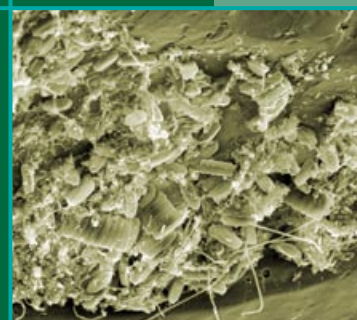




# Biological filtration processes for the removal of algal metabolites



Research Report

64

# **Biological filtration processes for the removal of algal metabolites**

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Biological filtration processes for the removal of algal metabolites

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## FOREWORD

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## EXECUTIVE SUMMARY

This project has identified biological filtration as a viable water treatment option for the removal of the algal metabolites 2-methylisoborneol (MIB), geosmin, cylindrosperopsin (CYN) and the microcystin toxins. Effective biological filtration of MIB and geosmin was observed at the Morgan water treatment plant in South Australia, and this was confirmed through laboratory-scale experiments. *Cylindrospermopsis raciborskii* and CYN removals were evident through a biologically-active filtration pilot plant located at North Pine Dam in Queensland, with removal of CYN shown to be through biodegradation. Microcystin toxins were shown to be effectively biodegraded through laboratory-scale columns containing sand sourced from various water treatment plants, under both slow and rapid sand filtration conditions.

Bacteria responsible for the degradation of geosmin, CYN and microcystin within biological filters were isolated and identified, and also shown to have the ability to degrade their respective metabolites in natural waters in planktonic states. A consortium comprising a *Sphingopyxis* sp., *Novosphingobium* sp. and *Pseudomonas* sp. were shown to be responsible for degrading geosmin, with degradation evident only when all three organisms were present. In contrast, individual bacteria were shown to be responsible for the degradation of microcystin (*Sphingopyxis* sp. LH21) and CYN (*Sinorhizobium* sp.).

Lag periods were shown to exist prior to the onset of degradation of the metabolites. However, upon re-exposure of the organisms to the metabolites, lag periods were reduced, and in some cases eliminated. For example, a lag period of 2 days was evident in planktonic batch studies prior to the degradation of microcystins commencing; however, after continuous exposure of the bacteria to microcystins the lag period was eliminated with complete degradation of microcystins observed within 5 hours.

The complete removal of MIB and geosmin through the sand filters of the Morgan WTP confirms that removal of these metabolites can be achieved under normal WTP operating conditions. However, this was only observed when no disinfectant was introduced into the filters. In addition, it appears that the size of the sand filter particles may play an important role in efficient MIB and geosmin removal, with greater removal with smaller particle size. The situation at the Morgan WTP should be of particular interest to SA Water and potentially other water utilities as this plant has demonstrated effective biofiltration of MIB and geosmin. Furthermore, the sand from the filter beds of Morgan WTP was also shown to be highly effective in removing microcystin toxins in laboratory-scale experiments, highlighting the diverse metabolite-degrading microbial community that can exist within the biofilm of WTP sand filters.

In laboratory-scale experiments microcystin appeared to be readily removed through a range of sand filters under both slow and rapid sand filtration conditions, confirming that removal of this metabolite could occur under normal plant operating conditions. Furthermore, the fact that no cytotoxic by-products of microcystin biodegradation were detected demonstrates the feasibility of biodegradation as a possible removal option for the microcystins.

The development of molecular tools such as PCR has allowed for the detection of the genes involved in the degradation of microcystin. Using this technology we now have the capability to evaluate biological filters in terms of their capacity to remove microcystin, prior to impending microcystin-producing blooms. This is of enormous value to water authorities as it will enable them to make confident decisions as to whether they can rely on their filters as an effective treatment barrier for these toxins.

## TABLE OF CONTENTS

<b>FOREWORD</b> .....	3
<b>EXECUTIVE SUMMARY</b> .....	4
<b>TABLE OF CONTENTS</b> .....	5
<b>LIST OF FIGURES</b> .....	9
<b>LIST OF TABLES</b> .....	12
<b>ABBREVIATIONS</b> .....	13
<b>Chapter 1: Introduction</b> .....	14
1.1 Background .....	14
1.2 Approach .....	16
1.3 Objectives .....	17
<b>Chapter 2: Biological removal of MIB and geosmin through rapid gravity filters</b> .....	18
2.1 Introduction .....	18
2.2 Full-scale study .....	19
2.2.1 Morgan water treatment plant .....	19
2.2.2 Geosmin in Morgan water treatment plant .....	20
2.3 Validation by laboratory scale biological sand filters .....	21
2.3.1 Materials and methods .....	21
2.3.2 Results and discussion .....	22
2.4 Conclusions .....	24
2.5 References .....	24
<b>Chapter 3: Removal of geosmin and MIB through biologically-active sand filters</b> .....	25
3.1 Introduction .....	25
3.2 Materials and methods .....	25
3.2.1 Experimental design and apparatus .....	25
3.2.2 Analytical methods .....	26
3.3 Results and discussion .....	27
3.3.1 Columns with established biofilm .....	27
3.3.2 Columns with developing biofilm .....	28
3.3.3 Biofilm studies .....	30
3.4 Summary and conclusions .....	30
3.5 References .....	31
<b>Chapter 4: Biodegradation rates of MIB and geosmin through sand filters and in bioreactors</b> .....	32
4.1 Introduction .....	32
4.2 Materials and methods .....	33
4.2.1 Chemicals and reagents .....	33
4.2.2 Laboratory column experiments .....	34
4.2.3 Bacterial enumeration .....	34
4.2.4 Batch bioreactor experiments .....	34
4.2.5 Identification of MIB and geosmin degraders .....	34
4.2.6 Analysis of MIB and geosmin .....	35
4.3 Results and discussion .....	35
4.3.1 Biological filtration of MIB and geosmin .....	35
4.3.2 Batch biodegradation of MIB and geosmin .....	38

4.3.3 Identification of MIB and geosmin degraders.....	40
4.4 Conclusions .....	42
4.5 References.....	42
<b>Chapter 5: Cooperative biodegradation of geosmin by a consortium comprising three gram-negative bacteria isolated from the biofilm of a sand filter column .....</b>	<b>44</b>
5.1 Introduction .....	44
5.2 Materials and methods.....	44
5.2.1 Bacterial enumeration .....	44
5.2.2 Geosmin analysis.....	45
5.2.3 Removal of geosmin through a biologically-active sand filter column .....	45
5.2.4 Enrichment of geosmin-degrading bacteria .....	45
5.2.5 Denaturing gradient gel electrophoresis analysis of the enrichment culture .....	45
5.2.6 Phylogenetic analysis of isolated bacteria .....	46
5.2.7 Geosmin degradation by bacterial isolates.....	46
5.3 Results .....	46
5.3.1 Removal of geosmin through a biologically-active sand filter column .....	46
5.3.1 Enrichment of geosmin-degrading bacteria .....	46
5.3.2 DGGE analysis of the enrichment culture.....	47
5.3.3 Phylogenetic analysis of isolated bacteria .....	48
5.3.4 Geosmin degradation by bacterial isolates .....	49
5.4 Discussion.....	49
5.5 References.....	50
<b>Chapter 6: Enhancing the biofiltration of geosmin by seeding sand filter columns with a consortium of geosmin-degrading bacteria .....</b>	<b>52</b>
6.1 Introduction .....	52
6.2 Experimental procedures .....	52
6.2.1 Materials.....	52
6.2.2 Culture of geosmin-degrading bacteria.....	53
6.2.3 Flow cytometry for bacterial enumeration .....	53
6.2.4 Laboratory-scale sand column experiments .....	53
6.2.5 Measurements of bacterial attachment.....	54
6.3 Results and discussion .....	55
6.3.1 Geosmin removal through sand columns with pre-sterilised biofilm.....	55
6.3.2 Geosmin removal through sand columns in the absence of an existing biofilm .....	57
6.3.3 Geosmin removal through sand columns containing an active biofilm.....	58
6.4 Conclusions .....	60
6.5 References.....	60
<b>Chapter 7: Bacterial degradation of microcystin toxins within a biologically-active sand filter .....</b>	<b>62</b>
7.1 Introduction .....	62
7.2 Experimental methods .....	63
7.2.1 Materials and reagents.....	63
7.2.2 Laboratory column experiments.....	63
7.2.3 Analysis of microcystin.....	64
7.2.4 Molecular techniques .....	65
7.3. Results and discussion .....	65
7.3.1 Biological filtration of microcystin .....	65
7.3.2 Detection of the microcystin degrading gene, <i>mlrA</i> .....	68
7.4 Conclusions .....	68

7.5 References.....	68
<b>Chapter 8: Degradation of microcystin through biological sand filters .....</b>	<b>71</b>
8.1 Introduction .....	71
8.2 Materials and methods.....	72
8.2.1 Chemicals and reagents .....	72
8.2.2 Biological filtration experiments .....	72
8.2.3 Identification of microcystin degraders.....	73
8.2.4 Microcystin analysis .....	73
8.3 Results and discussion .....	73
8.3.1 Biological filtration of microcystin .....	73
8.3.2 Isolation of microcystin-degrading bacteria.....	76
8.4 Conclusions .....	77
8.5 References.....	78
<b>Chapter 9: Discriminating and assessing the adsorption and biodegradation removal mechanisms during granular activated carbon filtration of microcystins .....</b>	<b>80</b>
9.1 Introduction .....	80
9.2 Experimental methods .....	80
9.2.1 Materials and reagents.....	80
9.2.2 Laboratory column experiments.....	81
9.2.3 Biodegradation experiments .....	81
9.2.4 Microcystin analyses .....	82
9.2.5 Dissolved organic carbon analysis.....	82
9.3 Results and discussion .....	83
9.3.1 Microcystin removal through the laboratory columns .....	83
9.3.2 Modelling the adsorption of microcystin.....	85
9.3.3 Assessing the biodegradation of microcystin.....	87
9.4 Conclusions .....	89
9.5 References.....	89
<b>Chapter 10: Isolation and identification of a microcystin-degrading bacterium from a biological sand filter.....</b>	<b>91</b>
10.1 Introduction .....	91
10.2 Experimental procedures .....	93
10.2.1 Materials and reagents.....	93
10.2.2 Analysis of microcystins .....	93
10.2.3 Isolation of microcystin-degrading bacteria.....	93
10.2.4 Detection of genes involved in microcystin degradation .....	93
10.2.5 Phylogenetic analysis of isolated bacteria .....	94
10.2.6 Batch degradation experiments .....	94
10.2.7 Effect of temperature and bacterial density on microcystin degradation .....	95
10.3 Results and discussion .....	95
10.3.1 Isolation of microcystin-degrading bacteria.....	95
10.3.2 Phylogenetic analysis of isolate LH21 .....	95
10.3.3 Microcystin degradation in batch experiments.....	96
10.4 Conclusions .....	102
10.5 References.....	103
<b>Chapter 11: Bacterial degradation of microcystin toxins in drinking water eliminates their toxicity .....</b>	<b>104</b>
11.1 Introduction .....	104



## BIOLOGICAL FILTRATION PROCESSES FOR THE REMOVAL OF ALGAL METABOLITES

11.2 Experimental procedures .....	104
11.3 Results and discussion .....	105
11.4 Conclusions .....	107
11.5 References .....	107
<b>Chapter 12: Biological filtration processes for the removal of cylindrospermopsin .....</b>	<b>108</b>
12.1 Introduction .....	108
12.2 Materials and methods .....	108
12.2.1 Study site – North Pine Dam .....	108
12.2.2 Biofiltration of <i>C. raciborskii</i> cells and CYN .....	110
12.2.3 Batch biodegradation of CYN .....	110
12.3 Results and discussion .....	110
12.3.1 Biofiltration of <i>C. raciborskii</i> cells .....	110
12.3.2 Biofiltration of dissolved CYN .....	111
12.3.3 Batch biodegradation of CYN .....	112
12.4 Conclusions .....	114
12.5 References .....	114
<b>Chapter 13: Isolation and identification of a cylindrospermopsin-degrading bacterium from a biological filtration plant .....</b>	<b>116</b>
13.1 Introduction .....	116
13.2 Materials and methods .....	116
13.2.1 Enrichment and isolation of CYN-degrading bacteria .....	116
13.2.2. Phenotypic identification of CYN-degrading bacteria .....	117
13.2.2 Genotypic identification of CYN-degrading bacteria .....	117
13.3 Results and discussion .....	118
13.3.1 Enrichment and isolation of CYN-degrading bacteria .....	118
13.3.2. Identification of the CYN-degrading bacterium SW1 .....	120
13.4 Conclusions .....	121
13.5 References .....	121
<b>Chapter 14: Summary and conclusions .....</b>	<b>123</b>
<b>Acknowledgements .....</b>	<b>125</b>
<b>Appendix I: Publications arisen from this project .....</b>	<b>126</b>
Peer reviewed international journal articles .....	126
Peer reviewed industry journal articles .....	126
Conference proceedings and/or presentations .....	126
Theses, reports, book chapters .....	127

## LIST OF FIGURES

Figure 1.1 Molecular structures of MIB and geosmin.....	14
Figure 1.2 Molecular structure of microcystin-LR, highlighting the variable amino acids leucine and arginine.....	15
Figure 1.3 Molecular structure of cylindrospermopsin. ....	15
Figure 2.1 Morgan WTP schematic prior to chloraminated backwash.....	19
Figure 2.2 SEM of Morgan filter sand, August 2004. ....	20
Figure 2.3 Geosmin concentration in Morgan WTP. ....	21
Figure 2.4 MIB and geosmin removal in Morgan filter sand sampled before CBW. ....	22
Figure 2.5 MIB and geosmin removal in Morgan filter sand taken during CBW. ....	23
Figure 2.6 MIB and geosmin removal in Morgan filter sand taken post-CBW, January 2007. ....	23
Figure 3.1 Geosmin removal in established biofilm columns. ....	27
Figure 3.2 MIB removal in established biofilm columns. ....	28
Figure 3.3 Geosmin removal in developing biofilm columns.....	29
Figure 3.4 MIB removal in developing biofilm columns.....	29
Figure 4.1 Removal of taste and odour (T&O) compounds MIB and geosmin through: (a) unsterilised sand filter and (b) pre-sterilised sand filter.....	36
Figure 4.2 MIB and geosmin removal between days 22 and 56 in the pre-sterilised sand filter. Insert: Pseudo-first-order kinetic plots for the MIB and geosmin removal curves.....	37
Figure 4.3 Batch degradation of: (a) MIB and (b) geosmin in treated Morgan water.....	39
Figure 4.4 DGGE analysis of the enrichment culture supplemented with geosmin as the sole carbon source. M; reference marker, Neg; negative control, band 1 <i>Pseudomonas</i> sp., band 2 <i>Alphaproteobacterium</i> , band 3 <i>Sphingomonas</i> sp., and band 4 <i>Acidobacteriaceae</i> . ....	41
Figure 5.1 Active bacterial abundance and geosmin concentration during (a) the enrichment of geosmin-degrading bacteria and (b) the study of geosmin degradation by the bacterial consortium comprising Geo24, Geo25 and Geo33. Error bars represent standard deviations of triplicate analyses. ....	47
Figure 5.2 Denaturing gradient gel electrophoresis (DGGE) profiles of 16S rRNA gene-directed PCR fragments from bacteria within the enrichment culture at various stages of incubation (left panel) and isolates Geo24, Geo25 and Geo33 (right panel). ....	48
Figure 5.3 Degradation of geosmin in reservoir water by the bacterial consortium. Error bars represent standard deviations of triplicate analyses. ....	49
Figure 6.1 Schematic of the laboratory sand column apparatus.....	54
Figure 6.2 Geosmin removal through the sand columns containing an autoclaved pre-existing biofilm (trial 1 and trial 1 repeat). ....	56

## BIOLOGICAL FILTRATION PROCESSES FOR THE REMOVAL OF ALGAL METABOLITES

Figure 6.3 Biomass activities in the sand columns containing an autoclaved pre-existing biofilm (trial 1). Error bars represent standard deviations from triplicate analyses. ....	57
Figure 6.4 Geosmin removal through the virgin sand columns without an existing biofilm (trial 2). ....	58
Figure 6.5 Biomass activities in the virgin sand columns without an existing biofilm (trial 2). Error bars represent standard deviations from triplicate analyses. ....	58
Figure 6.6 Geosmin removal and biomass activities in the sand columns containing an active pre-existing biofilm (trial 3). Error bars for the ATP data represent standard deviations from triplicate analyses. ....	59
Figure 7.1 Schematic of the laboratory sand column experiments. ....	64
Figure 7.2 Biological filtration of microcystin-LR (MCLR) and –LA (MCLA) through: (a) laboratory sand columns A and B; (b) laboratory sand column C. Error bars represent standard deviations from duplicate measurements. ....	66
Figure 7.3 Biomass concentration and leucine aminopeptidase (LAP) enzyme activity as a function of three separate sand portions of column A. Fresh sand signifies sand obtained from the Morgan WTP which was not used in the column experiments. Error bars represent standard deviations from triplicate measurements. ....	67
Figure 8.1 Removal of microcystin-LR (MCLR) through biological filters TM1 (autoclaved Morgan sand/Myponga Reservoir water) and TM2 (Morgan sand/Myponga Reservoir water). ....	74
Figure 8.2 Removal of microcystin-LR (MCLR) through biological filters BM1 (Morgan sand/Morgan treated water), BM2 (Clean Morgan sand/Morgan treated water) and BM3 (Clean Myponga sand/Morgan treated water). ....	75
Figure 8.3 Removal of microcystin-LR (MCLR) through biological filter JW1 (Happy Valley sand/Happy Valley treated water). ....	75
Figure 8.4 Removal of microcystin-LR (MCLR) through biological filter BS1 (autoclaved Morgan sand/Myponga Reservoir water at 10°C). ....	76
Figure 8.5 Detection of <i>mlrA</i> gene by PCR: Lane 1 - DNA reference marker (100 bp ladder); Lane 2 - <i>Sphingomonas</i> sp. ACM-3962 (positive control); Lane 3 - <i>Sphingopyxis</i> sp. Geo24; Lane 4 - <i>Novosphingobium</i> sp. Geo25; Lane 5 - Isolate LH21 from TM2 filter biofilm; Lane 6 - Milli Q water; Lane 7 - DNA reference marker (100 bp ladder). ....	77
Figure 9.1 Schematic of the laboratory column experiments. ....	82
Figure 9.2 Removal of MCLR (above) and MCLA (below) through the conventional GAC, sterile GAC and sand columns; the dashed area represents the onset of biodegradation in the sand column. ....	84
Figure 9.3 Dissolved organic carbon (DOC) removal through the conventional GAC, sterile GAC and sand columns. ....	84
Figure 9.4 Scanning electron micrographs (SEM) of the surface of the sand (left, 284x magnification) and the GAC (right, 289x magnification) particles. ....	86
Figure 9.5 Conceptual illustration of a GAC particle in the sterile GAC and conventional GAC columns. ....	86
Figure 9.6 Homogenous surface diffusion model (HSDM) fit of the sterile GAC and the conventional GAC experimental data. ....	87

Figure 9.7 Biodegradation of MCLR in bioreactors incubated at 22, 25, 30 and 40°C. Each bioreactor employed an initial bacterial inoculum concentration of $7.6 \times 10^6$ active bacteria mL <sup>-1</sup> .....	88
Figure 9.8 Biodegradation of MCLR using initial bacterial inoculum concentrations of $7.6 \times 10^7$ , $3.8 \times 10^7$ , $1.5 \times 10^7$ , $7.6 \times 10^6$ , $3.0 \times 10^6$ and $1.5 \times 10^6$ active bacteria mL <sup>-1</sup> . Each bioreactor was incubated at 25°C.....	89
Figure 10.1 Neighbour-joining phylogenetic tree showing the position of isolate LH21 (bold) in relation to other closely related species, including previously reported microcystin-degrading bacteria (underlined). Accession numbers correspond to partial sequences of 16S rRNA. Numerical tree values represent bootstrap support. Scale bar represents expected changes per site.....	97
Figure 10.2 Batch degradation of microcystin variants: (A) MCLR and (B) MCLA, in Myponga Reservoir water using isolate LH21 as the sole bacterial inoculum. Error bars represent standard error from duplicate measurements. ....	98
Figure 10.3 Batch degradation of microcystin variants: (A) MCLR and (B) MCLA, in Myponga Reservoir water using <i>Sphingomonas</i> ACM-3962 as the sole bacterial inoculum. Error bars represent standard error from duplicate measurements.....	98
Figure 10.4 Effect of temperature on the batch degradation of microcystin variants: (A) MCLR and (B) MCLA in Myponga Reservoir water. Effect of bacterial density on the batch degradation of microcystin variants: (C) MCLR and (D) MCLA in Myponga Reservoir water. Error bars represent standard error from duplicate measurements. ....	100
Figure 10.5 Bacterial abundance (as determined by flow cytometry) of isolate LH21 during batch degradation of MCLR and MCLA in Myponga Reservoir water. Error bars represent standard error from duplicate measurements.....	101
Figure 10.6 Batch degradation of microcystin variants: (A) MCLR and (B) MCLA in Myponga Reservoir water using isolate LH21 as the sole bacterial inoculum on day 15. Error bars represent standard error from duplicate measurements. ....	102
Figure 10.7 Active bacterial abundance and MCLR concentration during the batch degradation of MCLR in B3 using isolate LH21 as the sole bacterial inoculum. The temperature was increased to 22°C on day 15. Error bars represent standard error from duplicate measurements.....	102
Figure 11.1 Microcystin-LR and -LA (MCLR and MCLA) concentrations as a function of time in bioreactors BR1 and BR2. Inset: Zoom of BR1 data at a lower y-axis scale.....	106
Figure 11.2 Comparison of microcystin detection by high performance liquid chromatography (HPLC), protein phosphatase 2A (PP2A) and cytotoxicity (MTT) assays. Results presented as percent of control at zero-time. Error bars represent standard deviation from triplicate analyses. ....	106
Figure 12.1 Biologically-active filtration plant (BAFP) at North Pine Dam, Queensland showing reservoir holding tank, roughing filter (RF) and sand filter (SF).....	109
Figure 12.2 Aggregates (3 different sizes, RF1-20mm, RF2-10mm, RF3-5mm) located in horizontal roughing filter.....	109
Figure 12.3 Monitoring of <i>C. raciborskii</i> cells through the BAFP.....	111
Figure 12.4 Monitoring of dissolved CYN through the BAFP.....	112
Figure 12.5 The time taken for complete biodegradation of CYN over a 12 month period in laboratory batch experiments using inocula sourced from the BAFP. ....	113
Figure 12.6 Acclimation (lag) periods prior to the biodegradation of CYN commencing over a 12 month period in laboratory batch experiments using inocula sourced from the BAFP. ....	114

Figure 13.1 Enrichment of CYN-degrading organisms. ....	119
Figure 13.2 Degradation of CYN by bacterial isolate SW1. ....	120
Figure 13.3 Neighbour-joining phylogenetic tree showing the position of isolate SW1 in relation to other <i>Sinorhizobium</i> species based on 16S rRNA gene sequence. ....	121

## LIST OF TABLES

Table 3.1 Sand filter columns. ....	26
Table 3.2 Bacterial numbers on developing biofilm columns. ....	30
Table 4.1 Microorganisms implicated in the biodegradation of MIB and geosmin. ....	32
Table 4.2 Characteristics of treated Morgan water and Morgan filter sand. ....	33
Table 4.3 Bioreactor conditions employed for the batch biodegradation of MIB and geosmin. ....	34
Table 4.4 Pseudo-first-order rate constants ( $k$ ) for the biodegradation of MIB and geosmin in treated Morgan water. Correlation coefficients ( $R^2$ ) presented in parentheses. ....	40
Table 6.1 Geosmin-degrading consortium numbers employed during inoculation into sand column trials. ....	54
Table 7.1 Characteristics of Myponga Reservoir water and Morgan filter sand. ....	63
Table 7.2 Laboratory sand column conditions. ....	64
Table 8.1. Microorganisms implicated in the degradation of microcystin toxins. ....	71
Table 8.2 Characteristics of sample waters and sands. ....	72
Table 8.3 Conditions employed for the laboratory biological filtration experiments. ....	73
Table 10.1 Bacteria implicated in the degradation of microcystin toxins. ....	92
Table 10.2 Oligonucleotide primer sequences and their characteristics for the genes <i>mlrB</i> , <i>mlrC</i> and <i>mlrD</i> and spanning genes <i>mlrC-mlrA</i> , <i>mlrA-mlrD</i> and <i>mlrD-mlrB</i> . ....	94

**ABBREVIATIONS**

ATP	Adenosine triphosphate
BAFP	Biologically-active filtration plant
CYN	Cylindrospermopsin
DGGE	Denaturing gradient gel electrophoresis
DOC	Dissolved organic carbon
EBCT	Empty bed contact time
GAC	Granular activated carbon
HPLC	High performance liquid chromatography
MCLR	Microcystin-LR
MCLA	Microcystin LA
MIB	2-methylisoborneol
NOM	Natural organic material
NPD	North Pine Dam
PAC	Powdered activated carbon
PCR	Polymerase chain reaction
SEM	Scanning electron microscopy
T&O	Taste and odour
WTP	Water treatment plant

## CHAPTER 1: INTRODUCTION

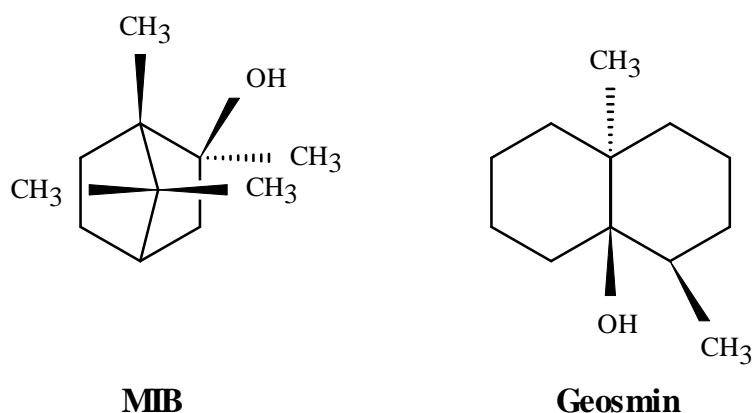
### 1.1 Background

This project addresses the important issue of biological treatment of blue-green algal (cyanobacterial) metabolites, in particular filtration through biologically-active media. With the ongoing concern regarding the addition of chemicals to our water supply, and the potential by-products of oxidation processes, biological treatment techniques are becoming more attractive to water suppliers and the general public. However, for the confident application of biological techniques to the removal of cyanobacterial metabolites it is essential that the optimum conditions are known, and the complete removal of the potentially harmful organic compounds is guaranteed. At present this is not possible, and this lack of knowledge is directly addressed within this report.

The major compounds of interest within this project are the algal metabolites:

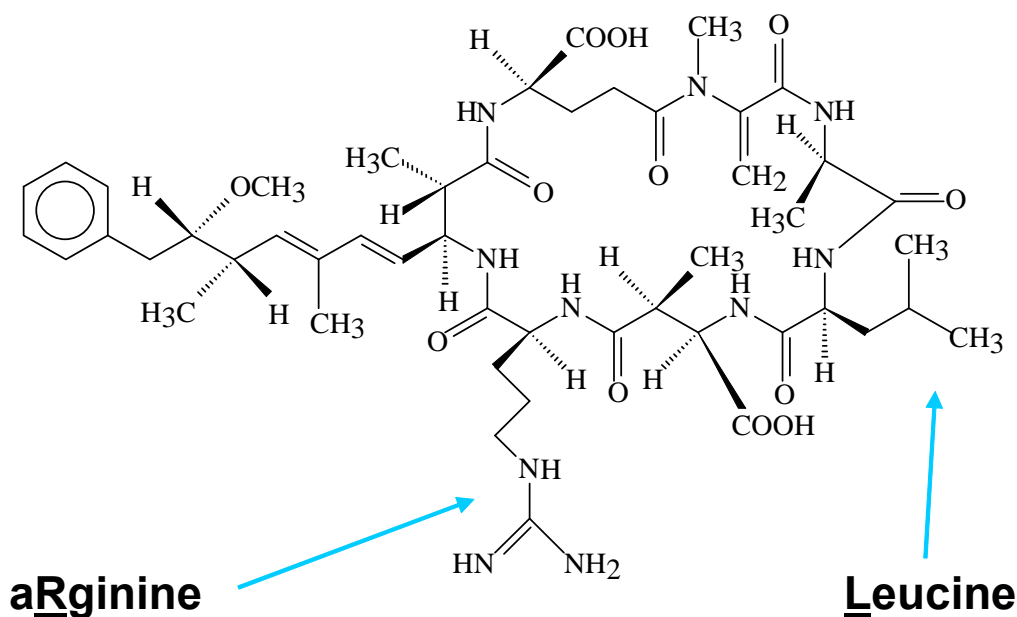
- 2-methylisoborneol (MIB) and geosmin
- microcystin toxins
- cylindrospermopsin

MIB and geosmin are cyclic aliphatic tertiary alcohols (see Figure 1.1) produced by a range of organisms as secondary metabolites. They are the most common naturally occurring taste and odour compounds worldwide, and are particularly problematic as they can be perceived by the consumer in drinking water at levels as low as 5-10 ng L<sup>-1</sup>. Oxidation processes such as chlorination and ozonation are not entirely effective for the removal of these compounds, and activated carbon adsorption is adversely affected by the presence of natural organic material (NOM). NOM is present at much higher levels than the odour compounds (mg compared with ng levels) and competes very strongly for adsorption sites reducing the lifetime of granular activated carbon (GAC) filters, and increasing the doses of powdered activated carbon (PAC) required to remove these compounds.



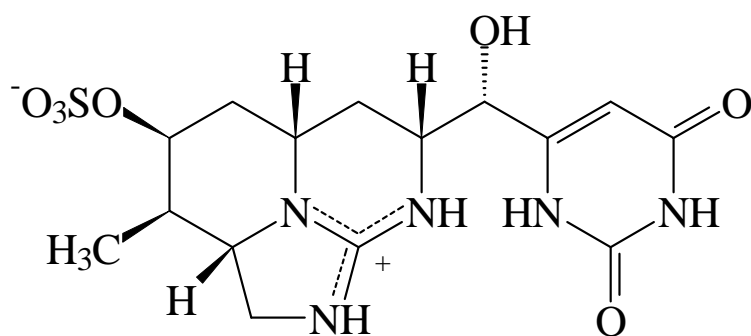
**Figure 1.1** Molecular structures of MIB and geosmin.

The microcystins are the most commonly reported of the cyanobacterial toxins world-wide. They are cyclic heptapeptides consisting of seven amino acid groups, two of which are variable. Minor variations to the other amino acids are also seen in some variants. The main mode of toxicity is liver damage due to protein phosphatase inhibition; however, microcystins have also been shown to promote the formation of cancerous tumours. The most common of the over seventy known variants of the toxin, microcystin-LR (MCLR), incorporates Leucine and arginine in the variable positions (Figure 1.2). Whilst this variant is the most common, the great majority of the cyanobacterial blooms producing microcystins will produce a range of the toxins. Some blooms have been found to contain no MCLR, while others have some of the other variants as the major components. Therefore, any investigation into the effect of water treatment processes on microcystins should include a range of the most commonly found variants.



**Figure 1.2** Molecular structure of microcystin-LR, highlighting the variable amino acids leucine and arginine.

Cylindrospermopsin (CYN) is an alkaloid cytotoxin produced mainly by the freshwater cyanobacteria *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*. The presence of high levels of CYN in drinking water can cause liver, kidney and gastrointestinal damage. It has also recently been implicated as a cancer-causing agent. Originally thought to be mainly an issue in tropical areas, this toxin is now reported regularly in more temperate regions. Figure 1.3 shows the molecular structure of cylindrospermopsin.



**Figure 1.3** Molecular structure of cylindrospermopsin.

Microcystins and CYN are susceptible to oxidation processes, given sufficiently high doses and contact times; however for compounds of such significant health concern, a multi-barrier approach to treatment of at least two levels is essential for the confident provision of toxin-free water.

These three groups of metabolites are considered to be of major interest to water suppliers internationally, and a low cost, low technology, reliable treatment option such as biological filtration would be of enormous value to the international water community.

There are many advantages to this type of treatment. Biological processes generally:

- Are of low technology, requiring relatively little maintenance and are therefore potentially of significant interest to regional and/or remote communities, and less advanced communities internationally



- Require relatively low infrastructure and running costs
- Do not require additional treatments and are therefore processes involving the *removal* of contaminants without the *addition* of chemicals that in themselves may have potential, or perceived health effects, or may produce by-products that are undesirable in drinking water.

Factors that have a major impact on the removal of organic compounds through biological filtration processes include:

- Variety and numbers of microorganisms present, biomass
- Temperature
- Water quality (pH, dissolved organic carbon character and concentration, alkalinity)
- Filter contact time, hydraulic loading
- Filter media

## 1.2 Approach

The questions that were addressed within this project, and important issues related to each are given below:

### **1. Can we identify individual bacteria capable of degrading the compounds? Are they present in a range of waters?**

*Issues:* Clearly the most important aspect of biological removal of organic compounds is the microbiology of the filter itself. If the biofilm is effectively degrading the compounds then the aim must be to identify the microorganisms, and determine the mechanism of degradation, ie. whether the degradation takes place by one particular microorganism, a number of individual microbes, or whether a mixed population is required for effective removal. In addition, whether the microorganisms are present in most waters, or different microorganisms will be responsible for the removal in different waters.

### **2. Will there be a "lag period", or delay, between when the biofilm first "sees" the compounds, and when it is capable of degrading them?**

*Issues:* A lag period of up to several weeks is often reported for the biodegradation of microcystins, and has been seen for the degradation of the other metabolites. This is of course a major hindrance to the confident application of biological filtration processes to the removal of transient contaminants such as algal metabolites. An effort was made to determine the origin of the lag period,. For example, it could be caused by the acclimation of individual microbes to the use of the compounds as a secondary substrate, or by the increase in numbers of degrading bacteria. In the latter case it might be expected that the metabolites are being used as a primary substrate in contrast to the secondary substrate role most microcontaminants are thought to play. The aim was to understand the cause of the lag period, and thereby work to minimise, or preferably eliminate, delay in biodegradation.

### **3. Under what conditions is biofiltration likely to be a viable option for removal of these metabolites? And can we predict whether a biological filter would be effective for the removal of a transient episode of the metabolites?**

*Issues:* These are perhaps the most important questions to be answered on this topic. If we cannot answer them, biofiltration could not be recommended as a reliable treatment method for the removal of CYN, for example. When we have established the parameters required in 2, above, we will still require tools to predict whether the biofilm will be capable of removing transient loads of the metabolites, as under normal circumstance they would not be present continuously. Application and development of methods for the measurement of biomass and identification and enumeration of microorganisms within the biofilms were required. The effect of operational parameters such as contact time, temperature, water quality were investigated in relation to the effective removal of the metabolites. The aim of this section was to produce guidelines for the use of biofiltration for the removal of these particular compounds.

### **4. Does ozone pre-treatment enhance the removal?**

*Issues:* Ozone has been shown to increase the biodegradability of NOM, often leading to enhanced removal of NOM across biologically-active filters. There is no evidence to date to suggest that these treatments would result in improved removal of other compounds, although the assumption among

water authorities is that they should. Removal of the metabolites across biologically-active filters was investigated in the presence and absence of ozone pre treatment.

***5. Is it possible to modify the biofilm and/or conditions to produce maximum removal in less than optimal conditions?***

*Issues:* This question was investigated during a range of seeding experiments using known degrading bacteria.

### **1.3 Objectives**

The major objectives of this project are:

- The identification of the microorganisms largely responsible for degradation of the metabolites in biological filters
- The establishment of design criteria/operating guidelines for the optimisation of biological filtration processes for the removal of algal metabolites
- The determination of the feasibility of "artificially" encouraging filters to function in an optimised biological treatment mode, either by seeding with appropriate bacteria, or spiking with a substrate known to promote the assimilation of the metabolites.

## CHAPTER 2: BIOLOGICAL REMOVAL OF MIB AND GEOSMIN THROUGH RAPID GRAVITY FILTERS\*

### 2.1 Introduction

The presence of adverse tastes and odours is a constant challenge to water treatment facilities. Consumers generally judge drinking water by aesthetic qualities such as taste, odour, colour and clarity. Problems with taste and odour often lead to decreased confidence in municipal drinking water supplies and increased complaints to the water authorities. Of particular concern are the musty-earthly odours produced by the tertiary alcohols 2-methylisoborneol (MIB) and geosmin. MIB and geosmin are produced by a range of cyanobacteria (blue-green algae) and actinomycetes and are found in surface waters all over the world. Although non-toxic, they create many difficulties for plant operations. The conventional treatment process of coagulation, flocculation, sedimentation, rapid filtration and disinfection is quite effective for removing intact cyanobacterial cells and hence intracellular MIB and geosmin. However, dissolved (extracellular) MIB and geosmin are not efficiently removed and can be detected by some consumers at below 10 ng L<sup>-1</sup>. Additional treatment processes capable of targeting very low concentrations are required.

The most common treatment used in Australia for the removal of these compounds is powdered activated carbon (PAC). PAC is an adsorption medium, and is generally added before, or during, the flocculation step of the conventional treatment process. It can then be removed downstream by sedimentation and filtration. PAC is advantageous as it can be used only as required and at doses specific to the inlet concentration of MIB or geosmin. However, PAC has disadvantages in that its effectiveness is greatly hampered by competition for adsorption sites with the more predominant natural organic material (NOM), and removal does not always occur as predicted. This means that large volumes of PAC are required, causing increased sludge load for the plant and operational costs as high as \$4000 - \$8000 per day (based on a production capacity of 200 ML per day and PAC costs of \$20-40 per ML, depending on water quality, inlet concentrations and the type of PAC used). The problems associated with PAC are increased as Australia's current drought situation leads to decreased water quality and subsequently more frequent algal blooms. Clearly, a cheaper, more reliable method for taste and odour removal is required.

A number of studies have shown the promise of biological treatment for MIB and geosmin removal (Hrudey et al., 1995; Huck et al., 1995; Nerenberg et al., 2000; Elhadi et al., 2004b; Elhadi et al., 2006). It is well established that MIB and geosmin can be biodegraded by a variety of naturally occurring microorganisms (Danglot et al., 1983; Izaguirre et al., 1988; Ishida et al., 1992; Hoefel et al., 2006). An ideal method to implement this process is biological filtration. Here, the filter medium is the carrier for the bacteria in the form of a biofilm. Biological filtration has many advantages. It does not require the addition of any compounds which may themselves produce toxic or odourous by-products. It is also a low maintenance process and requires few modifications to existing plant operation. Additionally, biological filtration aids in the removal of biodegradable compounds which can affect biological re-growth in the distribution system, thus reducing the chlorine demand of the product water.

The type of medium used in biological filtration will greatly impact its efficacy. A large proportion of studies on biological filtration have been conducted on granular activated carbon (GAC), as its porous, rough surface makes it an ideal carrier for biomass. However, GAC is expensive. Biological rapid sand filtration is a much more cost-effective option and is already the filtration process used in the majority of Australian water filtration plants. Unfortunately, sand is not the optimum media for biological filtration, as its smooth surface texture and the lack of crevices provide little opportunity for biofilm attachment. However, a number of studies have shown that it has promise. A recent study (Metz et al., 2006) showed that a rapid sand filter with no pre-chlorination was capable of 80-90% removal of MIB and 50% removal of geosmin over a period of 6 years. In another study, a biologically-active rapid

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\* This chapter is based on the following manuscript:

McDowall B., Ho L., Saint C.P. and Newcombe G. (2007) Biological removal of MIB and geosmin through rapid gravity filters. *Water* **34**(7), 48-54.

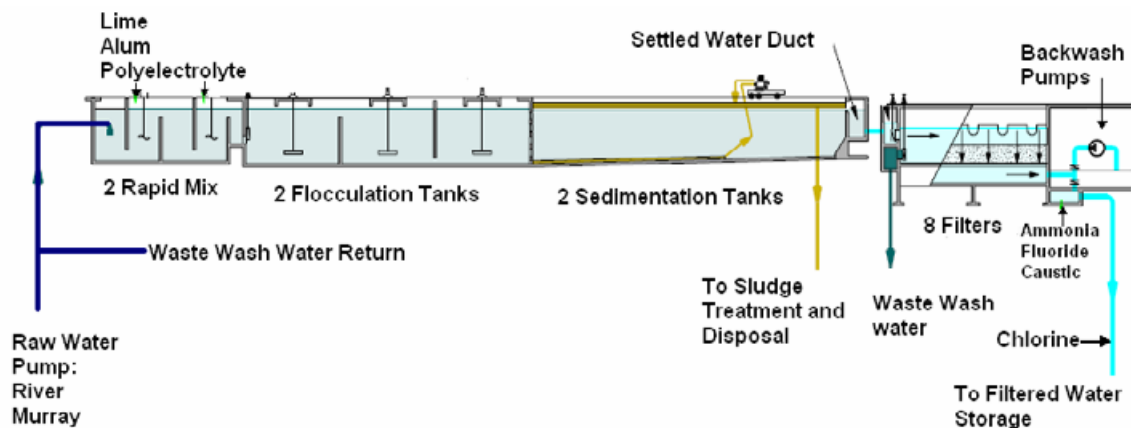
sand filter with 2 year old sand was capable of 50% removal of MIB at the end of a 4 month study (Summers et al., 2006). A Canadian study found that a laboratory scale filter utilising anthracite-sand media removed only 14% of the influent geosmin after a shorter period of 65 d (Elhadi et al., 2006). These previous studies suggest that biologically-active sand filtration is a promising alternative to PAC; however, studies over long time frames are required. Until now, no full-scale study has been carried out over a sufficient time period as to verify the potential of the process.

This paper describes a South Australian water treatment plant (WTP) which is capable of removing geosmin without the need for PAC treatment. Laboratory-scale experiments were used to validate the conclusion that biological activity in the rapid gravity filters is responsible for the taste and odour removal.

## 2.2 Full-scale study

### 2.2.1 Morgan water treatment plant

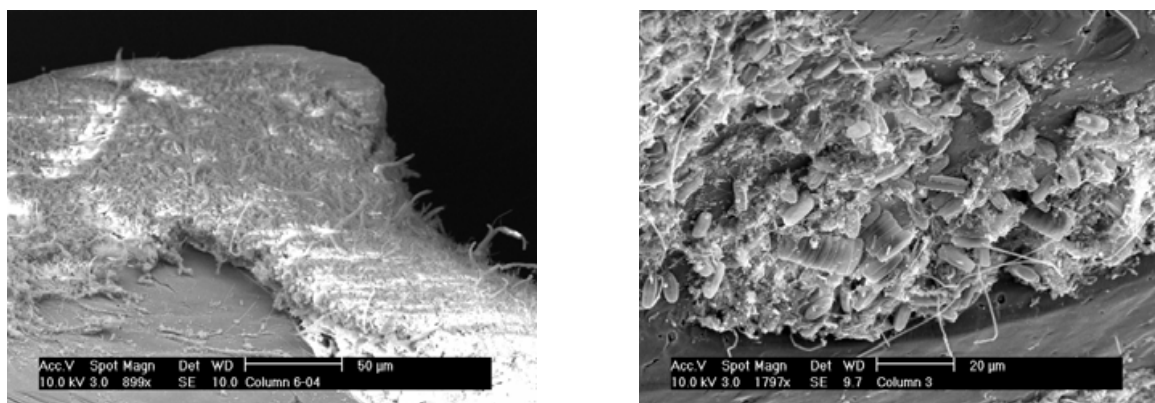
Morgan WTP was built in 1986 and services a large portion of South Australia's regional centres, such as Whyalla, Port Pirie and Port Augusta. It has a capacity of 200 ML per day. The plant operates with conventional treatment comprising of coagulation, flocculation, sedimentation, filtration and chloramine disinfection. A simplified plant schematic can be seen in Figure 2.1.



**Figure 2.1** Morgan WTP schematic prior to chloraminated backwash.

Morgan WTP utilises dual-media anthracite-sand rapid gravity filters, with no pre-chlorination. The filters operate with a hydraulic loading rate of  $10 - 13.7 \text{ m h}^{-1}$ , and an approximate empty bed contact time (EBCT) of 3 min. The sand has an effective size of 0.55 mm, and has not been replaced since the plant's commissioning in 1986.

Filter run times range from 10 to 50 h, depending on water quality. The backwashing process involves air scour for 5 min at approximately  $36 \text{ m}^3 \text{ h}^{-1}$ , followed by water wash for 12.5 min at  $470 - 850 \text{ L s}^{-1}$ . Up until 17th December 2004, the backwash water consisted of un-chlorinated filtered water after the addition of ammonia, fluoride, and caustic. Despite the filters not being designed as biological filters, the absence of chlorine and the long period of time in operation resulted in the filters containing a diverse, thriving biological community. This can be seen in scanning electron microscopy (SEM) images taken in 2004, shown in Figure 2.2. The biofilm is composed of many organisms, including bacteria and protozoa, and is held together by extracellular polymeric material. It is not possible to identify individual organisms from SEM alone.



**Figure 2.2** SEM of Morgan filter sand, August 2004.

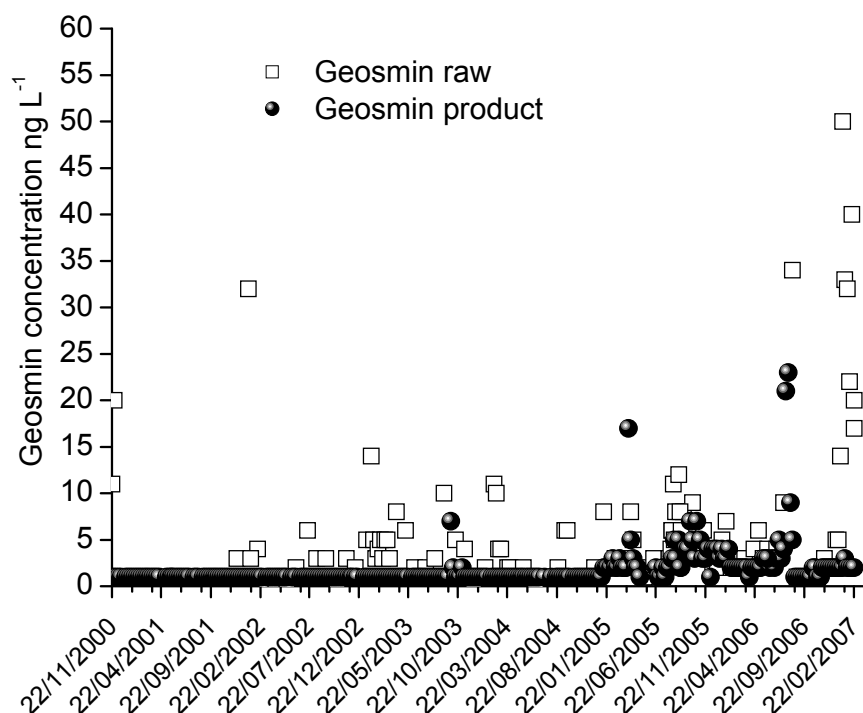
## 2.2.2 Geosmin in Morgan water treatment plant

Morgan WTP receives its water from the River Murray, which has frequent algal blooms and constant low to mid levels of geosmin. SA Water monitoring from 2000-2007 has shown an average of  $15.4 \text{ ng L}^{-1}$  ( $\pm 64\%$ ,  $n = 123$ ) geosmin in the raw water. Despite the constant levels of geosmin in the raw water, the Morgan WTP has historically produced water with geosmin levels below  $2 \text{ ng L}^{-1}$ .

On the 17th December 2004, for unrelated operational reasons the chlorine dosing point was moved to the backwash supply sump, resulting in chloraminated backwash water. As the filters were not deliberately being operated as biological filters, the effect of the chloramine on the biological activity was not of primary concern. In fact, chlorinated backwash water is often thought to enhance filtration efficiency. Australian WTPs often include a chlorination step prior to filtration as it aids in reducing particle counts and increases filter run times. The change in plant operation directly coincided with an increase in geosmin levels in the product water. Figure 2.3 shows geosmin levels in the raw and product waters of Morgan WTP over the period to 22/11/2000 to 6/02/2007. The figure shows that up until the change in backwashing conditions, levels of geosmin in the product water were consistently below  $2 \text{ ng L}^{-1}$ . However, with the implementation of the chloraminated backwash regime, significant breakthrough of geosmin occurred.

Backwashing with un-chloraminated water was resumed in late August 2006. During this time, the geosmin levels entering the plant increased markedly. This increase coincided with continuing drought conditions in the Murray-Darling basin resulting in poorer water quality in the River Murray. Interestingly, despite 2007 providing the second highest average geosmin inlet level since data was collected, the plant was able to consistently produce water with geosmin levels of  $5 \text{ ng L}^{-1}$  and below. Cessation of chloraminated backwashing directly resulted in the plant once again being able to remove geosmin without the need for PAC addition.

The above data suggests that biological activity in the rapid gravity filters was responsible for the removal of geosmin through Morgan WTP. The implementation of chloraminated backwash water resulted in a direct increase of geosmin in the finished water. Resumption of chlorine-free backwashing resulted in the filters once again removing geosmin.



**Figure 2.3** Geosmin concentration in Morgan WTP.

## 2.3 Validation by laboratory scale biological sand filters

Laboratory sand column experiments were conducted concurrently with the changes in backwashing conditions of the Morgan WTP to further validate the hypothesis that geosmin removal was occurring by biological activity in the rapid gravity filters. This study also included MIB. MIB is often present in the Morgan raw water; however, at much lower concentrations than geosmin.

### 2.3.1 Materials and methods

Glass columns with a bed height of 15 cm and interior diameter of 2.5 cm were used to simulate biologically-active sand filters. Influent water was taken from the settled water duct of the Morgan WTP and filtered through a 1  $\mu\text{m}$  Polypure Capsule (Pall Life Sciences, USA) prior to being used in the experiments. The water was stored in 20 L glass bottles and fed to the filters using an adjustable peristaltic pump (Gilson Miniplus 3, France). All tubing used was Tygon Lab tubing (Masterflex, USA).

MIB and geosmin stock solutions were used to spike the filter influents with 100  $\text{ng L}^{-1}$  of MIB and geosmin. Sample ports were located just prior to and just after the water sample left the filter to avoid system losses, which are known to occur in MIB and geosmin studies (Elhadi et al., 2004a). Each filter was backwashed weekly with deionised water for 2 min at 10% bed expansion. All experiments were conducted at room temperature ( $20 \pm 2^\circ\text{C}$ ).

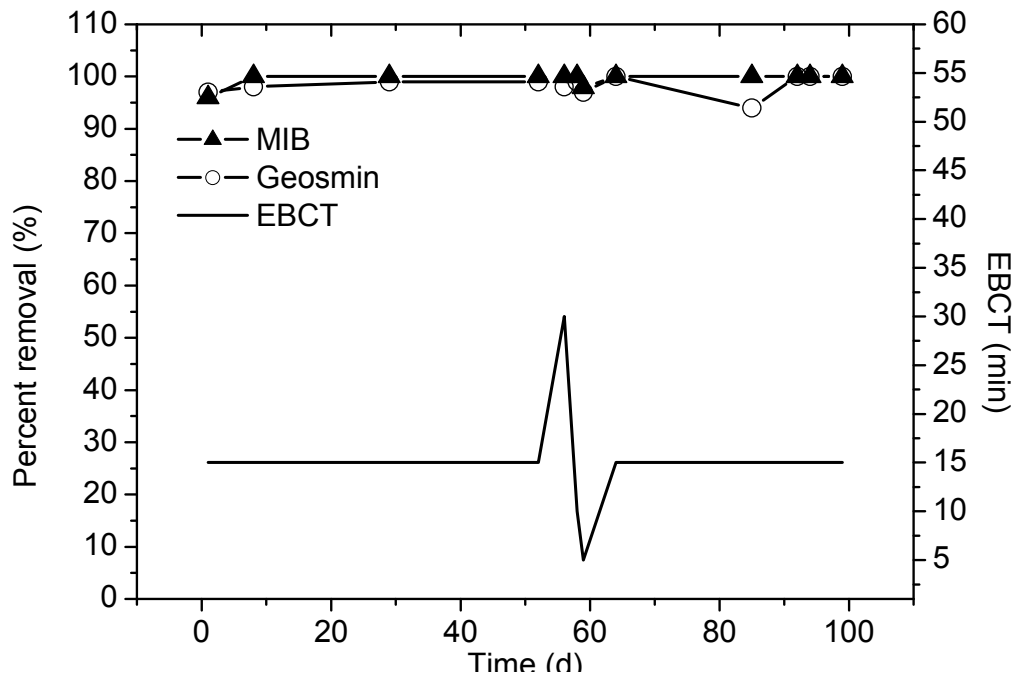
The filters were packed with sand taken from the Morgan filter beds during each stage of the backwash changes. Firstly, sand was taken prior to the chloraminated backwash (CBW). Secondly, sand was taken during the CBW period. Finally, sand was taken in January 2007, approximately 4 months after CBW was ceased.

EBCT was used as the primary filtration parameter. Unless stated otherwise, the columns were run at an EBCT of 15 min, which correlates to a loading rate of  $0.6 \text{ m h}^{-1}$ . Loading rates in the range of the full scale system were not feasible in the small-scale laboratory apparatus; however, EBCTs corresponding to that of the full-scale could be used for short periods.

### 2.3.2 Results and discussion

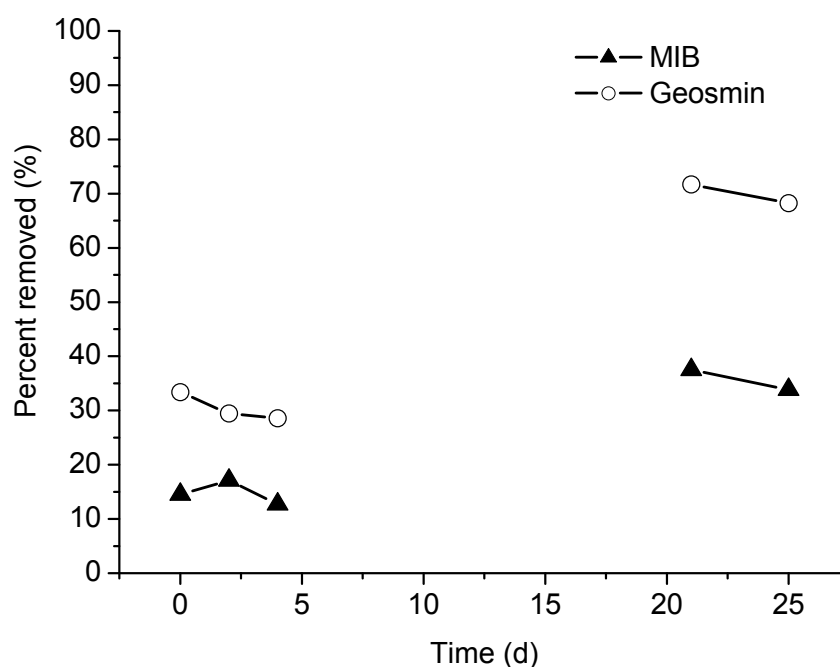
Figure 2.4 shows that Morgan filter sand taken prior to the chloramination period was capable of removing geosmin to below analytical detection limit. It was also capable of removing MIB, which is often also present in the raw water at low levels. This aids in validating the theory that MIB and geosmin removal was occurring through the rapid gravity filters at Morgan WTP, prior to the implementation of the CBW.

The laboratory filter was run at a baseline EBCT of 15 min. After 58 d of operation, the EBCT was sequentially decreased to 10, 5 and 3.5 min, to simulate full-scale conditions. Removal of MIB and geosmin remained at >95%. Thus, it was shown that Morgan filter sand taken prior to the CBW was capable of excellent removal of MIB and geosmin at EBCTs under rapid sand filtration conditions.



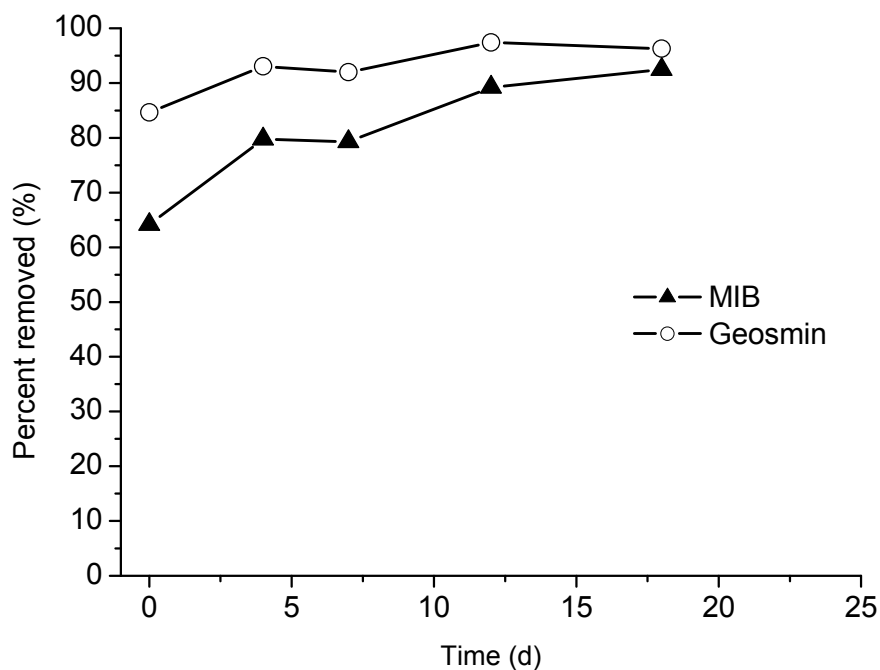
**Figure 2.4** MIB and geosmin removal in Morgan filter sand sampled before CBW.

In Figure 2.5 it is evident that sand taken during the CBW period was not as effective at removing MIB and geosmin. Initial removal was approximately 10% for MIB and 30% for geosmin, over a 5 d spiking period. Spiking was then stopped for a period of 15 d. After the resumption of spiking, removal had increased to 40% for MIB and 70% for geosmin. This increase suggests that the chloraminated backwashing did not completely destroy the biofilm, and it was able to re-establish in the absence of chlorine. Although the biomass would have been damaged with each backwash, it had time to recover to a certain extent during the filter run cycles. The ability of a biofilter to recover after chlorinated backwash has been described before (Miltner et al., 1995). In that study, backwashing an anthracite-sand biofilter resulted in a loss of 22% of the biomass, but biomass levels built back up to the pre-backwashing level after a 40 h filter run time.



**Figure 2.5** MIB and geosmin removal in Morgan filter sand taken during CBW.

Figure 2.6 shows the results from filter sand that was collected approximately 4 months after the cessation of the CBW. Here, initial removal of MIB and geosmin was at 64% and 84%, respectively. After a period of 18 d, the sand was removing >90% MIB and geosmin. This suggests that reverting to the original backwash process allowed the biofilm to rebuild. However, the biofilm was not as well developed as that prior to the CBW and took some time to acclimate to the presence of the taste and odour compounds.



**Figure 2.6** MIB and geosmin removal in Morgan filter sand taken post-CBW, January 2007.



## 2.4 Conclusions

This study highlighted the potential of biologically-active rapid sand filtration for taste and odour removal. Full-scale data showed that the Morgan WTP rapid sand filters were effectively removing geosmin to below the detection limit of consumers. However, the implementation of a chloraminated backwashing regime resulted in significant breakthrough of geosmin. After the cessation of the chloraminated backwash, the plant was again capable of excellent geosmin removal without the need for PAC usage. Laboratory-scale filter experiments validated the hypothesis that biological activity in the filter sand was responsible for the removal of geosmin through the plant. Filter sand taken before, during and after the chloraminated backwash periods showed the significant effect of the chloramine on the removal of geosmin. The laboratory studies also showed that the Morgan filter sand was capable of MIB removal.

These are encouraging results, as biological processes can save water utilities considerable costs by significantly decreasing the amount of PAC required.

## 2.5 References

- Danglot C., Amar G. and Vilagines R. (1983) Ability of bacillus to degrade geosmin. *Water Science & Technology* **15**(6/7), 291-299.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2004a) Determination of system losses of geosmin and MIB in bench-scale filtration apparatus. *Water Quality Research Journal of Canada* **39**(3), 207-212.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2004b) Removal of geosmin and 2-methylisoborneol by biological filtration. *Water Science & Technology* **49**(9), 273-280.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2006) Factors affecting the removal of geosmin and MIB in drinking water biofilters. *Journal of the American Water Works Association* **98**(8), 108-119.
- Hoefel D., Ho L., Aunkofer W., Monis P.T., Keegan A., Newcombe G. and Saint C.P. (2006) Cooperative biodegradation of geosmin by a consortium comprising three gram-negative bacteria isolated from the biofilm of a sand filter column. *Letters in Applied Microbiology* **43**(4), 417-423.
- Hrudey S.E., Huck P.M., Mitton M.J. and Kenefick S.L. (1995) Evaluation of odour removal by pilot-scale biological treatment process trains during spring runoff in an ice-covered stream. *Water Science & Technology* **31**(11), 195-201.
- Huck P.M., Kenefick S.L., Hrudey S.E. and Zhang S. (1995) Bench-scale determination of the removal of odour compounds with biological treatment. *Water Science & Technology* **31**(11), 203-209.
- Ishida H. and Miyaji Y. (1992) Biodegradation of 2-methylisoborneol by oligotrophic bacterium isolated from a eutrophied lake. *Water Science & Technology* **25**(2), 269-276.
- Izaguirre G., Wolfe R.L. and Means E.G. (1988) Bacterial degradation of 2-methylisoborneol. *Water Science & Technology* **20**(8/9), 205-210.
- Metz D.H., Pohlman R.C., Vogt J. and Summers R.S. (2006) *Removal of MIB and geosmin by full-scale biological sand filters*. In: Gimbel R., Graham N.J.D. and Collins M.R. (Eds.). *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. IWA Publishing, London, UK.
- Miltner R.J., Summers R.S. and Wang J.Z. (1995). Biofiltration performance: Part 2, Effect of backwashing. *Journal of the American Water Works Association* **87**(12), 64-70.
- Nerenberg R., Rittmann B.E. and Soucie W.J. (2000) Ozone/biofiltration for removing MIB and geosmin. *Journal of the American Water Works Association* **92**(12), 85-95.
- Summers R.S., Chae S., Kim S.M. and Ahn H.W. (2006) *Biodegradation of MIB and geosmin in biological sand and BAC filters: Acclimation, steady-state and varying influent*. In: Gimbel R., Graham N.J.D. and Collins M.R. (Eds.). *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. IWA Publishing, London, UK.

## **CHAPTER 3: REMOVAL OF GEOSMIN AND MIB THROUGH BIOLOGICALLY-ACTIVE SAND FILTERS\***

### **3.1 Introduction**

Geosmin and 2-methylisoborneol (MIB) can be biodegraded by bacteria that naturally occur in water sources (Danglot et al., 1983; Izaguirre et al., 1988). This biodegradation can be applied in the process of biological filtration. Biological filtration is relatively common practice in Europe, and is gaining attention in the world-wide water industry as it is a low cost, low maintenance process which does not require the addition of treatment chemicals which may themselves produce toxic or odorous by-products. It is generally accepted that the most effective biological process for odour removal is the ozone/granular activated carbon (GAC) system. Ozone and GAC have a synergistic relationship in the removal of taste and odour (T&O) compounds. Ozone breaks down larger natural organic material (NOM) fractions into compounds which are more easily assimilable by bacteria, creating water with greater biological instability. The greater instability aids in the development of the filter biomass, which can in turn enhance the removal of the remaining smaller NOM compounds which could lead to biological regrowth in the distribution system. Ozone is also capable of oxidising a portion of MIB and geosmin, with the remainder removed by the biological filter. A problem with the ozone/GAC system is that it is expensive, and implementation requires significant modifications to conventional treatment processes. Biological rapid sand filtration is a much more cost-effective option as it simply requires the elimination of disinfectants in the filter. Australian water treatment plants (WTPs) generally include a chlorination step prior to filtration in order to prevent biomass growth. Excessive build up of biomass can compromise the conventional filtration goals of particle removal and maximum filtration cycle length. However, these problems can be avoided with careful management of the filtration cycle. A number of studies have shown the potential of rapid sand filtration for MIB and geosmin removal (Ashitani et al., 1988; Metz et al., 2006; Summers et al., 2006). However, the removal is unpredictable and does not occur in all situations. Some potential problems associated with this process are its response to transient odour episodes, and whether it is capable of sufficient odour removal (Elhadi et al., 2004a). It is also important that the bacteria responsible for the degradation are present in the water source.

This paper outlines initial results from an experimental programme which was designed to find the conditions in which removal of MIB and geosmin will occur in a biologically-active sand filter. Identification of these conditions will enable WTPs to confidently implement biological treatment for removal of MIB and geosmin with minimal changes to the plant operation. The length of time required for the development of a biofilm capable of MIB and geosmin degradation was studied as it is important in the commissioning of a new biological facility. It is also important in cases where a biological filter may have had its biological activity compromised. The work was based upon laboratory-scale sand filtration columns with established and developing biofilms.

### **3.2 Materials and methods**

#### **3.2.1 Experimental design and apparatus**

Sand samples with established biofilms were taken from two South Australian WTPs, Plant A and Plant B. Until recently, Plant A operated rapid sand filters with no pre-chlorination. Long term data has shown that despite regular occurrences of geosmin in the plant influent, no geosmin was found in the product water. In 2005, chloraminated backwash water was introduced to the filtration cycle. From this time, the plant no longer effectively removed geosmin. This suggests that the removal was occurring through the rapid sand filters until the biofilm was damaged by the inclusion of chloramine. Plant B

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\* This chapter is based on the following manuscript:

McDowall B., Ho L., Saint C. and Newcombe G. (2007) Removal of geosmin and 2-methylisoborneol through biologically active sand filters. *International Journal of Environment and Waste Management* 1(4), 311-320.

receives water from a natural catchment, and utilises dissolved air flotation instead of the conventional flocculation/sedimentation step. Plant B does not pre-chlorinate its filters; however there is no data to suggest that the plant removes MIB and geosmin biologically. The major differences in the sands from Plant A and Plant B are the size and the length of time in the filters. Plant B filter media is replaced every two years and has an effective size of 2.0 mm. Plant A filter media has been in place since the plant's commissioning in the mid 1980s and has an effective size of 0.55 mm.

Small-scale sand filters were used to simulate biologically-active sand filters. The columns had a bed height of 15 cm and an internal diameter of 2.5 cm. Influent water was stored in 20 L glass bottles and fed to the columns using an adjustable peristaltic pump (Gilson Miniplus 3, France). All tubing used was Tygon Lab tubing (Masterflex, USA). MIB and geosmin stock solutions (Ultrafine Chemicals, UK) were spiked directly into the glass bottle at a target concentration of 100 ng L<sup>-1</sup>. Problems can often occur with losses of MIB and geosmin through experimental apparatus (Elhadi et al., 2004a). Therefore, samples for MIB and geosmin were taken just prior to the column and just as the product left the column to avoid system losses. Influent water was stored at room temperature and filtered through a 1 µm Polypure Capsule (Pall Life Sciences, USA) prior to being used in the experiment. The small scale of the apparatus meant that the filtration rate was limited, thus empty bed contact time (EBCT) was chosen as the primary filtration parameter. The columns were run at a baseline EBCT of 15 min, equivalent to a filtration rate of 0.6 m h<sup>-1</sup>. Each filter was backwashed once per week, using deionised water. Backwashing was carried out for 2 min with 10% bed expansion.

Four columns were constructed, using sand with and without a biofilm. Biofilm was removed by adding 40 mL of a 2 M sodium hydroxide solution to a vessel containing the sand and 100 mL of Milli-Q water (Millipore Pty Ltd, USA). The solution was agitated on a mixer-roller overnight. After this time, the sand was rinsed and the process was repeated. The presence/absence of biofilm on the sand was verified by scanning electron microscopy before and after sodium hydroxide treatment. All columns were fed with Morgan settled water. Morgan WTP receives its water from the River Murray. It has a typical dissolved organic carbon (DOC) concentration of 2.5 – 4.0 mg L<sup>-1</sup> and UV absorbance (at 254 nm) of 0.05 cm<sup>-1</sup>. The columns are described in Table 3.1.

**Table 3.1** Sand filter columns.

Column	Sand	Biofilm	Effective size of sand
1	Plant A	Yes	0.5 mm
2	Plant B	Yes	2.0 mm
3	Plant A	No	0.5 mm
4	Plant B	No	2.0 mm

### 3.2.2 Analytical methods

Flow cytometry was used to determine the number of viable bacteria in biofilm samples. Biofilm was removed by shaking and vortexing samples of 0.5-1.0 g of sand for 15 min. The biofilm samples were stained using the LIVE/DEAD® BacLight™ kit (Molecular Probes Inc, USA) and subsequently enumerated by a FACSCalibur flow cytometer (Becton Dickinson, USA). The method is described in more detail elsewhere (Hoefel et al., 2003).

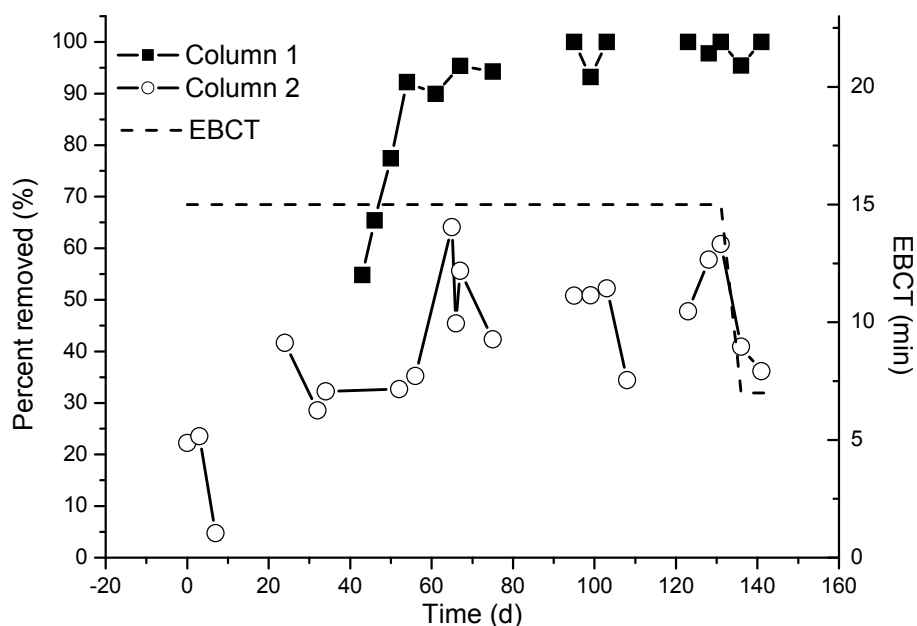
MIB and geosmin analysis was carried out at the Australian Water Quality Centre, South Australia. Sample concentration was achieved by solid phase microextraction using a polydimethylsiloxane-divinylbenzene syringe fibre (Supelco, Australia). Analysis was conducted on a Hewlett Packard 5890 Series II Gas Chromatograph with Hewlett Packard 5971 Series Mass Selective Detector (Agilent Technologies, Australia) against qualified labelled internal standards (Ultrafine Chemicals, UK). The method has a detection limit of 4 ng L<sup>-1</sup> for MIB and geosmin.

### 3.3 Results and discussion

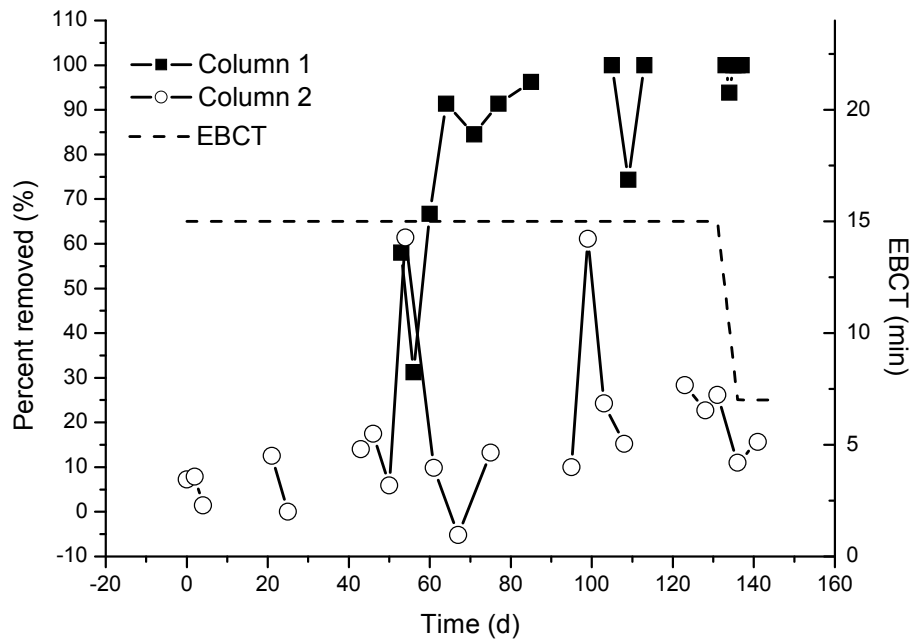
#### 3.3.1 Columns with established biofilm

Column 1 was run for 100 d with three spiking periods. The initial spike of 32 d showed that the filter was capable of removing greater than 90% of the influent geosmin and MIB within 11 d. This lag period is common to biological systems, with the bacteria adjusting to different substrates and environmental conditions (Ho et al., 2007). The initial spike was followed by a period of 20 d with no MIB and geosmin. This had no negative impact on removal, and MIB and geosmin removal increased to below the analytical detection limit for the second MIB and geosmin spike of 13 d. A further break of 15 d also had no negative impact on removal. The final spiking period included decreasing the EBCT to 7.5 min (filtration rate  $1.3 \text{ m h}^{-1}$ ) for 5 d. Removal remained at 100%.

Column 2 was run for a period of 141 d, with 5 spiking periods. It showed poor removal of MIB and geosmin throughout the experiment. The first spike of 4 d was followed by 16 d with no MIB or geosmin. The break in the spiking had no negative impact on removal. In fact, geosmin removal increased when spiking was resumed. MIB removal remained negligible. Over the course of the 5 spiking periods, geosmin removal increased from less than 25% to between 40 and 50%. This suggests that there was some form of biological removal. However, the majority of the removal was attributed to adsorption and/or volatilisation in the filtration apparatus. The increase in EBCT resulted in a decrease of MIB and geosmin removal. Figures 3.1 and 3.2 show the percentage removals of geosmin and MIB in the established biofilm columns. Note that column 1 was started 40 d after column 2. For ease of comparison, day 1 of column 1 has been plotted as day 40 of column 2.



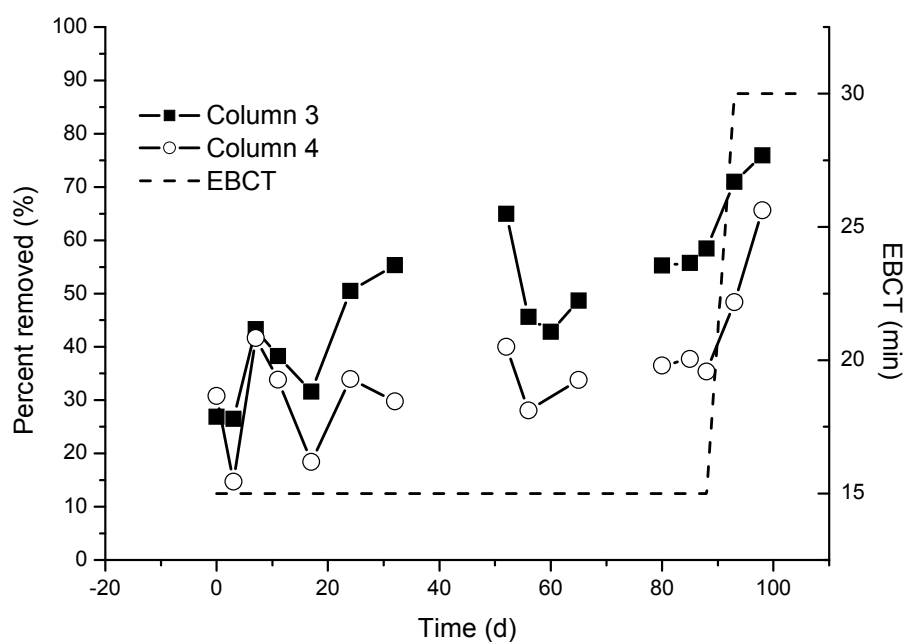
**Figure 3.1** Geosmin removal in established biofilm columns.



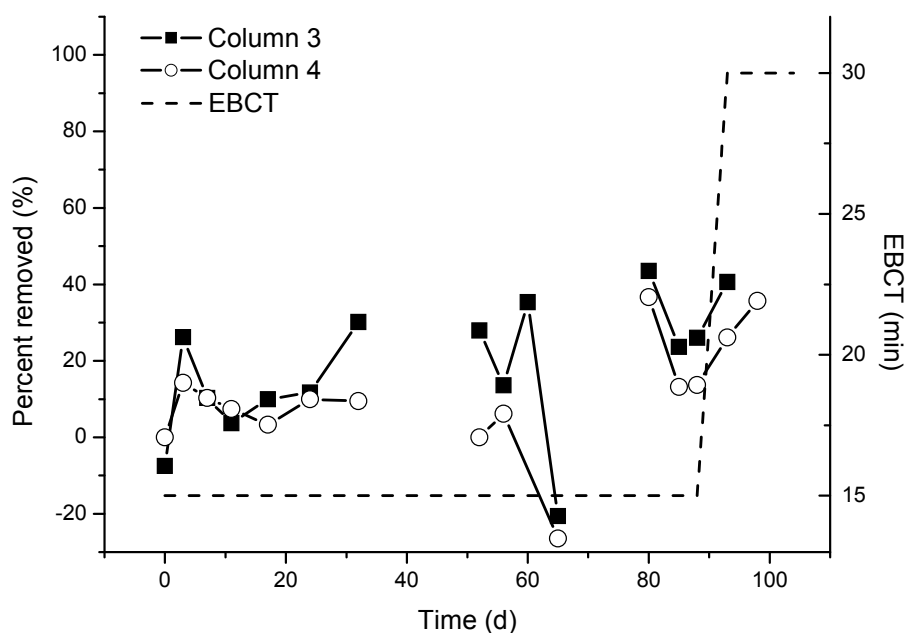
**Figure 3.2** MIB removal in established biofilm columns.

### 3.3.2 Columns with developing biofilm

The columns with developing biofilm were run for 154 d with 3 spiking periods. Removal of MIB and geosmin for the first 21 d was similar for both sands, between 20 and 40%. After this time column 3 began to show superior performance for geosmin removal, increasing to 50% after 80 d. This is still not sufficient, as the product water was above the OTC. Breaks in spiking had negligible impact on any biological removal. At day 90, the EBCT was increased to 30 min to allow greater contact with the biofilm. Geosmin removal in both columns increased by approximately 10% but the effluent was still above the OTC. Figures 3.3 and 3.4 show the percentage removals of geosmin and MIB in the developing biofilm columns.



**Figure 3.3** Geosmin removal in developing biofilm columns.



**Figure 3.4** MIB removal in developing biofilm columns.

In all studies, MIB removals were lower than that of geosmin. This is in agreement with other studies on biological degradation of MIB and geosmin (Ashitani et al., 1988; Elhadi et al., 2004b; Ho et al., 2007). The greater geosmin removal in the later stages suggests that a biofilm was developing which was capable of geosmin degradation but not MIB. This suggests that removal of T&O may not occur in a biological filter unless specific organisms responsible for degradation are present in the biofilm. A consortium comprising of three gram-negative bacteria was isolated and identified from a similar laboratory sand filter column (Hoefel et al., 2006). It was found that these bacteria could only degrade

geosmin when all three were present together. No other bacteria in this biofilm were shown to degrade geosmin.

### 3.3.3 Biofilm studies

At the completion of the experiment, the biofilm was removed from the entire developing biofilm columns to determine differences in the amount of biomass. A known amount of sterile Milli-Q water (Millipore Pty Ltd, USA) was added to the sand and the biofilm was detached by intermittent vortexing and shaking for a period of 15 min. The supernatant was removed and bacterial numbers were enumerated using flow cytometry. It is recognised that the detachment process may not have removed all bacterial species from the sand; however, the process allows for comparison between the two sands. The results are shown in Table 3.2, below.

**Table 3.2** Bacterial numbers on developing biofilm columns.

	Cells cm <sup>-3</sup> filter media	Cells per grain of sand	% of sand surface covered by bacteria
Column 3	1.2 x 10 <sup>7</sup>	1,395	0.15
Column 4	7.3 x 10 <sup>6</sup>	15,595	0.15

Columns utilising sand taken from Plant A (columns 1 and 3) showed consistently superior performance in terms of geosmin and MIB removal. The biofilm analysis on the developing biofilm columns showed that there is a greater amount of bacteria per unit filter media on the sand from Plant A. The smaller particle size of the sand gives a greater surface area per unit filter media, hence a greater quantity of biofilm. When bacterial numbers are related to sand size, however, it is seen that the percentage of sand surface covered by bacteria is the same for both sands.

The greater removal of geosmin in the sand from Plant A can be attributed to the greater amount of biofilm in the filter. In the established columns, the greater removal in the Plant A column can be attributed to its longer time in the filter. It has had over 20 years to develop a geosmin-degrading biofilm as opposed to the sand from column 2 which had only 2 years.

## 3.4 Summary and conclusions

This work has shown that complete removal of MIB and geosmin is possible through biological sand filtration, with a number of factors influencing the degree of efficacy.

A laboratory filter column utilising 0.5 mm effective size sand taken from a 26 year old biological rapid sand filter demonstrated the potential of the process. The filter was capable of removing MIB and geosmin to below the detection limit of the analytical method with a minimal acclimation period. It could also withstand periodic T&O episodes and a decrease in EBCT. In contrast, a similar filter utilising 2 mm effective size sand taken from a 2 year old filter was not capable of sufficient removal of the compounds, removing only 60% of geosmin and 30% of MIB after 141 d.

The above results show that the effective size of the filter sand is an important factor in the removal of MIB and geosmin. Sand with a smaller particle size is more effective as it allows for a greater amount of biomass per unit filter media.

The stage of development of the biofilm was also shown to be important. It may take a significant period of time before T&O removal occurs in filters utilising new media, or in cases where the biological activity of the filter had been damaged. This was seen in the columns utilising sand with developing biofilm, which were capable of removing a maximum of 60% of the influent geosmin and 40% of the influent MIB after 154 d in operation.

### 3.5 References

- Ashitani K., Hishida Y. and Fujiwara K. (1988) Behaviour of musty odorous compounds during the process of water treatment. *Water Science & Technology* **20**(8/9), 261-267.
- Danglot C., Amar G. and Vilagines R. (1983) Ability of *Bacillus* to degrade geosmin. *Water Science & Technology* **15**(6/7), 291-299.
- Elhadi S.L.N., Huck, P.M. and Slawson R.M. (2004a) Determination of system losses of geosmin and MIB in bench-scale filtration apparatus. *Water Quality Research Journal of Canada* **39**(3), 207-212.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2004b) Removal of geosmin and 2-methylisoborneol by biological filtration. *Water Science & Technology* **49**(9), 273-280.
- Ho L., Hoefel D., Bock F., Saint C.P. and Newcombe G. (2007) Biodegradation rates of 2-methylisoborneol (MIB) and geosmin through sand filters and in bioreactors. *Chemosphere* **66**(11), 2210-2218.
- Hoefel D., Grooby W.L., Monis P.T., Andrews S. and Saint C.P. (2003) Enumeration of water-borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiological Methods* **55**(3), 585-597.
- Hoefel D., Ho L., Aunkofer W., Monis P.T., Keegan A., Newcombe G. and Saint C.P. (2006) Cooperative biodegradation of geosmin by a consortium comprising three gram-negative bacteria isolated from the biofilm of a sand filter column. *Letters in Applied Microbiology* **43**(4), 417-423.
- Izaguirre G., Wolfe R.L. and Means E.G. (1988) Bacterial degradation of 2-methylisoborneol. *Water Science & Technology* **20**(8/9), 205-210.
- Metz D.H., Pohlman R.C., Vogt J. and Summers R.S. (2006) *Removal of MIB and geosmin by full-scale biological sand filters*. In: Gimbel R., Graham N.J.D. and Collins M.R. (Eds.). *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. IWA Publishing, London, UK.
- Summers R.S., Chae S., Kim S.M. and Ahn H.W. (2006) *Biodegradation of MIB and geosmin in biological sand and BAC filters: Acclimation, steady-state and varying influent*. In: Gimbel R., Graham N.J.D. and Collins M.R. (Eds.). *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. IWA Publishing, London, UK.



## CHAPTER 4: BIODEGRADATION RATES OF MIB AND GEOSMIN THROUGH SAND FILTERS AND IN BIOREACTORS\*

### 4.1 Introduction

Geosmin and 2-methylisoborneol (MIB) are susceptible to biological degradation (biodegradation) with several studies having implicated a variety of microorganisms responsible for their removal from water (see Table 4.1). The susceptibility of both MIB and geosmin to biodegradation can be attributed to their structures which are similar to biodegradable alicyclic alcohols and ketones (Trudgill, 1984; Rittmann et al., 1995). As yet, no definitive pathways for the biodegradation of MIB have been proposed, although Tanaka et al. (1996) were able to identify two possible dehydration products, 2-methylcamphene and 2-methylenebornane. However, it is possible that the biodegradation pathway of MIB may be similar to camphor, a bicyclic ketone which has been documented to be biodegraded by a strain of *Pseudomonas* and by *Mycobacterium rhodochrous* through the biological Baeyer-Villiger reaction (Trudgill, 1984). In this process, the ring structures of camphor are cleaved through a sequence of intermediate reactions which are catalysed by monooxygenase enzymes, resulting in the formation of unstable lactones. Oikawa et al. (1995) confirmed this by excising the entire *cam* operon from a camphor degrading *Pseudomonas putida*, where its subsequent transformation into *Escherichia coli* demonstrated the acquired ability of that *E. coli* to degrade MIB.

**Table 4.1** Microorganisms implicated in the biodegradation of MIB and geosmin.

MIB		Geosmin	
Microorganisms	Literature sources	Microorganisms	Literature sources
<i>Pseudomonas</i> spp.	Izaguirre et al. (1988), Egashira et al. (1992), Tanaka et al. (1996)	<i>Bacillus cereus</i>	Silvey et al. (1970), Narayan and Nunez (1974)
<i>Pseudomonas aeruginosa</i>	Egashira et al. (1992)	<i>Bacillus subtilis</i>	Narayan and Nunez (1974), Yagi et al. (1988)
<i>Pseudomonas putida</i>	Oikawa et al. (1995)	<i>Arthrobacter atrocyaneus</i>	Saadoun and El-Migdadi (1998)
<i>Enterobacter</i> spp.	Tanaka et al. (1996)	<i>Arthrobacter globiformis</i>	Saadoun and El-Migdadi (1998)
<i>Candida</i> spp.	Sumitomo (1988)	<i>Rhodococcus moris</i>	Saadoun and El-Migdadi (1998)
<i>Flavobacterium multivorum</i>	Egashira et al. (1992)	<i>Chlorophenolicus</i> strain N-1053	Saadoun and El-Migdadi (1998)
<i>Flavobacterium</i> spp.	Egashira et al. (1992)		
<i>Bacillus</i> spp.	Ishida and Miyaji (1992), Lauderdale et al. (2004)		
<i>Bacillus subtilis</i>	Yagi et al. (1988)		

No definitive pathways have been elucidated for the biodegradation of geosmin. Saito et al. (1999) were able to identify four possible biodegradation products of geosmin, two of which were identified as 1,4a-dimethyl-2,3,4,4a,5,6,7,8-octahydronaphthalene and enone. Interestingly, these two products have also been used in the chemical synthesis of (-)-geosmin (Marshall and Hochstetler, 1968; Saito et al., 1996). It is possible that geosmin may be biodegraded by a pathway similar to that of cyclohexanol. Trudgill (1984) documented that strains of *Acinetobacter* and *Nocardia* were capable of degrading cyclohexanol via monooxygenase enzymes, similar to the biological Baeyer-Villiger reaction. Cyclohexanol is initially oxidised to an alicyclic ketone. The insertion of a ring oxygen atom

\* This chapter is based on the following manuscript:

Ho L., Hoefel D., Bock F., Saint C.P. and Newcombe G. (2007) Biodegradation rates of 2-methylisoborneol (MIB) and geosmin through sand filters and in bioreactors. *Chemosphere* **66**(11), 2210-2218.

follows via a monooxygenase enzyme. The resultant lactone is unstable, causing the lactone to be hydrolysed into a diacid.

The biodegradability of MIB and geosmin in water indicates that there is potential for using biological filtration processes as a viable treatment option for removing these taste and odour (T&O) compounds. Biological filtration systems for the removal of contaminants are becoming more attractive to water suppliers as they are generally of low technology, requiring little maintenance and infrastructure. Furthermore, such systems are able to remove these contaminants without the addition of other chemicals that may have the potential to produce by-products that are undesirable in drinking water.

Of the biological filtration studies conducted on MIB and geosmin, the majority have used sand or granular activated carbon (GAC) media (Ashitani et al., 1988; Hattori, 1988; Lundgren et al., 1988; Yagi et al., 1988; Hrudey et al., 1995; Sumitomo, 1998; Elhadi et al., 2004b). However, the use of porous media in such experiments complicates the removal processes due to its ability to act as a solid support for biofilm, in addition to its adsorptive properties. A non-porous medium, such as sand, is required if experiments are designed to accurately study the extent of MIB and/or geosmin biodegradation.

Currently, studies regarding the biodegradation rates of MIB and geosmin are limited. Westerhoff et al. (2005) conducted batch studies in lake water and modelled MIB and geosmin biodegradation as a pseudo-zero-order reaction. In contrast, Rittmann et al. (1995) determined that MIB and geosmin would be utilised as secondary substrates in natural water, due to the presence of natural organic material (NOM) which is present at much higher concentrations than the T&O compounds. Consequently, they determined the biodegradation of MIB and geosmin in natural water to be a second-order reaction.

In this study, the major objective was to evaluate the biodegradation of MIB and geosmin within biologically-active sand filters by conducting bench-scale column and batch bioreactor experiments. A further objective was to determine the rates of biodegradation of MIB and geosmin in both sets of experiments. Finally, 16S rRNA gene analysis and genus identification were applied to isolates implicated in the biodegradation of both T&O compounds.

## 4.2 Materials and methods

### 4.2.1 Chemicals and reagents

All reagents used were analytical grade or better unless otherwise stated. Separate stock solutions of MIB and geosmin (Ultrafine Chemicals, UK) were prepared by dissolving in Milli-Q water (Millipore Pty Ltd, USA), then stored, head-space free at 4°C prior to use. Treated water (coagulation, flocculation and sedimentation) was collected from the Morgan water treatment plant (WTP), South Australia. Sand was obtained from the filter beds at the Morgan WTP. Characteristics of the treated water and filter sand from the Morgan WTP are presented in Table 4.2. All glassware and equipment was sterilised (autoclaved at 121°C for 20 min) prior to use.

**Table 4.2** Characteristics of treated Morgan water and Morgan filter sand.

	pH	UV absorbance at 254 nm (cm <sup>-1</sup> )	DOC (mg L <sup>-1</sup> )
Morgan water	7.5	0.043	2.4
	Effective particle size (mm)	Particle density (kg m <sup>-3</sup> )	Surface area in column (cm <sup>2</sup> )
Morgan sand	0.83	2585	3168

### 4.2.2 Laboratory column experiments

Filter sand, collected from the Morgan WTP, was packed into a laboratory glass column (length of 30 cm, I.D. of 2.5 cm) at a bed height of 15 cm. In addition, a second column was prepared in a similar fashion, with the exception of the sand, which had been pre-sterilised prior to packing. The purpose of this sterilised column was to initially identify the level of removal of MIB and geosmin in the absence of an active biofilm. Both columns were fed with same influent, treated Morgan water spiked with MIB and geosmin at target concentrations of 100 ng L<sup>-1</sup> each. The initial empty bed contact time (EBCT) of the columns was 15 min which corresponded to a filtration rate of 0.6 m h<sup>-1</sup>. Samples were taken from the column influents and effluents at regular intervals for MIB and geosmin analyses. Experiments were conducted at room temperature (20±2°C).

At the completion of the column experiments, sand (5 g wet weight) was removed from the unsterilised sand filter and the biofilm detached by periodic vortexing for 15 min in 30 mL of sterile phosphate buffered (0.01 M) saline solution. The supernatant was collected and any carry over of sand was pelleted by slow speed centrifugation at 1,000 g for 30 s. The supernatant was then washed twice by centrifugation at 1,000 g for 15 min with re-suspension of the pellet each time in sterile phosphate buffered saline solution. The resulting supernatant, containing bacteria from the biofilm, was used as the inocula for batch bioreactor experiments.

### 4.2.3 Bacterial enumeration

Numbers of active bacteria were determined by staining with the BacLight™ bacterial viability kit (Molecular Probes Inc, USA), and subsequent enumeration performed on those active bacteria using a FACSCalibur flow cytometer (Becton Dickinson, USA), using procedures described previously (Hoefel et al., 2003).

### 4.2.4 Batch bioreactor experiments

Four glass bioreactors, each containing 2 L of sterilised (autoclaved at 121°C for 20 min) treated Morgan water, were prepared for batch bioreactor experiments. MIB and geosmin were spiked into three of the bioreactors (A, C and D) at target concentrations of 200 ng L<sup>-1</sup> each, while bioreactor B was spiked with the T&O compounds at target concentrations of 50 ng L<sup>-1</sup> each. The sand filter biofilm was inoculated into each of the bioreactors at a dose of 1 x 10<sup>5</sup> active biofilm-associated bacteria mL<sup>-1</sup> (bioreactors A, B and D) and 1 x 10<sup>3</sup> active biofilm-associated bacteria mL<sup>-1</sup> (bioreactor C). Prior to inoculation into bioreactor D, the biofilm was inactivated by autoclaving at 121°C for 20 min. This provided a control to assess the removal of the T&O compounds by alternative routes such as adsorption to the cells or glass, or due to volatilisation. Table 4.3 outlines the conditions used for each of the bioreactors. The bioreactors were constantly stirred with a sterilised magnetic stirrer bar and incubated aerobically at room temperature (20±2°C). Samples were taken aseptically from each bioreactor at regular intervals for MIB and geosmin analyses.

**Table 4.3** Bioreactor conditions employed for the batch biodegradation of MIB and geosmin.

Bioreactor	Target MIB concentration (ng L <sup>-1</sup> )	Target geosmin concentration (ng L <sup>-1</sup> )	Sand filter biofilm concentration (cells mL <sup>-1</sup> )
A	200	200	1 x 10 <sup>5</sup>
B	50	50	1 x 10 <sup>5</sup>
C	200	200	1 x 10 <sup>3</sup>
D	200	200	1 x 10 <sup>5</sup> *

\*Prior to inoculation into bioreactor D, the biofilm was deactivated by autoclaving at 121°C for 20 min

### 4.2.5 Identification of MIB and geosmin degraders

Biofilm was detached from the sand in a similar manner to that described in section 4.2.2 with the exception of the re-suspension solution, in this set of experiments 30 mL of sterile Bushnell-Haas (BH)

minimal liquid medium (0.1% (w/v)  $\text{NH}_4\text{NO}_3$ , 0.1% (w/v)  $\text{K}_2\text{HPO}_4$ , 0.1% (w/v)  $\text{KH}_2\text{PO}_4$ , 0.01% (w/v)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01% (w/v)  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and 0.001% (w/v)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) was used. To enrich for either MIB or geosmin degrading bacteria, a total of  $1 \times 10^5$  active biofilm-associated bacteria  $\text{mL}^{-1}$  were inoculated in sterile BH medium, supplemented with 29  $\text{mg L}^{-1}$  of geosmin or 35  $\text{mg L}^{-1}$  of MIB (final culture volume of 20 mL). An equivalent number of active biofilm-associated bacteria were inoculated into 20 mL of BH minimal medium without geosmin or MIB, to control for any bacterial growth on alternative contaminating carbon sources within the minimal medium. To control for any losses of geosmin or MIB due to factors other than biodegradation, a portion of previously washed bacteria were inactivated by autoclaving ( $121^\circ\text{C}$  for 20 min) and added at a concentration of  $1 \times 10^5$  bacteria  $\text{mL}^{-1}$  to BH medium, which was supplemented with either 30  $\text{mg L}^{-1}$  geosmin or 37  $\text{mg L}^{-1}$  of MIB (final culture volume of 20 mL). Each culture was incubated at  $22^\circ\text{C}$  with shaking at 100 rpm for 35 d.

During enrichment, duplicate 0.25 mL aliquots were periodically taken and concentrated by centrifugation at 10,000 g for 10 min with re-suspension of the bacterial pellet in 50  $\mu\text{L}$  of 10 mM Tris-HCl (pH 7.5). Following cell membrane disruption by rapid freeze-thawing, a fragment of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primer set 357F-GC/518R, and the products of the reaction analysed by denaturing gradient gel electrophoresis (DGGE) (D-GENE™ Denaturing Gel Electrophoresis System, Bio-Rad, USA) as reported previously (Hoefel et al., 2005). Predominance of bands on the DGGE gel for time points equivalent to increases in bacterial abundance provided an indication of those bacteria involved in the biodegradation of the T&O compounds. Those bands were excised and DNA sequence analysis performed as reported previously (Hoefel et al., 2005).

#### 4.2.6 Analysis of MIB and geosmin

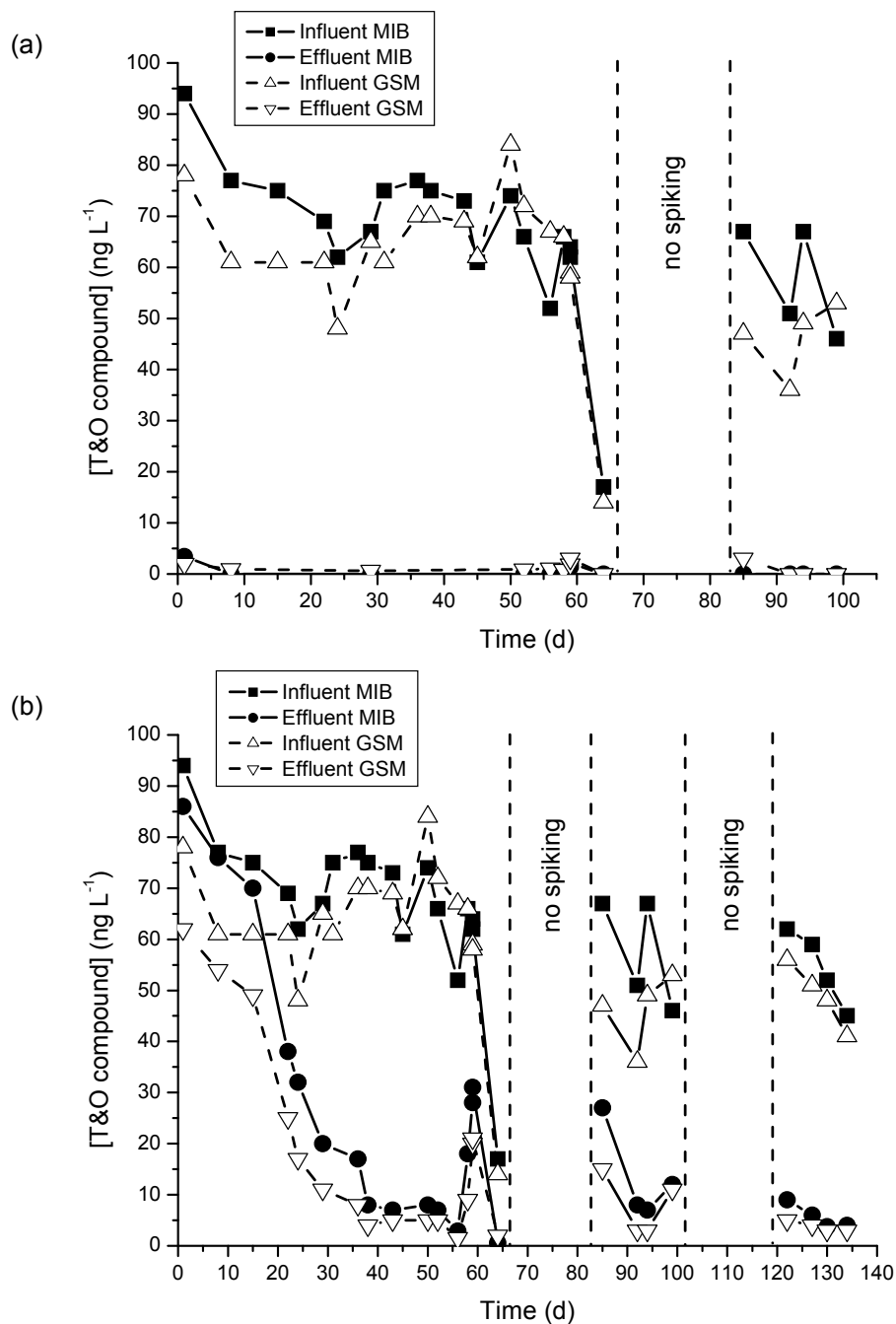
Samples for MIB and geosmin analyses were pre-concentrated using a solid phase microextraction syringe fibre (Supelco, Australia) and analysed on a 5890 Series II Gas Chromatograph with 5971 Series Mass Selective Detector (Hewlett Packard, Australia) against quantified labelled internal standards (Ultrafine Chemicals, UK). Full details of this analysis have been documented by Hayes and Burch (1989). The method had a detection limit of 1.6  $\text{ng L}^{-1}$  for MIB and 0.6  $\text{ng L}^{-1}$  for geosmin with a relative precision of 8% for MIB and 4% for geosmin.

### 4.3 Results and discussion

#### 4.3.1 Biological filtration of MIB and geosmin

The Morgan WTP sources water from the River Murray and is consistently challenged with MIB and geosmin (Hayes and Burch, 1989). However, the product water of the plant has historically been free of MIB and/or geosmin. This is quite unique considering the plant only operates under conventional mode. The removal of the T&O compounds has always been presumed to be through biological processes within the rapid sand filters, but this has yet to be substantiated.

Two laboratory columns containing Morgan WTP filter sand were assessed for their ability to remove MIB and geosmin (Figures 4.1a and b). Both columns were fed with treated Morgan water spiked with MIB and geosmin; however, the sand in one of the columns was pre-sterilised prior to commissioning. Throughout the experiment, negligible MIB and geosmin was observed in the effluent of the unsterilised sand filter (Figure 4.1a), consistent with observations at the Morgan WTP. Furthermore, negligible breakthrough of MIB or geosmin was evident between days 58 and 64 when the EBCT was sequentially reduced from 15 min down to 2.5 min and then increased back up to 15 min. These conditions approximate both slow and rapid sand filtration conditions, further highlighting the efficacy of the biofilm for MIB and geosmin removal. A substantial reduction of the influent MIB and geosmin concentrations (17 and 14  $\text{ng L}^{-1}$ , respectively) on day 64 had no effect on the removal of both T&O compounds.



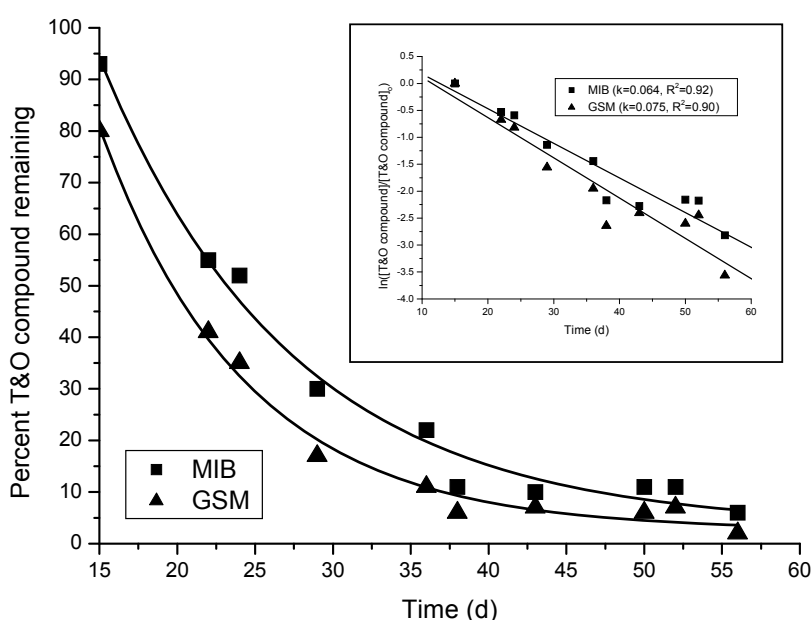
**Figure 4.1** Removal of taste and odour (T&O) compounds MIB and geosmin through: (a) unsterilised sand filter and (b) pre-sterilised sand filter.

On day 65 spiking of the T&O compounds was paused for a period of 21 d during which time only Morgan water was passed through the filter. Following the abatement, MIB and geosmin spiking recommenced (at an EBCT of 15 min). This was conducted to simulate transient conditions which are generally observed for T&O episodes. Upon recommencement of the spiking (for a further 14 d) MIB and geosmin was shown to be removed to below the limit of detection of the analytical method.

In contrast to the unsterilised sand filter, breakthrough of MIB and geosmin was immediately evident with the pre-sterilised sand filter with approximately 10% and 20% removal of MIB and geosmin,

respectively (Figure 4.1b). This initial removal of MIB and geosmin was attributed to physical losses such as adsorption and/or volatilisation. Larger losses were observed for geosmin than MIB, similar to findings by Elhadi et al. (2004a). This is due to the more volatile nature of geosmin which exhibits higher vapour pressure and Henry's law constant values than MIB (Pirbazari et al., 1992). As both sand filters shared the same influent water, this result strongly suggests that the removal of MIB and geosmin through the unsterilised sand filter was predominantly through biological action.

Between days 22 and 56 the removal of MIB and geosmin steadily increased suggesting that an active biofilm had established and was degrading both T&O compounds. During this period of time, the decrease of MIB and geosmin was modelled as a pseudo-first-order reaction with rate constants of  $0.064 \text{ d}^{-1}$  ( $R^2 = 0.92$ ) for MIB and  $0.075 \text{ d}^{-1}$  ( $R^2 = 0.90$ ) for geosmin (Figure 4.2). Whilst Westerhoff et al. (2005) showed that MIB and geosmin biodegradation was a pseudo-zero-order reaction in batch degradation studies, the findings in this study are consistent with those of Schmidt et al. (1985) and Anderozzi et al. (2006) who determined that the aerobic biodegradation of organic compounds (which are utilised as secondary substrates) was a pseudo-first-order reaction based on a simplified Michaelis-Menten equation. It is more likely that the biodegradation of MIB and geosmin in natural water follows pseudo-first-order kinetics as the concentration of the T&O compounds is considerably lower than that of the primary substrate, NOM.



**Figure 4.2** MIB and geosmin removal between days 22 and 56 in the pre-sterilised sand filter. Insert: Pseudo-first-order kinetic plots for the MIB and geosmin removal curves.

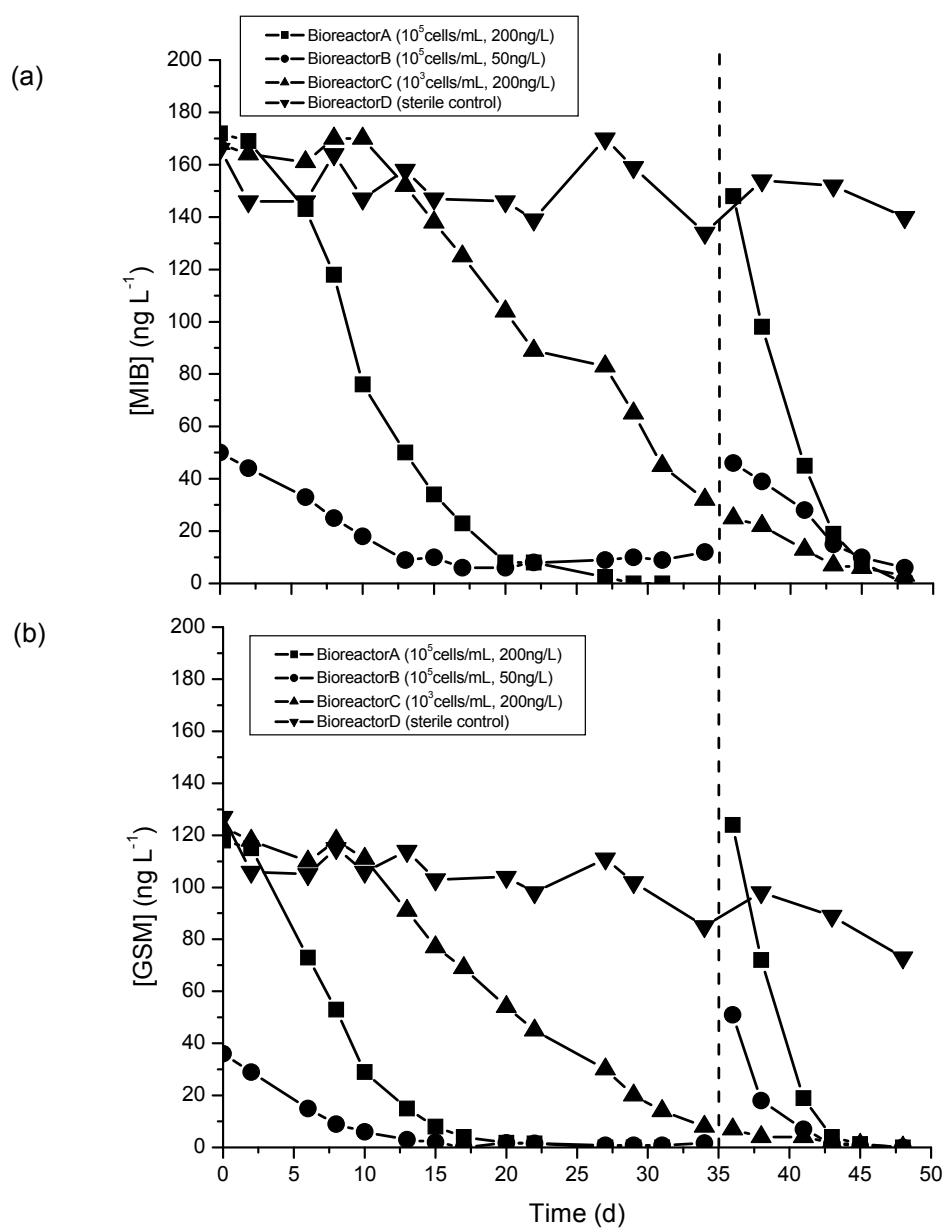
Geosmin appeared to be slightly easier to degrade than MIB, although the difference may be attributed to the higher volatility of geosmin as mentioned previously. Interestingly, geosmin has also been found to be more readily oxidised by ozone (Ho et al., 2002, 2004) and more easily adsorbed by activated carbon (Newcombe and Cook, 2002). These authors attributed the ease of geosmin removal to its more planar molecular structure and its lower solubility, making it more amenable to oxidant attack and adsorption.

During days 58-64 the EBCT of the pre-sterilised sand filter was sequentially reduced from 15 min down to 2.5 min and then increased back up to 15 min; identical to the conditions of the unsterilised sand filter. However, up to 30% breakthrough of the T&O compounds was observed during the decrease in EBCT. This suggests that the re-established biofilm was not as robust as the biofilm in the pre-sterilised sand filter, possibly due to lower numbers of MIB and geosmin degrading organisms. The unsterilised sand filter biofilm had established over a 30 year period within the Morgan WTP, and therefore acclimatised to several T&O episodes, in contrast to the biofilm on the pre-sterilised sand

filter which had been established in 58 d. Between days 64 and 85 no spiking of MIB and geosmin occurred, similar to that of the unsterilised filter. A second break in T&O spiking was also conducted between days 99 and 122. After the first abatement removals of MIB and geosmin ranged between 60 and 95%. However, removals had increased to >85% after the second abatement, strongly suggesting that the biofilm was becoming acclimatised to MIB and geosmin during those transient periods.

### **4.3.2 Batch biodegradation of MIB and geosmin**

Sand filter biofilm from the unsterilised sand filter was used in batch bioreactor studies to assess the ability of those bacteria to degrade MIB and geosmin in the absence of the solid support, and to assess the associated rates of removal. The removals of MIB and geosmin in each of the bioreactors are presented in Figures 4.3a and b, respectively. The lack of MIB and geosmin removals in bioreactor D (containing sterilised biofilm inoculum) indicates that the removals in the other bioreactors were predominantly through biodegradation processes. The biodegradation of both MIB and geosmin in Morgan water was determined to be a pseudo-first-order reaction, similar to the sand filter experiments. Rate constants for the biodegradation of the T&O compounds in bioreactors A, B and C are presented in Table 4.4. These rate constants were up to three times higher than those obtained with the sand filter experiments. This is possibly due to the bacteria having greater mobility within the aqueous suspension of the bioreactor and hence greater and consistent contact with MIB and geosmin molecules, in contrast to the immobilised bacteria within the sand filter biofilm where mass transfer resistance would be prevalent (Rittmann et al., 1995). In addition, rate constants were greater for geosmin compared with MIB, similar to the sand filtration experiments, implying that geosmin is potentially more assimilable than MIB.



**Figure 4.3** Batch degradation of: (a) MIB and (b) geosmin in treated Morgan water.



**Table 4.4** Pseudo-first-order rate constants ( $k$ ) for the biodegradation of MIB and geosmin in treated Morgan water. Correlation coefficients ( $R^2$ ) presented in parentheses.

Bioreactor	$k$ (d <sup>-1</sup> )	
	MIB	Geosmin
A	0.18 (0.97)	0.24 (0.98)
B	0.14 (0.96)	0.21 (0.99)
C	0.10 (0.95)	0.12 (0.96)
A*	0.36 (0.98)	0.58 (0.99)
B*	0.20 (0.98)	0.50 (0.97)

\*After re-spike of additional MIB and geosmin into the respective bioreactors

The rate of MIB and geosmin removal did not appear to be affected by the initial concentration of each T&O compound, as similar rate constants were obtained at both high and low T&O concentrations. This is in contrast to Izaguirre et al. (1988) who determined that MIB degradation was affected by the initial concentration of MIB. The authors showed more rapid degradation with the lower concentration of MIB. However, the experimental conditions employed by Izaguirre et al. (1988) were quite different to this study. Izaguirre et al. (1988) used different inocula for experiments conducted with the high (2 mg L<sup>-1</sup>) and low (290 ng L<sup>-1</sup>) MIB concentrations, whilst in this study, the same inoculum was employed for each of the MIB concentrations. In addition, Izaguirre et al. (1988) conducted experiments in minimal salts medium with MIB as the sole carbon source (or primary substrate). In this study, MIB and geosmin (present at ng L<sup>-1</sup>) would have been utilised as secondary substrates due to the presence of NOM which was present at mg L<sup>-1</sup> quantities. It is also possible that a very high MIB concentration of 2 mg L<sup>-1</sup> in the Izaguirre et al. (1988) study may have resulted in some degree of bacterial inhibition, thereby reducing the rate of biodegradation.

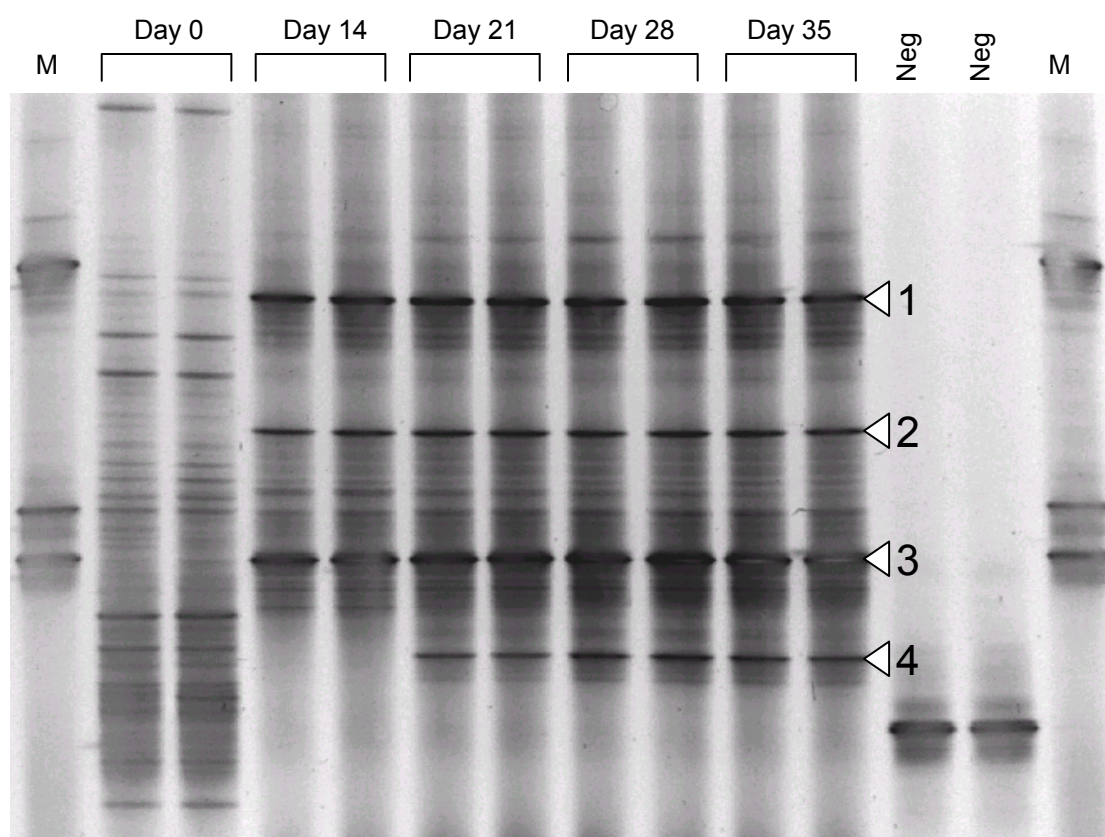
In our study, the initial biofilm inoculum concentration was shown to influence the biodegradation rate of MIB and geosmin. Bioreactor C employed an initial concentration of  $1 \times 10^3$  active biofilm-associated bacteria mL<sup>-1</sup> compared with  $1 \times 10^5$  active biofilm-associated bacteria mL<sup>-1</sup> in bioreactors A and B. The lower initial inoculum concentration in bioreactor C resulted in a distinct lag period (between days 0 to 10) prior to MIB and geosmin biodegradation commencing. On day 10, bioreactor C was found to contain  $6.5 \times 10^5$  active biofilm-associated bacteria mL<sup>-1</sup> which suggests that biodegradation of both T&O compounds may have been dependent upon the population density. Furthermore, MIB and geosmin biodegradation rate constants for bioreactor C were almost half that of bioreactor A. It is unclear why this is the case, since one would have expected similar rate constants once the bacterial numbers in bioreactor C approached those with bioreactor A. It is possible that the bacteria responsible for MIB and geosmin biodegradation in bioreactor C may have not reached the same numbers as that of bioreactor A due to the proliferation of other bacteria which may have comprised a larger ratio of the total active bacterial numbers than that of bioreactor A.

On day 36, additional MIB and geosmin was spiked into bioreactors A and B. Both T&O compounds were again biodegraded with removal determined to be a pseudo-first-order reaction. Interestingly, the rate constants obtained after the T&O re-spike were considerably higher than the original T&O spike, in some cases more than double (see Table 4.4). It is possible that as the genes encoding the enzymes responsible for the degradation of MIB and geosmin were recently induced (by the original T&O spike), then this resulted in more efficient up regulation of those genes following the addition of more MIB and geosmin, and hence more efficient biodegradation.

#### 4.3.3 Identification of MIB and geosmin degraders

Attempts were made to identify those bacteria responsible for the biodegradation of MIB and geosmin through the unsterilised sand filter and in the bioreactors. This was performed by enrichment of the degrading bacteria in a minimal medium supplemented with either MIB or geosmin as the sole carbon source, and subsequent profiling of the bacterial community during enrichment by 16S rRNA gene-directed PCR-DGGE. Enrichment of the geosmin degrading bacteria revealed the onset of biodegradation occurring 14 d following inoculation. By day 35, a total of 12.9 mg L<sup>-1</sup> of geosmin had been degraded (compared with the control culture) resulting in an increase in bacterial abundance of 1.97 log<sub>10</sub> (data not shown). The control culture with no added carbon source did not reveal any significant increase in bacterial abundance during this time.

DGGE analysis of the liquid culture revealed a shift in community composition from the initial biofilm inoculum to three major bands by day 14 (Figure 4.4). DNA sequencing of the excised bands revealed these to be representative of a *Pseudomonas* sp. (band 1, 100% sequence similarity to a 194 bp sequence of accession number AY269867), a member of the *Alphaproteobacteria* (band 2, 100% sequence similarity to a 194 bp product of accession number AJ011162) and a *Sphingomonas* sp. (band 3, 100% sequence similarity 114 bp sequence of AY129784). By day 21 an additional band representative of a member of the family *Acidobacteriaceae* (band 4, 99% sequence similarity to a 194 bp sequence of accession number AJ583203) also became predominant on the DGGE gel. As the DGGE analysis suggested that these bacteria became predominant during the increase in bacterial abundance associated with the biodegradation of geosmin in the enrichment culture, it is hypothesised that these bacteria were those responsible for the biodegradation of the geosmin in the enrichment culture. We recently isolated three Gram-negative bacteria from a sand filter which were capable of cooperatively degrading geosmin (Hoefel et al., 2006). From this it may be concluded that these bacteria may have played a vital role in the biodegradation of geosmin within the sand filter and also in the bioreactors. Experiments involving the application of stable isotope probing, using labelled geosmin, would be required to conclusively demonstrate that these were the only bacteria responsible for the biodegradation of geosmin in the sand filter and bioreactors.



**Figure 4.4** DGGE analysis of the enrichment culture supplemented with geosmin as the sole carbon source. M; reference marker, Neg; negative control, band 1 *Pseudomonas* sp., band 2 *Alphaproteobacterium*, band 3 *Sphingomonas* sp., and band 4 *Acidobacteriaceae*.

A similar approach was performed in an attempt to identify the key bacteria involved in the biodegradation of MIB within the sand filter and also within the bioreactors. However, attempts failed to enrich for any MIB degraders within the minimal medium supplemented with 35 mg L<sup>-1</sup> of MIB. These experiments were repeated using MIB as the sole carbon source at a concentration of 17 mg L<sup>-1</sup> but again failed to show any degradation of MIB or any increase in bacterial abundance. It is concluded that those bacteria responsible for the biodegradation of MIB may have required a growth factor(s) within the Morgan water that were absent within the minimal medium used for the enrichment culture, or that the concentrations of MIB used for enrichment had an inhibitory effect on the degrading bacteria.

## 4.4 Conclusions

MIB and geosmin were shown to be readily removed through bench-scale sand filters with removal shown to be predominantly through biodegradation processes, confirming the findings at the Morgan WTP. Biodegradation of both MIB and geosmin was determined to be a pseudo-first-order reaction, with rates influenced by the initial concentration of the biofilm but not the initial concentration of the T&O compounds. Furthermore, biodegradation rates were increased when the biofilm had been re-exposed to MIB and geosmin. Four bacteria, a *Pseudomonas* sp., an *Alphaproteobacterium*, a *Sphingomonas* sp. and an *Acidobacteriaceae* member were identified as microorganisms most likely involved in the biodegradation of geosmin within the sand filters and also the bioreactors. Experiments are currently underway in an attempt to isolate these microorganisms and to verify their ability to degrade geosmin individually.

## 4.5 References

- Andreozzi R., Cesaro R., Marotta R. and Pirozzi F. (2006) Evaluation of biodegradation kinetic constants for aromatic compounds by means of aerobic batch experiments. *Chemosphere* **62**(9), 1431-1436.
- Ashitani K., Hishida Y. and Fujiwara K. (1988) Behavior of musty odorous compounds during the process of water treatment. *Water Science & Technology* **20**(8/9), 261-267.
- Egashira K., Ito K. and Yoshiy Y. (1992) Removal of musty odour compound in drinking water by biological filter. *Water Science & Technology* **25**(2), 307-314.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2004a) Determination of system losses of geosmin and MIB in bench-scale filtration apparatus. *Water Quality Research Journal of Canada* **39**(3), 207-212.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2004b) Removal of geosmin and 2-methylisoborneol by biological filtration. *Water Science & Technology* **49**(9), 273-280.
- Hattori K. (1988) Water treatment systems and technology for the removal of odor compounds. *Water Science & Technology* **20**(8/9), 237-244.
- Hayes K.P. and Burch M.D. (1989) Odorous compounds associated with algal blooms in South Australian waters. *Water Research* **23**(1), 115-121.
- Ho L., Croué J.-P. and Newcombe G. (2004) The effect of water quality and NOM character on the ozonation of MIB and geosmin. *Water Science & Technology* **49**(9), 246-255.
- Ho L., Newcombe G. and Croué J.-P. (2002) Influence of the character of NOM on the ozonation of MIB and geosmin. *Water Research* **36**(3), 511-518.
- Hoefel D., Grooby W.L., Monis P.T., Andrews S. and Saint C.P. (2003) Enumeration of water-borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiological Methods* **55**(3), 585-597.
- Hoefel D., Ho L., Aunkofer W., Monis P.T., Keegan A., Newcombe G. and Saint C.P. (2006) Cooperative biodegradation of geosmin by a consortium comprising three Gram-negative bacteria isolated from the biofilm of a sand filter column. *Letters in Applied Microbiology* **43**(4), 417-423.
- Hoefel D., Monis P.T., Grooby W.L., Andrews S. and Saint C.P. (2005) Profiling bacterial survival through a water treatment process and subsequent distribution system. *Journal of Applied Microbiology* **99**(1), 175-186.
- Hrudey S.E., Huck P.M., Mitton M.J. and Kenefick S.L. (1995) Evaluation of odour removal by pilot-scale biological treatment process trains during spring runoff in an ice-covered river. *Water Science & Technology* **31**(11), 195-201.
- Ishida H. and Miyaji Y. (1992) Biodegradation of 2-methylisoborneol by oligotrophic bacterium isolated from eutrophied lake. *Water Science & Technology* **25**(2), 269-276.
- Izaguirre G., Wolfe R.L. and Means, III E.G. (1988) Degradation of 2-methylisoborneol by aquatic bacteria. *Applied & Environmental Microbiology* **54**(10), 2424-2431.
- Lauderdale C.V., Aldrich H.C. and Lindner A.S. (2004) Isolation and characterization of a bacterium capable of removing taste- and odor-causing 2-methylisoborneol from water. *Water Research* **38**(19), 4135-4142.
- Lundgren B.V., Grimvall A. and Sävenhed R. (1988) Formation and removal of off-flavour compounds during ozonation and filtration through biologically active sand filters. *Water Science & Technology* **20**(8/9), 245-253.

- Marshall J.A. and Hochstetler A.R. (1968) The synthesis of ( $\pm$ )-geosmin and the other 1,10-dimethyl-9-decalol isomers. *Journal of Organic Chemistry* **30**(6), 3642-3646.
- Narayan L.V. and Nunez, III W.J. (1974) Biological control: Isolation and bacterial oxidation of the taste and odour compound geosmin. *Journal of the American Water Works Association* **66**(9), 532-536.
- Newcombe G. and Cook D. (2002) Influences on the removal of tastes and odours by PAC. *Journal of Water Supply Research & Technology - AQUA* **51**(8), 463-474.
- Oikawa E., Shimizu A. and Ishibashi Y. (1995) 2-methylisoborneol degradation by the CAM operon from *Pseudomonas putida* PpG1. *Water Science & Technology* **31**(11), 79-86.
- Pirbazari M., Borow H.S., Craig S., Ravindran V. and McGuire M.J. (1992) Physical chemical characterization of five earthy-musty-smelling compounds. *Water Science & Technology* **25**(2), 81-88.
- Rittmann B.E., Gantzer C.J. and Montiel A. (1995) *Biological treatment to control taste-and-odor compounds in drinking water treatment*. In: Suffet I.H., Mallevalle J., Kawczynski E. (Eds.). *Advances In Taste-and-Odor Treatment and Control*. American Water Works Association Research Foundation, Denver, USA, pp. 209-246.
- Saadoun I. and El-Migdadi F. (1998) Degradation of geosmin-like compounds by selected species of Gram-positive bacteria. *Letters in Applied Microbiology* **26**(2), 98-100.
- Saito A., Tanaka A. and Oritani T. (1996) A practical synthesis of enantiomerically pure (-)-geosmin via highly diastereoselective reduction of (4aS,8S)-4,4a,5,6,7,8-hexahydro-4a,8-dimethyl-2(3H)-naphthalenone. *Tetrahedron: Asymmetry* **7**(10), 2923-2928.
- Saito A., Tokuyama T., Tanaka A., Oritani T. and Fuchigami K. (1999) Microbiological degradation of (-)-geosmin. *Water Research* **33**(13), 3033-3036.
- Schmidt S.K., Simkins S. and Alexander M. (1985) Models for the kinetics of biodegradation of organic compounds not supporting growth. *Applied & Environmental Microbiology* **50**(2), 323-331.
- Silvey J.K.G., Henley A.W., Nunez W.J. and Cohen R.C. (1970) Biological control: Control of naturally occurring taste and odors by microorganisms. In: *Proceedings of the National Biological Congress, Detroit, USA*.
- Sumitomo H. (1988) Odor decomposition by the yeast *Candida*. *Water Science & Technology* **20**(8/9), 157-162.
- Sumitomo H. (1998) pH and Mg/Ca control for biological treatment of an offensive flavour (2-MIB). *Water Science & Technology* **37**(10), 101-106.
- Tanaka A., Oritani T., Uehara F., Saito A., Kishita H., Niizeki Y., Yokota H. and Fuchigami K. (1996) Biodegradation of a musty odour component, 2-methylisoborneol. *Water Research* **30**(3), 759-761.
- Trudgill P.W. (1984) *Microbial degradation of the alicyclic ring: Structural relationships and metabolic pathways*. In: Gibson D.T. (Ed.). *Microbial Degradation of Organic Compounds*. Marcel Dekker Inc., New York, USA, pp. 131-180.
- Westerhoff P., Rodriguez-Hernandez M., Baker L. and Sommerfeld M. (2005) Seasonal occurrence and degradation of 2-methylisoborneol in water supply reservoirs. *Water Research* **39**(20), 4899-4912.
- Yagi M., Nakashima S. and Muramoto S. (1988) Biological degradation of musty odour compounds, 2-methylisoborneol and geosmin, in a bio-activated carbon filter. *Water Science & Technology* **20**(8/9), 255-260.

## CHAPTER 5: COOPERATIVE BIODEGRADATION OF GEOSMIN BY A CONSORTIUM COMPRISING THREE GRAM-NEGATIVE BACTERIA ISOLATED FROM THE BIOFILM OF A SAND FILTER COLUMN\*

### 5.1 Introduction

During water treatment, biofilm-associated bacteria attached to either sand (Ashitani et al., 1988; Lundgren et al., 1988) or granular activated carbon (GAC) filters (Yagi et al., 1988; Elhadi et al., 2004) have been reported to remove geosmin. Such approaches are attractive to water utilities as these process are generally low technology, requiring little maintenance and infrastructure, and operate without added chemicals. However, disadvantages include the need for an acclimatisation period during the establishment of a biofilter and the requirement for the degradative organisms within a biofilter to be retained during times of geosmin absence (Elhadi et al., 2004).

Biological degradation of geosmin-like compounds was first reported by Silvey and Roach (1964), who subsequently demonstrated that strains of *Bacillus cereus* were responsible for the degradation (Silvey et al., 1970). Since then, there have been conflicting reports, with Narayan and Nunez (1974) reporting that strains of *B. cereus* and *B. subtilis* isolated by soil-enrichment culture readily degraded geosmin; however, MacDonald et al. (1987) could not reproduce these results using the same strains. In addition, Danglot et al. (1983) reported the unsuccessful attempts to degrade geosmin using spent culture filtrates of *B. subtilis*. Since then, there has only been one other report of bacterial degradation of geosmin (Saadoun and El-Migdadi, 1998), where an alcohol transformation test was used to demonstrate that cultured strains of various Gram-positive bacteria, including *Arthrobacter atrocyaneus*, *Arthrobacter globiformis*, *Chlorophenolicus* strain N-1053 and *Rhodococcus maris*, were capable of degrading this compound in axenic culture with geosmin as the sole carbon source. In addition, there has been only one reported study which has attempted to investigate the various biodegradation products formed as part of the microbiological breakdown of geosmin (Saito et al., 1999).

To date there is a lack of understanding regarding the types of bacteria capable of degrading geosmin. In addition, there have been no studies to reveal if the action of a bacterial consortium, rather than single bacterial species acting alone, is responsible for degradation in natural water or filter biofilm. In this study we report, for the first time, the cooperative degradation of geosmin by a consortium comprising three Gram-negative bacteria. These results provide important information for water industry researchers trying to understand and harness the use of biological methods for the removal the taste and odour (T&O) compound, geosmin.

### 5.2 Materials and methods

#### 5.2.1 Bacterial enumeration

Numbers of active bacteria were determined by staining with the BacLight™ bacterial viability kit (Molecular Probes Inc., USA) and subsequent enumeration by flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, USA), as described previously (Hoefel et al., 2003).

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\* This chapter is based on the following manuscript:

Hoefel D., Ho L., Aunkofer W., Monis P.T., Keegan A., Newcombe G. and Saint C.P. (2006) Cooperative biodegradation of geosmin by a consortium comprising three Gram-negative bacteria isolated from the biofilm of a sand filter column. *Letters in Applied Microbiology* **43**(4), 417-423.

### 5.2.2 Geosmin analysis

Geosmin used in this study was purchased from Sigma-Aldrich (Sydney, Australia), as a racemic mixture and a stock solution prepared in sterile Milli-Q water (Millipore Ultra-Pure Water System) at a concentration of  $150 \text{ mg L}^{-1}$ . Samples for geosmin analysis were pre-concentrated using a solid phase microextraction syringe fibre (Supelco, Australia) and analysed on a 5890 Series II Gas Chromatograph with 5971 Series Mass Selective Detector (Hewlett Packard, Australia) against quantified labelled internal standards (Ultrafine Chemicals, UK), as reported previously (Hayes and Burch, 1989).

### 5.2.3 Removal of geosmin through a biologically-active sand filter column

Filter sand from Morgan water treatment plant (WTP) (South Australia) was packed into pre-autoclaved ( $121^\circ\text{C}$  for 20 min) laboratory glass columns (length 30 cm, internal diameter 2.5 cm) at a bed height of 15 cm, using apparatus as described by Ho et al. (2006). Columns were incubated at room temperature ( $20 \pm 2^\circ\text{C}$ ) and reservoir water (Myponga, South Australia,  $9.56 \text{ mg L}^{-1}$  dissolved organic carbon, pH 7.0), spiked with geosmin (target concentration of  $100 \text{ ng L}^{-1}$ ), was passed through the column at an average empty bed contact time (EBCT) of 15 min. Geosmin analysis was performed at regular intervals on samples taken from the column influent and effluent.

### 5.2.4 Enrichment of geosmin-degrading bacteria

Sand (5 g wet weight) was removed from the biologically-active filter column following 80 d, a time of 100% geosmin removal. Biofilm was detached from the sand by 15 min of periodic vortexing (four sets of continuous vortexing for 3 min with 1 min settling time in between) in 30 mL of sterile Bushnell-Haas (BH) minimal liquid medium (0.1% (w/v)  $\text{NH}_4\text{NO}_3$ , 0.1% (w/v)  $\text{K}_2\text{HPO}_4$ , 0.1% (w/v)  $\text{KH}_2\text{PO}_4$ , 0.01% (w/v)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01% (w/v)  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and 0.001% (w/v)  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ). Bacteria within the supernatant were then washed twice by centrifugation at 1,000 g for 15 min with re-suspension of the bacterial pellet each time in sterile BH medium. To enrich for geosmin degrading bacteria, a total of  $6.0 \times 10^5$  active biofilm-associated bacteria  $\text{mL}^{-1}$  were inoculated in sterile BH medium, supplemented with  $29.0 \text{ mg L}^{-1}$  of geosmin (final culture volume of 20 mL). An equivalent number of active biofilm-associated bacteria were inoculated into 20 mL of BH minimal medium without geosmin, to control for any bacterial growth on alternative contaminating carbon sources within the minimal medium. To control for any losses of geosmin due to factors other than biological degradation, a portion of previously washed bacteria were inactivated by autoclaving ( $121^\circ\text{C}$  for 20 min) and added at a concentration of  $6.0 \times 10^5$  bacteria  $\text{mL}^{-1}$  to BH medium, which was supplemented with  $29.5 \text{ mg L}^{-1}$  of geosmin (final culture volume of 20 mL). Each culture was incubated at  $22^\circ\text{C}$  with shaking at 100 rpm for 51 d.

### 5.2.5 Denaturing gradient gel electrophoresis analysis of the enrichment culture

During enrichment, duplicate 0.25 mL aliquots were periodically taken and concentrated by centrifugation at 10,000 g for 10 min with re-suspension of the bacterial pellet in 50  $\mu\text{L}$  of 10 mmol  $\text{L}^{-1}$  Tris-HCl (pH 7.5). Following cell membrane disruption by rapid freeze-thawing, a fragment of the 16S rRNA gene was PCR amplified using the primer set 357F-GC/518R, and the products of the reaction analysed by DGGE (D-GENE™ Denaturing Gel Electrophoresis System, Bio-Rad, USA) as reported previously (Hoefel et al., 2005).

Isolation of the predominant bacteria identified by DGGE was achieved by inoculating 10 fold dilutions of the enrichment culture onto solid R2A medium and selecting predominant colony types that had formed following 7 d incubation at  $22^\circ\text{C}$ . R2A medium is commonly used for the culture of bacteria of environmental origin (Hoefel et al., 2005). These colonies, denoted as Geo24, Geo25 and Geo33, were re-suspended in 100  $\mu\text{L}$  of 10 mmol  $\text{L}^{-1}$  of Tris-HCl (pH 7.5), amplified by PCR (as described previously) and the products analysed by DGGE, in parallel with the day 35 sample from the enrichment culture. Co-migration and subsequent DNA sequence analysis confirmed that the three isolated bacteria were the most abundant bacteria represented by the DGGE bands in the day 35 sample.

## 5.2.6 Phylogenetic analysis of isolated bacteria

The DNA sequence for a larger fragment of the 16S rRNA gene from Geo24, Geo25 and Geo33 was determined following PCR-amplification with the primer set 27F/1492R as reported previously (Hoefel et al., 2005). Sequence similarity searches were conducted using the National Center for Biotechnology Information BLAST network service (blastn). Similar sequences, from previously cultured bacteria, were obtained from GenBank and aligned against the DNA sequences of Geo24, Geo25 and Geo33 using ClustalX version 1.64b software (Thompson et al., 1997). Neighbour-joining analysis with 1,000 bootstrap replicates (MEGA version 2.1; Arizona State University, USA) was then performed.

## 5.2.7 Geosmin degradation by bacterial isolates

Isolates Geo24, Geo25 and Geo33 were inoculated into BH medium either individually, in every combination of two, and as a mixture of each of the three bacteria, at final concentrations between  $6.0 \times 10^5$  to  $1.0 \times 10^6$  active bacteria  $\text{mL}^{-1}$ . Cultures containing mixtures of bacteria were prepared such that each bacterium was added in equal proportion. Geosmin was supplemented as the sole carbon source in each culture at a final target concentration of  $20.0 \text{ mg L}^{-1}$  in a total volume of 20 mL. For each culture, any losses of geosmin due to factors other than biological degradation, and any growth of the bacteria utilising organic contaminants in the minimal medium, were controlled for as described above for the enrichment culture. Bacterial cultures were incubated at  $22^\circ\text{C}$  with shaking at 100 rpm for 56 d.

The ability of Geo24, Geo25 and Geo33 to degrade geosmin under more environmentally relevant conditions was also confirmed by experiments performed in  $0.2 \mu\text{m}$  filter sterilised/autoclaved reservoir water (Myponga, South Australia;  $12.5 \text{ mg L}^{-1}$  dissolved organic carbon,  $\text{UV}_{254} 0.472 \text{ cm}^{-1}$ , pH 7.5) spiked with geosmin concentrations of 37 and  $131 \text{ ng L}^{-1}$ , using the same combinations and controls as described previously. These cultures were continually stirred and incubation was performed at room temperature ( $20 \pm 2^\circ\text{C}$ ).

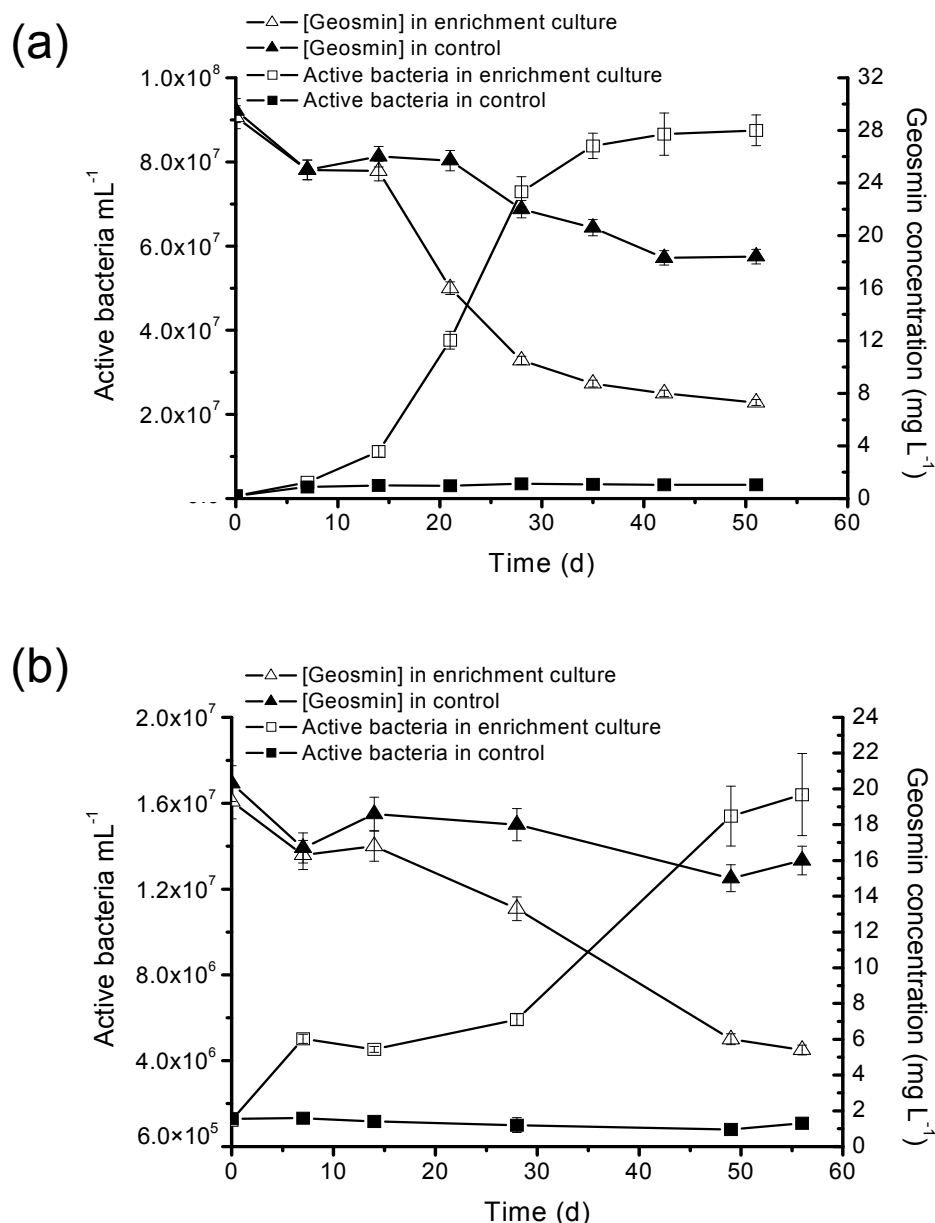
## 5.3 Results

### 5.3.1 Removal of geosmin through a biologically-active sand filter column

Initial geosmin removals at day 0 were equivalent to 46%, due to a combination of physical adsorption, losses due to volatilisation and biological removal by biofilm bacteria initially present on the filter sand. Following the passage of geosmin-spiked reservoir water through the column for 80 d, additional biofilm had established and the apparent acclimatisation of the biofilm resulted in 100% removal of geosmin. This sand filter biofilm provided a source of inocula for subsequent isolation of geosmin-degrading bacteria.

### 5.3.1 Enrichment of geosmin-degrading bacteria

Biofilm was taken from the sand filter medium and enrichment of geosmin-degrading bacteria was performed in BH broth supplemented with geosmin as the sole carbon source. As shown in Figure 5.1a, measurable geosmin degradation began following 14 d of incubation, where the greatest removal occurred between days 14 and 28, during which time bacterial growth was also at a maximum. By day 51,  $21.7 \text{ mg L}^{-1}$  of the original  $29 \text{ mg L}^{-1}$  geosmin had been degraded, resulting in a  $2.12 \log_{10}$  increase in active bacterial numbers. These results were confirmed in a replicate experiment.

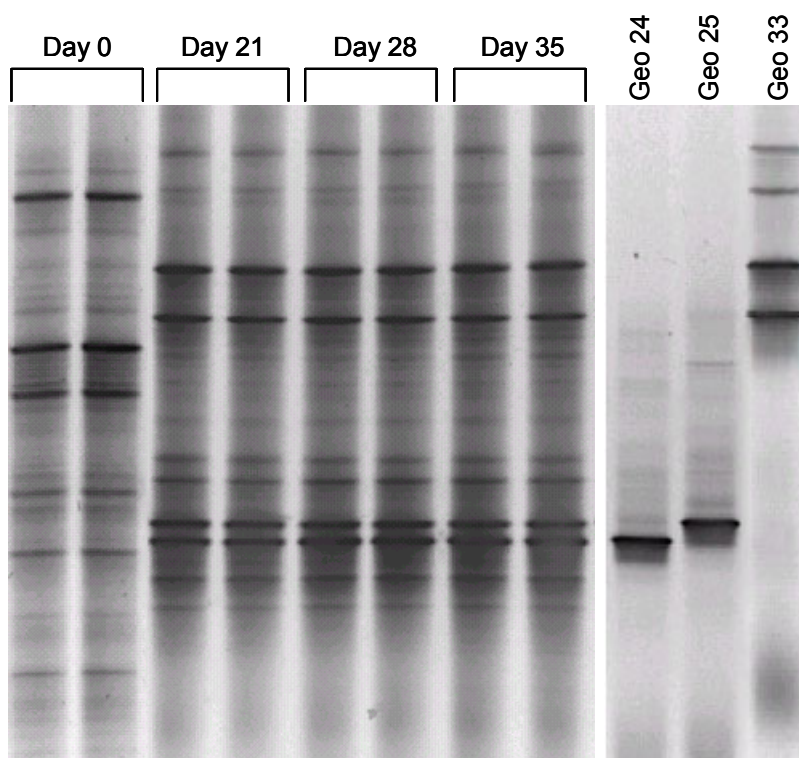


**Figure 5.1** Active bacterial abundance and geosmin concentration during (a) the enrichment of geosmin-degrading bacteria and (b) the study of geosmin degradation by the bacterial consortium comprising Geo24, Geo25 and Geo33. Error bars represent standard deviations of triplicate analyses.

### 5.3.2 DGGE analysis of the enrichment culture

Samples for DGGE were taken throughout the course of the enrichment culture to reveal the predominance of any particular bacteria during the onset of geosmin degradation. As shown in Figure 5.2, there was a distinct change in community composition from that of the original sand filter biofilm inoculum (day 0) to that during the period of maximum geosmin degradation and greatest bacterial growth (day 21). Following day 21, geosmin degradation and bacterial growth continued (Figure 5.1a); however, the bacterial community profile appeared to remain constant (Figure 5.2). It was hypothesised that those bacteria represented by the major DGGE bands that appeared at day 21, and remained predominant for the duration of the culture, were those involved in the degradation of geosmin.





**Figure 5.2** Denaturing gradient gel electrophoresis (DGGE) profiles of 16S rRNA gene-directed PCR fragments from bacteria within the enrichment culture at various stages of incubation (left panel) and isolates Geo24, Geo25 and Geo33 (right panel).

The 16S rRNA gene products of three bacteria (Geo24, Geo25 and Geo33) isolated from the enrichment culture revealed identical DGGE band migration to the predominant DGGE bands in the day 35 sample (Figure 5.2). Band excision and subsequent DNA sequence analyses confirmed each isolate band as having identical DNA sequence to that of its corresponding day 35 sample band. Single bands were observed by DGGE for isolates Geo24 and Geo25; however, multiple bands were observed for isolate Geo33 (Figure 5.2). Heterogeneous copies of the 16S rRNA gene were identified as the cause of the more complex banding profile for isolate Geo33 (data not shown) and upon analysis of additional colonies the same profile was reproducibly observed.

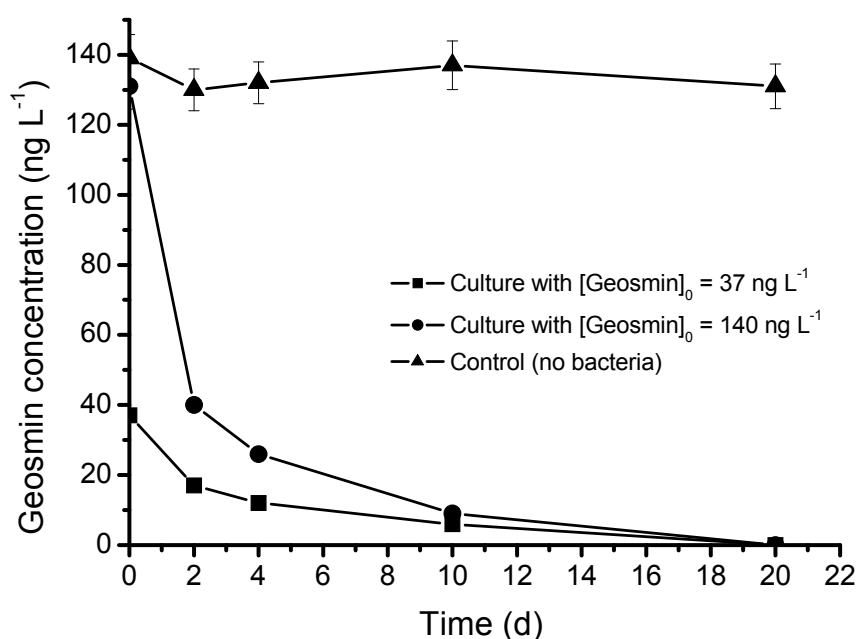
### 5.3.3 Phylogenetic analysis of isolated bacteria

Phylogenetic analysis revealed that isolate Geo24 clustered with *Sphingopyxis alaskensis*, *Sphingopyxis wittfariensis*, *Sphingopyxis composta*, *Sphingopyxis macrogoltabida*, *Sphingomonas taejonensis* and *Sphingomonas chilensis* (94% bootstrap support). The Geo24 sequence was 99.9% similar to a 1357 bp sequence of GenBank accession number AY177357 (a phenanthrene-degrading bacterium M20), but of the previously cultured bacteria was most similar to *S. alaskensis* (97% sequence similarity to a 1411 bp sequence). Isolate Geo25 was 99.9% similar to a 1405 bp sequence of GenBank accession number AJ575708 (an uncultured alpha-proteobacterium) but of the previously cultured bacteria was most similar to *Novosphingobium stygiae* (97% sequence similarity for a 1405 bp sequence of the 16S rRNA gene), and also clustered strongly with that bacterium (96% bootstrap support). Isolate Geo33 was identified as belonging to the fluorescent pseudomonad group and clustered with *Pseudomonas veronii* (93% bootstrap support), having 99.9% sequence similarity to a 1459 bp sequence of the 16S rRNA gene of that organism. The DNA sequences of the 16S rRNA gene fragments for Geo24, Geo25 and Geo33 were deposited to GenBank under accession numbers DQ137852, DQ137853 and DQ137854, respectively.

### 5.3.4 Geosmin degradation by bacterial isolates

Isolates Geo24, Geo25 and Geo33 were assessed for their capacity to degrade geosmin. During the incubation period, neither geosmin degradation nor an increase in active bacterial abundance was detected in any of the cultures containing the individual isolates or for any of the cultures containing mixtures of two bacteria. Identical results were obtained upon repeating these experiments at both 1 to 100  $\mu\text{g L}^{-1}$  and 40 to 150  $\text{ng L}^{-1}$  concentrations, suggesting that none of the isolates alone could perform the initial conversion of geosmin into a primary metabolite. However, geosmin degradation and an increase in active bacterial abundance was reported following a 14 d lag period within the culture containing a mixture of the three isolates (Figure 5.1b). Following 56 d of incubation a total of 13.9  $\text{mg L}^{-1}$  of geosmin had been degraded resulting in an increase of active bacterial abundance equivalent to 1.13  $\log_{10}$ . These results were confirmed in a replicate experiment. Whilst an increase in active bacterial abundance during geosmin degradation was reported (Figure 5.1b), DGGE analysis of samples taken throughout this period failed to reveal the predominance of any one member of the consortium (data not shown).

The isolated organisms were also assessed for their ability to degrade geosmin under more environmentally relevant geosmin concentrations in reservoir water. This was performed with each organism individually, and in the various combinations as mentioned above. As shown in Figure 5.3, following 20 d of incubation only the consortium mixture consisting of Geo24, Geo25 and Geo33 was able to degrade geosmin from initial geosmin concentrations of 37 and 131  $\text{ng L}^{-1}$  to below the limit of detection ( $<2 \text{ ng L}^{-1}$ ).



**Figure 5.3** Degradation of geosmin in reservoir water by the bacterial consortium. Error bars represent standard deviations of triplicate analyses.

## 5.4 Discussion

Until now, geosmin degradation had only been reported to occur by Gram-positive bacteria (Silvey et al., 1970; Narayan and Nunez, 1974; Saadoun and El-Migdadi, 1998), and this is the first documented case of geosmin degradation by a bacterial consortium of three Gram-negative bacteria isolated from a sand filter column. Cooperative metabolic activities amongst bacterial consortia during degradation of organic compounds in nature are widespread (Slater and Lovatt, 1984), where such cooperation generally relies on either of two mechanisms (Dejonghe et al., 2003). The first involves associated metabolism, in which cross feeding with metabolites from the degradation pathway occurs between

members of the consortium. Examples of this mechanism have been demonstrated in the cooperative catabolic pathways proposed for the degradation of the herbicide atrazine (De Souza et al., 1998; Smith et al., 2005). In such instances, it would be expected that at least one member of the consortium would be capable of initiating degradation in isolation, but this was shown not to occur in our study for any member of the geosmin-degrading consortium when inoculated individually with geosmin as the sole carbon source. The second most common mechanism for degradation of organic compounds by bacterial consortia is by complementation of metabolic deficiencies, where a bacterium contains all of the necessary genes for degradation but requires essential growth factors or nutrients delivered by secondary strains. One such example of this was demonstrated by Sørensen et al. (2002), who showed that the auxotrophic *Sphingomonas* sp. strain SRS2 could efficiently degrade the herbicide isoproturon when L-methionine was delivered to SRS2 by a second bacterium. The data from our study, where degradation and bacterial growth only occurred in the presence of all three members of the consortium, suggested that this second mechanism, or possibly a combination of associated metabolism and complementation of metabolic deficiencies, occurred in this instance.

This study has reported, for the first time, the cooperative degradation of geosmin by a consortium comprising three Gram-negative bacteria. These results are important for researchers currently employing molecular-based approaches to further understand the biodegradation of geosmin by bacteria; as such, studies may be complicated by the discovery of geosmin degradation occurring by a consortium. This study also advances the knowledge surrounding the types of bacteria capable of degrading the T&O compound, as investigations to date regarding this are limited. Further investigations are currently underway to elucidate the precise mechanism for the cooperative degradation of geosmin reported here, including the bacterial genes involved. Such information will provide a significant step, but only one of many more required before more successful strategies are developed for establishing and maintaining the degradative capacity of biologically-active filters during water treatment. Such information will also allow for the development of molecular-based tools, such as quantitative PCR, for assessing the degradative potential of such filters. In addition, it will provide a platform for the development of gene-based biosensors for the more rapid and cost effective analysis of geosmin in source waters.

## 5.5 References

- Ashitani K., Hishida Y. and Fujiwara K. (1998) Behavior of musty odorous compounds during the process of water treatment. *Water Science & Technology* **20**(8/9), 261-267.
- Danglot C., Amar G. and Vilagines R. (1983) Ability of *Bacillus* to degrade geosmin. *Water Science & Technology* **15**(5/6), 291-299.
- De Souza M.L., Newcombe D., Alvey S., Crowley D.E., Hay A., Sadowsky M.J. and Wackett L.P. (1998) Molecular basis of a bacterial consortium: interspecies catabolism of Atrazine. *Applied & Environmental Microbiology* **64**(1), 178-184.
- Dejonghe W., Berteloot E., Goris J., Boon N., Crul K., Maertens S., Höfte M., De Vos P., Verstraete W. and Top E.M. (2003) Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovorax* strain. *Applied & Environmental Microbiology* **69**(3), 1532-1541.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2004) Removal of geosmin and 2-methylisoborneol by biological filtration. *Water Science & Technology* **49**(9), 273-280.
- Hayes K.P. and Burch M.D. (1989) Odorous compounds associated with algal blooms in South Australian waters. *Water Research* **23**(1), 115-121.
- Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.
- Hoefel D., Grooby W.L., Monis P.T., Andrews S. and Saint C.P. (2003) Enumeration of water-borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiological Methods* **55**(3), 585-597.
- Hoefel D., Monis P.T., Grooby W.L., Andrews S. and Saint C.P. (2005) Profiling bacterial survival through a water treatment process and subsequent distribution system. *Journal of Applied Microbiology* **99**(1), 175-186.
- Lundgren B.V., Grimvall A. and Savenhed R. (1988) Formation and removal of off-flavour compounds during ozonation and filtration through biologically active sand filters. *Water Science & Technology* **20**(8/9), 245-253.

- MacDonald J.C., Bock C.A. and Slater G.P. (1987) Evaluation of *Bacillus* as a practical means for degradation of geosmin. *Applied Microbiology & Biotechnology* **25**(4), 392-395.
- Narayan L.V. and Nunez W.J. (1974) Biological control: Isolation and bacterial oxidation of the taste and odor compound geosmin. *Journal of the American Water Works Association* **66**(9), 532-536.
- Saadoun I. and El-Migdadi F. (1998) Degradation of geosmin-like compounds by selected species of Gram-positive bacteria. *Letters in Applied Microbiology* **26**(2), 98-100.
- Saito A., Tokuyama T., Tanaka A., Oritani T. and Fuchigami K. (1999) Microbial degradation of (-)-geosmin. *Water Research* **33**(13), 3033-3036.
- Silvey J.K.G., Henley A.W., Nunez W.J. and Cohen R.C. (1970) Biological control: Control of naturally occurring taste and odors by microorganisms, In: *Proceedings of the National Biological Congress, Detroit, USA*.
- Silvey J.K.G. and Roach A.W. (1964) Studies on microbiotic cycles in surface water. *Journal of the American Water Works Association* **56**, 60-72.
- Slater J.H. and Lovatt D. (1984) *Biodegradation and the significance of microbial communities*. In: Gibson D.T. (Ed.). *Microbial Degradation of Organic Compounds*. Marcel Dekker Inc., New York, USA, pp. 439-485.
- Smith D., Alvey S. and Crowley D.E. (2005) Cooperative catabolic pathways within an atrazine-degrading enrichment culture isolated from soil. *FEMS Microbiology Ecology* **53**(2), 265-273.
- Sørensen S.R., Ronen Z. and Aamand J. (2002) Growth in coculture stimulates metabolism of the phenylurea herbicide isoproturon by *Sphingomonas* sp. strain SRS2. *Applied & Environmental Microbiology* **68**(7), 3478-3485.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G. (1997) The CLUSTAL\_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**(24), 4876-4882.
- Yagi M., Nakashima S. and Muramoto S. (1988) Biological degradation of musty odor compounds, 2-methylisoborneol and geosmin, in a bio-activated carbon filter. *Water Science & Technology* **20**(8/9), 255-260.

## CHAPTER 6: ENHANCING THE BIOFILTRATION OF GEOSMIN BY SEEDING SAND FILTER COLUMNS WITH A CONSORTIUM OF GEOSMIN-DEGRADING BACTERIA\*

### 6.1 Introduction

Biological filtration (biofiltration) is a promising method for the treatment of geosmin. A number of biofiltration studies for the removal of geosmin have been reported, many of which were conducted on granular activated carbon (GAC) media (Yagi et al., 1988; Hruvey et al., 1995; Elhadi et al., 2004b), with some using alternative media such as glass beads, porous ceramic and plastic media (Namkung and Rittmann, 1987; Egashira et al., 1992; Hruvey et al., 1995; Terauchi et al., 1995; Sugiura et al., 2003). A useful approach for biofiltration is to use water treatment plant (WTP) sand filters as a medium. Here, only minimal changes to plant operation are required, such as the elimination of a pre-chlorination step before the filters; a process commonly used to enhance the particle removal and filtration cycle length. A number of studies have shown the potential of biological sand filtration for geosmin removal (Ashitani et al., 1988; Lundgren et al., 1988; Ho et al., 2007; McDowall et al., 2007b). However, it has been shown that the biodegradation process not only requires a significant period of time to commence, it is also highly dependent upon the types of organisms present (Hoefel et al., 2006; Summers et al., 2006; Ho et al., 2007; McDowall et al., 2007a). In addition, studies have shown that although geosmin removals can occur within a biologically-active sand filter, the level of geosmin removal is often not sufficient to produce water of an acceptable quality (Elhadi et al., 2006; Metz et al., 2006).

One approach for enhancing the removal of geosmin through sand filters is to artificially inoculate or “seed” sand filters with organisms capable of degrading geosmin. This approach may minimise the extended start-up period and also increase geosmin removals. Feakin et al. (1995) showed increased removals of s-triazine pesticides through GAC filters seeded with s-triazine degrading bacteria, demonstrating that this approach may be feasible for other organic compounds, such as geosmin. To date, there has only been one reported attempt to enhance the biofiltration of geosmin, where Yagi et al. (1988) inoculated GAC, sand and zeolite filters with a strain of *Bacillus subtilis* thought to be a geosmin-degrader. Results of that study revealed no significant enhancement of geosmin removal where only 50% of the loaded geosmin was removed, presumed to be through biodegradation processes.

The aim of this study was to enhance the biofiltration of geosmin by seeding sand filters with a fully characterised consortium of geosmin-degrading bacteria previously isolated by Hoefel et al. (2006). Sand was obtained from the filter bed of a South Australian WTP and tested in laboratory-scale columns.

### 6.2 Experimental procedures

#### 6.2.1 Materials

Settled water was collected from the Morgan WTP in South Australia and used as the influent water for all experiments in this study. Before collection, the settled water had been treated by coagulation, flocculation and sedimentation but no disinfection. It had a dissolved organic carbon (DOC) concentration of between 2.5–4.0 mg L<sup>-1</sup> and UV absorbance at 254 nm of between 0.05–0.06 cm<sup>-1</sup>. Following collection, this water was filtered through a 1 µm Polypure Capsule (Pall Life Sciences, USA) and autoclaved (121°C for 15 min) prior to use.

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\* This chapter is based on the following manuscript:

McDowall B., Hoefel D., Newcombe G., Saint C.P. and Ho L. Enhancing the biofiltration of geosmin by seeding sand filter columns with a consortium of geosmin-degrading bacteria. *Water Research* (submitted).

Geosmin was received as a yellow oil (Ultrafine Chemicals, UK), and prepared by dissolving in Milli-Q water (Millipore Pty Ltd., USA). The final stock solution was transferred to 100 mL amber glass bottles and stored headspace free in the dark at 4°C. This geosmin stock was used to spike Morgan settled water at a target geosmin concentration of 100 ng L<sup>-1</sup>. Geosmin analysis was carried out by solid phase microextraction gas chromatography-mass spectrometry. Sample concentration was performed using solid phase micro extraction syringe fibres (Supelco, Australia) and analysis conducted on a Hewlett Packard 5890 Series II Gas Chromatograph with Hewlett Packard 5971 Series Mass Selective Detector (Agilent Technologies, Australia) against qualified labelled internal standards (Ultrafine Chemicals, UK). The detection limit was 0.6 ng L<sup>-1</sup> geosmin. Full details of the method are reported elsewhere (Hayes and Burch, 1989).

### 6.2.2 Culture of geosmin-degrading bacteria

The bacteria used in this study were *Sphingopyxis* sp. Geo24, *Novosphingobium* sp. Geo25 and *Pseudomonas* sp. Geo33. All three bacteria were isolated from a previous sand filter column study by our group, and subsequently shown to cooperatively degrade geosmin (Hoefel et al., 2006). Each organism was cultured separately in 50 mL of R2A liquid medium (0.025% (w/v) tryptone, 0.05% (w/v) yeast extract, 0.075% (w/v) peptone, 0.05% (w/v) glucose, 0.05% (w/v) starch, 0.03% (w/v) K<sub>2</sub>HPO<sub>4</sub>, 0.0024% (w/v) MgSO<sub>4</sub> and 0.03% (w/v) sodium pyruvate, pH 7.2) for 5 d at 22°C with continuous shaking. The cultures then underwent centrifugation at 1,000 g for 15 min, the supernatant was aspirated and the bacterial pellet re-suspended in 10 mL of sterile Morgan settled water. The numbers of active bacteria for each culture were then determined by flow cytometry.

### 6.2.3 Flow cytometry for bacterial enumeration

Flow cytometry was used in this study to determine the number of active bacteria in the inoculum. Bacteria were stained using the LIVE/DEAD® BacLight™ kit (Molecular Probes Inc., USA) and subsequently enumerated using a FACSCalibur flow cytometer (Becton Dickinson, USA) as described previously (Hoefel et al., 2003).

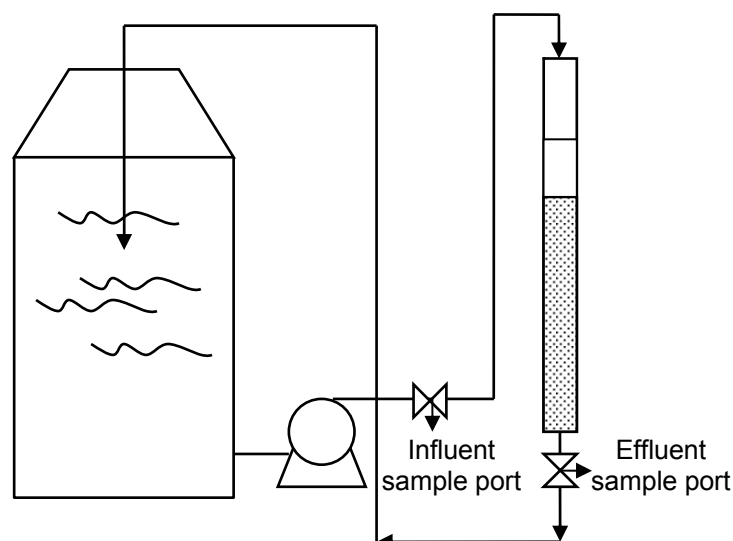
### 6.2.4 Laboratory-scale sand column experiments

Laboratory-scale sand filter columns were used to simulate the sand filtration process that occurs within WTPs. These columns had an internal diameter of 2.5 cm with a media height of 15 cm. Each laboratory column trial employed selected sand media. The first trial utilised sand which contained a pre-existing biofilm. This sand was sampled from the filter beds at the Morgan WTP in South Australia and shown to contain a flourishing biofilm (McDowall et al., 2007b). After collection, the sand was passed through a 1 mm sieve to remove any gravel and anthracite, giving a final effective particle size of 0.55 mm. Prior to loading in the laboratory columns, the sand was autoclaved at 121°C for 15 min to inactivate the existing biofilm. The autoclaving procedure was employed as the biofilm of this sand had previously been shown to harbour organisms capable of degrading geosmin (Hoefel et al., 2006; Ho et al., 2007).

The second trial utilised virgin sand which did not contain any biofilm. This sand was purchased new from a commercial supplier (Riversands, Australia) and shown to have the same particle size characteristics as the Morgan WTP sand. Finally, the third trial employed sand with a pre-existing biofilm which was not capable of removing geosmin through biological action. This sand was taken from a biofiltration pilot plant which had been operating for 780 d and had not shown effective geosmin removals (unpublished data). It was the same sand as that purchased for the virgin sand trial.

Autoclaved Morgan settled water was stored in 10 L glass containers and fed to the laboratory sand columns using an adjustable peristaltic pump (Gilson Miniplus 3, France). All tubing used was Tygon® Lab tubing (Masterflex, USA). The laboratory sand columns were set up as shown in Figure 6.1. Prior to inoculation with the geosmin-degrading consortium, the columns were drained so that the head water was approximately 1 cm above the top of the filter bed. Inoculum volumes of 20 mL of each

organism were seeded into the top of the column. The resulting number of each organism in the 10 L glass container is shown in Table 6.1.



**Figure 6.1** Schematic of the laboratory sand column apparatus.

**Table 6.1** Geosmin-degrading consortium numbers employed during inoculation into sand column trials.

Organisms	Inoculum concentration in 10 L container (active cells mL <sup>-1</sup> )		
	Trial 1- Sand with sterilised pre-existing biofilm	Trial 2- Virgin sand with no biofilm	Trial 3 – Sand with non-sterilised pre-existing biofilm
Geo 24	5.1 x 10 <sup>6</sup> (4.2 x 10 <sup>6</sup> )	2.3 x 10 <sup>6</sup>	5.9 x 10 <sup>6</sup>
Geo 25	1.9 x 10 <sup>6</sup> (4.1 x 10 <sup>6</sup> )	3.9 x 10 <sup>6</sup>	2.5 x 10 <sup>6</sup>
Geo 33	1.5 x 10 <sup>6</sup> (2.9 x 10 <sup>6</sup> )	3.6 x 10 <sup>6</sup>	2.6 x 10 <sup>6</sup>

Data in parentheses represent numbers in a repeat experiment of trial 1

After bacterial inoculation, autoclaved Morgan settled water was recirculated through the sand columns at a rate of 1 mL min<sup>-1</sup>, corresponding to an empty bed contact time (EBCT) of 75 min, for a period of 4 d during which the bacterial consortium was expected to attach to the sand surface. The filtration rate was then increased to 5 mL min<sup>-1</sup> (15 min EBCT) and recirculation was ceased, allowing the inoculated water to pass out of the system to waste. The 10 L container and tubing were then cleaned thoroughly with hot tap water followed by rinsing with Milli-Q water. The cleaned 10 L container was filled with autoclaved Morgan settled water spiked with 100 ng L<sup>-1</sup> of geosmin, which was passed through the system at a continuous rate of 5 mL min<sup>-1</sup>. All experiments were conducted at room temperature (20±2°C). Samples for geosmin analysis were taken at regular intervals during this study from the column sample ports located just prior to and just after the product left the filter to avoid system losses which are known to occur in geosmin studies (Elhadi et al., 2004a). Non-inoculated control sand columns were carried out in parallel to each sand column study. These control columns were conducted in an identical manner to the inoculated columns, with the exception of the geosmin-degrading consortium.

### 6.2.5 Measurements of bacterial attachment

During the laboratory sand column trials, sand was periodically sampled from the upper layers of the columns to determine the extent of bacterial attachment using the ATPlite Luminescence ATP Detection Assay System (Perkin Elmer, USA). Sand was aseptically sampled (in triplicate) and placed

in pre-weighed sterile 1.5 mL tubes. Excess water was removed by pipetting, and the 'wet weight' of the sand then determined (approximately 0.3-0.4 g was sampled each time). Following this, 200  $\mu\text{L}$  of the cell lysis solution was added to each sand sample and the mixture was vortexed for 2 min to release ATP from the cells within the biofilm. Optimisation of the method had revealed that a vortex time of 2 min resulted in maximum ATP release (data not shown). All liquid was then transferred to a new sterile 1.5 mL tube and any particulate matter was then pelleted by centrifugation at 10,000 g for 4 min. Following this, 50  $\mu\text{L}$  of the supernatant (containing the ATP) was then transferred to a new 1.5 mL tube, to which 100  $\mu\text{L}$  of sterile Milli-Q water was added in addition to 50  $\mu\text{L}$  of the enzyme substrate solution.

ATP standards were prepared by adding 250  $\mu\text{L}$  of cell lysis solution to approximately 0.5 g of autoclaved (121°C for 15 min) Morgan WTP sand. Method validation had previously revealed that this autoclaving destroyed all measurable ATP from within the sand biofilm (data not shown). To prepare the standards, 50  $\mu\text{L}$  of the sand biofilm supernatant was taken, to which 80  $\mu\text{L}$  of sterile Milli-Q water was added, followed by a range of ATP concentrations ( $10^{-6}$  M ATP to  $10^{-9}$  M ATP) delivered in a volume of 20  $\mu\text{L}$  solution. Finally, 50  $\mu\text{L}$  of enzyme substrate solution was added. The use of autoclaved sand supernatant accounted for any interference due to excess colour from the sand biofilm.

Samples and standards were analysed in a 96 well Isoplate using a Wallac 1420 multilabel counter system (Perkin Elmer, USA). The luminescence outputs of the samples were compared to ATP standard curves. Final ATP concentrations were multiplied by four to account for the dilution of sample in 200  $\mu\text{L}$  of cell lysis solution. This value was divided by the wet weight of the sand to obtain nmol ATP g $^{-1}$  sand (wet weight). The final data is presented as nmol ATP cm $^{-3}$  filter media. A media density of 1.54 g cm $^{-3}$  was used to convert the weight values to unit filter volumes.

## 6.3 Results and discussion

### 6.3.1 Geosmin removal through sand columns with pre-sterilised biofilm

The first trial involved inoculation of the geosmin-degrading consortium into a sand column containing a pre-sterilised biofilm. This biofilm was pre-sterilised as it had previously been shown to harbour indigenous organisms capable of degrading geosmin (Hoefel et al., 2006; Ho et al., 2007), and this would have effectively masked any geosmin removal observed as a result of the inoculation procedures employed in this study. The procedure employed for inoculation of the geosmin-degrading consortium into the sand column was similar to that of Feakin et al. (1995), who inoculated pesticide-degrading bacteria into GAC filters. However, in our study we also employed a novel recirculation step which allowed for maximum attachment of the geosmin-degrading bacteria onto the sand/biofilm surface. A non-inoculated control sand column was also prepared in parallel to assess the losses of geosmin in the absence of the geosmin-degrading consortium. Both columns were fed with autoclaved Morgan settled water spiked with geosmin.

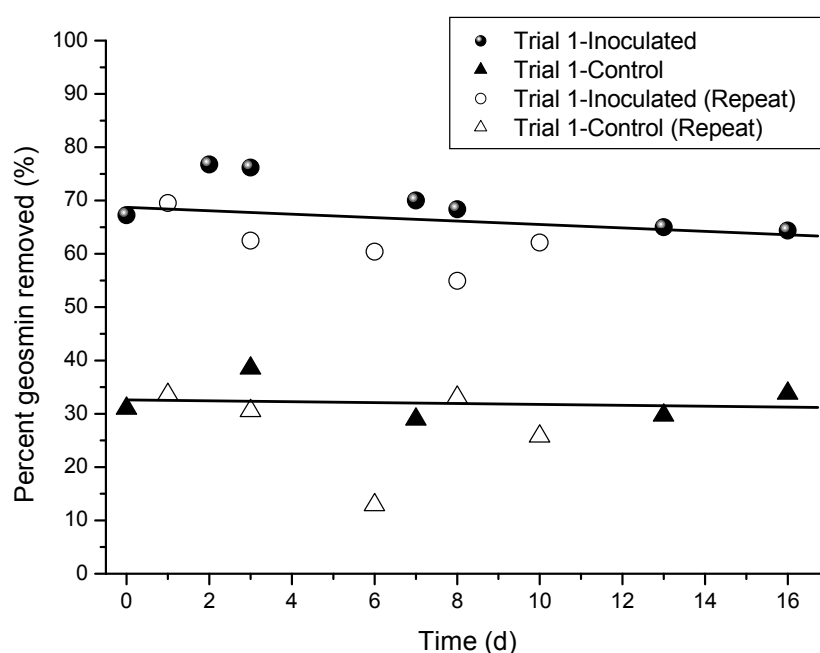
The removal of geosmin in trial 1 is presented in Figure 6.2. The concentrations of geosmin in the influent varied between 60 and 120 ng L $^{-1}$ . Geosmin removal through the non-inoculated sand column averaged 32%. Previous studies conducted by our research group have shown comparable removals through similar control columns and attributed the losses of geosmin to physical/abiotic processes, including volatilisation and adsorption (Ho et al., 2007). In contrast, geosmin removal through the inoculated sand column was considerably higher, an average of 70% throughout the trial. The enhanced removal of geosmin through this sand column is attributed to the presence of the inoculated bacterial consortium which has been shown previously to be capable of biodegrading geosmin (Hoefel et al., 2006).

This first trial was repeated to assess the reproducibility of the results. Geosmin removals in the repeat trial (see open symbols in Figure 6.2) were similar, demonstrating the repeatability of the inoculation and laboratory column procedures.

As observed in Figure 6.2, complete geosmin removal through the inoculated sand columns did not occur. Hoefel et al. (2006) previously reported complete geosmin removal by the same bacterial consortium but in a planktonic state within batch bioreactor studies following 20 d incubation in



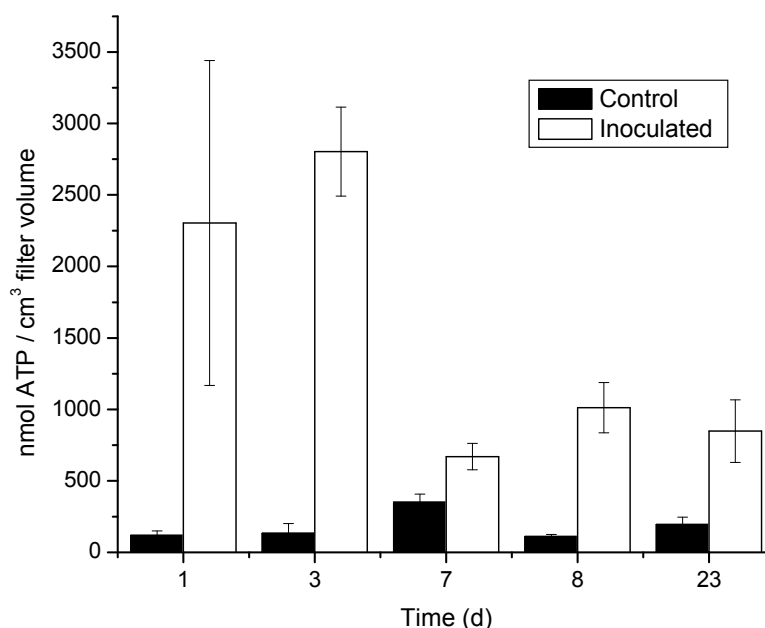
reservoir water. These differences may be due to the decreased contact time between consortium members and geosmin within the biofilm of the sand column, compared with the planktonic batch reactor studies where consortium members and geosmin were in a closed system throughout the experiment. However, it is also known that bacteria within biofilms can exhibit different behaviours when they are present in a planktonic state (Watnick and Kolter, 2000; Hall-Stoodley et al., 2004; Hansen et al., 2007), and this may provide an additional explanation for the differences in geosmin removals between both studies. A further explanation for the reduced geosmin removal in the sand columns compared with the planktonic study (Hoefel et al., 2006), may be due to the distribution of the three organisms within the biofilm of the sand column. It has been demonstrated that all three bacteria are required to degrade geosmin (Hoefel et al., 2006); most likely resulting in a cascade of degradation products, and a range of signalling mechanisms, between the consortium members. These processes may have been hindered in this biofilm study here by the specific (and static) distribution of the three organisms within the sand column biofilm, compared with the free dispersion of cells within the planktonic batch reactor study.



**Figure 6.2** Geosmin removal through the sand columns containing an autoclaved pre-existing biofilm (trial 1 and trial 1 repeat).

The biomass activities, as measured by the ATP assay, of the sand columns in trial 1 are shown in Figure 6.3. There was a distinct difference between the biomass activity of the inoculated sand column and the non-inoculated sand column, strongly suggesting that the bacterial consortium had effectively attached to the sand/biofilm surface. The ATP concentrations of the inoculated column were at a maximum on days 1 and 3 (2300 and 2800 nmol ATP cm<sup>-3</sup>, respectively) after which levels decreased and stabilised between 670 and 1000 nmol ATP cm<sup>-3</sup> (day 7 onwards). This stabilising of ATP may have been due to the organisms adopting a 'biofilm associated' level of metabolism following the 7 d post inoculation. It may be expected that organisms recently cultured using nutrient rich medium would retain a higher level of metabolism, but this would eventually plateau when the organisms were exposed to a comparatively lower nutrient (oligotrophic) environment such as sand filter biofilm. An alternate explanation for the reduction in ATP may have been the detachment (or sloughing) of the biofilm from the sand surface at day 7, although there were no significant decreases in geosmin removal at those times to support this. This highlights the potential limitations of using biomass measurements, such as ATP, to assess the performance of biofilters for the removal of target contaminants. In this study the ATP assay was employed only as an indicator of the bacterial attachment to the sand/biofilm surface. This application was unique for this study since the influent

waters and sand had been pre-autoclaved (121°C for 15 min); therefore, increases in ATP levels could be attributed to the presence of the geosmin-degrading consortium alone.

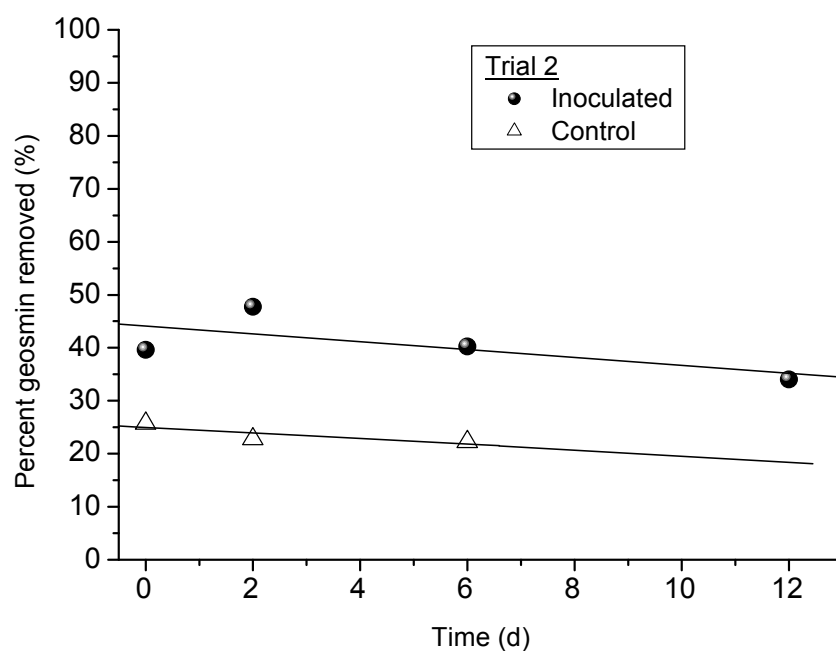


**Figure 6.3** Biomass activities in the sand columns containing an autoclaved pre-existing biofilm (trial 1). Error bars represent standard deviations from triplicate analyses.

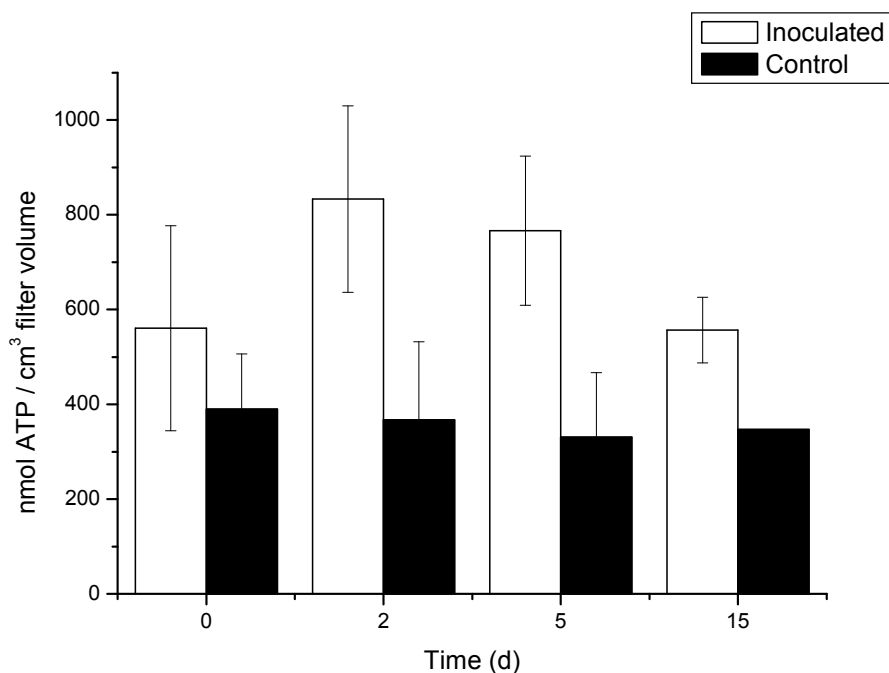
### 6.3.2 Geosmin removal through sand columns in the absence of an existing biofilm

A second trial was conducted where the geosmin-degrading consortium was inoculated into a laboratory column containing virgin sand with no existing biofilm. The method employed was identical to trial 1, with a minor exception in the duration of the recirculation step, which was increased to 5 d (compared with 4 d).

Percentage removals of geosmin for the inoculated and non-inoculated sand columns are shown in Figure 6.4. The concentrations of geosmin in the influent varied between 70 and 100 ng L<sup>-1</sup>. The data from day 12 of the non-inoculated column was omitted as it was deemed to be an outlier, since it showed 0% geosmin removal. Once this point was omitted, the removal of geosmin through the non-inoculated sand column averaged 25%, which is comparable with the previous trial and literature (Ho et al., 2007). The removal of geosmin through the inoculated sand column averaged 40%, which is considerably lower than that observed for the sand columns with the pre-sterilised biofilm (Figure 6.2). This suggests that the inoculated organisms may not have attached to the virgin sand with the same success as the previous sand with the pre-sterilised biofilm. Results of the biomass ATP activities from this trial lend support to this contention with lower overall ATP levels in the inoculated virgin sand (Figure 6.5; ranging 560 to 850 nmol ATP cm<sup>-3</sup>) compared with higher levels of ATP (up to 2800 nmol ATP cm<sup>-3</sup>) in the inoculated sand with the pre-sterilised biofilm (Figure 6.3).



**Figure 6.4** Geosmin removal through the virgin sand columns without an existing biofilm (trial 2).



**Figure 6.5** Biomass activities in the virgin sand columns without an existing biofilm (trial 2). Error bars represent standard deviations from triplicate analyses.

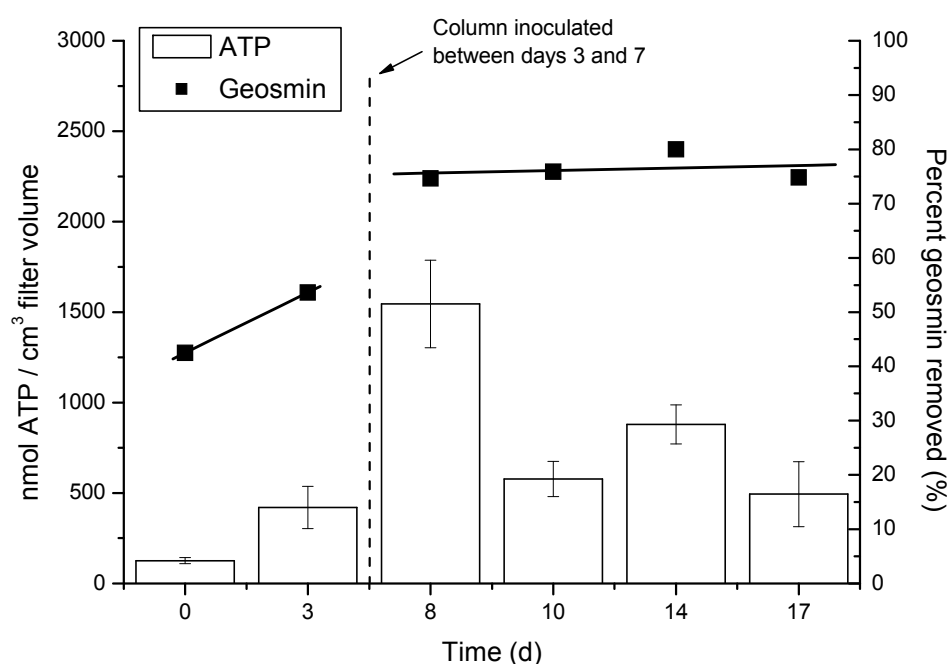
### 6.3.3 Geosmin removal through sand columns containing an active biofilm

A third trial was conducted using the same methodology to the previous two trials; with the exception of the sand column containing an active pre-existing biofilm (non-sterilised). This sand was sampled

from a pilot plant which was shown to be incapable of effectively removing geosmin even after 780 d of operation (unpublished data). A non-inoculated control was not run in parallel with the inoculated column in this trial as the previous two trials showed comparable geosmin removals which were attributed to abiotic losses. Instead, this trial was designed so that abiotic losses of geosmin could be observed prior to day 3. This is possible as inoculation of the sand column with the bacterial consortium occurred between days 3 and 7.

Figure 6.6 shows the percentage removals of geosmin, in addition to the ATP levels, during trial 3. The concentrations of geosmin in the influent varied between 60 and 80 ng L<sup>-1</sup>. The removal of geosmin before inoculation of the bacterial consortium was 40 and 50% for days 0 and 3, respectively. These abiotic losses were slightly higher than observed from the first trial (32%). This was possibly due to the active biofilm present on this sand possessing a greater adsorptive capacity for geosmin compared with an autoclaved biofilm. Previous studies by Lahti et al. (1997) and Holst et al. (2003) indicated that adsorption of the cyanotoxin microcystin was lower or insignificant with dead cells and organic matter compared with active cells. Another possible explanation for the differences may be due to the distinct biofilm compositions of the two sands as both sands were obtained from separate sources; therefore, it is likely that both sands would have contained different biofilm properties, in terms of its physical and chemical composition. In addition, the autoclaving process may have also altered the physical and chemical nature of the biofilm, making it less amenable to geosmin adsorption.

The ATP levels during days 0 and 3 were low (below 500 nmol ATP cm<sup>-3</sup>). These values were similar to the values of the non-inoculated columns of the first two trials, and suggest that the indigenous biofilm was low in either biomass or overall activity.



**Figure 6.6** Geosmin removal and biomass activities in the sand columns containing an active pre-existing biofilm (trial 3). Error bars for the ATP data represent standard deviations from triplicate analyses.

After inoculation of the bacterial consortium, geosmin removal increased to 75% and remained at this level for the duration of the trial (Figure 6.6). This enhanced removal of geosmin was similar to the removal levels observed in the inoculated sand columns of trial 1 and can be attributed to the action of the geosmin-degrading bacterial consortium. Similar to trial 1, a greater ATP level was observed immediately after inoculation of the bacterial consortium which then decreased and stabilised to levels between 500 and 900 nmol ATP cm<sup>-3</sup> in the subsequent days.

The differences observed between the results with the virgin sand and with the sands containing a pre-existing biofilm (autoclaved or non-autoclaved) can be related to the presence/absence of the biofilm. The data presented here suggest that attachment of the geosmin-degrading consortium was aided by the presence of an existing biofilm, resulting in enhanced geosmin removal. Biofilms are held together by extracellular polymeric substances (EPS) which have been shown to enhance bacterial attachment (Sutherland, 2001; Alpkvist et al., 2006). The physical and chemical structure of a biofilm has also been shown to affect attachment of bacteria within fixed beds (Leon-Morales et al., 2004; Liu and Li, 2008). While the consortium members may have formed a biofilm themselves upon the virgin sand, it may be expected that the biofilm was not as developed as that of the sands with the pre-existing biofilms. The sand from trial 1 had been in Morgan WTP filter beds for over 25 years, while the sand in trial 3 had been in operation in a biofiltration pilot plant for 780 d. The results in trial 2 suggest that the geosmin-degrading consortium was able to attach to the virgin sand and possibly initiate the formation of a biofilm, since geosmin removal was increased by an average of 15% when the consortium was inoculated compared with the non-inoculated sand column. However, further work is required to substantiate this. This could involve a similar experiment undertaken over a prolonged timeframe. Nevertheless, the results from this study do indicate that there is potential to enhance geosmin removal through sand filtration by appropriately seeding geosmin-degrading bacteria into these filters. This could potentially create a further treatment barrier which would be cost-effective for water authorities since most WTPs contain a sand filtration process. In addition, this would decrease the reliance on activated carbon as the major treatment barrier, thereby reducing capital costs. Work is also being carried out to isolate a single geosmin-degrading organism that would make consistent seeding of sand filters easier and possibly result in higher removal of geosmin across the filter.

## 6.4 Conclusions

This is the first study which has demonstrated enhanced geosmin removal (up to an additional 38%) through sand filters by the inoculation of geosmin-degrading bacteria. The extent of the bacterial attachment was monitored using an ATP assay, where the attachment of the bacterial consortium, and subsequent geosmin degradation, was shown to be enhanced by the presence of a pre-existing biofilm. Negligible difference in geosmin removal was observed when the geosmin-degrading bacteria were inoculated into the sand columns containing either a sterilised pre-existing biofilm or non-sterilised pre-existing biofilm. This information is valuable for water treatment operators who wish to use such approaches for the enhanced removal of taste and odour compounds via biofiltration processes, as the seeding of virgin sand may not result in desirable levels of enhancement.

## 6.5 References

- Alpkvist E., Picioreanu C., van Loosdrecht M.C.M. and Heyden A. (2006) Three-dimensional biofilm model with individual cells and continuum EPS matrix. *Biotechnology and Bioengineering* **94**(5), 961-979.
- Ashitani K., Hishida Y. and Fujiwara K. (1988) Behavior of musty odorous compounds during the process of water treatment. *Water Science & Technology* **20**(8/9), 261-267.
- Egashira K., Ito K. and Yoshiy Y. (1992) Removal of musty odour compound in drinking water by biological filter. *Water Science & Technology* **25**(2), 307-314.
- Elhadi S.L.N., Huck, P.M. and Slawson R.M. (2004a) Determination of system losses of geosmin and MIB in bench-scale filtration apparatus. *Water Quality Research Journal of Canada* **39**(3), 207-212.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2004b) Removal of geosmin and 2-methylisoborneol by biological filtration. *Water Science & Technology* **49**(9), 273-280.
- Elhadi S.L.N., Huck P.M. and Slawson R.M. (2006) Factors affecting the removal of geosmin and MIB in drinking water biofilters. *Journal of the American Water Works Association* **98**(8), 108-119.
- Feakin S.J., Blackburn E. and Burns R.G. (1995) Inoculation of granular activated carbon in a fixed bed with s-triazine-degrading bacteria as a water treatment process. *Water Research* **29**(3), 819-825.
- Hall-Stoodley L., Costerton J.W. and Stoodley P. (2004) Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology* **2**(2), 95-108.
- Hansen S.K., Rainey P.B., Haagensen J.A.J. and Molin S. (2007) Evolution of species interactions in a biofilm community. *Nature* **445**(7127), 533-536.

- Hayes K.P. and Burch M.D. (1989) Odorous compounds associated with algal blooms in South Australian waters. *Water Research* **23**(1), 115-121.
- Ho L., Hoefel D., Bock F., Saint C.P. and Newcombe G. (2007) Biodegradation rates of 2-methylisoborneol (MIB) and geosmin through sand filters and in bioreactors. *Chemosphere* **66**(11), 2210-2218.
- Hoefel D., Grooby W.L., Monis P.T., Andrews S. and Saint C.P. (2003) Enumeration of water-borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiological Methods* **55**(3), 585-597.
- Hoefel D., Ho L., Aunkofer W., Monis P.T., Keegan A., Newcombe G. and Saint C.P. (2006) Cooperative biodegradation of geosmin by a consortium comprising three Gram-negative bacteria isolated from the biofilm of a sand filter column. *Letters in Applied Microbiology* **43**(4), 417-423.
- Holst T., Jørgensen N.O.G., Jørgensen C. and Johansen A. (2003) Degradation of microcystin in sediments at oxic and anoxic, denitrifying conditions. *Water Research* **37**(19), 4748-4760.
- Hrudey S.E., Huck P.M., Mitton M.J. and Kenefick S.L. (1995) Evaluation of odour removal by pilot-scale biological treatment process trains during spring runoff in an ice-covered stream. *Water Science & Technology* **31**(11), 195-201.
- Lahti K., Rapala J., Färdig M., Niemälä M. and Sivonen K. (1997) Persistence of cyanobacterial hepatotoxin, microcystin-LR in particulate material and dissolved in lake water. *Water Research* **31**(5), 1005-1017.
- Leon-Morales C.F., Leis A.P., Strathmann M. and Flemming H.-C. (2004) Interactions between laponite and microbial biofilms in porous media: Implications for colloid transport and biofilm stability. *Water Research* **38**(16), 3614-3626.
- Liu Y. and Li J. (2008) Role of *Pseudomonas aeruginosa* biofilm in the initial adhesion, growth and detachment of *Escherichia coli* in porous media. *Environmental Science & Technology* **42**(2), 443-449.
- Lundgren B.V., Grimvall A. and Sävénhed R. (1988) Formation and removal of off-flavour compounds during ozonation and filtration through biologically active sand filters. *Water Science & Technology* **20**(8/9), 245-253.
- McDowall B., Ho L., Saint C.P. and Newcombe G. (2007a) Removal of geosmin and 2-methylisoborneol through biologically active sand filters. *International Journal of Environment and Waste Management* **1**(4), 311-320.
- McDowall B., Ho L., Saint C.P. and Newcombe G. (2007b) Biological removal of MIB and geosmin through rapid gravity filters. *Water: Journal of the Australian Water Association* **34**(7), 48-54.
- Metz D.H., Pohlman R.C., Vogt J. and Summers R.S. (2006) Removal of MIB and geosmin by full-scale biological sand filters. In: Gimbel R., Graham N.J.D. and Collins M.R. (Eds.). *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. IWA Publishing, London, UK.
- Namkung E. and Rittmann B.E. (1987) Removal of taste- and odour-causing compounds by biofilms grown on humic substances. *Journal American Water Works Association* **79**(7), 107-112.
- Sugiura N., Isoda H. and Takaaki M. (2003) Degradation of potential musty odour in a drinking water source by a biofilm method. *Journal of Water Supply: Research and Technology - AQUA* **52**(3), 181-187.
- Summers R.S., Chae S., Kim S.M. and Ahn H.W. (2006) Biodegradation of MIB and geosmin in biological sand and BAC filters: Acclimation, steady-state and varying influent. In: Gimbel R., Graham N.J.D. and Collins M.R. (Eds.). *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. IWA Publishing, London, UK.
- Sutherland I.W. (2001) Exopolysaccharides in biofilms, flocs and related structures. *Water Science & Technology* **43**(6), 77-86.
- Terauchi N., Ohtani T., Yamanaka K., Tsuji T., Sudou T. and Ito K. (1995) Studies on a biological filter for musty odour removal in drinking water treatment processes. *Water Science & Technology* **31**(11), 229-235.
- Watnick P. and Kolter R. (2000) Biofilm, city of microbes. *Journal of Bacteriology* **182**(10), 2675-2679.
- Yagi M., Nakashima S. and Muramoto S. (1988) Biological degradation of musty odour compounds, 2-methylisoborneol and geosmin, in a bio-activated carbon filter. *Water Science & Technology* **20**(8/9), 255-260.

## CHAPTER 7: BACTERIAL DEGRADATION OF MICROCYSTIN TOXINS WITHIN A BIOLOGICALLY-ACTIVE SAND FILTER\*

### 7.1 Introduction

The occurrence of cyanobacterial blooms and their associated toxins can be traced as far back as 1878 (Francis, 1878). Since then, extensive monitoring of these blooms has shown that up to 70% are potentially toxic (NRA, 1990; Codd, 1995). Among the most common of the cyanobacterial toxins are the microcystins, a group of monocyclic heptapeptide hepatotoxins produced predominantly by species of *Microcystis*, *Anabaena*, *Nostoc* and *Planktothrix* (Carmichael, 1992; Codd, 1995; Keil et al., 2002). They have a general structure comprised of five amino acids with minor variations (D-alanine, D-erythro- $\beta$ -methyl aspartic acid, D-glutamic acid, N-methyldehydroalanine and Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid)) and a pair of variable L-amino acids. The most commonly studied, and one of the most toxic variants, is microcystin-LR (L and R represent the variable amino acids Leucine and aRginine, respectively) which has an LD<sub>50</sub> value of 0.05 mg kg<sup>-1</sup> in mice by intraperitoneal injection (Carmichael, 1988). The potency of microcystin-LR in humans was demonstrated in 1996 when fifty patients at a haemodialysis centre in Brazil died as a result of acute hepatic failure. It was discovered that the water used for dialysis had been contaminated with microcystin-LR at concentrations high enough to cause severe liver damage (Dunn, 1996; Jochimsen et al., 1998). As a result of these concerns about the effect of microcystins, a guideline value of 1.0  $\mu$ g L<sup>-1</sup> for microcystin-LR in drinking water has been issued by the World Health Organisation (WHO, 1997).

Microcystins are chemically stable in water (Jones and Orr, 1994; Tsuji et al., 1994) and have been documented to be recalcitrant to conventional water treatment processes (Hoffman, 1976; Keijola et al., 1988; Himberg et al., 1989; Lahti and Hiisvirta, 1989). Activated carbon adsorption and ozone oxidation have been shown to be successful in their removal from drinking water (Jones et al., 1993; Rositano et al., 2001; Newcombe et al., 2003; Ho, 2004). However, the presence of natural organic material (NOM) reduces the effectiveness of both of these treatment processes for the removal of microcystin. For activated carbon, NOM decreases the adsorption capacity for these metabolites through competitive adsorption and/or pore blockage mechanisms (Lambert et al., 1996; Newcombe et al., 2003), while NOM can consume ozone thereby reducing its concentration in solution (Rositano et al., 2001; Ho, 2004).

Many studies have reported biological degradation of microcystin in natural lakes and reservoirs (Jones and Orr, 1994; Rapala et al., 1994; Cousins et al., 1996; Christoffersen et al., 2002). Only a few studies have implicated degradation of microcystin in biologically-active sand filters. Biological filtration systems for the removal of problematic contaminants, such as microcystin toxins, are becoming more attractive to water suppliers as they are generally of low technology, requiring little maintenance and infrastructure. Such systems are also able to remove contaminants without the addition of other chemicals that may have the potential to produce undesirable by-products. Lahti and Hiisvirta (1989) found up to 86% removal of microcystin was achieved in a pilot-scale slow sand filter. The results of Sherman et al. (1995) and Grützmacher et al. (2002) also suggested biological degradation of microcystin occurred in their slow sand filters. However, these studies were unable to conclusively prove that the removal of microcystin was through biological degradation rather than physical processes, such as adsorption to sand. Furthermore, only slow sand filtration studies have been documented and to date no removal of microcystin has been reported under rapid sand filtration conditions.

Perhaps the most important aspect of any biological degradation process is the identification of the degrading organisms. To date, only a few strains of the genus *Sphingomonas* have been reported to

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\* This chapter is based on the following manuscript:

Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.

degrade microcystin (Bourne et al., 1996, 2001; Park et al., 2001; Saito et al., 2003; Harada et al., 2004; Ishii et al., 2004). Bourne et al. (1996, 2001) identified a gene cluster in *Sphingomonas* sp. ACM-3962 which was responsible for the degradation of microcystin-LR. Recently, Saito et al. (2003) developed a polymerase chain reaction (PCR) assay for the detection of one of these genes, *mlrA*, which encodes the enzyme responsible for cleaving the cyclic structure of microcystin.

The objective of this study was to determine whether biologically-active sand filters were capable of effectively removing microcystin toxins under slow and rapid sand filtration conditions. An additional objective was to confirm that the removal was primarily through biological degradation rather than any physical processes. A PCR assay was used to provide additional evidence that removal was through biological degradation by detection of the *mlrA* gene within bacteria attached to the sand filter.

## 7.2 Experimental methods

### 7.2.1 Materials and reagents

Microcystin-LR (MCLR) and -LA (MCLA) were isolated from a natural bloom of *Microcystis aeruginosa* that occurred in the Torrens Lake in South Australia during the summer of 1998-99. The isolation procedure involved freeze-thawing the bloom material in water and methanol, followed by preparative reverse phase flash chromatography and preparative high performance liquid chromatography (HPLC).

Sample water was collected from the Myponga Reservoir, South Australia and immediately filtered through a 1 µm filter cartridge to remove particulate matter. Sand was obtained from the filter beds at the Morgan water treatment plant (WTP) where no pre-chlorination is practiced. Characteristics of the sample water and sand are outlined in Table 7.1.

**Table 7.1** Characteristics of Myponga Reservoir water and Morgan filter sand.

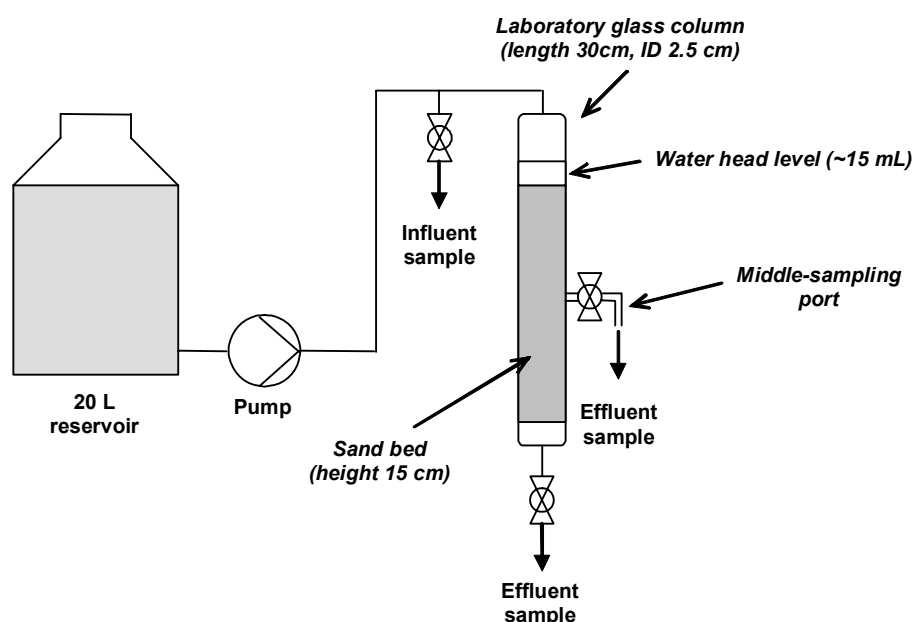
	pH	UV absorbance at 254 nm (cm <sup>-1</sup> )	DOC (mg L <sup>-1</sup> )
Myponga water	6.7	0.412	11.8
	Effective particle size (mm)	Particle density (kg m <sup>-3</sup> )	Surface area in column (cm <sup>2</sup> )
Morgan sand	0.83	2585	3168

### 7.2.2 Laboratory column experiments

Morgan WTP filter sand was packed into three separate glass columns (length 30cm, internal diameter 2.5 cm) at a bed height of 15 cm. A schematic of the column set up is presented in Figure 7.1, while the conditions of the column experiments are listed in Table 7.2. Column A had been in operation for approximately 12 months prior to the commencement of this study, during which time it was continuously fed with Myponga Reservoir water (MRW) with periodic spikings of MCLR and MCLA. During this time column A was able to effectively remove both microcystin variants (unpublished data). Prior to the commencement of this study, column A had not been exposed to either of the microcystin variants for a period of six months. The purpose of this column was to determine whether it still had the ability to remove microcystin after a prolonged break from microcystin exposure. This simulated a realistic situation as microcystin episodes are usually of a transient nature.

Column B was commissioned at the commencement of this study using filter sand directly obtained from the Morgan WTP. Therefore, the sand had an existing biofilm which was not acclimatised to the new experimental regime. Column B was fed with the same influent water as that of column A. In contrast to columns A and B, sand in column C was autoclaved prior to the commencement of the experiments to inactivate the existing biofilm.





**Figure 7.1** Schematic of the laboratory sand column experiments.

**Table 7.2** Laboratory sand column conditions.

EBCT (min)	Filtration rate (m h <sup>-1</sup> )	Time employed (d)		
		Column A	Column B	Column C
30	0.3	0-6, 28-29, 35-39	0-20, 28-29, 35-36	0-5
15	0.6	7-20	21-27	-
10	0.9	21-27	30-34	-
7.5	1.2	30-34	37-39	-

Each column was fed with MRW spiked with MCLR and MCLA at target concentrations of 20 µg L<sup>-1</sup> each. However, different empty bed contact times (EBCTs) were employed for each column. For column C, a constant EBCT of 30 min was used for the 5 d experiment, whereas for columns A and B the EBCT was varied as shown in Table 7.2. Duplicate water samples were taken from the influent and effluent of each column at regular intervals for microcystin analysis. Experiments were conducted at room temperature (20±2°C).

At the completion of the column experiments, sand was removed from column A for further characterisation. Samples were obtained from different layers of column A and analysed using a leucine aminopeptidase (LAP) assay to assess the biological activity in the columns. Full details of the LAP assay have been documented elsewhere (Meyn, 2004). For DNA extraction procedures, sand (10 g wet weight) was removed from column A and the biofilm detached by periodic vortexing for 15 min in pre-autoclaved (121°C for 20 min) MRW. The supernatant, containing the biofilm, was collected and any carry-over of sand was removed by slow speed centrifugation at 1,000 g for 30 s.

The apparatus for column B, including the sand, underwent a solvent extraction procedure using acetonitrile and methanol to desorb any microcystin which may have adsorbed during the experiment. Details of this procedure have been reported previously (Meyn, 2004).

### 7.2.3 Analysis of microcystin

Prior to HPLC analysis, microcystins were concentrated from sample waters by C18 solid phase extraction according to the methods described by Nicholson et al. (1994). A HPLC system consisting

of a 600 pump controller, 717plus autosampler and 996 photodiode array detector (Waters Pty Ltd, Australia) was employed. Sample volumes of 50  $\mu\text{L}$  were injected into a 150 x 4.6 mm Luna C18 column (Phenomenex, Australia) at a flow rate of 1  $\text{mL min}^{-1}$ . Two mobile phases were used for the gradient run (30% acetonitrile/0.05% trifluoroacetic acid and 55% acetonitrile/0.05% trifluoroacetic acid). Concentrations of MCLR were determined by calibration of the peak areas (at 238 nm) with that of external standards (Sapphire Bioscience Pty Ltd, Australia). All MCLA concentrations were expressed in terms of MCLR equivalents. The HPLC method has a detection limit of 0.5  $\mu\text{g L}^{-1}$ . Microcystin recoveries were greater than 95% with a relative precision of 10%.

## 7.2.4 Molecular techniques

DNA from the column biofilm was extracted and prepared using the InstaGene™ Matrix DNA Preparation Kit according to the manufacturer's instructions (Bio-Rad, Australia). The DNA was then used as a template for PCR targeting the *mlrA* gene using the specific oligonucleotide primer set MF, 5'-GACCCGATGTTCAAGATACT-3' and MR, 5'-CTCCTCCCACAAATCAGGAC-3' (Saito et al., 2003). DNA was also extracted, using the procedure above, from three sphingomonads: *Sphingomonas* sp. ACM-3962 (NCBI accession number AF411072), *Sphingopyxis* sp. Geo24 (NCBI accession number DQ137852) and *Novosphingobium* sp. Geo25 (NCBI accession number DQ137852). DNA from these sphingomonads were used as controls for the PCR since previous reports of microcystin degradation have implicated only members of this group.

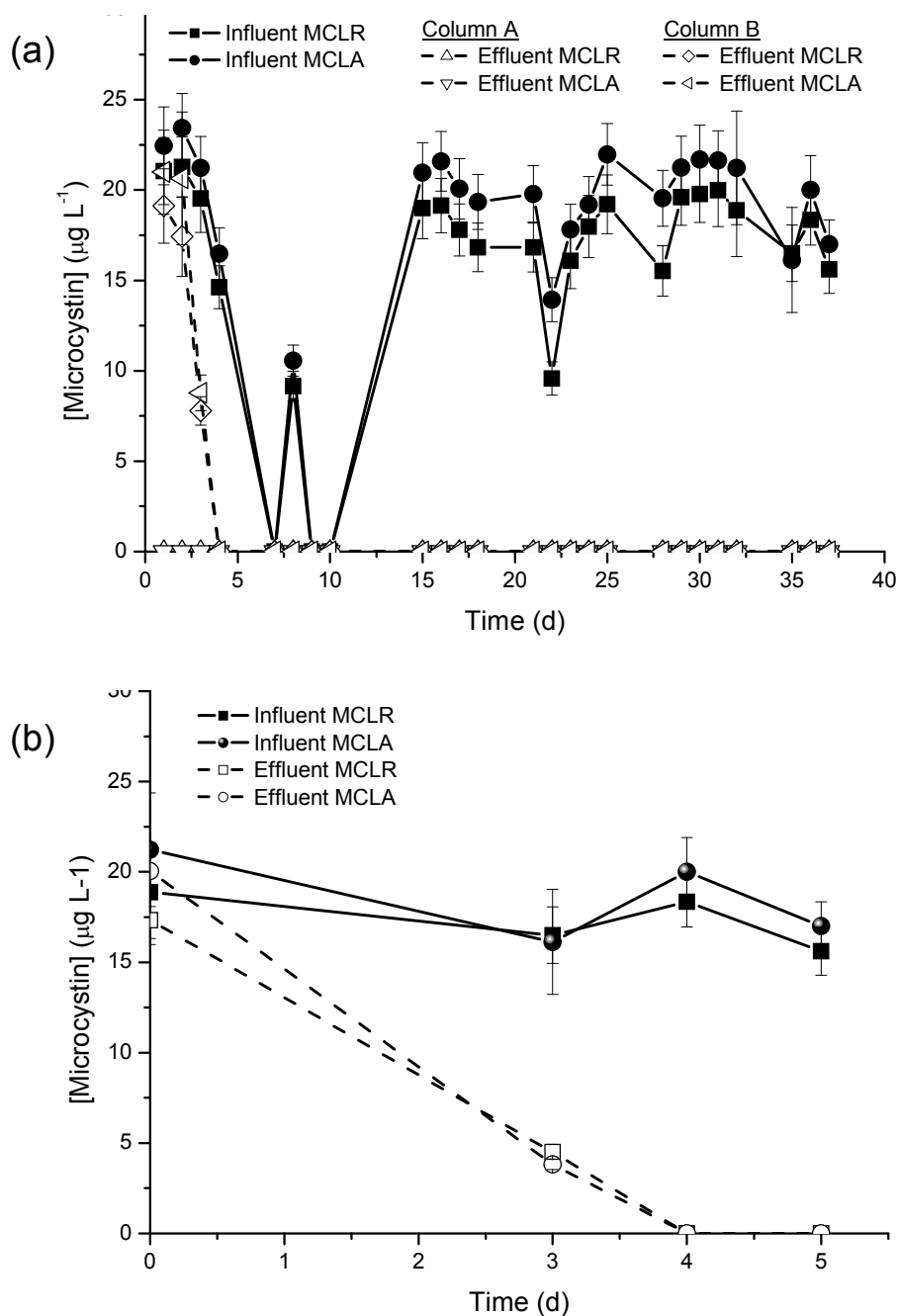
Amplifications were performed in 50  $\mu\text{L}$  volumes, containing 1 pmol of each primer, 2.5 mM  $\text{MgCl}_2$  (Perkin Elmer, Australia), 1 x PCR Buffer II (Perkin Elmer, Australia), 200  $\mu\text{M}$  deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics Pty Ltd, Australia), 1.25 units of Ampli Taq Gold DNA Polymerase (Perkin Elmer, Australia) and 10  $\mu\text{L}$  of genomic DNA template. A GeneAmp® 2400 PCR System (Perkin Elmer, Australia) was used for the amplifications under the following conditions: 94°C for 10 min; 35 cycles of 94°C for 20 s, 60°C for 10 s and 72°C for 30 s; and 72°C for 10 min. PCR products were separated by 1% agarose gel electrophoresis with the resultant DNA bands visualised on a Dark Reader illuminator (Clare Chemical Research, USA).

The PCR products were cloned using the TOPO TA Cloning® Kit (Invitrogen™, USA) and the resulting plasmid DNA purified using the QIAprep® Miniprep Kit (QIAGEN, USA). The nucleotide sequence of cloned DNA inserts was determined using the Big Dye version III Sequencing Kit (Applied Biosystems, Australia) and analysed on a Perkin Elmer 3700 Capillary DNA Sequence Analyser (Applied Biosystems, Australia). Oligonucleotide primers (M13) flanking the insert were used to sequence both the forward and reverse strands of DNA. Sequences of DNA were aligned using the SeqMan™ II and EditSeq™ software programs (DNASTAR, USA). Identification of the amplified DNA fragment was performed using the Nucleotide-nucleotide BLAST® network service on the NCBI GenBank® database (<http://www.ncbi.nlm.nih.gov/BLAST>).

## 7.3. Results and discussion

### 7.3.1 Biological filtration of microcystin

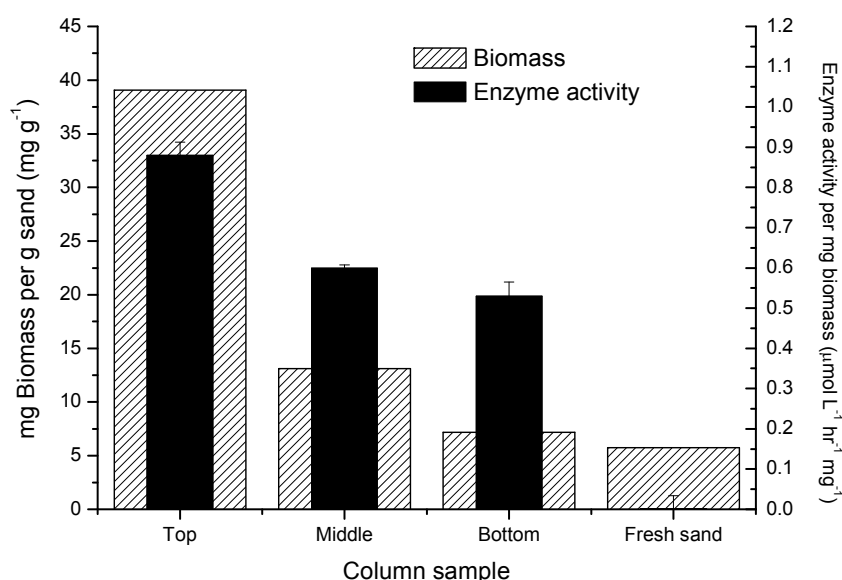
Three laboratory sand columns were employed for the biological filtration of MCLR and MCLA. All three columns contained sand sourced from the filters at the Morgan WTP; however each were commissioned differently as outlined in section 7.2.2. Results of the biological filtration of the microcystins through each of the columns are shown in Figures 7.2a and b. It was difficult to maintain consistent microcystin influent concentrations between days 7-10 of the experiment due to degradation of the compounds in the influent water of columns A and B. This was resolved by cleaning the influent water reservoir and tubing at weekly intervals to minimise any biological activity occurring prior to the sand filters. After this point of time (day 15 onwards), microcystin concentrations in the influent water remained relatively consistent.



**Figure 7.2** Biological filtration of microcystin-LR (MCLR) and -LA (MCLA) through: (a) laboratory sand columns A and B; (b) laboratory sand column C. Error bars represent standard deviations from duplicate measurements.

No microcystin was detected in the effluent of column A at any time during the experiment (Figure 7.2a). Furthermore, no microcystin was detected in regular samples obtained from a sampling port approximately mid way up the column bed (data not shown). Samples from this port effectively halve the EBCT of the sand bed; consequently, the lowest EBCT of approximately 4 min still yielded no toxin breakthrough. This low EBCT corresponds to a filtration rate of approximately  $2.4 \text{ m h}^{-1}$  which is similar to conditions of a rapid sand filter. To date, no data has been published in reference to the degradation of microcystin under rapid sand filtration conditions. This is a significant finding as it indicates that removal of microcystin can occur through rapid sand filters, which are commonly used in Australian WTPs.

Since no microcystin was detected in samples taken from the middle port of column A at any time during the experiment, then this indicates that all the degradation of microcystin occurred in the top portion of the sand column. In general, biomass densities tend to be greater in the upper regions of sand filters with numbers decreasing with depth (Eighmy et al., 1993; Urfer and Huck, 2003). The LAP assay employed in this study not only showed greater biomass abundance in the upper portions of sand column A, but also showed greater biomass activity in the upper portions of the column (Figure 7.3). As the LAP enzyme hydrolyses a large number of peptides and amino acids, similar to the groups present in the microcystin structure, it is probable that a higher concentration of microcystin-degrading organisms would have been present in the upper regions of sand column A.



**Figure 7.3** Biomass concentration and leucine aminopeptidase (LAP) enzyme activity as a function of three separate sand portions of column A. Fresh sand signifies sand obtained from the Morgan WTP which was not used in the column experiments. Error bars represent standard deviations from triplicate measurements.

In contrast to column A, breakthrough of MCLR and MCLA was observed in the first 3 d of the effluent of column B (Figure 7.2a). However, after this point of time no microcystin was detected in the effluent for the duration of the experiment. Similar results were observed for column C which employed a constant EBCT of 30 min (Figure 7.2b). The results suggested that the time required for a biofilm to establish and acclimatise to microcystin in this set of experiments was quite short (less than 4 d). This is in contrast to other studies which have documented lag periods of up to 16 d prior to degradation of microcystin commencing (Miller and Fallowfield, 2001). Furthermore, the initial breakthrough of microcystin in columns B and C suggested that a majority of the removal of microcystin through the columns was through biological degradation and not any other physical processes such as adsorption to the sand or glass columns. Solvent (acetonitrile/methanol) extraction of the apparatus of column B, including the sand, at the completion of the experiments yielded no microcystin, confirming that no physical removal occurred.

The immediate removal of microcystin in column A suggested that pre-exposure of the biofilm to microcystin was able to eliminate the lag period prior to degradation commencing, since breakthrough was initially observed in column B. However, this could also have been due to the more advanced bacterial community within the biofilm of column A, compared with that of column B which had not been acclimatised to the experimental conditions. Studies by Rapala et al. (1994), Christoffersen et al. (2002) and Holst et al. (2003) have shown that lag periods in microcystin degradation studies could be substantially reduced, in some circumstances removed, when bacteria had been pre-exposed to microcystin.

### 7.3.2 Detection of the microcystin degrading gene, *mlrA*

A PCR assay was used to identify the presence of a gene involved in microcystin degradation, *mlrA*, from within the biofilm of column A, which effectively removed microcystin. *Sphingomonas* sp. ACM-3962, a microcystin degrading bacterium originally isolated from an Australian water body by Jones et al. (1994), was used as a positive control for the PCR assay since the MF-MR primer set was initially designed from its *mlrA* gene sequence (Saito et al., 2003). This generated a PCR product of approximately 800 bp in size (data not shown). *Sphingopyxis* sp. Geo24 and *Novosphingobium* sp. Geo25, sphingomonads which were previously isolated from WTP sand filters, were employed as negative controls for the PCR assay. These two sphingomonads were previously shown to be incapable of degrading microcystin. The absence of any amplification products for these two organisms indicated that the *mlrA* gene is not ubiquitous to the sphingomonad group, rather it appears to be unique to microcystin degraders, as described by Saito et al. (2003). A product of equivalent size to the positive control was evident for the DNA extract from the biofilm of column A. This product was cloned and found to have a DNA sequence of 98% similarity to the 807 bp nucleotide sequence of the *mlrA* gene from *Sphingopyxis* sp. C-1 (NCBI accession number AB161685). The next closest match in the NCBI GenBank® database was a 91% similarity to the *mlrA* gene from *Sphingomonas* sp. ACM-3962 (NCBI accession number AF411068). Experiments are currently underway in an attempt to isolate and identify the bacterium which contains this *mlrA* gene homologue within the biofilm of column A.

To date, no other studies have reported detection of the bacterial *mlrA* gene in biological sand filters. The detection of the homologous *mlrA* gene in the biofilm extract of column A suggests that the removal of microcystin through this column most likely followed a degradation pathway similar to one previously reported by Bourne et al. (1996, 2001) where the enzyme encoded by the *mlrA* gene cleaves the cyclic structure of microcystin. The resultant linear structure of microcystin is then sequentially degraded by two additional enzymes, encoded by the *mlrB* and *mlrC* genes, respectively.

## 7.4 Conclusions

Under conditions employed in this study, biological sand filtration was determined to be an effective treatment process for the complete removal of two microcystin analogues, MCLR and MCLA. Furthermore, complete removal of the microcystins was evident under rapid sand filtration conditions, which has not been previously reported.

To date, most of the literature has anecdotally implicated biological degradation of microcystin in sand filters. In this study, the removal of microcystin through the sand filters was shown to be primarily through biological degradation rather than any physical processes. In addition, this is the first study to report detection of the bacterial *mlrA* gene in sand filters which were removing microcystin. This study demonstrates the potential of the PCR assay to be used to assess the microcystin-degrading capability of biologically-active sand filters prior to an impending episode of microcystin.

## 7.5 References

- Bourne D.G., Jones G.J., Blakeley R.L., Jones A., Negri A.P. and Riddles P. (1996) Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR. *Applied & Environmental Microbiology* **62**(11), 4086-4094.
- Bourne D.G., Riddles P., Jones G.J., Smith W. and Blakely R.L. (2001) Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin LR. *Environmental Toxicology* **16**(6), 523-534.
- Carmichael W.W. (1988) *Toxins of freshwater algae*. In: Tu A.T. (Ed.). *Handbook of Natural Toxins, Marine Toxins and Venoms*. Marcel Dekker, Inc., New York, USA, pp. 121-147.
- Carmichael W.W. (1992) Cyanobacteria secondary metabolites - The cyanotoxins. *Journal of Applied Bacteriology* **72**(6), 445-459.
- Christoffersen K., Lyck S. and Winding A. (2002) Microbial activity and bacterial community structure during degradation of microcystins. *Aquatic Microbial Ecology* **27**(2), 125-136.

- Codd G.A. (1995) Cyanobacterial toxins: Occurrence, properties and biological significance. *Water Science & Technology* **32**(4), 149-156.
- Cousins I.T., Bealing D.J., James H.A. and Sutton A. (1996) Biodegradation of microcystin-LR by indigenous mixed bacterial populations. *Water Research* **30**(2), 481-485.
- Dunn J. (1996) Algae kills dialysis patients in Brazil. *British Medical Journal* **312**(7040), 1183-1184.
- Eighmy T.T., Collins M.R., Malley, Jr. J.P., Royce J. and Morgan D. (1993) *Biologically enhanced slow sand filtration for removal of natural organic matter*. AWWA Research Foundation Report, American Water Works Association, USA.
- Francis G. (1878) Poisonous Australian lake. *Nature (London)* **18**, 11-12.
- Grützmacher G., Böttcher G., Chorus I. and Bartel H. (2002) Removal of microcystins by slow sand filtration. *Environmental Toxicology* **17**(4), 386-394.
- Harada K.-I., Imanishi S., Kato H., Masayoshi M., Ito E. and Tsuji K. (2004) Isolation of Adda from microcystin-LR by microbial degradation. *Toxicon* **44**(1), 107-109.
- Himberg K., Keijola A.-M., Hiisvirta L., Pyysalo H. and Sivonen K. (1989) The effect of water treatment processes on the removal of hepatotoxins from *Microcystis* and *Oscillatoria* cyanobacteria: A laboratory study. *Water Research* **23**(8), 979-984.
- Hoffman J.R.H. (1976) Removal of *Microcystis* toxins in water purification processes. *Water SA* **2**(2), 58-60.
- Ho L. (2004) *The removal of cyanobacterial metabolites from drinking water using ozone and granular activated carbon*. PhD Dissertation, University of South Australia, Adelaide, Australia.
- Holst T., Jørgensen N.O.G., Jørgensen C. and Johansen A. (2003) Degradation of microcystin in sediments at oxic and anoxic, denitrifying conditions. *Water Research* **37**(19), 4748-4760.
- Ishii H., Nishijima M. and Abe T. (2004) Characterization of degradation process of cyanobacterial hepatotoxins by a Gram-negative aerobic bacterium. *Water Research* **38**(11), 2667-2676.
- Jochimsen E.M., Carmichael W.W., An J., Cardo D.M., Cookson S.T., Holmes C.E.M., Antunes M.B.C., Filho D.A.M., Lyra T.M., Barreto V.S.T., Azevedo S.M.F.O. and Jarvis W.R. (1998) Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *The New England Journal of Medicine* **338**(13), 873-878.
- Jones G., Minatol W., Craig K. and Naylor R. (1993) Removal of low level cyanobacterial peptide toxins from drinking water using powdered and granular activated carbon and chlorination – Results of laboratory and pilot plant studies. In: *Proceedings of the 15th Federal AWWA Convention, April 18-23, 1993, Gold Coast, Queensland, Australia*, pp. 579-586.
- Jones G.J., Bourne D.G., Blakeley R.L. and Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Natural Toxins* **2**(4), 228-235.
- Jones G.J. and Orr P.T. (1994) Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Research* **28**(4), 871-876.
- Keijola A.-M., Himberg K., Esala A. L., Sivonen K. and Hiisvirta L. (1988) Removal of cyanobacterial toxins in water treatment processes: Laboratory and pilot-scale experiments. *Toxicity Assessment: An International Journal* **3**, 643-656.
- Keil C., Forchert A., Fastner J., Szewzyk U., Rotard W., Chorus I. and Krätke R. (2002) Toxicity and microcystin content of extracts from a *Planktothrix* bloom and two laboratory strains. *Water Research* **36**(8), 2133-2139.
- Lahti K. and Hiisvirta (1989) Removal of cyanobacterial toxins in water treatment processes: Review of studies conducted in Finland. *Water Supply* **7**(4), 149-154.
- Lambert T.W., Holmes C.F.B. and Hruidey S.E. (1996) Adsorption of microcystin-LR by activated carbon and removal in full scale water treatment. *Water Research* **30**(6), 1411-1422.
- Meyn T. (2004) *Biological removal of microcystin LR and LA: Laboratory studies to assess filtration, degradation kinetics and microbiological characterisation*. Diploma Thesis Dissertation, Technical University of Berlin, Berlin, Germany.
- Miller M.J. and Fallowfield H.J. (2001) Degradation of cyanobacterial hepatotoxins in batch experiments. *Water Science & Technology* **43**(12), 229-232.
- Newcombe G., Cook D., Brooke S., Ho L. and Slyman N. (2003) Treatment options for microcystin toxins: Similarities and differences between variants. *Environmental Technology* **24**(3), 299-308.
- Nicholson B.C., Rositano J. and Burch M.D. (1994) Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *Water Research* **28**(6), 1297-1303.
- NRA (1990) *Toxic blue-green algae*. Report of the National Rivers Authority, Water Quality Series No. 2, Bristol, UK.

## BIOLOGICAL FILTRATION PROCESSES FOR THE REMOVAL OF ALGAL METABOLITES

- Park H.-D., Sasaki Y., Maruyama T., Yanagisawa E., Hiraishi A. and Kato K. (2001) Degradation of cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. *Environmental Toxicology* **16**(4), 337-343.
- Rapala J., Lahti K., Sivonen K. and Niemälä S.I. (1994) Biodegradability and adsorption on lake sediments of cyanobacterial hepatotoxins and anatoxin-a. *Letters in Applied Microbiology* **19**(6), 423-428.
- Rositano J., Newcombe G., Nicholson B. and Sztajn bok P. (2001) Ozonation of NOM and algal toxins in four treated waters. *Water Research* **35**(1), 23-32.
- Saito T., Okana K., Park H.-D., Itayama T., Inamori Y., Neilan B.A., Burns B.P. and Sugiura N. (2003) Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes. *FEMS Microbiology Letters* **229**(2), 271-276.
- Sherman P., Tully I. and Gibson H. (1995) Removal of cyanobacterial cells and toxins from drinking water with biologically active filters. In: *Proceedings of the 16th Federal AWWA Convention, April 2-6, 1995, Sydney, Australia*, pp. 587-592.
- Tsuji K., Naito S., Kondo F., Ishikawa N., Watanabe M.F., Suzuki M. and Harada K.-I. (1994) Stability of microcystins from cyanobacteria: Effect of light on decomposition and isomerization. *Environmental Science & Technology* **28**(1), 173-177.
- Urfer D. and Huck P.M. (2001) Measurement of biomass activity in drinking water biofilters using a respirometric method. *Water Research* **35**(6), 1469-1477.
- WHO (1997) *Report of the working group meeting on chemical substances in drinking water*. World Health Organisation, Geneva.

## CHAPTER 8: DEGRADATION OF MICROCYSTIN THROUGH BIOLOGICAL SAND FILTERS\*

### 8.1 Introduction

In dissolved (extracellular) form, microcystins are not well removed by conventional water treatment processes (Himberg et al., 1989; Lahti and Hiisvirta, 1989). In some cases, conventional water treatment processes have been shown to lyse cyanobacterial cells, resulting in the release of additional intracellular toxins (Pietsch et al., 2002). In Australia activated carbon is the major treatment option used to remove cyanobacterial metabolites from drinking water. However, activated carbon can be adversely affected by the presence of natural organic material (NOM) which can reduce the adsorption of these compounds through pore blockage or competitive adsorption mechanisms, thereby reducing the lifetime of granular activated carbon filters, and increasing the doses of powdered activated carbon required to remove these metabolites (Newcombe et al., 1997; Cook et al., 2001).

For the effective removal of microcystins from potable water, it is imperative for water suppliers to have confidence in the application of successful treatment options. In most cases, a multi-barrier treatment approach of at least two barriers would be required to ensure effective removal of these toxins. As mentioned previously, activated carbon is not always entirely effective; however, microcystins are susceptible to biological degradation (biodegradation) with several studies having implicated a variety of microorganisms for their removal from water (Table 8.1). Bourne et al. (1996, 2001) identified a gene cluster in *Sphingomonas* sp. ACM-3962 which was shown to be involved in the degradation of microcystin. In addition, Saito et al. (2003) detected one of these genes, *mlrA*, within two microcystin-degrading bacteria, *Sphingomonas* sp. MD-1 and *Sphingomonas* sp. Y2. The *mlrA* gene encodes the enzyme responsible for cleaving the cyclic structure of microcystin. Therefore, it is possible that this gene may be specific to microorganisms which have the ability to degrade microcystin.

**Table 8.1.** Microorganisms implicated in the degradation of microcystin toxins.

Microorganisms	Reference(s)
<i>Paucibacter toxinivorans</i>	Rapala et al. (2005)
<i>Poterioochromonas</i> sp.	Ou et al. (2005)
<i>Pseudomonas aeruginosa</i>	Takenaka and Watanabe (1997)
<i>Ralstonia solanacearum</i>	Yan et al. (2004)
<i>Sphingomonas</i> sp. ACM-3962	Jones et al. (1994); Bourne et al. (1996, 2001)
<i>Sphingomonas</i> sp. Y2	Park et al. (2001); Maruyama et al. (2003, 2006)
<i>Sphingomonas</i> sp. MD-1	Saitou et al. (2003); Saito et al. (2003)
<i>Sphingomonas</i> sp. B9	Harada et al. (2004); Imanishi et al. (2005)
<i>Sphingomonas</i> sp. 7CY	Ishii et al. (2004)
<i>Sphingomonas</i> sp. MDB2	Maruyama et al. (2006)
<i>Sphingomonas</i> sp. MDB3	Maruyama et al. (2006)
<i>Sphingomonas</i> sp. CBA4	Valeria et al. (2006)

To date, most of the literature regarding the biodegradation of microcystins has been through batch degradation experiments or in natural water supplies (Jones and Orr, 1994; Rapala et al., 1994; Cousins et al., 1996; Christoffersen et al., 2002). Little has been published with respect to biological filtration of these toxins. Biological filtration techniques are becoming more attractive for water authorities as they incorporate natural biodegradation principles and do not produce deleterious by-

\* This chapter is based on the following manuscript:

Ho L., Hoefel D., Saint C.P. and Newcombe G. (2007) Degradation of microcystin-LR through biological sand filters. *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management* 11(3), 191-196.



products. Of the biological filtration studies conducted on microcystin toxins, only a few have been via sand media (Lahti and Hiisvirta, 1989; Grützmacher et al., 2002; Ho et al., 2006b).

The main objective of this study was to determine whether different biological sand filters were capable of effectively removing microcystin from a variety of water sources. An additional objective was to isolate the microorganism(s) responsible for the degradation of microcystin using molecular genetic techniques, in particular, to detect the presence of the *mlrA* gene, previously shown to be involved in the degradation of microcystin.

## 8.2 Materials and methods

### 8.2.1 Chemicals and reagents

All reagents used were analytical grade or better unless otherwise stated. All glassware and equipment were sterilised (autoclaved 121°C for 15 min) prior to use. Microcystin-LR (MCLR) was extracted from a bloom of *Microcystis aeruginosa* using methods documented previously (Ho et al., 2006b). Water was collected from the Myponga Reservoir, South Australia. In addition, treated waters (coagulation/flocculation/sedimentation) were sampled from the Morgan and Happy Valley water treatment plants (WTPs), South Australia. All sample waters were stored at 4°C when not in use. Sand was obtained from the filter beds at the Morgan, Myponga and Happy Valley WTPs. Characteristics of the sample waters and sands are presented in Table 8.2.

**Table 8.2** Characteristics of sample waters and sands.

Water	pH	UV absorbance at 254 nm (cm <sup>-1</sup> )	DOC (mg/L)
Myponga Reservoir	6.7	0.412	11.8
Morgan treated	7.9	0.040	2.9
Happy Valley treated	6.8	0.044	4.0
Sand	Effective particle size (mm)	Uniformity coefficient	Particle density (g cm <sup>-3</sup> )
Myponga	1.25	1.4	1.62
Morgan	0.55	1.3	1.54
Happy Valley	0.60	1.3	1.46

In some instances, sand samples were initially autoclaved (121°C for 15 min) prior to commissioning. In addition, some sand samples were stripped of any biofilm or dissolved matter by NaOH treatment. This process involved adding sand samples to a vessel containing 2 M NaOH solution which was then agitated overnight to ensure thorough mixing. Sand samples were then rinsed with Milli-Q water (Millipore Pty Ltd, USA) and the process repeated. The presence/absence of biofilm on the sand was verified by scanning electron microscopy before and after NaOH treatment.

### 8.2.2 Biological filtration experiments

Sand was collected from various WTP filter beds and packed into laboratory glass columns (length of 30 cm, I.D. of 2.5 cm) at a bed height of 15 cm. The columns were fed with various waters spiked with MCLR at concentrations ranging from 3 to 20 µg L<sup>-1</sup>. Table 8.3 lists the conditions for each of the laboratory columns tested. Each biological filter was operated at an empty bed contact time (EBCT) of 15 min which corresponded to a filtration velocity of 0.6 m h<sup>-1</sup>. Samples were taken from the filter influents and effluents at regular intervals for microcystin analyses. Experiments were conducted at room temperature (20±2°C) unless otherwise stated.

**Table 8.3** Conditions employed for the laboratory biological filtration experiments.

Column	Sand	Influent water	[MCLR] <sub>0</sub> ( $\mu\text{g L}^{-1}$ )	Temperature (°C)
TM1	Autoclaved Morgan	Myponga Reservoir	20	20
TM2	Morgan	Myponga Reservoir	20	20
BM1	Morgan	Morgan treated	10	20
BM2	Clean (NaOH) Morgan	Morgan treated	10	20
BM3	Clean (NaOH) Myponga	Morgan treated	10	20
JW1	Happy Valley	Happy Valley treated	3	20
BS1	Autoclaved Morgan	Myponga Reservoir	10	10

### 8.2.3 Identification of microcystin degraders

At the completion of the biological filtration experiments, sand was removed from specific filters and the biofilm detached by periodic vortexing for 15 min. The supernatant was collected and any carry over of sand was pelleted by slow speed centrifugation at 1,000 g for 30 s. The resulting supernatant, containing the biofilm bacteria, was inoculated onto solid R2A medium (Oxoid, Australia) and incubated at 25°C for 7 d, after which selected colonies were resuspended in 100  $\mu\text{L}$  of sterile Milli-Q water. DNA from the bacterial suspensions was extracted by three equivalent cycles of boiling at 100°C for 5 min followed by freezing in liquid nitrogen for 1 min. Each bacterial preparation was then screened for the *mcrA* gene using polymerase chain reaction (PCR). Full details of the PCR assay and conditions have been reported previously (Ho et al., 2006b).

Positive PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, USA) and the resulting plasmid DNA purified using the QIAprep Miniprep Kit (QIAGEN Pty Ltd, USA). The purified DNA then underwent a sequencing reaction using the Big Dye v2.0 Terminator Cycle Sequencing Kit (PE Applied Biosystems, USA). Sequences of DNA were aligned using the SeqMan II and EditSeq software programs (DNASTAR, USA). Identification of the amplified DNA fragment was performed online using the Nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) network service on the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>).

### 8.2.4 Microcystin analysis

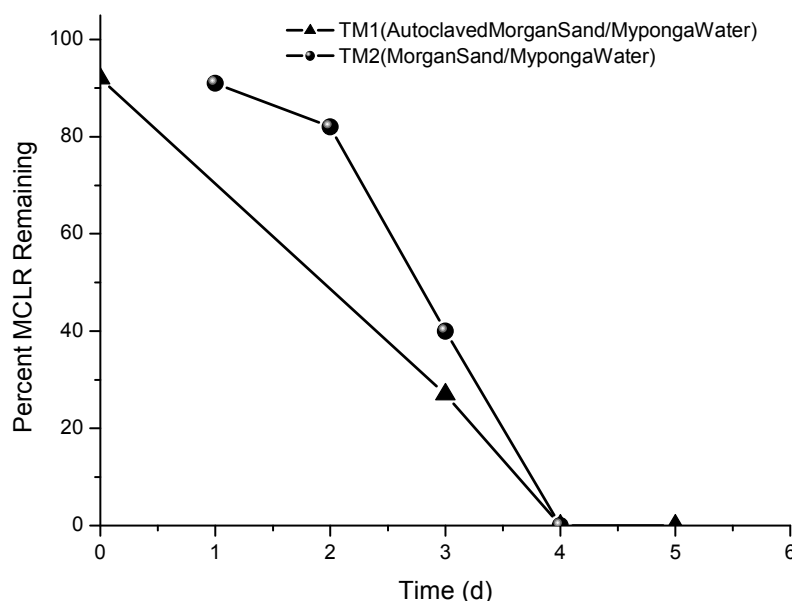
Prior to high performance liquid chromatography (HPLC) analysis, MCLR was concentrated from sample waters by C18 solid phase extraction according to the methods described by Nicholson et al. (1994). A HPLC system consisting of a 600 pump controller, 717plus autosampler and 996 photodiode array detector (Waters Pty Ltd, USA) was employed. Sample volumes of 50  $\mu\text{L}$  were injected into a 150 x 4.6 mm Luna C18 column (Phenomenex, USA) at a flow rate of 1  $\text{mL min}^{-1}$ . Two mobile phases (mobile phase A: 30% acetonitrile/0.05% trifluoroacetic acid and mobile phase B: 55% acetonitrile/0.05% trifluoroacetic acid) were used for the gradient run (0 min - 100% A; 12.5 min - 50% A, 50% B; 15 min - 100% B; 21 min - 100% B; 23 min - 100% A; 32 min - 100% A). Concentrations of MCLR were determined by comparison of the peak areas with that of an external standard (Sapphire Bioscience Pty Ltd, Australia). Confirmation of MCLR presence was determined at a wavelength of 238 nm of the absorption spectra. The HPLC method has a detection limit of 0.5  $\mu\text{g L}^{-1}$ . Microcystin recoveries were greater than 95% with a relative precision of 10%.

## 8.3 Results and discussion

### 8.3.1 Biological filtration of microcystin

Seven biological filters were commissioned for this study, with each filter employing different conditions as described previously in Table 8.3. Figure 8.1 shows the removal of MCLR (at an initial concentration of 20  $\mu\text{g L}^{-1}$ ) through biological filters TM1 and TM2, where the combination of Morgan sand and Myponga Reservoir water was employed. The sand sourced from the Morgan WTP had been in the filter beds for over 25 years, hence it contained an established biofilm; however, for TM1

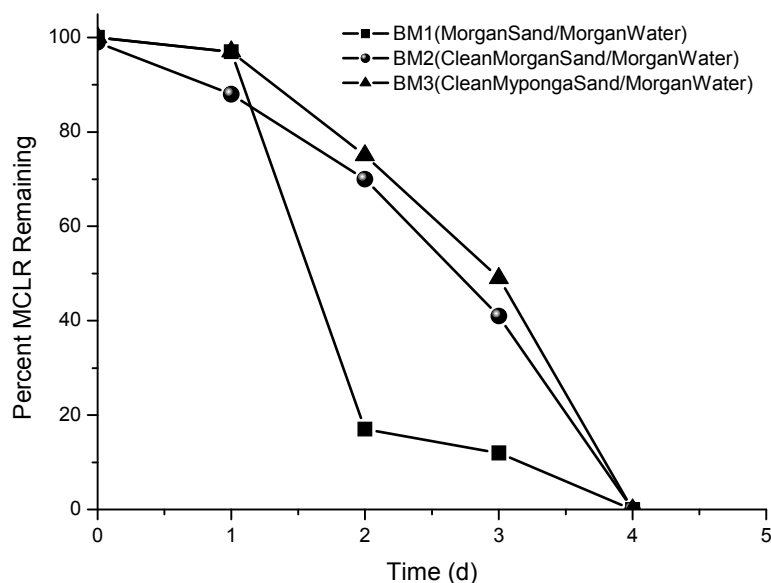
this sand was initially autoclaved to de-activate the biofilm. The results from TM1 indicated that the time required for a biofilm to establish and remove MCLR to below HPLC detection was rapid (4 d), the same time as that of TM2 where an existing biofilm was present.



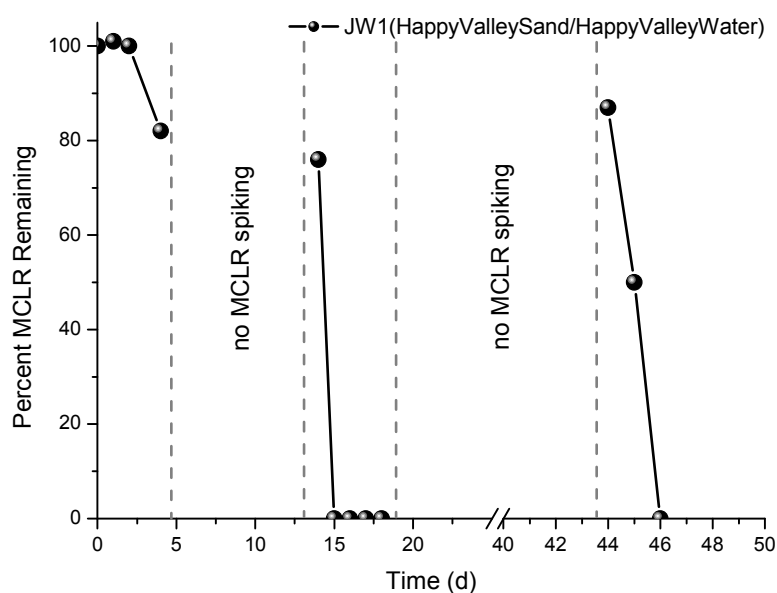
**Figure 8.1** Removal of microcystin-LR (MCLR) through biological filters TM1 (autoclaved Morgan sand/Myponga Reservoir water) and TM2 (Morgan sand/Myponga Reservoir water).

Figure 8.2 shows the removal of MCLR through BM1, BM2 and BM3. These three filters were fed with Morgan treated water, although each employed different sand: BM1 used Morgan sand (the same sand as TM2); BM2 used clean (NaOH treated) Morgan sand; and BM3 used clean (NaOH treated) Myponga sand. In addition, these filters were fed with a lower MCLR concentration ( $10 \mu\text{g L}^{-1}$ ) than the TM filters ( $20 \mu\text{g L}^{-1}$ ). Complete MCLR removal was observed within the same timeframe as the TM filters, indicating that the behaviour of the filters for MCLR removal was similar regardless of whether they initially contained an active biofilm. However, the initial absence of the biofilm in BM2 and BM3 resulted in a different mechanism of MCLR removal than BM1, where in BM1 approximately 80% of MCLR was removed by day 2, with complete removal by day 4, whilst a more gradual decrease in MCLR was observed through BM2 and BM3. The more rapid initial removal of MCLR through BM1 was thought to be due to the initial presence of a biofilm which has been shown to enhance bacterial attachment due to the presence of extracellular polymeric substances (Sutherland, 2001; Alpkvist et al., 2006).

The JW1 filter used sand (which had an existing biofilm) and water sampled from the Happy Valley WTP. Figure 8.3 shows results of MCLR spiking trials through this filter. Lower MCLR removal (maximum of approximately 20% removal on day 4) was observed in the first spiking trial of this filter compared with the TM and BM filters, where by day 4 no MCLR was detected in the TM and BM filter effluents. However, in the second and third spiking trials, conducted after 10 and 26 d abatements in MCLR spiking, respectively, MCLR was shown to be removed to below HPLC detection. This implies that the microorganisms responsible for the degradation of MCLR may have required an initial period of acclimation prior to the onset of degradation. Once the microorganisms were re-exposed to MCLR in subsequent spiking trials, no lag period was observed similar to findings by Rapala et al. (1994), Christoffersen et al. (2002) and Ho et al. (2006a), where lag periods were substantially reduced (in some cases removed) after re-exposure of microcystins. It is also possible that there may have been insufficient numbers of microcystin-degrading microorganisms during the initial stages of the first spiking trial.



**Figure 8.2** Removal of microcystin-LR (MCLR) through biological filters BM1 (Morgan sand/Morgan treated water), BM2 (Clean Morgan sand/Morgan treated water) and BM3 (Clean Myponga sand/Morgan treated water).

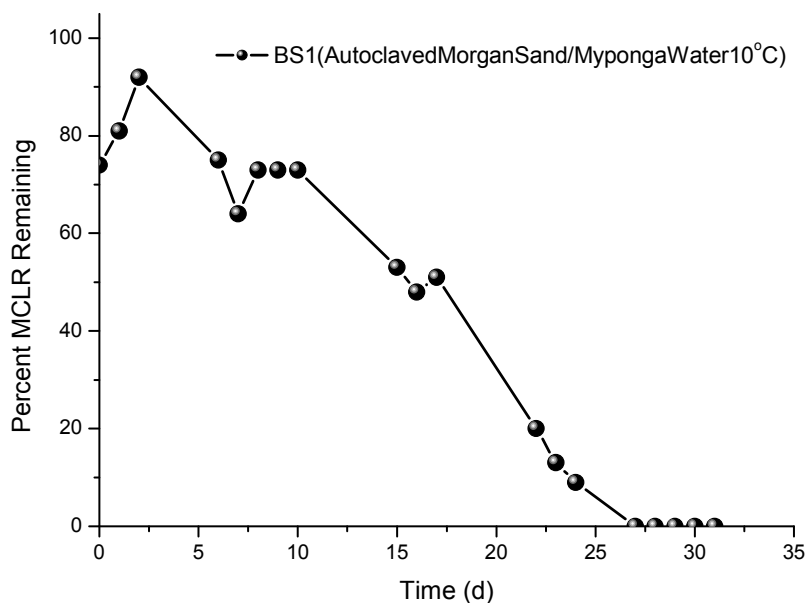


**Figure 8.3** Removal of microcystin-LR (MCLR) through biological filter JW1 (Happy Valley sand/Happy Valley treated water).

The lack of MCLR removal in the initial few days of the first spiking trial provides strong evidence that the complete MCLR removals in the TM and BM filters were through biological degradation and not some other physical processes. We have shown previously that complete removals of MCLR through these filter experiments were through biological processes and not any other physical processes such as adsorption to the sand/biofilm or experimental apparatus (Ho et al., 2006b).

Filter BS1 was commissioned to observe the effect of a lower temperature on the degradation of MCLR (Figure 8.4). BS1 employed similar conditions to TM1; however, this filter was maintained at a

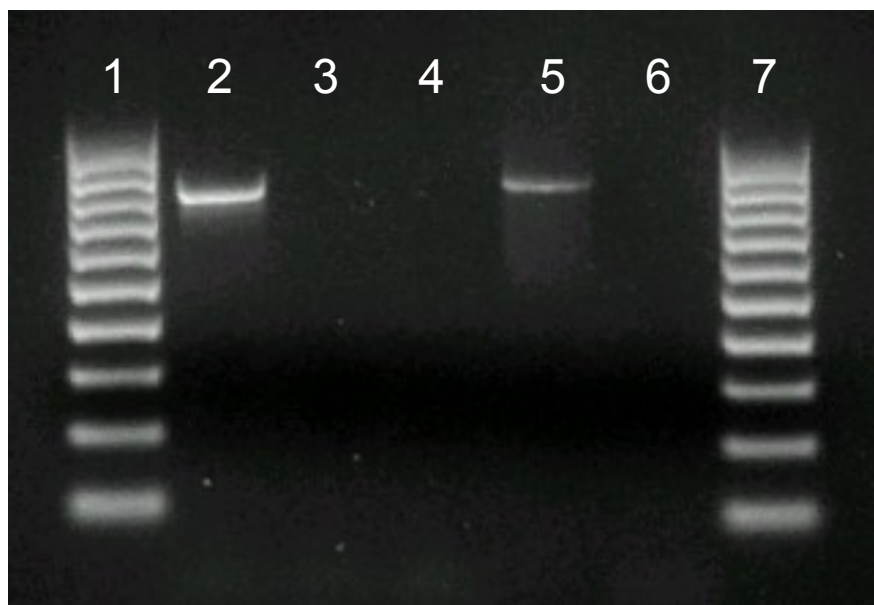
temperature of 10°C as opposed to 20°C. In addition, the influent MCLR concentration of the BS1 filter was approximately half that of TM1. An average of 25% MCLR removal was observed in the first 10 d of the experiment. This initial removal was attributed to small physical losses through the experimental system. After day 10, a gradual increase in MCLR removal was observed with no MCLR detected in the filter effluent by day 27. The prolonged timeframe required for MCLR removal in BS1 suggests that the microorganisms (or enzymes produced by the organisms) responsible for the degradation were inhibited by the lower temperature. This is a significant finding as Australian waters do not generally fall to this temperature compared with those of Europe and the United States, and suggests that the colder climates may not be conducive for rapid biodegradation of MCLR. However, as different microcystin-degrading organisms are present in other countries (see Table 8.1), it is possible that these different organisms may have adapted to these colder climates.



**Figure 8.4** Removal of microcystin-LR (MCLR) through biological filter BS1 (autoclaved Morgan sand/Myponga Reservoir water at 10°C).

### 8.3.2 Isolation of microcystin-degrading bacteria

Biofilm bacteria were detached from the TM2 filter and inoculated onto solid R2A medium. Thirty two isolates from the R2A medium were screened for the *mcrA* gene using PCR. Of these, only one isolate, LH21, was shown to contain this gene. Figure 8.5 shows results of the *mcrA* detection by PCR. Lane 2 corresponds to DNA extracted from *Sphingomonas* sp. ACM-3962, a microcystin-degrading bacterium previously shown to contain the *mcrA* gene (Bourne et al., 1996, 2001; Saito et al., 2003). Consequently, DNA from this bacterium was used as a positive control for the PCR assay in this study. Lanes 3 and 4 correspond to DNA from *Sphingopyxis* sp. Geo24 and *Novosphingobium* sp. Geo25, respectively. Both of these bacteria were previously isolated from WTP sand filters and neither possesses the ability to degrade microcystin; therefore, both were employed as negative controls for the PCR assay. A product of equivalent size (approximately 800 bp) to the positive control was evident for the DNA extract from isolate LH21 (lane 5). This product was cloned and found to have a sequence of 98% similarity to a putative *mcrA* gene from *Sphingopyxis* sp. C-1 (NCBI accession number AB161685). The next closest match in the database was a 91% similarity to the *mcrA* gene from *Sphingomonas* sp. ACM-3962 (NCBI accession number AF411068).



**Figure 8.5** Detection of *mlrA* gene by PCR: Lane 1 - DNA reference marker (100 bp ladder); Lane 2 - *Sphingomonas* sp. ACM-3962 (positive control); Lane 3 - *Sphingopyxis* sp. Geo24; Lane 4 - *Novosphingobium* sp. Geo25; Lane 5 - Isolate LH21 from TM2 filter biofilm; Lane 6 - Milli Q water; Lane 7 - DNA reference marker (100 bp ladder).

The PCR assay was then used to screen for the *mlrA* gene in DNA extracts from the biofilm of filters BM2 and BM3. Both DNA extracts yielded positive amplification products for the *mlrA* gene. These products were found to have DNA sequences of 90% similarity to the putative *mlrA* gene from *Sphingopyxis* sp. C-1. The *mlrA* sequences from BM2 and BM3 were aligned and shown to have 100% sequence similarity. Since both filters initially contained biofilm-free sand (through NaOH treatment) and were fed with the same influent water, then this suggests that the same microcystin-degrading bacteria may have been present in both filters. Efforts are currently being made to isolate the *mlrA* containing bacteria from within these filters.

In addition, the sequences of both PCR products from BM2 and BM3 were found to be 91% similar to the *mlrA* gene detected in isolate LH21 from TM2. As the TM and BM filters were fed with different influent waters (Myponga and Morgan, respectively) then this result implies that the microcystin-degrading bacteria from TM2 and the BM filters were different although closely related, based on the *mlrA* sequence similarity. Further work is required to substantiate this supposition.

The detection of homologous *mlrA* genes within the biofilms of TM2, BM2 and BM3 provided additional evidence that the removal of MCLR through the biological sand filters was through biodegradation.

## 8.4 Conclusions

The diverse range of biological sand filters in this study were shown to be capable of effectively removing MCLR. The initial influent concentration of MCLR (from 3 to 20  $\mu\text{g L}^{-1}$ ) did not appear to affect the removal of MCLR through the filters, rather the temperature was determined to be a limiting factor, with slower degradation at the lower temperature. The *mlrA* gene was detected in three of the filters providing strong evidence that the removal of MCLR through the filters was through bacterial degradation. A putative microcystin-degrading bacterium, containing an *mlrA* gene homologue, was isolated from one of the filters.

## 8.5 References

- Alpkvist E., Picioreanu C., van Loosdrecht M.C.M. and Heyden A. (2006) Three-dimensional biofilm model with individual cells and continuum EPS matrix. *Biotechnology and Bioengineering* **94**(5), 961-979.
- Bourne D.G., Jones G.J., Blakeley R.L., Jones A., Negri A.P. and Riddles P. (1996) Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR. *Applied & Environmental Microbiology* **62**(11), 4086-4094.
- Bourne D.G., Riddles P., Jones G.J., Smith W. and Blakeley R.L. (2001) Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin LR. *Environmental Toxicology* **16**(6), 523-534.
- Christoffersen K., Lyck S. and Winding A. (2002) Microbial activity and bacterial community structure during degradation of microcystins. *Aquatic Microbial Ecology* **27**(2), 125-136.
- Cook D., Newcombe G. and Sztajn P. (2001) The application of powdered activated carbon for MIB and geosmin removal: Predicting PAC doses in four raw waters. *Water Research* **35**(5), 1325-1333.
- Cousins I.T., Bealing D.J., James H.A. and Sutton A. (1996) Biodegradation of microcystin-LR by indigenous mixed bacterial populations. *Water Research* **30**(2), 481-485.
- Grützmacher G., Böttcher G., Chorus I. and Bartel H. (2002) Removal of microcystins by slow sand filtration. *Environmental Toxicology* **17**(4), 386-394.
- Harada K.-I., Imanishi S., Kato H., Masayoshi M., Ito E. and Tsuji K. (2004) Isolation of Adda from microcystin-LR by microbial degradation. *Toxicon* **44**(1), 107-109.
- Himberg K., Keijola A.-M., Hiisvirta L., Pyysalo H. and Sivonen K. (1989) The effect of water treatment processes on the removal of hepatotoxins from *Microcystis* and *Oscillatoria* cyanobacteria: A laboratory study. *Water Research* **23**(8), 979-984.
- Ho L., Hoefel D., Meyn T., Saint C.P. and Newcombe G. (2006a) *Biofiltration of microcystin toxins: An Australian perspective*. In: Gimbel R., Graham N.J.D. and Collins M.R. (Eds.). *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. IWA Publishing, London, UK, pp. 162-170.
- Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006b) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.
- Imanishi S., Kato H., Mizuno M., Tsuji K., and Harada K.-I. (2005) Bacterial degradation of microcystins and nodularin. *Chemical Research in Toxicology* **18**(3), 591-598.
- Ishii H., Nishijima M. and Abe T. (2004) Characterization of degradation process of cyanobacterial hepatotoxins by a Gram-negative aerobic bacterium. *Water Research* **38**(11), 2667-2676.
- Jones G.J., Bourne D.G., Blakeley R.L. and Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Natural Toxins* **2**(4), 228-235.
- Jones G.J. and Orr P.T. (1994) Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Research* **28**(4), 871-876.
- Lahti K. and Hiisvirta (1989) Removal of cyanobacterial toxins in water treatment processes: Review of studies conducted in Finland. *Water Supply* **7**(4), 149-154.
- Maruyama T., Kato K., Yokoyama A., Tanaka T., Hiraishi A. and Park H.-D. (2003) Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*. *Microbial Ecology* **46**(2), 279-288.
- Maruyama T., Park H.-D., Ozawa K., Tanaka Y., Sumino T., Hamana K., Hiraishi A. and Kato K. (2006) *Sphingosinicella microcystinivorans* gen. nov., sp. nov., a microcystin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology* **56**(1), 85-89.
- Newcombe G., Drikas M. and Hayes R. (1997) Influence of characterised natural organic material on activated carbon adsorption: II. Effect of pore volume distribution and adsorption of 2-methylisoborneol. *Water Research* **31**(5), 1065-1073.
- Nicholson B.C., Rositano J. and Burch M.D. (1994) Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *Water Research* **28**(6), 1297-1303.
- Ou D.Y., Song L.R., Gan N.Q. and Chen W. (2005) Effects of microcystins on and toxin degradation by *Poteroochromonas* sp. *Environmental Toxicology* **20**(3), 373-380.
- Park H.-D., Sasaki Y., Maruyama T., Yanagisawa E., Hiraishi A. and Kato K. (2001) Degradation of cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. *Environmental Toxicology* **16**(4), 337-343.
- Pietsch J., Bornmann K. and Schmidt W. (2002) Relevance of intra- and extracellular cyanotoxins for drinking water treatment. *Acta Hydrochimica et Hydrobiologica* **30**(1), 7-15.

- Rapala J., Berg K.A., Lyra C., Niemi R.M., Manz W., Suomalainen S., Paulin L. and Lahti K. (2005) *Paucibacter toxinivorans* gen. nov., sp. nov., a bacterium that degrades cyclic cyanobacterial hepatotoxins microcystins and nodularin. *International Journal of Systematic and Evolutionary Microbiology* **55**(4), 1563-1568.
- Rapala J., Lahti K., Sivonen K. and Niemälä S.I. (1994) Biodegradability and adsorption on lake sediments of cyanobacterial hepatotoxins and anatoxin-a. *Letters in Applied Microbiology* **19**(6), 423-428.
- Saito T., Okana K., Park H.-D., Itayama T., Inamori Y., Neilan B.A., Burns B.P. and Sugiura N. (2003) Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes. *FEMS Microbiology Letters* **229**(2), 271-276.
- Saitou T., Sugiura N., Itayama T., Inamori Y. and Matsumura M. (2003) Degradation characteristics of microcystins isolated bacteria from Lake Kasumigaura. *Journal of Water Supply: Research and Technology - AQUA* **52**(1), 13-18.
- Sutherland I.W. (2001) Exopolysaccharides in biofilms, flocs and related structures. *Water Science & Technology* **43**(6), 77-86.
- Takenaka S. and Watanabe M.F. (1997) Microcystin LR degradation by *Pseudomonas aeruginosa* alkaline protease. *Chemosphere* **34**(4), 749-757.
- Valeria A.M., Ricardo E.J., Stephan P. and Alberto W.D. (2006) Degradation of microcystin-RR by *Sphingomonas* sp. CBA4 isolated from San Roque reservoir (Córdoba - Argentina). *Biodegradation* **17**(5), 447-455.
- Yan H., Pan G., Zou H., Li X.L., and Chen H. (2004) Effective removal of microcystins using carbon nanotubes embedded with bacteria. *Chinese Science Bulletin* **49**(16), 1694-1698.



## CHAPTER 9: DISCRIMINATING AND ASSESSING THE ADSORPTION AND BIODEGRADATION REMOVAL MECHANISMS DURING GRANULAR ACTIVATED CARBON FILTRATION OF MICROCYSTINS\*

### 9.1 Introduction

Granular activated carbon (GAC) has been shown to be successful in the removal of microcystins from drinking water (Falconer et al., 1989; Lahti and Hiisvirta, 1989; Jones et al., 1993; Newcombe et al., 2003). However, excess loading of natural organic material (NOM) causes the reduction of the operational lifetime of a GAC and consequently reduces its adsorption capacity for microcystin (Lambert et al., 1996). Craig and Bailey (1995) showed significant breakthrough of microcystin after five months operation of a GAC filter using an empty bed contact time (EBCT) of 15 min. In addition, they showed that an EBCT of 6 min resulted in significant microcystin breakthrough after 1 month operation.

The presence of a biofilm within a GAC filter may increase its lifetime for the removal of problematic compounds such as the microcystins. The bacteria, immobilised on the surface of the GAC, may remove microcystins via the process of biological degradation (biodegradation). To date, only a few studies have implicated biodegradation of microcystin in GAC filters (Hart and Stott, 1993; Carlile, 1994; UKWIR, 1996). However, these studies were unable to conclusively demonstrate that the removal of microcystin was through biodegradation rather than physical processes, such as adsorption.

The adsorption and biodegradation mechanisms are known to be the predominant factors contributing to microcystin removal during the GAC filtration process. However, it is difficult to identify the relative importance of the two mechanisms at different operational stages of GAC filters. Consequently, operating guidelines that could optimise the filtration process remain unknown, and the development of mathematic models to simulate the removal efficiency of GAC filters is not possible without understanding the individual removal processes.

The main objective of this study was to provide a clearer insight into the individual adsorption and biodegradation removal mechanisms of two microcystin variants in GAC filters by discriminating the removal achieved by each process. A further objective was to assess the adsorption behaviour of the microcystins using a mathematical model. Finally, this study aimed to conclusively demonstrate that biodegradation of microcystins could occur within GAC filters.

### 9.2 Experimental methods

#### 9.2.1 Materials and reagents

A natural spiking toxin solution containing both microcystin-LR (MCLR) and microcystin-LA (MCLA) was extracted from a *Microcystis aeruginosa* bloom which occurred in a lake in Gippsland, Victoria, Australia. The extraction procedure involved freeze-thawing the bloom material in water and methanol, followed by preparative reverse phase flash chromatography and preparative high performance liquid chromatography (HPLC).

Conventionally treated water was sourced from the Myponga water treatment plant (WTP), South Australia. The water was obtained after alum coagulation, dissolved air flotation and rapid sand

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\* This chapter is based on the following manuscript:

Wang H., Ho L., Lewis D.M., Brookes J.D. and Newcombe G. (2007) Discriminating and assessing adsorption and biodegradation removal mechanisms during granular activated carbon filtration of microcystin toxins. *Water Research* **41**(18), 4262-4270.

filtration, but prior to final chlorination. The water has an average dissolved organic carbon (DOC) concentration of  $6 \text{ mg L}^{-1}$  and an average UV absorbance (at 254 nm) of  $0.1 \text{ cm}^{-1}$ .

A commercially available GAC, A6 (PICA Carbons, Australia) was selected in this study. It is a coal-based, steam-activated GAC, and contains a high volume of micropores and mesopores, characteristics which have previously been shown to favour the adsorption of microcystins (Donati et al., 1994; Cook and Newcombe, 2002). A desired GAC particle size of between 1.0-1.4 mm was achieved by screening the GAC sample between 1.0 mm and 1.4 mm sieves, followed by rinsing in Milli-Q water (Millipore Pty Ltd, USA) and drying at  $100^{\circ}\text{C}$  overnight. The resultant GAC was stored in a desiccator prior to use.

Virgin sand (River Sands Pty Ltd, Australia) of the same particle size as the GAC was obtained for this study. The sand is non-porous, and thus has limited adsorption capacity compared with the GAC. Pre-treatment for the sand was performed following the same procedures as the GAC.

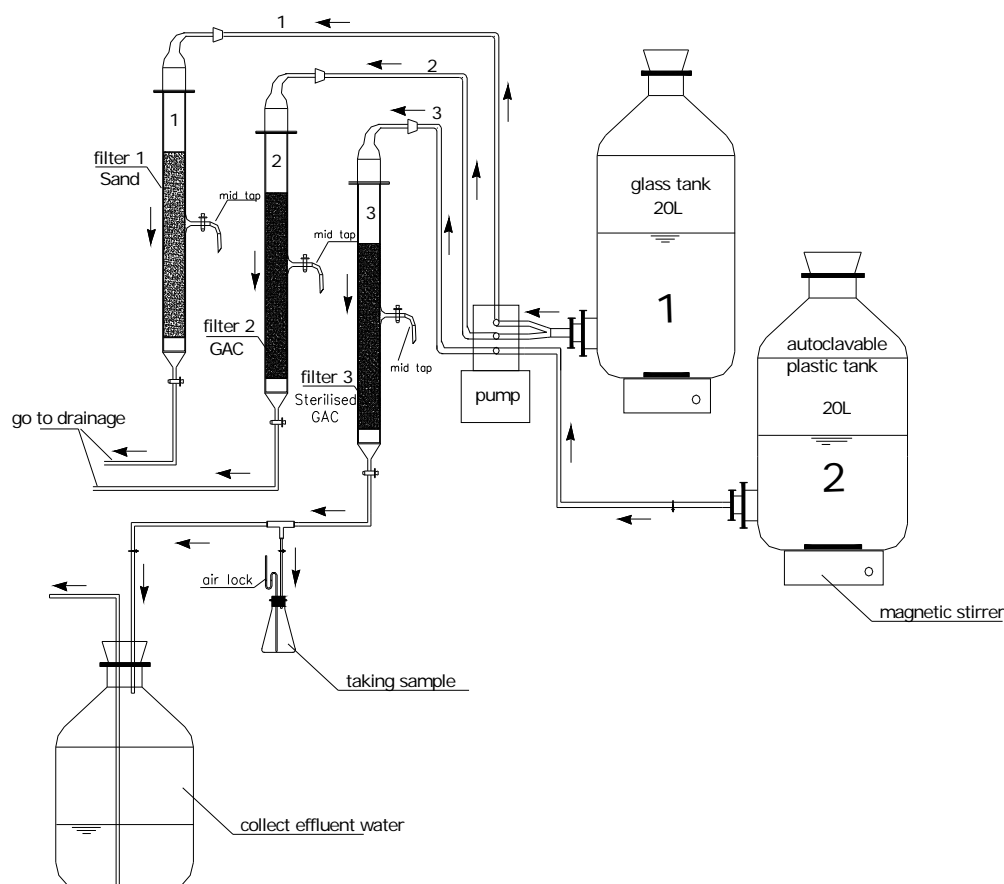
### 9.2.2 Laboratory column experiments

Three laboratory glass columns (25 mm I.D. and 15 cm bed depth) were prepared in parallel to discriminate the adsorption and biodegradation removal mechanisms of microcystin during GAC filtration. Figure 9.1 shows a schematic of the experimental set-up. Microcystin (MCLR and MCLA) spiked Myponga treated water was continuously fed through the three columns at a target EBCT of 15 min which corresponded to a filtration velocity of  $0.6 \text{ m h}^{-1}$ . The target influent concentration of each microcystin analogue was  $5 \text{ } \mu\text{g L}^{-1}$ . Samples were regularly collected from the influent and effluent of each of the columns and analysed for microcystin concentrations. One of the columns contained GAC, whilst the other two columns contained sand and sterilised GAC, respectively. The purpose of the conventional GAC column was to simulate the processes of both adsorption and biodegradation, while the sand column was used to simulate biodegradation only. The sterilised GAC column was prepared to function only in adsorption mode. In order to maintain sterile conditions throughout the experiment for the sterilised GAC system, regular (weekly) autoclaving (at  $121^{\circ}\text{C}$ , 500 kPa for 20 min) of the GAC, associated experimental apparatus and influent water was conducted. All column experiments were conducted at a temperature of  $25^{\circ}\text{C}$ .

To assess the sterility of the sterile GAC system, heterotrophic plate counts (HPCs) were conducted using solid R2A medium (Oxoid, Australia). A volume of  $100 \text{ } \mu\text{L}$  of the effluent sample was routinely taken from the sterile GAC system and spread onto the surface of the agar medium. Colony examination was carried out after incubation at  $25^{\circ}\text{C}$  for 7 d.

### 9.2.3 Biodegradation experiments

Batch biodegradation experiments were conducted in 2 L glass bioreactors containing sterilised (autoclaved at  $121^{\circ}\text{C}$ , 500 kPa for 20 min) Myponga treated water spiked with MCLR at a target concentration of  $5 \text{ } \mu\text{g L}^{-1}$ . Bacterial inocula were sourced from the biofilm of the conventional GAC column. At the completion of the column experiment, GAC media (5 g wet weight) was removed from this column and the bacteria within the biofilm detached from the GAC by periodic vortexing for 15 min in deionised water. The supernatant, containing the bacteria, was collected and used as the bacterial inocula. The bacterial solution was inoculated in the bioreactors at a concentration of  $7.6 \times 10^7$  active bacteria  $\text{mL}^{-1}$  (unless otherwise stated) and incubated aerobically. The number of active bacteria was enumerated using a FACSCalibur flow cytometer (Becton Dickinson, USA) following staining of the bacteria with the BacLight™ bacterial viability kit (Molecular Probes Inc., USA), as described previously (Hoefel et al., 2003).



**Figure 9.1** Schematic of the laboratory column experiments.

To assess the effects of temperature on the biodegradation of MCLR, four bioreactors were prepared as above and incubated at 22, 25, 33 and 40°C. To evaluate the impact of initial bacterial concentration on the biodegradation of microcystin, six bioreactors were prepared using different bacterial inoculum concentrations of  $7.6 \times 10^7$ ,  $3.8 \times 10^7$ ,  $1.5 \times 10^7$ ,  $7.6 \times 10^6$ ,  $3.0 \times 10^6$  and  $1.5 \times 10^6$  active bacteria  $\text{mL}^{-1}$ . Each bioreactor was incubated at 25°C.

Samples were taken from each bioreactor at regular intervals and analysed for MCLR concentrations. For each bioreactor, any losses of microcystin due to factors other than biodegradation were controlled for.

## 9.2.4 Microcystin analyses

Prior to HPLC analysis, water samples containing the microcystins were concentrated using C18 solid phase extraction cartridges (Waters Pty Ltd, Australia) according to the methods documented by Nicholson et al. (1994). Full details of the HPLC analysis have been described previously by Ho et al. (2006). The HPLC method has a detection limit of  $0.5 \mu\text{g L}^{-1}$ . Microcystin recoveries were greater than 95% with a relative precision of 10%.

## 9.2.5 Dissolved organic carbon analysis

Prior to analysis, samples were passed through  $0.45 \mu\text{m}$  cellulose nitrate membrane filters (Schleicher and Schuell, Germany). DOC measurements were made on an 820 Total Organic Carbon Analyser (Sievers Instruments Inc., USA).

## 9.3 Results and discussion

### 9.3.1 Microcystin removal through the laboratory columns

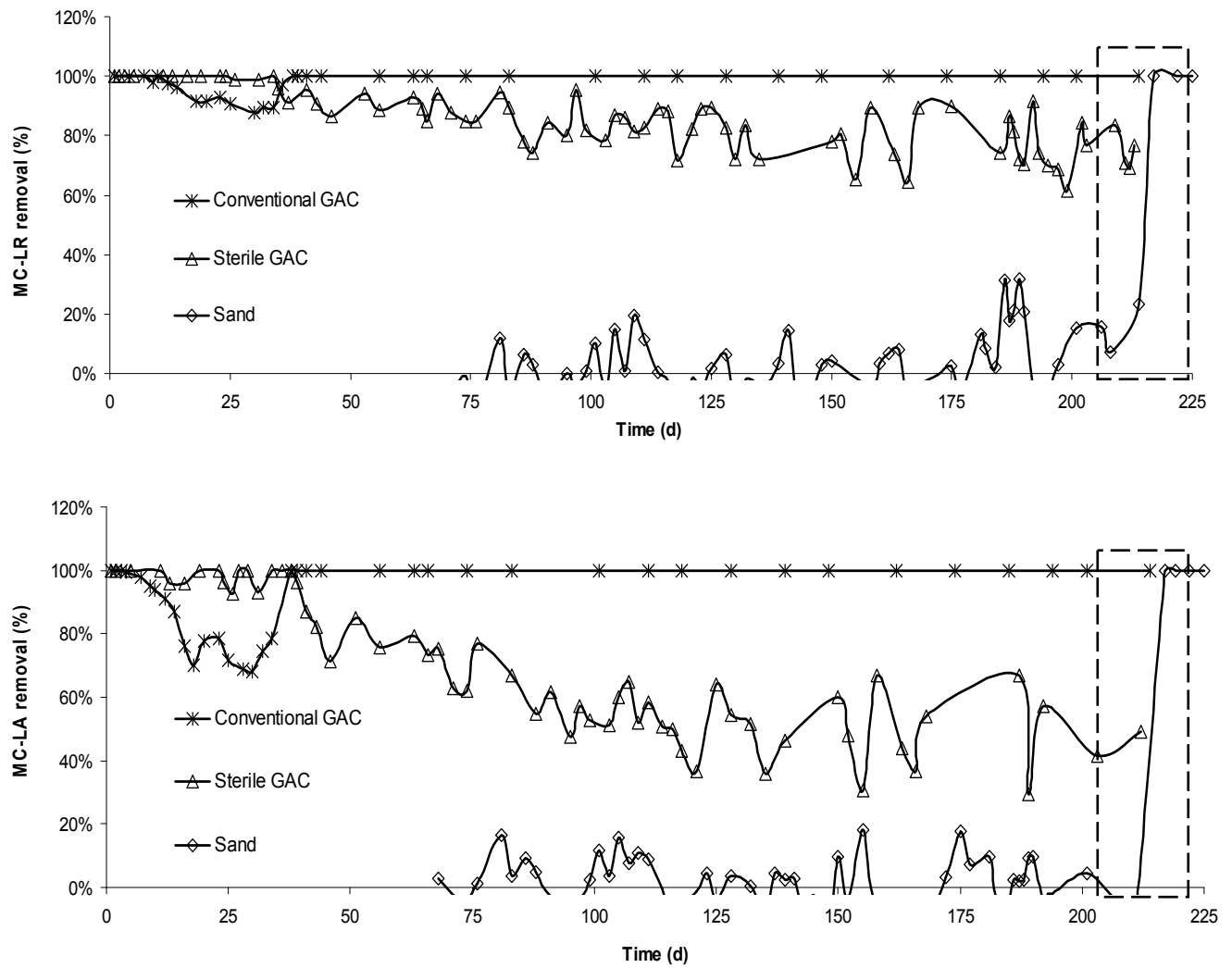
To separate the adsorption and biodegradation removal mechanisms of microcystins in GAC filtration, three laboratory columns were operated in parallel, as described previously. Results of the removal of MCLR and MCLA through each of the columns are shown in Figure 9.2. In the sterile GAC column, only adsorption was responsible for any microcystin removal, since regular HPCs showed no bacterial growth. The microcystin removal capacity of the sterile GAC column gradually decreased from initial complete removal in the first few days to approximately 70% (MCLR) and 40% (MCLA) removals after 6 months. Interestingly, MCLA did not adsorb as well as MCLR. Similar observations have been reported by Newcombe et al. (2003). The reason for this is unclear since MCLA has a lower molecular weight and is more hydrophobic than MCLR, characteristics which should result in greater adsorption of MCLA. Bjelopavic et al. (1998) showed that strong repulsion between polyions adsorbed on activated carbon led to a decrease in the surface coverage. Consequently, a possible explanation for the lower adsorption of MCLA may be electrostatic repulsion between the adsorbed MCLA molecules within the pores of the GAC. Newcombe et al. (2003) attempted to reduce the electrostatic interaction by increasing the ionic strength of the solution containing the microcystin variants. They found that the adsorption of MCLA was significantly increased while the adsorption of the other variants remained the same. This suggests that electrostatic interactions may be a factor in the low adsorption of MCLA. In addition, the conformation and size of the microcystin molecules in solution cannot be disregarded.

The decrease in microcystin removal capacity in the sterile GAC column was attributed to the NOM in the water which could compete with the toxins for adsorption sites on the surface of the GAC. Furthermore, NOM could also reduce the adsorption capacity for microcystin by pore blockage mechanisms. Using the Freundlich adsorption model and experimentally-derived adsorption kinetic parameters, Ho (2004) demonstrated that pore blockage was the major mechanism by which NOM acted to decrease the GAC adsorption capacity for microcystin.

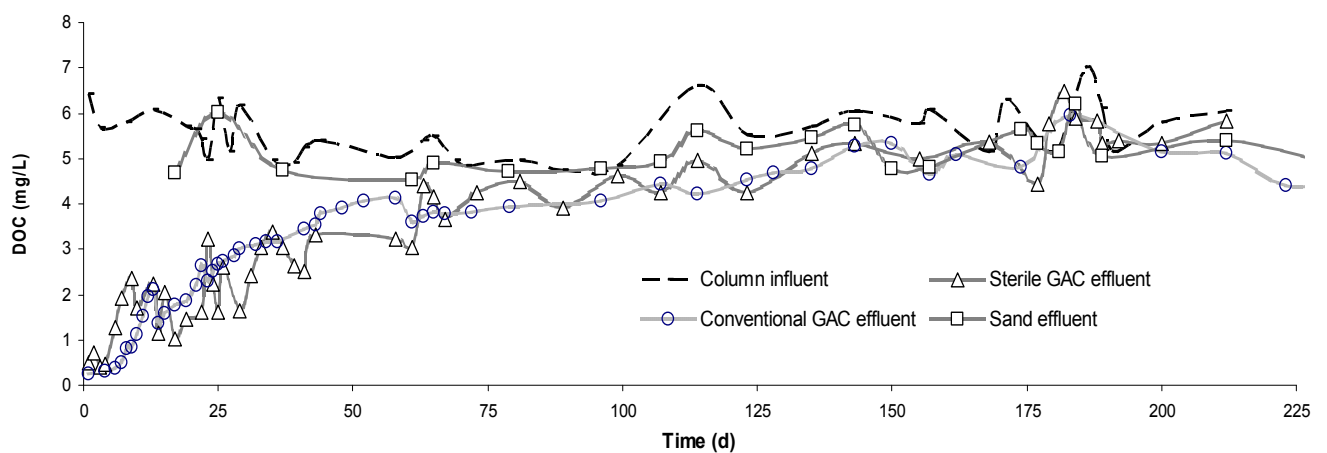
The adsorption of NOM (expressed as DOC) by the sterile GAC is illustrated in Figure 9.3. After approximately 6 months, complete breakthrough of DOC was observed. However, as the level of detection of DOC was in milligram per litre concentrations, it is possible that some removal of DOC was still occurring although at lower concentrations (eg. microgram per litre concentrations). This is highlighted in the adsorption of microgram per litre concentrations of the microcystins (as determined by HPLC), as approximately 70% and 40% removal of MCLR and MCLA were still observed, respectively.

In the conventional GAC column both the adsorption and biodegradation mechanisms would have been prevalent for microcystin removal. No microcystin was detected in the effluent of the conventional GAC column during the first few days after which breakthrough commenced and was observed up to day 38 (Figure 9.2). The breakthrough commenced at an earlier stage when compared with the sterile GAC column (1 week compared with 5 weeks). This was unexpected as it was presumed that the conventional GAC would have initially behaved similarly to the sterile GAC since adsorption would have been the major removal mechanism. In addition, the discrepancy in the breakthrough behaviour of MCLR and MCLA was indicative of adsorption occurring during the initial stages of the conventional GAC. The reason for the dissimilar adsorption performances for both GACs is unclear, although it is possible that the active biofilm that had grown on the surface of the conventional GAC may have restricted transfer of the microcystins into the pores of the GAC. This phenomenon will be discussed in detail in section 9.3.2.

## BIOLOGICAL FILTRATION PROCESSES FOR THE REMOVAL OF ALGAL METABOLITES



**Figure 9.2** Removal of MCLR (above) and MCLA (below) through the conventional GAC, sterile GAC and sand columns; the dashed area represents the onset of biodegradation in the sand column.



**Figure 9.3** Dissolved organic carbon (DOC) removal through the conventional GAC, sterile GAC and sand columns.

From day 38 onward, both microcystin variants were not detected by HPLC in the effluent of the conventional GAC column. This sudden increase in the removal of microcystin was attributed to biodegradation, as it was highly unlikely that the adsorption of microcystin could be improved during

this timeframe. It has been suggested that the microcystins may have adsorbed to the cells of dead microorganisms or organic material; however, studies by Holst et al. (2003) and Ho et al. (2006) have shown this to be insignificant. The effluent DOC concentrations from this column were shown to be very similar to that of the sterile GAC column (Figure 9.3) indicating that the presence of the active biofilm was unable to effectively remove any measurable DOC (milligram per litre concentrations).

In the sand column, negligible removal of the microcystins was observed in the first 7 months (Figure 9.2). However, between days 211 and 217 the removal of both microcystin variants increased dramatically, suggesting the onset of biodegradation. The column was continued for a further week during which time no microcystins were detected in the effluent of the sand column. This result is in contrast to that of Ho et al. (2006) who showed that 4 d was the time required for a biofilm to establish, acclimatise and biodegrade the same microcystin variants in similar sand column experiments sourcing water from the same WTP. The only difference between the work of Ho et al. (2006) and this study was the sand media, as Ho et al. (2006) employed sand with a smaller diameter (effective particle size of 0.83 mm), compared with a particle size range of 1.0-1.4 mm in this study. Furthermore, the sand used by Ho et al. (2006) had an existing biofilm which had been inactivated prior to commissioning. It is presumed that these differences may have resulted in the longer time required for the sand column in this study to develop an efficient biofilm. The smaller sand particle size would result in a larger surface area for microorganisms to colonise within the column compared with a larger particle size which would result in a smaller surface area. Additionally, the existing biofilm would contain extracellular polymeric substances (EPS) which has been shown to enhance bacterial attachment (Sutherland, 2001; Alpkvist et al., 2006). These factors may provide some answers as to why different lag periods, prior to the onset of microcystin degradation, have been observed in other studies (Sherman et al., 1995; Miller and Fallowfield, 2001; Grützmacher et al., 2002).

Both the sand and the GAC in this study shared similar particle sizes and therefore were thought to represent similar surface areas for biofilm growth. However, as mentioned above, the time required for a biofilm to establish and efficiently degrade microcystin was unexpectedly longer in the sand column (7 months) than in the conventional GAC column (1 month). This may be attributed to the different surface characteristics of the two substrates as shown in the scanning electron microscopic (SEM) images in Figure 9.4. The surface of the sand is smooth and non-porous, while the GAC shows a much rougher surface with widely distributed crevasses and ridges. The rougher surface of the GAC has been shown to enhance bacterial attachment compared with smoother surfaces (Hattori, 1988). Moreover, the amorphous GAC structures could help protect newly attached bacteria from shear forces which could have been a major hindrance for biofilm development. The different elemental compositions of both surfaces may also have influenced bacterial attachment.

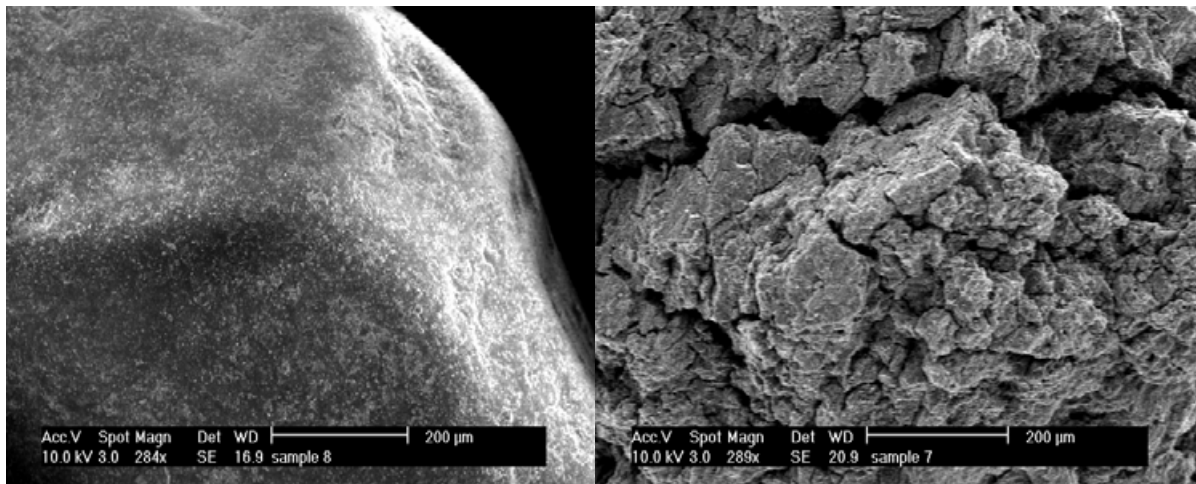
Negligible removal of DOC was observed through the sand column (Figure 9.3), confirming its non-porous nature. This lack of DOC removal also confirms the findings with the conventional GAC, indicating that the active biofilms on the respective media were unable to effectively remove milligram per litre quantities of DOC.

### 9.3.2 Modelling the adsorption of microcystin

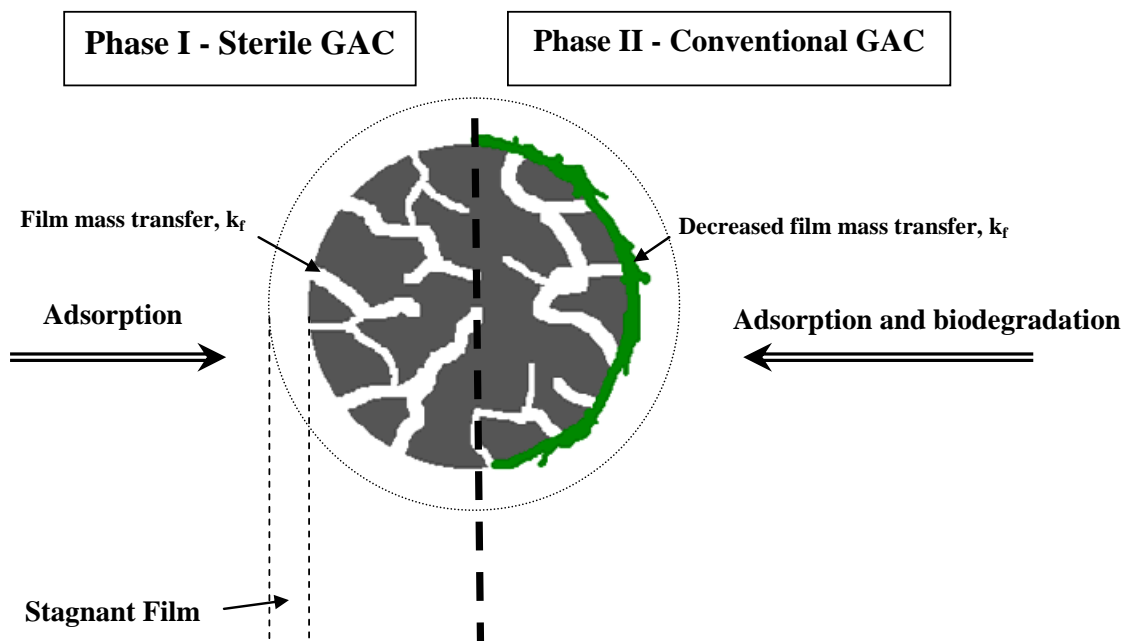
It has generally been presumed that once the adsorption mode of GAC has been exhausted then biodegradation would commence. To date, this anecdotal presumption has yet to be experimentally confirmed. The results in this study indicate that the adsorption of microcystin would still play an important role even after complete breakthrough of DOC was observed, as up to 70% removal of MCLR was apparent after 6 months. Nevertheless, once biodegradation commences, it also acts as a predominant mechanism for microcystin removal as shown in the conventional GAC and sand columns. However, as mentioned previously, there was an anomaly with the adsorption mechanism in the conventional GAC column when an active biofilm was present. The earlier microcystin breakthrough (compared with the sterile GAC) suggested that the active biofilm may hinder the transfer of the microcystin molecule into the pores of the GAC. In order to fully comprehend this phenomenon, a conceptual illustration of a GAC particle was developed as depicted in Figure 9.5.

The microcystin removal process in GAC filtration can be divided into two phases, Phase I (adsorption) and Phase II (adsorption and biodegradation). Phase I can be used to represent the sterile GAC system where adsorption is the primary removal mechanism. Adsorption in this system

can be described using the homogeneous surface diffusion model (HSDM), which has been used to successfully predict the adsorption kinetics of atrazine and 2-methylisoborneol (MIB) by activated carbon (Knappe et al., 1998, 1999; Gillogly et al., 1999). Full details of the HSDM have been described previously (Weber and Liu, 1980; Traegner and Sudan 1989; Knappe et al., 1994).



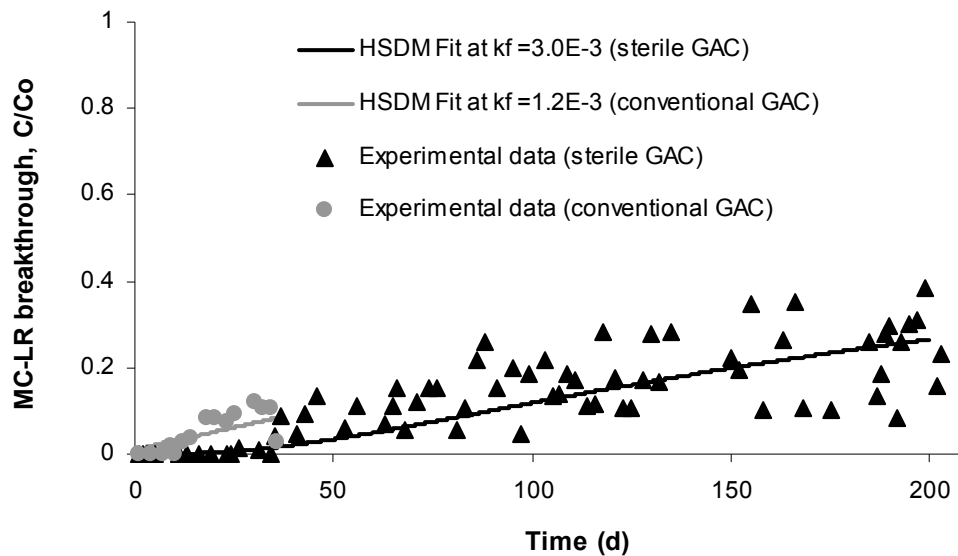
**Figure 9.4** Scanning electron micrographs (SEM) of the surface of the sand (left, 284x magnification) and the GAC (right, 289x magnification) particles.



**Figure 9.5** Conceptual illustration of a GAC particle in the sterile GAC and conventional GAC columns.

Two computer programs, FS-SI and FS, developed by Yuasa (1982), have been used to numerically solve the HSDM. These programs require experimentally derived input parameters, namely the Freundlich isotherm constants,  $K$  and  $1/n$ , and the mass transfer kinetic parameters,  $D_s$  (surface diffusion coefficient) and  $k_f$  (film mass transfer coefficient). These parameters have previously been determined for the adsorption of MCLR using GAC (Ho, 2004). Using these input parameters, in addition to the fixed parameters in this study (eg. particle diameter, influent toxin concentration, experimental run time, etc.) it was possible to predict the breakthrough of MCLR in the sterile and conventional GAC columns using the FS program (Figure 9.6). The prediction involved minimising the difference between the experimental data and model output by intuitively varying the  $k_f$  value whilst keeping all other parameters constant. The  $k_f$  values, derived from the HSDM predictions, were

determined to be  $3.0 \times 10^{-3} \text{ cm s}^{-1}$  and  $1.2 \times 10^{-3} \text{ cm s}^{-1}$  for the sterile and conventional GAC columns, respectively.



**Figure 9.6** Homogenous surface diffusion model (HSDM) fit of the sterile GAC and the conventional GAC experimental data.

In theory,  $k_f$  is a function of both particle diameter and flow rate; therefore, it is intuitive to expect an active biofilm to increase the dimensions of a GAC particle. Consequently, the lower  $k_f$  value derived for the conventional GAC provides strong evidence that the active biofilm on the surface of the GAC did indeed hinder transport of the microcystin molecules onto the surface of the GAC. A similar effect has also been documented by Olmstead (1989) who showed that the  $k_f$  value of trichloroethylene decreased with increasing biomass growth on the surface of a GAC.

Phase II can be used to describe the conventional GAC system where adsorption still occurs although to a lesser extent, and biodegradation commences. Ho (2004) showed the adsorption of MCLR and MCLA decreased with increasing preloading time and attributed this to the co-adsorption of NOM which reduced the adsorption capacity for microcystin through pore blockage mechanisms.

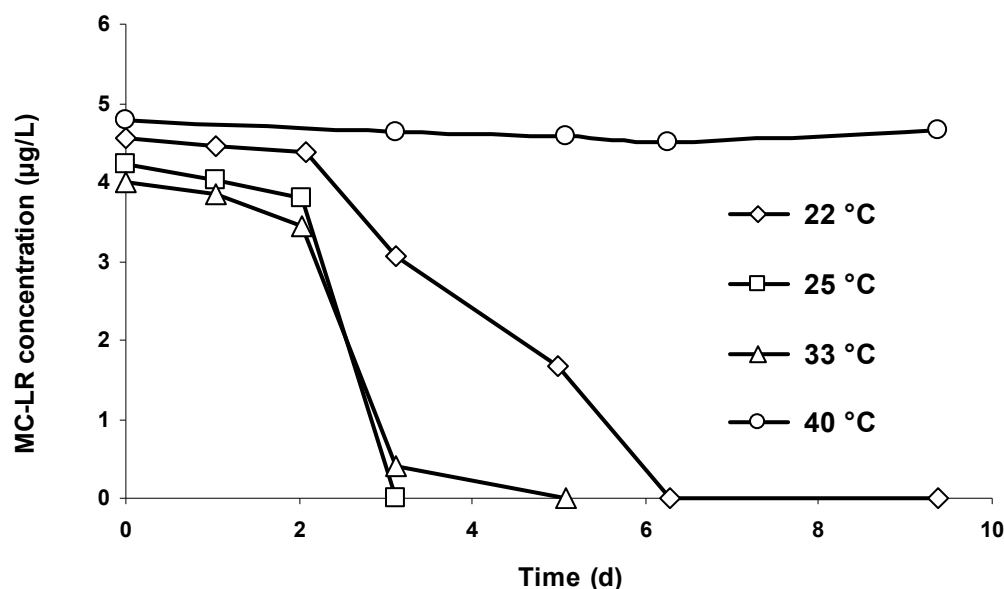
A major removal mechanism of microcystin in phase II would be biodegradation where microorganisms located on the surface of the GAC would act to utilise microcystin as a secondary substrate; NOM would be utilised as a primary substrate due to its higher concentration, milligram per litre concentrations compared with microgram per litre concentrations of microcystin. The biodegradation mechanism is discussed further in section 9.3.3.

### 9.3.3 Assessing the biodegradation of microcystin

Results from the column experiments suggested that biodegradation of microcystins occurred in the conventional GAC and sand columns. A recent study by Ho et al. (2006) conclusively demonstrated biodegradation of microcystin through sand column experiments; however, no studies to date have been able to demonstrate biodegradation of microcystin through GAC columns. In this study, batch biodegradation experiments were conducted using bacteria sourced from the biofilm of the conventional GAC column to verify that biodegradation was taking place. The effect of temperature on the biodegradation of MCLR is shown in Figure 9.7. No removal of MCLR was observed in the bioreactor which was incubated at 40°C suggesting that this temperature may have inactivated the microcystin-degrading bacteria. Subsequent HPC analysis conducted from a sample of this bioreactor showed no growth of heterotrophic bacteria confirming this supposition. Consequently, this bioreactor was used as a control to demonstrate that any removal of MCLR through the other bioreactors was through biological action and not any physical processes, such as adsorption.



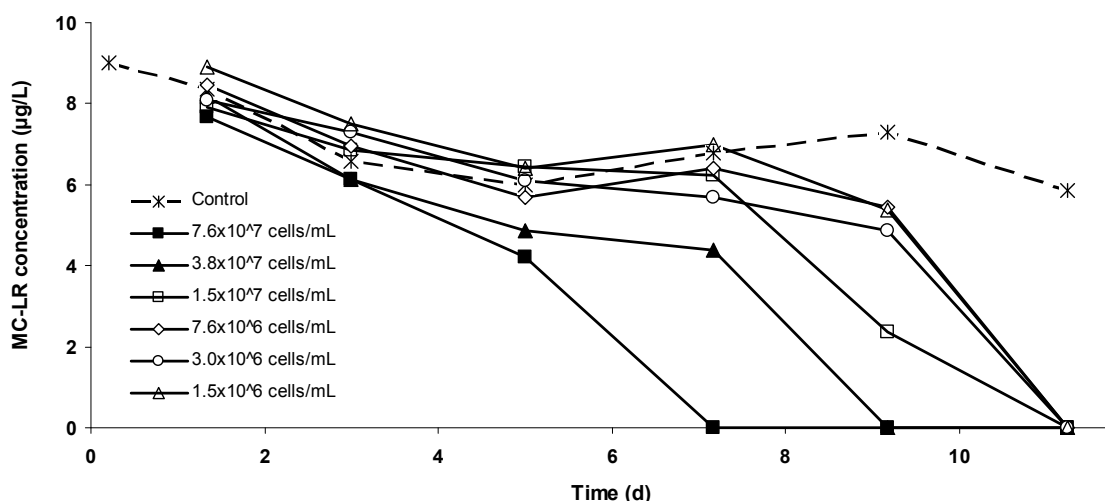
Biodegradation of MCLR was observed in the remaining three bioreactors (22, 25 and 30°C), with a lag period of 2 d prior to biodegradation commencing in each case. The results demonstrate that biodegradation of MCLR was dependent upon temperature with a higher rate of degradation observed at 25°C and 30°C. At both these temperatures, negligible difference was observed with no detection of MCLR in the bioreactors by day 3, whilst 6 d was required for similar microcystin removal in the 22°C bioreactor. The lower biodegradation rate in the 22°C bioreactor was not surprising since the column experiments were conducted at 25°C. Therefore, the bacteria within the biofilm sourced from the conventional GAC column would have been more acclimatised to the higher temperature.



**Figure 9.7** Biodegradation of MCLR in bioreactors incubated at 22, 25, 30 and 40°C. Each bioreactor employed an initial bacterial inoculum concentration of  $7.6 \times 10^6$  active bacteria  $\text{mL}^{-1}$ .

The initial bacterial concentration was shown to influence the biodegradation of MCLR with more rapid biodegradation with the higher initial bacterial inoculum concentrations (Figure 9.8). Larger differences were apparent for the three bacterial concentrations of  $10^7$  active bacteria  $\text{mL}^{-1}$  (7.6, 3.8 and 1.5) with the rate decreasing with decreasing cell numbers. Similar observations were documented by Ho et al. (2007) where biodegradation rates of MIB and geosmin increased with higher initial bacterial inoculum concentration. However, no observable difference was evident when using the three bacterial concentrations of  $10^6$  active bacteria  $\text{mL}^{-1}$  (7.6, 3.0 and 1.5) with complete MCLR removal within 11 d. These results indicate that efficient microcystin degradation may be dependent upon a specific bacterial concentration. This interpretation would help in explaining why there was such an extended period of time (7 months) prior to biodegradation of microcystin occurring in the sand column. As mentioned previously, the surface characteristics of the sand medium may not have initially been conducive for enhanced bacterial attachment. However, once a biofilm began to establish, then higher bacterial numbers may have been able to attach, resulting in efficient biodegradation of microcystin.

It should be noted that this study is by no means universal in that the presence of an active biofilm at a specified concentration will always result in biodegradation of microcystin; rather, successful biodegradation of microcystin is highly dependent upon the type of bacteria and how these bacteria are able to respond and/or produce the degrading enzymes under environmental conditions (Ho et al. 2006). In this study, it was fortuitous to obtain an indigenous source of microcystin-degrading bacteria which were able to colonise within the biofilm of the columns. A study is currently underway to identify these microcystin-degrading bacteria within the water source studied as well as within the biofilm of the biologically-active filters.



**Figure 9.8** Biodegradation of MCLR using initial bacterial inoculum concentrations of  $7.6 \times 10^7$ ,  $3.8 \times 10^7$ ,  $1.5 \times 10^7$ ,  $7.6 \times 10^6$ ,  $3.0 \times 10^6$  and  $1.5 \times 10^6$  active bacteria  $\text{mL}^{-1}$ . Each bioreactor was incubated at  $25^\circ\text{C}$ .

## 9.4 Conclusions

This study demonstrates the effectiveness of GAC filtration for efficient microcystin removal with its dual removal mechanism of both adsorption and biodegradation. Furthermore, this is the first study to discriminate and assess the adsorption and biodegradation of microcystins in GAC filtration. Once it commenced, biodegradation was shown to be an efficient mechanism for microcystin removal, with biodegradation shown to be dependent upon the temperature and initial bacterial density.

Adsorption of microcystins in the GAC columns was modelled using the HSDM, and this model was used to provide evidence that an active biofilm present on the surface of the GAC would hinder transfer of microcystin into the internal adsorption sites of the GAC. Adsorption was shown to still play a vital role in the removal of microcystin with up to 70% removal of MCLR and 40% removal of MCLA after 6 months operation of the sterile GAC column. Results from this study will allow for the development of mathematical models to simulate the adsorption and biodegradation mechanisms for the removal of microcystins.

## 9.5 References

- Alpkvist E., Picioreanu C., van Loosdrecht M.C M. and Heyden A. (2006) Three-dimensional biofilm model with individual cells and continuum EPS matrix. *Biotechnology and Bioengineering* **94**(5), 961-979.
- Bjelopavlic M., Newcombe G. and Hayes R. (1998) Adsorption of NOM onto activated carbon: Effect of surface charge, ionic strength and pore volume distribution. *Journal of Colloid and Interface Science* **210**(2), 271-280.
- Carlile P.R. (1994) *Further studies to investigate microcystin-LR and anatoxin-a removal from water*. Foundation for Water Research Report, FR 0458, Swindon, UK.
- Cook D. and Newcombe G. (2002) Removal of microcystin variants with powdered activated carbon. *Water Science & Technology: Water Supply* **2**(5/6), 201-207.
- Craig K. and Bailey D. (1995) Cyanobacterial toxin microcystin-LR removal using activated carbon – Hunter Water Corporation Experience. In: *Proceedings of the 16th Federal AWWA Convention, April 2-6, 1995, Sydney, Australia*.
- Donati C., Drikas M., Hayes R. and Newcombe G. (1994) Microcystin-LR adsorption by powdered activated carbon. *Water Research* **28**(8), 1735-1742.
- Falconer I.R., Runnegar M.T.C., Buckley T., Huyn Y.L. and Bradshaw P. (1989) Using activated carbon to remove toxicity from drinking water containing cyanobacterial blooms. *Journal of the American Water Works Association* **81**(2), 102-105.

- Gillogly T.E.T., Snoeyink V.L., Newcombe G. and Elarde J.R. (1999) A simplified method to determine the powdered activated carbon does required to remove methylisoborneol. *Water Science & Technology* **40**(6), 59-64.
- Grützmacher G., Böttcher G., Chorus I. and Bartel H. (2002) Removal of microcystins by slow sand filtration. *Environmental Toxicology* **17**(4), 386-394.
- Hart J. and Stott P. (1993) *Microcystin-LR removal from water*. Foundation for Water Research Report, FR 0367, Swindon, UK.
- Hattori K. (1988) Water treatment systems and technology for the removal of odor compounds. *Water Science & Technology* **20**(8/9), 237-244.
- Ho L. (2004) *The removal of cyanobacterial metabolites from drinking water using ozone and granular activated carbon*. PhD Dissertation, University of South Australia, Adelaide, Australia.
- Ho L., Hoefel D., Bock F., Saint C.P. and Newcombe G. (2007) Biodegradation rates of 2-methylisoborneol (MIB) and geosmin through sand filters and in bioreactors. *Chemosphere* **66**(11), 2210-2218.
- Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.
- Hoefel D., Grooby W.L., Monis P.T., Andrews S. and Saint C.P. (2003) Enumeration of water-borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiological Methods* **55**(3), 585-597.
- Holst T., Jørgensen N.O.G., Jørgensen C. and Johansen A. (2003) Degradation of microcystin in sediments at oxic and anoxic, denitrifying conditions. *Water Research* **37**(19), 4748-4760.
- Jones G., Minatol W., Craig K. and Naylor R. (1993) Removal of low level cyanobacterial peptide toxins from drinking water using powdered and granular activated carbon and chlorination – Results of laboratory and pilot plant studies. In: *Proceedings of the 15th Federal AWWA Convention, April 18-23, 1993, Gold Coast, Queensland, Australia*, pp. 579-586.
- Knappe D.R.U., Matsui Y., Snoeyink V.L., Roche P., José Prados M. and Bourbigot M.-M. (1998) Predicting the capacity of powdered activated carbon for trace organic compounds in natural waters. *Environmental Science & Technology* **32**(11), 1694-1698.
- Knappe D.R.U., Snoeyink V.L., Matsui Y., José Prados M. and Bourbigot M.-M. (1994) Determining the remaining life of a granular activated carbon (GAC) filter for pesticides. *Water Supply* **14**, 1-14.
- Knappe D.R.U., Snoeyink V.L., Roche P., José Prados M. and Bourbigot M.-M. (1999) Atrazine removal by preloaded GAC. *Journal of the American Water Works Association* **91**(10), 97-109.
- Lahti K. and Hiisvirta (1989) Removal of cyanobacterial toxins in water treatment processes: Review of studies conducted in Finland. *Water Supply* **7**(4), 149-154.
- Lambert T.W., Holmes C.F.B. and Hudey S.E. (1996) Adsorption of microcystin-LR by activated carbon and removal in full scale water treatment. *Water Research* **30**(6), 1411-1422.
- Miller M.J. and Fallowfield H.J. (2001) Degradation of cyanobacterial hepatotoxins in batch experiments. *Water Science & Technology* **43**(12), 229-232.
- Newcombe G., Cook D., Brooke S., Ho L. and Slyman N. (2003) Treatment options for microcystin toxins: Similarities and differences between variants. *Environmental Technology* **24**(3), 299-308.
- Nicholson B.C., Rositano J. and Burch M.D. (1994) Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *Water Research* **28**(6), 1297-1303.
- Olmstead K.P. (1989) *Microbial interference with the adsorption of target organic contaminants by granular activated carbon*. PhD Dissertation, University of Michigan, Ann Arbor, Michigan, USA.
- Sherman P., Tully I. and Gibson H. (1995) Removal of cyanobacterial cells and toxins from drinking water with biologically active filters. In: *Proceedings of the 16th Federal AWWA Convention, April 2-6, 1995, Sydney, Australia*, pp. 587-592.
- Sutherland I.W. (2001) Exopolysaccharides in biofilms, flocs and related structures. *Water Science & Technology* **43**(6), 77-86.
- Traegner U.K. and Suidan M.T. (1989) Evaluation of surface and film diffusion coefficients for carbon adsorption. *Water Research* **23**(3), 267-273.
- UKWIR (1996) *Pilot scale GAC tests to evaluate toxin removal*. UK Water Industry Research Ltd. Report No. 96/DW/07/1, London, UK.
- Weber W.J. and Liu K.T. (1980) Determination of mass transport parameters for fixed bed adsorbers. *Chemical Engineering Communications* **6**(1-3), 49-60.
- Yuasa A. (1982) *A kinetic study of activated carbon adsorption processes*. PhD Dissertation, Hokkaido University, Sapporo, Japan.

## CHAPTER 10: ISOLATION AND IDENTIFICATION OF A MICROCYSTIN-DEGRADING BACTERIUM FROM A BIOLOGICAL SAND FILTER\*

### 10.1 Introduction

There are a growing number of isolated bacteria reported as having the capacity to degrade microcystin in water (Table 10.1) and so far these appear to be limited to the family *Sphingomonadaceae*. Within the genome of the first isolated microcystin-degrading bacterium, *Sphingomonas* sp. ACM-3962, Bourne et al. (1996, 2001) identified a gene cluster, *mlrA*, *mlrB*, *mlrC* and *mlrD*, responsible for the degradation of MCLR. The authors determined that the *mlrA* gene encoded an enzyme responsible for the hydrolytic cleaving of the cyclic structure of MCLR. The resultant linear MCLR molecule was then sequentially hydrolysed by peptidases encoded by the *mlrB* and *mlrC* genes. The final gene, *mlrD*, encoded for a putative transporter protein that may have allowed for active transport of microcystin and/or its degradation products into or out of the cell. Recently, Saito et al. (2003) designed a polymerase chain reaction (PCR) assay for detection of *mlrA* from *Sphingomonas* sp. ACM-3962. Using this technique, Saito et al. (2003) reported gene homologues of *mlrA* in two microcystin-degrading bacteria, *Sphingomonas* sp. MD-1 and *Sphingomonas* sp. Y2, both of which were previously isolated from separate Japanese lakes.

Currently there is a lack of understanding regarding the types of bacteria within biologically-active filters that are capable of degrading microcystin toxins. In a recent study, we reported the use of a sand filter which was shown to effectively remove two microcystin variants, MCLR and MCLA, from source water and demonstrated that removal occurred predominantly through biological degradation processes (Ho et al., 2006). In that study we also employed PCR as a screening tool to detect a gene homologous to *mlrA* within a biofilm-associated bacterium attached to the sand filter medium (Ho et al., 2006).

The major objectives of this study were to: (1) isolate and identify the bacterium containing the *mlrA* gene from the sand filter reported previously (Ho et al., 2006); (2) determine if this bacterium contained each of the genes, *mlrA*, *mlrB*, *mlrC* and *mlrD*, previously reported to be involved in the degradation of MCLR by *Sphingomonas* sp. ACM-3962; and (3) investigate the capacity of this novel bacterium to degrade two microcystin variants under environmentally relevant conditions. Results from this study will provide insights into the mechanisms of microcystin degradation that may occur within sand filters actively removing microcystin. This will provide an important step in the development of more successful strategies aimed at eliminating start-up lag time for new filters by potentially seeding filters with known degraders, and permit the screening of filters for the presence of known degraders when a microcystin event is imminent.

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\* This chapter is based on the following manuscript:

Ho L., Hoefel D., Saint C.P. and Newcombe G. (2007) Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. *Water Research* **41**(20), 4685-4695.

**Table 10.1** Bacteria implicated in the degradation of microcystin toxins.

Bacterium	Source	GenBank Accession Number <sup>a</sup>	Reference(s)	Degradable Variants <sup>b</sup>	Non-degradable Variants <sup>b</sup>
<i>Sphingomonas</i> sp. ACM-3962	Murrumbidgee River, Australia	AF401172	Jones et al. (1994)	MCLR, MCRR	Nodularin
<i>Sphingomonas</i> sp. Y2	Lake Suwa, Japan	AB084247	Bourne et al. (1996, 2001) Park et al. (2001)	MCLR, MCRR, MCYR, 6(Z)-Adda-MCLR	-
<i>Sphingomonas</i> sp. MD-1	Lake Kasumigaura, Japan	AB1110635	Maruyama et al. (2003, 2006) Saitou et al. (2003)	MCLR, MCRR, MCYR	Nodularin
<i>Sphingomonas</i> sp. B9	Lake Tsukui, Japan	AB159609	Saito et al. (2003) Harada et al. (2004) Imanishi et al. (2005)	MCLR, MCRR, 3-DMMCLR <sup>c</sup> , DHMCLR <sup>d</sup> , MCLR-Cys <sup>e</sup> , Nodularin	MCLF, 6(Z)-Adda-MCLR, 6(Z)-Adda-MCRR
<i>Sphingomonas</i> sp. 7CY	Lake Suwa, Japan	AB076083	Ishii et al. (2004)	MCLR, MCRR, MCLY, MCLW, MCLF	Nodularin-Har <sup>f</sup>
<i>Sphingomonas</i> sp. MDB2	Tenryu River, Japan	AB219940	Maruyama et al. (2006)	-	-
<i>Sphingomonas</i> sp. MDB3	Tenryu River, Japan	AB219941	Maruyama et al. (2006)	-	-
<i>Sphingomonas</i> sp. CBA4	San Roque reservoir, Argentina	AY920497	Valeria et al. (2006)	MCRR	-

<sup>a</sup> GenBank accession numbers for partial 16S rRNA gene sequences

<sup>b</sup> including nodularin

<sup>c</sup> 3-desmethylmicrocystin-LR

<sup>d</sup> dihydromicrocystin-LR

<sup>e</sup> MCLR cysteine conjugate

<sup>f</sup> a variant of nodularin possessing a homoarginine in place of an arginine

## 10.2 Experimental procedures

### 10.2.1 Materials and reagents

Two microcystin variants were used in this study, MCLR and MCLA. Both were isolated from a natural bloom of *Microcystis aeruginosa* that occurred in the Torrens Lake in South Australia during the summer of 1998-99. The isolation procedure involved freeze-thawing the bloom material in water and methanol, followed by preparative reverse phase flash chromatography and preparative high performance liquid chromatography (HPLC). Sample water was collected from the Myponga Reservoir in South Australia and immediately filtered through a 1 µm filter cartridge to remove particulate matter. *Sphingomonas* sp. ACM-3962 was purchased from the Australian Collection of Microorganisms, University of Queensland, Australia.

### 10.2.2 Analysis of microcystins

Prior to HPLC analysis, microcystins were concentrated from sample waters by C18 solid phase extraction according to the methods described by Nicholson et al. (1994). A HPLC system consisting of a 600 pump controller, 717plus autosampler and 996 photodiode array detector (Waters Pty Ltd, Australia) was employed. Sample volumes of 50 µL were injected into a 150 x 4.6 mm Luna C18 column (Phenomenex, Australia) at a flow rate of 1 mL min<sup>-1</sup>. Two mobile phases were used for the gradient run (30% acetonitrile/0.05% trifluoroacetic acid and 55% acetonitrile/0.05% trifluoroacetic acid). Concentrations of MCLR were determined by calibration of the peak areas (at 238 nm) with that of an external standard (Sapphire Bioscience Pty Ltd, Australia). All MCLA concentrations were expressed in terms of MCLR equivalents. The HPLC method has a detection limit of 0.5 µg L<sup>-1</sup>. Microcystin recoveries were greater than 95% with a relative precision of 10%.

### 10.2.3 Isolation of microcystin-degrading bacteria

Full details of a biological sand filter shown to effectively remove MCLR and MCLA, have been reported previously (Ho et al., 2006). At the completion of the filter experiment, sand (10 g wet weight) was removed from the filter and the bacteria within the biofilm detached from the sand by periodic vortexing for 15 min in sterilised (autoclaved at 121°C for 20 min) Myponga Reservoir water. The supernatant, containing the bacteria, was collected and any carry over of sand was pelleted by slow speed centrifugation at 1,000 g for 30 s. The resulting supernatant was inoculated onto solid R2A medium (Oxoid, Australia) and incubated at 25°C for 7 d, after which colonies of differing morphologies were resuspended in 100 µL of sterile Milli Q water. DNA from the bacterial suspensions was extracted by three equivalent cycles of boiling at 100°C for 5 min followed by freezing in liquid nitrogen for 1 min. Each bacterial preparation was then screened for the genes involved in microcystin degradation using PCR (see below). In addition, the preparations were used as a template for phylogenetic characterisation.

### 10.2.4 Detection of genes involved in microcystin degradation

A specific oligonucleotide primer set MF, 5'-GACCCGATGTTCAAGATACT-3' and MR, 5'-CTCCTCCACAAATCAGGAC-3' (Saito et al., 2003) was used in PCR to screen bacterial isolates for the *mlrA* gene. Amplifications were conducted on a GeneAmp 2400 PCR System (Perkin Elmer, USA) under conditions previously documented by Ho et al. (2006). PCR products were separated by electrophoresis on a 1% agarose gel containing 0.3 x Gelstar nucleic acid stain (Cambrex Bio Science, USA). Resultant DNA bands were visualised on a Dark Reader transilluminator (Clare Chemical Research Inc., USA).

Using Primer Premier 5 software (Premier Biosoft, USA), new oligonucleotide primer sets, *mlrBf1-mlrBr1*, *mlrCf1-mlrCr1* and *mlrDf1-mlrDr1* were designed respectively from the *mlrB*, *mlrC* and *mlrD* gene sequences of *Sphingomonas* sp. ACM-3962. In addition, primer sets were designed to span regions between the *mlrC* and *mlrA* genes, *mlrA* and *mlrD* genes and *mlrD* and *mlrB* genes. Details of

each of the primers sets are presented in Table 10.2. Amplifications were performed in 50  $\mu$ L volumes, containing 1 pmol of each primer, 2.5 mM  $MgCl_2$  (Perkin Elmer, USA), 1 x PCR Buffer II (Perkin Elmer, USA), 200  $\mu$ M deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics Pty Ltd., Australia), 1.25 units of Ampli Taq Gold DNA Polymerase (Perkin Elmer, USA) and 10  $\mu$ L of genomic DNA template. A GeneAmp 2400 PCR System (Perkin Elmer, USA) was used for the amplifications under the following conditions: 95°C for 10 min; 40 cycles of 95°C for 20 s, 60°C for 10 s and 72°C for 30 s; and 72°C for 10 min. PCR products were analysed by gel electrophoresis as described above. The DNA sequence for each of the PCR fragments of expected size were subsequently determined as described previously (Hoefel et al., 2005).

**Table 10.2** Oligonucleotide primer sequences and their characteristics for the genes *mlrB*, *mlrC* and *mlrD* and spanning genes *mlrC-mlrA*, *mlrA-mlrD* and *mlrD-mlrB*.

Gene	Primer	Sequence (5' - 3')	Length (bp)	T <sub>m</sub> (°C)	Position <sup>a</sup>
<i>mlrB</i>	mlrBf1	CGACGATGAGATACTGTCC	19	51	99-117
	mlrBr1	CGTGCGGACTACTGTTGG	18	52	530-547
<i>mlrC</i>	mlrCf1	TCCCCGAAACCGATTCTCCA	20	54	98-117
	mlrCr1	CCGGCTCACTGATCCAAGGCT	21	58	744-764
<i>mlrD</i>	mlrDf1	GCTGGCTGCGACGGAATG	19	55	51-69
	mlrDr1	ACAGTGTGCGGAGCTGCTCA	21	56	702-722
<i>mlrC-mlrA</i> <sup>b</sup>	mlrCf2	CATTGCCCGTCGCAGTTGA	19	53	1153-1171
	mlrAr2	TGTTGAGGTGAGCGTGCGTCT	21	56	129-149
<i>mlrA-mlrD</i> <sup>c</sup>	mlrAf2	GTAAACGTCACCTGCCGAATGGG	22	57	802-823
	mlrDr2	GAGTAGCGGGCAGAGTTGGAAG	22	58	352-373
<i>mlrD-mlrB</i> <sup>d</sup>	mlrDf2	TTGGGCTCGCACTGGTTATCC	21	56	932-952
	mlrBr2	CCGAAATGAAACGCTTGGA	20	52	172-191

<sup>a</sup>On respective gene of *Sphingomonas* sp. ACM-3962

<sup>b</sup>spanning genes between *mlrC* and *mlrA*

<sup>c</sup>spanning genes between *mlrA* and *mlrD*

<sup>d</sup>spanning genes between *mlrD* and *mlrB*

### 10.2.5 Phylogenetic analysis of isolated bacteria

The primer set 27F-1492R was used in PCR amplification of the 16S rRNA gene fragment of isolate LH21 using conditions described previously (Lane, 1991). Sequence similarity searches were conducted using the National Center for Biotechnology Information BLAST network service (blastn). Similar 16S rRNA gene sequences, from previously cultured bacteria, were downloaded from GenBank and manually checked for ambiguous sites. Using ClustalX version 1.64b software (Thompson et al., 1997), alignments were then performed against the 16S rRNA gene sequence of isolate LH21, where the pair-wise deletion option for gaps was employed. The alignment data was then used for neighbour-joining analysis with 1,000 bootstrap replicates (MEGA version 2.1; Arizona State University, USA).

### 10.2.6 Batch degradation experiments

Isolate LH21 was grown overnight in Luria-Bertani (LB) liquid medium (1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0) at 30°C, then washed twice by centrifugation at 1,000 g for 15 min with re-suspension of the bacterial pellet each time in sterile 0.01 M phosphate buffered saline. The resulting supernatant, containing isolate LH21, was inoculated into 2 L Pyrex vessels (Corning, USA) containing 2 L of 0.2  $\mu$ m filter sterilised/autoclaved (121°C for 20 min) Myponga Reservoir water at concentrations of  $1.0 \times 10^6$  active bacteria  $mL^{-1}$ . The number of active bacteria was enumerated using a FACSCalibur flow cytometer (Becton Dickinson, USA) following staining of the bacteria with the BacLight bacterial viability kit (Molecular Probes Inc., USA), as described previously (Hoefel et al., 2003). MCLR and MCLA, each at target concentrations of 25  $\mu$ g  $L^{-1}$ , 10  $\mu$ g  $L^{-1}$ , and 5  $\mu$ g  $L^{-1}$ , were spiked into separate vessels. Any losses of microcystin due to factors other than biological degradation were controlled for using a sterilised vessel containing inactivated (autoclaved at 121°C for 20 min) inoculum. Each vessel was incubated aerobically at 22°C with constant stirring. Parallel

experiments were conducted using *Sphingomonas* ACM-3962 as the sole active bacterial inoculum. This isolate was chosen for comparison with isolate LH21 due to its availability. Sterile conditions were maintained throughout the batch experiments with samples taken aseptically from the vessels at regular intervals for microcystin analyses.

### 10.2.7 Effect of temperature and bacterial density on microcystin degradation

To evaluate the effect of temperature and bacterial density on the degradation of microcystin, additional sets of batch experiments were conducted using the same experimental procedures outlined in section 10.2.6. To assess the effect of temperature, two identical vessels, B2 and B3, containing Myponga Reservoir water spiked with MCLR and MCLA (each at target concentration of  $5 \mu\text{g L}^{-1}$ ) were inoculated with isolate LH21 at a concentration of  $1.0 \times 10^6$  active bacteria  $\text{mL}^{-1}$ . B2 and B3 were incubated aerobically at temperatures of  $30^\circ\text{C}$  and  $4^\circ\text{C}$ , respectively. Samples were taken aseptically from the vessels at regular intervals for microcystin analyses.

To assess the effect of bacterial density, a vessel, B4, containing Myponga Reservoir water spiked with MCLR and MCLA (each at a target concentration of  $5 \mu\text{g L}^{-1}$ ) was inoculated with isolate LH21 at an initial concentration of  $1.0 \times 10^4$  active bacteria  $\text{mL}^{-1}$  and incubated aerobically at  $22^\circ\text{C}$ . Samples were taken aseptically from B4 at regular intervals for microcystin analyses. During both sets of experiments, samples were also taken aseptically at regular intervals for bacterial enumeration using flow cytometry as described in section 10.2.6. The data obtained from both sets of experiments (effect of temperature and bacterial density) was directly compared with the LH21 batch experiment in section 10.2.6, B1, where Myponga Reservoir water (containing  $5 \mu\text{g L}^{-1}$  of MCLR and  $5 \mu\text{g L}^{-1}$  of MCLA) was inoculated with LH21 at  $1.0 \times 10^6$  active bacteria  $\text{mL}^{-1}$  and incubated aerobically at  $22^\circ\text{C}$ .

For re-spike experiments, MCLR and MCLA (each at target concentrations of  $10 \mu\text{g L}^{-1}$ ) were added into B1, B2 and B4 on days 6 and 15 and microcystin concentrations monitored on a daily and hourly basis, respectively. The re-spike experiments were conducted to determine if previous exposure of isolate LH21 to microcystin had the ability to enhance degradation.

## 10.3 Results and discussion

### 10.3.1 Isolation of microcystin-degrading bacteria

A total of 32 isolates from the R2A medium were screened for the *mlrA* gene using PCR. Of these, only one isolate, LH21, was shown to contain this gene. This *mlrA* gene sequence for isolate LH21 was shown to be identical to that of the *mlrA* gene sequence previously detected in the biofilm of the sand filter described by Ho et al. (2006). Using PCR, isolate LH21 was then shown to contain homologues to each of *mlrB*, *mlrC* and *mlrD*, where these sequences were 92%, 89% and 88% similar to the respective genes from *Sphingomonas* sp. ACM-3962. These sequences for isolate LH21 were deposited to GenBank as: *mlrB* accession number DQ423530; *mlrC* accession number DQ423531; and *mlrD* accession number DQ423532. In addition, using PCR primers designed to span regions between each of these genes, the order and orientation for each gene within isolate LH21 was shown to be identical to that reported by Bourne et al. (1996, 2001) for *Sphingomonas* sp. ACM-3962. These results suggest that isolate LH21 may have degraded microcystin via a similar pathway to that of *Sphingomonas* sp. ACM-3962. The DNA sequences for each of the gene spanning PCR products from *Sphingomonas* sp. ACM-3962 and isolate LH21 were deposited under the following accession numbers: ACM-3962 *mlrC-mlrA* genes DQ423533; LH21 *mlrC-mlrA* genes DQ423534; ACM-3962 *mlrA-mlrD* genes DQ423535; LH21 *mlrA-mlrD* genes DQ423536; ACM-3962 *mlrD-mlrB* genes DQ423537; and LH21 *mlrD-mlrB* genes DQ423538.

### 10.3.2 Phylogenetic analysis of isolate LH21

Based on a 1454-bp fragment of the 16S rRNA gene, isolate LH21 was shown to be 98% similar to *Sphingopyxis wittflariensis*, and this DNA sequence was deposited to GenBank under accession number DQ112242. Neighbour-joining phylogenetic analysis using 16S rRNA gene fragments for each

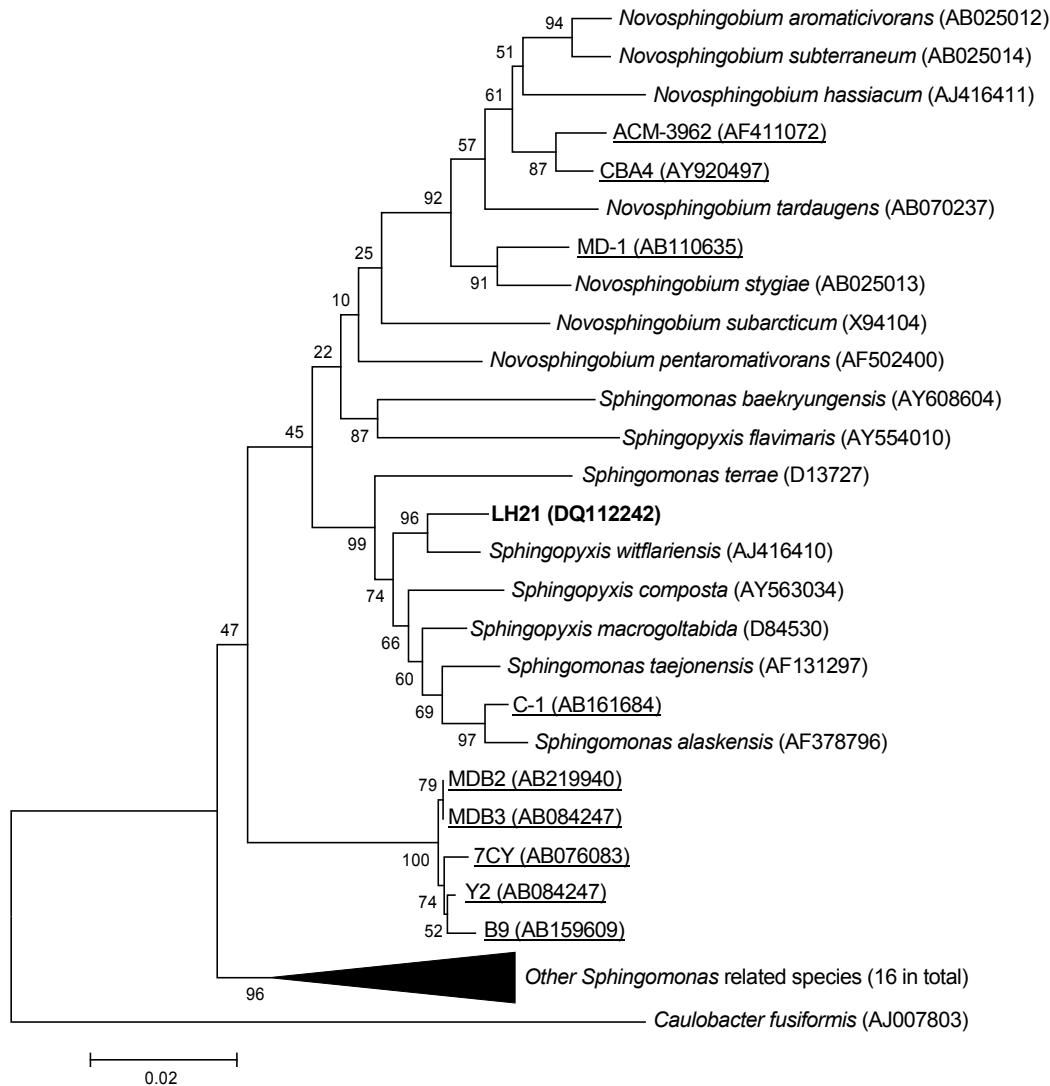


of the previously reported microcystin-degrading bacteria to date, and close relatives, was then performed (Figure 10.1). This revealed isolate LH21 clustered strongly with *S. witflariensis* (96% bootstrap support), but of the previously described microcystin-degrading bacteria was most closely related to isolate C-1 (accession number AB161684). Recent investigations have revealed the isolation of many different microcystin-degrading bacteria, and phylogenetic analyses of these were also performed in this study (Figure 10.1). This revealed a distinct cluster of microcystin-degrading bacterial isolates including MDB2, MDB3, 7CY, Y2 and B9. These five microcystin-degrading bacteria were all isolated from different Japanese water bodies, such as Lake Suwa (7CY and Y2), Lake Tsukui (B9) and the Tenryu River (MDB2 and MDB3), where each water body frequently yields toxic *Microcystis* blooms.

The first reported microcystin-degrading bacterium, ACM-3962, which was isolated from the Murrumbidgee River in Australia, appears to cluster with CBA4, another microcystin-degrading bacterium recently isolated from the San Roque reservoir in Córdoba, Argentina. The remaining microcystin-degrading bacteria highlighted in Figure 10.1, including isolate LH21, cluster with other cultured organisms rather than known microcystin-degraders.

### 10.3.3 Microcystin degradation in batch experiments

Figure 10.2 shows the degradation of two microcystin variants by isolate LH21 in sterilised Myponga Reservoir water, at three different initial concentrations. No losses of MCLR and MCLA were evident in a parallel sterile Pyrex vessel containing inactivated (autoclaved) LH21 inoculum, confirming that the removals in the subsequent unsterilised vessels were through biological degradation processes. At the higher initial microcystin concentrations ([MCLR]  $\sim 25 \mu\text{g L}^{-1}$ ; [MCLA]  $\sim 30 \mu\text{g L}^{-1}$ ) a lag period of 10 d was evident prior to isolate LH21 initiating degradation of both variants, with complete removal observed by day 12. A shorter lag period of 2 d was evident using an initial microcystin concentration of  $10 \mu\text{g L}^{-1}$  (with complete removal by day 4), while no apparent lag period was observed using the lowest microcystin concentrations ([MCLR]  $\sim 3 \mu\text{g L}^{-1}$ ; [MCLA]  $\sim 5 \mu\text{g L}^{-1}$ ) with no microcystin detected by day 2. It should be noted that experiments for the three unsterilised vessels with different initial concentrations of microcystin were conducted using Myponga Reservoir water which was sampled at different times. Although some water quality parameters, namely pH, dissolved organic carbon (DOC), UV absorbance (at 254 nm), turbidity, total nitrogen, total hardness (as  $\text{CaCO}_3$ ), were consistent during the different sampling times (results not shown), other non-measured aqueous constituents may have affected the growth of isolate LH21 resulting in the differences in the lag periods prior to the degradation of the microcystins. Amor et al. (2001) determined that the presence of metals, such as cadmium, nickel and zinc inhibited the degradation of alkylbenzene. In this study, elevated levels of inhibitory substances, such as heavy metals, may have been present in Myponga Reservoir water when spiked with the higher initial concentrations of microcystins, resulting in the extended lag period prior to the onset of degradation. Nevertheless, once degradation commenced after the extended lag period, only 2 d was required for complete removal of both microcystins variants.

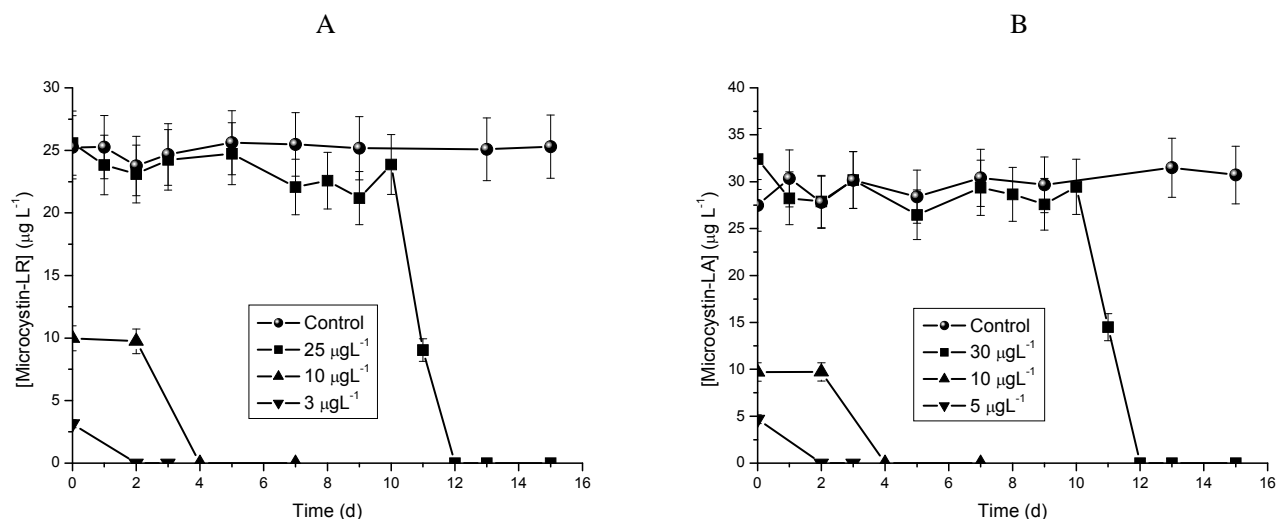


**Figure 10.1** Neighbour-joining phylogenetic tree showing the position of isolate LH21 (bold) in relation to other closely related species, including previously reported microcystin-degrading bacteria (underlined). Accession numbers correspond to partial sequences of 16S rRNA. Numerical tree values represent bootstrap support. Scale bar represents expected changes per site.

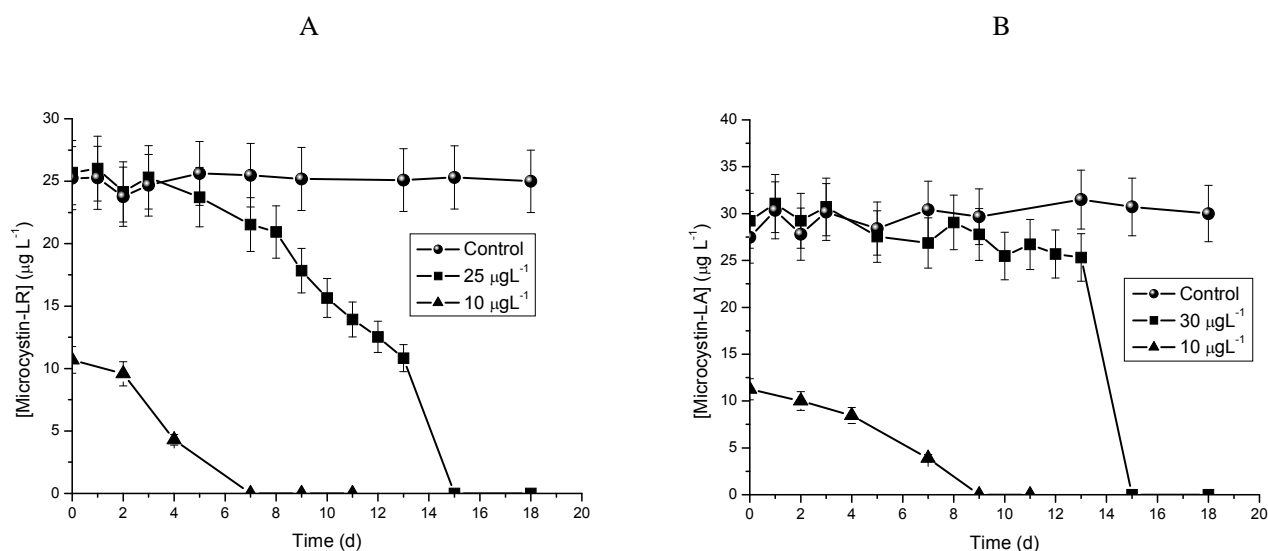
Parallel batch experiments were conducted using *Sphingomonas* ACM-3962 as the sole bacterial inoculum; however, only the high ([MCLR]  $\sim 25 \mu\text{g L}^{-1}$ ; [MCLA]  $\sim 30 \mu\text{g L}^{-1}$ ) and intermediate ([MCLR]  $\approx$  [MCLA]  $\sim 10 \mu\text{g L}^{-1}$ ) initial microcystin concentrations were evaluated (Figure 10.3). Once again, the higher initial microcystin concentrations resulted in an extended period prior to complete degradation when compared with the intermediate initial microcystin concentration (15 d compared with 7 d).

A few observations of note were apparent with the *Sphingomonas* ACM-3962 experiments in comparison with the LH21 experiments. The degradation pattern of MCLR (at the higher initial concentration) appeared to vary for the two organisms. Furthermore, isolate LH21 appeared to completely degrade both microcystin variants more rapidly than *Sphingomonas* ACM-3962 under equivalent conditions. The difference in degradation characteristics between the two organisms may be due to the different physiological state of both organisms when inoculated into the vessels. The transfer of the organisms from the nutrient rich media into Myponga Reservoir water may have had a distinct effect on bacterial metabolism resulting in the different degradation characteristics.

## BIOLOGICAL FILTRATION PROCESSES FOR THE REMOVAL OF ALGAL METABOLITES



**Figure 10.2** Batch degradation of microcystin variants: (A) MCLR and (B) MCLA, in Myponga Reservoir water using isolate LH21 as the sole bacterial inoculum. Error bars represent standard error from duplicate measurements.



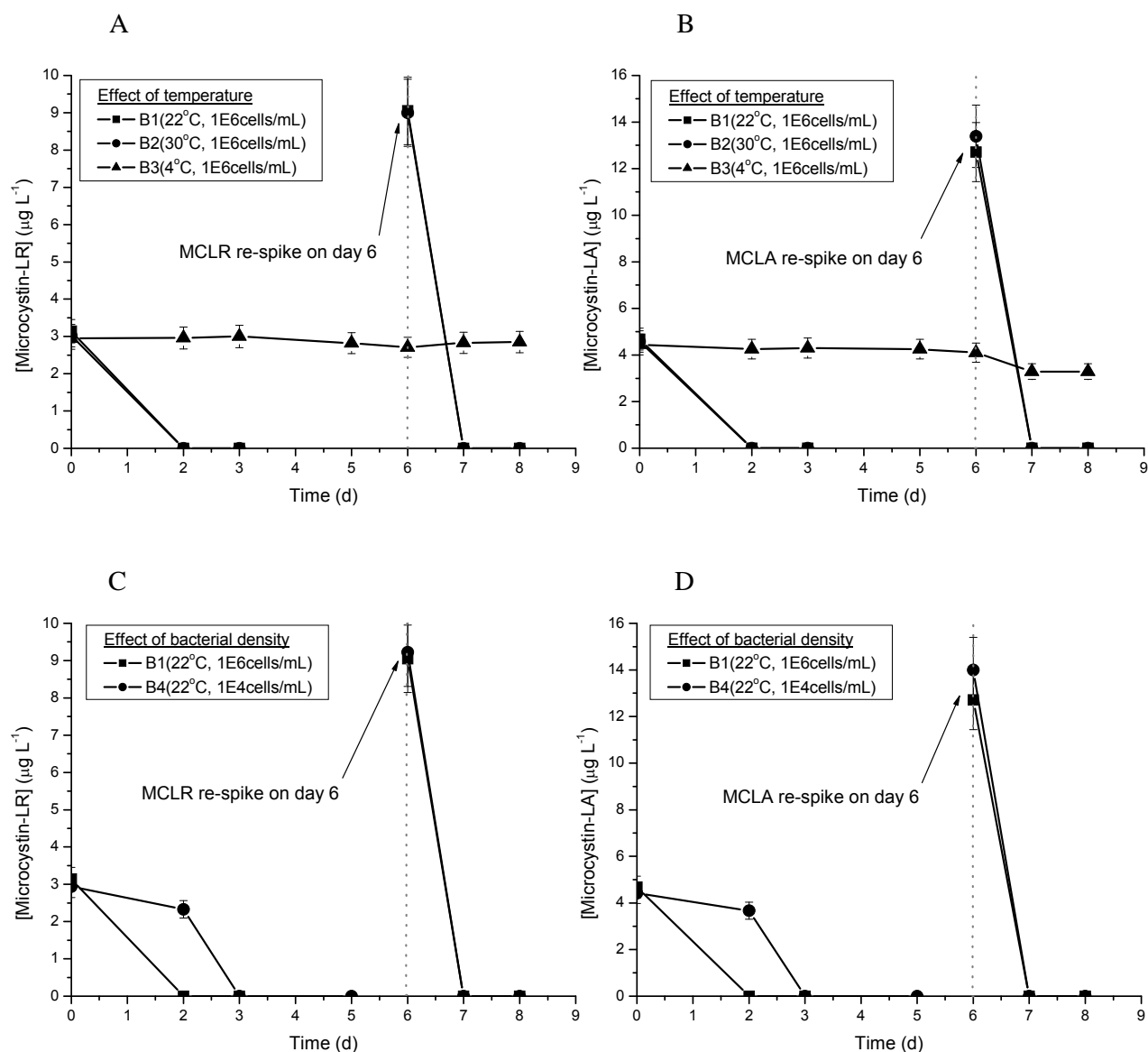
**Figure 10.3** Batch degradation of microcystin variants: (A) MCLR and (B) MCLA, in Myponga Reservoir water using *Spingomonas* ACM-3962 as the sole bacterial inoculum. Error bars represent standard error from duplicate measurements.

The disparity in MCLR and MCLA degradation patterns by *Spingomonas* ACM-3962 may be due to some form of substrate specificity, in particular, the absence of the arginine group in MCLA. Bourne et al. (1996, 2001) characterised the degradation of MCLR and determined that the enzyme encoded by the *mlrA* gene was the first enzyme to initiate microcystin degradation by cleaving the structure of MCLR at the arginine and Adda peptide bond. As the arginine group is replaced by the alanine group in MCLA, then it is possible that this may account for the discrepancy in the degradation pattern of both analogues by *Spingomonas* ACM-3962. Imanishi et al. (2005) also observed differences in the degradation of various microcystin variants using *Spingomonas* sp. B9. They showed that this bacterium was able to degrade MCLR using a similar mechanism to that of *Spingomonas* ACM-3962. In addition, the authors also suggested that strain B9 was only able to degrade variants which contained the arginine-Adda peptide bond, including nodularin which also contains this specific peptide bond. Interestingly, Jones et al. (1994) and Bourne et al. (1996) showed that *Spingomonas*

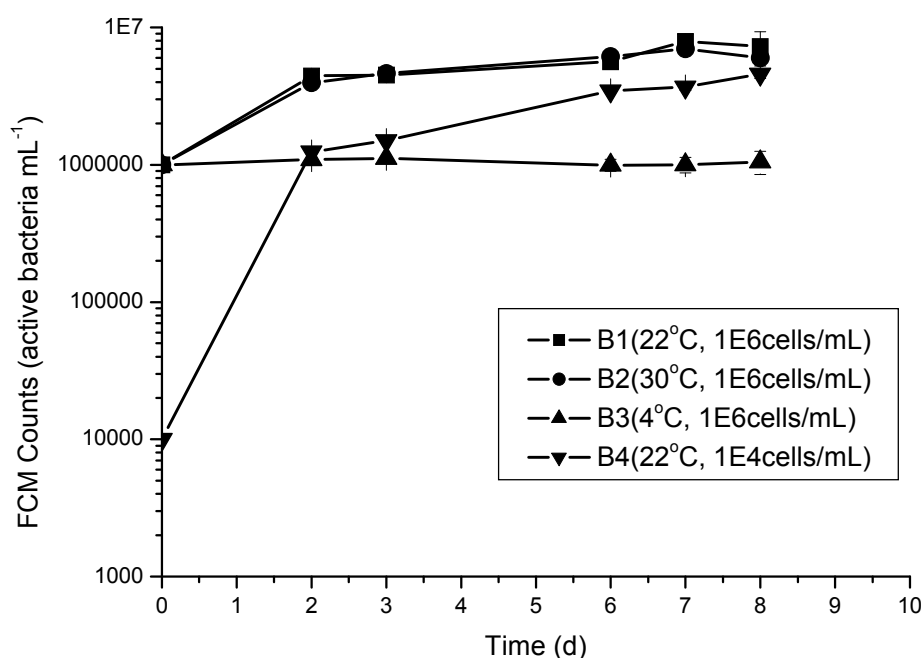
ACM-3962 was unable to degrade nodularin. Table 10.1 lists the documented microcystin-degrading bacteria and the microcystin variants (including nodularin) they are capable or incapable of degrading. The previous contention by Imanishi et al. (2005) that degradation of microcystin variants may be dependent upon the arginine-Adda peptide bond does not appear to be universal for all microcystin-degraders as documented in the case of strain 7CY (Ishii et al., 2004) which was able to degrade microcystin variants which did not contain this specific peptide bond, in particular, MCLW, MCLF and MCLY. Likewise in this study, isolate LH21 and *Sphingomonas* ACM-3962 were able to degrade MCLA which also does not contain the arginine-Adda peptide bond. It should be noted that this is the first report of bacteria being able to degrade the variant MCLA which has significant implications in Australia where MCLA, which is equal in its toxicity to MCLR, is commonly isolated from Australian *Microcystis* blooms. Further work is required to elucidate the differences in the degradation of various microcystin variants. This could involve extraction of the proteins from the different strains and examining their substrate specificity.

An additional set of batch experiments in Myponga Reservoir water, using isolate LH21 as the sole bacterial inoculum, was conducted to observe the effects of temperature and initial bacterial numbers on the degradation of MCLR and MCLA (Figure 10.4). B1, B2 and B3 represent Pyrex vessels which were incubated at 22, 30 and 4°C, respectively. All three vessels employed an initial LH21 concentration of  $1.0 \times 10^6$  active bacteria  $\text{mL}^{-1}$ . In contrast, isolate LH21 was inoculated into B4 at a concentration of  $1.0 \times 10^4$  active bacteria  $\text{mL}^{-1}$ , and this vessel was incubated at 22°C. No observable difference was discerned between B1 and B2 with complete degradation of both microcystin variants by day 2. In addition, no observable difference was apparent in the growth of isolate LH21 in B1 and B2 with numbers increasing to approximately  $4.4 \times 10^6$  active bacteria  $\text{mL}^{-1}$  and  $4.0 \times 10^6$  active bacteria  $\text{mL}^{-1}$  by day 2, respectively (Figure 10.5). An additional day was required for complete degradation of both microcystins in B4, indicating that degradation was dependent upon bacterial numbers. This was confirmed with the flow cytometric counts in B4 with no significant degradation observed until bacterial numbers reached approximately  $1.5 \times 10^6$  active bacteria  $\text{mL}^{-1}$ , lower than the numbers in B1, but the same order of magnitude. In contrast, no degradation was observed in B3 throughout the experiment, suggesting that the microcystin-degrading enzymes (and cell metabolism in general) were not active at the low temperature (4°C). Bacterial numbers remained consistent in B3, implying that isolate LH21 remained dormant during the experiment.

On day 6 MCLR and MCLA were re-spiked into B1, B2 and B4, each at a target concentration of  $10 \mu\text{g L}^{-1}$ . Consequently, each vessel had not been exposed to microcystin for at least 3 d. No microcystin was detected in these three vessels on the following day indicating that isolate LH21 had acclimatised to the microcystins. This suggests that once the genes have been previously activated and/or induced, then efficient production of the microcystin-degrading enzymes can proceed once re-exposed to the toxins, irrespective of the initial concentration of microcystin. Between days 3 and 6, bacterial numbers approximately doubled in B4 while an increase of approximately 20-25% was observed in B1 and B2; however, during the microcystin re-spike (between days 6 and 7) only slight increases were observed in the three vessels, with numbers plateauing by day 8.



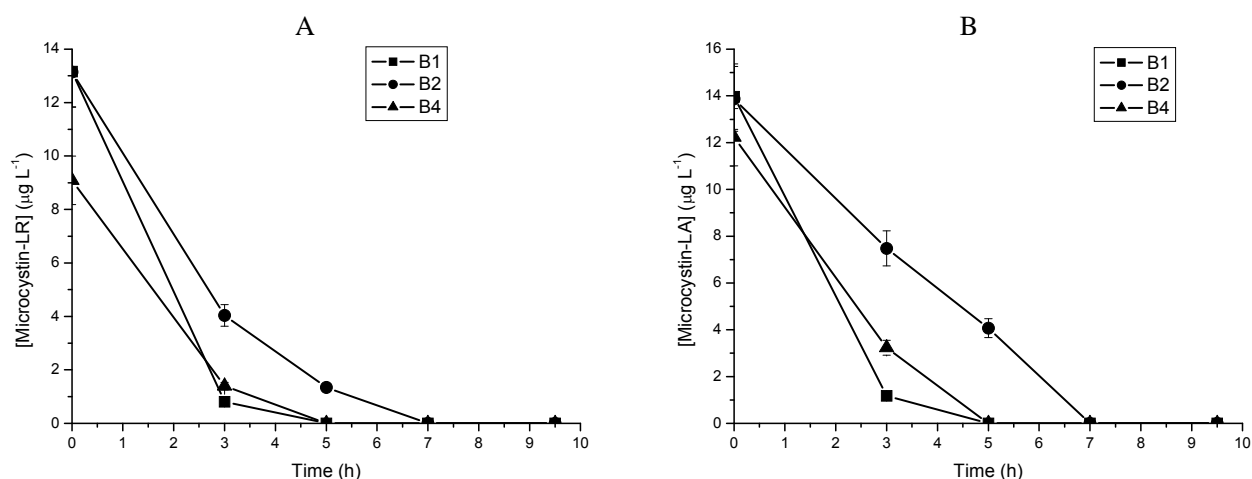
**Figure 10.4** Effect of temperature on the batch degradation of microcystin variants: (A) MCLR and (B) MCLA in Myponga Reservoir water. Effect of bacterial density on the batch degradation of microcystin variants: (C) MCLR and (D) MCLA in Myponga Reservoir water. Error bars represent standard error from duplicate measurements.



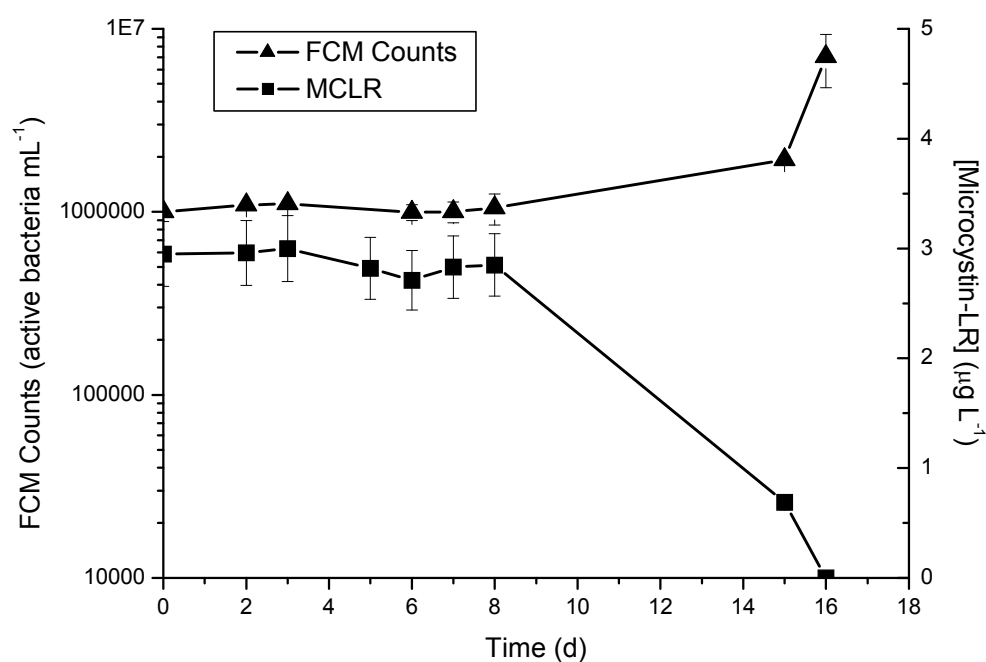
**Figure 10.5** Bacterial abundance (as determined by flow cytometry) of isolate LH21 during batch degradation of MCLR and MCLA in Myponga Reservoir water. Error bars represent standard error from duplicate measurements.

An identical re-spiking of MCLR and MCLA was conducted on day 15 in the same vessels, 7 d after the previous spiking of microcystin (Figure 10.6); however, in this spiking trial, microcystin levels were monitored at hourly intervals as opposed to daily intervals. Within 3 h microcystin degradation commenced in each vessel, with complete degradation by the 5th hour in B1 and B4. Interestingly, 7 h was required for complete degradation of MCLR and MCLA in B2, suggesting that the higher temperature may have reduced the rate of degradation. This was confirmed in the first-order rate constants for MCLR which were estimated to be  $1.0 \text{ h}^{-1}$  and  $0.4 \text{ h}^{-1}$  for B1 and B2, respectively; and for MCLA,  $0.9 \text{ h}^{-1}$  and  $0.3 \text{ h}^{-1}$  for B1 and B2, respectively. Bacterial numbers were not monitored during this final microcystin spiking trial.

On day 15 B3 was removed from the  $4^\circ\text{C}$  incubator and incubated at  $22^\circ\text{C}$ . Degradation of MCLR commenced immediately with no MCLR detected by day 16 (Figure 11.7). The degradation coincided with a significant increase in bacterial numbers with numbers reaching  $7.1 \times 10^6$  active bacteria  $\text{mL}^{-1}$  by day 16 (from  $1.1 \times 10^6$  active bacteria  $\text{mL}^{-1}$  on day 8). Similar trends were observed for MCLA (data not shown). This suggests that whilst cell numbers did not increase during incubation at  $4^\circ\text{C}$ , some form of gene expression may have occurred for the genes associated with microcystin degradation. It is therefore possible that the microcystin-degrading enzymes may not be active at this low temperature as degradation of both microcystin variants was observed immediately upon incubation at  $22^\circ\text{C}$ .



**Figure 10.6** Batch degradation of microcystin variants: (A) MCLR and (B) MCLA in Myponga Reservoir water using isolate LH21 as the sole bacterial inoculum on day 15. Error bars represent standard error from duplicate measurements.



**Figure 10.7** Active bacterial abundance and MCLR concentration during the batch degradation of MCLR in B3 using isolate LH21 as the sole bacterial inoculum. The temperature was increased to  $22^\circ\text{C}$  on day 15. Error bars represent standard error from duplicate measurements.

## 10.4 Conclusions

This is the first report of a microcystin-degrading bacterium, LH21, being isolated from a sand filter, providing further evidence that degradation of these toxins can indeed occur within biologically-active sand filters. Phylogenetic analysis of the 16S rRNA gene sequence of isolate LH21 revealed it to cluster most closely to *Sphingopyxis wittflariensis* and additional analyses were also performed to examine the relationship between each of the known microcystin-degrading bacteria reported to date.

In addition, isolate LH21 was shown to contain homologues to the four genes previously associated with the degradation of MCLR. Batch experiments using isolate LH21 as the sole bacterial inoculum demonstrated effective degradation of MCLR and MCLA under environmentally relevant conditions with complete removal observed within 5 h.

## 10.5 References

- Amor L., Kennes C. and Veiga M.C. (2001) Kinetics of inhibition in the biodegradation of monoaromatic hydrocarbons in the presence of heavy metals. *Bioresource Technology* **78**(2), 181-185.
- Bourne D.G., Jones G.J., Blakeley R.L., Jones A., Negri A.P. and Riddles P. (1996) Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR. *Applied & Environmental Microbiology* **62**(11), 4086-4094.
- Bourne D.G., Riddles P., Jones G.J., Smith W. and Blakely R.L. (2001) Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin LR. *Environmental Toxicology* **16**(6), 523-534.
- Harada K.-I., Imanishi S., Kato H., Masayoshi M., Ito E. and Tsuji K. (2004) Isolation of Adda from microcystin-LR by microbial degradation. *Toxicon* **44**(1), 107-109.
- Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.
- Hoefel D., Grooby W.L., Monis P.T., Andrews S. and Saint C.P. (2003) Enumeration of water-borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiological Methods* **55**(3), 585-597.
- Hoefel D., Monis P.T., Grooby W.L., Andrews S. and Saint C.P. (2005) Profiling bacterial survival through a water treatment process and subsequent distribution system. *Journal of Applied Microbiology* **99**(1), 175-186.
- Imanishi S., Kato H., Mizuno M., Tsuji K., and Harada K.-I. (2005) Bacterial degradation of microcystins and nodularin. *Chemical Research in Toxicology* **18**(3), 591-598.
- Ishii H., Nishijima M. and Abe T. (2004) Characterization of degradation process of cyanobacterial hepatotoxins by a Gram-negative aerobic bacterium. *Water Research* **38**(11), 2667-2676.
- Jones G.J., Bourne D.G., Blakeley R.L. and Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Natural Toxins* **2**(4), 228-235.
- Lane D.J. (1991) *16S/23S rRNA sequencing*. In: Stackebrandt, E., Goodfellow, M. (Eds.), *Nucleic acid techniques in bacterial systematics*, Academic Press, New York, USA, pp. 115-175.
- Maruyama T., Kato K., Yokoyama A., Tanaka T., Hiraishi A. and Park H.-D. (2003) Dynamics of microcystin-degrading bacteria in mucilage of *Microcystis*. *Microbial Ecology* **46**(2), 279-288.
- Maruyama T., Park H.-D., Ozawa K., Tanaka Y., Sumino T., Hamana K., Hiraishi A. and Kato K. (2006) *Sphingosinicella microcystinivorans* gen. nov., sp. nov., a microcystin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology* **56**(1), 85-89.
- Nicholson B.C., Rositano J. and Burch M.D. (1994) Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *Water Research* **28**(6), 1297-1303.
- Park H.-D., Sasaki Y., Maruyama T., Yanagisawa E., Hiraishi A. and Kato K. (2001) Degradation of cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. *Environmental Toxicology* **16**(4), 337-343.
- Saito T., Okana K., Park H.-D., Itayama T., Inamori Y., Neilan B.A., Burns B.P. and Sugiura N. (2003) Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes. *FEMS Microbiology Letters* **229**(2), 271-276.
- Saitou T., Sugiura N., Itayama T., Inamori Y. and Matsumura M. (2003) Degradation characteristics of microcystins isolated bacteria from Lake Kasumigaura. *Journal of Water Supply: Research and Technology - AQUA* **52**(1), 13-18.
- Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G. (1997) The CLUSTAL\_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**(24), 4876-4882.
- Valeria A.M., Ricardo E.J., Stephan P. and Alberto W.D. (2006) Degradation of microcystin-RR by *Sphingomonas* sp. CBA4 isolated from San Roque reservoir (Córdoba - Argentina). *Biodegradation* **17**(5), 447-455.



## CHAPTER 11: BACTERIAL DEGRADATION OF MICROCYSTIN TOXINS IN DRINKING WATER ELIMINATES THEIR TOXICITY\*

### 11.1 Introduction

Microcystins have been shown to be readily degraded in water by a range of aquatic organisms. Moreover, the majority of these microcystin-degrading organisms have been identified as Sphingomonads (Bourne et al., 1996, 2001; Park et al., 2001; Saito et al., 2003; Harada et al., 2004; Ishii et al., 2004; Ho et al., 2006; Valeria et al., 2006). However, before biological treatment can be considered a feasible option for effective removal of the microcystins, there is a need to ascertain if any toxic biodegradation by-products are generated.

Only a few studies have identified possible by-products from the biodegradation of microcystin-LR. Bourne et al. (1996) and Harada et al. (2004) identified two intermediate products from the bacterial degradation of microcystin-LR by *Sphingomonas* sp. ACM-3962 and *Sphingomonas* sp. B9, respectively. Both studies identified linearised microcystin-LR (NH<sub>2</sub>-Adda-Glu-Mdha-Ala-Leu-MeAsp-Arg-OH) and a tetrapeptide (NH<sub>2</sub>-Adda-Glu-Mdha-Ala-OH) as the intermediate products, with Harada et al. (2004) also isolating Adda as one of the final degradation products. The authors determined that both these intermediate products were less active than the parent microcystin-LR using protein phosphatase inhibition and mouse bioassays. However, to date no studies have assessed the cytotoxicity of the biodegradation products of microcystin. Furthermore, only the biodegradation by-products of one microcystin variant (microcystin-LR) have been assessed for toxicity.

The major objective of this study was to determine whether the bacterial degradation of two microcystin variants, microcystin-LR and -LA, generated any cytotoxic by-products. Furthermore, this study aimed at correlating the cytotoxicity data with that of a protein phosphatase inhibition assay and high performance liquid chromatography (HPLC), thereby allowing for the cytotoxicity assay to be used as a possible method of microcystin detection.

### 11.2 Experimental procedures

*Sphingopyxis* sp. LH21, a microcystin-degrading bacterium recently isolated and characterised by Ho et al. (2007), is capable of effectively degrading microcystin-LR (MCLR) and -LA (MCLA) (at  $\mu\text{g L}^{-1}$  levels) and contains homologues to four genes, *mlrA*, *mlrB*, *mlrC* and *mlrD* previously shown to be involved in the degradation of microcystin (Bourne et al., 1996, 2001). This bacterium was inoculated into a 1 L glass bioreactor (BR1) containing 1 L of sterilised Happy Valley Reservoir water (South Australia) at a concentration of  $1.3 \times 10^7$  active bacteria  $\text{mL}^{-1}$ . The water was collected after the coagulation step in the water treatment process and sterilised by filtration (0.2  $\mu\text{m}$ ) and autoclaving (121°C for 20 min) prior to bacterial inoculation. The number of active bacteria was enumerated using a FACSCalibur flow cytometer (Becton Dickinson, USA) following staining of the bacteria with the BacLight bacterial viability kit (Molecular Probes Inc., USA), as described previously (Hoefel et al., 2003). MCLR and MCLA (both semi-purified from a natural bloom of *Microcystis aeruginosa*) were spiked into BR1 at target concentrations of 2  $\text{mg L}^{-1}$  and 3  $\text{mg L}^{-1}$ , respectively. A second bioreactor (BR2) was prepared in a similar fashion without the bacterial inoculum to assess for any losses of microcystin due to factors other than biodegradation. Each bioreactor was incubated aerobically at 25°C with constant stirring. Samples were taken aseptically from each bioreactor at regular intervals for HPLC and toxicity analyses.

Prior to HPLC analyses, microcystins were concentrated from sample waters by C18 solid phase extraction according to the methods described by Nicholson et al. (1994). A HPLC system consisting of a 600 pump controller, 717plus autosampler and 996 photodiode array detector (Waters Pty Ltd.,

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\* This chapter is based on the following manuscript:

Ho L., Gaudieud A.-L., Fanok S., Newcombe G. and Humpage A.R. (2007) Bacterial degradation of microcystin toxins in drinking water eliminates their toxicity. *Toxicon* **50**(3), 438-441.

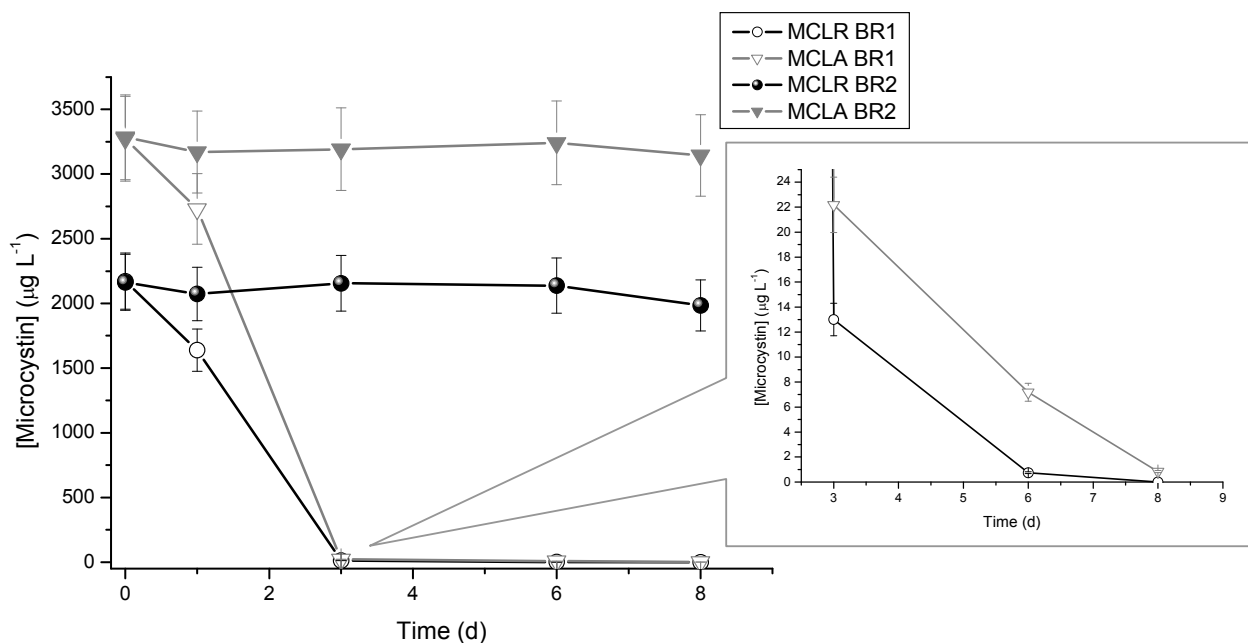
Australia) was employed using methods described by Ho et al. (2006). A multi-assay approach was selected for toxicity assessment to offset bias often experienced with single toxicity tests, particularly as UV absorbance and protein phosphatase 2A (PP2A) inhibitory activity both rely on the same factor, that is, an intact Adda moiety. A number of studies have coupled the PP2A assay with the mouse bioassay to assess toxicity (Bourne et al., 1996; Harada et al., 2004; Brooke et al., 2006), but the mouse bioassay cannot provide the sensitivity of a cell-based cytotoxicity assay. Inhibition of serine/threonine PP2A (Promega Corporation, Australia) was determined using *p*-nitrophenol phosphate as the substrate. Full details of this method have been described previously (Heresztyn and Nicholson, 2001). The cytotoxicity evaluation was based on the procedure described in AS/NZS 4020:2005 (Standards Australia, 2005; Fanok et al., 2007), with modifications. Ten-fold concentrated culture medium (Dulbecco's Modified Eagle's Medium) was diluted 1:9 in the water extracts. The VERO cell line (African green monkey kidney, ATCC # CCL-81) was seeded into 96-well microtitre plates at 5000 cells per well and grown in medium for 7 d, after which the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine remaining cell viability (Standards Australia, 2005; Fanok et al., 2007). The detection limit of the assay was determined to be 0.5  $\mu\text{M}$  for total microcystin.

### 11.3 Results and discussion

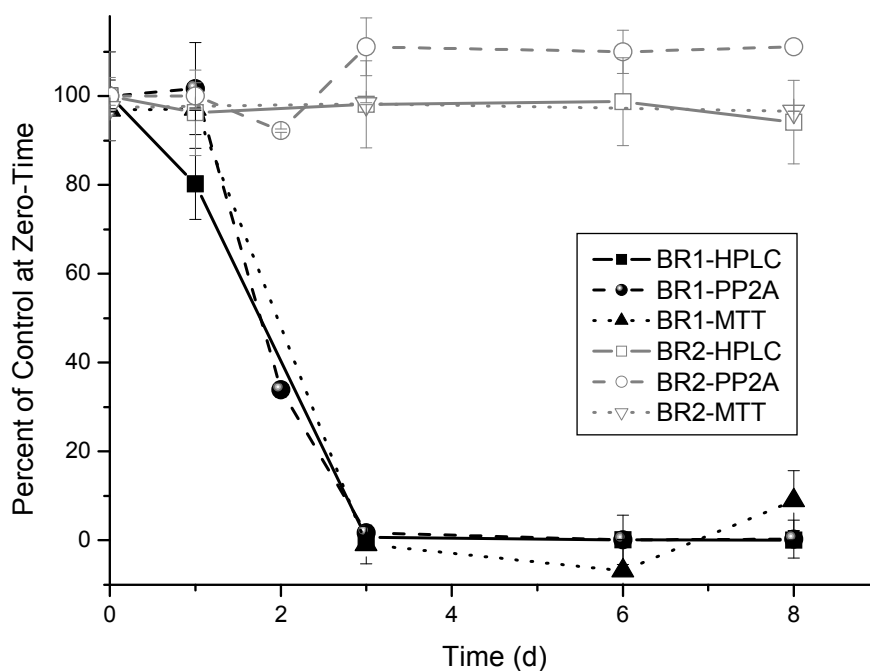
Figure 11.1 shows the biodegradation of MCLR and MCLA by *Sphingopyxis* sp. LH21 in treated Happy Valley Reservoir water. Biodegradation of both microcystin variants commenced immediately with reductions of 17% for MCLR and 25% for MCLA by the first day. On day 3, 99% reduction in both variants was evident; however, this corresponded to remaining concentrations of 13 and 22  $\mu\text{g L}^{-1}$  for MCLR and MCLA, respectively. It wasn't until day 8 when both variants were removed to below the WHO guideline value of 1  $\mu\text{g L}^{-1}$  (see inset of Figure 11.1). No losses of the toxins were evident in a parallel bioreactor containing no bacterial inoculum (BR2), providing strong evidence that removal of the toxins in BR1 was through biodegradation processes.

Samples were also taken from BR1 and BR2 at regular intervals for PP2A and cytotoxicity analyses. Figure 11.2 shows results of the PP2A and cytotoxicity assays compared with the HPLC data (sum of MCLR and MCLA) for BR1 and BR2. In each case, results were presented as percent of control at zero-time. In BR2, microcystin levels remained constant throughout the experiment, as detected by HPLC. This was confirmed with the PP2A and cytotoxicity assays where toxicity levels also remained constant.

In BR1, the PP2A and cytotoxicity results were in good agreement with the HPLC results. The decrease in toxicity, in parallel with the decrease in microcystin concentrations, indicates that no cytotoxic by-products were generated from the biodegradation of MCLR and MCLA by *Sphingopyxis* sp. LH21. Although this study did not attempt to isolate and identify any by-products, it is hypothesised that *Sphingopyxis* sp. LH21 degraded microcystin via a similar pathway to that proposed by Bourne et al (1996, 2001), yielding similar by-products since it has previously been shown to contain similar genes associated in the degradation of microcystin (Ho et al., 2007). This is the first study which has correlated HPLC, PP2A and VERO cytotoxicity data for the detection of microcystin toxins and their biodegradation by-products.



**Figure 11.1** Microcystin-LR and -LA (MCLR and MCLA) concentrations as a function of time in bioreactors BR1 and BR2. Inset: Zoom of BR1 data at a lower y-axis scale.



**Figure 11.2** Comparison of microcystin detection by high performance liquid chromatography (HPLC), protein phosphatase 2A (PP2A) and cytotoxicity (MTT) assays. Results presented as percent of control at zero-time. Error bars represent standard deviation from triplicate analyses.

## 11.4 Conclusions

The fact that no cytotoxic by-products of MCLR and MCLA biodegradation were detected demonstrates the feasibility of biodegradation as a possible removal option for the microcystins. In particular, biological filters may be an attractive water treatment option as most water treatment plants employ filters of some kind. Furthermore, this study demonstrates that the bacterium, *Sphingopyxis* sp. LH21, was insensitive to extremely high concentrations ( $\text{mg L}^{-1}$ ) of these toxins. Whilst it is uncommon to find extracellular (dissolved) microcystins at these concentrations in water bodies, these levels can occur within the cells of microcystin-producing cyanobacteria and could be released locally within filters when cells lyse.

## 11.5 References

- Bourne D.G., Jones G.J., Blakeley R.L., Jones A., Negri A.P. and Riddles P. (1996) Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR. *Applied & Environmental Microbiology* **62**(11), 4086-4094.
- Bourne D.G., Riddles P., Jones G.J., Smith W. and Blakeley R.L. (2001) Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin LR. *Environmental Toxicology* **16**(6), 523-534.
- Brooke S., Newcombe G., Nicholson B. and Klass G. (2006) Decrease in toxicity of microcystins LA and LR in drinking water by ozonation. *Toxicon* **48**(8), 1054-1059.
- Fanok S., Frosio S. and Humpage A. (2007) Improved cytotoxicity testing of products in contact with drinking water. *Water* **34**(5), 55-58.
- Harada K.-I., Imanishi S., Kato H., Masayoshi M., Ito E. and Tsuji K. (2004) Isolation of Adda from microcystin-LR by microbial degradation. *Toxicon* **44**(1), 107-109.
- Heresztyn T. and Nicholson B.C. (2001) Determination of cyanobacterial hepatotoxins directly in water using a protein phosphatase inhibition assay. *Water Research* **35**(13), 3049-3056.
- Ho L., Hoefel D., Saint C.P. and Newcombe G. (2007) Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. *Water Research* **41**(20), 4685-4695.
- Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.
- Hoefel D., Grooby W.L., Monis P.T., Andrews S. and Saint C.P. (2003) Enumeration of water-borne bacteria using viability assays and flow cytometry: A comparison to culture-based techniques. *Journal of Microbiological Methods* **55**(3), 585-597.
- Ishii H., Nishijima M. and Abe T. (2004) Characterization of degradation process of cyanobacterial hepatotoxins by a Gram-negative aerobic bacterium. *Water Research* **38**(11), 2667-2676.
- Nicholson B.C., Rositano J. and Burch M.D. (1994) Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *Water Research* **28**(6), 1297-1303.
- Park H.-D., Sasaki Y., Maruyama T., Yanagisawa E., Hiraishi A. and Kato K. (2001) Degradation of cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. *Environmental Toxicology* **16**(4), 337-343.
- Saito T., Okana K., Park H.-D., Itayama T., Inamori Y., Neilan B.A., Burns B.P. and Sugiura N. (2003) Detection and sequencing of the microcystin LR-degrading gene, *mlrA*, from new bacteria isolated from Japanese lakes. *FEMS Microbiology Letters* **229**(2), 271-276.
- Standards Australia (2005) *Testing of products for use in contact with drinking water*. Australian/New Zealand Standard AS/NZS 4020:2005, pp. 42-50.
- Valeria A.M., Ricardo E.J., Stephan P. and Alberto W.D. (2006) Degradation of microcystin-RR by *Sphingomonas* sp. CBA4 isolated from San Roque reservoir (Córdoba - Argentina). *Biodegradation* **17**(5), 447-455.

## CHAPTER 12: BIOLOGICAL FILTRATION PROCESSES FOR THE REMOVAL OF CYLINDROSPERMOPSIN\*

### 12.1 Introduction

In drinking water treatment, biologically-active filters are operated for the dual purpose of particle removal and removal of biodegradable soluble organic materials. Slow sand filtration technology is one of the earliest forms of potable water treatment and remains an important process for water purification throughout the world. Although it often has been replaced by faster and more advanced high-rate filtration methods, its low cost, ease of operation, minimal maintenance requirements, and success in removing pathogenic microorganisms make slow sand filtration an attractive option for rural communities and developing nations. Biological filtration systems for the removal of cyanobacterial toxins are increasingly important in the water industry as they incorporate natural degradation principles and do not have the potential to produce chemical disinfection by-products. Many studies have been conducted on biological degradation of cyanobacterial toxins in natural lakes and reservoirs (Jones and Orr, 1994; Rapala et al., 1994; Cousins et al., 1996; Christoffersen et al., 2002). Only few studies have demonstrated degradation of cyanobacterial toxins in biologically-active sand filters. Earlier experiments carried out by Smith (2005) have reported that a biologically-active filtration plant (BAFP) at North Pine Dam (NPD) was successful in removing both *Cylindrospermopsis raciborskii* cells and soluble cylindrospermopsin (CYN). Results of that study also demonstrated that the high elimination efficiency of soluble CYN was attributed to biological action, and not by adsorption of CYN into the abiotic matrix of the filtration plant. This study was conducted to provide information on the efficiency of the BAFP to eliminate CYN at NPD over a 12 month period.

### 12.2 Materials and methods

#### 12.2.1 Study site – North Pine Dam

This research project was undertaken at the BAFP at NPD in Brisbane, Queensland, Australia. The dam was constructed in 1974 by Queensland Department of Natural Resources and Mines and it is owned and managed by the South East Queensland Water Corporation. The average depth of the dam is 10 metres and its storage capacity is 215,000 megalitres. The water is treated by Brisbane City Council and distributed to the northern areas of Brisbane City, Parts of Pine Rivers Shire and Redcliffe City.

The filtration plant was originally designed and constructed by Queensland Department of Natural Resources and Mines and donated to National Research Centre for Environmental Toxicology (EnTox) for this study. The filtration plant consisted of 3 parts including a reservoir holding tank to which water was pumped from NPD, a roughing filter (RF) and a sand filter (SF). The RF was 5 m x 0.5 m and 1 m in depth, and consisted of filter aggregates (predominantly gravel) that reduced in size; approximately 20 mm, 10mm, and 5 mm (Figures 12.1 and 12.2). The SF was 1.6 m x 1.6 m and 2.4 m in depth and consisted of sand with an effective size between 0.25-0.35 mm. The water was pumped from NPD to the reservoir holding tank and passed through the RF horizontally and then through the SF. The water flowed through the filtration plant at 8 L h<sup>-1</sup> with a contact time of approximately 6 h. The plant was running for a period of approximately 12 months before sampling began. It was presumed that the CYN-degrading bacteria would colonise within the filters during that time.

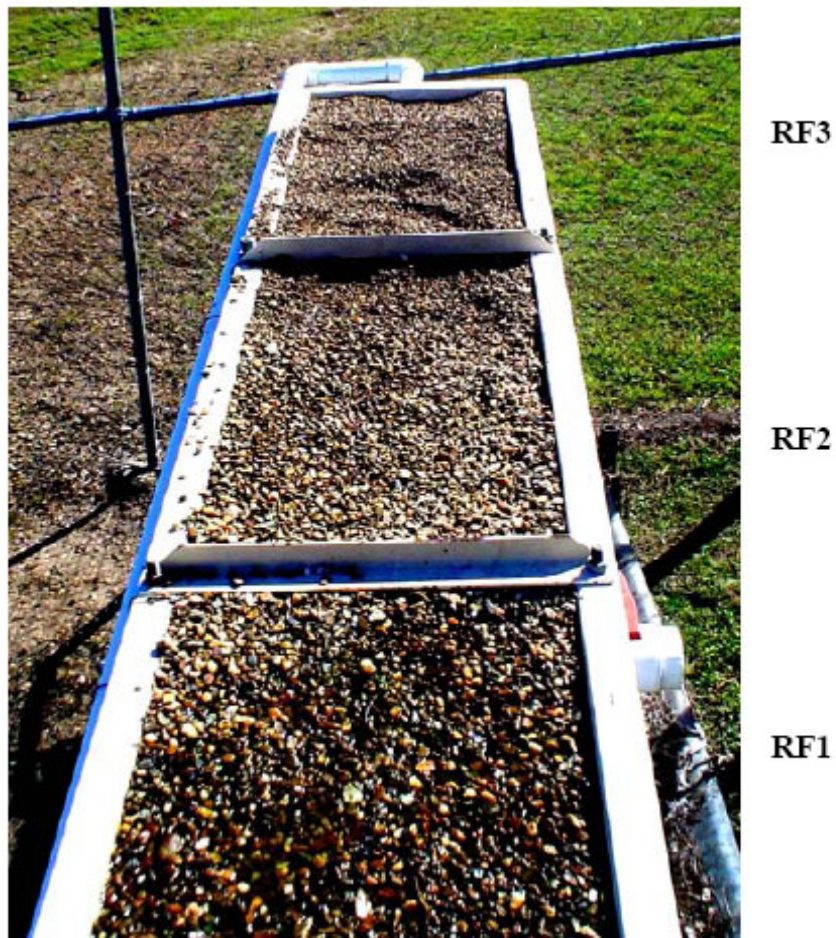
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\* This chapter is based on a component of the following PhD thesis:

Wijesundara S. (2008) *Biological filtration processes for removal of the cyanobacterial toxin, cylindrospermopsin*. PhD thesis. University of Queensland, Queensland, Australia.



**Figure 12.1** Biologically-active filtration plant (BAFP) at North Pine Dam, Queensland showing reservoir holding tank, roughing filter (RF) and sand filter (SF).



**Figure 12.2** Aggregates (3 different sizes, RF1-20mm, RF2-10mm, RF3-5mm) located in horizontal roughing filter.



### 12.2.2 Biofiltration of *C. raciborskii* cells and CYN

Water samples were taken at the inlet point of the pilot plant (just prior to the RF) and at the outlet point for *C. raciborskii* cell and soluble CYN determinations. The outlet samples were taken approximately 6 h after the inlet sample as it was estimated it would take this period of time for the water to pass through the filters. The water samples were collected in 250 mL detergent washed plastic bottles.

Samples for *C. raciborskii* cell determination and enumeration were preserved with acidified Lugol's solution (1mL of acidified Lugol's solution per 100 mL of sample) prior to analysis. *C. raciborskii* cells were identified and enumerated in Sedgwick Rafter chambers under a phase contrast microscope (100x and 400x magnification).

Soluble (dissolved) CYN was measured by high performance liquid chromatography (HPLC) coupled to a tandem mass spectrometer (MS/MS). Prior to analysis, samples were filtered through 0.22 µm sterile filters. The HPLC/MS/MS consisted of an LC-200 series pump, series 200 auto sampler and API 300 MS/MS with turbo ion spray interface. An Altima C18 (250 x 4.6 mm, 5 µm) column at 40°C was used. Chromatography consisted of a linear gradient from 1 to 60% over 5 min, with a final isocratic stage holding at 60% methanol for 1 min. Total flow rate was 1.1 mL min<sup>-1</sup>. The mobile phase was buffered to 5 mM with ammonium acetate. Post column splitting was used to submit only 20% of the column effluent to the MS/MS interface. The transition from the M+H ion (416 m/z) to the 194 m/z fragment was monitored for quantification using the multiple reactant monitoring (NRM) modes. Analytical standards were prepared by using methanol extracted lyophilized *C. raciborskii* cultures (Eaglesham et al., 1999). CYN analysis was performed by Geoff Eaglesham, QHSS, Queensland, Australia.

### 12.2.3 Batch biodegradation of CYN

Aggregate samples from the RF and the SF were aseptically collected at monthly intervals in order to determine if the biofilm-associated organisms within the filters possessed the ability to biodegrade CYN under laboratory conditions. Sterile Milli Q water (50 mL) was added separately to 3 different sizes of aggregates from the RF (approximately 20 mm, 10 mm and 5 mm) and to sand from the SF. The samples were shaken to remove attached bacteria; the supernatants were transferred to 50 mL sterile containers and spiked with *C. raciborskii* cell-free extract at a concentration of 50 µg L<sup>-1</sup> CYN. Control experiments were prepared using the sterilised aggregates following the same method. The samples were incubated at 30°C in the dark. At regular intervals a sub-sample (1 mL) of each culture was filtered through a 0.22 µm sterile filter and the CYN concentration was determined using HPLC/MS/MS as described above.

## 12.3 Results and discussion

### 12.3.1 Biofiltration of *C. raciborskii* cells

The ability of the BAFP to remove cells was assessed over a 12 month period (Figure 12.3). *C. raciborskii* numbers in the inlet samples varied between 2,210 to 41,898 cells mL<sup>-1</sup>, with the highest cell count detected during July 2005. Throughout the trial no whole or fragmented *C. raciborskii* cells were detected in the effluents of the RF and SF indicating that the BAFP was able to eliminate *C. raciborskii* cells when operated at a flow rate of 8 L h<sup>-1</sup> with a contact time of approximately 6 h. It is presumed that removal of the cells was attributed to physical straining through the media. Hijnen et al. (2004) showed that slow sand filters were efficient in removing organisms larger than viruses with removal shown to be predominantly through physical straining of the organisms. Furthermore, biological processes occurring in the upper layers of sand filters (schmutzdecke) have been shown to aid in the entrapment of larger organisms (Huisman and Wood, 1974; Wotton, 2002).

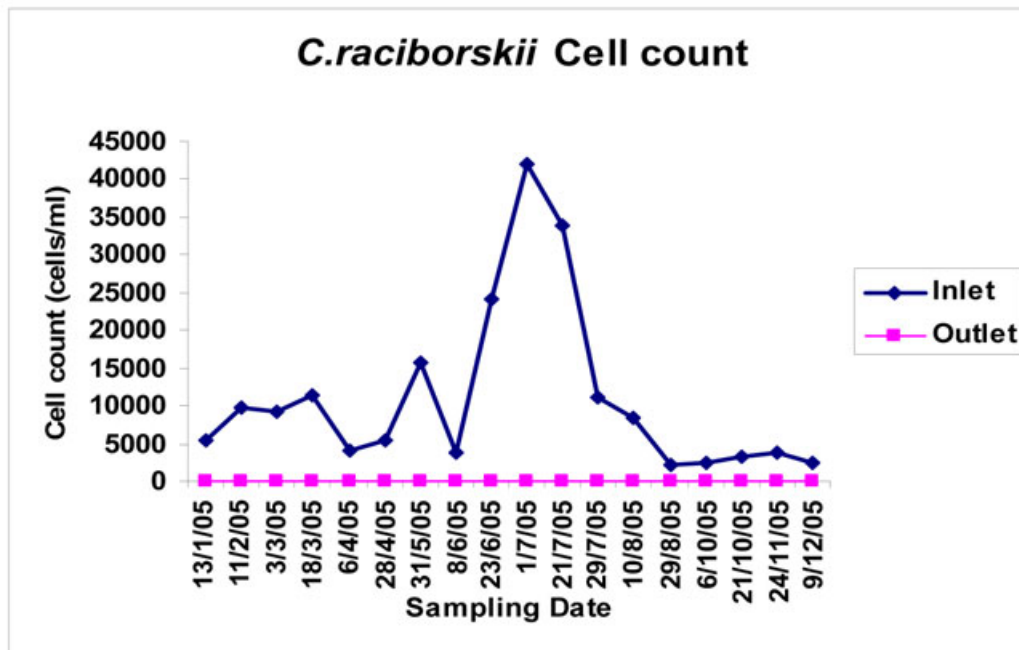


Figure 12.3 Monitoring of *C. raciborskii* cells through the BAFP.

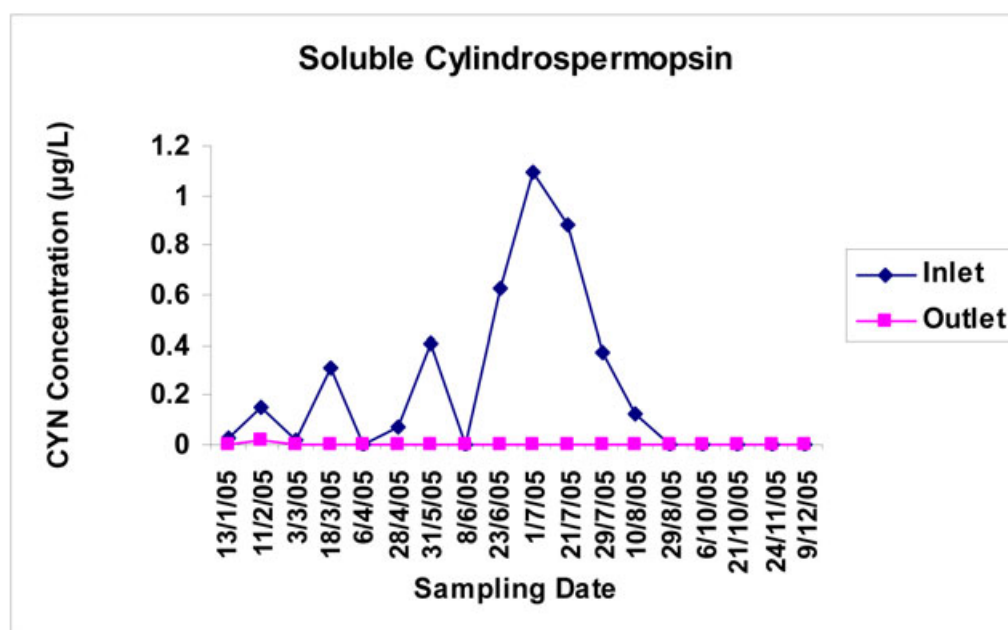
### 12.3.2 Biofiltration of dissolved CYN

The levels of dissolved CYN in the BAFP inlet water samples ranged from 0 to  $1.1 \mu\text{g L}^{-1}$ . The highest CYN concentration was detected during July 2005, which coincided with the highest *C. raciborskii* cell numbers in Figure 12.3. In fact, similar trends were apparent between the *C. raciborskii* cell numbers and CYN concentration during the 12 month period. As shown in Figure 12.4, dissolved CYN was completely removed through the BAFP during the 12 month study. Smith (2005) observed similar CYN removals at the BAFP in a previous study. In that study, total and dissolved CYN was completely removed through the RF and SF. Smith (2005) showed CYN removal through the BAFP was primarily through biodegradation processes rather than physical processes, such as adsorption to sand and/or aggregates.

Other studies have also demonstrated that biodegradation was the major removal mechanism for the removal of another cyanotoxin, microcystin, through biologically-active sand filters. Ho et al. (2006) showed complete removal of two microcystin variants, microcystin-LR and -LA, was primarily through biodegradation rather than any physical processes. Similarly, Miller et al. (2001) also observed very little adsorption of microcystin toxins to sandy material with effective elimination of the toxins attributed to biodegradation in batch experiments. Lahti and Hiisvirta (1989) found up to 86% removal of microcystin was achieved in a pilot-scale slow sand filter with removal determined to be predominantly through biodegradation. Grützmacher et al. (2002) also showed biodegradation of microcystin occurred in their slow sand filters with negligible adsorption occurring through these filters.

Although an intensive experiment was not conducted in this study to determine the possibilities of adsorption/absorption of CYN by the filter matrix, batch experiments were conducted to ascertain whether extracted organisms from within the biofilm of the BAFP were capable of effectively removing CYN (see section 12.3.3). Results from this will provide evidence that CYN removal through the BAFP was through biological processes rather than abiotic processes.



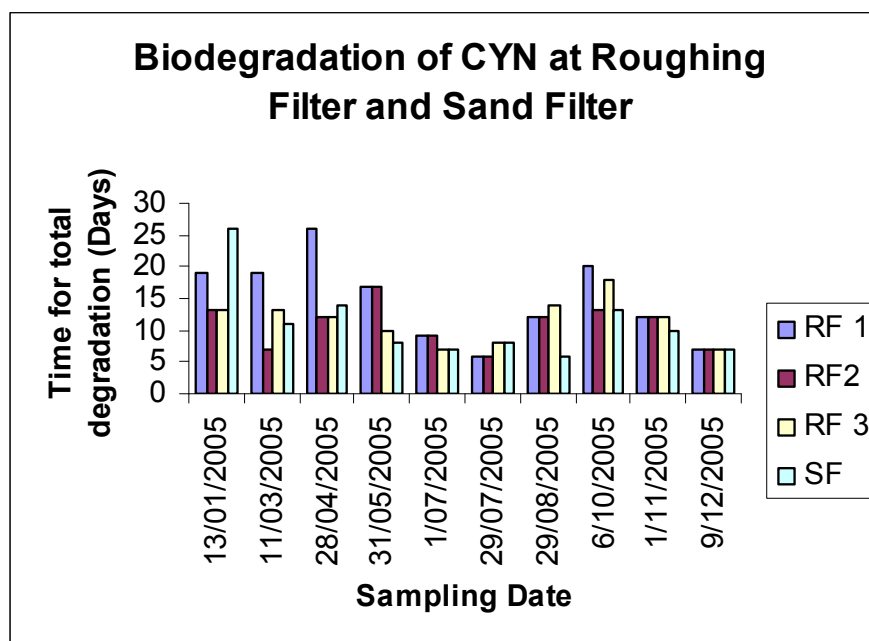


**Figure 12.4** Monitoring of dissolved CYN through the BAFP.

### 12.3.3 Batch biodegradation of CYN

Batch CYN biodegradation experiments were conducted using extracted biofilm from the RF and SF aggregates as the inocula. Complete biodegradation of CYN (initial concentration of  $50 \mu\text{g L}^{-1}$ ) was observed in each monthly batch of aggregate samples taken from the RF and SF throughout the year (Figure 12.5). These results suggest that the complete biodegradation of CYN at the BAFP was not affected by seasonal/temporal variations. Furthermore, the results indicate that the organisms responsible for CYN degradation were ubiquitous within the BAFP as degradation was observed in samples taken from RF1, RF2, RF3 and SF. The CYN concentration in the sterilised control samples remained relatively consistent even after 30 d of the experiment, providing strong evidence that the removal of CYN in the non-sterilised samples was through biodegradation processes.

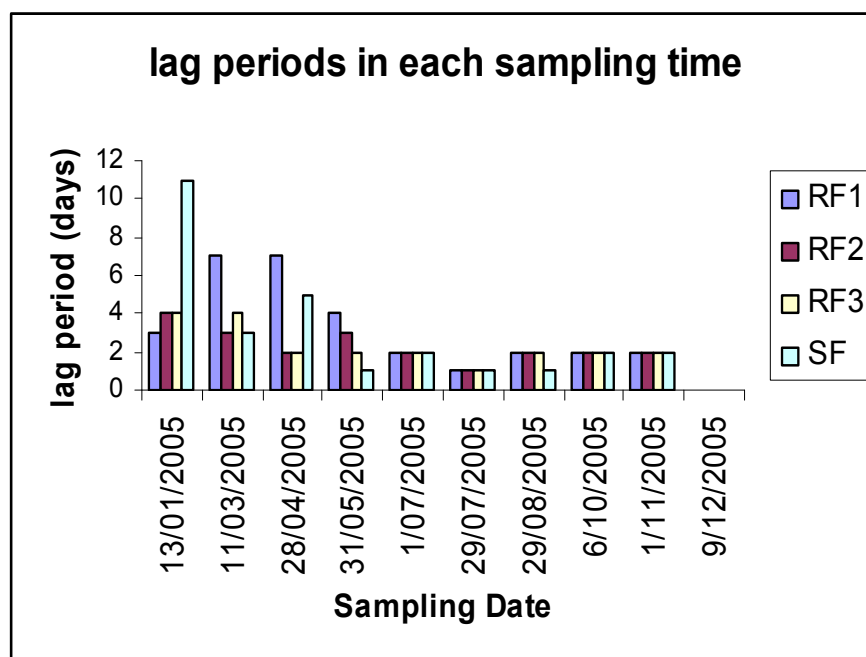
The time taken for complete degradation of CYN varied between 6 to 26 d during the 12 month period, similar timeframes to Smith (2005) who demonstrated that CYN, at an initial concentration  $120 \mu\text{g L}^{-1}$ , was completely degraded within 14-20 d at the same BAFP. In this current study the most rapid degradation of CYN was observed in July and December. Interestingly the highest CYN concentration in NPD was evident in July during the study period. Therefore, it is possible that the high concentration of CYN during this period could have stimulated the activity of CYN-degrading organisms within NPD and subsequently the BAFP. Smith et al. (2008) suggested that the rate of CYN degradation was dependent upon the initial concentration of CYN, with more rapid degradation observed with higher CYN concentration. The authors also hypothesised that this dependence on CYN concentration may imply that CYN acts as an inducer, that is, a molecule which activates the genes which synthesise the enzymes involved in the degradation process. It is unclear why rapid CYN degradation was also observed in December, since the CYN concentration was low in this period. However, other factors, such as temperature and the numbers of CYN-degrading organisms, have also been documented to impact the rate of CYN degradation (Smith et al., 2008).



**Figure 12.5** The time taken for complete biodegradation of CYN over a 12 month period in laboratory batch experiments using inocula sourced from the BAFP.

Acclimation (lag) periods were observed during the study prior to biodegradation of CYN commencing. When comparing the length of the lag period in each month, it was found that the length of the lag period of the later months of the study period was shorter than at the initial months (Figure 12.6). The longer lag periods in the initial stages of the study may be due to the time required for CYN-degrading organisms to establish and disperse within the aggregates of the BAFP. It is also possible that this microbial population may not have attained a critical biomass required within the aggregates at early stages of the study period. Studies also suggest that the lag period may be due to the length of time required for the enzymes responsible for degradation to be induced (Torstensson et al., 1975; Stephenson et al., 1984). Other possible reasons for the lag period include an insufficient supply of inorganic compounds, the preferential assimilation of other organic compounds before the target compound of interest, or the time required for acclimatisation to -or removal of -inhibitors present in the environment (Vashon et al., 1982; Kuiper and Hanstveit, 1984; Lewis et al., 1986).

In December, CYN degradation was very rapid and no recorded lag period was observed. The reduction and elimination of the lag period observed in this study are consistent with other studies. Smith et al. (2008) reported a lag period reduction from 6 d to <2 d when CYN had been respiked into an induced sample from NPD. Similar decreases in lag periods have also been reported for the degradation of microcystin toxins (Jones et al., 1994; Rapala et al., 1994; Christoffersen et al., 2002; Holst et al., 2003; Ho et al., 2006, 2007b) and other micropollutants, such as *p*-nitrophenol (Spain et al., 1980; Wiggins et al., 1987). These results suggest that biodegradation of CYN may commence more rapidly in water bodies that have frequent exposure to CYN-producing cyanobacterial blooms.



**Figure 12.6** Acclimation (lag) periods prior to the biodegradation of CYN commencing over a 12 month period in laboratory batch experiments using inocula sourced from the BAFP.

## 12.4 Conclusions

This study showed efficient removal of soluble CYN and CYN-producing *C. raciborskii* cells in NPD water through the RF and SF of the BAFP over a 12 month period, suggesting that the removal was independent of seasonal variations. Biodegradation was shown to be the predominant removal mechanism of CYN through the BAFP as biofilm-associated organisms, extracted from the RF and SF, were able to completely degrade CYN in batch experiments.

During these batch experiments lag periods were observed prior to complete degradation of CYN, and these were found to decrease with time, with no lag period observed at the end of the trial. This suggested that the organisms responsible for CYN degradation may have required an initial period of time to establish and reach a critical biomass to initiate CYN degradation. However, once exposed to CYN, these organisms were able to degrade CYN more rapidly.

For a better understanding of the mechanism of CYN degradation, more in-depth experiments are required, including identification of the CYN-degrading organism(s). This will be discussed in the next chapter.

## 12.5 References

- Christoffersen K., Lyck S. and Winding A. (2002) Microbial activity and bacterial community structure during degradation of microcystins. *Aquatic Microbial Ecology* **27**(2), 125-136.
- Cousins I.T., Bealing D.J., James H.A. and Sutton A. (1996) Biodegradation of microcystin-LR by indigenous mixed bacterial populations. *Water Research* **30**(2), 481-485.
- Eaglesham G.K., Norris R.L., Shaw G.R., Smith M.J., Chiswell R.K., Davis B.C., Neville G.R., Seawright A.A. and Moore M.R. (1999) Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. *Environmental Toxicology* **14**(1), 151-154.
- Grützmacher G., Böttcher G., Chorus I. and Bartel H. (2002) Removal of microcystins by slow sand filtration. *Environmental Toxicology* **17**(4), 386-394.
- Hijnen W.A.M., Schijven J.F., Bonne P., Visser A. and Medema G.J. (2004) Elimination of viruses, bacteria and protozoan oocysts by slow sand filtration. *Water Science & Technology* **50**(1), 147-154.

- Ho L., Hoefel D., Saint C.P. and Newcombe G. (2007) Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. *Water Research* **41**(20), 4685-4695.
- Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.
- Holst T., Jørgensen N.O.G., Jørgensen C. and Johansen A. (2003) Degradation of microcystin in sediments at oxic and anoxic, denitrifying conditions. *Water Research* **37**(19), 4748-4760.
- Huisman L. and Wood W. E. (1974) *Slow sand filtration*. Geneva: World Health Organisation, pp. 20-46.
- Jones G.J., Bourne D.G., Blakeley R.L. and Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Natural Toxins* **2**(4), 228-235.
- Jones G.J. and Orr P.T. (1994) Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Research* **28**(4), 871-876.
- Kuiper J. and Hanstveit A.O. (1984) Fate and effects of 4-chlorophenol and 2,4-dichlorophenol in marine plankton communities in experimental enclosures. *Ecotoxicology and Environmental Safety* **8**(1), 15-33.
- Lahti K. and Hiisvirta (1989) Removal of cyanobacterial toxins in water treatment processes: Review of studies conducted in Finland. *Water Supply* **7**(4), 149-154.
- Lewis D.L., Kollig H.P. and Hodson R.E. (1986) Nutrient limitation and adaptation of microbial populations to chemical transformations. *Applied & Environmental Microbiology* **51**(3), 598-603.
- Miller M.J., Critchley M.M., Hutson J. and Fallowfield H.J. (2001) The adsorption of cyanobacterial hepatotoxins from water onto soil during batch experiments. *Water Research* **35**(6), 1461-1468.
- Rapala J., Lahti K., Sivonen K. and Niemälä S.I. (1994) Biodegradability and adsorption on lake sediments of cyanobacterial hepatotoxins and anatoxin-a. *Letters in Applied Microbiology* **19**(6), 423-428.
- Smith M. (2005) *Biodegradation of the cyanotoxin cylindrospermopsin*. PhD thesis, University of Queensland, Brisbane, Queensland, Australia.
- Smith M.J., Shaw G.R., Eaglesham G.K., Ho L. and Brookes J.D. (2008) Elucidating the factors influencing the biodegradation of cylindrospermopsin in drinking water sources. *Environmental Toxicology* **23**(3), 413-421.
- Spain J.C., Prichard P.H. and Bourquin A.W. (1980) Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. *Applied & Environmental Microbiology* **40**(4), 726-734.
- Stephenson T., Lester J.N. and Perry R. (1984) Acclimatisation to nitrilotriacetic acid in the activated sludge process. *Chemosphere* **13**(9), 1033-1040.
- Torstensson N.T.L., Stark J. and Goransson B. (1975) The effect of repeated applications of 2,4-D and MCPA on their breakdown in soil. *Weed Research* **15**(3), 159-164.
- Vashon R.D., Jones W.J. and Payne A.G. (1982) The effect of water hardness on nitrilotriacetate removal and microbial acclimation in activated sludge. *Water Research* **16**(9), 1429-1432.
- Wiggins B.A., Jones S.H. and Alexander M. (1987) Explanations for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. *Applied & Environmental Microbiology* **53**(4), 791-796.
- Wotton R.S. (2002) Water purification using sand. *Hydrobiologia* **469**(1-3), 193-201.

## CHAPTER 13: ISOLATION AND IDENTIFICATION OF A CYLINDROSPERMOPSIN-DEGRADING BACTERIUM FROM A BIOLOGICAL FILTRATION PLANT\*

### 13.1 Introduction

Much of the information about the genetics, metabolism and physiology of microorganisms has been derived from pure culture techniques under well-defined conditions. In the environment, a range of organisms are present and the effects on the growth and metabolism of an organism under environmentally-relevant conditions are difficult to study as the results are often confounded by the growth of other competing organisms (Alexander, 1994). Pure cultures are often used to study such effects and then relate these findings to explain the changes in the population of mixed cultures. Additionally, pure cultures are often used for physiological studies such as biodegradation pathways or enzyme inductions. Enrichment culture techniques are often followed by isolation of pure cultures of particular microorganisms.

A common procedure that relies on the ability of microorganisms to use organic compounds as source of carbon and energy for growth is known as the enrichment culture technique (Alexander, 1994). The enrichment culture technique has been the basis for the isolation of pure cultures of microorganisms that are able to utilise a large range of organic molecules as an energy source. Once a microbial community has been enriched, its component members can be isolated in pure cultures by traditional agar plate technique. Microscopic examination of both the community and the isolates, together with observation (microscopic and plate growth) of a community reconstructed from the pure isolates, can help considerably in ensuring that all the community members have been isolated (Parkes, 1982). Accurate identification of bacterial isolates is an essential task for applying biodegrading organisms in industries. Traditional phenotypic identification methods are sometimes followed by interpretation of test results (Stager and Davis, 1992). Phylogenetic analysis such as 16S rRNA based molecular identification could achieve correct identification for reasons including its universal distribution among bacteria and the presence of species-species variable regions (Weisburg et al., 1991). These methods have been used to identify cyanobacterial toxin-degrading organisms. In particular, microcystin-degrading bacteria have been identified as *Pseudomonas* species and *Sphingomonas* species by utilising molecular techniques (Jones et al., 1994; Takenaka and Watanabe, 1997; Park et al., 2001; Saitou et al., 2003; Ishii et al., 2004; Tsuji et al., 2006; Valeria et al., 2006; Ho et al., 2007). To date, only one bacterium has been isolated and shown to degrade cylindrospermopsin (CYN) and this was shown to belong to the genus *Delftia* (Smith, 2005).

The aim of this study was to isolate and identify organisms capable of CYN biodegradation at the biologically-active filtration plant (BAFP) at North Pine Dam.

### 13.2 Materials and methods

#### 13.2.1 Enrichment and isolation of CYN-degrading bacteria

A gravel sample from the BAFP was selected for isolation of CYN-degrading organisms. Jaworski's (JW) medium (50 mL) was inoculated with the organisms and this culture was spiked with 50  $\mu\text{g L}^{-1}$  CYN (cell free extract). The sample was incubated at 30°C in the dark. At regular intervals a sub sample (1 mL) of each culture was filtered through a 0.22  $\mu\text{m}$  sterile filter and the CYN concentration was determined using HPLC/MS/MS (as described in Chapter 13). Once the CYN concentration reached below the detection limit, the entire medium was centrifuged; supernatant was decanted and

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\* This chapter is based on a component of the following PhD thesis:

Wijesundara S. (2008) *Biological filtration processes for removal of the cyanobacterial toxin, cylindrospermopsin*. PhD thesis. University of Queensland, Queensland, Australia.

the bacterial pellet was washed three times with sterile JW medium. The washed pellet was re-suspended in 50 mL of sterile JW medium containing CYN cell free extract ( $50 \mu\text{g L}^{-1}$ ) and incubated  $30^\circ\text{C}$  in the dark. At regular intervals CYN concentration was determined using HPLC/MS/MS. This procedure was repeated four more consecutive sub-cultures to ensure that the enrichment of bacteria capable of degrading CYN occurred. On the fourth enrichment an aliquot was streaked onto solidified JW medium containing CYN cell free extract at  $50 \mu\text{g L}^{-1}$  and incubated  $30^\circ\text{C}$  in the dark. Single colonies from these plates were transferred back into sterile JW medium (50 mL) containing CYN cell free extract ( $50 \mu\text{g L}^{-1}$ ) and incubated at  $30^\circ\text{C}$  in the dark. At regular intervals an aliquot was taken from this culture and CYN degradation was monitored to ensure CYN-degrading bacteria were isolated. A control sample containing sterile JW medium spiked with CYN cell free extract at  $50 \mu\text{g L}^{-1}$  was also conducted.

### 13.2.2. Phenotypic identification of CYN-degrading bacteria

#### ***Wet mount motility test (Hanging drop method)***

A drop of bacterial isolate was placed in the middle of a clean cover glass. A thin layer of petroleum jelly was applied around the cover glass and a depression slide was turned upside down (depressed area facing down) and touched the cover glass. The prepared slide was viewed using light microscopy ( $\times 100$  magnification).

#### ***Gram stain***

A thin smear of bacterial suspension was made on a microscope slide and heat fixed. Crystal violet (1 mL) was dispensed onto the fixed smear, followed by 1 mL of iodine solution. The smear was then decolourised with 95% ethanol, counter stained with safranin and viewed using a light microscope ( $\times 1000$  magnification).

#### ***Catalase test***

Catalase is an extra cellular enzyme which catalyses the breakdown of hydrogen peroxide to water and oxygen. A portion of bacterial growth from a nutrient agar plate is added to a few drops of 5% hydrogen peroxide on a glass microscope slide. The evolution of gas is considered as a positive reaction.

#### ***Oxidase test***

The oxidase test determines the ability to oxidise aromatic amines to colour products. A portion of bacterial growth was smeared onto a filter paper that has been moistened with freshly prepared 1% aqueous *p*-aminodimethylaniline oxalate and 1%  $\alpha$ -naphthol in ethanol. A deep blue colour indicated a positive reaction.

### 13.2.2 Genotypic identification of CYN-degrading bacteria

#### ***DNA extraction***

The cell density was determined by measuring the absorbance in the bacterial cultures in order to determine the suitable cell density for DNA extraction. Extraction was carried out using DNeasy kit (QIAGEN, Australia) according to the manufacturer's instructions. DNA yield was determined by measuring the concentration of DNA in the sample by its absorbance at 260 nm.

#### ***16S rRNA amplification***

A polymerase chain reaction (PCR) assay was used to identify bacterial isolates by amplifying products from the 16S rRNA gene. Two oligonucleotide primers, 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTACGACT-3') (Sigma Genosys, Australia) were designed to amplify products from the 16S rRNA gene (Suzuki and Giovannoni, 1996). Each 50  $\mu\text{L}$  PCR reaction contained 2.5 U *Taq* polymerase (Invitrogen, Australia), 5  $\mu\text{L}$  of 10 x PCR buffer, 2.5mM of  $\text{MgCl}_2$ , 20 pmol of each primer, 400  $\mu\text{mol}$  of mix dNTPs and 13 ng of DNA template. PCR was performed with thermal cycling conditions of:  $95^\circ\text{C}$  for 10 min; 30 cycles of  $94^\circ\text{C}$  for 30 s,  $50^\circ\text{C}$  for 1 min and  $72^\circ\text{C}$  for 2 min; and  $72^\circ\text{C}$  for 5 min.

The amplified DNA products were run on a 1% (w/v) agarose gel in 1 x TBE buffer to visualise the PCR products. Ethidium bromide was incorporated in the gel when the gel was mounted.

Electrophoresis was conducted at 100 V for 1 h. The lengths of amplified products were estimated by comparison against a 1 kb DNA ladder. The gel was visualised under ultraviolet light and photographed.

### ***Purification of PCR products***

The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Australia) following the manufacturer's protocol.

### ***Sequencing of PCR products***

16S rRNA sequences were obtained using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., Australia) according to the manufacturer's instructions. The sequenced products were purified using DyeEx 2.0 Spin kit (QIAGEN, Australia) in order to remove unincorporated dye terminators directly from sequencing reactions. The purified PCR products were sent to DNA sequencing laboratory, School of Biomolecular and Biomedical Science, Griffith University, Nathan campus, Queensland for sequencing.

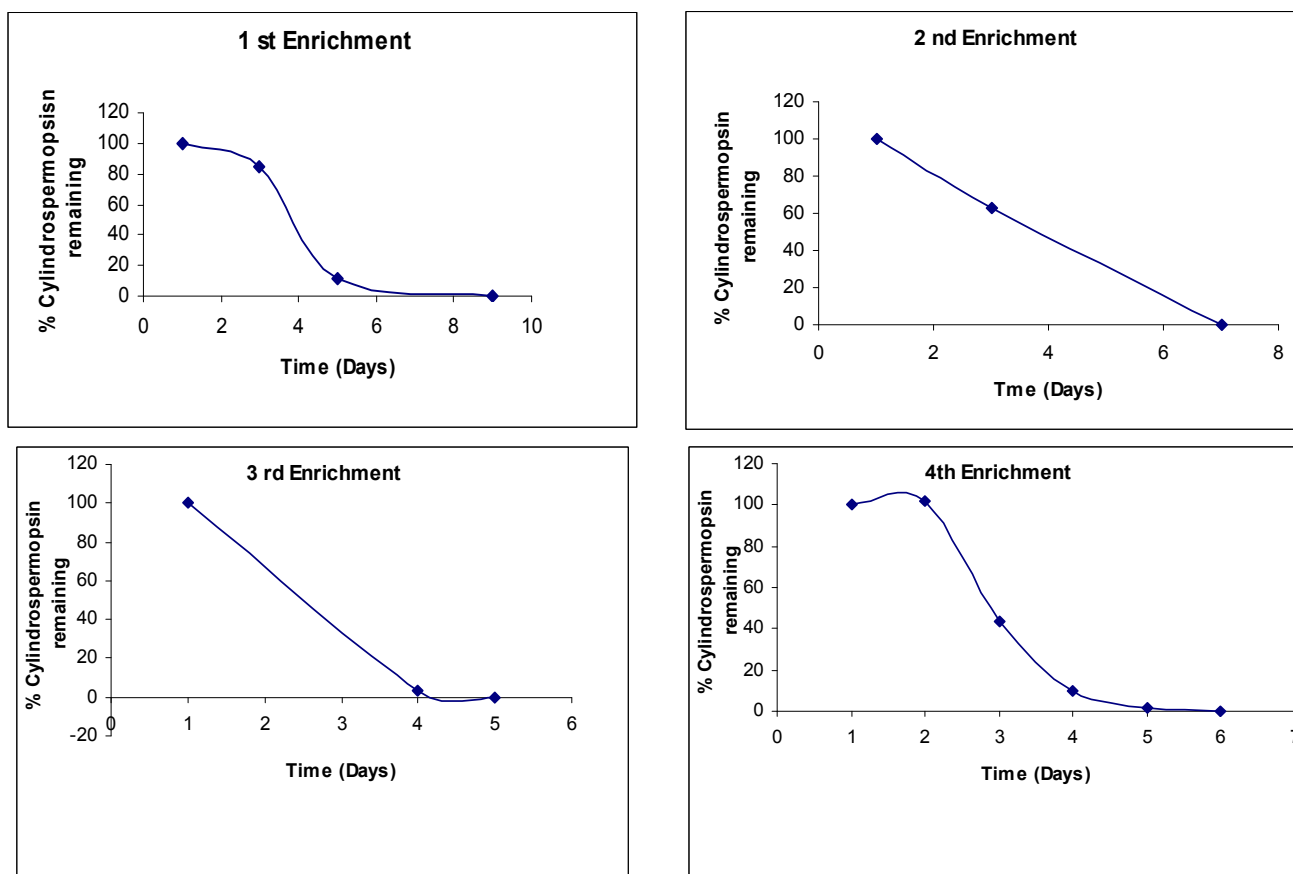
The sequencing results were analysed using Chromas version 2.31 software. This software automatically assembled the sequence data fragments in the correct linear fashion. The consensus sequence was then compared with a sequence database. This was achieved by using BLAST (available through the National Centre for Biotechnology Information) algorithm and querying the GenBank which was located on the following web site: <http://www.ncbi.nlm.nih.gov/BLAST/>.

## **13.3 Results and discussion**

### **13.3.1 Enrichment and isolation of CYN-degrading bacteria**

Conventional enrichment and isolation techniques are generally carried out under nutrient excess conditions which favour the selection of specific organisms with the most rapid and maximum specific growth rate (Slater and Lovatt, 1984). However, for samples obtained from environment, nutrients are traditionally low making it difficult to isolate specific organisms. In addition, the growth of other competing organisms can confound isolation processes. In this study, a consecutive enrichment procedure was employed to aid in the selection and subsequent isolation of CYN-degrading organisms from gravel samples obtained within the BAFP. Enrichment was performed four times as shown in Figure 13.1.

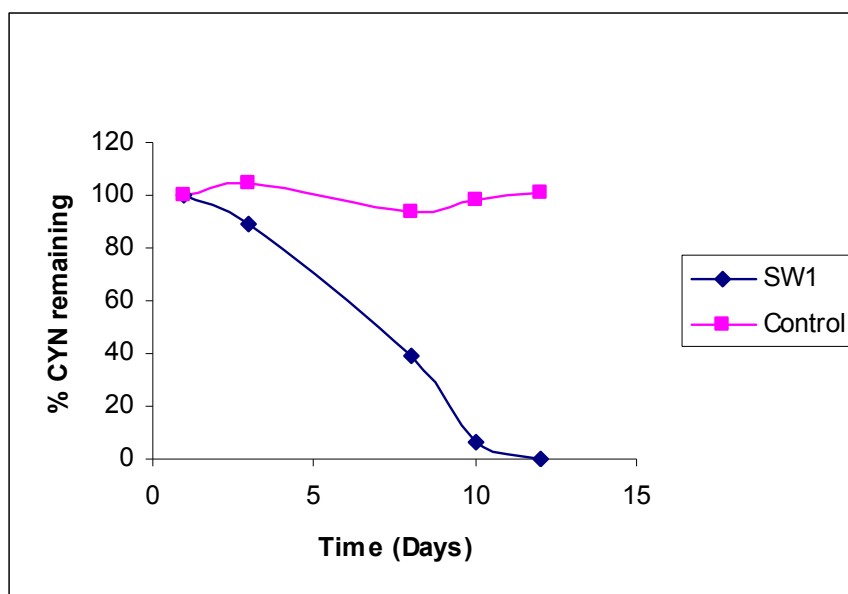
Noticeable CYN degradation began following 2 d of incubation, where the greatest degradation occurred between 2-4 d. A noticeable reduction of the time taken for complete CYN degradation was evident in each of the enrichment procedures, with the time decreasing from 9 d to approximately 5-6 d (Figure 13.1). This reduction in time for complete CYN degradation is consistent with the findings in Chapter 13 and with that of Smith (2005) who also showed a decrease in complete CYN degradation time when CYN was re-added to an induced sample.



**Figure 13.1** Enrichment of CYN-degrading organisms.

The final enrichment in this study also served for the isolation of small, slow-growing single colonies on solid JW medium. Individual colonies were placed into liquid JW medium spiked with  $50 \mu\text{g L}^{-1}$  CYN and CYN concentrations monitored at regular intervals. Only one isolate, designated as SW1, was able to degrade CYN (Figure 13.2). This isolate was able to completely degrade CYN within 12 d with no observable lag period. However, the time required for complete degradation of CYN by SW1 was longer than observed in the enrichment procedures (12 d compared with 5 d). It is unclear as to why this is the case, although it is possible that there may be synergistic interactions between SW1 and other bacteria present in the enrichment cultures which may have accelerated CYN degradation. Similar findings have been reported in the biodegradation of pesticides. Kumar et al. (2007) demonstrated that endosulfan was degraded by bacteria isolated from contaminated soils. However in that study, the degradation efficiencies of the individual pure cultures were far less when compared with those of the mixed culture. Similarly, Dejonghe et al. (2003) showed that linuron was more rapidly degraded in an enriched culture of bacteria when compared with a *Variovorax* sp. which was isolated from the same enriched culture.





**Figure 13.2** Degradation of CYN by bacterial isolate SW1.

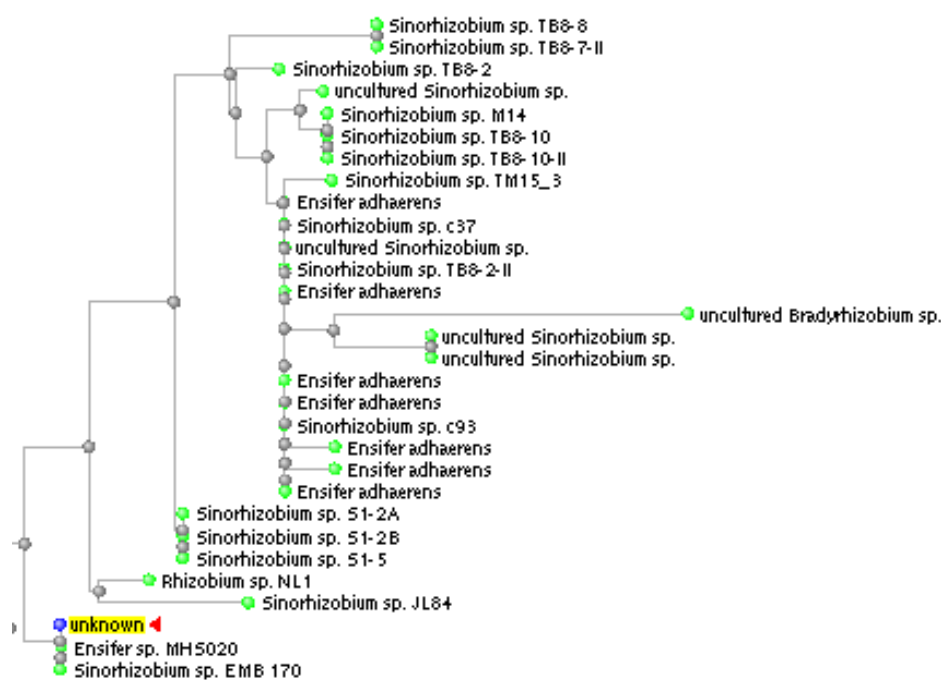
### 13.3.2. Identification of the CYN-degrading bacterium SW1

Based on phenotypic identification tests, isolate SW1 was determined to be a motile, Gram negative rod-shaped bacterium which was oxidase negative and catalase positive. The colonies of SW1 on solidified JW medium were white and mucoidal.

In order to obtain more definitive information on the taxonomic and phylogenetic position of isolate SW1, its 16S rRNA gene sequence was determined and compared with the sequences available in the ribosomal sequence database. Based on a fragment of the 16S rRNA gene, isolate SW1 was shown to be 99% similar to a *Sinorhizobium* sp. Figure 13.3 shows the neighbour-joining phylogenetic analysis of isolate SW1 in relation to other *Sinorhizobium* species based on 16S rRNA gene sequence.

This genus consists of soil bacteria that are capable of forming nitrogen-fixing nodules on leguminous plants (Chen et al. 1988). Because of their possible symbiosis with plants, rhizobial bacteria have been studied in relation to phytoremediation (Johnson et al. 2004). It is known that many species in *Rhizobiaceae* family including *Sinorhizobium* sp. have the ability to degrade various types of chemicals which are generally difficult to degrade, and can produce several specific enzymes adapted to them (Aitken et al., 1998). Bacteria in the family of *Rhizobiaceae* can be found in contaminated environments where various toxic chemicals are present (Ahmad et al., 1997). Therefore, it is possible that the genus *Sinorhizobium* is particularly widespread and may possess the enzymatic system necessary to degrade natural organic compounds such as CYN. A study by Keum et al. (2006) indicated that species of *Sinorhizobium* can utilise phenanthrene as a sole carbon source. Recent studies showed that several putative genes, which are usually found in polycyclic aromatic hydrocarbon degrading bacteria, are also present in *Sinorhizobium meliloti* (Ahmad et al., 1997). Furthermore, a recent study showed that some *Sinorhizobium* species have many genes encoding simple aromatic acid assimilation system (Galibert et al., 2001).

To date, only one bacterial isolate has been identified as a CYN-degrader and this isolate has been shown to be most closely related to a *Delftia* sp. (Smith, 2005). This CYN degrader was isolated from Hervey Bay retardation basin, which has had a history of toxic *C. raciborskii* blooms.



**Figure 13.3** Neighbour-joining phylogenetic tree showing the position of isolate SW1 in relation to other *Sinorhizobium* species based on 16S rRNA gene sequence.

## 13.4 Conclusions

The results of this study have several positive implications with respect to future development of biotechnological methods to eliminate CYN in drinking water. A bacterium was isolated from a biologically-active filtration plant and shown to be capable of effectively degrading CYN. Phylogenetic analysis, based on 16S rRNA gene sequence, revealed that the isolated CYN-degrading bacterium most likely belonged to the genus *Sinorhizobium*. This bacterial isolate may have a unique enzyme for the degradation of CYN; however, to advance the studies for CYN degradation, identification of the genes encoding the enzymes involved in the degradation of CYN is required. The understanding and application of the degradation mechanism and functions of these organisms will facilitate the solution of environmental problems caused by toxic *C. raciborskii* blooms.

## 13.5 References

- Ahmad D., Mehmannaavaz R. and Damaj M. (1997) Isolation and characterization of symbiotic N<sub>2</sub>-fixing *Rhizobium meliloti* from soils contaminated with aromatic and chloroaromatic hydrocarbons: PAHs and PCBs. *International Biodeterioration & Biodegradation* **39**(1), 33-43.
- Aitken M.D., Stringfellow W.T., Nagel R.D., Kazunga C. and Chen S.-H. (1998) Characteristics of phenanthrene-degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons. *Canadian Journal of Microbiology* **44**(8), 743-752.
- Alexander M. (1994) *Biodegradation and bioremediation*. Academic Press, San Diego, CA, USA.
- Chen W.X., Yan G.H. and Li J.L. (1988) Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *International Journal of Systematic Bacteriology* **38**(4), 392-397.
- Dejonghe W., Berteloot E., Goris J., Boon N., Crul K., Maertens S., Hofte M., De Vos P., Verstraete W. and Top E.M. (2003) Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovorax* strain. *Applied & Environmental Microbiology* **69**(3), 1532-1541.
- Galibert, F., Finan T.M., Long S.R., Puhler A., Abola P., Ampe F., Barloy-Hubler F., Barnett M.J., Becker A., Boistard P., Bothe G., Boutry M., Bowser L., Buhrmester J., Cadieu E., Capela D., Chain P., Cowie A., Davis R.W., Dreano S., Federspiel N.A., Fisher R.F., Gloux S., Godrie T., Goffeau A., Golding B., Gouzy J., Gurjal M., Hernandez-Lucas I., Hong A., Huizar L., Hyman R.W.,

- Jones T., Kahn D., Kahn M.L., Kalman S., Keating D.H., Kiss E., Komp C., Lalaure V., Masuy D., Palm C., Peck M.C., Pohl T.M., Portetelle D., Purnelle B., Ramsperger U., Surzycki R., Thebault P., Vandenbol M., Vorholter F.-J., Weidner S., Wells D.H., Wong K., Yeh K.-C. and Batut J. (2001) The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **293**(5530), 668-672.
- Ho L., Hoefel D., Saint C.P. and Newcombe G. (2007) Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. *Water Research* **41**(20), 4685-4695.
- Ishii H., Nishijima M. and Abe T. (2004) Characterization of degradation process of cyanobacterial hepatotoxins by a Gram-negative aerobic bacterium. *Water Research* **38**(11), 2667-2676.
- Johnson D.L., Maguire K.L., Anderson D.R. and McGrath S.P. (2004) Enhanced dissipation of chrysene in planted soil: the impact of a rhizobial inoculum. *Soil Biology and Biochemistry* **36**(1), 33-38.
- Jones G.J., Bourne D.G., Blakeley R.L. and Doelle H. (1994) Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Natural Toxins* **2**(4), 228-235.
- Keum Y.-S., Seo J.-S., Hu Y. and Li Q.X. (2006) Degradation pathways of phenanthrene by *Sinorhizobium* sp. C4. *Applied Microbiology & Biotechnology* **71**(6), 935-941.
- Kumar K., Devi S.S., Krishnamurthi K., Kanade G.S. and Chakrabarti T. (2007) Enrichment and isolation of endosulfan degrading and detoxifying bacteria. *Chemosphere* **68**(2), 317-322.
- Park H.-D., Sasaki Y., Maruyama T., Yanagisawa E., Hiraishi A. and Kato K. (2001) Degradation of cyanobacterial hepatotoxin microcystin by a new bacterium isolated from a hypertrophic lake. *Environmental Toxicology* **16**(4), 337-343.
- Parkes R. (1982) *Methods for enriching, isolating and analysing microbial communities in laboratory systems*. In *Microbial interactions and communities*. J.S.A. Bull (Ed.), Academic Press Inc., London, UK.
- Saitou T., Sugiura N., Itayama T., Inamori Y. and Matsumura M. (2003) Degradation characteristics of microcystins isolated bacteria from Lake Kasumigaura. *Journal of Water Supply: Research and Technology - AQUA* **52**(1), 13-18.
- Slater J.H. and Lovatt D. (1984) *Biodegradation and the significance of microbial communities*. In: Gibson D.T. (Ed.). *Microbial Degradation of Organic Compounds*. Marcel Dekker Inc., New York, USA.
- Smith M. (2005) *Biodegradation of the cyanotoxin cylindrospermopsin*. PhD thesis, University of Queensland, Brisbane, Queensland, Australia.
- Stager C.E. and Davis J.R. (1992) Automated systems for identification of microorganisms. *Clinical Microbiology Reviews* **5**(3), 302-327.
- Suzuki M.T. and Giovannoni S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied & Environmental Microbiology* **62**(2), 625-630.
- Takenaka S. and Watanabe M.F. (1997) Microcystin LR degradation by *Pseudomonas aeruginosa* alkaline protease. *Chemosphere* **34**(4), 749-757.
- Tsuji K., Asakawa M., Anzai Y., Sumino T. and Harada K. (2006) Degradation of microcystins using immobilized microorganism isolated in an eutrophic lake. *Chemosphere* **65**(1), 117-124.
- Valeria A.M., Ricardo E.J., Stephan P. and Alberto W.D. (2006) Degradation of microcystin-RR by *Sphingomonas* sp. CBA4 isolated from San Roque reservoir (Córdoba - Argentina). *Biodegradation* **17**(5), 447-455.
- Weisburg W.G., Barns S.M., Pelletier D.A. and Lane D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**(2), 697-703.

## CHAPTER 14: SUMMARY AND CONCLUSIONS

This project identified biological filtration as a viable treatment option for the removal of the algal metabolites MIB, geosmin, cylindrosperopsin (CYN) and the microcystin toxins. Effective biological filtration of MIB and geosmin was observed at the Morgan WTP in South Australia, and this was confirmed through laboratory-scale experiments. Removal of *Cylindrospermopsis raciborskii* and CYN was evident through a biologically-active filtration pilot plant located at North Pine Dam in Queensland, with removal of CYN shown to be through biodegradation. Microcystin toxins were shown to be effectively biodegraded through laboratory-scale columns containing either GAC or sand. Due to a limited supply of the microcystins, in addition to concerns regarding the safety of the microcystins, we were unable to conduct biofiltration of microcystin experiments at the pilot-scale level.

Outcomes of this project are described in the answers to the specific questions raised in the formulation of this project:

### **1. Can we identify individual bacteria capable of degrading the compounds? Are they present in a range of waters?**

Yes, bacteria were isolated from biological filters which were effectively removing each of the algal metabolites studied in this project. The only exception was MIB, where although effective removal of this compound was observed through biological filters, the methods employed in this study were unable to isolate the organism(s) responsible for its removal. Work is still in progress to isolate the MIB-degrading organism(s). The following lists the algal metabolite and the corresponding degrading bacteria:

Microcystin – *Sphingopyxis* sp. LH21

Cylindrosperopsin – *Sinorhizobium* sp.

Geosmin – consortium comprising a *Sphingopyxis* sp. (Geo24), *Novosphingobium* sp. (Geo25) and *Pseudomonas* sp. (Geo33).

Biodegradation of the metabolites by the isolated bacteria was also observed in batch studies conducted in a range of natural waters, confirming their ability to exist in planktonic state and subsequently degrade these contaminants. It is intuitive to expect these bacteria to be able to function accordingly in the planktonic state as this would have been their original habitat prior to colonising within the biofilm of the filters.

### **2. Will there be a "lag period", or delay, between when the biofilm first "sees" the compounds, and when it is capable of degrading them?**

In some cases lag periods were shown to exist prior to the onset of degradation of the metabolites. However, upon re-exposure of the organisms to the metabolites, lag periods were reduced, and in some cases eliminated. For example, a lag period of 2 days was evident in planktonic batch studies prior to the degradation of microcystins commencing; however, after continuous exposure of the bacteria to microcystins the lag period was eliminated with complete degradation of microcystins observed within 5 hours.

More work is required to fully comprehend why the lag period exists as there is still conjecture as to the origins of the lag period. The time required for small populations of the organism(s) responsible for the degradation to become sufficiently large to commence degradation has been cited as an explanation for the lag period. Other possible explanations for the lag period include an insufficient supply of inorganic compounds, the preferential assimilation of other organic compounds before the target compound of interest, or the time required for acclimatisation to- or removal of- inhibitors present in the environment. Some of the results obtained in this project suggest that the lag period may be due to the length of time required for the enzymes responsible for degradation of the metabolite to be induced. Moreover, it may be evident that the metabolite, itself, is an inducer.

### **3. Under what conditions is biofiltration likely to be a viable option for removal of these metabolites? And can we predict whether a biological filter would be effective for the removal of a transient episode of the metabolites?**

The complete removal of MIB and geosmin through the sand filters of the Morgan WTP confirms that removal of these metabolites can be achieved under normal WTP operating conditions. However, this

was only observed when no disinfectant was introduced into the filters, eg. prechlorination or backwashing with disinfected water. Whilst the practice of prechlorination of filters is employed to achieve other water quality targets, such as reducing iron and manganese issues downstream of filters, it is apparent that there needs to be a compromise if filters are going to be considered as an effective treatment barrier for the metabolites. In addition, it appears that the size of the sand filter particles may play an important role in efficient MIB and geosmin removal, with greater removal with smaller particle size. The situation at the Morgan WTP should be of particular interest to SA Water and potentially other water utilities as this plant has demonstrated effective biofiltration of MIB and geosmin. Furthermore, the sand from the filter beds of Morgan WTP was also shown to be highly effective in removing microcystin toxins in laboratory-scale experiments, highlighting the diverse metabolite-degrading microbial community that can exist within the biofilm of WTP sand filters.

In laboratory-scale experiments microcystin appeared to be readily removed through a range of sand filters under both slow and rapid sand filtration conditions, confirming that removal of this metabolite could occur under normal plant operating conditions. Furthermore, the fact that no cytotoxic by-products of microcystin biodegradation were detected demonstrates the feasibility of biodegradation as a possible removal option for the microcystins.

Effective removal of CYN was observed under slow sand filtration conditions at the pilot plant located at North Pine Dam.

The development of molecular tools such as PCR has allowed for the detection of the genes involved in the degradation of microcystin. Using this technology we now have the capability to evaluate biological filters in terms of their capacity to remove microcystin, prior to impending microcystin-producing blooms. This is of enormous value to water authorities as it will enable them to make confident decisions as to whether they can rely on their filters as an effective treatment barrier for these toxins.

#### ***4. Does ozone pre-treatment enhance the removal?***

Ozone pre-treatment was only evaluated at a pilot-scale biofiltration plant situated at the Happy Valley WTP in South Australia, and only for MIB and geosmin removal. Results of that study were not presented in this report, but are presented in an accompanying document, Bridget McDowall's PhD thesis. In short, the results demonstrated no enhancement of MIB and geosmin removal with preozonation.

#### ***5. Is it possible to modify the biofilm and/or conditions to produce maximum removal in less than optimal conditions?***

As mentioned previously, prior exposure of the metabolites appears to enhance degradation in terms of reducing or eliminating lag period. Seeding filters with the isolated degraders appears to be quite promising, in particular, using the geosmin-degrading consortium resulted in an enhancement (up to an additional 38%) in the removal of geosmin through sand filters.

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## APPENDIX I: PUBLICATIONS FROM THIS PROJECT

### Peer reviewed international journal articles

- Ho L., Meyn T., Keegan A., Hoefel D., Brookes J., Saint C.P. and Newcombe G. (2006) Bacterial degradation of microcystin toxins within a biologically active sand filter. *Water Research* **40**(4), 768-774.
- Ho L., Hoefel D., Aunkofer W., Meyn T., Keegan A., Brookes J., Saint C. and Newcombe G. (2006) Biological filtration for the removal of algal metabolites from drinking water. *Water Science & Technology: Water Supply* **6**(2), 153-159.
- Hoefel D., Ho L., Aunkofer W., Monis P.T., Keegan A., Newcombe G. and Saint C.P. (2006) Cooperative biodegradation of geosmin by a consortium comprising three Gram-negative bacteria isolated from the biofilm of a sand filter column. *Letters in Applied Microbiology* **43**(4), 417-423.
- Ho L., Hoefel D., Bock F., Saint C.P. and Newcombe G. (2007) Biodegradation rates of 2-methylisoborneol (MIB) and geosmin through sand filters and in bioreactors. *Chemosphere* **66**(11), 2210-2218.
- Ho L., Hoefel D., Saint C.P. and Newcombe G. (2007) Degradation of microcystin-LR through biological sand filters. *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management* **11**(3), 191-196.
- Ho L., Gaudieux A.-L., Fanok S., Newcombe G. and Humpage A.R. (2007) Bacterial degradation of microcystin toxins in drinking water eliminates their toxicity. *Toxicon* **50**(3), 438-441.
- Wang H., Ho L., Lewis D.M., Brookes J.D. and Newcombe G. (2007) Discriminating and assessing adsorption and biodegradation removal mechanisms during granular activated carbon filtration of microcystin toxins. *Water Research* **41**(18), 4262-4270.
- McDowall B., Ho L., Saint C. and Newcombe G. (2007) Removal of geosmin and 2-methylisoborneol through biologically active sand filters. *International Journal of Environment and Waste Management* **1**(4), 311-320.
- Ho L., Hoefel D., Saint C.P. and Newcombe G. (2007) Isolation and identification of a novel microcystin-degrading bacterium from a biological sand filter. *Water Research* **41**(20), 4685-4695.
- Wang H., Lewis D.M., Newcombe G., Brookes J.D. and Ho L. Separated adsorption and bacterial degradation of microcystins in GAC filtration. *International Journal of Environment and Waste Management* (submitted).
- McDowall B., Hoefel D., Newcombe G., Saint C.P. and Ho L. Enhancing the biofiltration of geosmin by seeding sand filter columns with a consortium of geosmin degrading bacteria. *Water Research* (submitted).

### Peer reviewed industry journal articles

- Ho L., Wijesundara S., Shaw G., O'Donohue M., Saint C. and Newcombe G. (2005) Biological filtration processes for the removal of algal metabolites. *Water: Journal of the Australian Water Association* **32**(5), 64-68.
- McDowall B., Ho L., Saint C.P. and Newcombe G. (2007) Biological removal of MIB and geosmin through rapid gravity filters. *Water: Journal of the Australian Water Association* **34**(7), 48-54.

### Conference proceedings and/or presentations

- Ho L., Meyn T., Keegan A., Brookes J., Saint C. and Newcombe G. (2005) Biological treatment of microcystin toxins. In *Proceedings of the AWA Ozwater Convention and Exhibition, May 8-12, Brisbane, Australia*.
- Ho L., Hoefel D., Aunkofer W., Meyn T., Keegan A., Brookes J., Saint C. and Newcombe G. (2005) Biological filtration for the removal of algal metabolites from drinking water. In *Proceedings of the 1st IWA-ASPIRE Conference, July 10-15, Singapore*.
- Wang H., Lewis D.M., Brookes J.D., Newcombe G. and Ho L. (2005) Combined adsorption and biodegradation of microcystins. In *Proceedings of the 10th International Conference on Applied Phycology, July 24-30, Kunming, China*.
- Hoefel D., Ho L., Aunkofer W., Bock F., Keegan A., Monis P., Saint C. and Newcombe G. (2005) Biofiltration and identification of bacteria involved in the removal of taste and odour compounds

- from water. In *Proceedings of the 7th IWA Symposium on Off-flavours in the Aquatic Environment, October 2-7, Cornwall, Ontario, Canada*.
- Ho L., Hoefel D., Meyn T., Saint C.P. and Newcombe G. (2006) Biofiltration of microcystin toxins: An Australian perspective. In *Proceedings of the 4th International Slow Sand and Alternative Biological Filtration Conference, May 3-5, Mülheim an der Ruhr, Germany*.
- Wijesundara S., Shaw G. and Newcombe G. (2006) Biodegradation of cyanobacterial toxin, cylindrospermopsin in drinking water. *Second International Conference on Environmental Science and Technology, August 19-22, Houston, Texas, USA*.
- Wang H., Lewis D.M., Brookes J.D., Newcombe G. and Ho L. (2006) Separated adsorption and bacterial degradation of microcystins in GAC filtration. In *Proceedings of the CHEMECA Conference, September 17-20, Auckland, New Zealand*.
- Ho L., Hoefel D., Saint C. P. and Newcombe G. (2006) Degradation of microcystin-LR through biological sand filters. In *Proceedings of the 2nd International Conference on Sustainable Water Environment: Water Resource and Quality Management, October 30-November 1, Taipei, Taiwan*.
- McDowall B., Ho L., Saint C.P., and Newcombe G. (2006) Removal of geosmin and 2-methylisoborneol (MIB) through biologically active sand filters. In *Proceedings of the 10th Annual Australian Environmental Research Event, December 10-13, Macquarie University, Sydney, Australia*.
- Ho L. and Newcombe G. (2007) Biological removal of taste and odour compounds. In *Proceedings of the AWA Water & Health Specialty Conference III, May 23-24, Sydney, Australia*.
- Ho L. and Newcombe G. (2007) Investigation into the performance of treatment processes in removing cyanobacteria and their metabolites at representative plants in South Australia. In *Proceedings of the AWA Water & Health Specialty Conference III, May 23-24, Sydney, Australia*.
- Ho L. and Newcombe G. (2007) Cyanobacteria: What are the issues and what are the solutions? *Annual Scientific Meeting & Exhibition of the Australian Society for Microbiology, July 9-13, Adelaide, Australia*.
- McDowall B., Ho L., Saint C.P. and Newcombe G. (2007) Removal of taste and odour compounds by biodegradation in the filter beds at Morgan Water Treatment Plant. In *Proceedings of the AWA South Australian Regional Conference, July 26, Adelaide, Australia*.
- Ho L., Hoefel D., Gaudieus A.-L., Humpage A.R., Saint C.P. and Newcombe G. (2007) Elimination of microcystins and associated toxicity through biofiltration processes. In *Proceedings of the AWWA Water Quality Technology Conference, November 4-8, Charlotte, North Carolina, USA*.
- Ho L. and Newcombe G. (2007) Removal of cyanobacteria and their metabolites through water treatment processes - South Australian case studies. In *Proceedings of the AWWA Water Quality Technology Conference, November 4-8, Charlotte, North Carolina, USA*.
- Ho L., Hoefel D., Saint C. P. and Newcombe G. (2008) Isolation and characterisation of biofilm-associated bacteria responsible for the degradation of cyanobacterial metabolites in drinking water. In *Proceedings of the IWA Biofilm Technologies Conference, January 8-10, Singapore*.

## Theses, reports, book chapters

- Meyn T. (2004) *Biological removal of microcystin LR and LA: Laboratory studies to assess filtration, degradation kinetics and microbiological characterisation*. Diploma thesis. Technical University of Berlin, Berlin, Germany.
- Bock F. (2005) *Biological filtration processes for the removal of algal metabolites*. Diploma thesis. Georg-Simon-Ohm-Fachhochschule Nürnberg, Nürnberg, Germany.
- Ho L., Hoefel D., Meyn T., Saint C.P. and Newcombe G. (2006) *Biofiltration of microcystin toxins: An Australian perspective*. In *Recent Progress in Slow Sand and Alternative Biofiltration Processes*. Gimbel R., Graham N.J.D. and Collins M.R. (Eds.), IWA Publishing, London, UK, pp. 162-170.
- Wang H. (2006) *Combined adsorption and biological degradation of microcystins*. Masters thesis. University of Adelaide, South Australia, Australia.
- Wijesundara S. (2008) *Biological filtration processes for removal of the cyanobacterial toxin, cylindrospermopsin*. PhD thesis. University of Queensland, Queensland, Australia.
- McDowall B. (2008) *Removal of geosmin and 2-methylisoborneol from drinking water through biologically active sand filters*. PhD thesis. University of Adelaide, South Australia, Australia.



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