# Investigations of microcystins (cyanobacterial peptide toxins) detection, purification and analysis.

COYLE, S.M..

1997

# INVESTIGATIONS OF MICROCYSTINS (CYANOBACTERIAL PEPTIDE TOXINS): DETECTION, PURIFICATION AND ANALYSIS

By

SADIE MARIA COYLE

A thesis submitted in partial fulfilment for the Degree of Doctor of Philosophy.

The Robert Gordon University, May 1997.

CONTENTS

## DEDICATION

*To my husband, Craig*

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. L.A. Lawton and Dr. C. Edwards (Biotage, UK) for their supervision and support throughout this study. Thanks also to the many people in the department for their help and advise over the years especially my fellow lab partners Jacqui and Ben. A special thanks to my husband and family for all their encouragement.

## ABSTRACT

Cyanobacteria (blue-green algae) occur commonly in British freshwaters sometimes producing extensive growths known as water blooms. This study was an investigation of microcystins, the cyclic heptapeptide toxins produced by cyanobacteria. The method of detection relied on high performance liquid chromatography (HPLC) analysis which led to the development of large-scale purification processes to provide material for use as standards in routine monitoring of natural waters and laboratory cultures. These methods were also employed to purify unknown microcystin variants for characterisation. The purified variants were used to constuct a spectral library, using a photodiode array detector, against which samples were screened and any microcystins present identified and quantified. The above detection method was used to monitor the occurrence of several microcystins present in loch water during a single day. This study observed considerable spatial and temporal variation in the occurrence of these microcystins but suggests how sampling techniques may help reduce this variability.

Ecological studies, such as that above, require a large number of samples. This led to the development of a simple and rapid sampling and extraction method for microcystins. It was specifically designed for suspensions of cells in open water and enabled rapid sample processing prior to analysis by HPLC. This method was also used in the development of a 14 day

bioassay to investigate environmental factors influencing the growth and toxin content of cyanobacteria. Once optimised the bioassay method was used to try and identify parameters limiting growth and toxin production in a selected freshwater loch.

# CHAPTER 1

## Introduction

Cyanobacteria are an ancient group of phototrophic prokaryotes which can be found in a wide range of habitats. The term 'blue-green algae' is commonly used when referring to these organisms as they possess an algal-like morphology and are capable of photosynthesis. A dilemma exists as to whether they should be classified under the International Code of Botanical Nomenclature (1972) or the International Code of Nomenclature of Bacteria (1975) (Skulberg *et al.*, 1993). These differences have led to the taxonomic treatment of these organisms as a separate and distinct group-: Class Cyanophyceae.

Fossil record shows that cyanobacteria existed 3.3 to 3.5 billion years ago and were the first organisms capable of photosynthesis. For this reason it is speculated that they may have played a role in the oxygenation of the atmosphere. It is also theorised that cyanobacteria were the origins of chloroplasts, the structure in plants responsible for photosynthesis. It is suggested that these early photosynthesisers may have been taken up by other microbes, lost their ability to function independently and became chloroplasts (Carmichael, 1994).

Cyanobacteria are capable of producing potent toxins which can be divided into three main groups: hepatotoxins, neurotoxins and lipopolysaccharide endotoxins.

The largest and most commonly encountered group of toxins in freshwater are hepatotoxins known as microcystins (Carmichael, 1994) which are the subject of this thesis. These are low molecular weight cyclic heptapeptides with a common structure consisting of three D-amino acids: alanine; erythro-β-methylaspartic acid and glutamic acid and two unusual amino acids namely N-methyldehydroalanine and a 20 carbon chain, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, often abbreviated to ADDA. Variations in the two L-amino acid, the degree of methylation/demethylation and variations in the structure of the ADDA gives rise to at least 50 microcystin variants characterised to date (Bell and Codd, 1996). Microcystins (MCYSTS) are named to indicate the two variable amino acids, for example, MCYST-LR contains leucine (L) and arginine (R) at positions X and Y (Fig. 1.1.) (Carmichael *et al.*, 1988(a)).

Another hepatotoxin group consists of the nodularins which are a closely related group of cyclic pentapeptide toxins which affects the same target organs and show similar hepatotoxicity in animals as microcystins (Carmichael *et al.*, 1988b). These are produced by the genus *Nodularia* and a range of nodularin variants have been identified (Namikoshi *et al.*, 1994). However a hydrophobic analogue of nodularin called motuporin has been identified in extracts of the marine sponge *Theonella*

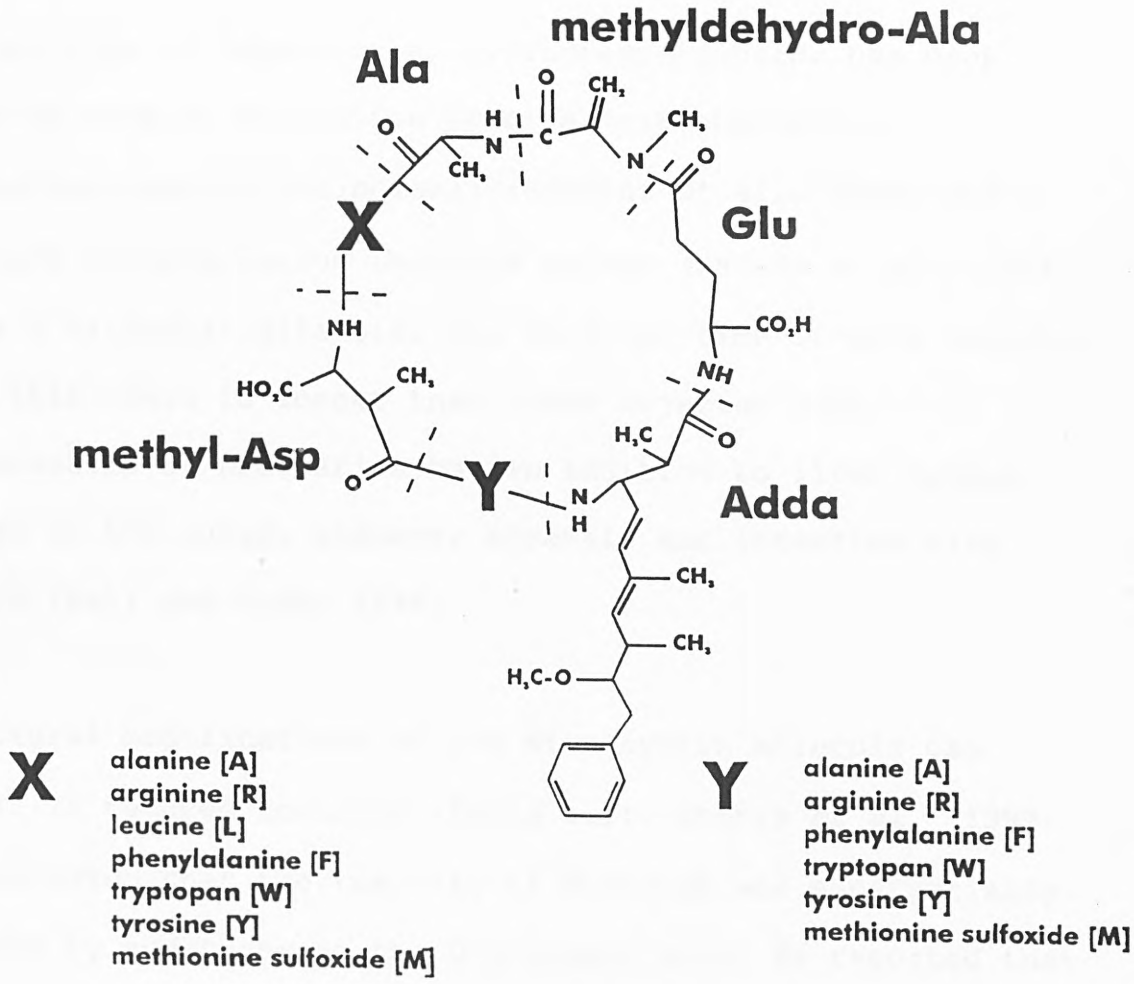



Fig. 1.1. The general structure of a microcystin (MCYST), the two variable amino acid positions labelled X and Y.

sp. and is the first representative of the microcystin class of toxins to be found in the marine environment (Andersen *et al.*, 1993).

Another type of hepatotoxin, cylindrospermopsin, has been isolated from an Australian tropical cyanobacterium *Cylindrospermopsis raciborskii* (Hawkins *et al.*, 1985) and a Japanese cyanobacterium *Umezakia natans* (Harada *et al.*, 1994). It is a tricyclic alkaloid. The survival time of mice injected with this toxin is longer than those injected with microcystins or nodularins but in addition to liver damage, damage to the lungs, kidneys, adrenals and intestine also occurs (Bell and Codd, 1996)

Structural modifications of the microcystin molecule can result in reduced toxicity (Table 1.1). Stotts *et al.* (1993) demonstrated that the toxicity of MCYST-LR was substantially reduced by additions to the D-glutamic acid. He reported that the most toxic variants he investigated during the study all had conserved the D-glutamic acid. Studies have shown that removal or saturation of the ADDA structure greatly reduces the toxicity of MCYST-LR (Dahlem, 1989). Harada *et al.* (1990) isolated a geometrical isomer, 4(E), 6(Z) isomer of the diene of ADDA portion of MCYST-LR and showed that the molecule did not retain its hepatotoxicity, demonstrating the importance of

Table 1.1. The toxicity of a range of microcystin toxins.

| TOXIN | $LD_{50}$ [a] | REF [b] |
|---|---|---|
| MCYST-LR | 25-150 | 1;2;3 |
| [DMAdda$^{5}$]MCYST-LR | 97 | 4 |
| [Dha7]MCYST-LR | 259 | 4 |
| MCYST-RR | 111-650 | 1;3;4 |
| MCYST-YR | 68-~171 | 3;4 |
| MCYST-YM | 56 | 5 |
| MCYST-LY | 91 | 1 |
| MCYST-LA | 39 | 1 |
| MCYST-WR | ~171 | 4 |
| MCYST-FR | ~249 | 4 |
| MCYST-AR | ~249 | 4 |
| MCYST-M(O)R | 750 | 4 |

a: Intraperitoneal mouse bioassay, µg purified toxin per kg body wt.

b: 1. Stoner *et al.*, 1989; 2. Fawell *et al.*, 1993; 3. Watanabe *et al.*,1988; 4. Stotts *et al.*, 1993; 5. Ellemon *et al.*, 1978;

steric configuration. Toxicity also appears to require that the peptide is cyclic (Rinehart *et al.*, 1994).

It is strongly suspected that microcystin toxicity is a result of the inhibition of important regulatory enzymes in the eukaryotic cell. It has been shown that microcystins are potent and specific inhibitors of protein phosphatases 1 and 2A (MacKintosh *et al.*, 1990). Runnegar *et al.* (1993) also demonstrated that inhibition of these enzymes was preceded or accompanied by clinical changes observed due to microcystin intoxication. Protein phosphatase enzymes play a very important role in the regulation of many cellular processes in eukaryotic cells by catalysing the dephosphorylation of intracellular phosphoproteins thereby reversing the actions of protein kinases. Protein kinases are responsible for promoting cellular division and their action is thereby kept in check by protein phosphatases.

Microcystins are also suspected to act as tumour promoters as they inhibit protein phosphatases in a similar manner to okadaic acid which is a potent tumour promoter (Rudolph-Böhner *et al.*, 1994). Intraperitoneal injection of MCYST-LR to rats caused the promotion of liver tumour cell growth after chemical initiation of the tumours (Nishiwaki-Matsushima *et al.*, 1992). Falconer (1991) applied a known carcinogen to the skin of mice which were then given either water or water with

*Microcystis* extract. After 52 days he observed a 7-fold increase in the weight of skin tumours in mice given the *Microcystis* extract compared to the control. Hepatocellular carcinoma is one of the major cancers in China and Yu (1994) observed that people who drank pond/ditch water had higher mortality rates from this type of cancer than people who drank deep-well water. He remarked that a very high number of all ponds sampled had high cyanobacterial populations during the summer and autumn periods.

It is evident that microcystin toxins specifically target the liver but the reasons for this are not clear although it is likely that the toxins are carried into hepatocytes by the bile acid carrier salt transport system, found in the liver (Runnegar *et al.*, 1991). Microcystin toxicity is characterised by damage to the cytoskeleton fibres of the liver and it is known that phosphorylation/ dephosphorylation reactions can greatly influence the structure of these fibres (Carmichael, 1994). Symptoms of microcystin and nodularin poisoning include weakness, cold extremities, diarrhoea and vomiting followed by death due to liver haemorrhage. These toxins are produced by members of the genus *Microcystis, Anabaena, Oscillatoria* (*Planktothrix*) and *Nostoc* (Bell and Codd, 1996).

The cyanobacterial neurotoxins include anatoxin-a, anatoxin-a(s), saxitoxin and neosaxitoxin. Anatoxin-a was the first

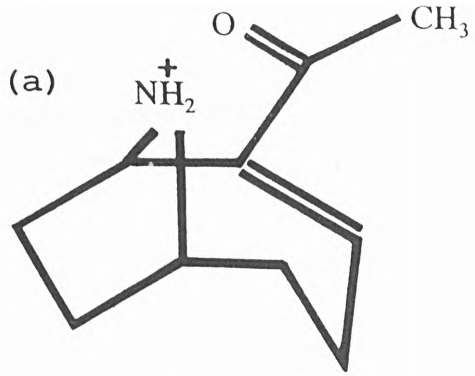
toxin characterised from a freshwater cyanobacterium (Devlin *et al.*, 1977). It is a secondary amine alkaloid, 2-acetyl-9-azabicyclo[4.2.1]non-2-ene (Fig. 1.2(a).) and is produced by members of the genus *Anabaena*, *Aphanizomenon flos-aqua* and *Oscillatoria* sp. (Bell and Codd, 1996; Carmichael and Falconer, 1993). It acts as a postsynaptic, cholinergic, nicotinic agonist resulting in overstimulation of the muscle cells. Death usually occurs as a result of respiratory arrest. Anatoxin-a(s) is a N-hydroxyguanidine methyl phosphate ester produced by *Anabaena* sp. (Fig. 1.2(b).). It acts by inhibiting acetylchloinesterase, thus impeding the degradation of acetylcholine. This also results in overstimulation of the muscle cells but it is four times more potent than anatoxin-a (Table 1.2).

Table 1.2. The toxicity of a range of neurotoxins.

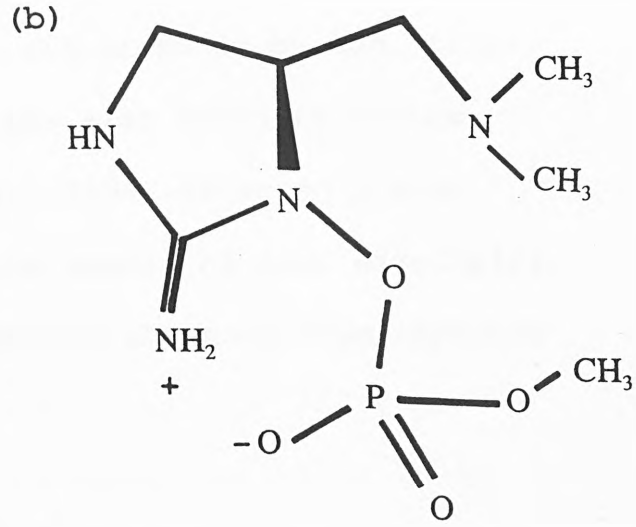
| TOXIN | $LD_{50}$ [a] | REF [b] |
|---|---|---|
| ANATOXIN-A | 200-250 | 1;2;3 |
| ANATOXIN-A(S) | 31-50 | 1;2;3 |
| SAXITOXIN | 10 | 1 |
| NEOSAXITOXIN | 10 | 1 |

a: Intraperitoneal mouse bioassay, µg purified toxin per kg body wt.

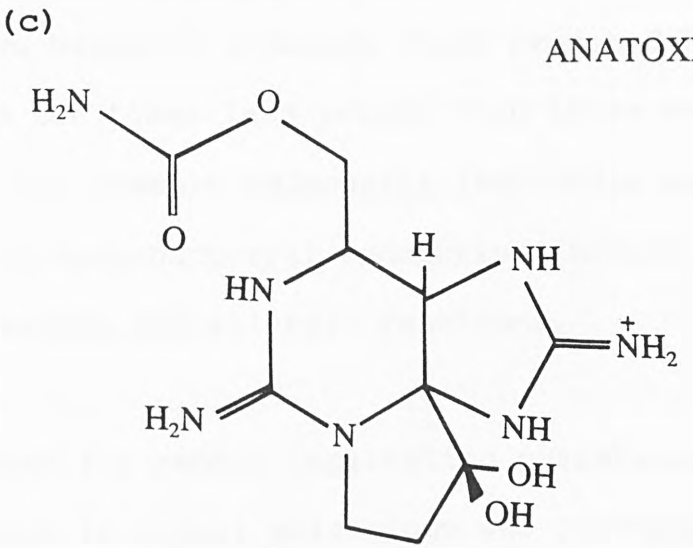
b: 1. Carmichael, 1988; 2. Carmichael, 1992; 3. Devlin *et al.*, 1977.




ANATOXIN-A




ANATOXIN-A(S)




SAXITOXIN

Fig. 1.2. The chemical structures of (a) anatoxin-a, (b) anatoxin-a(s) and (c) saxitoxin.

Saxitoxin is a member of a group of closely related tetrahydropurine compounds known as the paralytic shellfish toxins (PSP) (Fig. 1.2(c).). Derivatisation of saxitoxin results in a number of other natural toxins which includes neosaxitoxin (N-1 hydroxy (R1) saxitoxin) (Baden and Trainer, 1993). Saxitoxin and neosaxitoxin are produced by *Aph. flos-aqua* and *Anabaena circinalis* but are also found in marine dinoflagellates. Steffensen *et al.* (1994) detected a wide range of these PSP toxins in a scum sample of *Ana. circinalis*. These compounds act by blocking sodium channels thus impeding nerve conduction.

Lipopolysaccharide (LPS) endotoxins are commonly produced by many Gram-negative bacteria although those produced by cyanobacteria are ten times less potent than those produced by other pathogens, for example *Salmonella* (Raziuddin *et al.*, 1983). Symptoms of cyanobacterial endotoxins include vomiting, diarrhoea, skin rashes and allergic reactions.

The earliest scientific report implicating cyanobacteria as the causative agent in animal poisonings was published by Francis (1878). He observed cattle, horse and sheep fatalities after the ingestion of scum material of the cyanobacterium *Nodularia spumigena*, which had accumulated along the edge of Lake Alexandria in South Australia. Since then many animal poisonings attributed to cyanobacteria have been reported

world-wide. Codd *et al.* (1992) reported the deaths of dogs after ingesting neurotoxic benthic *Oscillatoria* sp. which had accumulated along the shoreline of several lochs in Scotland during 1990 and 1991. In 1989, 20 lambs and 15 dogs died at Rutland Water, Leicestershire, UK, after consuming shoreline scums of *Microcystis aeruginosa*. A range of microcystins were detected by HPLC in the rumen contents of one of the lambs that had died (*Lawton et al.*, 1995). A large fish kill in Loch Leven, Scotland, in 1992 occurred after a bloom of *Ana. flos-aquae* had begun to senesce. The bloom proved toxic by mouse bioassay and microcystins were also detected in the loch water. Examination of the dead fish showed extensive liver damage consistent with hepatotoxin poisoning (Rodger *et al.*, 1994).

There have been many reports also implicating cyanobacteria in human illnesses. The earliest public health report was in 1931 when a population using water from the Ohio river, USA, were afflicted with gastro-enteritis. It was observed that a cyanobacterial scum had accumulated in the side branch of the river used as a water source (Tisdale, 1931). In 1989 soldiers were hospitalised with atypical pneumonia after canoeing exercises on Rudyard Lake, Staffordshire, UK, which was experiencing a large bloom of *M. aeruginosa* at the time (Turner *et al.*, 1990). However, no fatal poisonings of humans had been confirmed until April, 1996, when 38 people died

after dialysis treatment using water contaminated by MCYST-LR (Dunn, 1996).

Poisoning events such as these have led to a greater awareness of the potential hazard of cyanobacteria. A large sampling program was carried out in the UK by the National Rivers Authority in 1989 in which they found that 68% of the blooms tested contained toxic cyanobacteria (National Rivers Authority, 1990). It has been shown that the toxicity of blooms can be very variable depending on site and time of collection (Carmichael and Gorham, 1981) and non-toxic blooms have been shown to become toxic after the first biomass increase (Benndorf and Henning, 1989). For these reasons it has been recommended that all cyanobacterial blooms are treated as toxic.

As microcystins are the most commonly occurring toxins, they have been the focus of several studies investigating the stability and fate of these toxins in the environment. It has been reported that pure microcystin has a high degree of thermal stability (National Rivers Authority, 1991; Coyle and Lawton, 1996). Microbial degradation plays an important role in the decomposition of cyanobacterial toxins in the natural environment. Cousin *et al.* (1996) observed degradation of MCYST-LR in reservoir water in less than a week. Watanabe *et al.* (1992) observed during induced decomposition experiments

using *Microcystis* cells that the cell density declined gradually to the 14th day and then rapidly decreased from the 14th to the 42nd day, accompanied by an increase in hetertrophic bacteria and release of microcystins into the filtrate. The equivalent amount of microcystin initially present in the *Microcystis* cells was detected in the filtrate on the 35th day of the experiment but decomposition of the toxin did not commence until the 42nd day.

Jones (1990) also reported the biodegradation of MCYST-LR in natural waters but only after an induction period lasting from 3 days to 3 weeks. However, re-addition of MCYST-LR to the water led to immediate decomposition without a lag phase. He suggested that microcystins may persist or decompose depending on the history of the waterbody and whether it has previously been exposed to cyanobacteria (Jones and Orr, 1994). Rapala *et al.* (1994) also reported that loss of hepatotoxins was fastest in vials inoculated with water and sediment samples taken from eutrophic lakes during or after a hepatotoxic bloom, although the loss of anatoxin-a occurred equally fast in all types of water and sediment inocula. It has also been reported that different microcystins decompose at different rates. Rapala *et al.* (1994) noted that MCYST-LR decomposed before variants of MCYST-RR and Watanabe *et al.* (1992) reported that MCYST-YR degraded more rapidly than MCYST-LR.

Conventional water treatment processes normally consist of coagulation; flocculation; sedimentation and filtration but many studies have shown that this is not effective in removing algal toxins (Drikas, 1994; Hoffmann, 1976; Rositano and Nicholson, 1994). Keijola *et al.* (1988) investigated the use of slow sand filtration for the removal of both neurotoxins and hepatotoxins. They demonstrated that it was possible to achieve 60-80% removal but cautioned that this may vary in different filters depending on the biofilm present and also the age of the filter. Many researchers have found that activated carbon treatment can be effective in toxin removal (Keijola *et al.*, 1988; Himberg *et al.*, 1989). Donati *et al.* (1994) demonstrated that the efficiency of toxin removal depended on brand and showed that wood-based carbons were the best.

Ozonation has been shown to completely and rapidly remove both hepatotoxins and anatoxin-a at low doses (Keijola et al., 1988; Rositano and Nicholson, 1994). Rositano and Nicholson (1994) also demonstrated that UV irradiation removed 90% of MCYST-LR after 30 minutes but the radiation needed was high. They suggested, however, that it may be practical to use this treatment in combination with an oxidising agent. The use of ozonation in combination with granular activated (GAC) filters has been proposed. Ozone oxidises organic matter to smaller more biodegradable compounds which would enable the more rapid

establishment of biofilms on the filters (Drikas, 1994). The main drawback to this method is the cost.

Cyanobacterial toxins are secondary metabolites i.e. compounds that are not used by the organisms for its primary metabolism. It is not clear why cyanobacteria produce these compounds but the most prevalent theory in the literature is that they play a defensive role against predation. Shiel and Green (1992) demonstrated in laboratory experiments that *Boeckella triarticulata* fed almost entirely on green algae even when presented with toxic cyanobacteria. Lawton *et al.* (1990) reported that exposing *Daphnia pulex* and *Daphnia magna* to pure MCYST-LR resulted in time and concentration dependent mortalities. Carmichael (1994) observed that zooplankton species generally do not consume toxic cyanobacteria unless there is no other available food and then they often modulate the amount they eat. Beveridge *et al.* (1993) demonstrated that phytoplanktivorous fish can discriminate between microcystin containing and non-microcystin containing cultures of *M. aeruginosa*. These fish stopped grazing when presented with toxic cyanobacteria but controls presented with non-toxic cyanobacteria continued to feed.

Other functions proposed for the production of these toxins include 1) antifouling and surfactant agents 2) metal acquisition and storage 3) unwanted excretion products (Codd,

1995) or 4) that they may once have had some critical function that they have since lost (Carmichael, 1994).

The widespread occurrence of toxin-producing cyanobacteria has led to the need for control and management strategies to be devised. As cyanobacteria thrive in nutrient-rich waters an obvious control method involves reducing nutrient input into waterbodies. Other more short-term strategies involve killing the blooms. The most commonly used algicide is copper sulphate but if a bloom is toxic this treatment results in a large release of toxin into the water. Australian water authorities have generally recommended a 7-10 day period during which water is not utilised following algicide treatment, however, as was discussed previously degradation of microcystins may take anything from 3 days to 3 weeks.

Cyanobacterial growth would be completely prevented by excluding light but this control strategy is only practical in the case of small service reservoirs and holding tanks. An alternative approach is artificial aeration which is used to mix and destratify waterbodies. This strategy also reduces the amount of light received by cyanobacteria and can provide a selective advantage for other phytoplankton such as diatoms. Aeration can also help reduce nutrient recycling that can arise when sediments become anoxic (Hrudey and Lambert, 1994).

Barley straw has been used in the UK to control the appearance of blooms (Newman and Barrett, 1993). It is thought that the decomposition of the straw releases compounds with algicidal properties into the water. However, its success has been variable. A toxic bloom was observed in Loch Rescobie, Scotland, during August 1994, even though straw bales were in place throughout the year (author's observation).

It has also been suggested that cyanobacterial populations could be controlled biologically by the introduction of predators although much research into this area is still required. Matveev and Jones (1994) observed that when *Daphnia carinata* King was presented with lake phytoplankton it consumed everything including colonies of cyanobacteria. However, as discussed earlier many zooplankton and planktivorous fish may avoid feeding on toxic cyanobacteria. The introduction of new species into waterbodies must also be strictly assessed and controlled.

It has been demonstrated that many environmental factors can affect the growth and toxicity of cyanobacteria (Sivonen, 1990; Van der Westhuizen and Eloff, 1985; Campbell, 1994; Utilken and Gjølme, 1995). However, many conflicting results exist in the literature. It has been observed that, at times, different strains or species of cyanobacteria are used to investigate the effects of environmental factors. Ambiguous results between research groups may be due to different

responses/adaptations of particular cyanobacteria. It was also noted that the range/concentrations of parameters such as temperature, light and nutrients used by researchers can also be quite variable. The majority of these studies employ a growth assay which is lengthy and very time consuming, usually involving weekly sampling over a period of eight to ten weeks.

This study set out to develop a simple and rapid bioassay approach with a fourteen day endpoint to replace this more traditional approach. This bioassay was then used to assess the impact of selected nutrients on toxin production by *M. aeruginosa* PCC 7820 cultured in both media and natural loch water. It was simple and rapid to perform and enabled many factors that affect cyanobacterial growth and toxin production to be investigated over a shorter period of time. It will also be used in future to investigate the affect of these parameters on a range of cyanobacteria species. This would provide more insight into the role these environmental parameters play in the dynamics of cyanobacteria populations.

There was a need also to develop a simple sampling and extraction procedure for the routine analysis of microcystins in both the environment and in laboratory cultures. The method had to be suitable for the collection of open water samples and the detection of microcystins in lochs not experiencing bloom conditions at the time. The methodology developed was simple to use and enabled the rapid collection and analysis of

a large number of samples. This allowed the investigation of both temporal and spatial variation of microcystin toxins in a freshwater loch and suggested how sampling strategies can effect this variablity.

Most purification methods developed to date only result in microgram to milligram amounts of pure microcystins. This study aimed to develop a large scale purification method to purify gram quantities of microcystins. This provided material for standards in the routine HPLC analysis of loch waters and also provided material for creating a spectral library of a range of microcystins. This library was used to identify microcystins occurring in the environment. This method was also used to purify and characterise a range of unidentified microcystins produced by a strain of *M. aeruginosa* which was originally purchased from a catalogue supplying schools with biological material.

# CHAPTER 2

## Development and application of a sampling and extraction procedure for the quantitative analysis of microcystins in cyanobacterial cells.

## 2.1. INTRODUCTION

There is an increasing requirement for routine methods to sample and monitor cyanobacterial toxins. Poisoning events involving animals have been reported since 1878 (Francis, 1878). There have been numerous reports which have implicated cyanobacterial toxins in human illnesses (Tisdale, 1931; Turner *et al.*, 1990; Dillenberg and Dehnel, 1960) and animal deaths (Codd *et al.*, 1992). However, no lethal poisonings of humans had been confirmed until April, 1996, when 38 people died after dialysis treatment using water contaminated by MCYST-LR (Dunn, 1996). Incidents such as these has led to an increased awareness of the danger these cyanobacterial toxins present in both recreational and potable water supplies.

There is a need for a simple sampling and extraction regime for microcystins in cyanobacterial cells which would allow the routine collection and analysis of large numbers of water samples. This would provide more meaningful ecological data about the presence of toxic cyanobacteria in a waterbody. The majority of extraction methods used to date have been primarily designed to facilitate the purification of microcystins to allow characterisation (Sivonen *et al.*, 1992; Harada *et al.*, 1988). With the exception of a few (Gjølme and Utkilen, 1994; Lawton *et al.*, 1994), extraction methodology has been designed for large amounts of cell material

(Namikoshi *et al.*, 1992) which has favoured the collection of heavy shoreline scums.

More recently, methods have been proposed which enable the analysis of cyanobacterial cells at much lower cell density. These methods allow the collection of open water samples. Gjølme and Utkilen (1994) proposed a simple protocol which involved filtering cells onto a GF/C filter, then a cycle of freeze/thawing to rupture the cells, followed by aqueous extraction. Similarly, Lawton *et al.* (1994) filtered cells onto GF/C disks but found methanolic extraction was more efficient at extracting the different microcystin variants present. However, both these procedures related the amount of microcystin present to the volume of sample filtered and this is difficult to compare to the conventional mouse bioassay which relates to biomass. To appreciate the toxicity of cyanobacteria it is important to relate the amount of toxin present to a specified biomass of cells.

This chapter presents a sampling and extraction procedure for the routine analysis of microcystins in cyanobacterial cells. It is specifically designed for suspensions of cells in open water or laboratory cultures and incorporates a drying step to allow the determination of cyanobacterial biomass. The method enables rapid sample processing prior to analysis by HPLC. Once optimised the method was applied to monitoring of freshwater lochs and routine screening of laboratory cultures.

The toxicity of certain cyanobacterial species can be quite variable. Carmichael and Gorham (1981) found that the toxicity, as well as density and species composition, varied greatly from site to site on a single day, from day to day, season to season and even at the same site at different times during the day. Bowling (1994) also observed considerable between-site variability in cyanobacterial numbers during two small blooms in Lake Cargelligo, New South Wales, but suggested that error due to less frequent sampling at the time and wind moving the bloom may have had significant impact.

The distribution of plankton both horizontally and vertically has often been reported as patchy (Fogg, 1965). Cyanobacteria possess gas vesicles and thus have an advantage over other plankton in being able to rapidly adjust their buoyancy so that they can move into areas of the water column more suitable for their growth. It has been observed that many species of cyanobacteria form well-defined populations at particular depths throughout the water column (Reynolds, 1971; Walsby and Klemer, 1974). These populations located throughout a lake may form seed populations of later surface blooms (Lindholm, 1992). This has been demonstrated by Reynolds (1971) who showed that surface accumulations of cyanobacteria in the Shropshire Mere, England, resulted from the concentration of colonies previously located several meters deep in the water column. Keller and Paerl (1980) also observed an *Anabaena* population migrating between surface and

sub-surface waters throughout a day. This movement occurred in an unpredictable manner and often resulted in the formation of scums.

This presents an obvious problem when sampling, both quantitatively and qualitatively, the plankton population of a waterbody. A large number of sites would need to be screened over a longer period of time to provide more meaningful ecological data on the distribution and occurrence of cyanobacteria. Cyanobacteria are also traditionally sampled by scooping cells from the surface layer of blooms accumulated near shore. This is particularly the case when obtaining samples for toxicity testing. Samples obtained in this manner are unlikely to be representative of the entire waterbody, as samples obtained from the surface of the water takes no account of any cyanobacteria populations that may be distributed within the water column. These surface accumulations are also subject to wind induced movement.

This chapter describes the variation in biomass and microcystin content of a freshwater cyanobacterial bloom at selected sites over the period of a day. Samples were obtained, by the traditional method of scooping cells from the surface layer of the water, at six sites. However, integrated samples of the water column between 0-1 m were obtained at a single site. It was observed on the day that the bloom moved continuously by gentle wind action and it was proposed that

samples taken from the water column may be more reproducible and representative than the surface samples.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Cultivation of cyanobacteria

Cultures of *Microcystis aeruginosa* PCC 7820 (Institut Pasteur, Paris) were grown in BG-11 medium (Stanier *et al.*, 1971) plus nitrate (8.8 mM $NaNO_3$) under continuous illumination of 20 μmol $m^{-2}s^{-1}$ by cool white fluorescent tubes (36 W) and sparged with sterile air throughout. Growth temperature ranged between 21 and 29°C. Cultures were grown to exponential phase before sub-culturing.

**BG-11 plus nitrate**

| | |
|---|---|
| $NaNO_3$ | 0.750 $gl^{-1}$ |
| $K_2HPO_4$ | 0.040 $gl^{-1}$ |
| $MgSO_4.7H_2O$ | 0.075 $gl^{-1}$ |
| $Na_2CO_3$ | 0.020 $gl^{-1}$ |
| $CaCl_2.2H_2O$ | 0.036 $gl^{-1}$ |
| EDTA | 0.001 $gl^{-1}$ |
| $FeSO_4.7H_2O$ | 0.006 $gl^{-1}$ |
| Citric acid | 0.006 $gl^{-1}$ |
| Trace element solution | 1 $ml l^{-1}$ |

**Trace element solution**

| | |
|---|---|
| $H_3BO_3$ | 2.680 $gl^{-1}$ |
| $MnCl_2.H_2O$ | 1.810 $gl^{-1}$ |

| | |
|---|---|
| $ZnSO_4.7H_2O$ | 0.222 $gl^{-1}$ |
| $Na_2MoO_4.2H_2O$ | 0.390 $gl^{-1}$ |
| $Cu(NO_3)_2.6H_2O$ | 0.079 $gl^{-1}$ |
| $Co(NO_3)_2.6H_2O$ | 0.049 $gl^{-1}$ |

Autoclaved at 120°C (pressure approx. 15 psi) for a time dependant on volume (approximately 20 minutes for 1 l volumes).

### 2.2.2. Assessment of drying protocol

**(a) Whole cells**

Aliquots (20 ml) of *M. aeruginosa* PCC 7820 were filtered through pre-weighed (to five decimal places) 7 cm GF/C filter disks. Each filter plus cells was placed in a Petri dish and dried at 70°C for 3 hours (**Hot-dry method**). The control consisted of filters plus cells prepared in an identical manner as before except frozen (-20°C) then defrosted prior to extraction without a drying step (**Freeze-thaw method**). Filters for each treatment were prepared in triplicate.

Once it had been established that the hot-dry method caused a decrease in recovery of microcystins a modified drying process was developed. This involved drying the filters at 55°C for 30 minutes, removing them from oven and allowing them to cool in

a dessicator, followed by a further 15 minutes at 55°C (**Cool-dry method**).

**(b) Purified microcystins**

The microcystin variants present in *M. aeruginosa* PCC 7820 are MCYST-LR; -LY; -LW; -LF (Lawton *et al.*, 1995). A mixture of these purified microcystins was prepared (see purification protocol, chapter 4) in a ratio similar to that found in the laboratory culture. The approximate concentration in the test solution was as follows: MCYST-LR, 1.5 $mgml^{-1}$; MCYST-LY, 0.07 $mgml^{-1}$; MCYST-LW, 0.17 $mgml^{-1}$; MCYST-LF, 0.26 $mgml^{-1}$. An aliquot (40 µl) of the test mixture was then applied to GF/C filters which were subsequently wetted with 200 µl distilled water to mimic conditions and drying time required when cyanobacterial cells were filtered. Triplicate filters were processed by each of the previously described protocols, i.e. hot-dry; cool-dry and freeze-thaw.

### 2.2.3. Extraction Protocol

All filtered samples used in the assessment of drying were processed by the following method: Each filter disk was placed in a glass beaker with 10 ml methanol and allowed to extract for one hour. The extract was then decanted into a 50 ml pear-shaped flask and rotary evaporated to dryness. A total of three consecutive extractions were performed with the liquor

from each filter being pooled to give a single sample. The sample was re-suspended in 2 x 250 µl methanol and analysed by HPLC as previously described (Lawton *et al.*, 1994).
Three filter disks plus cyanobacterial cells were extracted, without drying, at the outset of this investigation to confirm the suitability of the extraction methodology employed. To allow the effectiveness of the repeated extractions to be examined, the liquor from each was processed separately. All other aspects of the extraction and analysis were as described.

Finally, the optimum length of time required to extract the microcystins from cells was assessed. Filter disks plus *M. aeruginosa* PCC 7820 were prepared as before and cool-dried. Triplicate filters were extracted for 5, 30 and 60 minutes and extracted using the method described.

### 2.2.4. Analysis of natural samples

Open water samples were collected from several Scottish lochs on 23 August 1994 by filling a container from the sub-surface layer of the water. The samples were filtered through a sieve to remove large particles such as zooplankton and debris and examined microscopically to identify which genera of cyanobacteria were represented. Aliquots of the samples were then processed and analysed by the developed protocol.

The variation in toxicity at sites on a freshwater loch over a single day was also investigated. Sampling was carried out on the 31 August 1995 at Loch Rescobie, Scotland, which was experiencing bloom conditions at the time. Six sites were selected around a pier (Fig. 2.1.) and it was observed that quite thick surface scums were accumulating near the shore. Surface samples were collected every hour at these sites over a period from 11.20 a.m to 3.20 p.m. (Hours 1-5). Aliquots were filtered through pre-weighed GF/C filters using a hand-held vacuum pump. These filters were taken back to the laboratory, dried as described in section 2.2.2 and a dry weight of cells obtained.

One metre composite samples were also taken from 0-1 m in the water column every hour at site A (Fig. 2.1.). These samples were obtained using a 1 m pipe (3.5 cm diameter) which was lowered into the water column, the water being retained inside the pipe by insertion of a rubber bung. An aliquot (200 ml) was filtered onto pre-weighed GF/C filters and treated as described above. A sample was taken back to the laboratory for microscopic examination to identify the species of cyanobacteria present. It was a warm, cloudy day with a light wind. It was observed that the surface scum moved continuously but by the last sampling time (3.20 p.m) the wind had risen and dispersed the scum that had accumulated around the pier.

The filters were extracted and analysed for microcystins as described in section 2.2.3 and expressed as μg microcystin $g^{-1}$ dry weight of cells. Quantification was based on a MCYST-LR standard.

Microcystins were identified by their characteristic absorption spectra between 200 and 300 nm ( absorption maximum at 238 nm or 222 nm for variants containing the amino-acid tryptophan) using a 996 photodiode array detector (Waters Corp., Milford, MA). This model of detector has advanced spectral contrast software and can detect small variations in spectral shape (1.2 nm resolution). This enhanced resolution allows small differences in spectrum shape between the variants to be distinguished (Fig. 2.1.a). Spectra from a range of microcystin standards were used to create a library against which the spectra of unknown peaks in a sample could be compared. There are over 50 microcystin variants characterised to date and few are available as standards but unknown microcystins (i.e. when there was no standard) were identified on the basis of their similarity to spectra in the library. The software computes match angles and thresholds which indicate the closeness of the match between the unknown and the standards (Fallick and Romano, 1994).

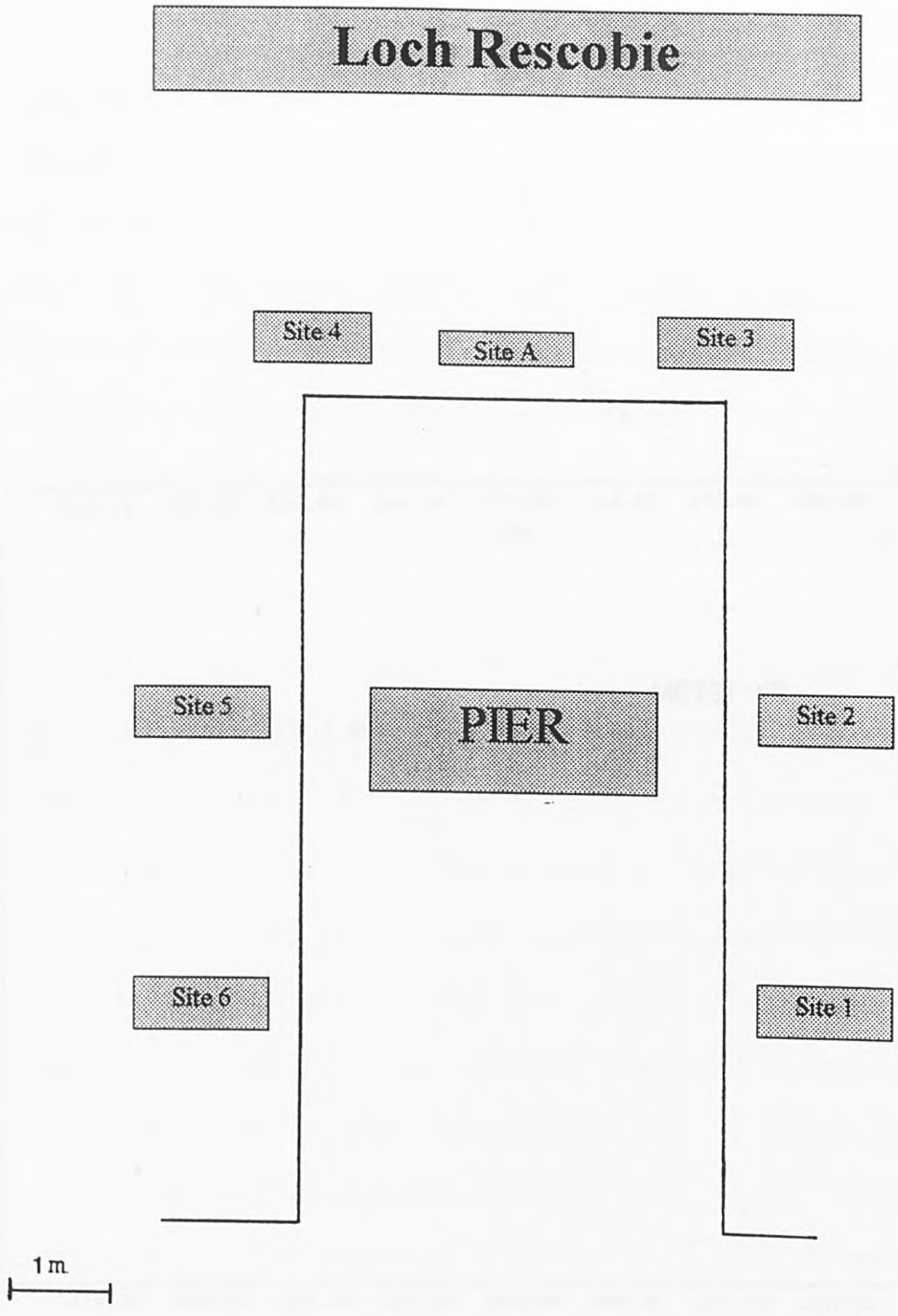



Fig. 2.1. Sites selected for sampling on 31st August 1995 around a pier at Loch Rescobie, Scotland.

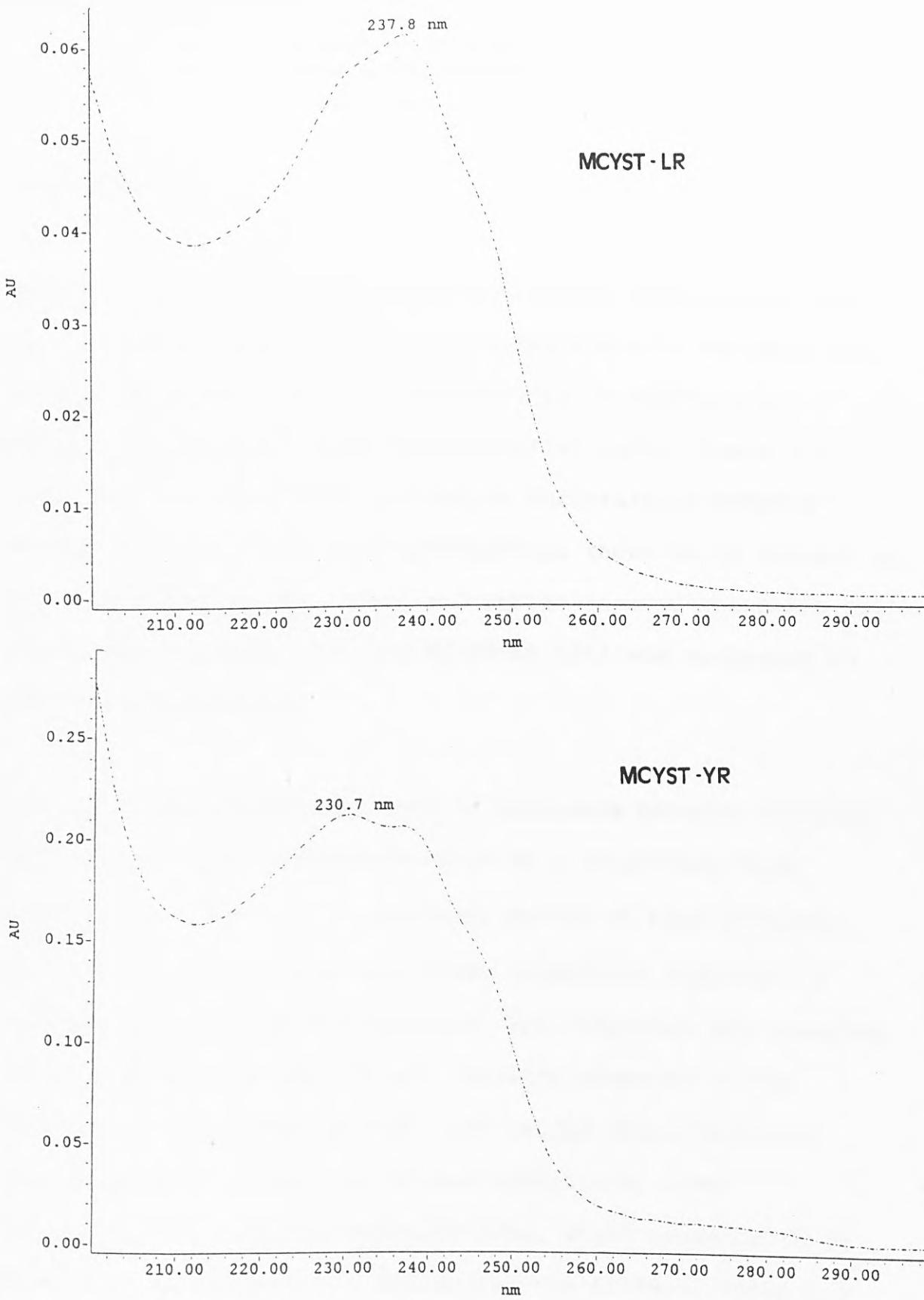



Fig. 2.1.a. Typical spectra (200-300 nm) of MCYST-LR and MCYST-YR showing small variations in spectral shape detected by a 996 photodiode array detector with 1.2 nm resolution.

## 2.3. RESULTS

Before assessment of the sample processing methodology, the use of three sequential one hour extractions in methanol was investigated to confirm its suitability in removing all microcystin variants from cyanobacterial cells. Table 2.1 indicates that the first extraction successfully removed between 86-91% of the four microcystins known to be present in the test culture. The second extraction removed between 9-12% of the microcystins but only MCYST-LR (2%) was recovered in the final extraction.

The first drying protocol used to determine biomass involved drying the filter disks plus cells at a relatively high temperature (70°C) for a prolonged period of time (3 hours). Table 2.2 clearly shows that these conditions resulted in considerable loss of microcystins. The reduction was observed to be greater than 60% for all variants compared to the controls (Freeze-thaw method). The second drying protocol investigated involved less severe conditions, lower temperature and shorter exposure time, which appeared to be much more appropriate for processing the filters. Table 2.3 indicates that there is no loss of microcystins when samples were dried at a cooler temperature for a shorter period of time. Table 2.4 suggests that purified microcystins were stable under the more extreme conditions of the hot-dry

method. There was no difference in yields of pure microcytins between the two drying methods and the control (Freeze-thaw method). It was noted that slightly less MCYST-LW was recovered from the freeze-thaw treated samples. There is no obvious explanation for this although it may be related to the fact that the water is not removed from these filters (i.e. they are not dried) and this may have in some way hampered the recovery of this hydrophobic compound.

Investigation of the extraction time confirmed that 60 minutes was the most suitable duration for optimum recovery of microcystins. It was found that in the first extraction 86-92% of microcystin variants were recovered compared with 77-98% in the 30 minute extraction and 58-69% in the five minute extraction (Fig. 2.2a.). As expected more microcystins were recovered for the reduced times in the second extraction step, 22-33% of the microcystins recovered after 5 minutes, 13-18% recovered after 30 minutes and 8-14% after 60 minutes (Fig. 2.2b.). In the third extraction step only one microcystin variant (MCYST-LR) was detected in the 60 and 30 minute extractions while all microcystins were still detectable in the 5 minute extraction and accounted for 7-10% of the total microcystin variants extracted (Fig. 2.2c.).

The 60 minute extraction gave only slightly better overall recovery of microcystins than the 30 minute extraction. In the 60 minute extraction, 4.80 ug MCYST-LR, 0.48 ug MCYST-LY, 0.94

ug MCYST-LW and 0.95 ug MCYST-LF were recovered in total compared to 4.48 ug MCYST-LR, 0.41 ug MCYST-LY, 0.89 ug MCYST-LW and 0.92 ug MCYST-LF in the 30 minute extraction (all values relate to $ugmg^{-1}$ dry weight of cells).

Application of the sampling and processing methodologies to natural open water samples was successful in detecting microcystin in one of four lakes sampled (Table 2.5). The amount of microcystin present was calculated equivalent to a MCYST-LR standard and recorded in relation to biomass as determined from the dry weight.

Considerable variation in toxicity was recorded at selected sites on Loch Rescobie during a single day (31/8/95). Microscopic examination of a sample taken from the loch revealed that approximately 50% of the sample was comprised of *Microcystis* spp. and 49% of *Anabaena* spp. A very small percentage was comprised of *Oscillatoria* spp.

The biomass of cells at sites 1-6, where samples were obtained from the surface of the bloom, varied considerably with time (Fig. 2.3.). At sites 1, 4 and 5 the greatest biomass was recorded on the first hour of sampling, whereas at sites 2, 3 and 6 the greatest biomass was observed on the second sampling hour. The greatest overall variation during the five hours was observed at sites 5 and 6.

**Table 2.1.** Recovery of microcystins (MCYST) from 20 ml suspensions of *Microcystis aeruginosa* PCC 7820 (not dried) filtered onto GF\C filter disks, extracted three times in methanol with the yield of microcystins at each step determined by HPLC analysis.

| | MCYST-LR | MCYST-LY | MCYST-LW | MCYST-LW |
|---|---|---|---|---|
| | % recovery of microcystin variants | | | |
| **1st Extraction** | | | | |
| Mean | 86 | 91 | 91 | 90 |
| % Error | 1 | 0.8 | 0.2 | 0 |
| **2nd Extraction** | | | | |
| Mean | 12 | 9 | 9 | 10 |
| % Error | 8 | 0.8 | 2 | 0 |
| **3rd Extraction** | | | | |
| Mean | 2 | 0 | 0 | 0 |
| % Error | 5 | 0 | 0 | 0 |

n=3

**Table 2.2.** Comparison of freeze-thaw (A) and hot-dry (B) methods of processing *Microcystis aeruginosa* PCC 7820 cells filtered onto GF/C filter disks, extracted in methanol with the yield of microcystin (MCYST) variants determined by HPLC analysis.

| Treatment | MCYST-LR | MCYST-LY | MCYST-LW | MCYST-LF |
|---|---|---|---|---|
| | μg microcystin variants/20 ml culture suspension | | | |
| Mean (A) | 46.41 | 3.81 | 3.76 | 9.52 |
| $\sigma_{n-1}$ | 3.32 | 0.36 | 0.27 | 0.74 |
| Mean (B) | 17.27 | 1.42 | 1.38 | 3.48 |
| $\sigma_{n-1}$ | 3.12 | 0.26 | 0.26 | 0.63 |

n=3

**Table 2.3.** Comparison of freeze-thaw (A) and cool-dry (B) methods of processing *Microcystis aeruginosa* PCC 7820 cells filtered onto GF/C filter disks, extracted in methanol with the yield of microcystin (MCYST) variants determined by HPLC analysis.

| | MCYST-LR | MCYST-LY | MCYST-LW | MCYST-LF |
|---|---|---|---|---|
| | μg microcystin variants/20 ml culture suspension | | | |
| Mean (A) | 41.31 | 2.79 | 2.58 | 6.30 |
| $\sigma_{n-1}$ | 3.49 | 0.22 | 0.17 | 0.35 |
| Mean (B) | 43.36 | 3.09 | 2.52 | 6.51 |
| $\sigma_{n-1}$ | 3.38 | 0.41 | 0.23 | 0.51 |

n=3

**Table 2.4.** Comparison of freeze-thaw, hot-dry and cool-dry methods of processing purified *Microcystis aeruginosa* PCC 7820 toxins placed on GF/C filter disks, extracted in methanol and the yield of microcystin (MCYST) variants determined by HPLC analysis.

| | MCYST-LR | MCYST-LY | MCYST-LW | MCYST-LF |
|---|---|---|---|---|
| | μg microcystin variants/filter disk | | | |
| **Freeze-Thaw** | | | | |
| Mean | 66.00 | 3.07 | 5.67 | 10.10 |
| $\sigma_{n-1}$ | 3.24 | 0.15 | 0.42 | 1.40 |
| **Hot-Dry** | | | | |
| Mean | 63.00 | 3.16 | 7.83 | 10.93 |
| $\sigma_{n-1}$ | 2.48 | 0.15 | 0.31 | 0.61 |
| **Cool-Dry** | | | | |
| Mean | 58.96 | 3.06 | 7.10 | 10.66 |
| $\sigma_{n-1}$ | 1.79 | 0.21 | 0.36 | 0.40 |

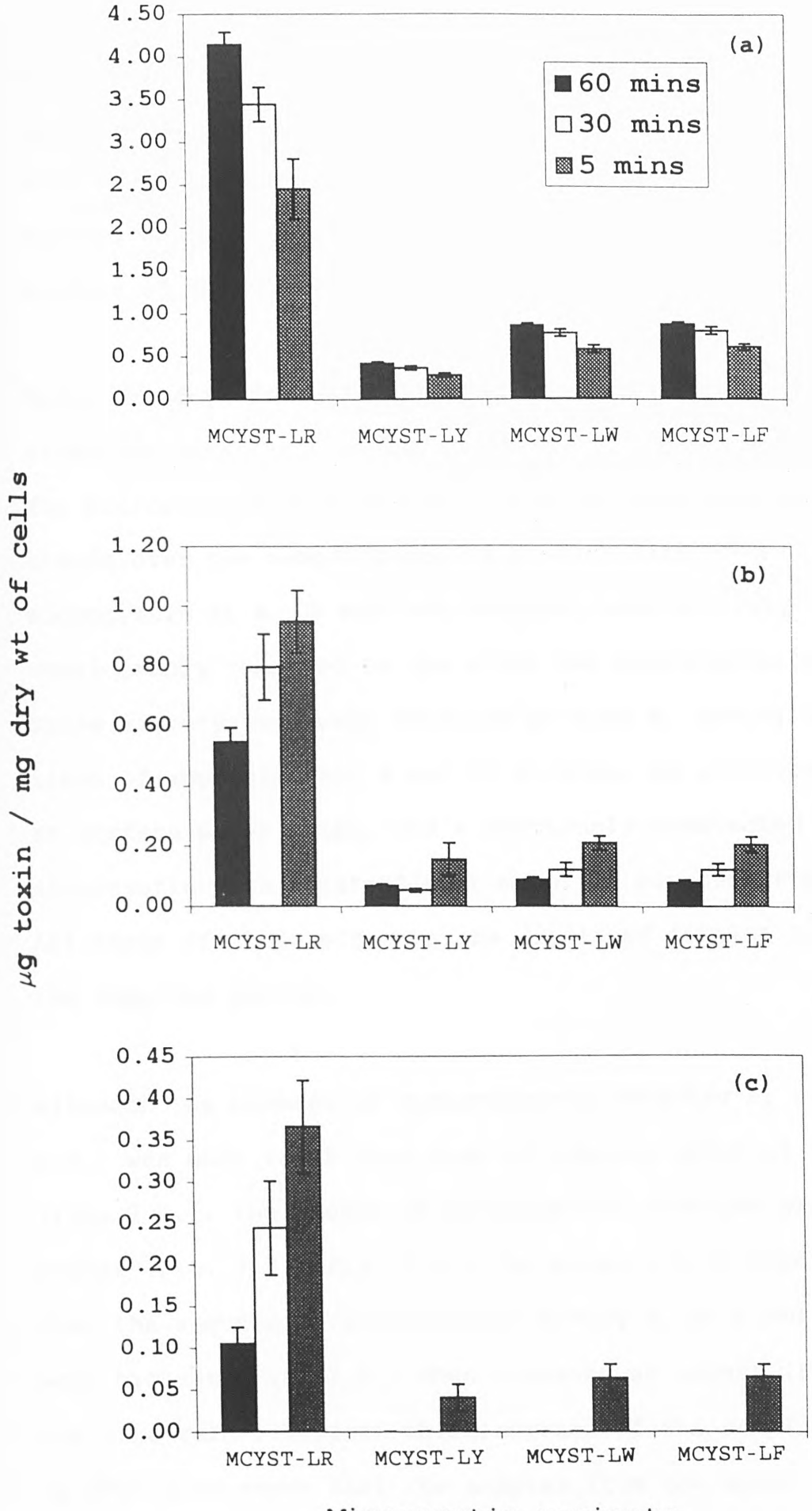
n=3

**Table 2.5.** Water samples from various Scottish lochs (23/8/94) filtered through GF\C filter disks, extracted in methanol and the amount of microcystin (MCYST) variants present determined by HPLC analysis.

| Loch | Mean dry wt (grms) | Organisms | Microcystin present ($\mu g mg^{-1}$ dry wt.) |
|---|---|---|---|
| Rescobie | 0.0065 ± 0.0005 | *Anabaena* spp.<br>*Microcystis* spp. | 0.37 ± 0.04 |
| Federat | 0.0127 ± 0.0029 | *Anabaena* spp. | ND |
| Butterstone 1 | 0.0114 ± 0.0042 | *Gomphosphaena* spp.<br>*Gleotrichia* spp.<br>*Microcystis* spp.<br>*Anabaena* spp. | ND |
| Butterstone 2 | 0.0012 ± 0.0003 | *Gomphosphaena* spp.<br>*Microcystis* spp.<br>*Gleotrichia* spp.<br>*Anabaena* spp | ND |

Butterstone 1 sample from open lake
Butterstone 2 from closed off inlet
ND - Not detected

Fig. 2.2. Comparison of the recovery of microcystin (MCYST) variants from three consecutive extractions (a) first (b) second (c) third of *Microcystis aeruginosa* PCC 7820 cells filtered onto GF/C filter disks and extracted for 60, 30 and 5 minutes in methanol. Bars represent sample standard deviation (n=3).


(a)
60 mins
30 mins
5 mins
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
MCYST-LR
MCYST-LY
MCYST-LW
MCYST-LF
(b)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
MCYST-LR
MCYST-LY
MCYST-LW
MCYST-LF
(c)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
MCYST-LR
MCYST-LY
MCYST-LW
MCYST-LF
μg toxin / mg dry wt of cells
Microcystin variants

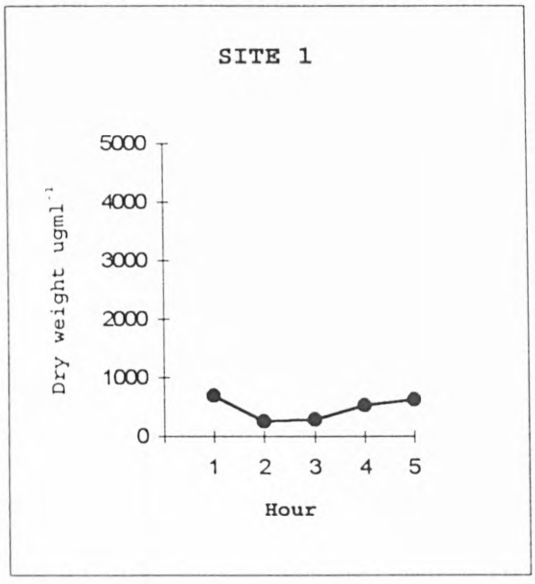
The biomass of cells at site A, where composite samples of the water column between 0-1 m were taken, did not display such wide variation over time (Fig. 2.4.). As might be expected the biomass was much lower than that of samples collected at the surface of the bloom.

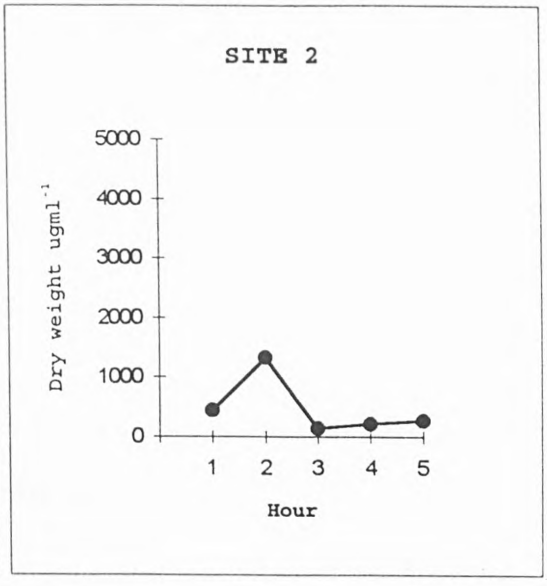
Three microcystins were detected at sites 1-6, having retention times ($R_t$) of approximately 8, 18 and 20 minutes. The microcystins at $R_t$ 8 and 18 minutes displayed very similar trends over the sampling period at each site (Fig. 2.5.). The microcystin at $R_t$ 20 minutes, however, did not vary considerably compared to the other two microcysins detected. Three microcystins were detected at site A, having retention times of approximately 8 and 18 minutes, as previously found at surface water sites, and a previously undetected microcystin with a retention time of 16 minutes (Fig. 2.6.). All three of these microcystins displayed similar trends over the sampling period.

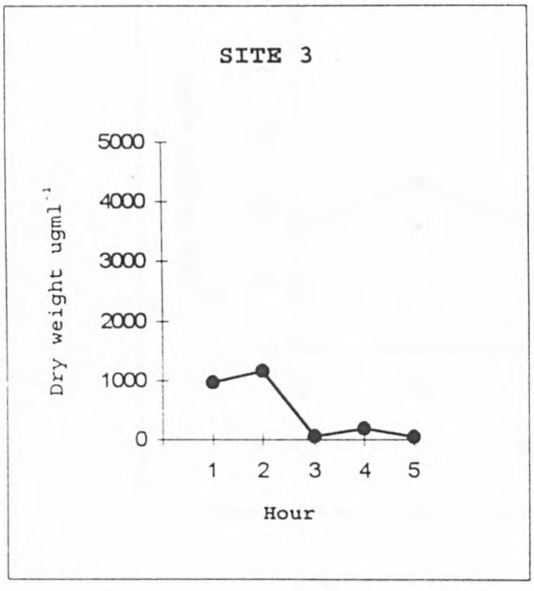
Although the biomass of cyanobacteria detected at site A (Fig. 2.4.) was much lower than that of samples taken at the surface (Fig. 2.3.), the amount of microcystins detected was generally higher (Fig. 2.5.; Fig. 2.6.). At sites 1-6 it also was noted that the amounts of microcystins having $R_t$ of 8 and 18 minutes were highest (Fig. 2.5.) when biomass was lowest (Fig. 2.3.) and vice versa. Typical chromarograms of the samples analysed by HPLC also shows that the samples from the water column

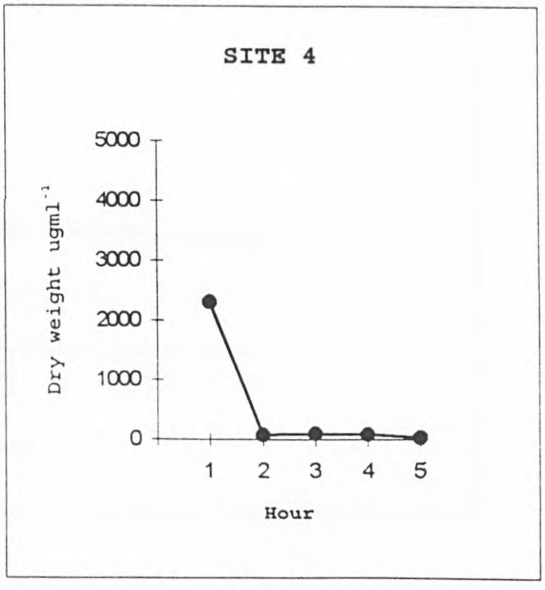
(site A) were much cleaner than the samples obtained from the surface of the bloom (Fig. 2.7; Fig. 2.8).

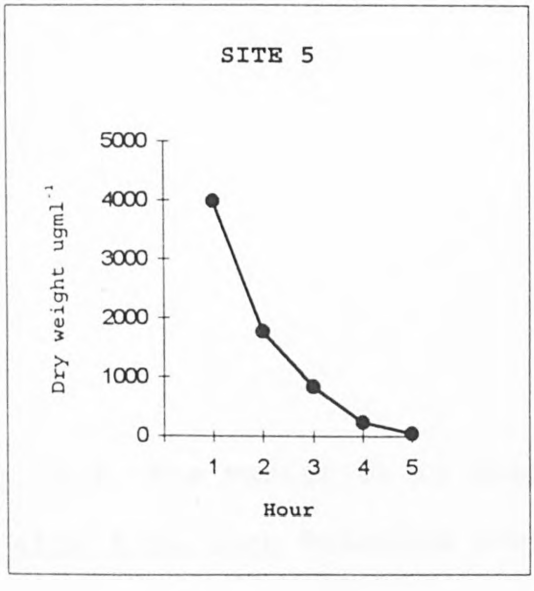
Fig. 2.3. The variation in biomass of a cyanobacterial bloom at six sites around a pier on Loch Rescobie over the period of five hours.

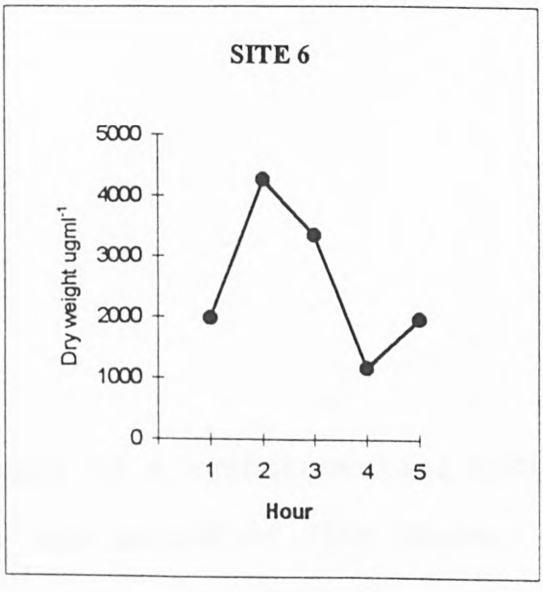

SITE 1
Dry weight ugml-1
5000
4000
3000
2000
1000
0
1 2 3 4 5
Hour


SITE 2
Dry weight ugml-1
5000
4000
3000
2000
1000
0
1 2 3 4 5
Hour


SITE 3
Dry weight ugml-1
5000
4000
3000
2000
1000
0
1 2 3 4 5
Hour


SITE 4
Dry weight ugml-1
5000
4000
3000
2000
1000
0
1 2 3 4 5
Hour


SITE 5
Dry weight ugml-1
5000
4000
3000
2000
1000
0
1 2 3 4 5
Hour


SITE 6
Dry weight ugml-1
5000
4000
3000
2000
1000
0
1 2 3 4 5
Hour

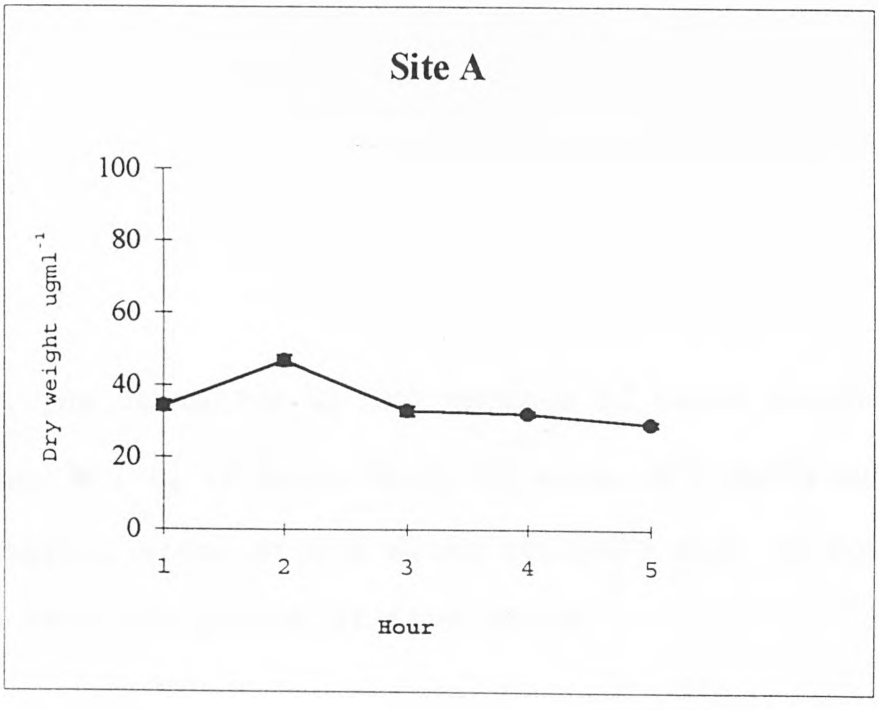
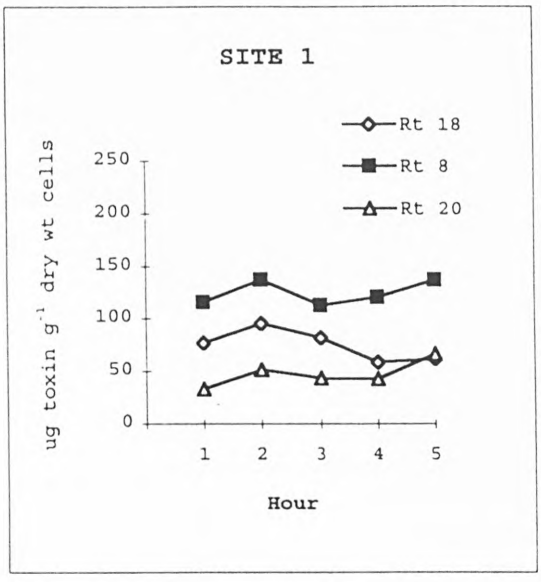
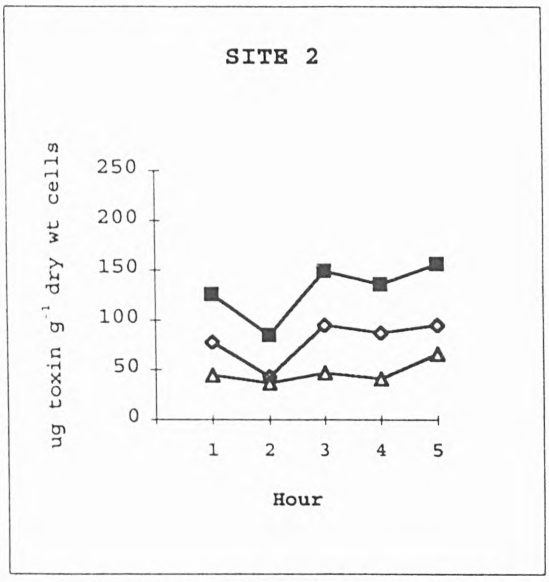
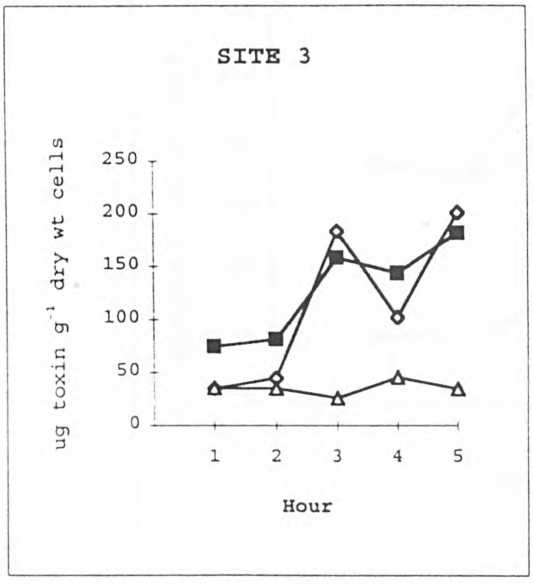


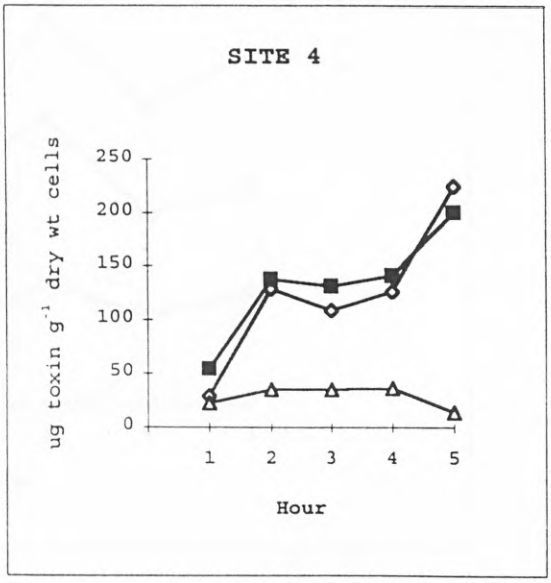

Fig. 2.4. The variation in biomass of a cyanobacterial bloom at site A on Loch Rescobie over the period of five hours.

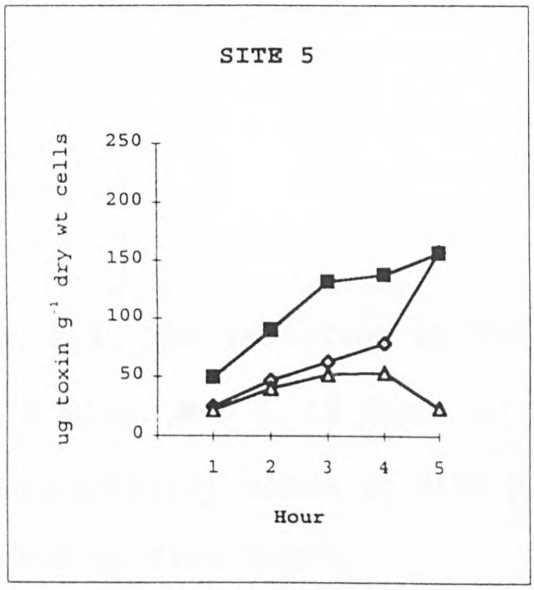
Fig. 2.5. The variation in the amounts of three microcystins ($R_t$ 8 mins, ■ ; $R_t$ 18 mins, ◊; $R_t$ 20 mins, Δ ) detected in a cyanobacterial bloom at six sites around a pier on Loch Rescobie over the period of five hours.

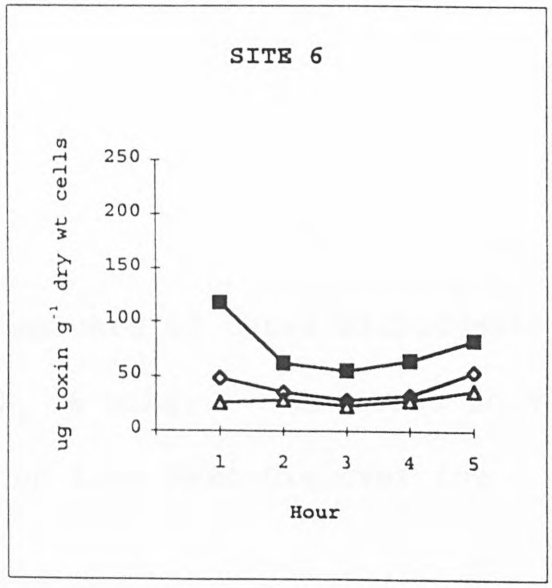

SITE 1
Rt 18
Rt 8
Rt 20
ug toxin g-1 dry wt cells
250
200
150
100
50
0
1
2
3
4
5
Hour


SITE 2
ug toxin g-1 dry wt cells
250
200
150
100
50
0
1
2
3
4
5
Hour


SITE 3
ug toxin g-1 dry wt cells
250
200
150
100
50
0
1
2
3
4
5
Hour


SITE 4
ug toxin g-1 dry wt cells
250
200
150
100
50
0
1
2
3
4
5
Hour


SITE 5
ug toxin g-1 dry wt cells
250
200
150
100
50
0
1
2
3
4
5
Hour


SITE 6
ug toxin g-1 dry wt cells
250
200
150
100
50
0
1
2
3
4
5
Hour

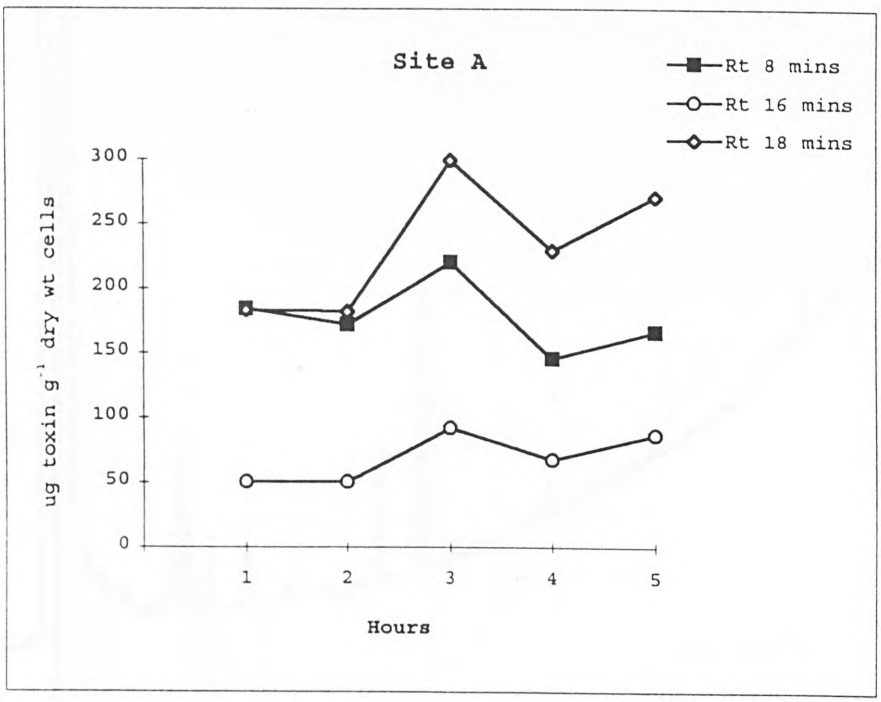



Fig. 2.6. The variation in the amounts of three microcystins ($R_t$ 8 mins, ■ ; $R_t$ 16 mins, o; $R_t$ 18 mins, ◊ ) detected in a cyanobacterial bloom at site A on Loch Rescobie over the period of five hours.

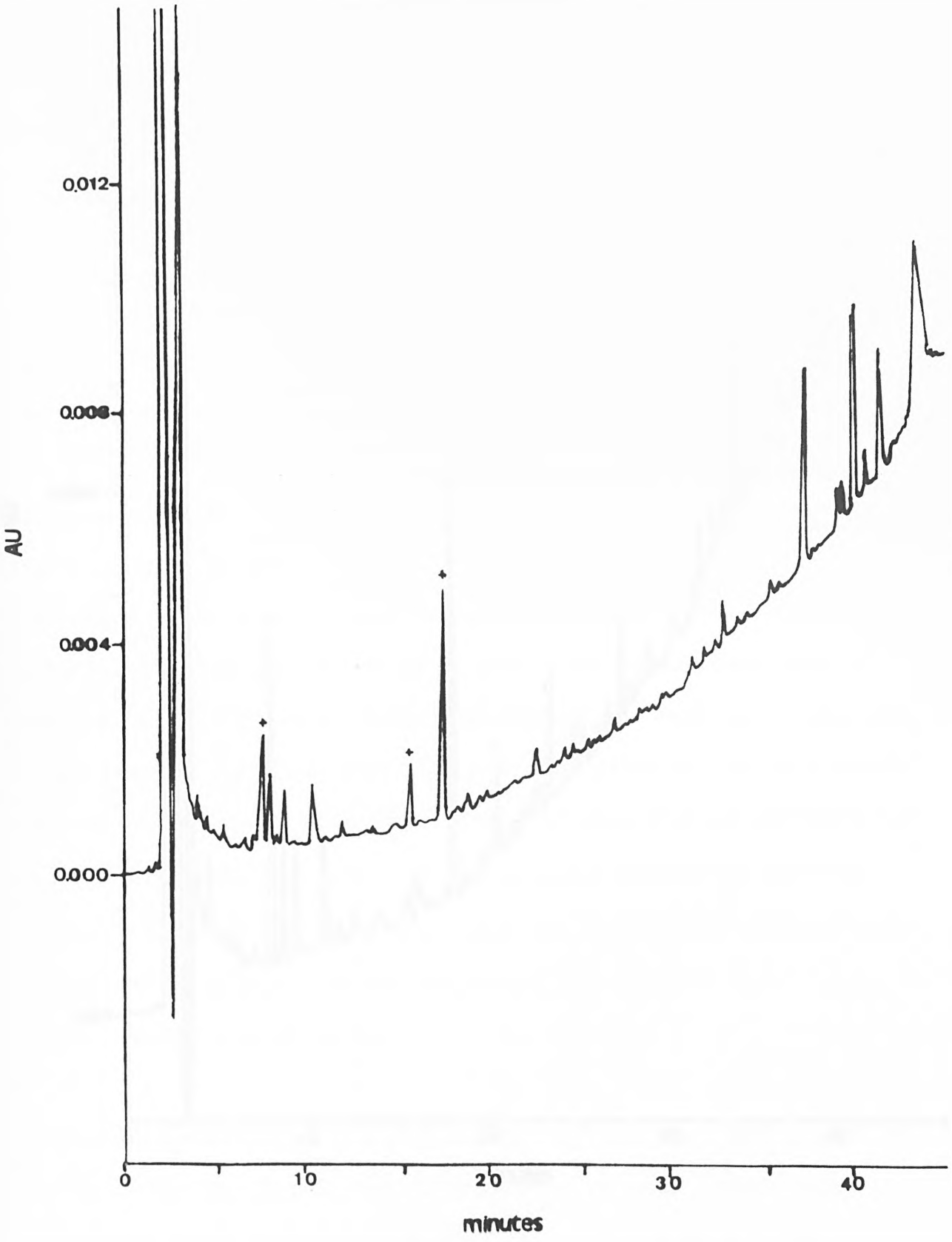



Fig. 2.7. Analysis of a methanolic extract of cyanobacteria sampled from the water column (site A) of Loch Rescobie analysed by reversed-phase HPLC with diode-array detection. Microcystins at $R_t$ 8, 16, and 18 minutes are indicated (+).

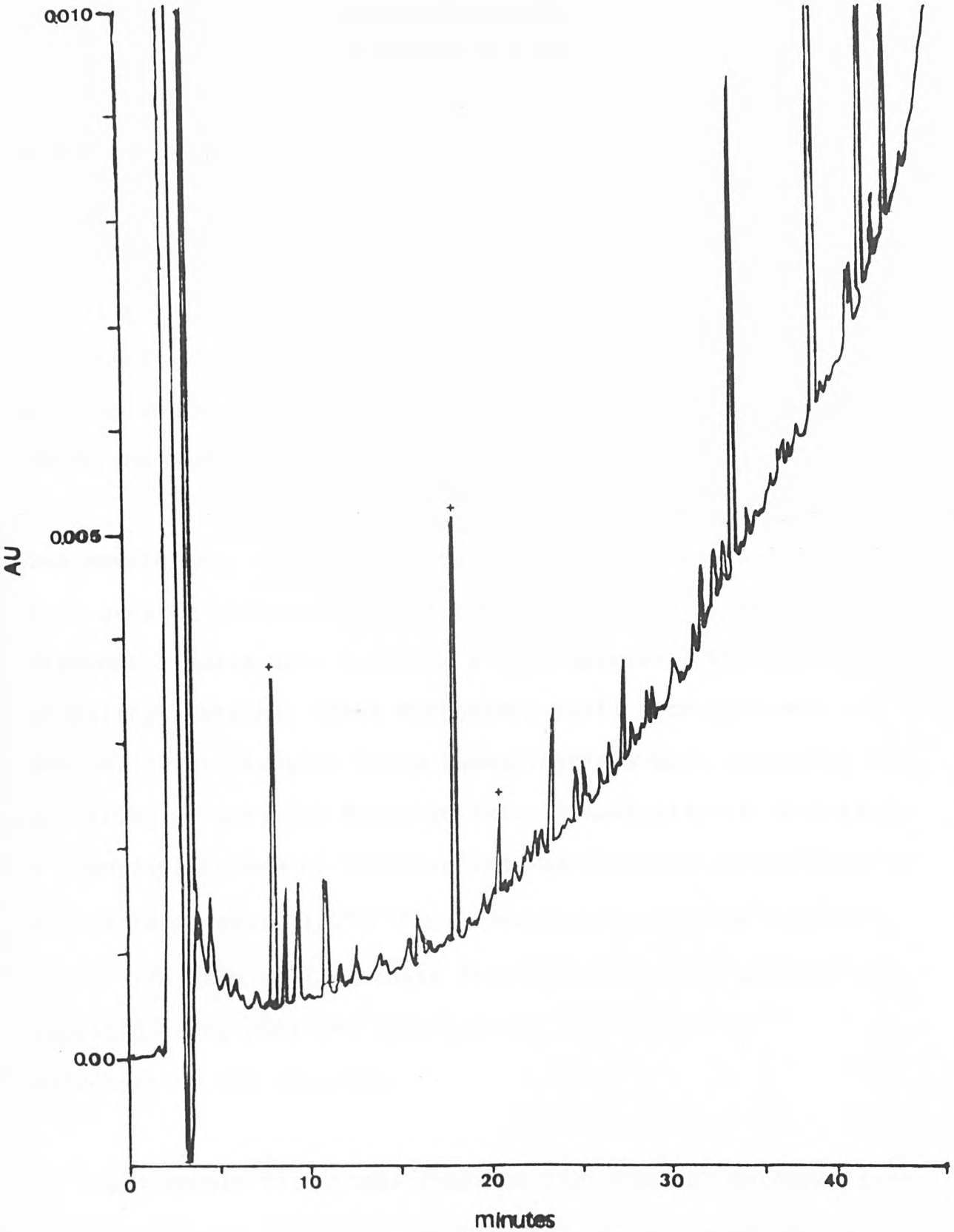



Fig. 2.8. Analysis of a methanolic extract of cyanobacteria sampled from the surface water (sites 1-6) of Loch Rescobie analysed by reversed-phase HPLC with diode-array detection. Microcystins at $R_t$ 8, 18 and 20 minutes are indicated (+).

## 2.4.DISCUSSION

The results presented here confirm that the use of methanol facilitates the extraction of a group of microcystins of differing polarity. Furthermore, it appears that three successive extractions is sufficient to exhaustively remove these compounds from intact cells.

The development of a drying step to provide biomass data led to a greater understanding of the stability of microcystins. Previous studies have reported a high degree of thermal stability (National River Authority, 1991; Weckesser and Martin, 1990), however these investigations were examining the stability of purified microcystins. In contrast, in this study a significant loss of microcystins was observed on exposure to a high temperature (70°C) for an extended period of time (3 hours). To help explain these findings the drying process was repeated using purified microcystins and no loss of microcystins was observed.

It would appear from these findings that thermal decomposition of microcystins occurs in the presence of cell components. There are some similarities with these findings and the observations of Tsuji *et al.* (1994). They found that the presence of photosynthetic pigments, a substantial component of the cell content, accelerated decomposition of MCYST-LR on

exposure to sunlight compared to controls containing only purified MCYST-LR.

Modification of the drying step to reduce the severity of conditions, lower temperature and shorter exposure to elevated temperature, was found to dry filter disks without causing decomposition. Hence it was concluded that this method was ideal for processing samples where it is desirable to know the biomass of cells present.

Analysis of the time allowed for extraction confirmed that a 60 minute extraction provided the most efficient recovery of microcystins. However, extraction for 30 minutes was only slightly less effective. The use of a 30 minute extraction period greatly reduces the time required to process samples, therefore in some instances it may be advantageous to adopt this, for example, where a large number of samples require processing or when the results of analysis are rapidly required.

Once finalised the method proved useful in the analysis of natural samples and although certain waterbodies sampled did not contain visible cyanobacterial blooms it was still possible, by means of collecting and filtering a small volume of open water, to detect and quantify microcystins present in the lochs. The methodology developed here is also being routinely employed in the laboratory in the investigation of

microcystin production under different environmental conditions.

To summarise, the method presented here can easily be adopted in routine monitoring and by incorporating the determination of biomass can help unify the quantification of microcystin. Data gathered in this manner could readily be compared from year to year and over a wide range of sites.

Employing this sampling and processing method a cyanobacterial bloom was monitored over the period of a day. Considerable variation in microcystin content when collecting cyanobacteria material for toxicity testing was observed during this study. The genus of cyanobacteria identified in the Loch Rescobie bloom during sampling are known to be capable of producing microcystins. The biomass and amount of microcystins detected in the bloom varied considerably between sites 1-6 at each sampling hour and also at individual sites over the entire sampling period. This variation may have been caused by wind-induced movement of the cyanobacterial bloom observed on the day. Bowling (1994) also concluded that wind moving a bloom he was studying may have been the cause of considerable between-site variability.

It was also observed that, in general, at sites 1-6 when biomass was low, the amounts of two of the microcystins ($R_t$ 8 and 18 minutes) were high and vice versa. Kotak et al. (1994)

also observed that peak abundance of *M. aeruginosa* occurred in August and September but the maximum concentration of MCYST-LR did not occur at these times. Watanabe *et al.* (1994) reported a decrease in the concentration of microcystins detected in a loch at times when maximum cell numbers of *Microcystis* were present. One possible explanation is that different strains of non-toxic *Microcystis* spp. are becoming dominant or are being selectively sampled. It has been shown that some field isolates of species from toxic bloom produce toxins while others do not (Watanabe *et al.*, 1991). However, it has also been demonstrated in laboratory based studies that conditions for optimal growth may not always be optimal for toxin production (Van der Westhuizen and Eloff, 1985). It is also a possibility that different areas of the bloom contain cyanobacterial cells at different cell cycle phases. Eloff and Van der Westhuizen (1981) found that toxicity of a *Microcystis* spp. increased during the exponential phase and then decreased at the beginning of the stationary phase. It is not known why cyanobacteria produce toxins but the prevelant theory in the literature is that they play a defensive role against predation. It may be that cyanobacteria produce less toxin when they occur in greater densities/colonies and are thus less vulnerable to predation.

In this study it was discovered that although the biomass of cells collected at site A were considerably lower than at

sites 1-6 sampled at the surface of the bloom, the total amount of microcystins detected there over the sampling period was greater. It may be that the cyanobacterial cells at the surface have lost their buoyancy control and are beginning to senesce and lyse, releasing their toxin into the surrounding water, hence there is less present in the cell biomass.

The detection of a microcystin in the water column samples which was absent from the surface samples suggests the presence of a toxin-producing cyanobacterial species distributed within the water column which was absent from the surface layer. Site A displayed less variability in biomass and the amount of microcystins detected during the sampling period. It is suggested that sampling the water column rather than the surface of the blooms may provide more meaningful and consistent data. It would also provide a method for assessing the toxicity of waterbodies not experiencing bloom conditions at the time of sampling and aid in the assessment of the potential hazards to users. Data gathered in this manner could also be more readily compared from year to year and over a wide range of sites.

# CHAPTER 3

## Variability of growth and microcystin content of *Microcystis aeruginosa* PCC 7820 under selected environmental factors

## 3.1. INTRODUCTION

It has been demonstrated that various environmental factors can greatly influence the toxicity of certain cyanobacteria. The appearance of cyanobacterial blooms in freshwaters is to a certain extent random and unpredictable. It is likely that in the natural environment a variety of factors are involved in both the growth and toxin production of cyanobacteria.

Temperature, light, nutrients and pH, among other factors have been shown to affect toxin production. Watanabe and Oishi (1985) observed a marked change in the toxicity of *M. aeruginosa* M228 in light intensity experiments. They found that toxin production was suppressed under low light conditions but a fourfold increase in toxicity was observed when the light intensity was increased from 7.53 to 30.1 μEinsteins $m^{-2}s^{-1}$. Van der Westhuizen and Eloff (1985) reported that toxicity of *M. aeruginosa* UV-006 tended to be less at very low and high light intensities. In contrast, Sivonen (1990) found that at low light intensities toxin production was greater than at high light intensities (12-95 μEinsteins $m^{-2}s^{-1}$) for two strains of *Oscillatoria agardhii* sp. Codd and Poon (1988) reported that light intensities of 5-50 μEinsteins $m^{-2}s^{-1}$ had no effect on toxin production by several strains of *M. aeruginosa*.

Van der Westhuizen and Eloff (1985) reported that optimum temperature for growth (32°C) differed from the optimum temperature for maximum toxin production (20°C). Their cultures of *M. aeruginosa* UV-006 were most toxic at 20°C but toxicity was not significantly reduced until temperature exceeded 28°C. Gorham (1964) also reported that optimum temperature for growth (30°C and 35°C) were different from the optimum temperature for toxicity (25°C) for a strain of *M. aeruginosa*. He found that the toxicity of *M. aeruginosa* to mice was greater after growth at 25°C than at 20°C and decreased at 30°C to an intermediate value. However, Sivonen (1990) found that *O. agardhii* strain 97 had an optimum temperature (25°C) for both growth and toxin production. Another strain (*O. agardhii* CYA 128) produced almost equal amounts of toxin at 15, 20 and 25°C. Toxin production, however, in both strains was lowest at 30°C.

Sivonen (1990) found that high nitrogen content in the culture medium favoured both growth and toxin production by two strains of *Oscillatoria*. Phosphorus did not have as great an effect. Toxin production was reduced at low phosphorus concentrations when growth was poor. Campbell (1994) also found that increasing levels of nitrate compared to base

levels resulted in an increase in toxicity. Codd & Poon (1988) transferred cultures of *M. aeruginosa* 7813 from complete growth medium to medium minus added nutrients. They found that phosphorus removal did not influence toxicity but nitrogen and inorganic carbon removal each caused a tenfold decrease in toxicity. However, it has been reported that *M. aeruginosa* 7813 in batch culture appeared to grow slightly better at reduced nitrate levels but no difference in the amount of MCYST-LR it produced was observed. In contrast, when this strain was grown in steady-state continuous culture it was observed that increasing amounts of MCYST-LR were produced when the nitrate level was reduced (National Rivers Authority, 1991).

Utilken and Gjølme (1995) found that nitrate- and phosphate-limited conditions had no effect on toxin production by *M. aeruginosa* but iron-limited conditions resulted in a decrease in the ratio of toxin content to protein content. In contrast, Lukac and Aegerter (1993) found that the toxin content increased as the iron concentration decreased. They concluded that in the absence or at low levels of iron the cells grew more slowly and produced 20-40% more toxin. Van der Westhuizen and Eloff (1983) reported *M. aeruginosa* UV-006 was more toxic at higher and lower pH values where the cells also grew more slowly.

The contrast and differences in toxin production under specific conditions may be due to, for example, species or strain of cyanobacterium used in each experiment; culture conditions; method of detection; culture media. An obvious drawback when relating these experiment to the environment is that these factors are more than likely acting in concert in nature to control the growth and toxicity of cyanobacteria.

In this chapter the effect of selected environmental factors was investigated using a developed bioassay. This bioassay allowed us to rapidly assess the impact of various conditions on the growth and toxin content of *M. aeruginosa* PCC 7820. This bioassay was also employed to assess the potential factors limiting growth and microcystin production in a selected freshwater loch. The principle behind this type of bioassay is that a factor is not limiting if an increase in that factor produces no significant stimulation in algal growth (Lund *et al.*, 1971) or microcystin production. The assay measured the growth and microcystin content of a cyanobacteria inoculated into media plus and minus selected nutrients. The above parameters were also assessed in filtered, natural water, otherwise unmodified or spiked with selected nutrients.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Effect of nitrate on the growth and toxin production of *Microcystis aeruginosa* PCC 7820

A 1 l culture of *M. aeruginosa* PCC 7820 was grown under standard conditions (chapter 2; 2.2.1) for 7 days prior to use in the bioassay, to ensure that the cells were growing exponentially. After this time the culture was centrifuged in a sterile 1 l centrifuge pot at 1500 x g for 60 minutes. The supernatant was decanted and the pellet of cells re-suspended in 400 ml sterile BG11 medium (Stanier *et al.* 1971) minus nitrate. This mixture was used as stock for inoculating the test flasks.

Flasks (250 ml Erlenmeyer) were prepared containing 100 ml of BG11 medium plus different concentrations of nitrate. Five replicates per concentration: 17.6 mM, 8.8 mM, 1.76 mM and 0 mM $NaNO_3$, were prepared. These were autoclaved for 30 minutes at 120°C. When cool 10 ml of stock *M. aeruginosa* PCC 7820 was asceptically inoculated into each flask. The flasks were then incubated in a waterbath at 25°C under continuous illumination for 14 days (Fig. 3.1.). The flasks were shaken twice each day.


Fig. 3.1. Bioassay flasks incubated in a waterbath at 25°C under continuous illumination.

After the incubation period the following parameters were measured.

*Optical density*

O.D. was measured at 720 nm using a 1 cm pathlength plastic cuvette in a spectrophotometer (Novaspec II, Pharmacia Biotech) with 1 ml of sample. The sample was thoroughly mixed before the reading was taken. Distilled water was used as a blank.

*Chlorophyll a*

A 1 ml sample was microfuged in an microcentrifuge tube (Eppendorf, UK), supernatant removed and 1 ml methanol added to the pellet. The sample was mixed and left in the dark for one hour. The sample was microfuged again and the supernatant read at 663 nm. The absorbance reading was multiplied by 12.63 to give $\mu g ml^{-1}$ chlorophyll *a* (Lobban *et al.*, 1988).

*Dry weight*

Aliquots (20 ml) of culture was filtered through dried and pre-weighed GF/C filters. The filters were dried for 30 minutes at 55°C, removed from the oven and cooled in a dessicator for 15 minutes. The filters were returned to the oven for a further 15 minutes at 55°C, cooled and re-weighed.

*Intracellular microcystin analyses*

Filters were extracted in 10 ml methanol for one hour. Three consecutive extractions were performed and the liquor pooled to give a single sample. The extract was rotary evaporated to dryness and re-suspended in 2 x 250 µl methanol and transferred to a microcentrifuge tube. The samples were analysed for microcystin toxins by HPLC as previously described (Lawton *et al.*, 1994). Quantification of the four microcystin variants (MCYST-LR, MCYST-LY, MCYST-LW and MCYST-LF) known to be produced by *M. aeruginosa* PCC 7820 was based on a MCYST-LR standard.

### 3.2.2. Effect of phosphate on the growth and toxin production of *M. aeruginosa* PCC 7820.

A bioassay was performed as previously described in section 3.2.1 with the following changes:

Three replicates per concentration: 0.230 mM, 0.115 mM, 0.023 mM and 0 mM $K_2HPO_4$, were prepared.

It is known that cyanobacteria can have phosphate reserves which they utilise at times when phospate is limiting in the environment. Therefore *M. aeruginosa* PCC 7820 was grown as described previously for 7 days but in phosphate deficient BG11 medium and the phosphate bioassay repeated using this

culture. The pellet of cells was resuspended in 200 ml sterile BG11 medium minus phosphate to provide the stock culture. It was presumed that any phosphate reserves would have been depleted by the culture after this treatment. Quantification of the four characterised microcystin variants present in *M. aeruginosa* PCC 7820 was based a MCYST-LR standard.

### 3.2.3. A bioassay to assess the potential factors limiting growth and microcystin content of *Microcystis aeruginosa* PCC 7820 in a freshwater loch

Composite water samples were collected seasonally from 0-1 m in the water column from Loch Rescobie, Scotland (1st February 1996; 17th April 1996; 3rd July 1996 and 16th November 1996) as described previously (chapter 2; section 2.2.4.). The samples were taken back to the laboratory, filtered through GF/C filters and frozen at -20°C until required.

The loch water was thawed and filter-sterilised (Stericup-GS; 0.22 μm) prior to use in this experiment. Aliquots (100 ml) of sterile loch water were added to sterile 250 ml Erlenmeyer flasks and four selected autoclaved nutrients added asceptically in amounts equivalent to that added when preparing BG11 mediium. These nutrients were added together or in varying combinations resulting in six treatments (Table 3.1).

Three replicates per treatment were prepared. *M. aeruginosa* PCC 7820 which had been growing for 7 days was centrifuged at 1500 x g for 60 minutes. The supernatant was removed and the pellet of cells re-suspended in 250 ml of filter-sterilised loch water, to avoid carry-over of BG11 nutrients. Each flask was inoculated with 10 ml of this stock culture and incubated in a waterbath at 25°C for 14 days. Flasks were analysed for parameters described previously (section 3.2.1.).

It was observed in the initial bioassay that the cells were growing together in clumps/colonies. It was suspected that there may have been some factor(s) present in the water which was inducing this clumping effect. Thus the above bioassay was repeated with the water sample collected on 1st February 1996 but on this occasion the loch water was sterilised by autoclaving for 30 minutes at 120°C instead of being filter-sterilised, to determine if the factor(s) was heat labile. The loch water and nutrient additions were autoclaved separately.

Table 3.1. Concentration of nutrients added to natural Loch Rescobie water used in bioassays.

| **Treatments** | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Nitrate (mM)* | - | 8.8 | 8.8 | - | 8.8 | 8.8 |
| Phosphate (mM)* | - | 0.23 | - | 0.23 | 0.23 | 0.23 |
| Trace elements(ml)† | - | 0.1 | 0.1 | 0.1 | 0.1 | - |
| Iron* | - | 0.022 | 0.022 | 0.022 | - | 0.022 |

* Nitrate = mM $NaNO_3$; Phosphate = mM $K_2HPO_4$; Iron = mM $FeSO_4.7H_2O$.

† BG11 trace elements stock solution.

### 3.2.4. Investigation of the role of *Daphnia* sp. in the stimulation of colony formation by *Microcystis aeruginosa*

An experiment was carried out to investigate if the presence of *Daphnia* sp. in lake water may be responsible for colony formation by *M. aeruginosa*, which was observed during an initial bioassay. *Daphnia magna* was cultured in *Daphnia* media (ASTM, 1980) for 21 days and fed daily with *Chlorella vulgaris*. Media without *D. magna* was used as a control. After this time the *Daphnia* water was filtered through GF/C filter disks. Additions of nutrients as in BG11 media were made to the *Daphnia* water and the control. Half the *Daphnia* water and control were pre-treated by sterile-filtration and the other half by autoclaving as described previously (each treatment prepared in triplicate). Aliquots (10 ml) of a *M. aeruginosa* culture were asceptically inoculated into all the flasks and incubated at 25°C under continuous illumination for 24 days. The flasks were observed for any colony formation during this incubation period and analysed for parameters as described in section 3.2.1.

## 2.5. Statistical analyses

All data was subjected to a two-way analysis of variance using Tukey's honestly significant difference test ($p=0.05$). However, at times the data was not normally distributed

(failed Levene's homogeneity of variance test) and a non-parametric (Kruskal-Wallis test) was used. This is equivalent of two-way analysis of variance when there are two or more treatments.

## 3.3. RESULTS

### 3.3.1. Effect of nitrate on the growth and toxin content of *Microcystis aeruginosa* PCC 7820

At the end of the 14 day bioassay it was observed that the cultures of *M. aeruginosa* grown in media minus nitrate were unhealthy. These cultures had significantly lower optical density readings ($p<0.05$; Anova), chlorophyll *a* content and dry weight ($p<0.05$; Kruskal-Wallis) compared to the other treatments. They also contained significantly lower amounts of microcystin variants (MCYST-LR; MCYST-LY; MYCST-LW and MYST-LF) known to be produced by this cyanobacterium ($p<0.05$; Kruskal-Wallis).

There was no significant difference in optical density readings ($p>0.05$; Anova) between cultures grown in media plus 1.76 mM, 8.8 mM and 17.6 mM nitrate but there was a significant difference in the chlorophyll *a* content ($p<0.05$; Anova) of these three treatments. Cultures grown in media with the highest nitrate addition (17.6 mM) had the greatest level of chlorophyll *a* followed by cultures grown in media plus 8.8 mM nitrate and then cultures grown in media plus 1.76 mM nitrate. It was observed that the latter cultures appeared more unhealthy at the end of the bioassay and were beginning to senesce.

Cultures grown in media plus 1.76 mM nitrate had significantly higher dry weights ($p<0.05$; Anova) than cultures grown in 8.8 mM and 17.6 mM nitrate which were not significantly different from each other ($p>0.05$; Anova).

There was a significant difference in toxin content between all treatments ($p<0.05$; Kruskal-Wallis). Cultures grown in 8.8 mM nitrate contained significantly more microcystins than cultures grown in the highest nitrate concentration ($p<0.05$; Anova) (Table 3.2).

### 3.2. Effect of phosphate on the growth and toxin content of *Microcystis aeruginosa* PCC 7820

The initial bioassay used a stock culture of *M. aeruginosa* grown in BG11 media plus phosphate (section 3.2.2). At the end of the 14 day bioassay it was observed that the cultures grown in media minus phosphate were very unhealthy in appearance. These cultures had significantly lower optical density readings, chlorophyll *a* content and dry weights ($p<0.05$; Kruskal-Wallis) compared to the other treatments. These cultures also produced significantly lower amounts of microcystins ($p<0.05$; Kruskal-Wallis).

There was no significant difference ($p>0.05$; Kruskal-Wallis) between the cultures grown in 0.023 and 0.115 mM phosphate as regards optical density readings, chlorophyll *a* content, dry

Table 3.2. Effect of varying nitrate concentration in BG11 media on the growth and toxin production of *Microcystis aeruginosa* PCC 7820.

| Nitrate ($NaNO_3$) concentration | O.D @ 720 nm | Chlorophyll a ($\mu gml^{-1}$) | Dry Weight ($\mu gml^{-1}$) | MCYST-LR ($\mu gg^{-1}$ dry wt) | MCYST-LY ($\mu gg^{-1}$ dry wt) | MCYST-LW ($\mu gg^{-1}$ dry wt) | MCYST-LF ($\mu gg^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **0 mM** | | | | | | | |
| Mean | 0.094 | 0.227 | 72 | 349 | 67 | 130 | 121 |
| $\sigma_{n-1}$ | 0.001 | 0.009 | 3 | 19 | 6 | 12 | 15 |
| **1.76 mM** | | | | | | | |
| Mean | 0.673 | 4.368 | 488 | 911 | 186 | 455 | 314 |
| $\sigma_{n-1}$ | 0.012 | 0.125 | 17 | 107 | 23 | 49 | 38 |
| **8.8 mM** | | | | | | | |
| Mean | 0.698 | 7.285 | 392 | 1637 | 359 | 712 | 569 |
| $\sigma_{n-1}$ | 0.066 | 0.288 | 31 | 87 | 16 | 28 | 38 |
| **17.6 mM** | | | | | | | |
| Mean | 0.687 | 7.795 | 412 | 1378 | 302 | 526 | 447 |
| $\sigma_{n-1}$ | 0.049 | 0.335 | 36 | 193 | 35 | 64 | 60 |

weights and microcystin content. However, cultures grown in 0.230 mM phosphate appeared to grow slightly less and contain less microcystins than cultures in 0.023 and 0.115 mM phosphate (Table 3.3).

Cyanobacteria can have phosphate reserves which they utilise at times when phosphate is limiting in the environment. The previous phosphate bioassay was repeated using a stock culture of *M. aeruginosa* which had been growing in BG11 media minus phoshate for seven days so that any phosphate reserves would be depleted after this time.

At the end of the 14 day bioassay the cultures grown in media minus phosphate appeared unhealthy. These cultures had significantly lower optical density readings ($p<0.05$; Kruskal-Wallis), chlorophyll *a* content and dry weights ($p<0.05$; Anova) than the other treatments. These cultures also contained significantly lower amounts of MCYST-LY, MCYST-LW and MCYST-LF ($p<0.05$; Kruskal-Wallis). However, the amount of MCYST-LR detected was not significantly different ($p>0.05$; Anova) from cultures grown in 0.115 and 0.230 mM phosphate.

There was no significant difference in optical density readings ($p>0.05$; Kruskal-Wallis) or dry weights ($p>0.05$; Anova) between cultures grown in 0.023, 0.115 and 0.230 mM phosphate. The chlorophyll *a* content of cultures grown in 0.115 and 0.230 mM phosphate were not significantly different

Table 3.3. Effect of varying phosphate concentration in BG11 media on the growth and toxin production of *Microcystis aeruginosa* PCC 7820.

| Phosphate ($K_2HPO_4$) concentration | O.D @ 720 nm | Chlorophyll a ($\mu gml^{-1}$) | Dry Weight ($\mu gml^{-1}$) | MCYST-LR ($\mu gg^{-1}$ dry wt) | MCYST-LY ($\mu gg^{-1}$ dry wt) | MCYST-LW ($\mu gg^{-1}$ dry wt) | MCYST-LF ($\mu gg^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **0 mM** | | | | | | | |
| Mean | 0.175 | 1.137 | 104 | 940 | 79 | 136 | 165 |
| $\sigma_{n-1}$ | 0.027 | 0.287 | 2 | 172 | 16 | 24 | 30 |
| **0.023 mM** | | | | | | | |
| Mean | 0.531 | 4.067 | 230 | 1920 | 221 | 404 | 501 |
| $\sigma_{n-1}$ | 0.001 | 0.072 | 5 | 32 | 4 | 2 | 1 |
| **0.115 mM** | | | | | | | |
| Mean | 0.539 | 4.149 | 212 | 1679 | 207 | 378 | 477 |
| $\sigma_{n-1}$ | 0.014 | 0.080 | 41 | 300 | 50 | 83 | 103 |
| **0.23 mM** | | | | | | | |
| Mean | 0.451 | 3.726 | 218 | 1183 | 167 | 298 | 381 |
| $\sigma_{n-1}$ | 0.025 | 0.121 | 9 | 112 | 13 | 22 | 29 |

* The stock culture of *M. aeruginosa* used as inoculum was initially grown in BG11 media plus phosphate

but cultures grown in 0.023 mM phosphate had significantly lower chlorophyll *a* than those grown in 0.230 mM phosphate ($p<0.05$; Anova). This indicated that cultures growing in 0.023 mM phosphate were growing slightly less than cultures grown in the highest concentration.

The amount of MCYST-LR was significantly higher in cultures grown in 0.023 mM phosphate ($p<0.05$; Anova) but no significant difference between the other treatments ($p>0.05$; Anova) was observed. The amount of MCYST-LY, MCYST-LW and MCYST-LF was also greater in cultures grown in 0.023 mM phosphate ($p<0.05$; Anova) but was significantly lower in those grown in 0 mM phosphate. There was no significant difference in the amount of these microcystin variants produced by cultures grown in 0.115 and 0.230 mM phosphate (Table 3.4).

### 3.3.3. A bioassay to assess the potential factors limiting growth and microcystin content of *Microcystis aeruginosa* in a freshwater loch

The concentrations of nitrite and phosphate in the water samples collected during the year are summarised in Table 3.5.

#### February bioassay

It was observed at the end of the bioassay that the cultures grown in treatments 3 (minus phosphate) and 5 (minus iron) had deteriorated considerably. Cultures grown in treatments 1

Table 3.4. Effect of varying phosphate concentration in BG11 media on the growth and toxin production of *Microcystis aeruginosa* PCC 7820.

| Phosphate ($K_2HPO_4$) concentration | O.D @ 720 nm | Chlorophyll a ($\mu gml^{-1}$) | Dry Weight ($\mu gml^{-1}$) | MCYST-LR ($\mu gg^{-1}$ dry wt) | MCYST-LY ($\mu gg^{-1}$ dry wt) | MCYST-LW ($\mu gg^{-1}$ dry wt) | MCYST-LF ($\mu gg^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **0 mM** | | | | | | | |
| Mean | 0.152 | 0.661 | 112 | 2043 | 113 | 181 | 179 |
| $\sigma_{n-1}$ | 0.004 | 0.007 | 8 | 166 | 11 | 14 | 15 |
| **0.023 mM** | | | | | | | |
| Mean | 0.615 | 3.642 | 249 | 2772 | 345 | 650 | 660 |
| $\sigma_{n-1}$ | 0.067 | 0.430 | 22 | 336 | 39 | 98 | 100 |
| **0.115 mM** | | | | | | | |
| Mean | 0.579 | 4.294 | 249 | 1856 | 201 | 353 | 444 |
| $\sigma_{n-1}$ | 0.021 | 0.284 | 9 | 167 | 10 | 22 | 31 |
| **0.23 mM** | | | | | | | |
| Mean | 0.561 | 4.353 | 251 | 1820 | 210 | 368 | 466 |
| $\sigma_{n-1}$ | 0.016 | 0.146 | 8 | 78 | 18 | 36 | 46 |

* The stock culture of *M. aeruginosa* used as inoculum was initially grown in BG11 media minus phosphate

(unenriched) and 4 (minus nitrate) had also begun to appear unhealthy. In contrast, cultures grown in treatments 6 (minus trace elements) and 2 (enriched) were still actively growing.

It was also observed that the *M. aeruginosa* cells had formed colonies or clumped together. *Microcystis* spp. grows in this manner in nature but this ability is lost/halted when they are grown as laboratory cultures. The bioassay was repeated but this time the loch water was sterilised by autoclaving instead of sterile-filtration. The clumping effect was not observed in any of the cultures.

Treatments 2 (enriched) and 6 (minus trace elements) had significantly higher optical density, chlorophyll *a* content and dry weights ($p<0.05$; Kruskal-Wallis) compared to the other treatments but they were not significantly different from each other ($p>0.05$; Kruskal-Wallis). Treatment 2 (enriched) had significantly higher levels of microcystins than the other treatments while treatment 5 (minus iron) had significantly less ($p<0.05$; kruskal-Wallis). There was no significance difference in toxin content between the remaining treatments (Table 3.6).

Table 3.5. The concentrations of nitrite and phosphate in Loch Rescobie water sampled seasonally during 1996.

| Date | Phosphate ($mgl^{-1}$ $PO_4$-P) | Nitrite ($mgl^{-1}$ $NO_2$-N) |
|---|---|---|
| 22/2/96 | 0.02-0.03 | 0.02 |
| 20/3/96 | 0.01-0.09 | - |
| 4/7/96 | 0.01-0.03 | 0.02 |
| 26/9/96 | 0.18-0.22 | <0.01 |

*Data supplied by SEPA east region

**April bioassay**

At the end of this bioassay, cultures grown in treatments 1 (unenriched), 4 (minus nitrate) and 5 (minus iron) appeared either dead or very unhealthy. These cultures had significantly lower optical density readings, chlorophyll *a* content and dry weights compared to the other treatments ($p<0.05$; Anova). These cultures also contained significantly lower amounts of microcystins ($p<0.05$; Anova).

There was no significant difference between treatments 2 (enriched), 3 (minus phosphate) and 6 (minus trace elements) in optical density readings or dry weights ($p<0.05$; Anova). However, it appeared that treatment 2 (enriched) seemed to be growing less when expressed as chlorophyll *a* content. These cultures produced significantly different amounts of MCYST-LR.

Table 3.6. Effect of varying selected nutrients in filter-sterilsed natural water, collected from Loch Rescobie on 2/2/96, on the growth and toxin production of *Microcystis aeruginosa* PCC 7820.

| Treatments | O.D @ 720 nm | Chlorophyll a ($\mu g ml^{-1}$) | Dry Weight ($\mu g ml^{-1}$) | MCYST-LR ($\mu g g^{-1}$ dry wt) | MCYST-LY ($\mu g g^{-1}$ dry wt) | MCYST-LW ($\mu g g^{-1}$ dry wt) | MCYST-LF ($\mu g g^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **Unenriched** | | | | | | | |
| Mean | 0.191 | 1.490 | 99 | 1355 | 144 | 339 | 585 |
| $\sigma_{n-1}$ | 0.045 | 0.588 | 9 | 136 | 19 | 33 | 82 |
| **Enriched** | | | | | | | |
| Mean | 0.290 | 2.298 | 195 | 2003 | 178 | 427 | 727 |
| $\sigma_{n-1}$ | 0.042 | 1.248 | 30 | 360 | 35 | 68 | 132 |
| **- Phosphate** | | | | | | | |
| Mean | 0.118 | 0.518 | 63 | 1191 | 82 | 238 | 324 |
| $\sigma_{n-1}$ | 0.020 | 0.125 | 5 | 484 | 33 | 95 | 137 |
| **- Nitrate** | | | | | | | |
| Mean | 0.181 | 1.065 | 164 | 1225 | 101 | 235 | 414 |
| $\sigma_{n-1}$ | 0.020 | 0.156 | 24 | 127 | 8 | 28 | 33 |
| **- Iron** | | | | | | | |
| Mean | 0.101 | 0.261 | 50 | 1011 | 61 | 135 | 246 |
| $\sigma_{n-1}$ | 0.011 | 0.057 | 10 | 445 | 18 | 40 | 33 |
| **- Trace element** | | | | | | | |
| Mean | 0.264 | 2.913 | 191 | 1340 | 124 | 288 | 488 |
| $\sigma_{n-1}$ | 0.001 | 0.138 | 8 | 196 | 16 | 31 | 45 |

Treatment 3 (minus phosphate) produced the greatest amount, followed by treatment 6 (minus trace elements) and then treatment 2 (enriched)($p<0.05$; Anova).

Treatment 3 (minus phosphate) produced significantly more MCYST-LY, MCYST-LW and MCYST-LF, followed by both treatment 2 (enriched) and 6 (minus trace elements) which were not significantly different from each other (Table 3.7).

**July bioassay**

It was observed at the end of this bioassay that cultures grown in treatments 3 (minus phosphate), 4 (minus nitrate) and 5 (minus iron) were all dead. These cultures had significantly lower optical density readings, chlorophyll *a* content and dry weights compared to the other treatments ($p<0.05$; KruskaL-Wallis). No microcystins were detected in these cultures.

Both optical density readings and chlorophyll *a* measurements show that treatments 1 (unenriched), 2 (enriched) and 6 (minus trace elements) are all significantly different from each other ($p<0.05$; Anova) with treatment 6 being significantly higher. Treatment 6 also had a significantly higher dry weight but the dry weights of treatments 1 and 2 were not significantly different from each other ($p>0.05$; Anova).

The microcystin content of treatments 2 (enriched) and 6 (minus trace elements) were not significantly different

Table 3.7. Effect of varying selected nutrients in filter-sterilsed natural water, collected from Loch Rescobie on 17/4/96, on the growth and toxin production of *Microcystis aeruginosa* PCC 7820.

| Treatments | O.D @ 720 nm | Chlorophyll a ($\mu gml^{-1}$) | Dry Weight ($\mu gml^{-1}$) | MCYST-LR ($\mu gg^{-1}$ dry wt) | MCYST-LY ($\mu gg^{-1}$ dry wt) | MCYST-LW ($\mu gg^{-1}$ dry wt) | MCYST-LF ($\mu gg^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **Unenriched** | | | | | | | |
| Mean | 0.384 | 1.457 | 308 | 675 | 86 | 127 | 176 |
| $\sigma_{n-1}$ | 0.006 | 0.062 | 26 | 64 | 3 | 6 | 6 |
| **Enriched** | | | | | | | |
| Mean | 0.655 | 3.065 | 378 | 1357 | 180 | 330 | 428 |
| $\sigma_{n-1}$ | 0.036 | 0.371 | 19 | 163 | 24 | 44 | 55 |
| **- Phosphate** | | | | | | | |
| Mean | 0.640 | 3.940 | 385 | 2357 | 363 | 588 | 739 |
| $\sigma_{n-1}$ | 0.071 | 1.08 | 38 | 144 | 26 | 47 | 62 |
| **- Nitrate** | | | | | | | |
| Mean | 0.342 | 0.930 | 292 | 622 | 76 | 113 | 179 |
| $\sigma_{n-1}$ | 0.004 | 0.120 | 19 | 134 | 10 | 19 | 31 |
| **- Iron** | | | | | | | |
| Mean | 0.104 | 0.229 | 102 | 292 | 32 | 47 | 62 |
| $\sigma_{n-1}$ | 0.018 | 0.084 | 7 | 91 | 13 | 19 | 26 |
| **- Trace element** | | | | | | | |
| Mean | 0.699 | 5.052 | 426 | 1886 | 214 | 349 | 503 |
| $\sigma_{n-1}$ | 0.054 | 0.847 | 30 | 152 | 6 | 10 | 24 |

from each other ($p>0.05$; Anova). However, treatment 1 (unenriched) contained significantly less microcystins ($p<0.05$; Anova) (Table 3.8).

**November bioassay**

It was observed that only cultures in media minus trace elements were healthy at the end of this bioassay and contained significantly more microcytins. Cultures growing without iron or phosphate additions were dead and no microcystin variants were detected. Cultures in unenriched, enriched and minus nitrate media also appeared unhealthy and variations between replicates was large. Only MCYST-LR was detected in unenriched and minus nitrate treatments whereas all four microcystin variants were detected in enriched cultures (Table 3.9).

### 3.3.4. Investigation of the role of *Daphnia* sp. in the stimulation of colony formation by *M. aeruginosa*

No colony formation by *M. aeruginosa* was observed after being cultured in *Daphnia* water for 24 days. There was no significant difference in chlorophyll *a* ($\mu g ml^{-1}$) and microcystin content between cultures grown in *Daphnia* water pre-treated by filter-sterilisation and a control which was not exposed to *Daphnia* sp. ($p>0.05$; Anova). There was also no significant difference between the *Daphnia* water pre-treated

Table 3.8. Effect of varying selected nutrients in filter-sterilsed natural water, collected from Loch Rescobie on 3/7/96, on the growth and toxin production of *Microcystis aeruginosa* PCC 7820.

| Treatments | O.D @ 720 nm | Chlorophyll a ($\mu gml^{-1}$) | Dry Weight ($\mu gml^{-1}$) | MCYST-LR ($\mu gg^{-1}$ dry wt) | MCYST-LY ($\mu gg^{-1}$ dry wt) | MCYST-LW ($\mu gg^{-1}$ dry wt) | MCYST-LF ($\mu gg^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **Unenriched** | | | | | | | |
| Mean | 0.098 | 0.345 | 114 | 645 | 73 | 117 | 176 |
| $\sigma_{n-1}$ | 0.009 | 0.045 | 4 | 34 | 7 | 21 | 28 |
| **Enriched** | | | | | | | |
| Mean | 0.240 | 0.993 | 142 | 1734 | 235 | 454 | 562 |
| $\sigma_{n-1}$ | 0.018 | 0.120 | 16 | 66 | 7 | 15 | 15 |
| **- Phosphate** | | | | | | | |
| Mean | 0.011 | ND | 28 | ND | ND | ND | ND |
| $\sigma_{n-1}$ | 0.001 | | 6 | | | | |
| **- Nitrate** | | | | | | | |
| Mean | 0.026 | ND | 27 | ND | ND | ND | ND |
| $\sigma_{n-1}$ | 0.003 | | 6 | | | | |
| **- Iron** | | | | | | | |
| Mean | 0.013 | ND | 42 | ND | ND | ND | ND |
| $\sigma_{n-1}$ | 0.001 | | 9 | | | | |
| **- Trace element** | | | | | | | |
| Mean | 0.347 | 2.105 | 224 | 1868 | 254 | 460 | 588 |
| $\sigma_{n-1}$ | 0.036 | 0.196 | 18 | 354 | 55 | 104 | 134 |

*ND: Not detected

Table 3.9. *Effect of varying selected nutrients in filter-sterilsed natural water, collected from Loch Rescobie on 16/11/96, on the growth and toxin production of Microcystis aeruginosa PCC 7820.*

| Treatments | O.D @ 720 nm | Chlorophyll a ($\mu g ml^{-1}$) | Dry Weight ($\mu g ml^{-1}$) | MCYST-LR ($\mu g g^{-1}$ dry wt) | MCYST-LY ($\mu g g^{-1}$ dry wt) | MCYST-LW ($\mu g g^{-1}$ dry wt) | MCYST-LF ($\mu g g^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **Unenriched** | | | | | | | |
| Mean | 0.077 | 0.261 | 50 | 98 | ND | ND | ND |
| $\sigma_{n-1}$ | 0.003 | 0.007 | 4 | 43 | | | |
| **Enriched** | | | | | | | |
| Mean | 0.130 | 0.707 | 83 | 602 | 121 | 215 | 238 |
| $\sigma_{n-1}$ | 0.045 | 0.342 | 17 | 330 | 52 | 70 | |
| **- Phosphate** | | | | | | | |
| Mean | 0.016 | ND | 29 | ND | ND | ND | ND |
| $\sigma_{n-1}$ | 0.004 | | 2 | | | | |
| **- Nitrate** | | | | | | | |
| Mean | 0.043 | 0.156 | 52 | 90 | ND | ND | ND |
| $\sigma_{n-1}$ | 0.027 | 0.070 | 17 | 68 | | | |
| **- Iron** | | | | | | | |
| Mean | 0.012 | ND | 41 | ND | ND | ND | ND |
| $\sigma_{n-1}$ | 0.001 | | 2 | | | | |
| **- Trace element** | | | | | | | |
| Mean | 0.272 | 1.705 | 174 | 1153 | 136 | 236 | 299 |
| $\sigma_{n-1}$ | 0.012 | 0.108 | 4 | 36 | 10 | 9 | 6 |

by autoclaving and its control ($p>0.05$; Anova). However, it was observed that cultures in water that had been pre-treated by autoclaving had grown on average three times better than those grown in water which had been filter-sterilised (Table 3.10), but no significant difference in MCYST-LR or MCYST-LY content was observed between the two treatments ($p>0.05$; Anova). The autoclaved cultures produced slightly more MCYST-LW and MCYST-LF than the filter-sterilised cultures.

Table 3.10. Effect of culturing *Microcystis aeruginosa* PCC 7820 in water pre-exposed to *Daphnia Magna*. Pre-treatment of both *Daphnia* water (+) and the control (-), (water not pre-exposed to *D. magna*), by autoclaving and filter-sterilisation were compared.

| Treatment | O.D @ 720 nm | Chlorophyll a ($\mu gml^{-1}$) | Dry Weight ($\mu gml^{-1}$) | MCYST-LR ($\mu gg^{-1}$ dry wt) | MCYST-LY ($\mu gg^{-1}$ dry wt) | MCYST-LW ($\mu gg^{-1}$ dry wt) | MCYST-LF ($\mu gg^{-1}$ dry wt) |
|---|---|---|---|---|---|---|---|
| **Autoclaved (+)** | | | | | | | |
| Mean | 1.002 | 10.700 | 325 | 2022 | 308 | 632 | 491 |
| $\sigma_{n-1}$ | 0.022 | 0.555 | 50 | 99 | 32 | 62 | 75 |
| **Autoclaved (-)** | | | | | | | |
| Mean | 1.215 | 13.220 | 345 | 2287 | 322 | 653 | 527 |
| $\sigma_{n-1}$ | 0.021 | 0.619 | 28 | 292 | 34 | 55 | 59 |
| **Filtered (+)** | | | | | | | |
| Mean | 0.673 | 4.205 | 220 | 1807 | 226 | 424 | 314 |
| $\sigma_{n-1}$ | 0.006 | 0.050 | 5 | 213 | 26 | 48 | 39 |
| **Filtered (-)** | | | | | | | |
| Mean | 0.582 | 3.827 | 192 | 1610 | 213 | 395 | 294 |
| $\sigma_{n-1}$ | 0.129 | 1.415 | 24 | 459 | 78 | 153 | 108 |

## 3.4. DISCUSSION

Algal bioassays have been used frequently to assess the potential fertility of waterbodies (Lund *et al.*, 1971, 1975; Reynolds and Butterwick, 1979; Couture *et al.*, 1985; Wurtsbaugh *et al.*, 1985; Van der Does and Klapwijk, 1987). The simplest bioassay design involves the addition of an alga/cyanobacterium to various water types which are then grown under constant conditions of light and temperature. The growth of the alga/cyanobacterium after a specified time gives an indication of how fertile the different waters are. However, Lund *et al.* (1971) suggest that more than one type of alga/cyanobacterium should be used as some waters may not support the growth of a particular species. In this study the cyanobacterium *M. aeruginosa* PCC 7820 was used as it was originally isolated from an adjacent loch (Stewart *et al.*, 1977) and even becomes dominant at times in the waters of Loch Rescobie. The bioassay can be taken a step further and used to investigate the potential fertility of waters. Nutrients and trace elements can be added alone or in combination to water to observe if a stimulation in the growth of the alga/cyanobacterium occurs. If addition of a particular nutrient causes significant growth compared to unenriched water it can be suspected that this nutrient may be limiting growth.

In this study the above bioassay was adapted to investigate the effect of nutrients on the growth and toxin content of *M. aeruginosa* PCC 7820 inoculated into either BG11 media or filtered natural water from Loch Rescobie, unmodified or spiked with selected nutrients. It was designed to replace the lengthy traditional approach used to assess the effect of environmental factors on growth and toxin production. This type of experiment is usually run from 8 to 10 weeks with weekly monitoring of the cultures. Problems experienced with this approach include the cultures being grown in larger volumes (1 - 5 l) to facilitate weekly sampling. These cultures must be aerated by sparging with sterile air and this usually leads to uneven evaporation of culture replicates. In the present study a bioassay was developed which had a 14 day endpoint at which time the cultures were assessed for growth and microcystin content. This endpoint was selected as cultures would not yet have entered stationary phase. This meant that the cultures could be grown in smaller volumes without the need for artificial aeration.

The method of Lund *et al.* (1971) involved the collection of a natural water sample which was filtered through a Whatman GF/C filter disk before addition of the test algae (*Asterionella formosa*). Lund *et al.* (1971) reported that filtration of the water through sintered glass, membrane filters (0.45 μm) or sterilisation under pressure all reduced the growth of *A.*

*formosa* in bioassays compared to filtration through GF/C filters. In this study, however, a bioassay was set up as described above using *M. aeruginosa* but it was soon observed that the cultures had become heavily contaminated by bacterial growth. It was decided to pre-treat natural loch water by filter-sterilisation (0.22 μm) prior to use in the bioassay.

Lund *et al.* (1971) also described considerable reduction in the growth of the algae in waters which had been autoclaved compared to waters which had been boiled. He also observed that boiling filtered lake water could on occasion increase its fertility. Couture *et al.* (1985) also remarks on the results of bioassays when the water is pre-treated by autoclaving or filtration.

In this study it was observed that cultures of *M. aeruginosa* had grown on average three times better (as expressed in $\mu g ml^{-1}$ chlorophyll *a*) in BG11 medium which had been pre-treated by autoclaving in comparison to filter-sterilisation (0.22 μm) after a 24 day incubation period. It was interesting to note that although the autoclaved cultures grew much better than the filter-sterilised ones there was no significant difference in production of MCYST-LR and -LY.

It was also observed during a bioassay of Loch Rescobie water collected in February that *M. aeruginosa* grew in

clumps/colonies when the water was pre-treated by filtration. However, when the bioassay was repeated using the same water pre-treated by autoclaving, this growth pattern was lost suggesting the factor(s) was heat labile. This colony formation was not observed in any of the bioassays thereafter. Hessen and Donk (1993) discovered that the green algae *Scenedesmus acutus*, which grows in unicellular form in laboratory cultures, formed colonies when exposed for 48 hours to a chemical released by *Daphnia magna*. They suggest that this colony formation may be protection from grazing. Lampert *et al.* (1994) also observed that the addition of filtered *Daphnia* water to a culture of *Scenedesmus* resulted in a significant increase in colony formation. They also observed that colony formation depended on the concentration of the *Daphnia* factor and actively feeding *Daphnia* produced more colony formation than starved ones. They discovered that the chemical activity was heat stable (60°C) but was destroyed by incineration. However, in this study no induction of colony formation was observed when *M. aeruginosa* was inoculated into medium prepared from *Daphnia* water.

Nitrate is the most common form of nitrogen in freshwaters and is the form most commonly used by phytoplankton (Horne and Goldman, 1994). This study observed that media deficient or low (1.76 mM $NaNO_3$; 150 $mgl^{-1}$) in nitrate significantly reduced both chlorophyll *a* and microcystin content of *M. aeruginosa*

PCC 7820 compared to cultures grown in higher nitrate (8.8 mM; 750 $mgl^{-1}$ and 17.6 mM;1500 $mgl^{-1}$ $NaNO_3$) concentrations. These results are in agreement with those of Codd and Poon (1988) who reported that cultures of *M. aeruginosa* PCC 7813 transferred from complete growth medium to medium minus nitrate resulted in a tenfold decrease in toxicity by mouse bioassay. In this study it was observed that cultures grown in complete medium (17.6 mM $NaNO_3$; 1500 $mgl^{-1}$) produced approximately four times the amount of MCYST-LR than cultures grown in medium minus nitrate. Utkilen and Gjølme (1995) also found that the amount of MCYST-RR (expressed as ng toxin $\mu g^{-1}$ dry weight) produced by *M. aeruginosa* CYA 228/1 decreased in nitogen-limited (0.6 mM; 51 $mgl^{-1}$ and 0.35 mM; 30 $mgl^{-1}$ $NaNO_3$) medium. However, when they expressed the microcystin content as ng toxin per ug protein they discovered that nitrate and phosphate-limitation had no effect on toxin content of cultures.

It was also observed in this study that cultures grown in the highest nitrate concentration (17.6 mM; 1500 $mgl^{-1}$ $NaNO_3$) grew slightly better than those in 8.8 mM; 750 $mgl^{-1}$ $NaNO_3$ but the latter produced slightly more microcystins. This is in contrast to the results of Campbell (1994) who reported that an increase in the level of nitrate in media resulted in an increase of both growth and MCYST-LR content in cultures of *M. aeruginosa* PCC 7813. However, the concentrations compared were

17.6 mM (1500 $mgl^{-1}$) and 1.76 mM (150 $mgl^{-1}$) $NaNO_3$. An increase in chlorophyll *a* and microcystin content was also observed in this study when these two concentrations were compared but optimum microcystin content was observed at 8.8 mM (750 $mgl^{-1}$) $NaNO_3$. Other researchers (National Rivers Authority, 1991) observed no difference in microcystin content between cultures of *M. aeruginosa* PCC 7813 in media plus 8.8 mM (750 $mgl^{-1}$) $NaNO_3$ and 17.6 mM (1500 $mgl^{-1}$) $NaNO_3$. However, in a steady-state chemostat system they observed that reducing nitrate levels from 17.6 mM to 1.76 mM $NaNO_3$ increased the level of MCYST-LR detected and cultures in 8.8 mM $NaNO_3$ produced significantly more MCYST-LR than those in 17.6 mM $NaNO_3$ which is comparable to the results of this study.

Sivonen (1990) reported that high nitrogen concentrations in media favoured the growth and toxin production of two strains of *Oscillatoria agardhii*, however, the nitrate levels she used were considerable lower (0.42-84 $mgl^{-1}$) than in this study (0-1500 $mgl^{-1}$; 0-17.6 mM $NaNO_3$). Increases in growth and microcystin production were also observed in this study up to 8.8 mM $NaNO_3$ (750 $mgl^{-1}$).

Phosphate is often the most common growth-limiting factor for phytoplankton in freshwater. Most phosphorus is held in biologically unavailable forms by paticulates and phytoplankton can only use soluble phosphate for growth.

Concentrations of phosphate less than 10 $\mu g l^{-1}$ will limit growth (Campbell, 1994). In this study it was observed that cultures of *M. aeruginosa* grown in medium minus phosphate grew less well and produced significantly less microcystins than cultures with phosphate additions except in the second phosphate bioassay (cells initially grown in BG11 medium minus phosphate) when phosphate deficient cultures produced similar amounts of MCYST-LR as cultures in 0.115 mM (20 $mg l^{-1}$) and 0.23 mM (40 $mg l^{-1}$) $K_2PO_4$ although the other variants were still lower. This is in contrast to Codd and Poon (1988) who reported that cultures of *M. aeruginosa* PCC 7813 transferred from complete growth medium (0.23 mM; 40 $mg l^{-1}$ $K_2PO_4$) to medium minus phosphate did not affect toxicity.

Sivonen (1990) observed that growth and toxin production by two strains of *Oscillatoria* did not seem to be affected by phosphate concentrations between 0.4 and 5.5 $mg g^{-1}$ and Watanabe and Oishi (1985) only observed a small decrease in toxicity of *M. aeruginosa* when grown in full strength medium and medium at 1/10 and 1/20 the original concentration. Other researchers (National Rivers Authority, 1991) observed that a culture of *Anabaena flos-aqua* NRC 525-17 (which was producing 8 microcystin variants) produced slightly more of one of the variants in medium with reduced phosphate concentration (0.023 mM; 4 $mg l^{-1}$ $K_2PO_4$) compared to higher concentrations (0.23 mM; 40 $mg l^{-1}$ $K_2PO_4$).

The three normally used criteria for monitoring growth of phytoplankton are optical density, chlorophyll *a* content and dry weight. However, it was observed at times in this study that these citeria did not correspond. In the nitrate bioassay the optical density readings of three nitrate concentrations did not vary significantly but the lowest concentration had less chlorophyll *a* and a higher dry weight. This suggest that adopting a single criterion for growth may be misleading. It is suggested that cell numbers should also be monitored.

In general nitrate and phosphate levels in freshwater lochs are normally higher during the winter than in the summer (Horne and Goldman, 1994). Phosphate levels less than 10 $\mu gl^{-1}$ and nitrate levels less than 100 $\mu gl^{-1}$ may limit phytoplankton growth. Phosphate levels during the sampling period ranged between 10 and 220 $\mu gl^{-1}$ and thus never fell below levels quoted to be limiting to growth. Rescobie is classified as a shallow eutrophic lake. Mean phosphate and chlorophyll a levels recorded during 1991-94 were 137 and 42 $\mu gl^{-1}$ respectively which would indicate that the loch is eutrophic (Clelland, 1995).

It was observed that both nitrate and iron were limiting growth and microcystin content of *M. aeruginosa* in Loch Rescobie water collected in February, April, July and November. Phosphate was also limiting except in the April

sample when both growth and microcystin production by *M. aeruginosa* were high in waters minus phosphate additions. The most severe limitation of growth caused by the lack of these nutrients was observed in the July and November water samples when cultures were completely dead at the end of the bioassay. It was suprising to find that phosphate was a limiting factor as the concentrations recorded in the loch are much greater than those required to sustain growth. Unfortunately no data on nitrate levels was available.

Reynolds and Butterwick (1979) observed that *Asterionella* was able to sustain growth in an unfertilised enclosure in a loch for longer than the filtered water would support growth in a bioassay. They suggested that reduction of dissolved nutrient concentrations through prior uptake by algae and sequestering of ionic species by particulates may lead to the underestimation of their immediate availability to natural populations. It is also known that algae can produce alkaline phosphatases that cleave the bond between phosphate and the particulate to which it is attached resulting in free phosphate for growth. This phosphate source would not be available to algae growing in a bioassay using filtered loch water. This presents a problem when using short-term bioassays to assess fertility of waters. It is important to have chemical analysis data of the loch water when interpreting bioassay results. Care must be taken when interpreting results

from bioassays used to assess the factors limiting both growth and toxin content of cyanobacteria in natural waters.
It was observed that cultures in natural unenriched loch water often grew slightly better and produced more microcystins than cultures in enriched water minus phosphate, nitrate or iron. It may be that addition of nutrients to the water causes an initial stimulation in growth which is soon exhausted by lack of a specific nutrient. The algae in natural unenriched water may grow more slowly also using up their reserves. However, at no time did growth or toxin content in unenriched natural water exceed that enriched with phosphate, nitrate, iron and trace elements. Cultures in enriched waters minus trace elements always grew as well if not better than cultures in total enriched waters. Rippka (1988) reported that high concentrations of trace elements in media may be toxic. She observed that the concentration of copper, for example, even in amounts as low as that in BG11 medium, can be inhibitory to the growth of some cyanobacteria.

In the February bioassay the growth of *M. aeruginosa* in both enriched and enriched minus trace elements treatments was not significantly different but the former produced more microcystins. In the April bioassay there was also no significant difference in the growth of cultures in these two treatments but the enriched minus trace element cultures produced more microcystins. In the July bioassay the enriched minus trace element cultures grew much better than those in

enriched media but the amounts of microcystins produced were not significantly different from each other. In November the cultures minus trace elements were the only healthy cultures at the end of the bioassay and produced significantly more microcystins compared to the other treatments. There was no significant difference in the ratio of the microcystin variants in any of the bioassays. A change in the ratio of microcystins is an effect more commonly observed with changes in temperature (National Rivers Authority, 1992).

Many factors alone and in combination affect the growth and toxin content of cyanobacteria. Traditional growth assays are time-consuming and laborious. The bioassay approach developed in this study provides a rapid and simple means of investigating a large number of these parameters and also enables a large range of cyanobacteria species to be investigated. This will provide more insight into the factors influencing growth and toxin content.

# CHAPTER 4

## Large-scale purification of microcystin variants from *Microcystis aeruginosa*

## 4.1. INTRODUCTION

To date there has been more than 50 microcystin variants identified (Bell and Codd, 1996). As yet these compounds have not been totally synthesised (Edwards *et al.*, 1996) and are normally obtained by purification from natural or laboratory cultures of cyanobacteria.

Most methods developed to purify these compounds (Poon *et al.*, 1987; Lawton *et al.*, 1995) only result in microgram to milligram amounts of microcystins. There is an increasing demand for these compounds for use as analytical standards, biochemical tools and for toxicological studies. Thus there exists a need to develop simple and rapid procedures for isolating and purifying these compounds in larger quantities.

Many different procedures have been proposed for the extraction and purification of microcystins but many involve a time consuming multi-step methodology (Brooks and Codd, 1986; Harada *et al.*, 1991; Namikoshi *et al.*, 1992). Lawton *et al.*, (1995) presented a simple method for the purification of microcystins from bloom samples. This involved a methanolic extraction of cells, dilution of the extract with distilled water and concentration on a 1 g $C_{18}$ Sep-pak cartridge. The microcystins were then eluted from the cartridge using a step gradient from 0% to 100% methanol in 10% increments. Each

fraction was analysed by HPLC to quantify the microcystins present. Fractions containing microcystins of similar polarity were combined resulting in several more simplified fractions. These fractions were further purified by semi-preparative HPLC.

This chapter describes how the above method was scaled up allowing purification of sub-gram quantities of microcystins, using reverse-phase flash chromatography and labscale preparative HPLC (*Method A*) (Edwards *et al.*, 1996). The cells were extracted in methanol and the supernatant obtained was rotary evaporated to remove the methanol before loading onto a flash chromatography system. This part of the procedure was the most time consuming and took almost 1 week to complete. The flash fractions also required analysis by HPLC before pooling and then the pooled fractions had to be rotary evaporated to dryness.

However, this chapter also presents an alternative approach to the above procedure (*Method B*) which simplified the extraction/concentration step and reduced the time required to perform the procedure resulting in a simple and relatively rapid means of purifying sub-gram quantities of microcystins from large amounts of cell material (Edwards *et al.*, 1996a).

The cyanobacterial material used was collected from Rutland Water in Leicestershire, UK, in 1989. This had previously been shown to contain several microcystins (Lawton *et al.*, 1995).

## 4.2. MATERIALS AND METHODS

*Method A*

### 4.2.1. Cyanobacterial material

Cyanobacterial material, collected from a bloom of *Microcystis aeruginosa* at Rutland Waters (Leicestershire, UK) in September 1989, was supplied by Anglian Water and stored at -20°C until required.

### 4.2.2. Extraction of microcystins

Approximately 7 l of cell material was thawed and extracted in an equal volume of 100% methanol (Fisons, Loughborough, UK) plus ammonium acetate (0.5%, w/v) which enhances pellet formation during centrifugation. Aliquots (1 ml) of the methanolic extraction were placed in pre-weighed vials and dried in an oven at 80°C for three hours. The vials were cooled in a dessicator and re-weighed to give the dry weight of cells per ml of extract. Thus, it was calculated that the 7 l of cell material was equivalent to 313 g dry weight of cells. Methanol had previously been shown to be the best solvent for extracting all microcystin variants from cells (Lawton *et al.*, 1995). The cells were left to extract for 30 minutes with regular stirring. The extract was then centrifuged at 1500 x g for 30 minutes, the supernatant

removed and the pellet of cells re-extracted a further two times in 400 ml methanol for 30 minutes. The supernatants were pooled and rotary-evaporated at 40°C to remove the methanol. An oily residue remained which was stored at -20°C until further processing.

### 4.2.3. Concentration and clean-up of microcystins

The extract was diluted at a ratio of 1:1:8 (extract-methanol-water) and the aqueous extract (15.2 l) filtered through GF/C disks (Whatman, Maidstone, Kent, UK). Microcystins were concentrated using a Biotage Flash 75 S system (Biotage, Charlottesville, PA, USA) (Fig. 4.1.). This system comprises a stainless steel solvent reservoir which under pressure (approx. 20 psi) drives the solvent/sample through a cartridge housed in a radial compression module. Radial compression has been shown to improve the flow of the mobile phase through cartridges and prevents channeling by highly compacting the bed of silica (Fig. 4.2.) (Column Productivity, 1995).

Two stationary phases, spherical Hyperprep $C_{18}$ (30 µm particle size, 125 Å; Shandon, Runcorn, UK) and irregular Bondapak $C_{18}$ (37-55 µm particle size, 125 Å; Waters, Watford, UK) were packed in 9 x 7.5 cm I.D. cartridges. The performance of both stationary phases were compared in this application. The cartridges were preconditioned with 2 l of methanol and water


Fig. 4.1. Biotage Flash 75S system comprised of a stainless steel solvent reservoir which under pressure (approx. 20 psi) drives the solvent/sample through a cartridge (packed with appropriate stationary phase) housed in a radial compression module.

# Biotage *FLASH 75* Compression Module

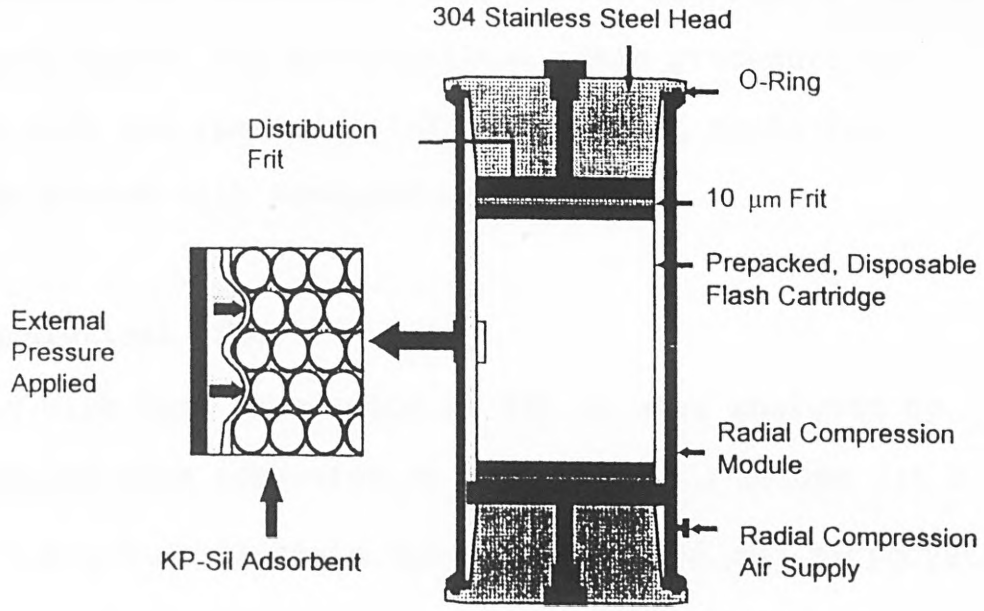



Fig. 4.2. A schematic diagram of a Biotage Flash 75 compression module housing a pre-packed disposable flash cartridge. The cartridge is subjected to radial pressure which compacts the bed of silica and prevents channeling of the sample.

before application of the aqueous extract.

A volume of 8 l of the aqueous extract was applied to the cartridge packed with Shandon Hyperprep $C_{18}$ at a flow-rate of 100 $mlmin^{-1}$. The microcystins were eluted using a stepwise gradient from 0 to 100% methanol in 10% increments using 2 l solvent per step. Fractions (200 ml) were collected manually. Each fraction was initially screened for microcystins using a spectrophotometer (Phillips, York, UK) at absorbance 238 nm (absorbance maxima for microcystins). This procedure was repeated with the remaining 7.2 l of extract using the cartridge packed with Bondapak $C_{18}$.

### 4.2.4. Analytical HPLC

Fractions with high absorbance at 238 nm were analysed by HPLC. Samples were separated on a Symmetry $C_{18}$ column (15 x 0.46 cm I.D.; 5 μm particle size; Waters, UK) and microcystins identified using a Waters 996 photodiode array detector. A mobile phase of (A) Milli-Q water (Millipore, Watford, UK) and (B) acetonitrile (Rathburns, Walkerburn, UK) both containing 0.1% trifluoroacetic acid (TFA) was used. Microcystins were separated using a linear gradient from 30% B to 35% B over 5 minutes followed by an increase to 60% B over the next 25 minutes.

### 4.2.5. Optimisation of separation methods using an analytical column

The combination of flash chromatography fractions containing microcystins of similar polarity resulted in three major fractions. Optimum conditions for separation of the microcystins to facilitate HPLC purification in each of these fractions was investigated using an analytical Shandon Hyperprep $C_{18}$ column (15 x 0.46 cm I.D.; 12 μm particle size; 100 Å pore size) using a mobile phase of (A) ammonium acetate (0.1%, w/v) and (B) acetonitrile at a flow rate of 1.5 $mlmin^{-1}$. This allowed the development of satisfactory separation methods without loss of much sample.

Once developed the process was scaled up using a preparative HPLC column packed with the same material as the analytical column. The eluent was monitored at 238 (absorption maxima for microcystins), 214, and 254 nm using a Waters 490 detector. Absorption at 254 nm aids in the identification of microcystins. If a compound has greater absorption at 254 nm than at 238 nm it is likely that it is not a microcystin. Many compounds absorb at 214 nm and this wavelength is monitored to observe how pure the sample is.

Three methods were developed on the analytical column which provided the best separation of microcystins contained in the three combined flash chromatography fractions:

*Method 1:* Fractions eluted from the flash column which contained more polar microcystins were combined to give fraction 1. These were separated isocratically using a mobile phase A:B (80:20).

*Method 2:* Fractions containing mainly MCYST-LR and MCYST-LY were pooled to give fraction 2. These were separated using a mobile phase A:B (78:22) with a step increase to 25% B after 12 minutes to elute MCYST-LY.

*Method 3*: Fractions containing the more hydrophobic microcystins were combined to give fraction 3. These were separated using a mobile phase A:B (76:24) with a step increase to 30% B after 8 minutes.

Next, increasing sample loads were injected onto the analytical column until resolution was lost. The amount of sample (mg) per gram of packing material which can be loaded without significant loss of resolution was calculated. This loading was scaled to a preparative column by keeping the ratio of mg of sample per gram of packing material constant.

### 4.2.6. Preparative HPLC

The preparative system used included a Kiloprep 100 laboratory scale HPLC with a KPCM 100 compression module (Biotage), a UV detector (Linear 205) and a Linear 1201 chart recorder (Thermoseparations, Stone, UK). Samples were injected through a 35 ml loop.

Separation was preformed on a 15 x 7.5 cm I.D. cartridge packed with Shandon HS BDS $C_{18}$ (12 μm particle size) and a mobile phase of (A) ammonium acetate (0.1%, w/v) and (B) acetonitrile under conditions described in methods 1-3 (Section 4.2.5) at a flow rate of 400 $mlmin^{-1}$. The eluent was monitored at 214 and 238 nm and fractions were collected manually. These fractions were analysed by analytical HPLC as decribed in section 4.2.4 and fractions containing pure compounds were combined and diluted with an equal volume of Milli-Q water. These fractions were desalted (removal of ammonium acetate) and concentrated by pumping onto the preparative HPLC cartridge at a flow rate of 200 $mlmin^{-1}$. The cartridge was washed with 1.5 l of Milli-Q water and microcystins eluted with 100% methanol.

Closed-loop recycling was used for separation of closely eluting compounds (MCYST-LW and MCYST-LF). This method involved diverting the column effluent from the detector back

onto the column via the pump. It was repeated several times to achieve adequate separation of the compounds. This procedure was performed at Biotage, UK, by Dr. C. Edwards.

*Method B*

### 4.2.7. Cyanobacterial material

Cyanobacterial material used was similar to that used in the development of method A (section 4.2.1.)

### 4.2.8. Extraction of microcystins

Cyanobacterial cells were extracted in methanol, centrifuged and the supernatants retained as described in section 4.2.2. Previously the supernatant was rotary evaporated to an oily residue which was diluted with 10% aqueous methanol and filtered through GF/C filter disks (to remove particulates) before loading onto a flash chromatography cartridge. Rotary evaporating the extract was very time consuming and took almost 1 week to process.

The aim, therefore, was to try and reduce this processing time. In place of rotary evaporation the concentration of methanol was reduced by diluting the supernatant to 20% (v/v)

with glass-distilled water and then filtered through GF/C filter disks.

### 4.2.9. Concentration and clean-up of microcystins

The resulting aqueous extract (80 l) was loaded onto a preconditioned Bondapak $C_{18}$ cartridge (9 x 7.5 cm I.D.; 37-55 μm particle size) housed in a radial compression module as described previously (section 4.2.3.) at a flow rate of 200 $mlmin^{-1}$. The waste eluent was monitored for breakthrough using analytical HPLC. The cartridge was washed with 10% and 20% methanol (2 l) and monitored for breakthrough. All the microcystins were eluted in 70% methanol (2 l) and the eluent concentrated to 200 ml by rotary evaporation at 40°C. This eliminated having a large number of flash fractions which have to be analysed by analytical HPLC as in method A.

### 4.2.10. Analytical HPLC

Microcystins were identified by HPLC as described in section 4.2.4.

### 4.2.11. Evaluation of suitable stationary phases for preparative HPLC

Four stationary phases commonly used for large-scale peptide separations were packed into analytical columns and assessed for use in this application. The aim was to develop a method to separate all the microcystins in the extract using a single gradient.

**(1).** YMC ODS (25 x 0.46 cm I.D.; 15 μm particle size, 120 Å pore size; YMC, Wilmington, DE, USA).

**(2).** Vydac $C_{18}$ (25 x 0.46 cm I.D.; 15-20 μm particle size, 300 Å pore size; Vydac, Hesperia, CA, USA).

**(3).** Shandon Hyperprep HS BDS $C_{18}$ (15 x 0.46 cm I.D.; 12 μm particle size, 100 Å pore size; Shandon HPLC, Runcorn, UK).

**(4).** Shandon Hyperprep HS BDS $C_{18}$ (15 x 0.46 cm I.D.; 8 μm particle size, 100 Å pore size).

The extract (0.1 mg) was separated on all four columns using a mobile phase of (A) ammonium acetate (0.1%, w/v) and (B) acetonitrile. The gradient was 22% B for the first 4 minutes followed by an increase to 35% B over the next 46 minutes (76 minutes for the 25 cm columns) at a flow rate of 1.5 $mlmin^{-1}$. The eluate was monitored at 238, 214 and 254 nm using a 490 detector (Waters, UK) (section 4.2.5.).

The maximum loading ($mgg^{-1}$ packing material) for the column was determined. The most appropriate stationary phase was chosen and increasing loads of sample were injected onto the column until resolution became unacceptable.

### 4.2.12. Preparative HPLC

The preparative HPLC system used is described in section 4.2.6. Separation was performed on a 15 x 7.5 cm I.D. cartridge packed with Hyperprep HS BDS $C_{18}$. This was found to be the best stationary phase after method development on analytical columns (see section 4.2.11.). A gradient starting at 20% acetonitrile and increasing to 22% over 4 minutes, followed by an increase to 32% over the next 36 minutes was used. The flow rate was 400 $mlmin^{-1}$ and the eluate was monitored at 238 and 214 nm. Fractions were collected manually and analysed by HPLC with diode-array to determine their purity.

Fractions containing purified microcystins were combined and desalted using the Biotage flash system. Fractions were diluted with an equal volume of Milli-Q water and loaded onto a new preconditioned $C_{18}$ flash cartridge (9 x 7.5 cm I.D.; Bondapak $C_{18}$ , 37-55 μm particle size) at a flow rate of 250 $mlmin^{-1}$. The cartridge was washed with 1.5 l of Milli-Q water and microcystins were eluted in 500 ml of 80% methanol.

## 4.3. Results

*Method A*

A total of fifteen microcystins were identified in the aqueous extract of *M. aeruginosa* by reversed-phase HPLC with diode array detection (Fig. 4.3.) which included previously characterised MCYST-LR (**5**), MCYST-LY (**8**), MCYST-LW (**13**) and MCYST-LF (**14**) and eleven unknown microcystin variants.

### 4.3.1 Flash chromatography

Flash fractions analysed by spectrophotometer displayed similar profiles for the two stationary phase cartridges with major absorbance (238 nm) occurring between fractions 45 and 75 (Fig. 4.4.). This corresponds to a methanol concentration between 30 and 70%. Although no replicates were used this pattern has repeatedly been observed in several other separations.

It was observed that fractions eluted in 0 and 10% methanol were yellow/orange in colour whilst fractions eluted in 80, 90 and 100% methanol were dark green (indicating the presence of chlorophyll a). Fractions eluted with 20-70% methanol were almost colourless (Fig. 4.5.) and contained the majority of the microcystins.

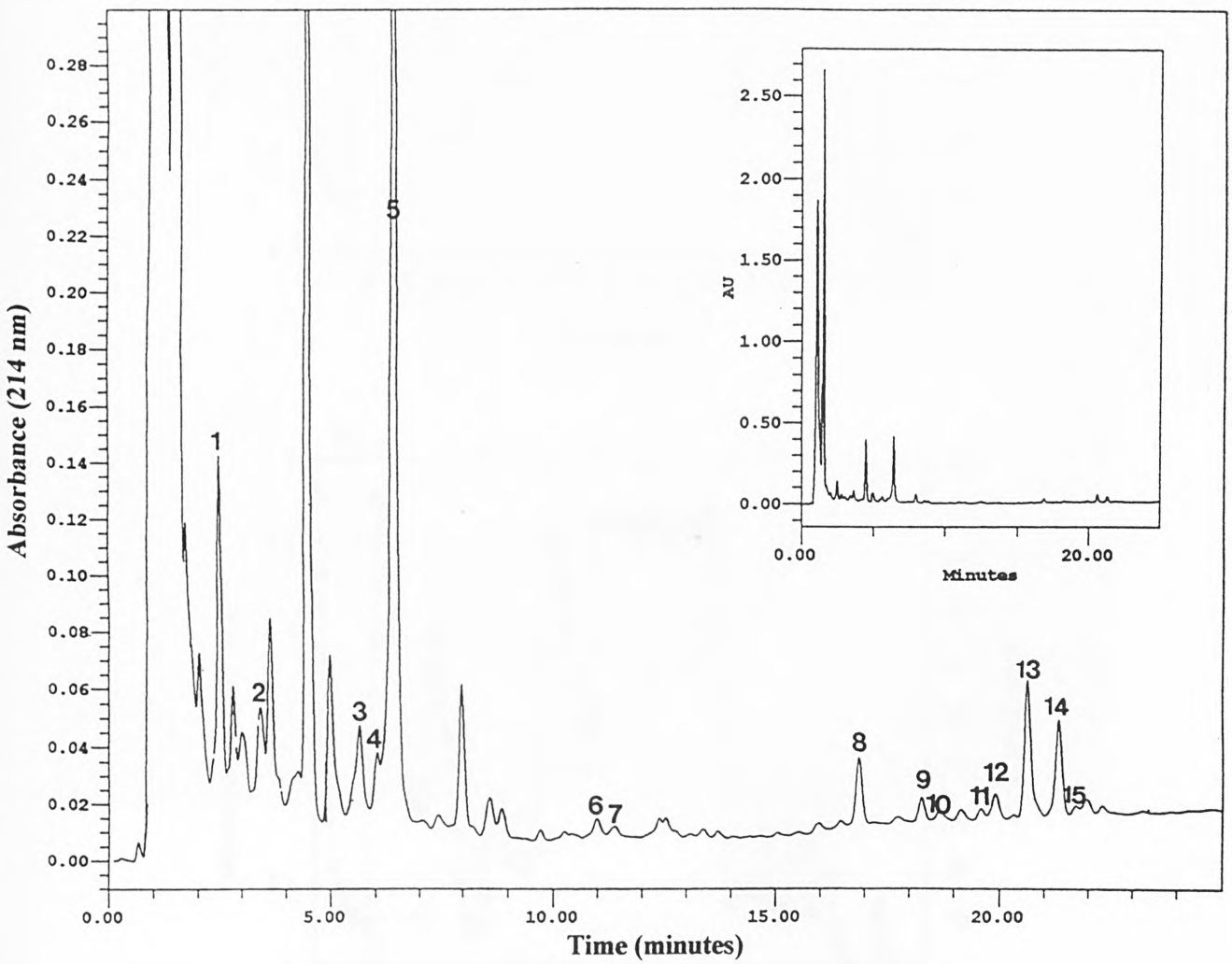



Fig. 4.3. Analysis of the aqueous methanolic extract of *Microcystis aeruginosa* by reversed-phase HPLC with diode-array detection. Microcystins (MCYST) are numbered 1-15.

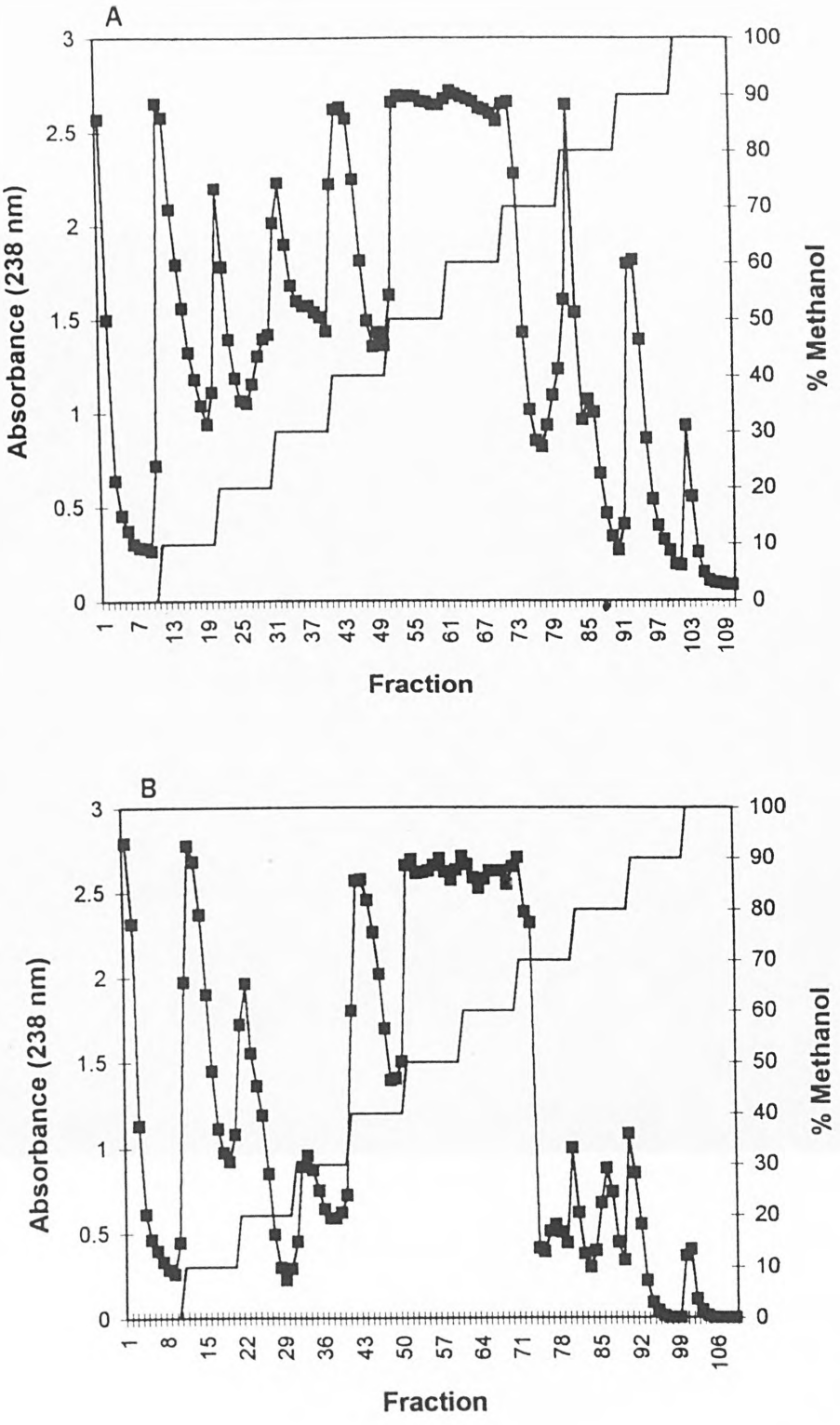



Fig. 4.4. Absorbance (238 nm) of fractions eluted from (a) Hyperprep $C_{18}$ and (b) Bondapak $C_{18}$ using a stepwise gradient from 0 to 100% methanol in 10% increments using 2 l solvent per step. Fractions (200 ml) were collected manually.

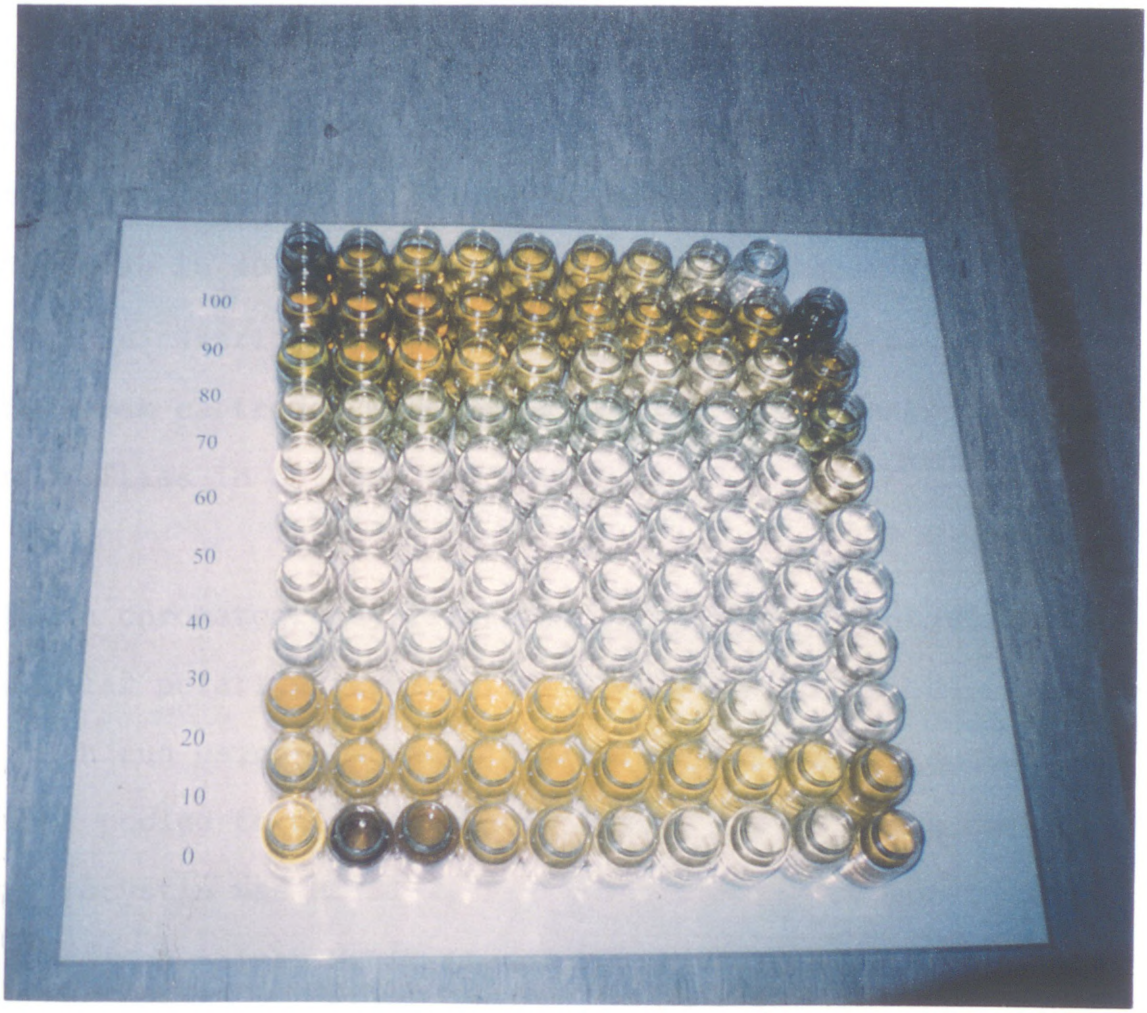



Fig. 4.5. Flash fractions eluted between 0 and 100% methanol in 10% increments (2 l solvent per step) showing the colour range of the fractions. Fractions eluted between 20-70% methanol were almost colourless and contained the majority of the microcystins.

Analysis of flash fractions showed the distribution of microcystins across the gradient for both cartridges (Table 4.1). The selectivity of the cartridges was very similar for most of the microcystins except MCYST (2) which was eluted from the Shandon cartridge in 30% methanol and from the Waters Bondapak in 40% methanol. MCYST (1) was eluted from the Shandon cartridge in 60% methanol compared to the Waters Bondapak cartridge were it was eluted in approximately equal quantities in 50% and 60% methanol.

Flash chromatography fractions containing microcystins of similar polarity resulted in three major fractions. From the flash run using a Shandon Hyperprep cartridge fractions 21-30 were pooled to give fraction 1 (Fig. 4.6a.) where the main microcystin was MCYST-2. Fractions 51-57 were pooled and contained mainly MCYST-LR and MCYST-LY (Fig. 4.6b.). Fractions 64-67 were pooled and contained MCYST-LW and MCYST-LF along with several unknown microcystins (Fig. 4.6c.). Fractions from the second flash run using a Waters Bondapak were pooled in a similar fashion (results not shown).

### 4.3.2. Optimisation of preparative HPLC loading using an analytical column

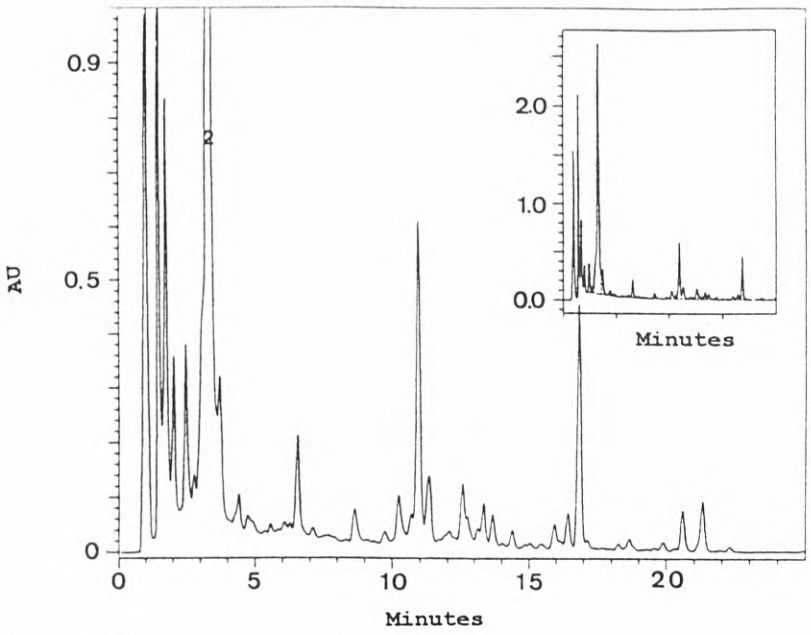
Increasing amounts of fraction 1 (0.05-0.25 mg per gram of packing material) were injected onto the analytical column (Shandon 15 x 0.46 cm I.D.)(Fig. 4.7.). Resolution

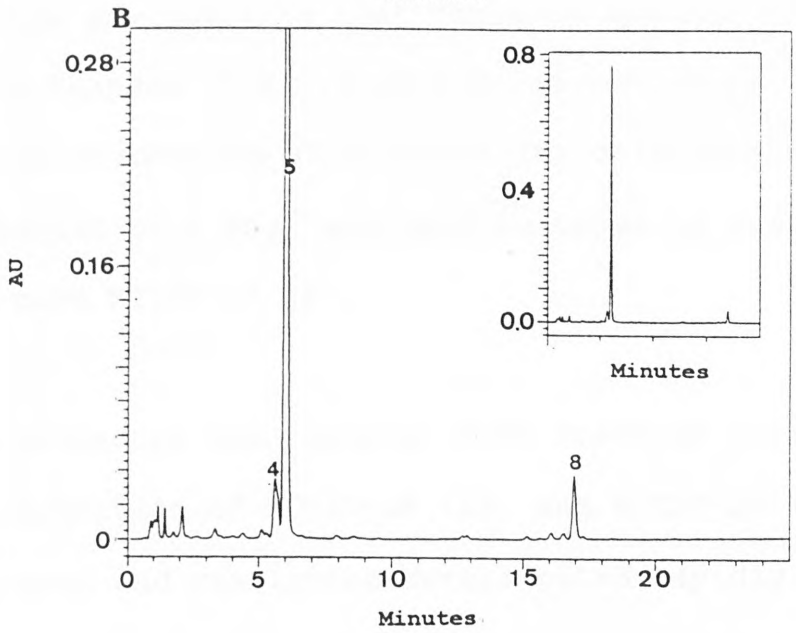
**Table 4.1.** Distribution of microcystins in reversed-phase flash chromatography fractions

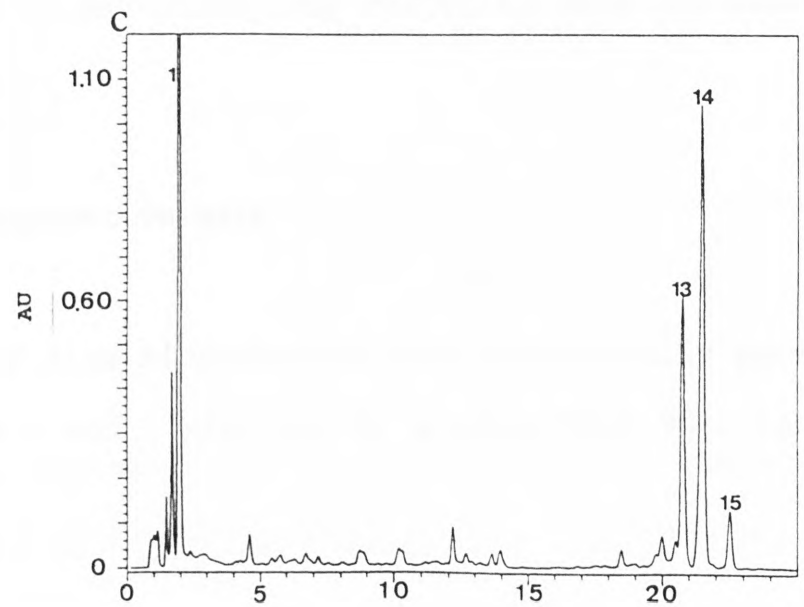
| | Yield of microcystin (mg)[a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % Methanol | 1[b] | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Hyperprep | | | | | | | | | | | | | | | |
| 30 | - | 30 | - | - | - | 7 | - | - | - | - | - | - | - | - | - |
| 40 | - | 4 | 2 | - | 17 | - | - | - | - | - | - | - | - | - | - |
| 50 | - | - | 26 | 22 | 617 | - | - | 20 | - | - | - | - | - | - | - |
| 60 | 107 | - | - | - | 38 | - | 4 | - | - | 7 | 7 | 17 | 53 | 41 | 7 |
| 70 | - | - | - | - | - | - | 11 | - | - | - | - | - | - | 3 | 7 |
| Bondapak | | | | | | | | | | | | | | | |
| 30 | - | 7 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 40 | - | 37 | 2 | - | 15 | 7 | - | - | - | - | - | - | - | - | - |
| 50 | 62 | - | 31 | 21 | 600 | - | - | 18 | - | - | - | - | - | - | - |
| 60 | 58 | - | - | - | - | - | 2- | - | 8 | 7 | 7 | 14 | 47 | 22 | 3 |
| 70 | - | - | - | - | - | - | 11 | - | - | - | - | - | 5 | 27 | 11 |

[a] As determined by reversed-phase HPLC where quantification was by external standards where available and unknowns were quantified based on a MCYST-LR standard.
[b] Numbers refer to peaks shown in Fig. 3.

Fig. 4.6. Analyses of the three main fractions, by reversed-phase HPLC with diode-array detection, resulting from pooled flash fractions containing microcystins of similar polarities: (a) fraction 1 contained one predominant microcystin (MCYST-**2**), (b) fraction 2 contained mainly MCYST-LR (**5**) and MCYST-LY (**8**) and (c) fraction 3 contained MCYST-LW (**13**) and MCYST-LF (**14**) along with other uncharacterised microcystins (MCYST-1; MCYST-12 and MCYST-15).


0.9
0.5
0
AU
2
2.0
1.0
0.0
Minutes
0
5
10
15
20
Minutes


B
0.28
0.16
0
AU
5
4
8
0.8
0.4
0.0
Minutes
0
5
10
15
20
Minutes


C
1.10
0.60
0
AU
1
14
13
15
0
5
10
15
20

deteriorated at low loading (0.25 $mgg^{-1}$). This may have been an effect of increased methanol reducing retention times of the more polar microcystins. Further optimisation would be necessary before scaling up the separation of fraction 1.

Increasing loads of fraction 2 from 0.05 to 0.75 mg MCYST-LR (**5**)(total load of approximately 0.1 to 1.5 mg per gram of packing material) were injected onto the analytical column to determine the maximum load that could be applied to the preparative Shandon 15 x 7.5 cm I.D. column (Fig. 4.8.). At 1.5 $mgg^{-1}$ the resolution of MCYST-LY (**8**) deteriorated and a maximum loading of 1 $mgg^{-1}$ was used in order to ensure maximum yields of pure MCYST-LY (**8**).

The above procedure was repeated with fraction 3 (Fig. 4.9.). Complete separation of MCYST-LW (**13**) and MCYST-LF (**14**) was never achieved and resolution deteriorated rapidly. It was necessary to use close-loop recycling when the separation was scaled up.

### 4.3.3. Preparative HPLC

A total of five microcystins were successfully purified from fractions 2 and 3 with purity greater than 90% (Table 4.2).

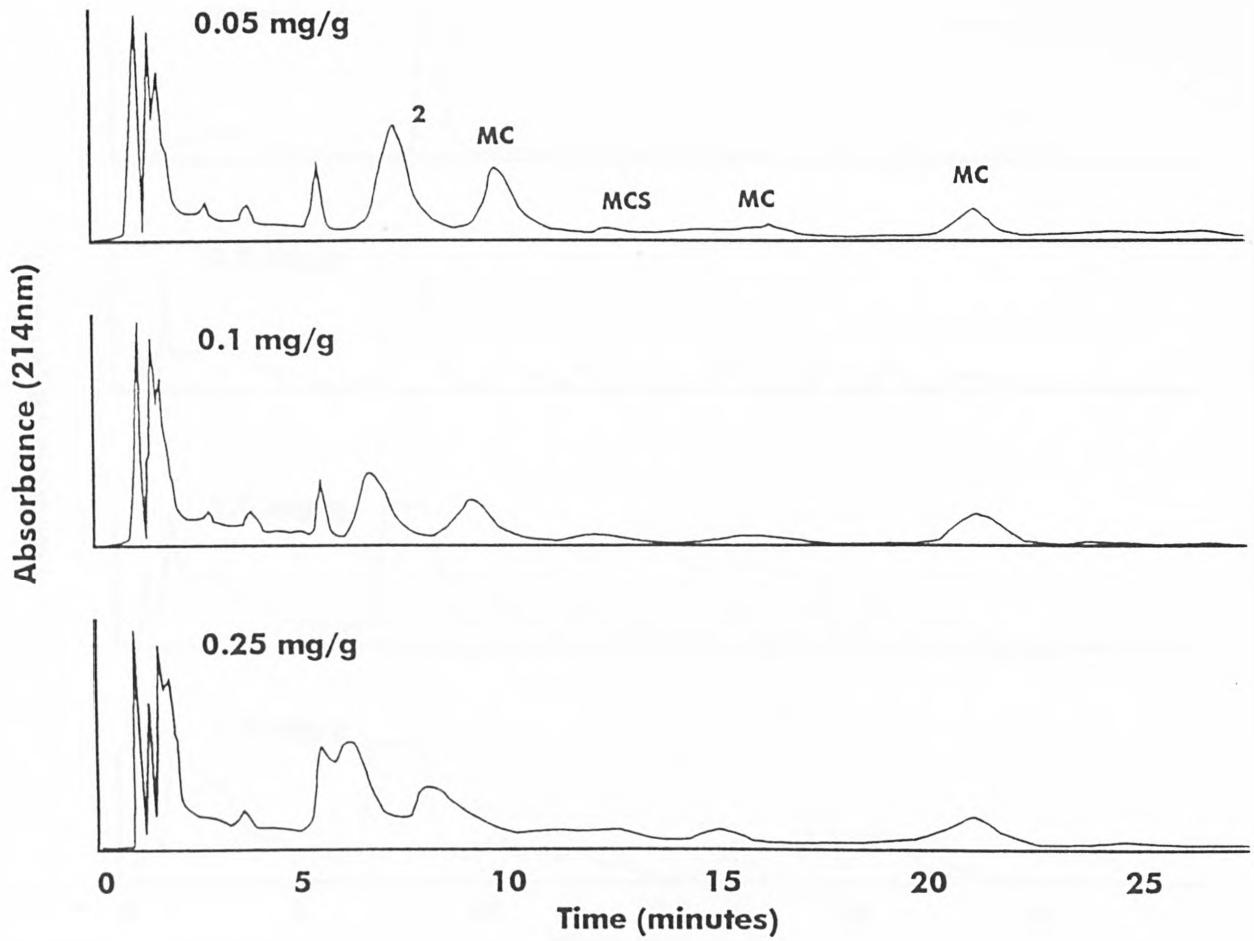



Fig. 4.7. Separation of fraction 1 and load optimisation on an analytical Hyperprep HS BDS $C_{18}$ (15 x 0.46 cm I.D.; 12 μm particle size).

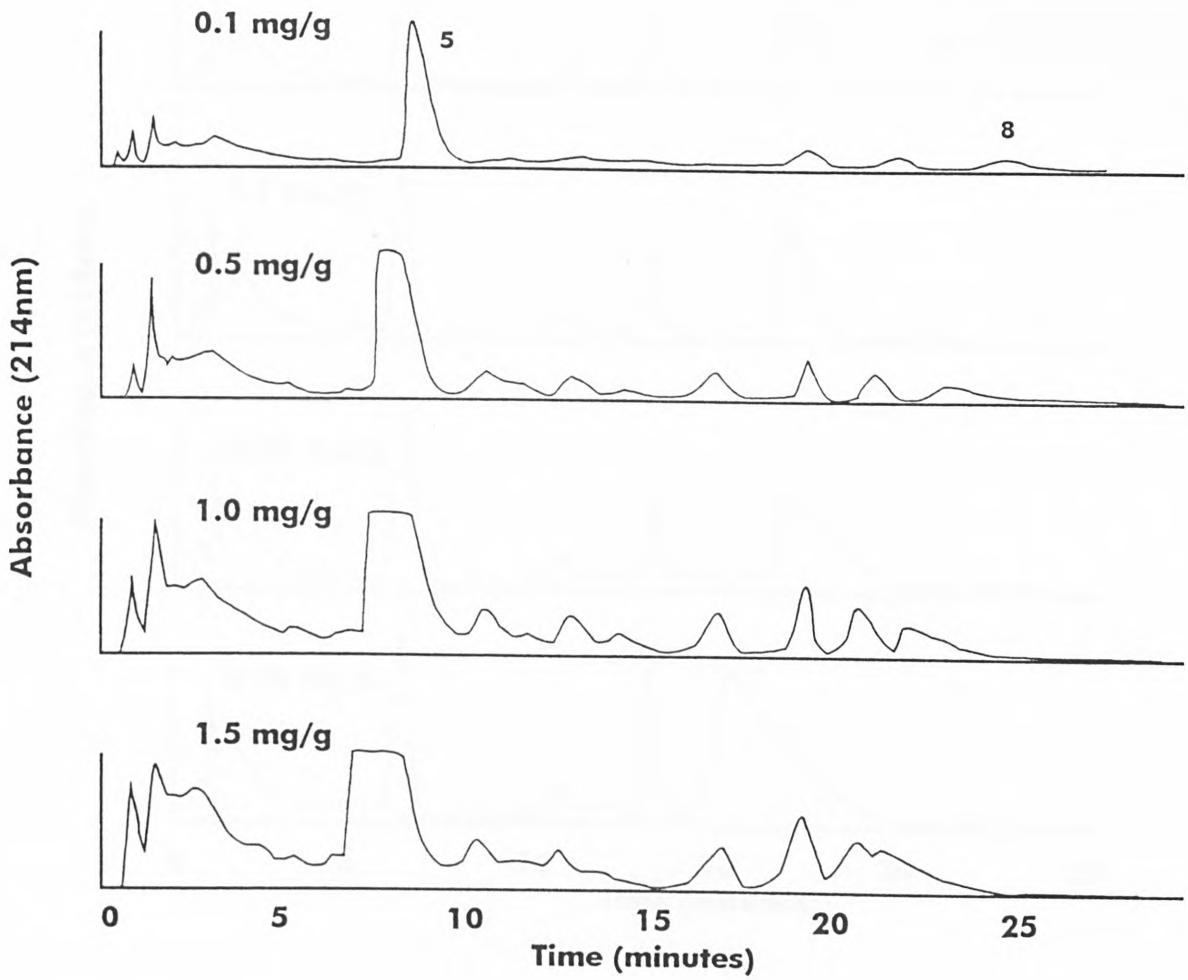



Fig. 4.8. Separation of fraction 2 and load optimisation on an analytical Hyperprep HS BDS $C_{18}$ (15 x 0.46 cm I.D.; 12 μm particle size).

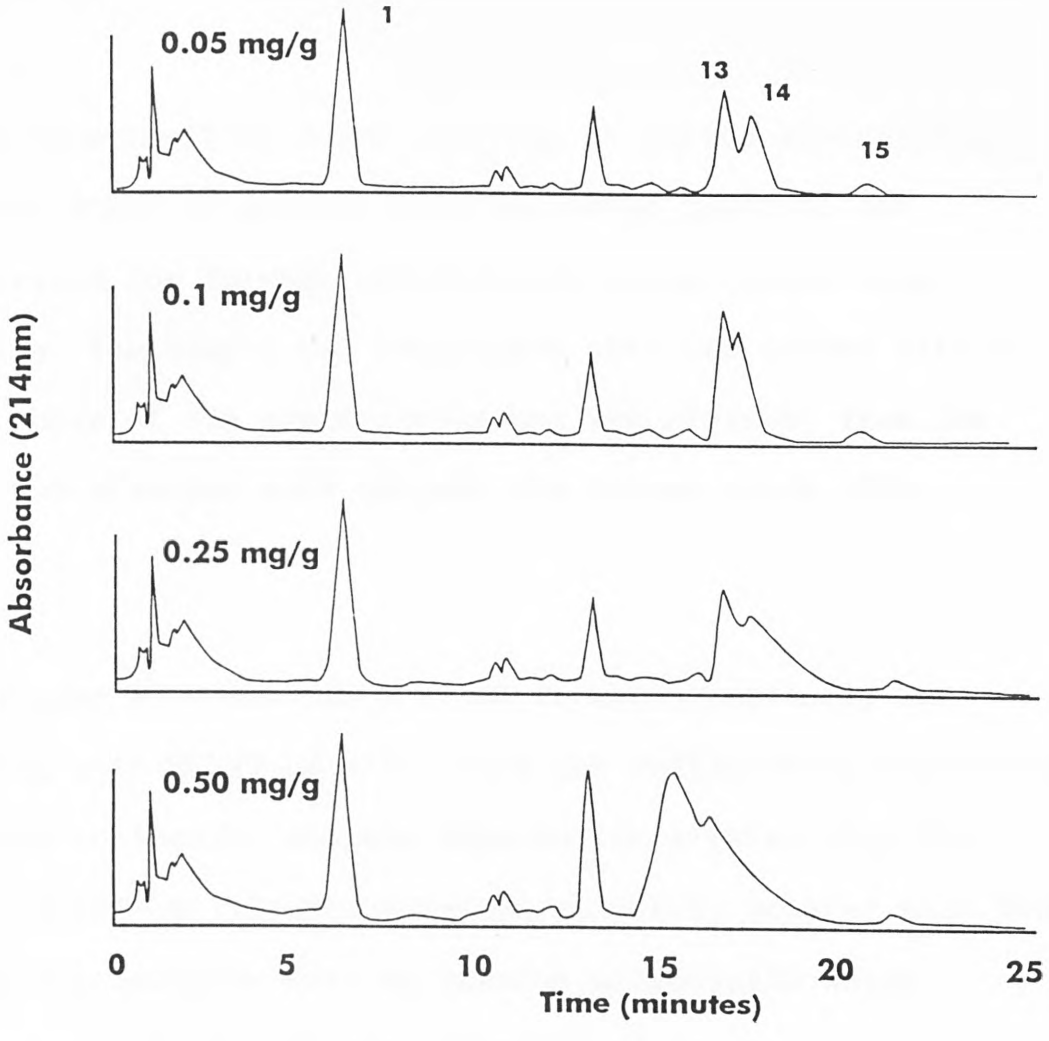



Fig. 4.9. Separation of fraction 3 and load optimisation on an analytical Hyperprep HS BDS $C_{18}$ (15 x 0.46 cm I.D.; 12 µm particle size).

Separation of fraction 2 was scaled up to a preparative column (15 x 7.5 cm) where 352 mg of MCYST-LR (**5**) (equivalent to a total load of 0.5-0.6 g) was injected. MCYST-LR was obtained at a purity greater than 95% where the main contaminant was a related microcystin (Fig. 4.10.). MCYST-LY (**8**) was also purified (> 80%) but was found to contain closely eluting microcystins.

MCYST-1 from fraction 3 was obtained at purity greater than 95% while MCYST-13 and -14 were collected together and concentrated for further purification using closed-loop recycling. The sample was reinjected onto the column with a mobile phase of 30% acetonitrile and the effluent from the column was diverted back through the column twice (Fig. 4.11.).

The effluent also contained MCYST-12 which initially was coeluting with MCYST-LW (**13**). This was sufficiently separated to enable collection and was obtained at greater than 95% purity. MCYST-LW (**13**) was obtained at purity greater than 90% but was contaminated with an unknown microcystin which coeluted. MCYST-LF (**14**) was collected at purity greater than 95%. This separation and purification of MCYST-LW (**13**) and MCYST-LF (**14**) would not be possible without the use of closed-loop recycling.

**Table 4.2.** Recovery and purity of microcystins from pooled flash fractions using laboratory-scale preparative HPLC.

| | 1[a] | 5 | 8 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|
| Fraction[b] | 3 | 2 | 2 | 3 | 3 | 3 |
| Total load (g)[c] | 0.2 | 0.6 | 0.6 | 0.1 | 0.1 | 0.1 |
| Recovery (%) | 93 | 76 | 86 | 75 | 70 | 90 |
| Purity (%)[d] | >95 | >95 | >80 | >95 | >90 | >95 |

[a] Number refer to peaks in Fig. 4.3

[b] Fractions from flash run as shown in Fig. 4.6

[c] Total load is approximate since it was not possible to quantify unknown components which were not microcystins.

[d] Purity as assessed by reversed-phase HPLC with diode-array detection

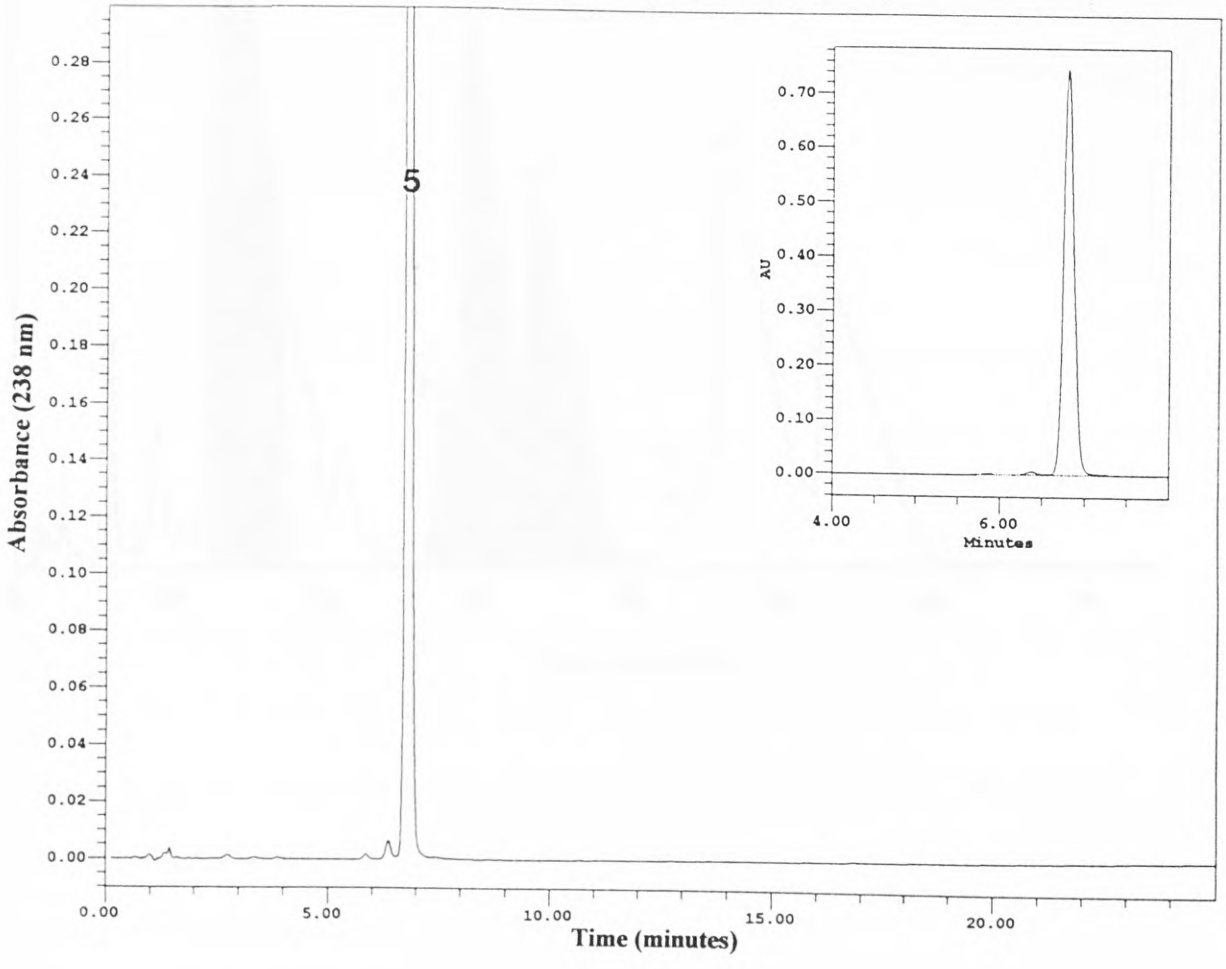



Fig. 4.10. Analysis of purified MCYST-LR (5) (obtained from laboratory preparative HPLC) by reversed-phase HPLC with diode-array detection having a purity greater than 95%.

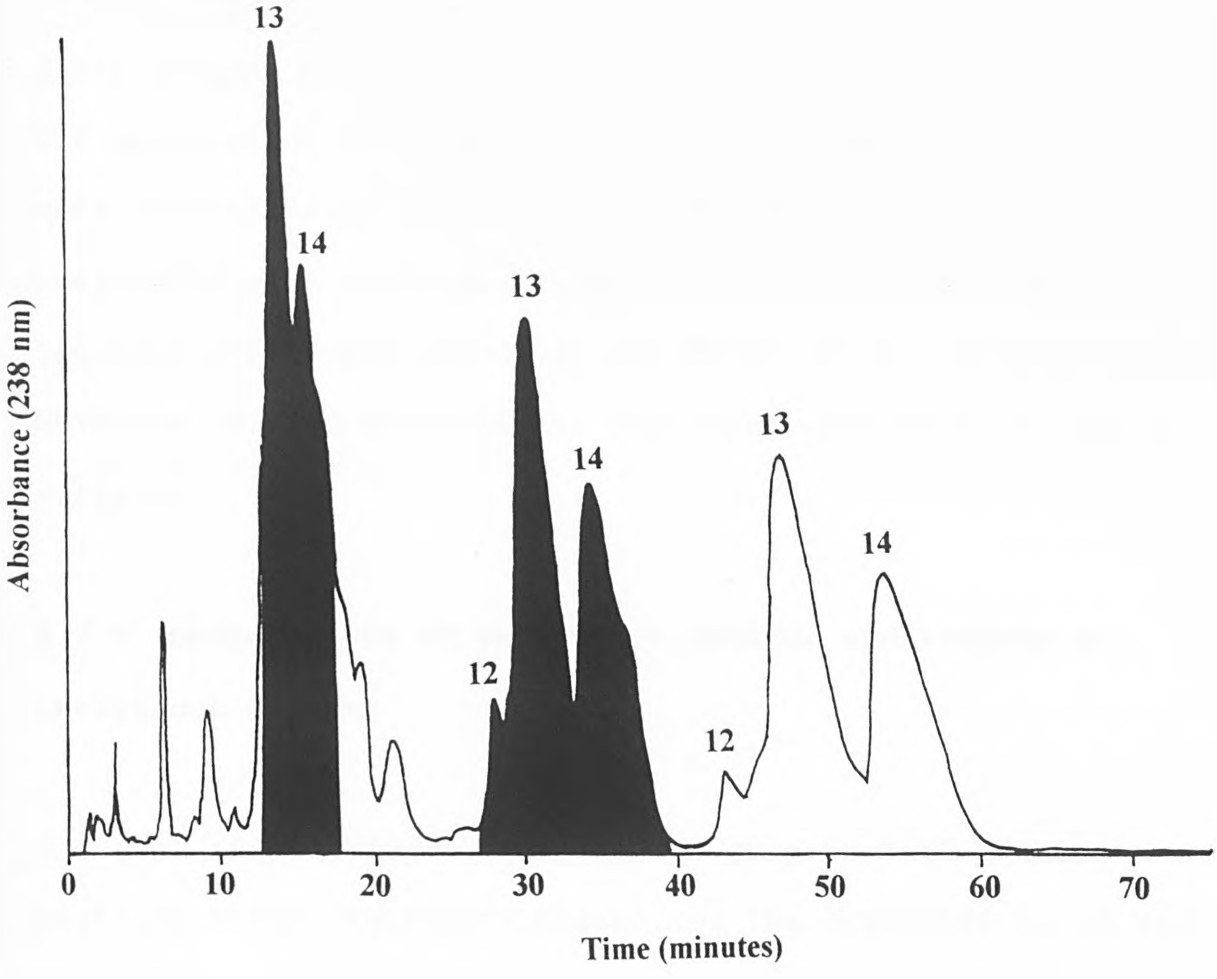



Fig. 4.11. Separation and purification of MCYST-LW (**13**) and MCYST-LF (**14**) using closed-loop recycling. An unknown microcystin (**12**) which originally coeluted with MCYST-LW (**13**) was observed. The dark area represents the effluent that was recycled.

*Method B*

### 4.3.4. Flash chromatography

It took 7 hours to load the 80 l of aqueous extract onto the flash column. No breakthrough of microcystins was observed in the waste after loading. A total of seven major microcystins were identified in the extract of *M. aeruginosa* by reversed-phase HPLC with diode-array detection (Fig. 4.12.) which included previously characterised MCYST-LR (**4**), MCYST-LY (**5**), MCYST-LW (**6**) and MCYST-LF (**7**) and three unknown microcystin variants.

### 4.3.5. Optimisation of separation methods and loading on analytical columns

The best resolution was achieved on columns with smaller particle sized stationary phase i.e. the Hyperprep $C_{18}$ (8 and 12 μm particle size) (Fig. 4.13.). The Hyperprep $C_{18}$ (8 μm particle size) stationary phase was chosen because MCYST-LR (**4**) had a greater retention time and MCYST-LW (**6**) and MCYST-LF (**7**) were slightly more resolved.

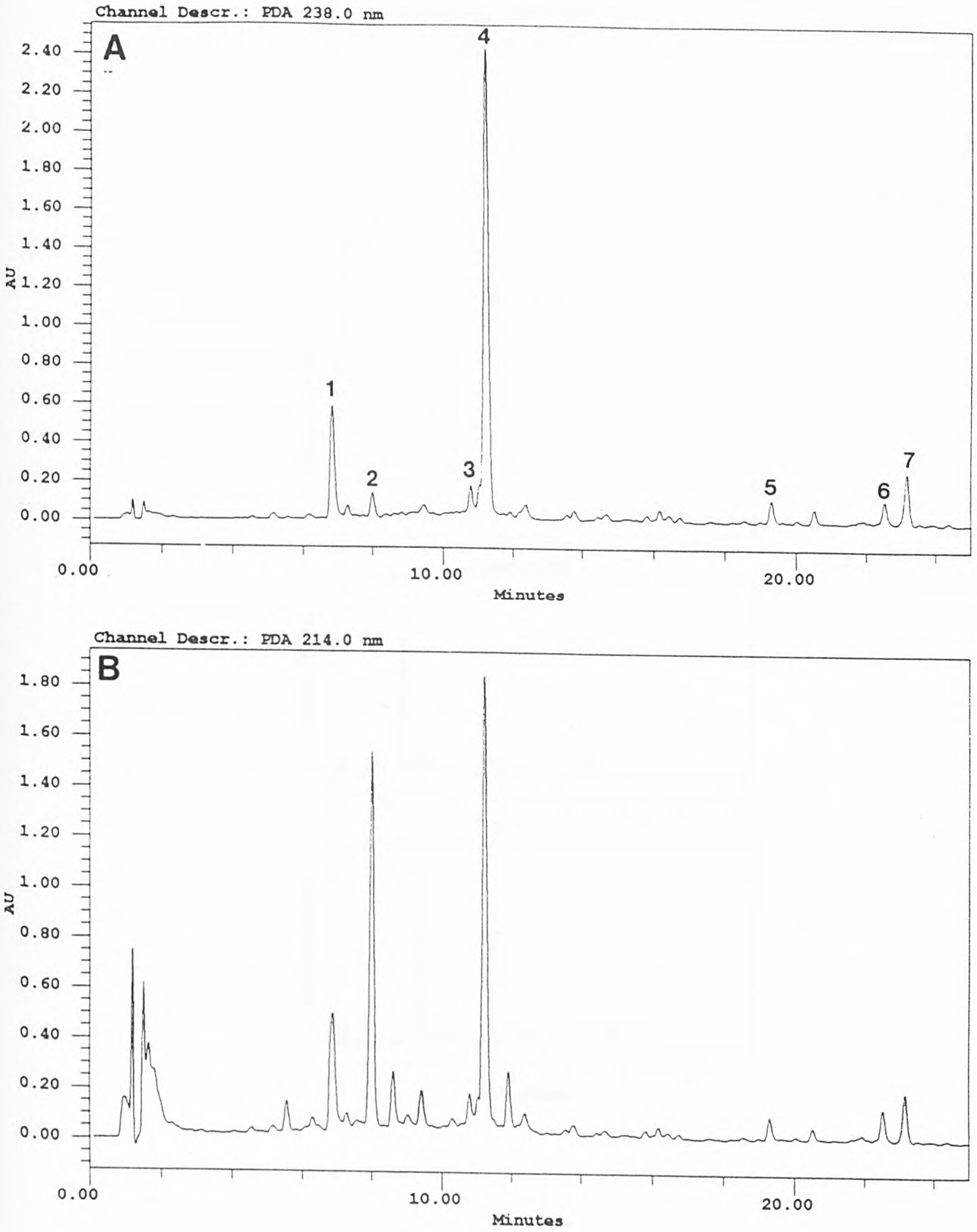



Fig. 4.12. Analysis of the post-flash extact by reversed-phase HPLC with diode-array detection. Microcystins are numbered 1-7 and include previously characterised MCYST-LR (**4**), MCYST-LY (5), MCYST-LW (6) and MCYST (7).

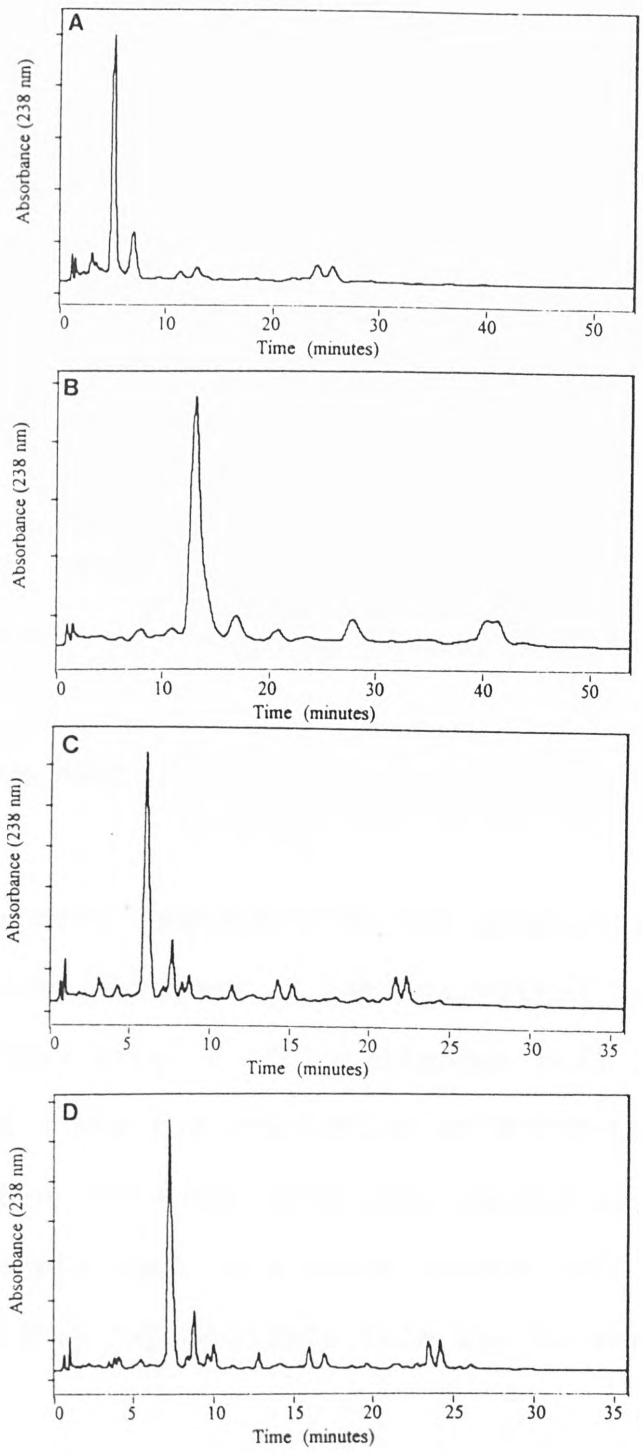



Fig. 4.13. Separation of microcystin extract (0.1 mg) on (A) Vydac $C_{18}$ (15-20 μm, 300 Å), (B) YMC ODS (15 μm, 120 Å), (C) Hyperprep HS BDS (12 μm, 100 Å) and (D) Hyperprep HS BDS $C_{18}$ (8 μm, 100 Å).

Increasing amounts of the extract (0.3, 0.75 and 1.5 $mgg^{-1}$ packing material) were injected onto the analytical Hyperprep $C_{18}$ (8 μm particle size) column (Fig. 4.14.). At 1.5 mg of extract per gram of packing material the separation of MCYST-LW (**6**) and MCYST-LF (**7**) was unacceptable and a load of 1 $mgg^{-1}$ was decided upon for scaling up to the preparative column. A load of 0.5 g was loaded onto the 15 x 7.5 cm I.D. column which contained 485 g of Hyperprep HS BDS $C_{18}$.

### 4.3.6. Preparative HPLC

The retention for most components on the preparative column was similar to those obtained on the analytical column used to scale-up the process (Fig. 4.15.), although peak 1 eluted after peaks 2 and 3 and the resolution of MCYST-LW and MCYST-LF had deteriorated. This may have been caused by loading the sample via the sample pump in a large volume (100 ml). The separation was highly reproducible from run to run (Table 4.3).

The pooled fractions containing MCYST-LR (**4**) were analysed by HPLC with diode-array detection (see section 4.2.4). Absorbance at 214 nm demonstrated the purity of this fraction (Fig. 4.16.). Yields of MCYST-LR (**4**) with purity greater than 95% were in excess of 80% (Table 4.4). However the purity of the other microcystins ranged between 60 and 80% and further purification would need to be carried out.

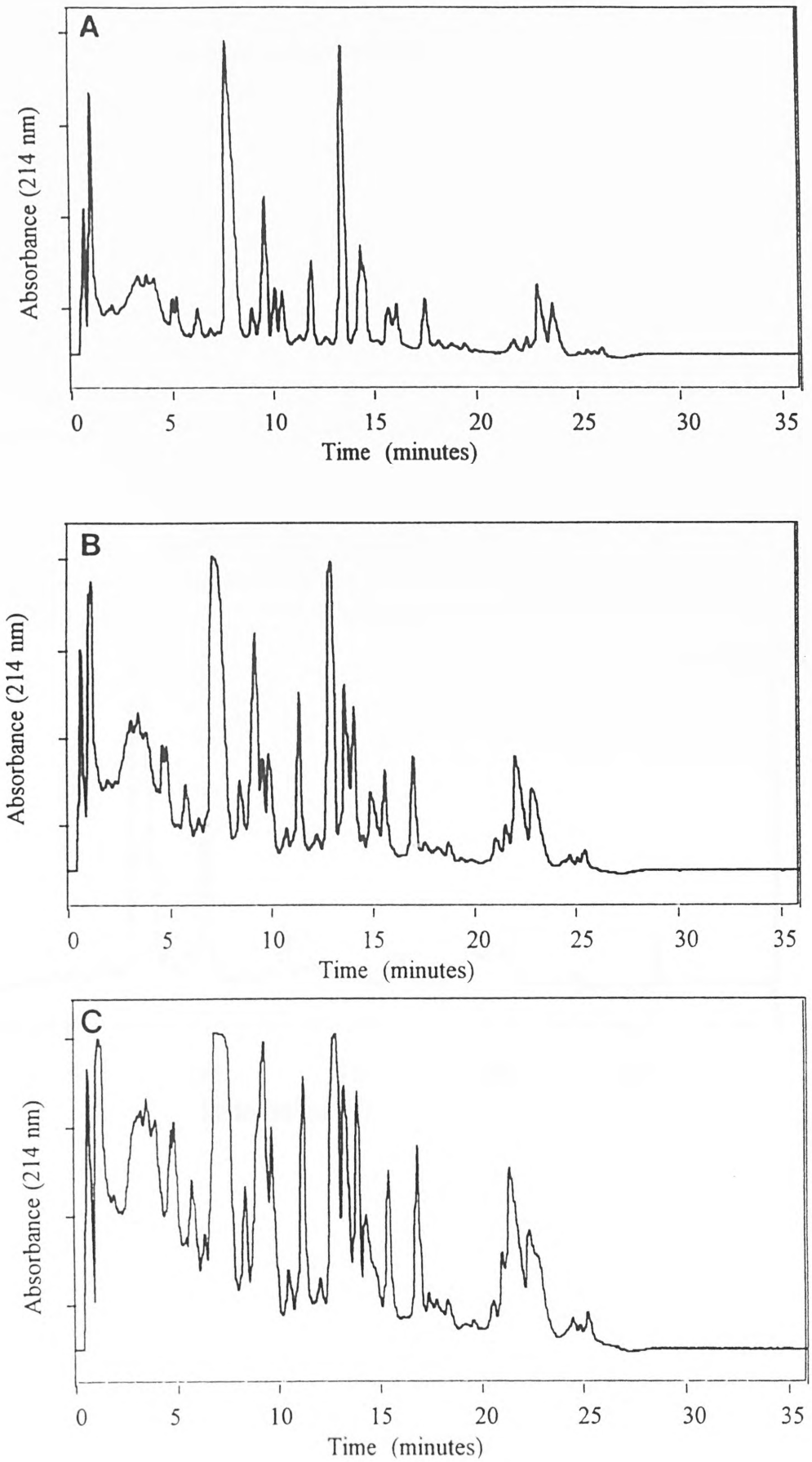



Fig. 4.14. Optimisation of loading on Hyperprep $C_{18}$ (15 x 0.46 cm; 8 μm particle size) using total loads of (A) 0.3, (B) 0.75 and (C) 1.5 $mgg^{-1}$ packing material.

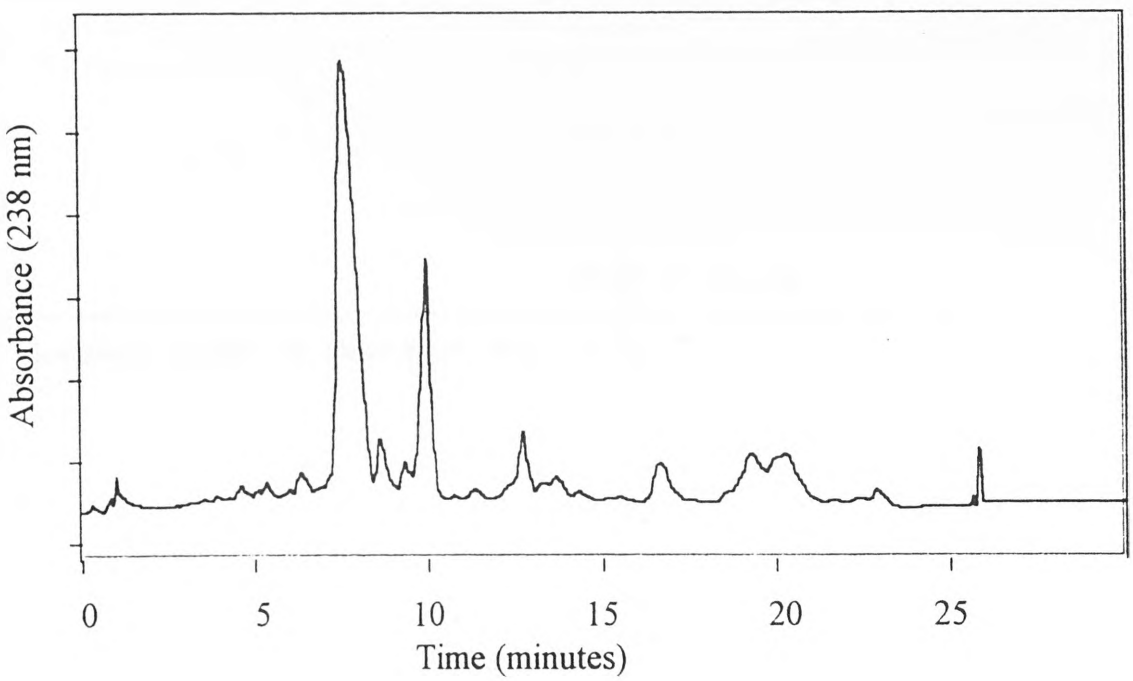



Fig. 4.15. Preparative separation of 0.5 g of extract on the 15 x 7.5 cm I.D. column.

Table 4.3. Mean retention in four preparative gradient separations.

| Peak No.[a] | Mean retention (secs.) ± S.D |
|---|---|
| 1 | 752 ± 6.0 |
| 2 | 861 ± 7.6 |
| 3 | 924 ± 4.6 |
| 4 | 993 ± 2.5 |
| 5 | 1669 ± 12.12 |
| 6 | 1928 ± 12.10 |
| 7 | 2025 ± 14.20 |

[a] Numbers refer to peaks in Fig. 4.12.

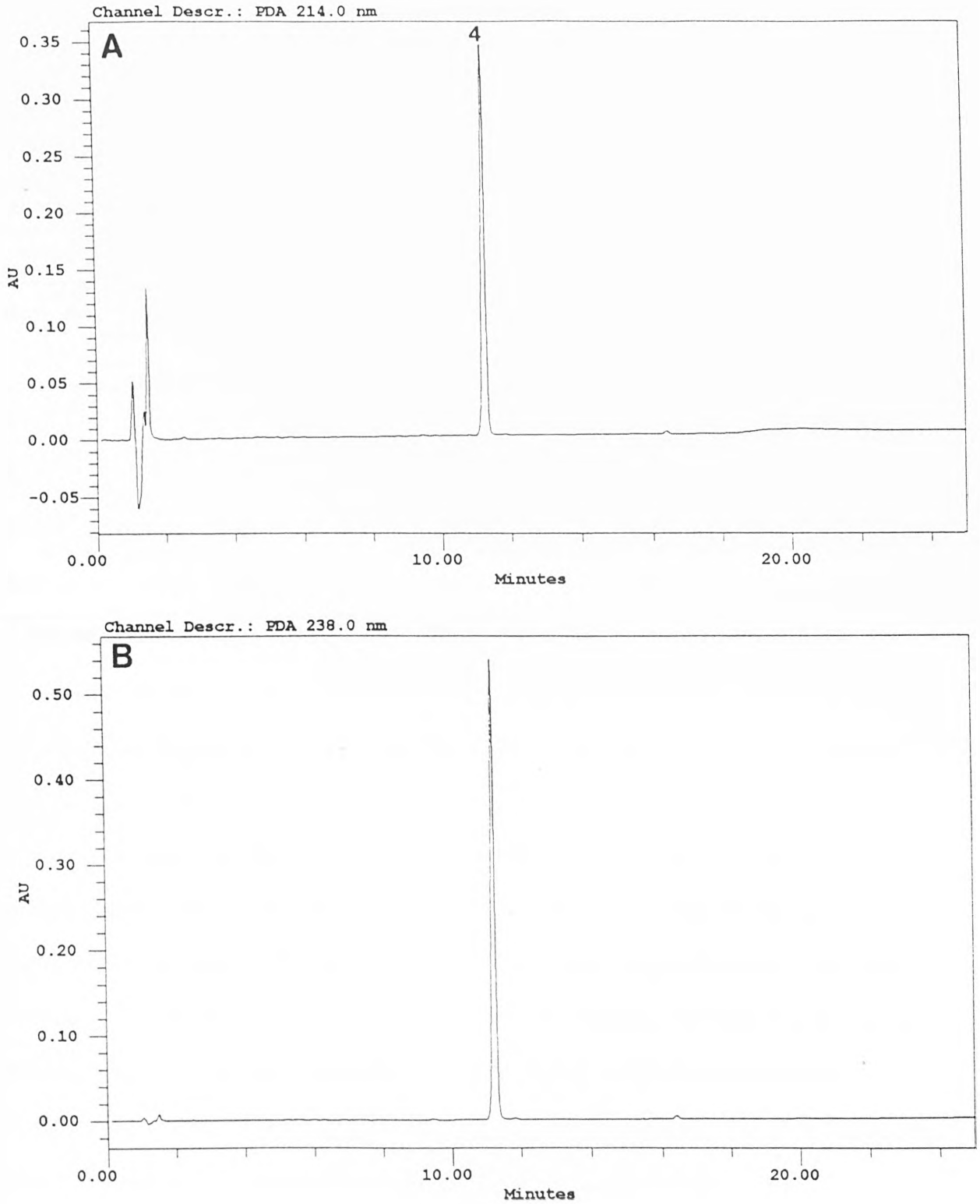



Fig. 4.16. Analysis of MCYST-LR (4) fraction collected from a preparative HPLC run by analytical HPLC with diode-array detection (see section 4.2.4).

Table 4.4. Recovery of MCYST-LR (**4**) from HPLC preparative gradient separations.

| Run No. | Total load (g) | Recovery (%) | Purity (%) |
|---|---|---|---|
| 1 | 0.3 | 84 | 98 |
| 2 | 0.5 | 83 | 98 |
| 3 | 0.5 | 82 | 96 |
| 4 | 0.5 | 85 | 97 |

[a] As determined by analytical HPLC with diode-array detection

## 4.4. DISCUSSION

This chapter presented a method of concentrating and cleaning-up gram quantities of microcystins using reversed-phase flash chromatography and laboratory-scale preparative HPLC.

The use of a Biotage Flash system which can house 500 g cartridges allows the processing of large quantities of cell extract. Most methods used to purify microcystins to date rely on the use of small (1 g) cartridges (Brooks and Codd, 1986; Sivonen *et al.*, 1992; Lawton *et al.*, 1995) which restricts the amount of material that can be used in a single purification.

Flash chromatography using prepacked cartridges resulted in simpified fractions which were more easily separated on preparative HPLC. No difference was observed between the two types of prepacked cartridges used in flash chromatography. Thus, there was no advantage in using a high-performance Hyperprep stationary phase in this application and Bondapak $C_{18}$ cartridges were used for any further clean-up of cyanobacterial extracts. Optimum conditions for separation of these simplified fractions was investigated using analytical columns, the advantage being that only small amounts of the samples were sacrificed during method development and also that various stationary phases could be assessed for use in this application. It also allowed the calculation of the

maximum loading of sample that could be applied to a preparative HPLC column.

In *method A* the supernatant from the extraction was rotary evaporated to an oily residue to remove the methanol and then diluted with water and filtered before application onto the flash system. The rotary evaporating part of the method was very time consuming (1 week) and this was altered in *method B* to reduce the processing time. The supernatant was diluted to 20% methanol, filtered and loaded onto the flash system. This increased the volume (80 l) of extract that had to be loaded onto the flash cartridge compared to *method A* but this took only seven hours to load and was still much less time consuming than rotary evaporating the supernatant. Further more, this reduces sample handling and exposure to toxic compounds. The Biotage system also has a large solvent/sample reservoir and the extract was left to load overnight. Another advantage of these methods is the rapid desalting of the microcystin-containing fractions by using the same preparative cartridge used for the separations. This allowed the microcystins to be eluted in a small volume which was easily dried down, thus avoiding the compounds being exposed to solvent and salts for long periods of time.

*Method A* enabled the purification of four of the microcystins (MCYST-1; MCYST-LR; MCYST-12 and MCYST-LF) at purity greater

than 95% with recoveries between 75-93%. It was possible to obtain 0.26 g MCYST-LR purified to 98% purity when a total load of 0.6 g of sample was injected onto the preparative column. The column was not overloaded at this stage and it would be possible to increase the total load to 1 g and thus purify 0.5 g MCYST-LR. The separation and purification of MCYST-LW and MCYST-LF would not of been possible without the use of closed-loop recycling but this method worked well and these microcystins were obtained at purities greater than 90%.

*Method B* resulted in one fraction containing all the microcystins, the aim being to purify as many as possible using a single gradient. This method avoided having many flash fractions which would have to be analysed by HPLC with diode-array detection before fractions of similar polarities could be combined. However the purities obtained were not as good as in *method A*. Yields of MCYST-LR with purity greater than 95% were obtained with recoveries greater than 80%, however, the purity of other microcystins ranged between 60 and 80% and further purification is necessary.

Many methods to date involve numerous steps between extraction and purification by HPLC. For example, Namikoshi *et al.* (1992) described the purification of microcystins from a methanolic extract of cells (240 g). The supernatant was passed through a series of three silica gel columns of varying mesh size before three more simplified fractions were obtained. Even at this

stage fraction 1 had to be subjected to another two column chromatography separations before repeated separation by preparative TLC (thin layer chromatography). The remaining two fractions also required more complex separations. Sivonen *et al.* (1992) described the purification of a methanolic extract of cells by elution from a $C_{18}$ silica gel column followed by a series of three further HPLC separations using semi-preparative $C_{18}$ silica gel columns before final purification on an analytical $C_{18}$ silica gel column.

The method proposed in this chapter simplifies the purification step considerably and is the first method described for the purification of large quantities of microcystins.

# Chapter 5

# Purification and characterisation of unknown microcystins in a laboratory culture of *Microcystis aeruginosa*

## 5.1. INTRODUCTION

The large-scale purification method developed previously was used to purify the microcystin variants known to be produced by a different strain of *Microcystis aeruginosa*. This strain was purchased from a school supplies catalogue and was discovered to be toxic during routine screening of laboratory cultures. This demonstrates the importance of screening all cyanobacteria.

The sample in this case proved more complex than the previous strain of *M. aeruginosa*, containing many closely eluting microcystin compounds. For this reason it was decided to elute the microcystins from the flash chromatography system in a stepwise gradient instead of elution in a single fraction. This resulted in two major fractions, one of which contained a single microcystin. The other fraction, however, contained the remaining microcystins and further purifications steps were required to allow characterisation by mass spectrometry and amino-acid analysis.

There are over 50 microcystin variants known to be produced by cyanobacteria but the majority of these are not commercially available as standards. It is important to identify the microcystins present in natural waters as this may give an indication of toxicity, as it is known that microcystin

variants can vary in potency. MCYST-LR is found to occur most frequently among the microcystins in samples from many different parts of the world. Namikoshi *et al.* (1992) found that MCYST-LR was the principle toxin (ca. 90% of the toxic component) isolated from *Microcystis* cyanobacteria collected from a loch in the USA. Vasconcelos *et al.* (1996) also reported that MCYST-LR was the most common microcystin isolated in a range of bloom samples in Portuguese freshwaters. MCYST-LR is also commonly reported in British freshwaters (Lawton et al., 1995).

Watanabe *et al.* (1988) reported that several strains of *M. aeruginosa* and *M. viridis* isolated from bloom samples in Japan contained mixtures of MCYST-LR, MCYST-RR and MCYST-YR. However, the main component of the toxins of these strains was MCYST-RR. The toxins produced by the strain of *M. aeruginosa* used in this study is more similar to that reported for Japanese strains of *Microcystis*. It is not known where this strain was originally isolated from but its microcystin profile is more unusual than that normally found in Europe.

## 5.2. MATERIALS AND METHODS

### 5.2.1 Cultivation of cyanobacteria

Cultures of *M. aeruginosa* (Sciento, Manchester) were grown in bulk under conditions previously described (chapter 2; section 2.2.1.). The cultures were harvested by centrifugation at 1500 x g for 60 mins. The supernatant was decanted and the pellets of cells transfered to plastic beakers and frozen at -20°C until required.

### 5.2.2 Extraction of microcystins

Approximately 4 l of cell material were thawed and extracted for an hour in an equal volume of 100% methanol (Rathburns, Walkerburn, UK) plus ammonium acetate (5 $gl^{-1}$) with regular stirring. Aliquots (1 ml) of the methanolic extraction were placed in pre-weighed vials and dried to give the dry weight of cells per ml of extract. Thus, it was calculated that the 4 l of cell material was equivalent to approximately 39 g dry weight of cells. The extract was centrifuged at 1500 x g for 45 minutes and the supernatant retained. The cells were extracted a further two times in 400 ml methanol for 30 minutes and each processed as before. The supernatants were pooled and diluted to 20% methanol with distilled water and filtered through GF/C filter disks.

### 5.2.3. Concentration and clean-up of microcystins

The resulting extract (approx. 28 l) was loaded onto a Biotage Flash 40 system (Biotage Inc., Charlottesville, USA) containing a Hyperprep $C_{18}$ flash cartridge. This is similar to the Biotage Flash 75 S system described previously (chapter 4; section 4.2.3.) except that it is smaller and uses smaller cartridges, thus reducing the cost of packing material.

Microcystins were eluted from the flash column using a step gradient from 0% to 100% in 10% increments (1 l solvent per step) and fractions (100 ml) were collected manually. These fractions were numbered 1-110. Fractions were monitored using a spectrophotometer (Novaspec II, Pharmacia Biotech) at 238 nm which is the characteristic absorbance maxima for microcystins. Fractions having a high absorbance at 238 nm were analysed by HPLC using a previously described method (Lawton *et al.*, 1994). Fractions containing significant quantities of microcystin(s) of similar polaritiy were then combined and rotary evaporated to dryness.

### 5.2.4. Optimisation of separation methods using an analytical column

The combination of similar flash chromatography fractions resulted in 2 major fractions. The aim was to purify

sufficient quantities of material to allow characterisation of the unknown microcystins. Optimum conditions for separation of the microcystin variants was investigated using an analytical Shandon Hyperprep HS BDS $C_{18}$ column (15 x 0.46 cm I.D.; 12 μm particle size) with a flow rate of 1.5 $mlmin^{-1}$. Once developed the process was scaled up using a preparative HPLC column packed with the same material as the analytical column.

*Method 1*: Fractions eluted from the flash column between 56 and 74 (Fraction 1) contained 1 microcystin which was sucessfully separated with a mobile phase of (A) Ammonium acetate (0.1% w/v) and (B) acetonitrile using an isocratic gradient of A:B (78:22). Fractions were collected over the peak and sufficiently pure fractions combined for characterisation.

*Method 2*: Fractions eluted from the flash column between 31 and 54 (Fraction 2) contained 4 main microcystins which were separated using a mobile phase of (A) Water (0.05% T.F.A) and acetonitrile (0.05% T.F.A). Best separation was achieved with a mobile phase of A:B (68:32), with a step increase after 20 minutes to A:B (65:35).

### 5.2.5. Preparative HPLC

An aliquot of fraction 2 was loaded onto a Kiloprep 100 labscale HPLC with a KPCM 100 compression module (Biotage, Charlottesville, U.S.A), a UV detector (Linear 205) and a linear 1201 chart recorder (Thermoseparations). The sample was separated on a 7.5 x 15 cm I.D. cartridge packed with Shandon HS BDS C18 (12 μm particle size) as described in method 2 (section 5.2.4.) with a flow rate of 400 $mlmin^{-1}$. The eluent was monitored at 238 and 214 nm and fractions were collected over a peak when it became visible on the chart recorder. Fractions were analysed by reversed-phase HPLC with diode array detection as described by Lawton *et al.* (1994) and pooled according to their purity to give five major fractions. Each fraction was rotary evaporated to dryness.

### 5.2.7. Liquid chromatography-mass spectrometry

Each of the five fractions was analysed using liquid chromatography-mass spectrometry by Mr. R. Boughtflower at Glaxowellcome, Stevenage, UK. Separation of fractions was performed on a 3.5 x 0.46 cm I.D. ABZ $C_{12}$ (3 μm particle size) column (Supelco, Poole, UK) using a mobile phase of (A) 0.1% formic acid (Sigma, UK) and (B) acetonitrile (Rathburns, Walkerburn, UK). Microcystins were separated using a linear

gradient from 0% to 95% (B) over 8 minutes and monitored by micromass platform using electrospray ionisation.

### 5.2.8. Amino-acid analysis

Several of the fractions, however, still contained trace contaminants of the other closely eluting microcystins and required further purification before amino-acid analysis could be performed. This was achieved by repetitive injections of the samples onto an analytical Symmetry $C_{18}$ column (15 x 0.46 cm. I.D.; 5 μm particle size) and collection of fractions over the peak using a fraction collector (Waters, UK).
Pure fractions were combined and subjected to amino-acid analysis using the following method which was adapted from Waters pico tag method.

*Materials*

HYDROLYSIS REAGENT

*Phenol crystals (1 g) were weighed into a small test tube and liquified over a waterbath at 50°C. The liquid phenol (1 ml) was added to 0.1 ml 6M HCL and mixed. Next, 0.1 ml of this solution was added to 10 ml 6M HCL.*

REDRYING REAGENT

*The redrying solution consisted of a 2:2:1 mixture (by volume) of methanol: water: triethylamine.*

DERIVATISATION REAGENT

*The derivatising solution consisted of a 7:1:1:1 mixture (by volume) of methanol: triethylamine: water: PITC (phenylispthiocyanate).*

Amino-acid analysis involved three steps namely hydrolysis of the peptide sample to free amino-acids followed by pre-column derivatisation and analysis of the sample by reversed-phased HPLC. Aliquots (approx. 10 µg) of sample were placed in sample tubes using an analytical syringe and dried under vacuum.

An aliqout (200 µl) of hydrolysis reagent was dispensed into the bottom of a reaction vial and the sample tubes placed inside, covered with a little glass wool and the reaction vial cap closed.

The reaction vial was then subjected to a total of three vacuum-nitrogen cycles to ensure that the reaction vial was free of oxygen. The reaction vial was placed in an oven at 150°C for three hours. The sample tubes were removed after cooling and dried under vacuum. When dry, 20 µl of redrying reagent was added to each sample tube and redried under vacuum. This step is necessary to adjust the pH of the derivatisation mixture to the required level.

The samples were then derivatised. An aliquot of derivatising reagent (20 µl) was added to each sample tube, vortex mixed and left to stand at room temperature for 20 minutes. The samples were dried again under vacuum to remove all traces of PITC.

The derivatised samples were analysed by HPLC on a Waters Nova-Pak $C_{18}$ (30 x 0.39 cm I.D.) column using the following gradient. The column oven temperature was set at 46°C and the detector at 254 nm. The samples were resuspended in 200 µl eluent A for analysis.

| Time (mins) | Flow ($mlmin^{-1}$) | % A | % B | Curve |
|---|---|---|---|---|
| 0 | 1 | 100 | 0 | - |
| 13.5 | 1 | 97 | 3 | 11 |
| 24 | 1 | 94 | 6 | 8 |
| 30 | 1 | 91 | 9 | 5 |
| 50 | 1 | 66 | 34 | 6 |
| 62 | 1 | 66 | 34 | 6 |
| 62.5 | 1 | 0 | 100 | 6 |
| 66.5 | 1 | 0 | 100 | 6 |
| 67 | 1 | 100 | 0 | 6 |
| 87 | 1 | 100 | 0 | 6 |

**Eluent A**

Sodium acetate trihydrate (9.525 g) was dissolved in 1 l water and the pH adjusted to 6.45 with 10% acetic acid. The solution was then filtered and 975 ml added to 25 ml acetonitrile.

**Eluent B**

This consisted of a mixture of 450 ml acetonitrile; 400 ml water and 150 ml water.

The amino acids present in the samples were identified and quantified by comparison to a standard solution of a series of amino-acids (2.5 $\mu molesml^{-1}$) (Sigma, UK). An injection volume (25 µl) was chosen to ensure 2.5 nmoles of each amino acid was loaded onto the column. The peak area of the amino acid alanine was used to quantify the amount of alanine present in the purified microcystin samples. Alanine was present in all of the samples analysed. The molecular weights of each sample was previously obtained by mass spectrometry and allowed the determination of the quantity of microcystin in each sample.

## 5.3. Results

Five main microcystins **(1-5)** and two minor closely eluting microcystins **(a & b)** were identified in the extract of *Microcystis aeruginosa* (Sciento) by reversed-phase HPLC with diode array detection (Fig. 5.1.).

### 5.3.1. Flash chromatography

Flash fractions analysed by spectrophotometer displayed two regions of high absorbance at 238 nm between fractions 31 and 54 and fractions 56 and 74 (Fig. 5.2.). Analysis of fractions by analytical HPLC showed that fractions 56 to 74 contained a single microcystin (MCYST-1) while fractions 31 to 54 contained four microcystins (MCYSTS 2-5) (Fig. 5.3.).

### 5.3.2. Optimisation of separation methods using an analytical column

Flash fractions 56 to 74 were pooled to give fraction 1 (Fig. 5.4.) and fractions 31 to 54 were pooled to give fraction 2 (Fig. 5.5.). Fraction 1 was successfully separated on the analytical Shandon Hyperprep column as described in method 1 (section 5.2.4.) (Fig. 5.6.) and generated a sufficient quantity for characterisation. The best separation of fraction 2 was achieved under conditions described in method 2 (section

5.2.4.) (Fig. 5.7.). It was suspected that MCYST-3 and MCYST-4 were coeluting and the peak before MCYST-5 was the two minor coeluting microcystins (MCYSTS a & b). This complex fraction required further purification.

### 5.3.3. Preparative HPLC

An aliquot of fraction 2 was loaded onto the preparative HPLC system described previously (chapter 4; section 4.2.6.) and the microcystins eluted under conditions described in method 2 (Fig. 5.8.). The eluent was monitored at 214 and 238 nm and fractions were collected manually over the eluting peaks. These fractions were analysed using HPLC with diode array detection (Lawton *et al.*, 1994) and fractions of acceptable purity were pooled resulting in five main fractions (Table 5.1).

### 5.3.5. Characterisation of microcystins

MCYST-2 and -4 were obtained at purities greater than 90% using this method. The other microcystin fractions ranged between 32-86% purity and further purification was necessary. The contaminates in these cases were closely related microcystins. Further purification of these fractions was attempted by separation on an analytical Symmetry $C_{18}$ column (15 x 0.46 cm I.D.; 5 μm particle size) and collection of

fractions using a waters fraction collector. However, it proved impossible to obtain MCYSTS -3 or -5 in sufficiently pure quantities for characterisation and it was proposed that closed-loop recycling (chapter 4; section 4.3.2) would be necessary.

Mass spectrometry tentatively identified MCYST-2 as MCYST-YR or -HtyR; MCYST-4 as MCYST-LR; MCYST-a and b as variants of MCYST-FR and MCYST-5 as MCYST-WR (Table 5.1). This identification was confirmed for several of the microcystins using amino-acid analysis which identified MCYST-1 as MCYST-RR (Fig. 5.9.), MCYST-2 as MCYST-YR (Fig. 5.10.) and MCYST-4 as MCYST-LR (Fig. 5.11.).

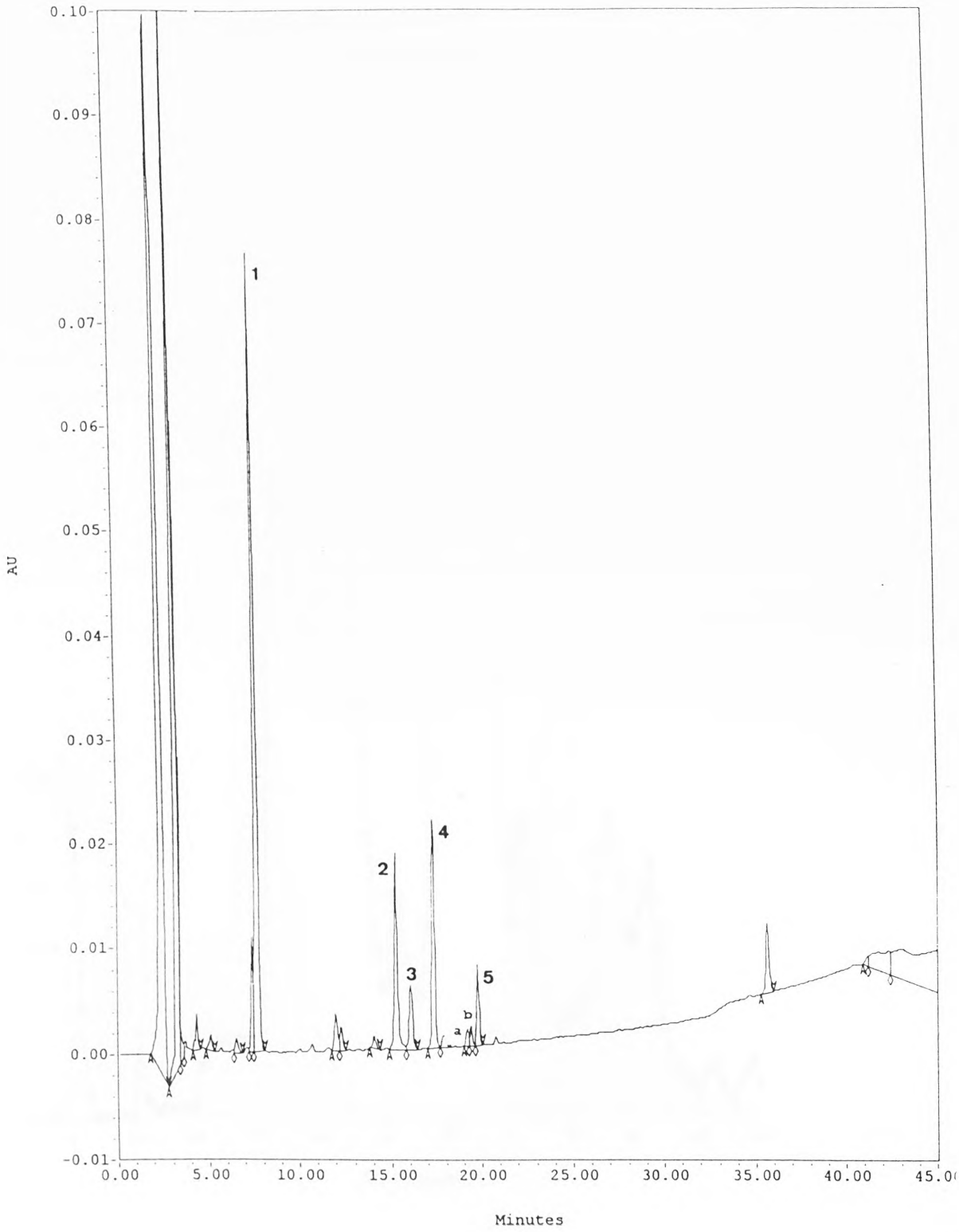



Fig. 5.1. Analysis of the methanolic extract of *Microcystis aeruginosa* (Sciento) by reversed-phase HPLC with diode array detection. Microcystins are labelled 1-5 and a-b.

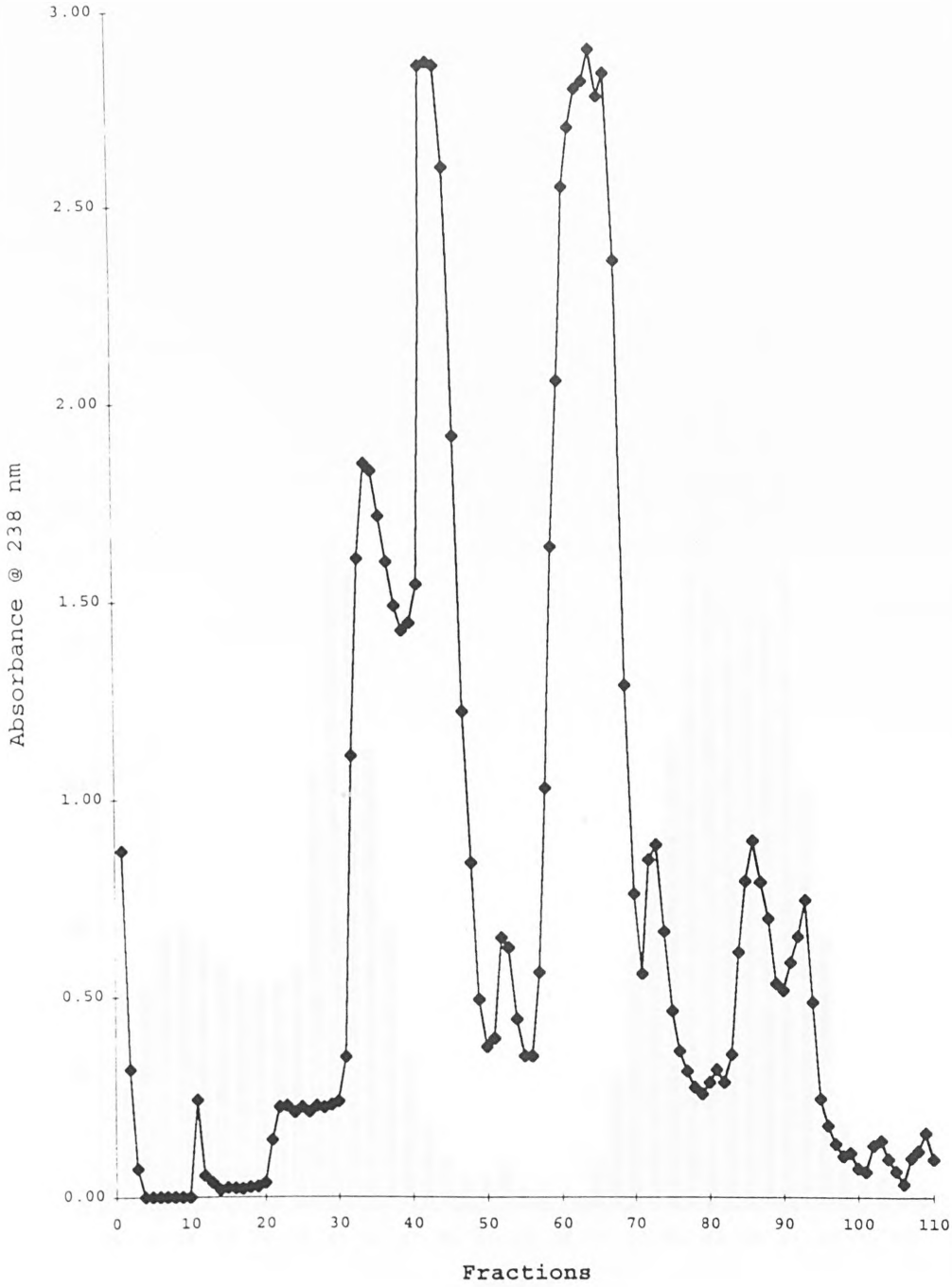



Fig. 5.2. Absorbance (238 nm) of fractions eluted from a Hyperprep $C_{18}$ flash cartridge using a stepwise gradient from 0 to 100% methanol in 10% increments using 1 l solvent per step. Fractions (100 ml) were collected manually.

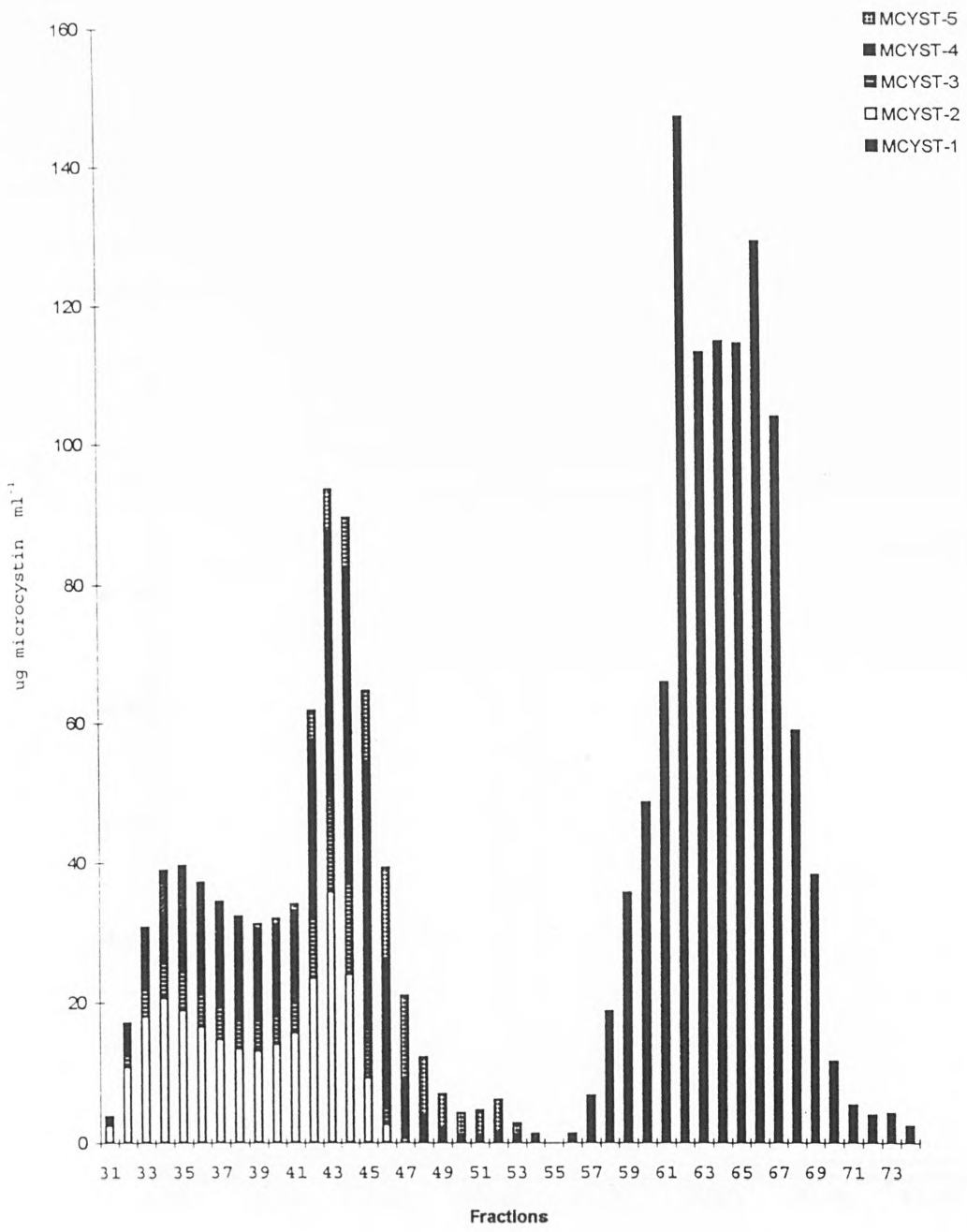



Fig. 5.3. Analysis of flash fractions by analytical HPLC with diode array detection showing the distribution of the five main (1-5) microcystins present in the extract of *Microcystis aeruginosa*.

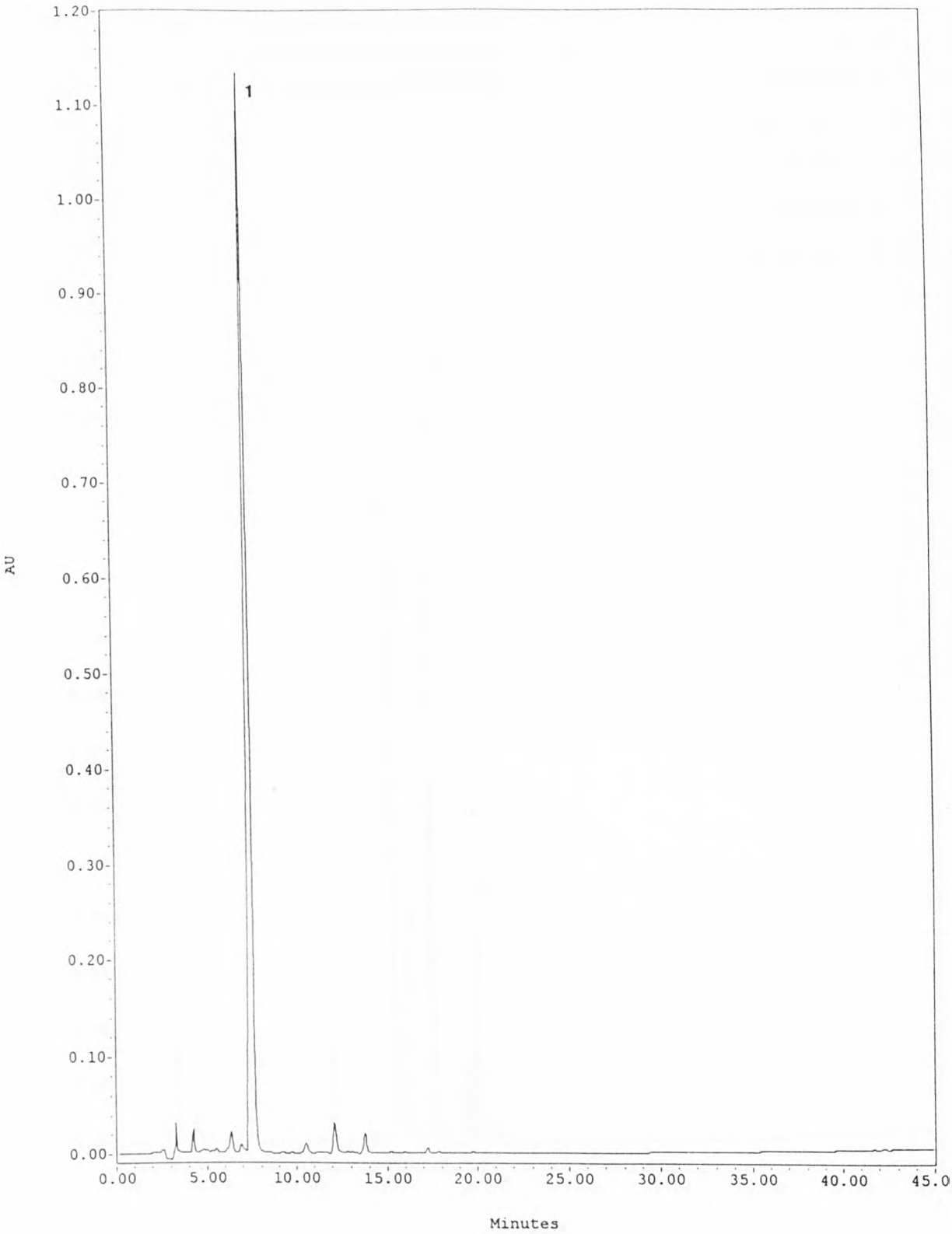



Fig. 5.4. Analysis of fraction 1 by reversed-phase HPLC with doide array detection showing the presence of one predominant microcystin (MCYST-1).

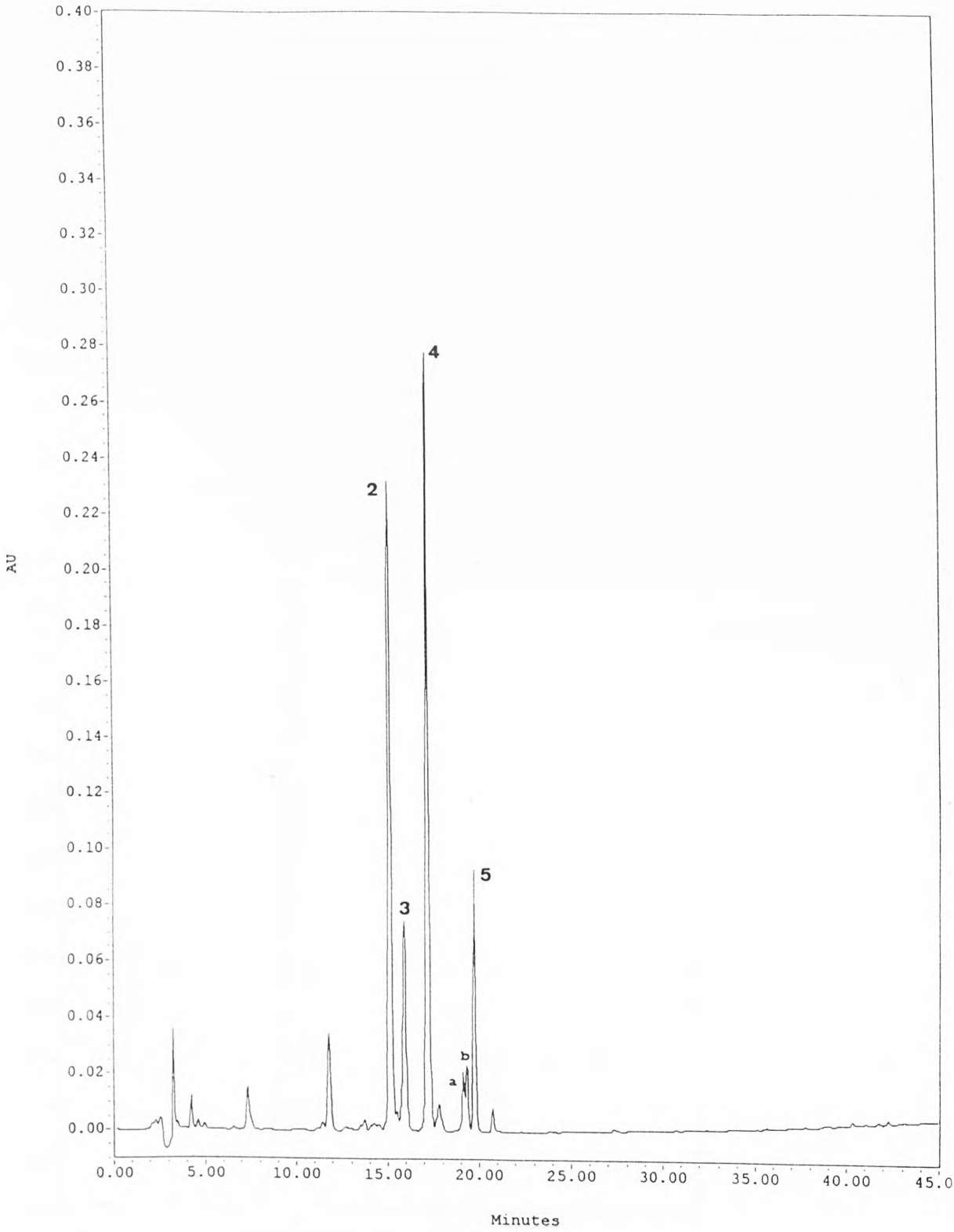



Fig. 5.5. Analysis of fraction 2 by reversed-phase HPLC with diode array detection showing the presence of four main microcystins (MCYST-2; -3; -4 and -5) and two coeluting microcystins (MCYST-a and -b).

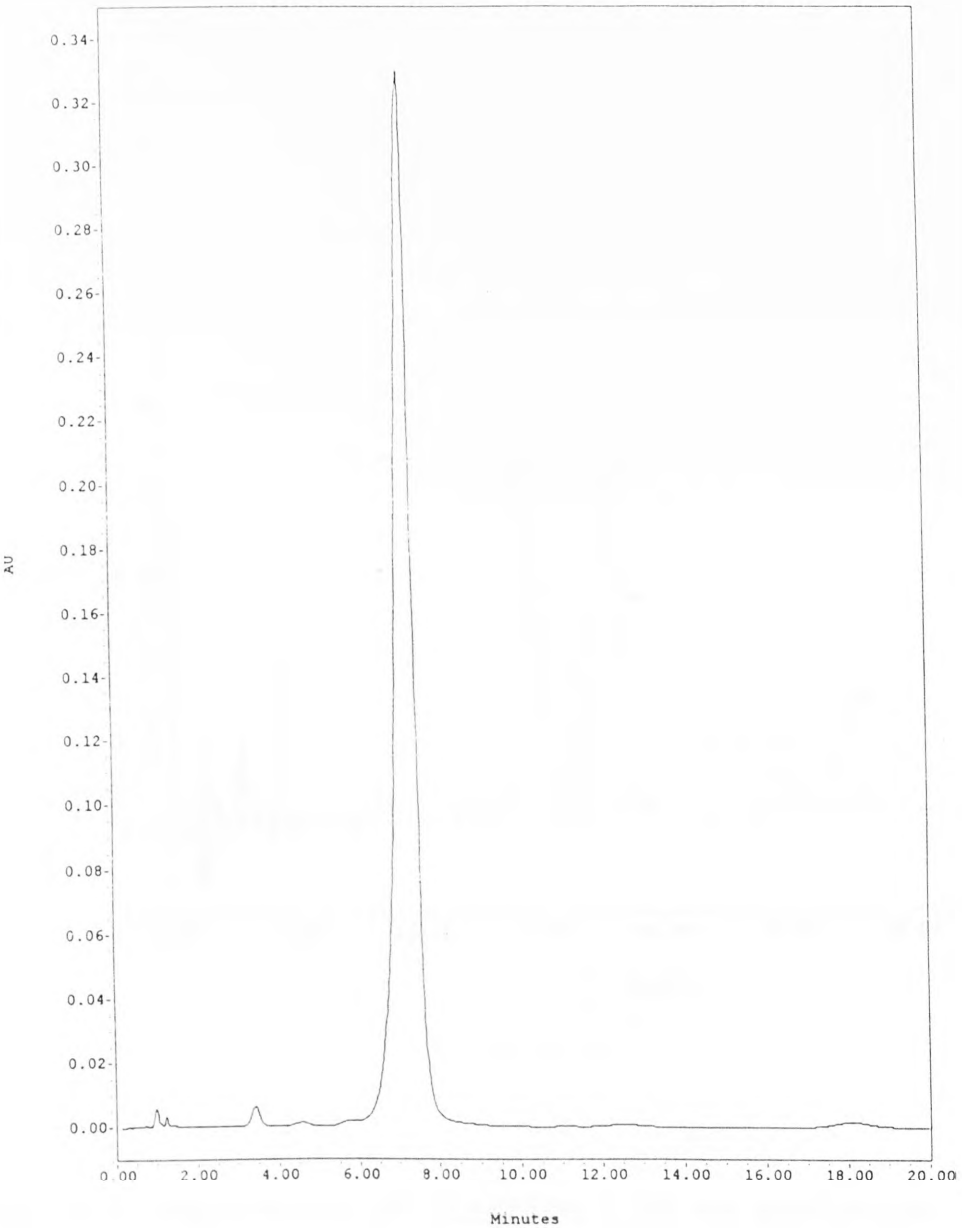



Fig. 5.6. Separation of fraction 1 on an analytical Shandon Hyperprep HS BDS $C_{18}$ Column (15 x 0.46 cm I.D.; 12 μm) using a mobile phase of (A) ammonium acetate (0.1%, w/v) and (B) acetonitrile, A:B (78:22).

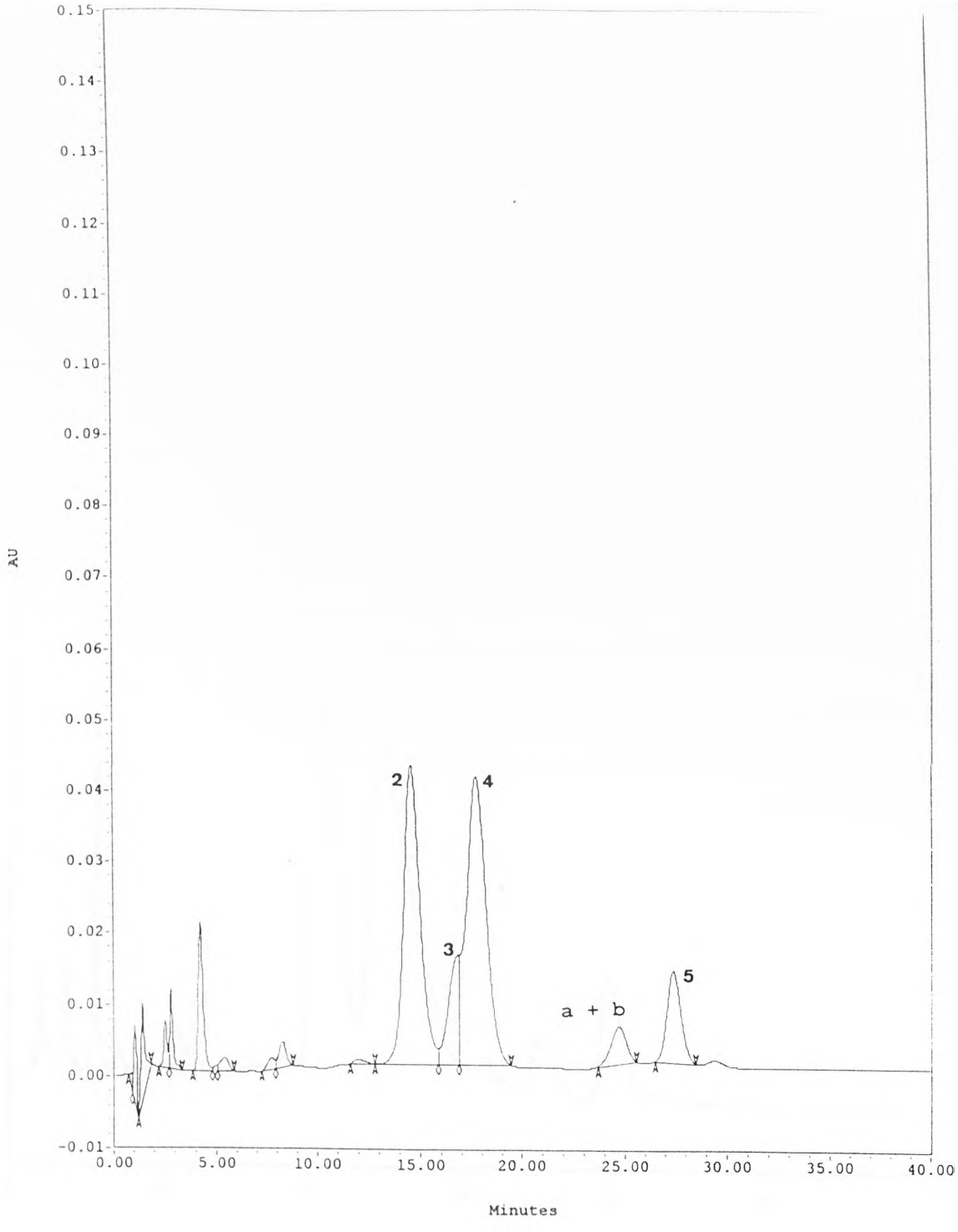



Fig. 5.7. Separation of fraction 2 on an analytical Shandon Hyperprep HS BDS $C_{18}$ column (15 x 0.46 cm I.D.; 12 µm) using a mobile phase of (A) water (0.05% TFA) and (B) acetonitrile (0.05% TFA). Best separation was achieved with a mobile phase of A:B (68:32) with a step increase after 20 minutes to A:B (65:35).

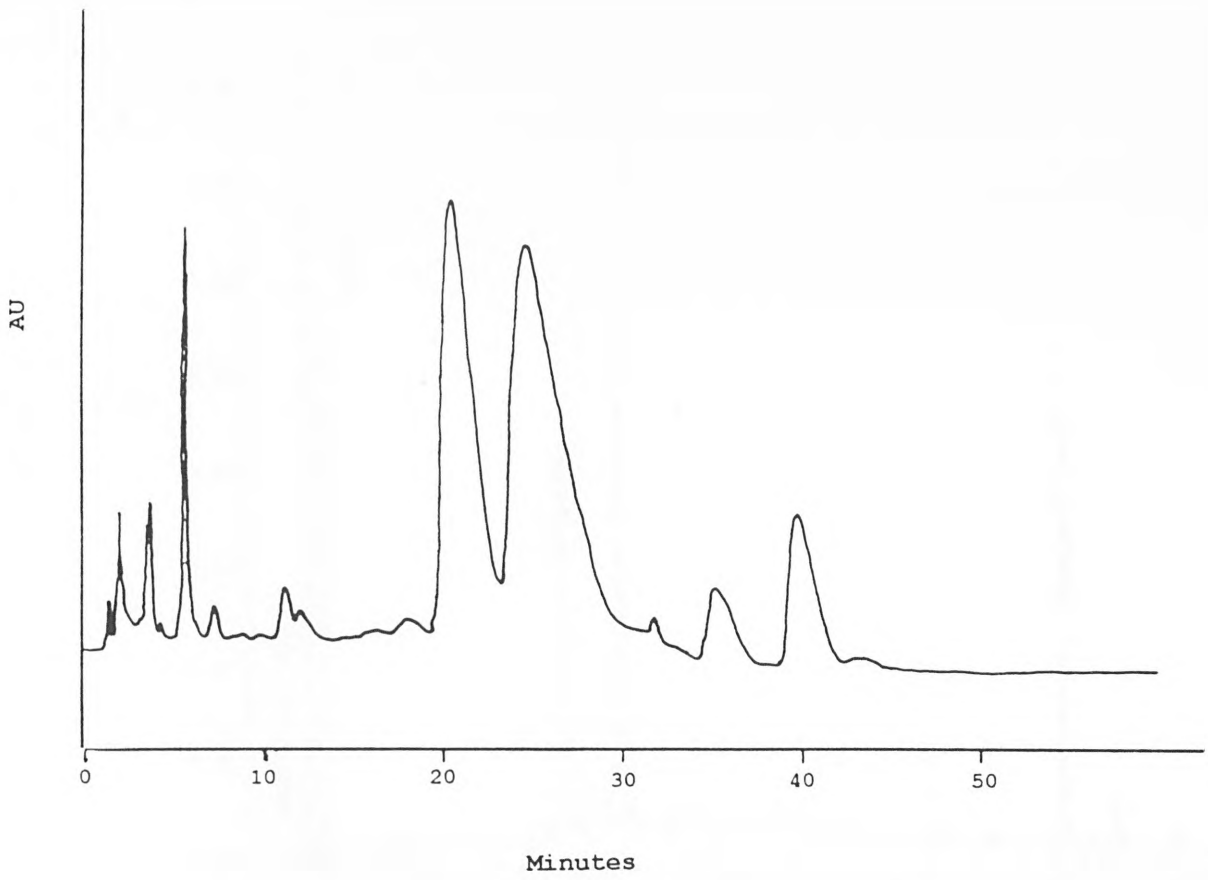



Fig. 5.8. Preparative separation of fraction 2 using a 7.5 x 15 cm I.D. cartridge packed with Shandon HS BDS $C_{18}$ (12 μm) at a flow rate of 400 $mlmin^{-1}$.

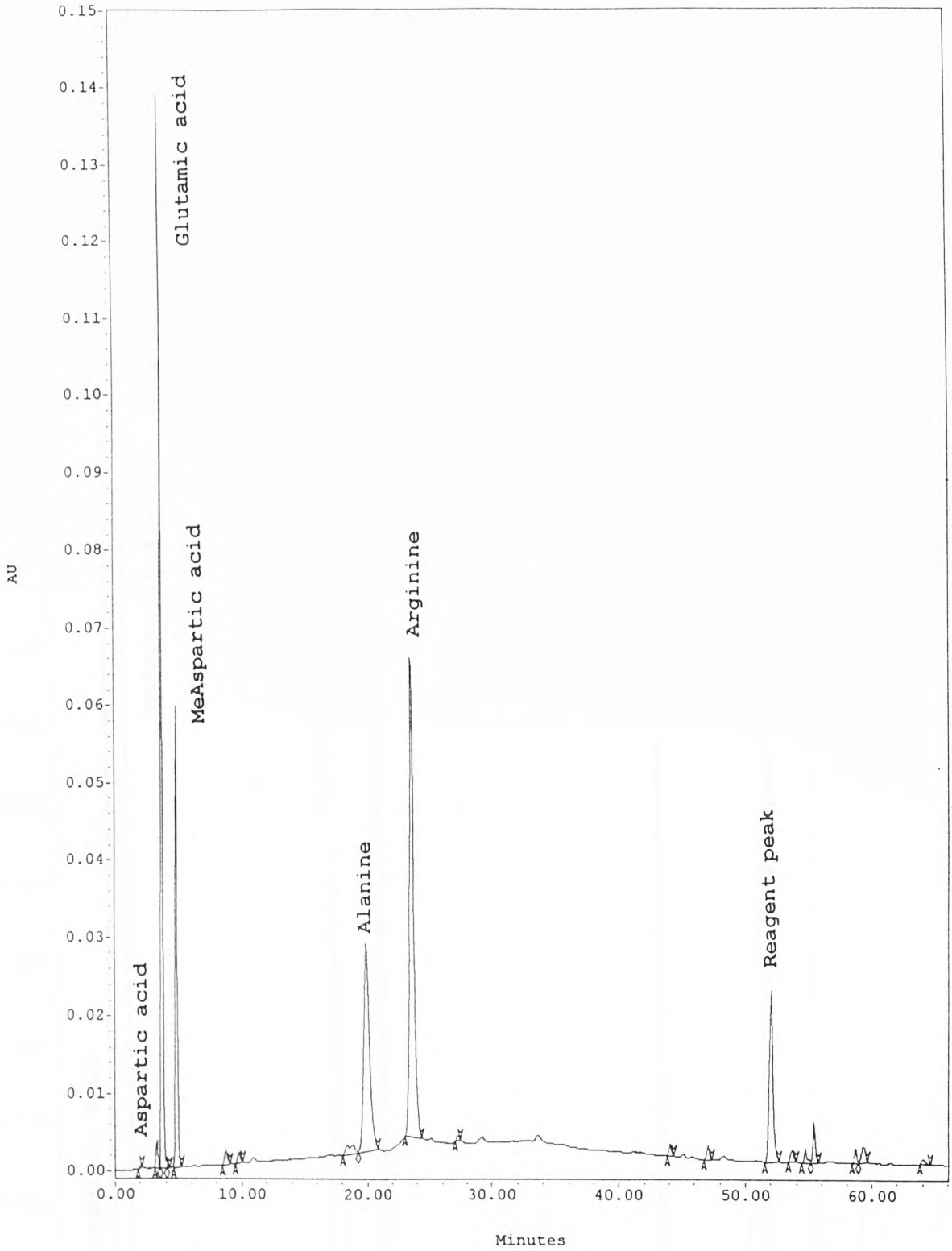



Fig. 5.9. Amino-acid analysis of MCYST-1. The identification of two arginine amino-acids in the X and Y positions of the microcystin molecule identify it as MCYST-RR.

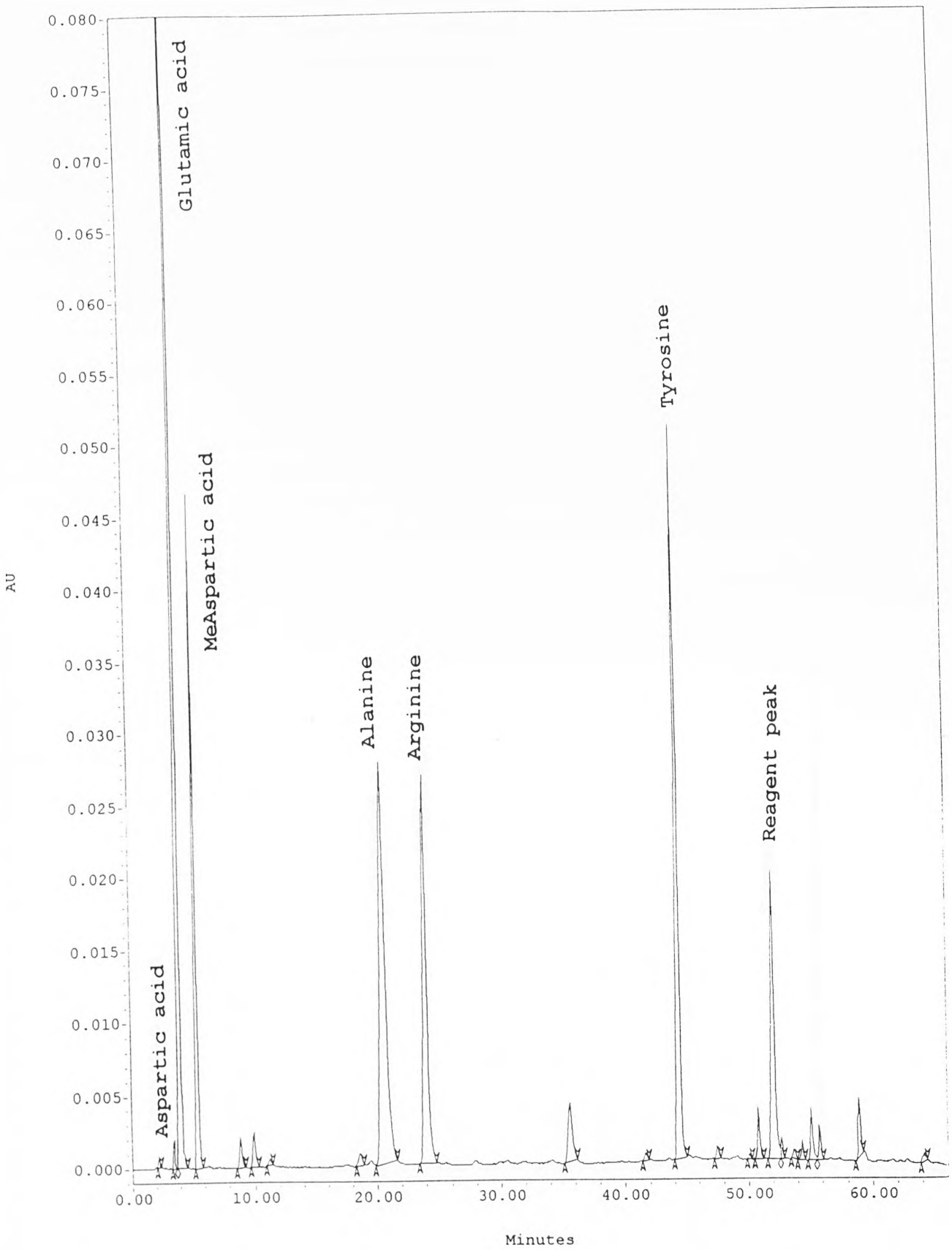



Fig. 5.10. Amino-acid analysis of MCYST-2. The identification of an arginine in the X position and a tyrosine in the Y positions of the microcystin molecule identify it as MCYST-YR.

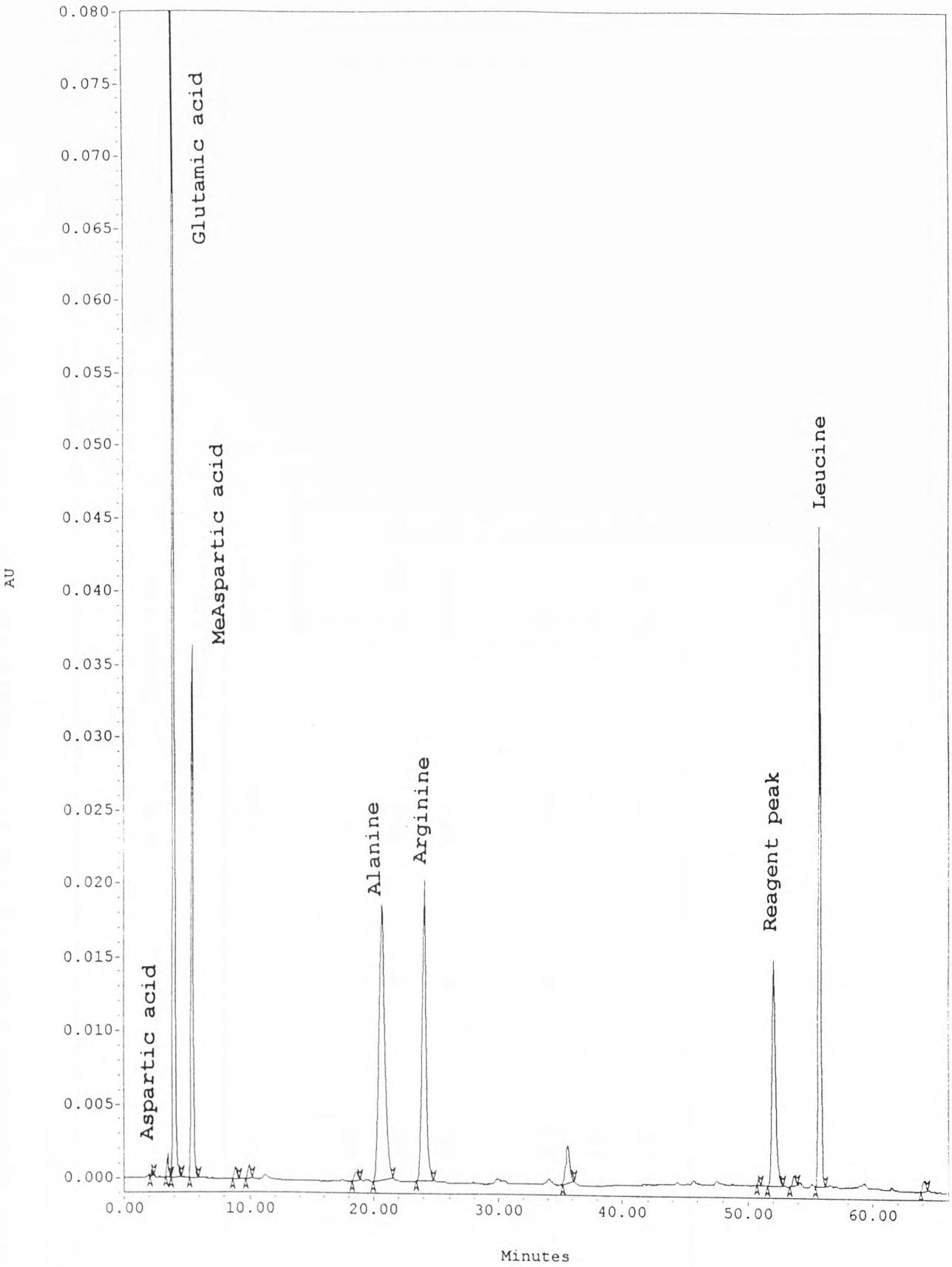



Fig. 5.11. Amino-acid analysis of MCYST-4. The identification of an arginine in the X position and a leucine in the Y positions of the microcystin molecule identify it as MCYST-LR.

Table 5.1. Mass spectrometry data of the five preparative HPLC fractions obtained from separation of fraction 2.

| Fraction | $R_t$ | Peak no. | M.W. | Tentative identification | Purity |
|---|---|---|---|---|---|
| 1 | 15.65 | 2 | 1044 | MCYST-YR<br>MCYST-HtyR | 91 |
| 2 | 16.48 | 3 | - | - | |
| 2 | 17.68 | 4 | 994 | MCYST-LR | 70 |
| 3 | 17.68 | 4 | 994 | MCYST-LR | 91.5 |
| 4 | 19.57 | a | 1028 | MCYST-FR | 65 |
| 4 | 19.74 | b | 1028 | MCYST-FR | 32 |
| 5 | 20.19 | 5 | 1068 | MCYST-WR | 86 |

## 5.4. DISCUSSION

By using the large-scale purification method developed previously it was possible to obtain and characterise unknown microcystins produced by a species of cyanobacteria. Although these microcystins have been previously identified they were not yet available in the quantities now obtained using this procedure. Several of these microcystins were obtained in sufficiently pure quantities and are available for use as standards or for building a spectral library against which natural samples can be screened. The closely eluting microcystins which were not successfully purified are available in sufficient quantities to allow purification by closed-loop recycling.

Future work will involve purifying further microcystin variants to add to the spectral library of microcystins. The ideal library would have spectra of all known variants and would provide a rapid means of obtaining a preliminary identification of microcystins in natural samples. If sufficient quantities of standards were also available this would allow coelution studies to be carried out.

If a microcystin variant(s) of interest is identified during screening of natural samples it can be isolated and grown in bulk to allow large scale purification. When obtained in sufficiently pure quantities the microcystins can be

tentatively identified and quantified by a combination of amino acid analysis and mass spectrometry. This initial analysis will identify previously characterised microcystins but also possible new variants. These new microcystins can then be further studied and their structure elucidated by more complex techniques such as nuclear magnetic resonance (NMR).

During the purification procedure it was observed that pH was affecting the elution profile of a certain microcystin identified later as MCYST-RR. Arginine is a polar amino acid with an extra ionizable N- containing group. It is possible that this characteristic could be used as a further means of separating microcystins by altering their charge and therefore their elution order under certain conditions.

This chapter demonstrates the application of a large scale purification process and the implications of having sufficient quantities of a wide range of microcystin variants available.

# Chapter 6

# Conclusions

The occurrence of cyanobacterial blooms is now recognised as a major world-wide problem in water management. Animal poisonings and recent human fatalities have highlighted the need for further research into these organisms and the toxins they produce. This study concentrated on the hepatotoxins known as microcystins which occur commonly in British freshwaters. These compounds have not yet been fully synthesised and the majority of the variants known to occur in nature are not commercially available as standards. This presents a problem when using HPLC as a technique to monitor the occurrence of these toxins in both the environment and in laboratory cultures. This study developed a large scale purification process which provided gram quantities of microcystins which could be used as standards in routine HPLC analysis.

The methodology was developed using a strain of *M. aeruginosa* were the four microcystins it produces had been previously characterised. These four variants were obtained at purities ranging from 80-95%. However, it was discovered that this species also produced eleven other microcystins although in much smaller quantities, two of these were obtained at greater than 95% purity and are available for characterisation. During routine screening of another strain of *M. aeruginosa* purchased from a school supplies catalogue it was discovered that this strain produced five main microcystin variants. Three of these were successfully purified and tentatively characterised by

mass spectrophotometry and amino-acid analysis. The next step would involve confirmation of the identifications by MS-MS and NMR spectrophotometry. The remaining variants require further purification by closed-loop recycling as they are contaminated by closely eluting microcystins.

The above methodology provided sufficient material to construct a spectral library, using a photodiode array detector, of a wide range of microcystin variants against which natural water samples were screened and any microcystins present identified. This was employed in routine monitoring of selected freshwater lochs and was also used to trace the occurrence of microcystins identified in a loch throughout the period of a day. This study observed considerable spatial and temporal variation of several microcystins that were identified in the loch water. It also provided an insight into how sampling techniques may affect this variation and how they may be improved to decrease this observed variability.

It was noted that cyanobacterial samples taken from the water column of a loch displayed less variation in both biomass and microcystin content than samples obtained from the surface of the bloom. It was interesting to note that although the biomass of cells obtained from water column samples was lower than samples taken at the surface, the total amount of microcystins quantified was greater. Samples for toxicity testing are normally obtained from the surface of

cyanobacterial blooms and it is suggested that samples taken in this manner may be misleading. Much research is still required to discover the best techniques for sampling cyanobacteria and assessing toxicity. From this study it appeared that sampling the water column was more reproducible than sampling from the surface. Representative and reproducible sampling techniques are essential for ecological studies comparing data from year to year and over a range of different sites. Future work will involve investigating the variation between water column samples throughout the loch and also at different depths.

Investigations of this type require that a large number of samples can be obtained and analysed. This led to the development of a simple and rapid sampling and extraction procedure for microcystins in loch water. This method allowed the collection of open water samples and was able to detect and quantify the presence of microcystins in lochs not experiencing bloom conditions at the time of sampling. The method related the amount of microcystins present to a specific biomass of cyanobacteria and could be important in monitoring lochs that may in the future develop a cyanobacterial bloom problem.

One of the main advantages of this methodology was that the loch samples could be processed on site and avoided the need to transport quantities of water back to the laboratory. It

also avoided the lengthy process of freeze-drying cyanobacteria cells before extraction for microcystins. The cyanobacteria filtered onto filter disks on site can be dried in one hour to obtain a dry weight and are ready for extraction. During development of a drying regime for the cyanobacterial samples it was discovered that the microcystins were not thermally stable at high temperatures in the presence of cellular components. This problem was overcome by drying the samples at a lower temperature for a shorter period of time. No loss of microcystins was observed when cells were dried in this manner.

The above methodology was also used in the laboratory to investigate environmental factors influencing growth and toxin content of cyanobacteria. A 14 day bioassay was developed at which time the growth and toxin content of the cells was assessed. The ability to rapidly process samples meant that a larger number of replicates could be used for each treatment investigated. It was observed that high nitrate levels in growth medium reduced the microcystin content of cultures of *M. aeruginosa* PCC 7820. In phosphate bioassays more microcystins were also detected in cultures grown in medium with the lowest additions of phosphate. Although it is known that cyanobacteria thrive in nutrient-rich waters the levels of nutrients used in growth medium may be too high and become inhibitory or toxic. This phenomenon as regards to trace elements has been reported by Rippka (1988). It has also been

reported that the type of growth medium used can have considerable effect on the growth of algae and cyanobacteria (Rao *et al.*, 1996). This is an obvious problem when investigating factors affecting growth and toxin production of cyanobacteria and trying to relate it to the natural environment.

To try and overcome this problem a series of bioassays were carried out using natural sterile loch water. The aim being to assess the factors limiting growth and toxin production in a particular waterbody. Again the bioassay involved monitoring *M. aeruginosa* PCC 7820 inoculated into loch water either unmodified or spiked with selected nutrients. It was discovered that both phosphate and nitrate were limiting growth and toxin content throughout the year except in April when both growth and microcystin content was high in flasks minus any phosphate additions. The water samples were obtained from Loch Rescobie which is classified as eutrophic (Clelland, 1995) and it was suprising to find that phosphate and nitrate were limiting factors in the bioassays. It is suspected that processing of the loch water (filtration; sterilisation) may be interfering with the bioassay and the sample may not be a true representation of the loch water. The cyanobacteria are also not subjected to the same light regime or water movement that play an important role in the dynamics of their populations in the environment. It is suggested that *in situ* experiments in parallel with bioassay studies may be the next

step for investigating the role environmental factors play in cyanobacteria development.

In summary, this study has developed methodologies for sampling, extraction, purification and detection of microcystins in both the environment and in laboratory cultures. Future work will concentrate on investigating a wider range of environmental parameters and developing field studies to further understanding of the occurrence of these compounds in nature.

# CHAPTER 7

# References

Andersen, R.J., D.L. Burgoyne, D.E. Williams, F.M. Kong, E.D. Desilva, S.C. Miao, T.M. Allen, C.F.B. Holmes and D. Chen (1993). New natural products from marine invertebrates. *Gaz. Chim. Ital.* **123**:293-299.

ASTM. (1980). Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians (Unpublished report E-729-80). American standards for testing and materials, Philadelphia, PA.

Baden, D.G. and V.L. Trainer. (1993). Mode of action of toxins of seafood poisoniong. *In*: **Algal Toxins in Seafood and Drinking Water**, pp:49-74. (ed.) Falconer, I.R. Academic Press.

Bell, S.G. and G.A. Codd. (1996). Detection, analysis and risk assessment of cyanobacterial toxins. *In*: **Agricultural chemicals and the environment.** (eds.) Hester, R.E. and R.M. Harrison. The Royal Society of Chemistry, UK.

Benndorf, J and M. Henning. (1989). *Daphnia* and toxic blooms of *Microcystis aeruginosa* in Bautzen Reservoir (GDR). *Rev. Gesamten Hydrobiol.* **74**:223-248.

Beveridge, M.C.M., D.J. Baird, S.M. Rahmatullah, L.A. Lawton, K.A. Beattie and G.A. Codd. (1993). Grazing rate on toxic and

non-toxic strains of cyanobacteria by *Hypophthalmichthys molitrix* and *Oreochromis niloticus*. *J. Fish Biol.* **43**:901-907.

Bowling, L. (1994). Occurrence and possible causes of a severe cyanobacterial bloom in Lake Cargelligo, New South Wales. *Aust. J. Mar. Freshwater Res.* **45**:737-745.

Brooks, W.P. and G.A. Codd. (1986). Extraction and purification of toxic peptides from natural blooms and laboratory isolates of the cyanobacterium *Microcystis aeruginosa*. *Lett. Appl. Microbiol.* **2**:1-3.

Campbell, P.L. (1994). **Laboratory and field investigations into the cyanobacterial hepatotoxin, microcystin-LR.** Ph.D thesis. University of Dundee, UK.

Carmichael, W.W. and P.R. Gorham. (1981). The mosaic nature of toxic blooms of cyanobacteria. *In*: **The Water Environment-Algal Toxins and Health**, Vol 3, pp:121-147. (ed.) Tu, A.T. Marcel Dekker, Inc., New York, Basel.

Carmichael, W.W. (1988). Toxins in freshwater algae. *In*: **Handbook of Natural Toxins**, Vol 3, pp:121-147. (ed.) Tu, A.T. Marcel Dekker, New York.

Carmichael, W.W., V.R. Beasley, D.L. Bunner, J.N. Eloff, I. Falconer, P. Gorham, K.-I. Harada, T. Krisnamurthy, Y. Min-Juan, R.E. Moore, K.L. Rinehart, M. Runnegar, O.M. Skulberg and M.F. Watanabe. (1988a). Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae). *Toxicon.* **26**:971-973.

Carmichael, W.W., J.T. Eschedor, G.L.M. Patterson and R.E. Moore. (1988b). Toxicity and partial structure of a hepatotoxic peptide produced by the cyanobacterium *Nodularia spumigena* Mertens emends. L575 from New Zealand. *Appl. Envir. Microbiol.* **54**:2257-2263.

Carmichael, W.W. (1992). **A status report on planktonic cyanobacteria (blue-green algae) and their toxins.** US. E.P.A. - EPA/600/R-92/079, pp:5-7.

Carmichael, W.W. and I.R. Falconer. (1993). Diseases related to freshwater blue-green algal toxins and control measurements. *In*: **Algal Toxins in Seafood and Drinking Water**, pp:187-209. (ed.) Falconer, I.R. Academic Press.

Carmichael, W.W. (1994). The toxins of cyanobacteria. *Sci. Amer.* January, 1994, pp:64-72.

Clelland, B. (1995). Loch water quality in Tayside and Fife. Poster presentation. Conference on freshwater quality: defining the indefinable? University of Stirling, 6-7 Sept. 1995.

Codd, G.A. and G.K. Poon. (1988). Cyanobacterial Toxins. *In*: **Biochemistry of the algae and cyanobacteria**, pp:283-296. (eds.) Rogers, L.J and J.R. Gallon. Proceedings of the Phytochemistry Society of Europe. Vol. 28. Oxford University Press, Oxford.

Codd, G.A., C. Edwards, K.A. Beattie, W.M. Barr and G.J. Gunn. (1992). Fatal attraction to cyanobacteria? *Nature*. **359**:110-111.

Codd, G.A. (1995). Cyanobacterial toxins: occurrence, properties and biological significance. *Water Sci. & Tech.* **32**:149-156.

Column productivity. 1995. Biotage, Charlottesville, VA.

Cousins, I.T., D.J. Bealing, H.A. James and A. Sutton. (1996). Biodegradation of microcysin-LR by indigenous mixed bacterial populations. *Water Res.* **30**:481-485.

Couture, P., S.A. Visser, R. van Collie and C. Blaise. (1985). Algal bioassays: their significance in monitoring water quality with respect to nutrients and toxicants. *Schweiz. Z. Hydrol.* **47**/2:127-158.

Coyle, S.M. and L.A. Lawton. (1996). Development of an extraction procedure for the quantitative analysis of microcystins in cyanobacterial cells. *Phycologia*. Suppl. **35**:57-61.

Dahlem, A.M. (1989). **Structure/toxicity relationships and fate of low molecular weight peptide toxins from cyanobacteria.** PhD. Thesis. University of Illinois, pp:135-148.

Devlin, J.P., O.E. Edwards, P.R. Gorham, N.R. Hunter, R.K. Pike and B. Stavric. (1977). Anatoxin-a, a toxic alkaloid from *Anabaena flos-aquae* NRC-44h. *Can. J. Chem.* **55**:1367-1371.

Dillenberg, H.O. and M.K. Dehnel. (1960). Toxic waterbloom in Saskatchewan, 1959. *Can. Med. Assoc. J.* **83**:151.

Donati, M., R. Drikas and G. Newcombe. (1994). Microcystin-LR adsorption by powdered activated carbon. *Water Res.* 28:1735-1742.

Drikas, M. (1994). Control and/or removal of algal toxins. *In*: **Toxic cyanobacteria: Current status of research and management**, pp:93-102. (eds.) Steffensen, D.A. and B.C. Nicholson. Proceedings of an international workshop, Adelaide, Australia, March 22-24, 1994.

Dunn,J. (1996). Algae kills dialysis patients in Brazil. *Br. Med. J.* **312**:1183-1184.

Edwards, C., L.A. Lawton, S.M. Coyle and P. Ross. (1996). Laboratory-scale purification of microcystins using flash chromatography and reversed-phase high-performance liquid chromatography. *J. Chromatogr. A.* **734**:163-173.

Edwards, C., L.A. Lawton, S.M. Coyle and P. Ross. (1996a). Automated purification of microcystins. *J. Chromatogr. A.* **734**:175-182.

Ellemon, T.C., I.R. Falconer, A.R.B. Jackson and M.T. Runnegar. (1978). Isolation, characterisation and pathology of the toxin from a *Microcystis aeruginosa* bloom. *Aust. J. Biol. Sci.* **31**:209-218.

Eloff, J.N. and A.J. van der Westhuizen. (1981). Toxicological studies on *Microcystis*. *In*: **The water environment- algal**

**toxins and health**, pp:343-364. (ed.) Carmichael, W.W. Plenum Press, New York.

Falconer, I.R. (1991). Tumor promotion and liver injury caused by oral consumption of cyanobacteria. *Env. Toxicol. Water Qual.* **6**:177-184.

Fallick, G. and J. Romano. (1994). Enhanced photodoide array detection. *Liq. Chromatogr. GC Mag.* **12**:pp 768-772.

Fawell, J.K., C.P. James and H.A. James. (1993). Toxins from blue-green algae: Toxicological assesssment of microcystin-LR and a method for its determination in water. Wrc Foundation for Water Research, Allen House, The Listons, Morlow, Bucks, UK SL7 IFD.

Fogg, G.E. (1965). The general features of phytoplankton growth in lakes and the sea. *In*: **Algal cultures and phytoplankton ecology**, 2nd ed., pp:62. (ed.) Fogg, G.E. London: Athlone Press

Francis, G. (1878). Poisonous Australian Lake. *Nature (london)*. **18**:11-12.

Gorham, P.R. 1964. Toxic algae. *In*: **Algae and Man**, pp:307-336. (ed.) Jackson, D.F. Plenum Press, New York.

Gjølme, N. and H. Utkilen. (1994). A simple and rapid method for extraction of toxic peptides from cyanobacteria. *In*: **Detection Methods for Cyanobacterial Toxins**, pp:168-171. (eds.) Codd, G.A., T.M. Jefferies, C.W. Keevil and E. Potter. The Royal Society of Chemistry, Great Britain.

Harada, K.-I., M. Suzuki, A.M. Dahlem, V.R. Beasley, W.W. Carmichael and K.L. Rinehart. (1988). Improved method for purification of toxic peptides produced by cyanobacteria. *Toxicon*. **26**:433-439.

Harada, K.-I., K. Matsuura, M. Suzuki, M.F. Watanabe, S. Oishi, A.M, Dahlem, V.R. Beasley and W.W. Carmichael. (1990). Isolation and characterisation of the minor components associated with microcystins LR and RR in the cyanobacterium (blue-green algae). *Toxicon*. **28**:55-64.

Harada, K-I., K. Ogawa, Y. Kimura, H. Murata and M. Suzuki. (1991). Microcystins from *Anabaena flos-aquae* NRC 525-17. *Chem. Res. Toxicol*. **4**:535-540.

Harada, K.-I., I. Ohtani, K. Iwamoto, M. Suzuki, M.F. Watanabe, M. Watanabe and K. Terao. (1994). Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. *Toxicon*. **32**:73-84.

Hawkins, P.R., M.T.C. Runnegar, A.R.B. Jackson and I.R. Falconer. (1985). Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated from a domestic water supply reservoir. *Appl. Envir. Microbiol.* **50**:1292-1295.

Hessen, D.O. and E. van Donk. (1993). Morphological changes in *Scenedesmus* induced by substances released from *Daphnia*. *Arch. Hydrobiol.* **127**:129-140.

Himberg, K., A.M. Keijola, L. Hiisvirta, H. Pyysalo and K. Sivonen. (1989). The effect of water treatment processes on the removal of hepatotoxins from *Microcystis* and *Oscillatoria* cyanobacteria: A laboratory study. *Water Res.* **23**:979-984.

Hoffman, J.R.H. (1976). Removal of *microcystis* toxins in water purification processes. *Water (S Afr)*. **2**:58-60.

Horne, A.J. and C.R. Goldman. (1994). **Limnology**, 2nd edition. McGraw-Hill, Inc.

Hrudey, S.E. and T.W. Lambert. (1994). Overview of treatment/management of cyanobacteria within raw water sources. *In*: **Toxic cyanobacteria: Current status of research and management**, pp:103-110. (eds.) Steffensen, D.A. and B.C.

Nicholson. Proceedings of an international workshop, Adelaide, Australia, March 22-24, 1994.

International Code of Botanical Nomenclature. (1972). International association for plant taxonomy.

International Code of Nomenclature of Bacteria. (1975). American society for microbiology, Washington, DC.

Jones, G.J. (1990). Biodegradation and removal of cyanobacterial toxins in natural waters, pp:33-36. *In*: **Proceedings of Sydney Water Board Blue-green Algae Seminar,** 21-22 Nov. 1990.

Jones, G.J. and P.T. Orr. (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 Res.* **28**:871-876.

Keijola, A.M., K. Himberg, A.L. Esala, K. Sivonen and L. Hiisvirta. (1988). Removal of cyanobacterial toxins in water treatment processes: Laboratory and pilot-scale experiments. *Toxicity Assess.* **3**:643-656.

Kellar, P.E. and H.W. Paerl. (1980). Physiological adaptations in response to environmental stress during $N_2$-fixing *Anabaena* bloom. *Appl. Envir. Microbiol.* **40**:587-595.

Kotak, B.G., A.K-Y. Lam and E.E. Prepas. (1994). Variability of the hepatotoxin microcystin-LR in hypereutrophic drinking water lakes. *J. Phycol.* **31**:248-263.

Lampert, W., K.O. Rothhaupt and E. von Elert. (1994). Chemical induction of colony formation in a green alga (*Scenedesmus acutus*) by grazers (*Daphnia*). *Limnol. Oceanogr.* **39**(7):1543-1550.

Lawton, L.A., S.P. Hawser, K. Jamel Al-Layl, K.A. Beattie, C. MacKintosh and G.A. Codd. (1990). Biological aspects of cyanobacterial microcystin toxins, pp:83-89. *In*: **Proceedings of the Second Biennal Water Quality Symposium: Microbiological Aspects.** (eds.) Castillo, G., V. Campos and L. Herrera, University of Santiago, Chile.

Lawton, L.A., C. Edwards and G.A. Codd. (1994). Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst*. **119**:1525-1530.

Lawton, L.A., C. Edwards, K.A. Beattie, S. Pleasance, G.J. Dear and G.A. Codd. (1995). Isolation and characterisation of microcystins from laboratory cultures and environmental samples of *Microcystis aeruginosa* and from an associated animal toxicosis. *Nat. Toxins*. **3**:50-57.

Lindholm, T. (1992). Ecological role of depth maxima of phytoplankton. *Arch. Hydrobiol. Beih. Ergebn. Limnol.* **31**:33-45.

Lobban, C.S., D.J. Chapman and B.P. Kremer. (1988). Experimental phycology: a laboratory manual. Cambridge University Press.

Lund, J.W.G., G.H.M. Jaworski and H. Bucka. (1971). A technique for bioassay of freshwater with special reference to algal ecology. *Acta Hydrobiol*. **13**:235-249.

Lund, J.W.G., G.H.M. Jaworski and C. Butterwick. (1975). Algal bioassay of water from Blelham Tarn, English Lake District and the growth of planktonic diatoms. *Arch. Hydrobiol*. Suppl. **49**:49-69.

Lukac, M. and R. Aegerter. (1993). Influence of trace metals on growth and toxin production of *Microcystis aeruginosa*. *Toxicon*. **31**:293-305.

MacKintosh, C., K.A. Beattie, S. Klumpp, P. Cohen and G.A. Codd. (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **264**:187-192.

Namikoshi, M., K.L. Rinehart and R. Sakai. (1992). Identification of 12 hepatotoxins from a Homer Lake bloom of the cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis* and *Microcystis wesenbergii*: Nine new microcystins. *J. Org. Chem.* **57**:866-872.

Namikoshi, M., B.W. Oishi, R. Sakai, F. Sun, K.L. Rinehart, W.W. Carmichael, W.R. Evans, P. Crue, M.H.G. Munro and J.W. Blunt. (1994). New nodularins- a general method for structure assignment. *J. Org. Chem.* **59**:2349-2357.

Matveeva, L. and G.J. Jones. (1994). Study of the ability of *Daphnia carinata* King to control phytoplankton ans resist cyanobacterial toxicity: Implications for biomanipulation in Australia. *Aust. J. mar. Freshwat. Res.* **45**:159-174.

National Rivers Authority. (1990). **Toxic blue-green algae.** The report of the National Rivers Authority, Water Quality series No. 2. National Rivers Authority, London.

National Rivers Authority. (1991). **Production and fate of blue-green algal toxins.** Progress report, R&D project 0271, May-Nov. Internal Report. University of Dundee, UK.

National Rivers Authoriy (1992). **Production and fate of blue-green algal toxins.** Progress report, R&D project 0271, May-Nov. Internal Report. University of Dundee, UK.

Newman, J.R. and P.R.F. Barrett. (1993). Control of *Microcystis aeruginosa* by decomposing barley straw. *J. Aquat. Plant Manage.* **31**:203-206.

Nishiwaki-Matsashima, R., S. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W.W. Carmichael and H. Fujiki. (1992). Liver tumour promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J. Cancer Res. Clin. Oncol.* **118**:420-424.

Poon, G.K., I.M. Priestly, S.M. Hunt, J.K. Fawell and G.A. Codd. 1987. Purification procedure for peptide toxins from the cyanobacterium *Microcystis aeruginosa* involving high-performance thin-layer chromatography. *J. Chromatogr.* **387**:551-555.

Rao, P.V.L., A.S.B. Bhaskar and R. Bhattacharya. (1996). Effects of nutrient media and culture duration on growth,

macromolecular composition and toxicity in batch cultures of *Microcystis aeruginosa*. *Microbios*. **86**:95-104.

Rapala, J., K. Lahti, K. Sivonen and S.I. Niemelä (1994). Biodegradability and adsorption on lake sediments of cyanobacterial hepatotoxins and anatoxin-a. *Lett. Appl. Microbiol*. **19**:423-426.

Raziuddin, S., H.W. Siegelman, T.G. Tornabene. (1983). Lipopolysaccharides in the cyanobacterium *Microcystis aeruginosa*. *Eur. J. Biochem*. **137**:333-336.

Reynolds, C.S. (1971). The ecology of the planktonic blue-green algae in the North Shropshire meres. *Fld. Stud.* **3**:409-432.

Reynolds, C.S. and C. Butterwick. (1979). Algal bioassay of unfertilised and artificially fertilised lake water, maintained in Lund tubes. *Arch. Hydrobiol*. Suppl. **56**:166-183

Rinehart, K.L., M. Namikoshi and B.W. Choi. (1994). Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). *J. Appl. Phycol*. **6**:159-176.

Rippka, R. (1988). Isolation and purification of cyanobacteria. *In*: **Methods in enzymology**, Vol. 167, pp: 3-27. (eds.) Packer, L. and A.N. Glazer. Academic Press, Inc. UK.

Rodger, H.D., T. Turnbull, C. Edwards and G.A. Codd. (1994). Cyanobacterial (blue-green algal) bloom associated pathology in brown trout, *Salmo trutta* L., in Loch Leven, Scotland. *J. Fish Dis.* **17**:177-181.

Rositano, J. and B.C. Nicholson. (1994). **Water treatment techniques for the removal of cyanobacterial peptide toxins from water.** ACWQR report 2/94.

Rudolph-Böhner, S., D.F. Mierke and L. Moroder. (1994). Molecular structure of the cyanobacterial tumor-promoting microcystins. *FEBS Lett.* **349**:319-323.

Runnegar, M.T., R.G. Gerdes and I.R. Falconer. (1991). The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. *Toxicon.* **29**:43-51.

Runnegar, M.T., S. Kong and N. Berndt. (1993). Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins. *Amer. J. Physiol.* **265**:G224-G230.

Shiel, R.J. and J.D. Green. (1992). Cyanobacteria: a problem in perspective? *Vic. Naturalist*. **109**:225-232.

Sivonen, K. (1990). Effects of light, temperature, nitrate, orthophosphate and bacteria on growth and hepatotoxin production by *Oscillatoria agardhii* strains. *Appl. Envir. Microbiol.* **56**:2658-2666.

Sivonen, K., M. Namikoshi, W.R. Evans, W.W. Carmichael, F. Sun, L. Rouhiainen, R. Luukkainen and K.L. Rinehart. (1992). Isolation and characterisation of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. *Appl. Envir. Microbiol.* **58**:2495-2500.

Skulberg, O.M., W.W. Carmichael, G.A. Codd and R. Skulberg. (1993). Taxonomy of toxic cyanophyceae (cyanobacteria). *In*: **Algal toxins in seafood and drinking water**, pp.145-164. (ed.) Falconer, I.R. Academic press.

Stanier, R.Y., R. Kunisawa, M. Mandel and G. Cohen-Bazine. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol. Rev.* **35**:171-205.

Stewart, R., C.C. Auchterlome, G.A. Codd. (1977). Studies on the subunit structure of ribulose-1.5-diphosphate carboxylases from the blue-green alga *Microcystis aeruginosa*. *Planta*. **136**:61-64.

Steffensen, D.A., A.R. Humpage, J. Rositano, A.H. Bretag, R. Brown, P.D. Baker and B.C. Nicholson. (1994). Neurotoxins *from Auatralian Anabaena*. *In*: **Detection Methods for Cyanobacterial Toxins**, pp:45-50. (eds.) Codd, G.A., C.W. Keevil and E. Potter. The Royal Society of Chemistry, UK.

Stoner, R.D., W.H. Adams, D.N. Slatkin and W. Harold. (1989). The effects of single L-amino acid substitutions on the lethal potencies of the microcystins. *Toxicon*. 27:825-828.

Stotts, R.R., M. Namikoshi, W.M. Haschek, K.L. Rinehart, W.W. Carmichael, A.M. Dahlem and V.R. Beasley. (1993). Structural modifications imparting reduced toxicity in microcystins from *Microcystis* spp. *Toxicon*. **31**:783-789.

Tisdale, E.S. (1931). Epidemic of intestinal disorders in Charleston, WV, occurring simultaneously with unprecedented water supply conditions. *Am. J. Public Health*. **21**:198-200.

Tsuji, K., S. Naito, F. Kondo, N. Ishikawa, M.F. Watanabe, M. Suzuki and K.-I. Harada. (1994). Stability of microcystins from cyanobacteria: effect of light on decomposition and isomerisation. *Envir. Sci. Technol*. **28**:173-177.

Turner, P.C., A.J. Gammie, K. Hollinrake and G.A. Codd. (1990). Pneumonia associated with contact with cyanaobacteria. *Br. Med. J.* **300**:1440-1441.

Utkilen, H. and N. Gjølme. (1995). Iron-stimulated toxin production in *Microcystis aeruginosa*. *Appl. Envir. Microbiol.* **61**:797-800.

Van der Does, J. and S.P. Klapwijk. (1987). Effects of phosphorus removal on the maximal algal growth in bioassay experiments with water from four Dutch lakes. *Inst. Revue ges. Hydrobiol.* **72**:27-39.

Van der Westhuizen, A.J. and J.N. Eloff. (1983). Effect of culture age and pH of the medium on the growth and toxicity of the blue-green alga *Microcystis aeruginosa*. *Z. Pflanzenphysiol.* Bd. **110**:157-163.

Van der Westhuizen, A.J. and J.N. Eloff. (1985). Effect of temperature and light on the toxicity of the blue-green alga *Microcystis aeruginosa* (UV-006). *Planta*. **163**:55-59.

Vasconcelos, V.M., K. Sivonen, W.R. Evans, W.W. Carmichael and M. Namikoshi. (1996). Hepatotoxic microcystin diversity in cyanobacterium blooms collected in Portuguese freshwaters. Wat. Res. 30:2377-2384.

Walsby, A.E. and A.R. Klemer. (1974). The role of gas vesicles in the microstratification of a population of *Oscillatoria agardhii* var *isothrix* in Deming Lake, Minnesota. *Arch. Hydrobiol.* **74**:375-392.

Watanabe, M.F. and S. Oishi. (1985). Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. *Appl. Envir. Microbiol.* **49**:1342-1344.

Watanabe, M.F., S. Oishi, K-I. Harada, K. Matsuura, H. Kawai and M. Suzuki. (1988). Toxins contained in the *Microcystis* species of cyanobacterium (blue-green algae). *Toxicon.* **26**:1017-1025.

Watanabe, M.F., M. Watanabe, T. Kato, K.-I. Harada and M. Suzuki (1991). Composition of cyclic peptide toxins among strains of *Microcystis aeruginosa* (blue-green algae: cyanobacteria). *Bot. Mag. Tokyo.* **104**:49-57.

Watanabe, M.F., K. Tsuji, Y. Watanabe, K.-I. Harada and M. suzuki. (1992). Release of heptapeptide toxin (microcystin) during the decomposition process of *Microcystis aeruginosa*. *Nat. Toxins.* **1**:48-53.

Watanabe, M.F., H.-D. Park and M. Watanabe. (1994). Composition of *Microcystis* species and heptapeptide toxins. *Verh. Int. Ver. Limnol.* **25**:2226-22229.

Weckesser, J. and C. Martin. (1990). Toxine aus cyanobakterien im wasser: Microcystin und verwandte peptide. *Forum Mikrobiol.* **7-8**:364-369.

Wurtsbaugh, W.A., W.F. Vincent, R. A. Tapia, C.L. Vincent and P.J. Richerson. (1985). Nutrient limitation of algal growth and nitrogen fixation in a tropical alpine lake, Lake Titicaca (Peru/Bolivia). *Freshwater Biol.* **15**:185-195.

Yu, S-Z. (1994). Blue-green algae and liver cancer. *In*: **Toxic cyanobacteria: Current status of research and management,** pp:75-85. (eds.) Steffensen, D.A. and B.C. Nicholson. Proceedings of an international workshop, Adelaide, Australia, March 22-24, 1994.