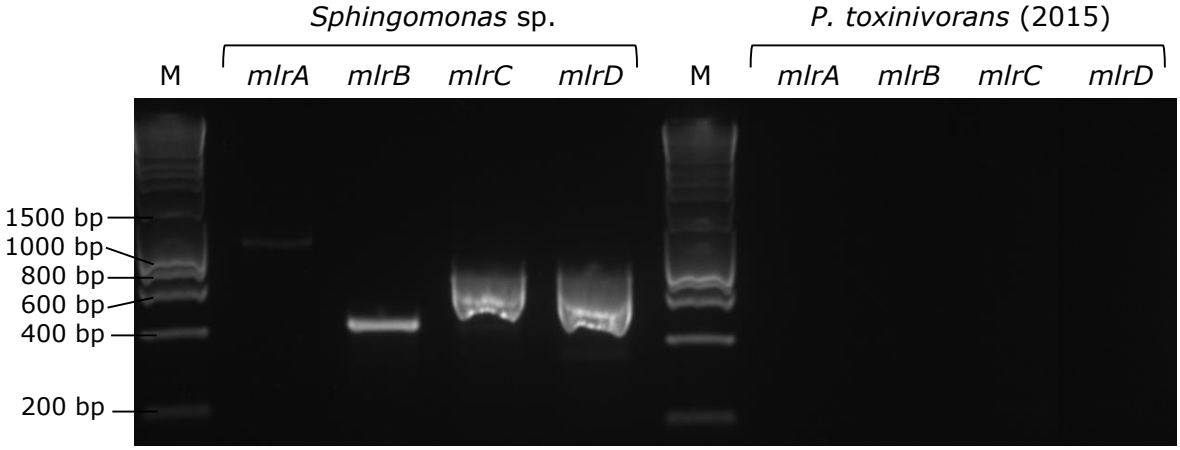


Figure 4.6 Gel electrophoresis of *mlrA,B,C,D* gene specific primer products amplified from DNA of *Sphingomonas* sp. strain ACM-3962 (positive control) and *P. toxinivorans* (2015). PCR products were run on a 1.5 % agarose gel for 60 min at 80 volt together with a marker (M) (HyperLadder 1 kb 100 lanes by Bioline).

4.4 Discussion

4.4.1 Comparison studies on *P. toxinivorans* Strains and Bacterial MC-LR Biodegradation in Different Growth Media

The presented study confirmed that repeated sub-culturing and long-term preservation drove physiological and functional drifts in *P. toxinivorans* (2007). This was evident from the difference in media preference (*P. toxinivorans* (2007) grew on LB agar unlike *P. toxinivorans* (2015)), MC-LR degradation (*P. toxinivorans* (2007) degraded MC-LR significantly less), oxidase activity (*P. toxinivorans* (2007) lacked oxidase activity unlike *P. toxinivorans* (2015)) as well as colony and cell morphology (*P. toxinivorans* (2007) formed cocci, *P. toxinivorans* (2015) formed rods). Cell growth, behaviour and morphology of *P. toxinivorans* (2015) were in agreement with observations by Rapala *et al.* (2005) who first described the organism. The findings suggested adaptation of *P. toxinivorans* (2007) to a nutrient rich environment provided by growth media such as NB and LB through genetic or gene regulatory variations that simultaneously reduced its MC-LR biodegradation and oxidase activity. These variations might have also driven cell and colony morphological changes. Another area of interest was to test the influence of nutrients on MC-LR degradation for which a simple and rapid 96-well plate based assay was developed that required minimal amounts of MC-LR. In the assay, *P. toxinivorans* (2015) degraded 100 % MC-LR in all growth media but 100 % concentrated LB broth which was known to inhibit the organism's growth. This demonstrated that MC-LR degradation is not impeded in the presence of different organic and inorganic nutrients of various concentrations.

P. toxinivorans (2007) was not only found to degrade MC-LR to a significantly smaller degree but also showed a nutrient-dependant MC-LR degradation activity. NB broth was revealed to contain most favourable nutrients and nutrient concentrations for MC-LR degradation. NB broth has a significantly higher concentration of organic compounds and also a higher concentration of sodium chloride compared to R2A broth, MSM and Carron water. Yet, these concentrations are half or less than half of what LB broth is composed of. Thereby, NB broth represents a media with medium organic and inorganic compound concentration compared to the remaining media used in the assay. As found in earlier biodegradation assays *Rhodococcus* sp. did not degrade MC-LR.

Also *Pseudomonas* sp. failed to remove MC-LR in earlier biodegradation studies. However, the current study found *Pseudomonas* sp. to degrade 28 % MC-LR in NB broth and an even higher amount of 56 % in LB broth. As enzyme activity is significantly dependent on temperature the increase from 25 °C to 29 °C, chosen according to *P. toxinivorans* (2015) optimal growth temperature, is likely to be a factor that promoted MC-LR degradation of *Pseudomonas* sp. As observed for *P. toxinivorans* (2007), MC-LR degradation by *Pseudomonas* sp. was promoted by growth media of higher nutrient concentration. This is in contrast to the above formulated hypothesis that bacterial maintenance in nutrient rich growth media reduces or impedes their MC-LR degradation activity.

Yet, many studies reported inhibition of biodegradation and an adaptation response to alternate nutrients as a result of preferential utilisation of more easily degradable carbon sources (Swindoll *et al.*, 1988; Chaineau *et al.*,

2005). However, some authors reported an enhanced biodegradation activity in the presence of nutrient rich media (Zhong *et al.*, 2007).

4.4.2 Partial Elucidation of the MC-LR Degradation Pathway in *P. toxinivorans*

Bourne *et al.* (1996) was the first to report the enzymatic pathway for bacterial MC-LR degradation using the Gram-negative *Sphingomonas* sp. strain ACM-3962. On the basis of MS analysis on MC-LR incubated with CEs of the *Sphingomonas* sp. strain (which were preincubated with protease inhibitors PMSF and EDTA; Bourne *et al.*, 1996) and genetic analysis (Bourne *et al.*, 2001) the enzymatic degradation pathway involving the *mlrABCD* gene cluster was unravelled. Since then the same degradation products and (parts of) the *mlrABCD* gene cluster have been reported in other *Sphingomonas* spp. as well as in bacteria of different genera (Dziga *et al.*, 2013).

The current study is the first to report MC-LR degradation products of *P. toxinivorans* and also one of the few to observe degradation products without the use of protease inhibitors. UPLC-MS analysis revealed a degradation product (A) that was remarkably similar in its parent and daughter ions to the first breakdown product identified by Bourne *et al.* (1996) which was linear MC-LR hydrolysed at the Arg-Adda peptide bond. This observation prompted investigations for the presence of the *mlrABCD* genes in *P. toxinivorans* (2015). However, *mlr* gene homologues could not be detected. This suggests that not identical but similar genes are present in *P. toxinivorans* (2015) that are responsible for the hydrolytic cleavage of the cyclic cyanobacterial peptides. Unlike described by Bourne *et al.* (1996) a second linear MC-LR intermediate product (B) was potentially present in the degradation samples of

P. toxinivorans (2015) which was eluted 50 seconds after the first intermediate product. Mass spectral analysis revealed several product ions (*m/z* 1013, 488, 304, 175, 135) also characteristic to the other linear MC-LR degradation product but also produced fragments that were not previously described (*m/z* 879, 588, 507). Based on the mass spectrum of degradation product B it is proposed that the product was the result of MC-LR linearisation not at the Arg-Adda but a different peptide bond. Potentially, however, product B could have also been a stereoisomer of product A since the mass spectrum fragmentation patterns were very similar. As shown by Bourne *et al.* (1996) linearisation of MC-LR reduces its toxic protein phosphatase inhibition 160 times.

Yet another MC-LR breakdown product (C) rarely reported in MC-LR biodegradation studies resulted from incubation of the cyanobacterial toxin with cells of *P. toxinivorans* (2015). Comparison to work done by Harada *et al.* (2004) identified degradation product C as the amino acid Adda. This brings additional insights into the enzymatic pathway of MC-LR degradation by *P. toxinivorans* as it demonstrates the cleavage of the Adda residue to be part of the MC-LR breakdown process. This finding is also important in regard to the toxicity of the intermediates generated during MC-LR degradation. Adda is essential for the characteristic toxic activity of MC-LR and by cleavage of the Adda portion the toxicity of MC-LR disappears completely (Harada *et al.*, 2004).

5 Testing Multiple Peptide Degradation by *P. toxinivorans* Using the 96-Well Plate Based Assay

5.1 Introduction

Rapala *et al.* (2005) reported that *P. toxinivorans* degraded the MC variants -LR and -YR as well as NOD. The MC-LR degradation activity of *P. toxinivorans* (2015) was confirmed here (chapter 4). The current chapter aimed to further investigate peptide degradation by *P. toxinivorans* (2015) by testing chemically and structurally diverse eukaryotic and prokaryotic peptides using the 96-well plate based assay. To give insight whether the degradation pathway found here was capable of degrading a broader range of MC variants MC analogues with variants in amino acids 2 (X) and 4 (Z) as well as demethylated analogues were included in the assay. Moreover, the cyclic cyanobacterial pentapeptide NOD which also contains the Adda residue and the cyclic cyanobacterial hexapeptides anabaenopeptin types A and B were tested. If *P. toxinivorans* was capable of degrading multiple MCs and other cyanotoxins it would be promising for the use in a water treatment system for the clean-up of contaminated water bodies.

Removal of yet another bacterial peptide, linear antimicrobial gramicidin A derived from *Bacillus brevis*, was evaluated in the assay to investigate possible antibiotic resistance mechanisms of *P. toxinivorans.*

To further assess whether *P. toxinivorans* specifically degrades cyanobacterial peptides or naturally occurring peptides in general, degradation of other small peptides was investigated which were unlikely to have been encountered by *P. toxinivorans* in its natural habitat. For this purpose the human peptides

fibrinopeptide B, leucine enkephalin acetate salt hydrate and oxytocin acetate salt hydrate were also included in the multiple peptide degradation assay.

5.2 Materials and Methods

5.2.1 Chemicals

DMC-LR (desmethyl MC-LR) standards were purified from batch cultures of *Microcystis aeruginosa* PCC 7806. NOD standards were purified from the cultures of *Nodularia spumigena* KAC66. MC-LY, MC-LF and MC-LW standards were obtained from batch cultures of *Microcystis aeruginosa* PCC 7813. MC-YR, MC-HtYR (MC-YR containing homotyrosine (HtY)), anabaenopeptin (AP) A and B, DMC-RR and MC-RR standards were obtained from batch cultures of *Microcystis aeruginosa* Sciento. Synthetic human [Glu^1]-fibrinopeptide B (FIB), leucine Enkephalin acetate salt hydrate (LEU) and oxytocin acetate salt hydrate (OXY) as well as bacterial gramicidin A (GRA) from *Bacillus brevis* were purchased from Sigma-Aldrich, Dorset, UK. All other chemicals were acquired as outlined before (section 2.2.1).

5.2.2 Ultra Performance Liquid Chromatography Chromatography for the Detection of the (Cyano-) Bacterial and Human Peptides

UPLC-PDA-MS analysis was conducted on all peptides as described in section 3.2.2. The only exception was gramicidin A; for the analysis of the highly hydrophobic peptide the gradient of acetonitrile was modified from 20 % - 70 % to 50 % - 100 %.

5.2.3 Culturing of *P. toxinivorans*

A single colony of *P. toxinivorans* (2015), maintained at 4 °C on R2A agar, was streaked onto a new R2A agar plate and incubated at 29 °C for 72 h.

5.2.4 Testing Multiple Peptide Degradation by *P. toxinivorans* Using the 96-Well Plate Based Assay

The methodology is very similar to that depicted in schemata 4.1. Cells of *P. toxinivorans* (2015) were transferred to 3 ml PBS and adjusted to a turbidity of A_{595} = 0.37. Under sterile conditions 140 µl NB broth were transferred to wells of 96-well plates (Figure 5.1, blue panel) and each inoculated with 5 µl of the OD adjusted *P. toxinivorans* (2015) culture. Each column of culture containing wells (4, 5, 6) was spiked with a different peptide (Table 5.1) by addition of 5 µl peptide stock per well to reach a final peptide concentration of 10 µg/ml. Control wells (Figure 5.1, red panel) contained 145 µl of NB broth spiked with 5 µl of the respective peptide but lacking bacteria to confirm loss of peptide concentration to be due to bacterial degradation. The plates were covered with the provided lids, placed in a closed sandwich box layered with wet paper towels and incubated at 29 °C, 150 rpm for 7 days as done earlier (chapter 4). After the incubation period all samples were prepared for UPLC-PDA-MS analysis as described previously (section 4.2.5) and analysed according to section 5.2.2.

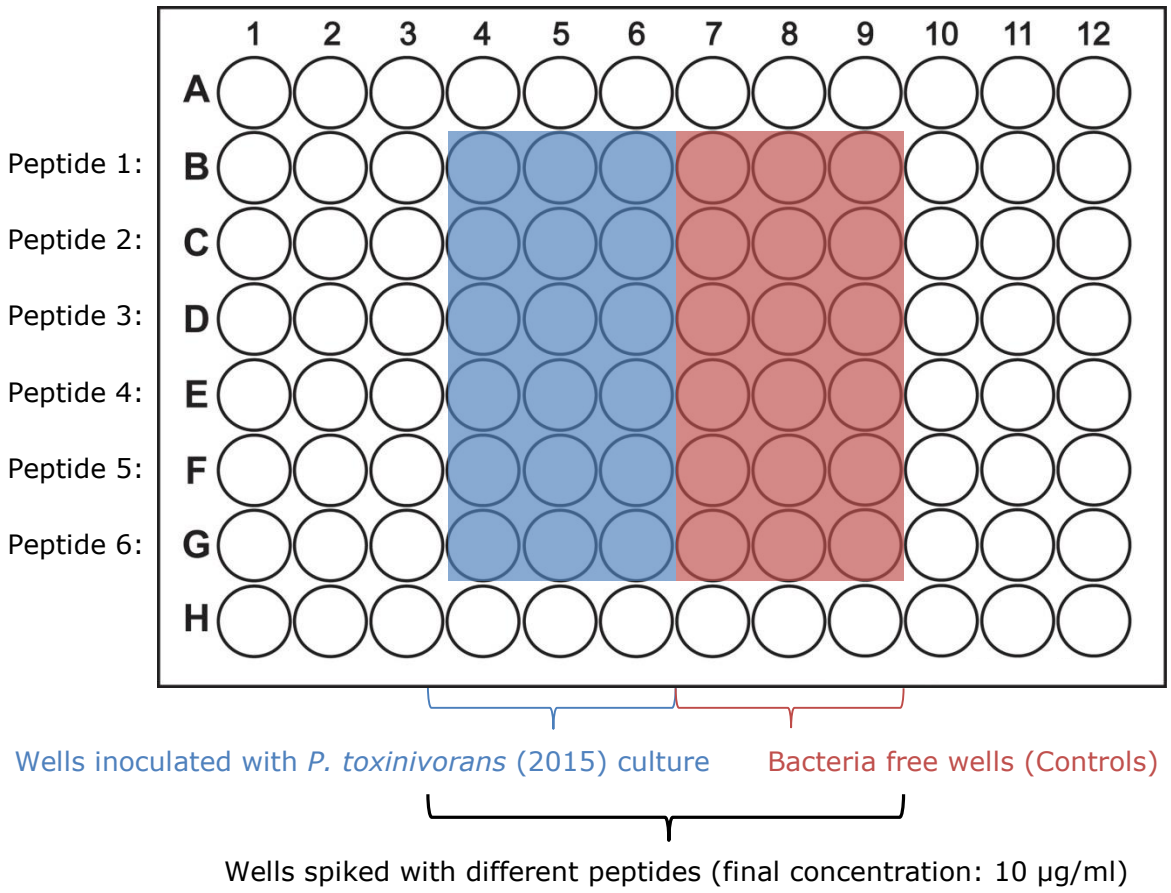
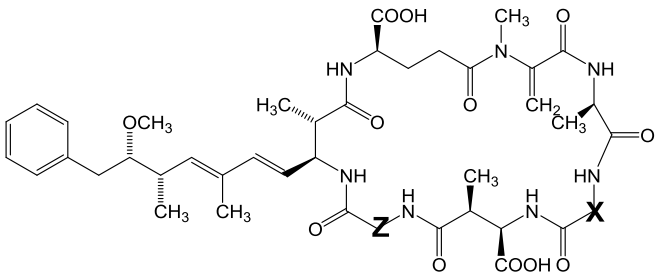


Figure 5.1 Schemata of the composition of the multiple peptide degradation assay carried out on *P. toxinivorans* (2015). Three 96 well plates were used. Each row of sample and control wells was spiked with a different peptide. Nine MC variants (-LR, DMC-LR, -LW, -RR, DMC-RR, -LF, -LY, -YR, -HtYR), three human peptides (FIB, LEU, OXY) and bacterial gramicidin A were tested (Table 5.1).

Table 5.1 Peptides used in the degradation assay on *P. toxinivorans* (2015) with their protonated ion mass (*m/*z), chemical structure and amino acid structure.

Cyanobacterial peptides

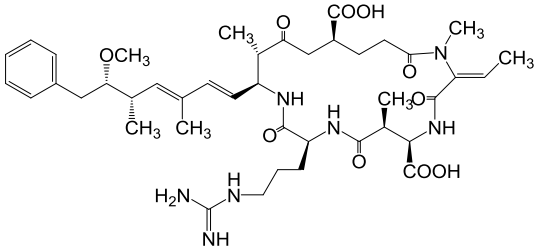


D-Ala-L-**X**-D-MeAsp-L-**Z**-Adda-D-Glu-Mdha

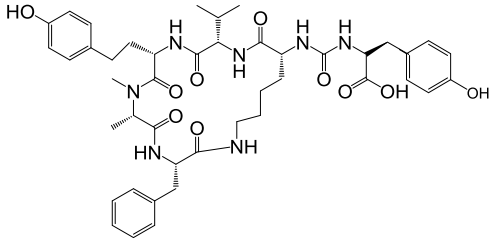
MC-LR ($C_{49}H_{74}N_{10}O_{12}$) *m/z* 996 $[M+H]^+$
DMC-LR ($C_{48}H_{72}N_{10}O_{12}$) *m/z* 982 $[M+H]^+$
MC-LW ($C_{54}H_{72}N_8O_{12}$) *m/z* 1026 $[M+H]^+$
MC-RR ($C_{49}H_{75}N_{13}O_{12}$) *m/z* 520 $[M+2H]^{2+}$
DMC-RR ($C_{48}H_{73}N_{13}O_{12}$) *m/z* 513 $[M+2H]^{2+}$
MC-LF ($C_{52}H_{71}N_7O_{12}$) *m/z* 987 $[M+H]^+$
MC-LY ($C_{52}H_{71}N_7O_{13}$) *m/z* 1003 $[M+H]^+$
MC-YR ($C_{52}H_{72}N_{10}O_{13}$) *m/z* 1046 $[M+H]^+$
MC-HtYR ($C_{53}H_{74}N_{10}O_{13}$) *m/z* 530 $[M+2H]^{2+}$

	X=	**Z=**
MC-LR/ DMC-LR	Leucine	Arginine
MC-LW	Leucine	Tryptophan
MC-RR/ DMC-RR	Arginine	Arginine
MC-LF	Leucine	Phenylalanine
MC-LY	Leucine	Tyrosine
MC-YR	Tyrosine	Argenine
MC-HtYR	Homotyrosine	Arginine

NOD ($C_{41}H_{60}N_8O_{10}$) *m/z* 825 $[M+H]^+$
D-MeAsp-L-Arg-Adda-D-Glu-Mdhb



AP A ($C_{44}H_{57}N_7O_{10}$) *m/z* 844 $[M+H]^+$
Tyr-CO-Lys-Val-Hty-MeAla-Phe



AP B ($C_{41}H_{60}N_{10}O_9$) *m/z* 419 $[M+2H]^{2+}$
Arg-CO-Lys-Val-Hty-MeAla-Phe

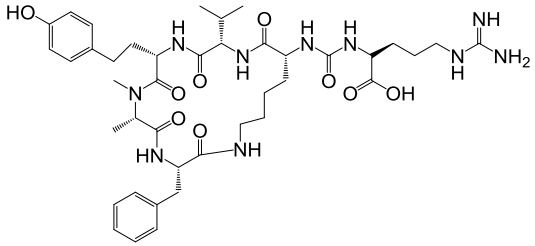
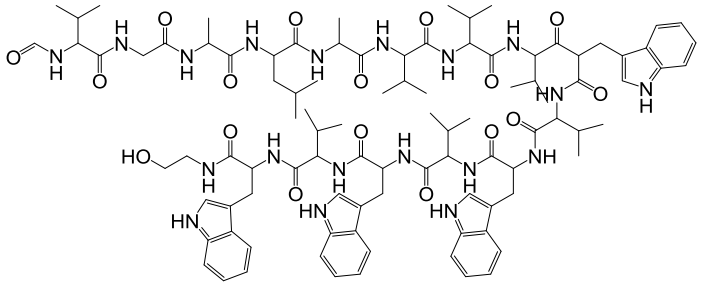
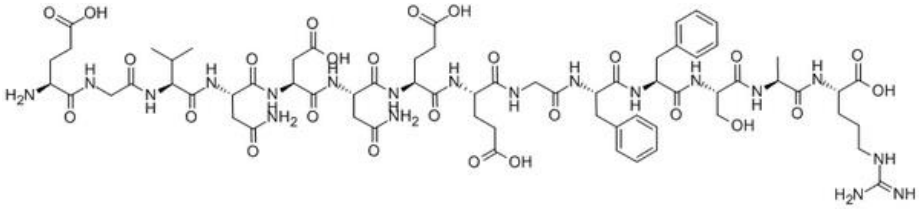
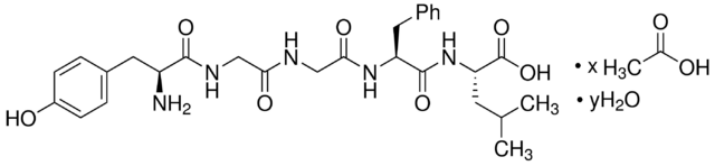
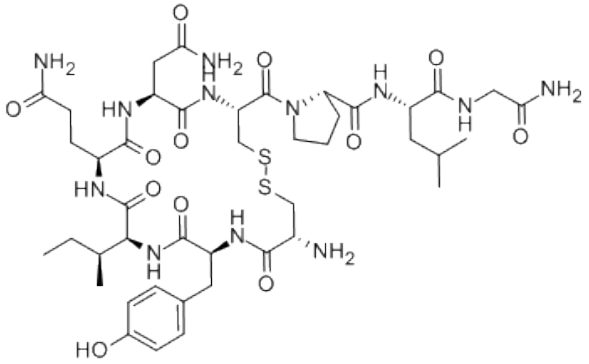


Table 5.1 Peptides used in the degradation assay on *P. toxinivorans* (2015) with their protonated ion mass (m/z), chemical structure and amino acid structure (continued).

<table>
<tr><td colspan="2">Bacterial peptide from Bacillus brevis (GRA) and human peptides (FIB, LEU, OXY)</td></tr>
<tr><td>GRA ($C_{99}H_{140}N_{20}O_{17}$) m/z 942 $[M+2H]^{2+}$
HCO-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-NHCH2CH2OH
  </td><td>FIB ($C_{66}H_{95}N_{19}O_{26}$) m/z 786 $[M+2H]^{2+}$
Glu-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg
  </td></tr>
<tr><td>LEU ($C_{28}H_{37}N_5O_7$) m/z 556 $[M+H]^+$
Tyr-Gly-Gly-Phe-Leu
  </td><td>OXY ($C_{43}H_{66}N_{12}O_{12}S_2$) m/z 1007 $[M+H]^+$
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2 [Disulfide Bridge: 1-6]
  </td></tr>
</table>

5.3 Results

All MC variants tested in the universal peptide degradation assay were degraded by *P. toxinivorans* (2015), although there were considerable differences in the amount of degradation. As observed in the previous 96-well plate based degradation assay, MC-LR was removed completely, demonstrating the reproducibility of the assay (Figure 5.2). The demethylated form of MC-LR, DMC-LR, was degraded to a similar degree (92 %) as the "standard" MC-LR. In contrast, MC-HtYR (92 %) and DMC-RR (95 %) were much more degraded than the "standard" forms MC-YR (59 %) and MC-RR (49 %) (Figure 5.2). The least polar MC variants -LF, -LY and -LW were with 42 %, 26 % and 22 % among the slowest degraded peptides (Figure 5.2). NOD was like many of the MC variants degraded to more than 50 % while the cyanobacterial peptide anabaenopeptin B was more resistant to degradation (37 %; Figure 5.2). The anabaenopeptin variant A which differs by an additional benzene ring was the least degraded cyanobacterial peptide tested in the assay (5 %; Figure 5.2). Unlike all other peptides, the *Bacillus brevis* derived antimicrobial peptide gramicidin A was not degraded by *P. toxinivorans* (2015) (Figure 5.2). Linear human peptides leucine enkephalin acetate salt hydrate and fibrinogen B, however, were found to be degraded to more than 95 % while the amount of degradation of the cyclic human peptide oxytocin acetate salt hydrate was with 17 % considerably less (Figure 5.2). Concerning the investigation of degradation products, it was interesting to see intermediates for all MC variants with the exception of MC-RR as well as for NOD indicative of the linear forms of the peptides (Figure 5.3). This was evident from the mass spectra that showed the characteristic fragments for the occurrence of hydrolysis (i.e. an increase of the respective *m/z* ratio by

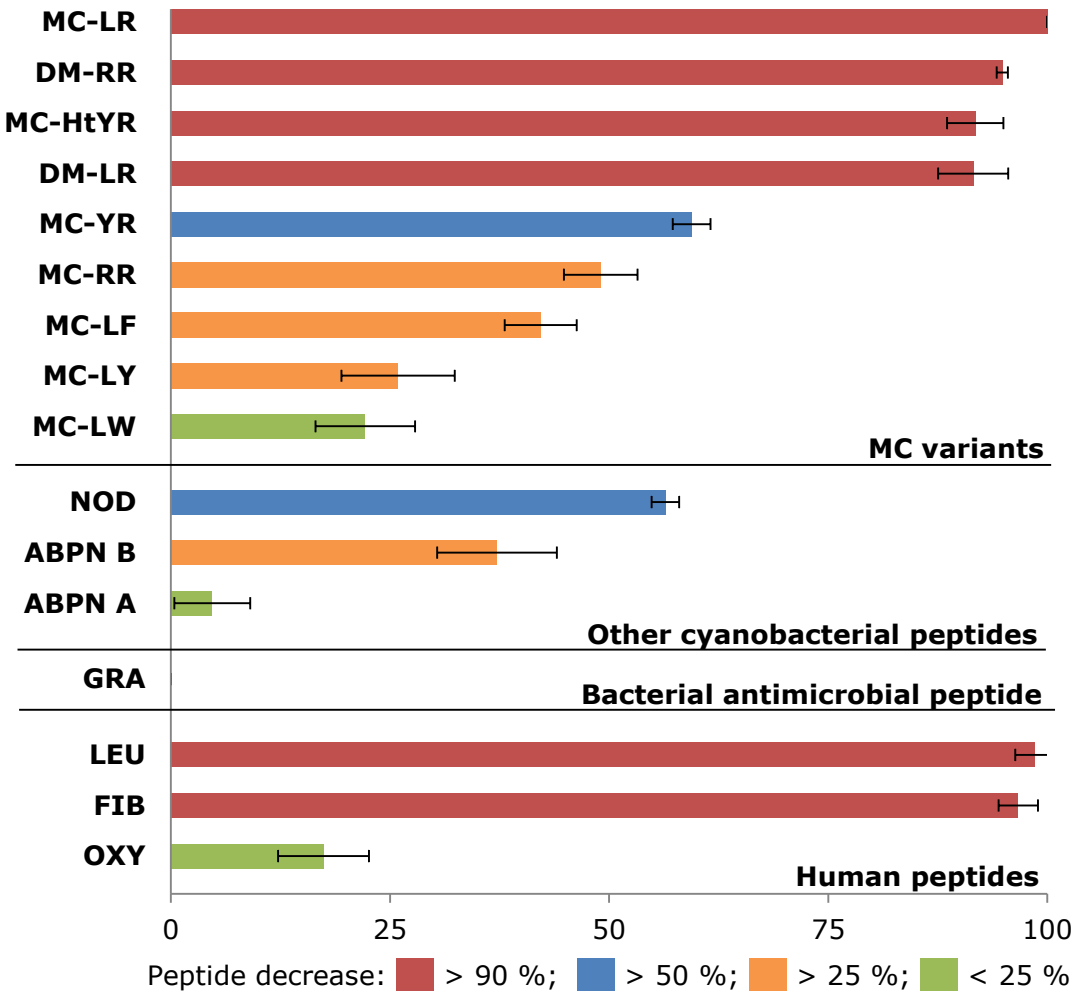


Figure 5.2 Percentage decrease of the different (cyano-) bacterial and human peptides that were incubated with *P. toxinivorans* (2015) in NB broth over 7 days. Controls contained peptide spiked media that lacked bacterial cultures. Standard errors are displayed (n = 3).

18 which is the molecular mass of H_2O). As observed before (section 4.3.2) degradation of MC-LR led to the accumulation of three degradation products, two open ring products and the Adda side chain (data not shown). Degradation of DMC-LR as well as MC-YR and MC-HtYR also resulted in the formation of two open ring products instead of one (Figure 5.3). No degradation products were found for the other peptides, i.e. for the anabaenopeptin variants and the human peptides.

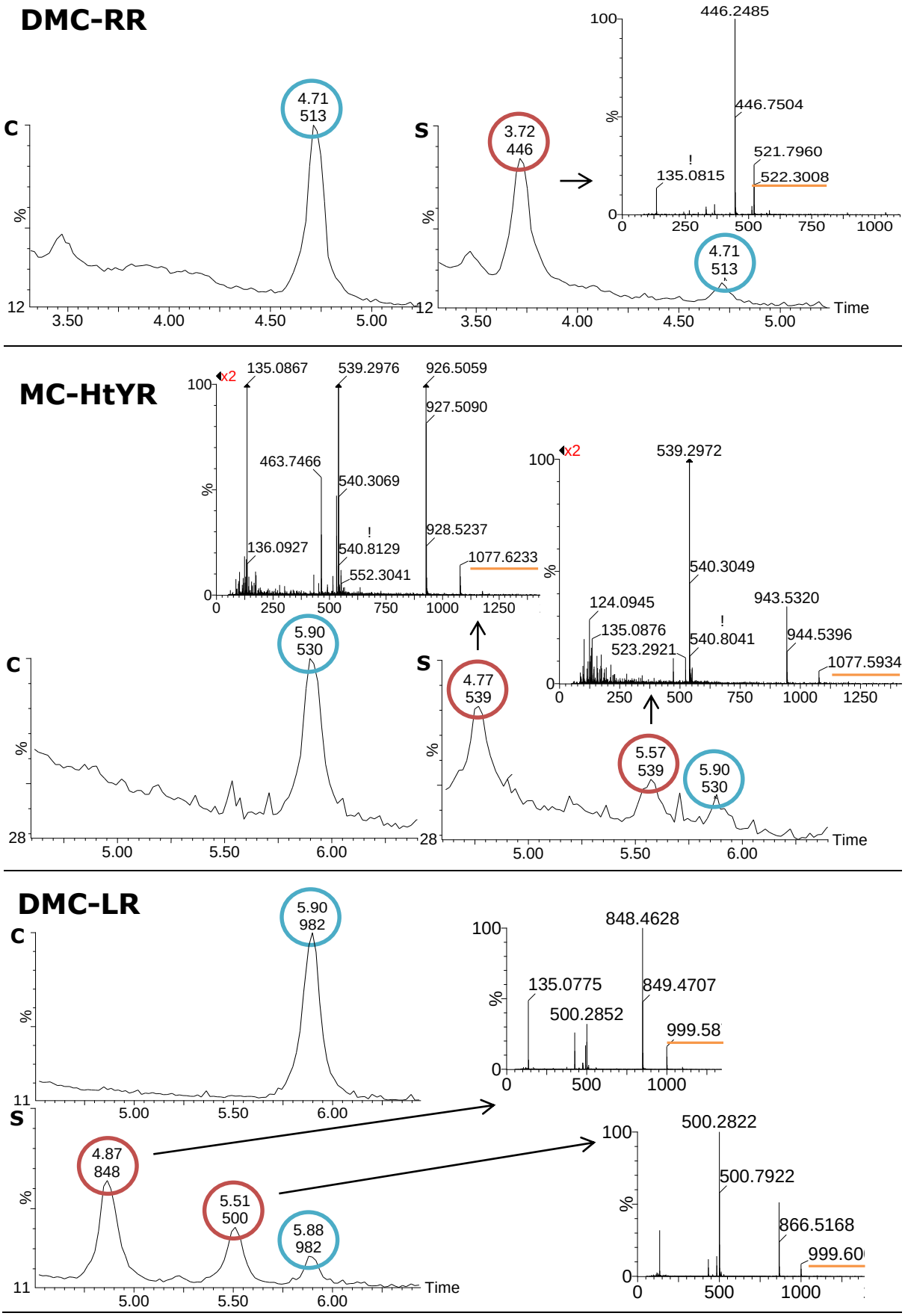


Figure 5.3 UPLC profiles of MC controls (**C**) and samples (**S**).

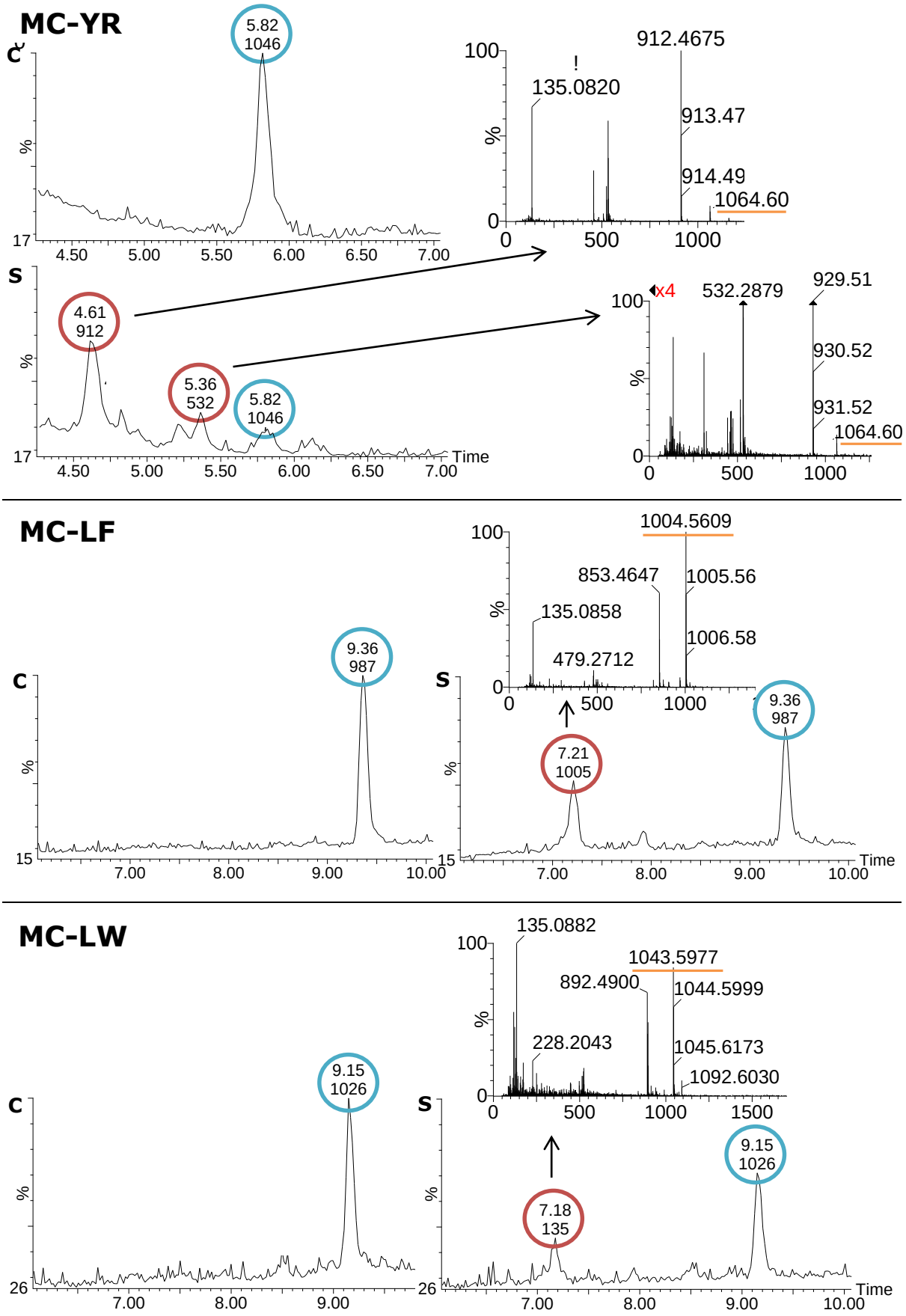


Figure 5.3 UPLC profiles of MC controls (**C**) and samples (**S**) (continued).

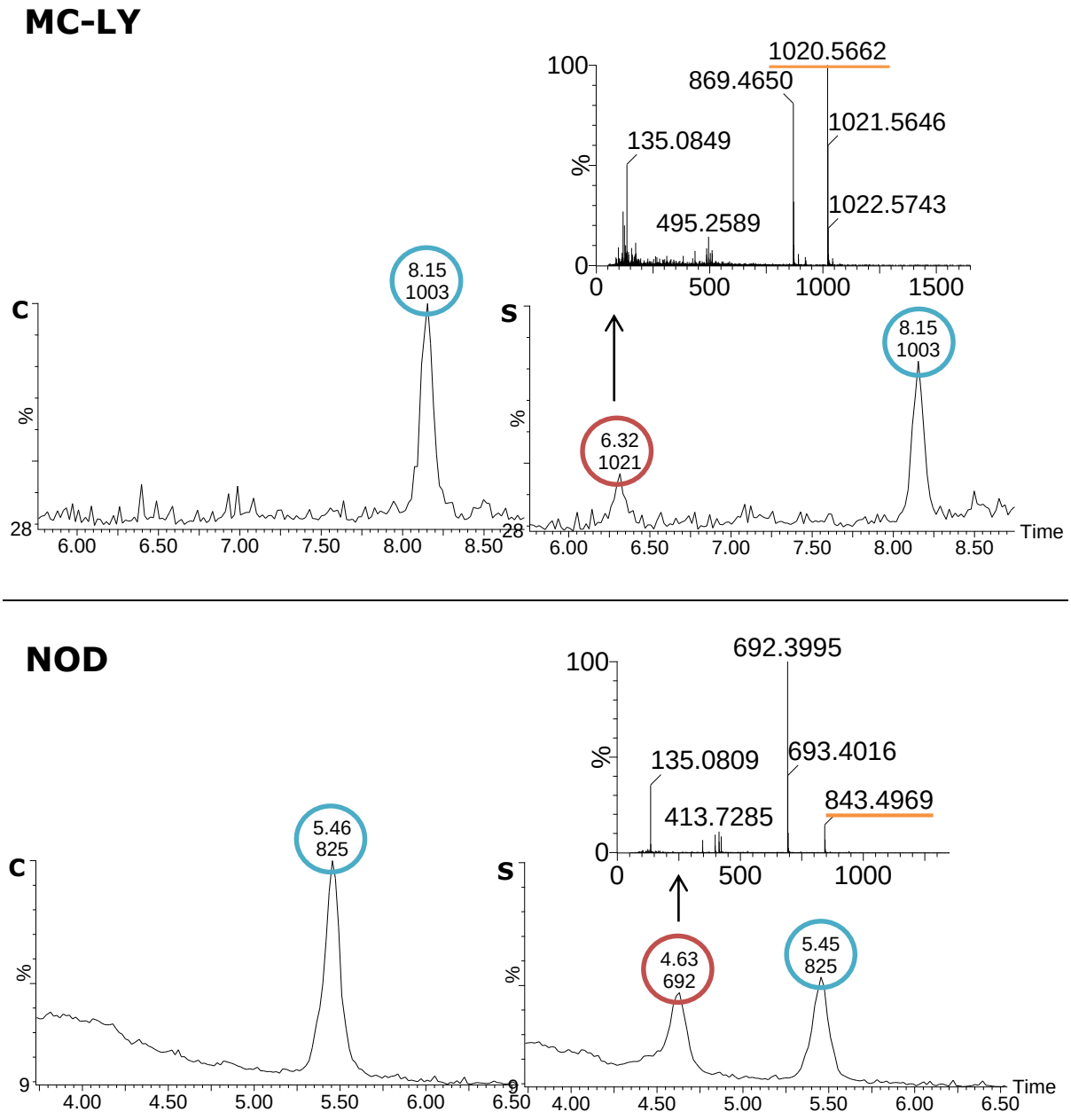


Figure 5.3 UPLC profiles of MC controls (**C**) and samples (**S**) (continued).

5.4 Discussion

P. toxinivorans (2015) successfully degraded the range of cyanobacterial and human peptides with exception of the *Bacillus brevis* derived antimicrobial peptide gramicidin A. However, significant differences in the amount of degradation were found for the different peptides.

Concerning cyanobacterial peptides, *P. toxinivorans* (2015) was shown to degrade the MC variants MC-LR, DMC-RR, MC-HtYR and DMC-LR to the highest degree. MC-YR was degraded to 59 % and slightly lower but yet degraded to more than 50 % was NOD. This confirmed findings of Rapala *et al.* (2005) which also demonstrated the ability of *P. toxinivorans* to degrade MC-LR, -YR and NOD. In contrast to the current study, Rapala *et al.* (2005) tested MC degradation using a mixture of MC-LR and -YR and found opposite degradation rates in that the mixture of MCs was with 4 – 16 µg/l/h much slower degraded than NOD (35 - 87 µg/l/h). The differing amount of degradation between single MCs and MC mixtures is interesting to note and has been reported in previous studies. Edwards *et al.* (2008) observed a reduced MC-LR degradation by river Rescobie bacteria in the presence of other MCs and NOD. In contrast, the addition of MCs and NOD to a water sample of Forfar Loch bacteria stimulated MC-LR degradation.

Using the Biolog MT2 assay Lawton *et al.* (2011) previously evaluated utilisation of a range of MCs (-LF, -LR, -LW, LY, -RR) and NOD by *P. toxinivorans* (2007). The Biolog screen revealed a slower utilisation of NOD compared to all MC variants tested. The small ring structure of NOD composed of five instead seven amino acids as found in MCs provides more chemical stability which is believed to hinder enzymatic degradation. Subsequent degradation studies on *P. toxinivorans* (2007) testing the same range of toxins

as used in the Biolog showed discrepancies to the current study in that MC variants -LF, -LY and -LW were degraded to a higher degree than -LR and -RR. The different response to MC variants between the previous and the current study was possibly due to the already long maintenance of *P. toxinivorans* (2007) under laboratory conditions prior to the degradation study.

A striking observation of the degradation assay performed here was that the five most degradable MCs all contain the Arg-Adda bond, whereas the less than 50 % degraded MC variants -LF, -LY and -LW contain the Phe-Adda, Trp-Adda or Tyr-Adda bond instead. The Arg-Adda peptide bond was previously shown to be the first side of attack by the hydrolytic enzyme microcystinase in *Sphingomonas* sp. strain ACM-3962 (Bourne *et al.*, 1996). Substrate specificity of the MC degrading enzymes to the Arg-Adda bond was also confirmed in another *Sphingomonas* sp., strain B9, by comparing Arg-Adda containing MCs (-LR, 3-DMC-LR, MC-LR-Cys, DHMC-LR, MC-RR) to MCs with a modified Adda residue (6(Z)-MC-LR, 6(Z)-MC-RR) or a substituted Arg residue (MC-LF) (Imanishi *et al.*, 2005). The Arg-Adda containing MCs were completely degraded after 48 hours, whereas Arg-6(Z)-Adda or Phe-Adda containing MCs were much less degraded (< 40 %). The current study also investigated degradation of the MC variant -RR and found it to be the least degraded MC (49 %) containing the Arg-Adda peptide bond. MC-RR contains two arginines in the variable positions and it has been suggested that this attribute causes steric hindrance and therefore reduces the rate of enzymatic attack (Lawton *et al.*, 2011).

The least degraded MC variants (-LF, -LW and -LY) were also the least polar MC variants. Polarity might play an important role in the cellular uptake of MCs for intracellular breakdown if the uptake of the MC peptides was to be a

prerequisite for their degradation. For most of the MC degraders it is unknown whether MC degradation occurs intracellular (hence, requires MC uptake) or extracellular. The study by Bourne *et al.* (2001) was the only study to give insights into the involvement of a transport mechanism in MC degradation. It was shown that *mlrD* of the *mlrABCD* gene cluster responsible for MC degradation in *Sphingomonas* sp. strain ACM-3962 encodes a protein that belongs to the PTR2 family of proton/oligonucleotide symporters. It was therefore predicted that MlrD is involved in the transport of MC-LR or its degradation products across the bacterial cell wall.

MC variants which had not been tested before were the demethylated forms DMC-RR and DMC-LR. It was surprising to see similar degradation between DMC-LR and the "standard" MC-LR while DMC-RR was much more degraded than the standard MC-RR. The current study was the first to test the degradation of the cyanobacterial protein phosphatase inhibitors anabaenopeptin A and B by *P. toxinivorans* (2015). These cyclic peptides are characterised by a phenylalanine in position 6 and homo-tyrosine in position 4 and vary in position 1 where type A contains tyrosine while type B contains arginine (Welker and Doehren, 2006). This structural difference might explain the lower degradation amount of anabaenopeptin A (5 %) compared to anabaenopeptin B (37 %) as tyrosine is less polar, possibly hindering cellular uptake.

To draw conclusion about the degradation of antimicrobial peptides and possible antibiotic resistance mechanisms in *P. toxinivorans* (2015) the cyclic heptapeptide antibiotic polymyxin B, structurally very similar to MCs, as well as gramicidin A were included in the degradation assay. Polymyxin B degradation however could not be evaluated due to insufficient solubility of the

peptide while degradation of gramicidin A did not occur. Also the cyclic fungal peptide cyclosporine A was included in the multiple peptide degradation assay but similar to polymyxin B evaluation of the peptide was hindered due to insufficient solubility.

What could however observed was the almost complete removal of the linear human peptides leucine enkephalin acetate salt hydrate and fibrinogen B. Their linear structure makes the peptides more accessible for bacterial degradation unlike the cyclic structure of oxytocin acetate salt hydrate which was with 17 % significantly less degraded.

To date only a few studies reported bacteria capable of degrading this wide range of MCs variants and NOD while other peptides of (cyano-) bacterial and human origin were mostly never evaluated. Imanishi *et al.* (2005) showed the ability of *Sphingomonas* sp. strain B-9 to rapidly degrade MC-LR, -RR, Mdha-modified -LR variants and NOD while similar to this study MC-LF was poorly degraded. Other bacteria, many of which were *Sphingomonas* sp., were also reported to degrade MCs but their range was limited or they lacked the ability to degrade NOD (Ishii *et al.*, 2004; Ho *et al.*, 2007; Chen *et al.*, 2010).

The present work demonstrated *P. toxinivorans* (2015) could degrade all twelve cyanobacterial peptides that were tested in the 96-well plate based degradation assay. This ability makes *P. toxinivorans* (2015) favourable for the use in water treatment applications, especially in waters where a range of cyanotoxins are recorded.

Findings of degradation intermediates revealed that indeed a single mechanism was involved in the breakdown of multiple MCs and NOD by *P. toxinivorans* (2015). This was evident from all intermediates showing the

occurrence of hydrolysis characteristic from the increase in *m/z* ratio by 18. The cyclic peptides were linearised making them less toxic and more susceptible to further degradation. Previous research demonstrated the bond between the Adda side chain and arginine in MCs and NOD to be most vulnerable (Bourne *et al.*, 1996; Edwards *et al.*, 2008). It is most probable that cleavage through hydrolysis occurred at this bond. As mentioned in chapter 4, MC degradation by *P. toxinivorans* (2015) showed the same characteristic breakdown products as previously described by Bourne *et al.* (1996) who's work focused on *Sphingomonas* sp. strain ACM-3962. The *mlr* genes of *Sphingomonas* sp. strain ACM-3962 involved in MC degradation however were not detected in *P. toxinivorans* (2015) (section 4.3.3).

6 Conclusion

The environmental conditions in the natural habitat or the laboratory is a main driving force that contributes to the shape of a bacterial genome and its regulation. Maintenance of bacteria under laboratory conditions, which often involves repeated sub-culturing and the use of nutrient rich growth media, can therefore alter bacterial behaviour. The current study showed the loss of MC-LR degradation activity by a number of phylogenetically different bacterial isolates following long-term sub-culturing in laboratory culture media. It was hypothesised that the provision of more readily available nutrients in the culture media induced a functional drift and that genes involved in MC-LR degradation were downregulated or subject to variation such as mutation. A simple and rapid 96-well plate based method was developed as a pivotal tool to test the effect of multiple culture media on MC-LR biodegradation using minimal amounts of materials such as MC-LR. Although, the influence of nutrients on bacterial MC-LR degradation was demonstrated, nutrient rich rather than poor media were shown to promote MC-LR degradation.
Evidence for functional and physiological drift of *P. toxinivorans* (2007) in response to prolonged laboratory maintenance was most striking. In addition to a significantly reduced capability to degrade MC-LR, the organisms' cell and colony morphology, its oxidase activity and its nutrient tolerance underwent changes. This was shown in comparison studies to the newly obtained *P. toxinivorans* (2015). The likelihood of a functional drift in bacterial MC-LR degradation supports the use of microbial consortia of various MC-LR degrading bacteria instead of single strains in a bioremediation perspective. MC-LR degradation assays on *P. toxinivorans* (2015) further allowed partial elucidation of its MC-LR degradation pathway through the discovery of MC-LR

breakdown products. One of the products corresponded to the complete Adda amino acid. The other two MC-LR breakdown products were identified as linearised (acyclo-) MC-LR of which one was remarkably similar to the hydrolysed MC-LR intermediate detected in *Sphingomonas* sp. strain ACM-3962 (Bourne *et al.*, 1996). This finding initiated screening for the *mlrABCD* genes involved in MC-LR degradation in the *Sphingomonas* sp. strain ACM-3962*,* however, no *mlr* gene homologues were detected. Further studies demonstrated the organism's capacity to degrade multiple MCs, NOD, anabaenopeptin-type peptides and structurally diverse human peptides and showed consistent MC hydrolysis as described by Bourne *et al.* (2001). This is the first study to give insights into the MC-LR biodegradation pathway of *P. toxinivorans,* potentially involving a new mechanism of MC and NOD degradation*.*

7 Future Outlook

Future studies aiming for the complete elucidation of the MC-LR degradation pathway in *P. toxinivorans* (2015) could make use of protease inhibitors to reveal further MC-LR intermediates and to classify the MC-LR degrading enzymes into general protease families.

Construction of a gene library and subsequent screening for expression of genes involved in MC-LR degradation would facilitate the identification of genes responsible for MC-LR degradation in *P. toxinivorans* (2015). Potentially, this would be the first study to report genes involved in MC-LR degrading other than the *mlr* genes. Their elucidation would further allow screening for genetic variations of gene homologues in *P. toxinivorans* (2007) which would explain its impaired MC-LR degradation activity. Alternatively, a transcriptional profile of *P. toxinivorans* (2015) delivered by gene expression microarrays or RNA seq (next-generation sequencing of cDNA) could be generated to elucidate genes involved.

To further investigate the organism's potential in cyanotoxin bioremediation degradation of MCs and NOD mixtures should be tested. This is of importance as many cyanobacterial blooms produce multiple cyanotoxin variants (Zurawell *et al.*, 2005). As shown in the current study, nutrient availability is also an influencing factor and it is crucial to determine nutrients that support bacterial growth and promote biodegradation. Nutrient availability in a bioremediation based process is influenced by its position in a water treatment cycle.

Concerning future studies on microorganisms promising for the utilisation in water treatment applications it is essential to preserve them prior to long-term laboratory maintenance to allow conservation of their activity. An additional approach could be supplementation of their growth media with peptides that

support expression of their MC degrading activity. Bourne *et al.* (1996) used peptone-yeast extract medium for this purpose.

8 References

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9 Appendix

Table 9.1 Components of media used in the 96-well plate based nutrient assay carried out on *P. toxinivorans* (2015), *P. toxinivorans* (2007), *Rhodococcus* sp. and *Pseudomonas* sp. (Chapter 4).

Luria-Bertani broth (LB) (Miller) (g/l)		**Nutrient broth (NB)** (Oxoid) (g/l)		**Reasoner´s 2A broth (R2A)** (LabM) (g/l)		**Mineral salt medium (MSM)** (Yang *et al.*, 2014) (g/l)	
Yeast Extract	5	Yeast Extract	2	Yeast Extract	0.5	$MgSO_4\ 7H_2O$	1.0
Sodium Chloride (NaCl)	10	Sodium Chloride (NaCl)	5	Sodium pyruvate	0.3	KH_2PO_4	0.5
Tryptone	10	Peptone	5	Meat Peptone	0.5	K_2HPO_4	4.0
		Lab-Lemco beef extract	1	Casamino Acids	0.5	NaCl	1.0
				Glucose	0.5	$CaCl_2$	0.02
				Starch	0.5	$FeSO_4$	0.005
				Dipotassium hydrogen		$MnCl_2\ 4H_2O$	0.005
				phosphate (K_2HPO_4)	0.3	Zink chloride ($ZnCl_2$)	0.005
				Magnesium sulphate		Copper(II) chloride	0.0005
				($MgSO_4$)	0.05	($CuCl_2$)	