The study of peptide toxins from freshwater cyanobacteria

by

Jittra Chaivimol B.Sc.

A thesis submitted for the degree of Doctor of Philosophy at the University of Warwick

Department of Biological Sciences
University of Warwick
Coventry CV4 7AL

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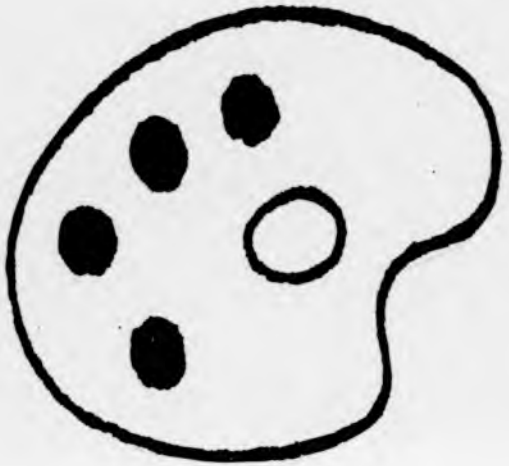
NUMEROUS ORIGINALS IN COLOUR



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ABBREVIATIONS

ACN	Acetonitrile
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
AcOH	Acetic acid
BSA	Bovine serum albumin
°C	Degree Celsius
CF-FAB	Continuous flow fast atom bombardment
cm	Centimetre
cpm	Counts per minute
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Disodium ethylene diamine tetraacetate·$2H_2O$
FAB	Fast atom bombardment
FID	Flame ionisation detector
g	Gramme
GGTP	γ-glutamyltransferase
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
L	Litre
lb	Pound
LD	Lethal Dose
M	Molar
$(M+H)^+$	Protonated molecular ion
m/z	Mass per charge
MALDI	Matrix assisted laser desorption ionisation
mg	Milligramme

min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MeOH	Methanol
NADH	β-nicotinamide adenine dinucleotide reduced form, disodium salt
ng	Nanogramme
nm	Nanometre
nM	Nanomolar
5'-NT	5'-nucleotidase
ODS	Octadesylsilyl
%	Percentage
P_i	Inorganic phosphate
PBS	Phosphate buffer saline
PCC	Pasteur Culture Collection
RP-HPLC	Reversed phase high performance liquid chromatography
rpm	Revolutions per minute
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
tR	Retention time
U	Enzyme activity
UV	Ultraviolet radiation
v/v	Volume by volume
w/v	Weight by volume
wt	Weight
μCi	Microcurie
μg	Microgramme

µl	Microlitre
µm	Micrometre
µmole	Micromole

Declaration

I hereby declare that the work described in this thesis is the result of original research by myself under the supervision of Dr C S Dow, unless otherwise stated. All sources of information have been acknowledged by means of reference. None of the work contained herein has been submitted for any previous degree.

Jittra Chaivimol

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PUBLICATIONS

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Summary

The freshwater reservoirs in the English Midlands were monitored during 1991 to 1993 for the diversity of cyanobacterial species and whether or not any of the species present produced toxins, emphasis being focused upon the toxic hepatopeptides. An HPLC analysis protocol has been developed which gives satisfactory resolution, both quantitative and qualitative, of "presumptive" cyanobacterial peptide toxins. Microcystin-LR, microcystin-RR and nodularin were used as the reference peptide toxins with detection limits of 50-100 ng under the employed conditions. *Microcystis aeruginosa* PCC 7806, a characterised hepatopeptide toxin producer, was taken as the reference toxic cell biomass.

The species isolated and characterised included *Oscillatoria*, *Aphanizomenon*, *Anabaena*, *Microcystis*, *Nodularia* and *Nostoc*. The *Oscillatoria* population present in Lower Shustoke reservoir were proved by mouse bioassay to be toxic throughout the period of evaluation. The lethal dose of cell lysate (LD_{100}) varied from 20 mg to 80 mg per kg body weight after intraperitoneal injection into mice. The toxic peptides from this strain were isolated and characterised by high performance liquid chromatography. There were three or four peptide toxins being expressed with spectral characteristics very close to those of the microcystins and nodularin.

Molecular weight and linearised fingerprints of reference peptide toxins were successfully analysed by FAB-MS using a Kratos MS50 and a Kratos Concept II HH four sector. The relative molecular masses of presumptive toxins were determined by matrix assisted laser desorption ionisation (MALDI) mass spectrometry to be 1023, 1022, 1043 and 981 Da.

The temporal variation of cellular expression of these presumptive toxins was monitored. There was considerable variation in the toxicity of the cellular biomass throughout the year and a corresponding variation in the cellular toxin content. The overall biomass toxicity was dependant on the ratio of the component toxins within the individual cells.

The variability in toxin expression/structure appeared to be linked to environmental factors. In this study, the concentration of hepatotoxins produced by the *Oscillatoria* species positively correlated with surface water temperature, cyanobacterial cell count, as well as concentration of ortho-phosphate and total phosphate while there was a negative correlation with total nitrate concentration. However, alteration in gene expression and/or the physiological status of the cells was not addressed.

Several alternatives to mouse bioassay have been examined for the determination of cyanobacterial toxicity. The protein phosphatase assay is one of the most promising procedures since phosphorylase phosphatase activity was strongly inhibited by the reference toxins when assayed against phosphorylase *a* substrate and crude mouse liver homogenate as sources of enzymes. Microcystin-LR is about 10 times more toxic than microcystin-RR when injected intraperitoneally into mice. However, they both have approximately the same IC_{50} (50% inhibition of phosphorylase phosphatase activity). This difference may be due to either permeability effects or differential activation of the two microcystins *in vivo*. *Drosophila melanogaster* bioassay was shown to be a useful indicator of toxicity.

CHAPTER 1

CHAPTER 1 : INTRODUCTION

1.1 CYANOBACTERIA OR BLUE-GREEN ALGAE IN GENERAL

The cyanobacteria, or "blue-green algae", are the largest group of photosynthetic prokaryotes (Whitton, 1992). Some authorities (Drouet, 1969; Lewin, 1976, 1979; Golubic, 1979) have categorised these organisms as algae because of the distinct differences between their photosynthetic mechanism and that of bacteria. The cyanobacteria contain chlorophyll a, which differs from the bacteriochlorophylls of the photosynthetic eubacteria, and they also carry out photosynthesis with the production of oxygen. Furthermore, they often display a characteristic gliding movement as seen in some eubacteria (Fogg *et al.*, 1973). However, Stanier *et al.* (1971) and Stanier (1977) considered the blue-green algae to be bacteria ("Cyanobacteria"), and advocated that they should be classified according to the principles of the Bacteriological Code rather than the Botanical Code (Stanier *et al.*, 1978). The inclusion of cyanobacteria in the prokaryotic kingdom has been justified because of their prokaryotic cellular organisation, as well as their ecology, reproduction, physiology and biochemistry. The name cyanobacteria came to replace the term blue-green algae as a means of distinguishing them from the eukaryotic algae. This name is derived from the Greek for blue (*Kyanos*) because of their characteristic blue-green colour. Cyanobacteria, rather than blue-green algae, is the name now well established in the literature, and is used throughout this thesis.

Cyanobacteria are ancient organisms with a fossil record extending back about 3.1 billion years into the Precambrian epoch of the Earth's history (Schopf, 1970; Schopf & Walter, 1982). Additional evidence is provided by the rocklike formations of limestone and other materials which trapped in layers colonies of cyanobacteria which have been dated as being 2.8 billion years old and are known as stromatolites (Taylor, 1981). Cyanobacteria are recognised as one of the first organisms able to carry out oxygenic photosynthesis, thus converting CO_2 into O_2, and are believed to have contributed to the early accumulation of oxygen in the Earth's original atmosphere (Knoll, 1985). Moreover, they are still undoubtedly responsible for a major part of the oxygenation process today. Photosynthesis in cyanobacteria is achieved in the same way as in eukaryotic plants and algae, which is distinct from that of photosynthetic eubacteria. Cyanobacteria carry out photosynthesis *via* photosystem I and II, while the photosynthetic eubacteria have a single photosystem and use a chemical reductant other than water. However, the use of hydrogen sulphide as an alternative hydrogen donor instead of water in an anoxygenic type of photosynthesis is found in some strains of cyanobacteria e.g. *Oscillatoria limnetica* (Cohen *et al.*, 1975).

The photosynthetic membranes, thylakoids, of cyanobacteria are located at the periphery of the cells as parallel concentric circles or in convoluted arrangements where they traverse most of the cell. This organisation is different from the chlorophyllous plants, where their thylakoids are enclosed in membrane-bound groups to form cellular organelles - chloroplasts (Lang & Rae, 1967). Thylakoids are the site of the photosynthetic pigments (Calvin & Lynch, 1952; Shatkin, 1960), including chlorophyll a (whereas most eukaryotic algae and plants use chlorophyll a and b), the accessory pigments and carotenoids such as β-carotene, echinenone and myxoxanthophyll. The accessory pigments used for light harvesting are the phycobilins consisting of C-phycocyanin, C-allophycocyanin and C-phycoerythrin. The phycocyanins are blue, and together with chlorophyll a give many cyanobacteria

a characteristic blue-green colour. The phycoerythrin, a red phycobilin, gives cyanobacteria a red or brown colour. The prefix C is applied to distinguish the cyanobacterial proteins from those of the red algae, which are given the prefix R (Svedberg & Katsurai, 1929). Each phycobilin absorbs a specific range of wavelengths of light, and the composition of phycobilins can vary widely among cyanobacteria. These pigments are organised in phycobilisomes found on the outer surface of the thylakoid membranes (Cohen-Bazire & Bryant, 1982), which is unlike the other eukaryotes where the light-harvesting complex is integrated into the chloroplast membrane and never appear as distinct structures above the membrane surface.

Cyanobacteria display two forms of movement. The first is gliding, a poorly understood type of movement found in some cyanobacteria which occurs when cells are in contact with a solid surface or other cells. The second is mediated by intracellular structures termed gas vesicles which are common to planktonic forms. These are hollow, cylindrical gas-filled structures. Cyanobacteria can regulate their buoyancy by means of the gas vesicles, thus enabling them to remain in, or move to, water depths where light is more abundant or adequate for growth.

There are three types of specialised cells formed by filamentous cyanobacteria in response to environmental conditions; hormogonia, akinetes and heterocysts. Heterocysts are involved in the fixation of atmospheric nitrogen. Hormogonia and akinetes are cell types associated with dispersal and survival of cyanobacteria. Hormogonia are the major reproductive forms in certain filamentous cyanobacteria and consist of short filaments of smaller cells that differentiate and fragment from the trichome. Akinetes are enlarged and thickened single cells analogous to cysts that are formed in response to unfavourable conditions.

Cyanobacteria are obligate photoautotrophs (organisms that utilise light as their energy source and CO_2 as the source of cell carbon). Thus they are found in nature only where light is available. Within this restriction, cyanobacteria have a

wide distribution in the natural environment and display a great variety of cellular forms. They occur in waters with a wide range of salinities (Humm & Wicks, 1980; Waterbury & Stanier, 1981), temperature and pH. In general, cyanobacteria thrive in neutral or slightly alkaline habitats with warm and nutrient-rich water. However, some species, e.g. *Chroococcus*, grow well in bogs at pH 4 (Bold & Wynne, 1985). Thermophilic cyanobacteria occur in alkaline hot springs where they tolerate temperatures up to 73°C (Castenholz, 1969; Bauld & Brock, 1974), in contrast, some cyanobacterial species grow in the frigid lakes of the Antarctic (Fogg *et al.*, 1973). Several cyanobacteria grow symbiotically in association with plants e.g. *Gloeocapsa*, *Nostoc* and *Anabaena* (Duckett *et al.*, 1975; Grilli-Caola, 1975; Nathanielsz & Staff, 1975; Tung & Shen, 1981). The biological advantage of these associations to the cyanobacteria is not clear since many of them are nonobligate and the cyanobacteria can be independently cultured. Cyanobacteria are also found widely in marine habitats and are often abundant on rocky shore areas, intertidal zones, salt marshes and marine muds. Although cyanobacteria are generally rather inconspicuous in open marine waters, the red filamentous cyanobacteria of the genus *Trichodesmium* are an exception. They can aggregate and form easily visible bundles near the surface of the water. The diversity of habitat occupied by cyanobacteria is due to their physiological characteristics - tolerance of high temperatures, high UV irradiation, desiccation and free sulphide, and the ability to utilise low light flux and CO_2 and to fix atmospheric N_2 (Whitton, 1992).

Initially the cyanobacteria were assigned to the eukaryotic algae class Cyanophyceae, having been classified by phycologists according to the Botanial Code using morphological and ecological characteristics of samples from nature. However, attempts to identify these organisms in axenic laboratory cultures using this system lead to many difficulties and ambiguities (Stanier *et al.*, 1971; Herdman *et al.*, 1979; Rippka *et al.*, 1979). In 1979 the cyanobacteria, class Cyanobacteriaceae, were classified into five sub-groups or orders, according to the

differences in their structure and development (Rippka *et al.*, 1979) (Table 1.1). Detailed descriptions for each genus has been given by Rippka *et al.* (1979) and Rippka (1988).

The ability to fix elemental (gaseous) nitrogen is one of the fascinating characteristic of cyanobacteria. They fix N_2 both in the free-living state and in symbiosis with a wide range of partners. Consequently they can thrive in the absence of combined nitrogen sources (Fogg *et al.*, 1973; Carr & Whitton, 1982). Cyanobacterial N_2-fixation is carried out by (1) filamentous heterocystous species (Tiwari, 1977), which fix nitrogen aerobically. These species have been investigated most extensively and are the most important contributors of combined nitrogen in aerobic environments; (2) aerobic nitrogen-fixing unicellular species (Rippka *et al.*, 1971); and (3) non-heterocystous filamentous species (Stewart, 1973), which do not fix nitrogen in air, but do so under microaerophilic conditions. This activity has been exploited to improve the fertility of rice fields by aggregating particles, adding organic matter and fixed nitrogen into soil (Bailey *et al.*, 1973).

In the natural environment, many cyanobacteria grow in situations that differ considerably from those favouring optimum growth (Whitton, 1992), and in the laboratory many cyanobacteria can tolerate considerable changes in the composition of the medium without any appreciable effects. Thus, it is extremely difficult to devise a general medium that fulfils the growth requirements of all cyanobacteria.

TABLE 1.1 List of sub-groups of cyanobacteria, (Rippka *et al.*, 1979)

Section I : Unicellular or nonfilamentous cyanobacteria; cells single or forming colonial aggregates held together by additional outer cell wall layers or gel-like matrix; reproduction by binary fission or by budding. e.g. *Synechococcus* and *Synechocystis*.
Section II : Unicellular or nonfilamentous cyanobacteria; cells single or forming colonial aggregates held together by additional outer cell wall layers or gel-like matrix; reproduction by internal multiple fissions producing baeocytes (small daughter cells), or by a mixture of multiple fission and binary fission. e.g. *Xenococcus* and *Chroococcidiopsis*.
Section III : Filamentous cyanobacteria; a trichome (chain of cells) grows by intercalary cell division; binary division in only one plane; trichomes do not form heterocystous (non-heterocystous cyanobacteria). e.g. *Spirulina*, *Oscillatoria* and *Pseudoanabaena*.
Section IV : Filamentous cyanobacteria; binary division in only one plane; in the absence of combined nitrogen, one or more cells per trichome differentiate into a heterocyst; some also produce akinetes. e.g. *Anabaena*, *Nodularia* and *Nostoc*.
Section V : Filamentous cyanobacteria; binary division in more than one plane, giving rise to multiseriate trichomes or trichomes with true branches or both; always possess capacity to form heterocysts. e.g. *Chlorogloeopsis* and *Fischerella*.

As with all microorganisms, cyanobacteria are found to be of benefit as well as being disadvantages to humans and the other living organisms. Firstly, cyanobacteria are the primary producers of organic matter and oxygen in the natural environment because of their photosynthetic activity. Moreover, they are a primary food source, which also includes secretion of substrates to organisms associated with them (Benson & Muscatine, 1974). In oriental and some western countries cyanobacteria and other algae have been used as sources of food for centuries (see Shelef & Soeder, 1980; Lobban & Wynne, 1981). It has also been suggested that cyanobacteria may be a valuable supplement to the modern diet. For example, *Spirulina* species, a renowned nutrient food source in present times is the richest natural source of vitamin B_{12} and relatively rich in the medicinally valuable γ-linolenic acid, moreover, they also have the highest protein content (60-70%) of any natural food (Richmond, 1992). Many cyanobacteria are of great importance in improving soil facility *via* their N_2-fixation characteristic, they are also important in erosion control since several genera form mats on the surface of bare soil and so cut down evaporation and add organic matter to the soil. Thus N_2-fixation, in combination with photosynthesis and growth under aerobic conditions, present cyanobacteria as having unique potential for contributing to productivity in a variety of agricultural and ecological situations.

Alternatively, there are many negative aspects associated with cyanobacteria. If present in large concentrations in lakes and reservoirs, the cyanobacterial blooms cause serious nuisance because they clog water treatment filters and may impart unpleasant tastes to drinking water, and offensive smells resulting from the odorous substances of their decay.

However, one of the major problems associated with cyanobacteria is that they may produce toxins that are effective against cattle, wildfowl, fish, and man. Many species of cyanobacteria have been observed to produce these toxins, which can be divided into three groups; peptide hepatotoxins, neurotoxins and

lipopolysaccharides. Reports of toxic activity have been published from around the world. Thus appropriate methods to detect and quantify these toxins in natural waters is very important. Equally, a variety of environmental factors are thought to promote the production of toxins by cyanobacteria, consequently in order to control the production of cyanobacterial toxins in the natural environment, these must be understood.

1.2 THE OCCURRENCE AND DISTRIBUTION OF TOXIC CYANOBACTERIAL BLOOMS

1.2.1 Historical study of cyanotoxins

The occurrence of cyanobacterial poisoning in aquatic environments was first reported from Lake Alexandrina in South Australia (Francis, 1878), the toxicity was ascribed to *Nodularia spumigena*. Later, during 1882-1933, five species of cyanobacteria; *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, *Coelosphaerium keutzigianum*, *Gloeotrichia echinulata* and *Microcystis aeruginosa* were implicated with the intoxication of animals and birds in Minnesota (Ingram & Prescott, 1954). A number of studies have also shown that cyanobacteria produce substances that are toxic to other algae (Proctor, 1957; Vance, 1965). However, most investigations of, and research into, cyanobacterial toxicity have been stimulated by poisoning incidents affecting animals, birds, fish and humans.

To date, poisoning and death of livestock, pets, birds and wildlife due to several genera of cyanobacteria have been reported in most countries of the world (Gorham & Carmichael, 1988). Most of the animal deaths in these cases have been implicated with blooms of fresh and brackish-water cyanobacteria e.g. *Microcystis*, *Anabaena*, *Aphanizomenon*, *Oscillatoria* and *Nodularia*. Death and/or illness occurs after consuming water contaminated by toxic cyanobacteria at high concentrations or

the ingestion of the scum which accumulates near the shore. Reports of human illnesses are only circumstantial (e.g. Schwimmer & Schwimmer, 1964; Billings, 1981; Falconer *et al.*, 1983a), and there are no confirmed human death caused by these toxins. However, heavy blooms of cyanobacteria, e.g. *Microcystis aeruginosa*, in public water supplies have been implicated in the primary cause of liver injury and gastrointestinal disorders (Falconer *et al.*, 1983a; Falconer, 1989). Moreover, the well known hepatotoxins; microcystins and nodularin, are suspected to be involved with promotion of primary liver cancer in humans exposed to long-term low doses of these toxins *via* drinking water (Yu, 1989; Carmichael, 1994). The increasing frequency of cyanobacterial blooms, particularly in water destined for human consumption, and the seriousness of the health risks, have raised public awareness and stimulated the water supply industry to address the problem.

A considerable number of papers on cyanobacterial toxins have been published covering the toxin producing species and improved procedures for purification of such cultures (Dierstein *et al.*, 1990; Shirai *et al.*, 1991), the structure and activity of toxins (Kungsuwan *et al.*, 1988; Krishnamurthy *et al.*, 1989; Harada *et al.*, 1990) and factors affecting bloom formation (Watanabe & Oishi, 1985; Wicks & Thiel, 1990). These taken together with improved methods of toxin detection (Meriluoto & Eriksson, 1988; Takeuchi *et al.*, 1988; Kondo *et al.*, 1992), uptake and toxicity effects (Adams *et al.*, 1988; Hooser *et al.*, 1991) provide the necessary background information for further studies.

1.2.2 Toxin producing cyanobacteria

Many cyanobacteria are known to produce from one or more toxic compounds that behave like endotoxins and are released only upon death or lysis of the cells (Humm & Wicks, 1980). Since the intoxication of animals and humans happens following the ingestion of bloom material, samples of such blooms have been collected and tested for toxicity. Species of cyanobacteria which have been confirmed in poisoning incidents are indicated in Table 1.2 (Utkilen, 1992). Most of the toxin-producing cyanobacteria are freshwater species, but some marine species are also toxic. *Anabaena*, *Aphanizomenon*, *Coelosphaerium*, *Gloeotrichia*, *Gomphosphaeria*, *Nodularia* and *Oscillatoria* are the most common freshwater and brackish-water species which have been implicated in poisoning episodes. The marine cyanobacteria; *Lyngbya*, *Schizothrix* and *Oscillatoria*, are most frequently encountered in toxic-marine incidents. Most of the toxin-producing species produce gas vacuoles, which often result in surface accumulation of cells, forming dense scums during calm weather, which by the action of gentle on-shore breezes, are washed ashore. Consequently an acute oral dose for animals is readily provided. It is also possible that animals and humans may obtain these toxic substances *via* the food chain. For example, a human disease called Ciguatera, caused by eating poisonous marine fish, is believed to be derived from toxic cyanobacteria in the food chain (Randell, 1958; Humm & Wicks, 1980).

The bloom-forming *Microcystis aeruginosa* has been the most frequent producer of freshwater cyanobacterial poisoning incidents to date. Its toxic substance "fast death factor" (FDF), a mixture of closely related peptides of low molecular weight (Bishop *et al.*, 1959), proved to be fatal upon administration to mice and rats (Runnegar & Falconer, 1982). A "very fast death factor", which is able to kill mice within 2 minutes, has been reported for cultures of *Anabaena flos-aquae* (Gorham *et al.*, 1964).

TABLE 1.2 Species of cyanobacteria which have been confirmed to have toxin-producing strains (Utkilen, 1992)

Species	Species
Anabaena circinalis Rabenh.	*Microcystis wesenbergii* Kom.
Anabaena flos-aquae (Lyngb.) Breb.	*Nodularia spumigena* Mertens
Anabaena hassallii (Kutz.) Wittr.	*Nostoc linckia* (Roth) Born. et Flah.
Anabaena lemmermanni P. Richt.	*Nostoc paludosum* Kutz.
Anabaena spiroides var. contracta Kleb.	*Nostoc rivulare* Kutz.
Anabaena variabilis Kutz.	*Nostoc zetterstedtii* Areschoug
Anabaenopsis milleri Woron.	*Oscillatoria acutissima* Kuff.
Aphanizomenon flos-aquae (L.) Ralfs	*Oscillatoria agardhii/rubescens* group
Coelosphaerium kutzingianum Nag.	*Oscillatoria formosa* Bory
Cylindrospermum Kutzing sp.	*Oscillatoria nigroviridis* Thwaites
Cylindrospermopsis raciborskii (Wolos.) Seenaya & Subla Raju	*Oscillatoria* Vaucher sp.
Fischerella epiphytica Ghose	*Pseudoanabaena catenata* Lauterb.
Gloeotrichia echinulata (J.E. Smith) P. Richter	*Schizothrix calcicola* (Ag.) Gom.
Gomphosphaeria lacustris Chod.	*Scytonema pseudohofmanni* Bharadw.
Gomphosphaeria nageliana (Unger) Lemm.	*Synechococcus* Nageli sp. (strain Miami BCII 6S)
Hormothamnion enteromorphoides Grun.	*Synechococcus* Nageli sp. (strain ATCC 18800)
Lyngbya majuscula Harvey	*Synechocystis* Sauvageau sp.
Microcystis aeruginosa Kutz.	*Tolypothrix byssoidea* (Hass.) Kirchn.
Microcystis cf. *botrys* Teil.	*Trichodesmium erythraeum* Ehrb.
Microcystis viridis (A. Br.) Lemm.	

The control of cyanobacterial blooms is rather complicated since the production of toxins is not constant. Cyanobacterial toxin levels per unit cyanobacterial biomass vary spatially and temporally at the same sampling location (Carmichael & Gorham, 1981; Codd & Bell, 1985). This variability is probably due to the existence of toxic and non-toxic strains or cells of the same strain giving differential expression. Moreover, the concentration of toxins is also found to be changeable within individual strains and cells (Carmichael & Gorham, 1977). These fluctuations in the toxicity have lead to significant problems with respect to hazard assessment associated with drinking water supplied to humans and animals and the assessment of the safety of water bodies used for recreation.

1.2.3 Factors affecting the incidence of cyanobacterial blooms

Cyanobacteria are widespread in numerous habitats. However, most are abundant in freshwater. The increase in freshwater cyanobacterial blooms introduces considerable problems e.g. interference with potable water treatment processes, imparting tastes and odours to the water. However it is freshwater cyanobacterial species that produce toxic substances that are of prime concern. This thesis is focussed upon these freshwater cyanobacterial species.

Mortality of domestic cattle, wildlife and pets by cyanobacterial toxins most often occurs during the summer or fall, usually when the cyanobacterial population forms a bloom. Under such circumstances, animals ingesting cyanobacterial scum when drinking can readily consume a fatal dose (Carmichael, 1994). The factors leading to blooms of cyanobacteria are many and complex. Physical factors, such as the size of the waterbody, the extent of mixing of the water layers and the depth of light penetration are important in determining the dominant species of cyanobacteria in particular lakes or reservoirs. The temperature and the nutrient availability are also influential on cyanobacterial growth. In order to understand the reasons for

bloom formation, it is necessary to consider those factors which most influence cyanobacterial growth and also the factors influencing the formation of surface scums.

1.2.3.1 Factors favouring cyanobacterial bloom formation

It is rather complicated, and indeed not possible, to specify the exact environmental factors that favour the growth of particular cyanobacterial species. No single factor is crucial to the growth of cyanobacterial populations, however, different types of habitat favour the success of particular species.

(i) Light

Planktonic cyanobacteria are similar to most photosynthetic plankton in that they require a certain minimum average light intensity for growth. Light absorption by the cyanobacteria is, however, enhanced by the phycobiliproteins, which absorb low light intensities over a wide band of the visible spectrum (NRA, 1990).

It has been reported that the filamentous cyanobacteria such as *Oscillatoria* prefer low light intensities in nature (Post *et al.*, 1985; Lindholm *et al.*, 1989) and they have been shown to occur in mass several meters below the water surface (Lindholm *et al.*, 1989). This does not seem to be the case with the colonial forms such as *Microcystis*, and the large *Anabaena* species which require more light to saturate their photosynthetic system than the smaller *Oscillatoria* species (Gibson & Smith, 1982), supporting the study of Harris (1978) that the efficiency of light absorption varies with the size of the cyanobacteria. The depth-dependence of light quality on the photosynthetic efficiency of some marine cyanobacteria and phytoplankton has also been reported (Wood, 1985).

(ii) Temperature

Ranges of temperature that are suitable for the growth of cyanobacteria are significantly wider than those for other algae. The optimum temperature for cyanobacteria is generally between 15-30°C. The thermophilic cyanobacteria require high temperatures, whereas some planktonic cyanobacteria from temperate regions will not tolerate temperatures above 15-23°C (Fogg *et al.*, 1973).

(iii) Nutrient enrichment

Striking increases in the abundance of cyanobacteria are often observed in nutrient-enriched (eutrophic) lakes with a reduction in nutrient load leading to a decrease in the cyanobacterial population (Gelin & Ripl, 1978). It is difficult to conclude that cyanobacteria are favoured by the higher nutrient concentrations directly, since they are often abundant at those times when external nutrient concentrations are low or undetectable, however, rates of nutrient supply may be of more importance than the actual concentrations (Healey, 1982).

It is widely accepted that phosphorus, nitrogen and carbon are the critical nutrients for the growth of cyanobacteria and that they play important roles as limiting nutrients. In lakes, small changes in the phosphorus levels markedly influence the growth of cyanobacteria (Sivonen, 1990). However, cyanobacteria can assimilate phosphorus in excess of their requirements and store it as polyphosphate which can be used under conditions of phosphorus deficiency. They are also able to increase the potential for phosphate uptake at low phosphate concentrations (Healey, 1982).

Cyanobacteria can utilise inorganic nitrogen compounds such as nitrate, nitrite and ammonium salts as sources of nitrogen for growth (Fogg *et al.*, 1973). Urea has also been reported to be an excellent nitrogen source for certain thermophilic unicellular (Allen, 1952) and marine cyanobacteria (Van Baalen, 1962). Some species, such as *Anabaena* and *Aphanizomenon*, can assimilate

gaseous nitrogen dissolved in water. Thus, when sources of fixed nitrogen are inadequate, these forms of cyanobacteria are at an advantage over the other planktonic algae. Although there may be no combined nitrogen in the waters, blooms of nitrogen-fixing cyanobacteria may still occur if phosphorus and other nutrients are available in sufficient quantity (Fogg *et al.*, 1973). Thus, the distribution and abundance of cyanobacteria appear to be controlled by the availability of phosphate, this being the major limiting nutrient.

The main source of cell carbon is atmospheric carbon dioxide. Cyanobacteria are more efficient with respect to carbon dioxide uptake at low concentrations than most green algae which in part leads to them dominating eutrophic waters. Furthermore, inorganic carbon can also be provided in the form of bicarbonate and certain species e.g. *Anabaena variabilis* can utilise organic carbon sources for growth in low light (Fogg *et al.*, 1973).

The growth of cyanobacteria has been shown to be dependent upon a number of trace elements e.g. magnesium, a component of the chlorophyll molecule, and iron, a constituent of cytochromes, ferrodoxins and nitrogenases. Trace amounts of manganese, boron, molybdenum, copper, zinc and cobalt are also essential for the growth of cyanobacteria but are toxic at elevated concentrations. The requirement for these trace elements are variable depending on the species, as well as other factors. However, the amounts of these trace elements in most natural water are generally more than sufficient.

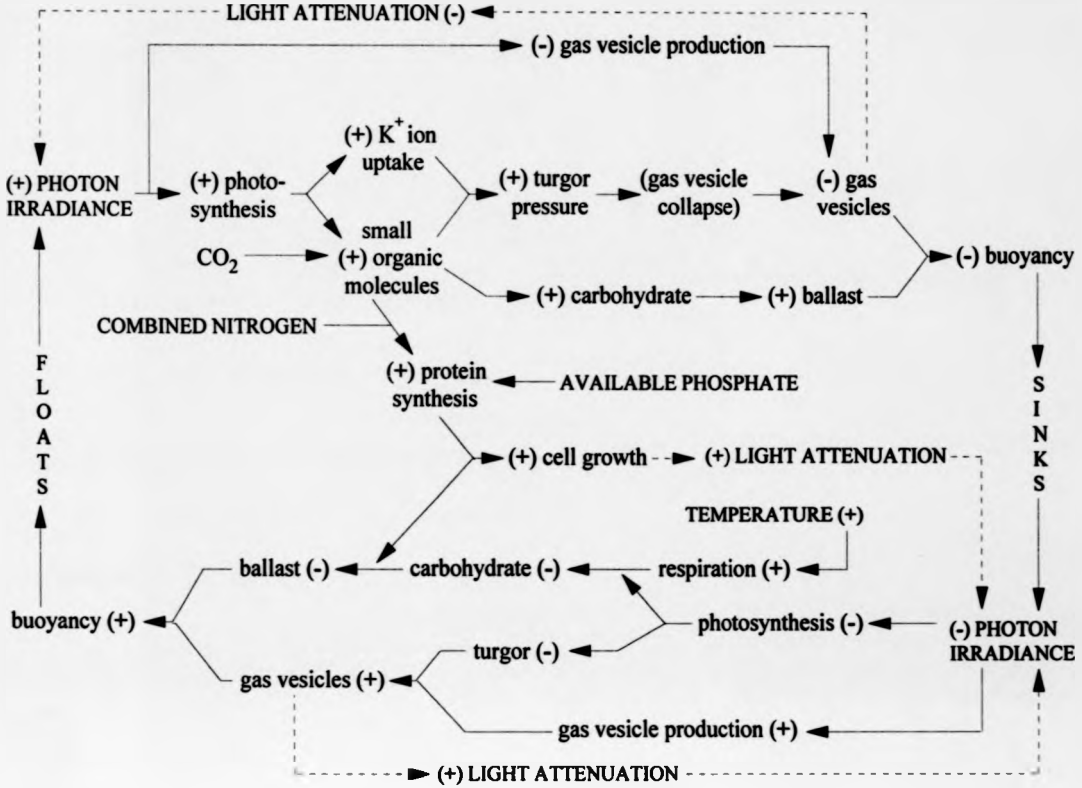
Another factor that supports the abundance of cyanobacteria in freshwater is that most are not extensively grazed by planktonic water fleas, copepods and protozoans, whereas many planktonic algae are. Although, cyanobacteria are susceptible to fungal, bacterial and viral attacks, the collapse of environmental populations due to these agents are not well known.

1.2.3.2 Buoyancy regulation

Under certain conditions, a number of cyanobacterial species are observed to form the layers at the surface of natural waters. All of those cyanobacteria possess gas vacuoles which are, in effect, buoyancy aids (Walsby, 1987). Since various factors, such as light, temperature and nutrient concentration are important for the growth of cyanobacteria and these show marked gradients with depth, buoyancy regulation *via* gas vacuoles enables the microbe to place itself at that point in the physico-chemical gradient most favourable for growth. Different cyanobacteria species respond to high light intensities at the surface in different ways. If cells receive too much light that causes inhibition of photosynthesis or damage to cell structure, buoyancy is reduced and the cells sink. In the opposite way, cells that receive too little light will become more buoyant and float upwards to where the light is stronger. However, it is not possible for an organism to regulate its buoyancy rapidly enough to stay precisely at the optimal depth; it is sufficient to maintain position in or around the preferred depth. Buoyancy regulation of planktonic cyanobacteria in response to light was first demonstrated in cultures of *Anabaena flos-aquae* (Reynolds & Walsby, 1975). Gas vacuoles are produced to make cells buoyant at low irradiance (Walsby, 1969), buoyancy being lost when cells are transferred to a high irradiance (Dinsdale & Walsby, 1972; Walsby, 1972). The mechanisms of buoyancy regulation in planktonic cyanobacteria are summarised in Figure 1.1 (Walsby, 1994).

Another factor affecting the speed of floating or sinking is that large bodies float or sink more quickly than smaller ones of the same density and spheres move faster than threads of the same volume and density. Thus many planktonic cyanobacteria aggregate together to form macroscopic colonies.

FIGURE 1.1 Summary of the factors involved in the regulation of gas vesicle content and buoyancy in planktonic cyanobacteria. The signs indicate whether the factor increases (+) or decreases (-) as a result of the change (+ or -) in the preceding factor (Walsby, 1994).



Under nutrient-rich conditions cyanobacteria increase their buoyancy and rise towards higher light intensities. On the other hand, gas vacuoles collapse and the cells sink to lower light intensities if nutrient levels supporting growth are depleted. Photosynthesis results in the accumulation of low molecular weight carbohydrates e.g. sucrose which accelerates buoyancy loss. However, the depletion of inorganic carbon has been shown to cause the opposite effect and it is probably one of the major factors in the formation of summer waterblooms in natural waters (Reynolds & Walsby, 1975). The supporting experimental evidence shows that under nutrient-rich conditions, *Anabaena* populations quickly exhaust dissolved inorganic carbon. Even the supply by diffusion of CO_2 from the atmosphere fails to keep up with the photosynthetic demands and eventually dissolved inorganic carbon becomes limiting. Consequently cells float to the water surface where the rate of arrival of carbon dioxide will be at its highest (Booker & Walsby, 1981).

1.2.3.3 Scum formation

With the appropriate environmental conditions, both physical and chemical, and an uninterrupted period of constant or regular mixing of water promotes cyanobacterial growth ultimately achieving a large population. In calm periods surface aggregation, i.e. scum, will form. However, as a consequence of wind action, buoyant cells may be retained in circulation so preventing them from accumulating at the top of the euphotic zone. If the wind drops abruptly, the mixing stops, and the cyanobacterial colonies start to rise and become concentrated at the surface forming semipermanent, thick crusted, floating mats. Moreover, the buoyancy of cyanobacteria will increase as a natural response of the regulatory mechanism as the cells are swirled through low mean irradiance, they then float up more rapidly before buoyancy can be reversed e.g. if the cyanobacteria are wind-mixed during the day and the wind abruptly falls off at night, they will float rapidly to the surface. Their buoyancy is not regulated actively during the night so that the opportunity to reverse excess buoyancy in darkness is non-existent. It is possible to say that surface scums are the result of a change in the weather acting on existing populations of cyanobacteria which had become dominant due to stable weather conditions (NRA, 1990). The scum can be redispersed by renewed wind mixing. However, if the winds are too light to break the surface, the scum can be pushed toward the lee shore causing toxic cyanobacteria to accumulate in a thick layer.

1.3 NATURE AND PROPERTIES OF CYANOTOXINS

There are a large number of reports concerning the production of toxins by cyanobacteria. These toxins have been confirmed to be responsible for the death of fish, wildlife and domestic animals, and the cause of human illness. However, cyanobacterial toxicity is variable both in time and in space. The toxins have been studied intensively to date, but only a few have been fully chemically characterised and their mode of action established (Gorham & Carmichael, 1988). Although, most of them are produced by freshwater cyanobacteria, marine species have also been reported to produce toxins. Toxin-producing cyanobacteria and their toxins are listed in Table 1.3. However, only the toxins produced by freshwater cyanobacteria are reviewed here.

The toxins produced by freshwater cyanobacteria are generally classified into three categories; neurotoxins, hepatotoxins and lipopolysaccharides, according to their mode of action. The assessment of their toxicity has usually been studied under laboratory conditions by giving doses to laboratory animals, with administration *via* the intra-peritoneal route or the oral route. The toxicological data are available by reference to LD_{50} or LD_{100} levels. However, toxicity depends upon the route of administration, the size of the dose and the period of exposure.

TABLE 1.3 Freshwater and brackishwater toxin-producing cyanobacteria and their toxins

Cyanobacterium	Toxin name	Toxin structure	Reference
Microcystis aeruginosa	microcystin or cyanoginosin	peptides	Runnegar & Falconer, 1981;
Microcystis viridis	or cyanoviridin		Santikarn *et al.*, 1983;
Microcystis wesenbergii			Botes, 1986; Kusumi *et al.*, 1987;
			Sivonen *et al.*, 1990b
Aphanizomenon flos-aquae	aphantoxins	alkaloids	Sasner *et al.*, 1981;
			Namikoshi *et al.*, 1992a
Anabaena flos-aquae	anatoxin *a*	alkaloid	Carmichael & Gorham, 1978;
(different strains)	anatoxin *b*	unknown	Krishnamurthy *et al.*, 1986;
Anabaena circinalis	anatoxin *c*	peptide	Sivonen *et al.*, 1992c
Anabaena lemmermannii	anatoxin *d*	unknown	
	anatoxin *a(s)*	organophosphate	
	anatoxin *b(s)*	unknown	
Oscillatoria agardhii	oscillatoria toxins	peptides	Skulberg *et al.*, 1984;
Oscillatoria rubescens			Eriksson *et al.*, 1988a;
Oscillatoria mougetii			Lindholm *et al.*, 1989;
			Bruno *et al.*, 1992;
			Luukkainen *et al.*, 1993
Nodularia spumigena	nodularin	peptide	Eriksson *et al.*, 1988b;
			Rinehart *et al.*, 1988
Gloeotrichia echinulata	gloeotrichia toxin	unknown	Codd & Bell, 1985
Synechocystis species	synechocystis toxin	unknown	Lincoln & Carmichael, 1981
Cylindrospermopsis raciborskii	cylindrospermopsin	peptide	Hawkins *et al.*, 1985;
			Ohtani *et al.*, 1992

1.3.1 Neurotoxins

Neurotoxins are generally cyclic or bicyclic alkaloids which contain nitrogen. These toxins are produced by several cyanobacterial species, such as *Anabaena*, *Aphanizomenon* and *Oscillatoria* (Carmichael & Gorham, 1978; Codd & Poon, 1988; Edwards *et al.*, 1992; Sivonen *et al.*, 1992b). Signs of poisoning of animals which have ingested scum containing cyanobacterial neurotoxins include paralysis, respiratory arrest, muscular tremor, salivation, staggering and convulsions. Several neurotoxins have been identified, some of which have a similar or identical structure to saxitoxin, which is produced by some marine dinoflagellates and causes paralytic shellfish poisoning (PSP).

1.3.1.1 Anatoxins

Six distinct anatoxins [Antx-a, Antx-a(s), Antx-b, Antx-b(s), Antx-c and Antx-d] are produced by strains of *Anabaena* (Carmichael & Gorham, 1978). They are distinguished on the basis of the strains that produce them and by reactions exhibited by mice, rats and chicks after intra-peritoneal injection (Carmichael & Gorham, 1978). Antx-a (Figure 1.2) of *Anabaena flos-aquae* NRC-44-1 was the first toxin from a freshwater cyanobacterium to be characterised, and it is the only anatoxin that has undergone extensive toxicological and pharmacological examination. It is a bicyclic secondary amine, 2-acetyl-9-azabicyclo (4.2.1) non-2-ene, with a molecular weight of 165 Daltons and acts as a potent postsynaptic depolarising neuromuscular blocking agent (Carmichael *et al.*, 1975; Devlin *et al.*, 1977). The toxin binds to the nicotinic acetylcholine receptors of *Torpedo* electric tissue with high affinity and at muscarinic acetylcholine receptors of rat brain with low affinity and regional selectivity (Aronstam & Witkop, 1981). Opisthotonous (head bent over backwards) is a characteristic feature of Antx-a poisoning in birds

(Carmichael, 1981). This toxin can induce muscle twitching and cramping, followed by fatigue and paralysis. If respiratory muscles are affected, the animal may suffer convulsions (from lack of oxygen to the brain) and die of suffocation. The toxin is a structural analogue of cocaine and can be synthesised by ring expansion of the 8-azabicyclo octane system of cocaine (Campbell *et al.*, 1977). The lethal dose (LD_{50}) of Antx-a after intra-peritoneal injection into mice is 250 $\mu g\ kg^{-1}$ (Devlin *et al.*, 1977). Since Antx-a is a small, water-soluble molecule, it is also readily absorbed by ingestion which accounts for the many cases of animal toxicity from blooms of *Anabaena flos-aquae*. Another, Antx-a(s) (Figure 1.3) from *Anabaena flos-aquae* strain 525-17 specifically causes viscous salivation and tear flow (sometimes bloody), as well as urinary incontinence and defecation in mice and rats in addition to the neuromuscular effects of anatoxins (Gorham & Carmichael, 1988). This toxin is a naturally occurring organophosphorus compound, the toxicity of which is higher than that of Antx-a after intra-peritoneal injection to mice (LD_{50}, 40-50 $\mu g\ kg^{-1}$). Antx-a(s) is also a potent inhibitor of acetylcholinesterase (an enzyme important in neurotransmission) (Carmichael, 1994). The other four anatoxins are recognised by their different physiological effects (Carmichael & Gorham, 1978; 1981). Antx-b and Antx-d have gross neuromuscular activity, but their pharmacology and structure have not been yet investigated. Antx-c is a mixture of peptide or peptide-containing toxins which are produced by many strains of *Anabaena flos-aquae*.

FIGURE 1.2 Structure of Anatoxin-A.

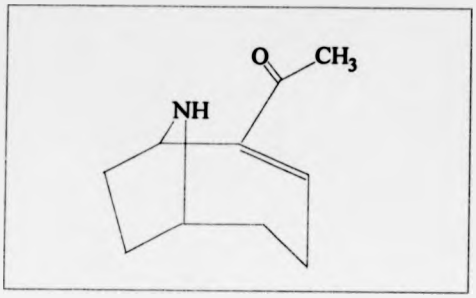
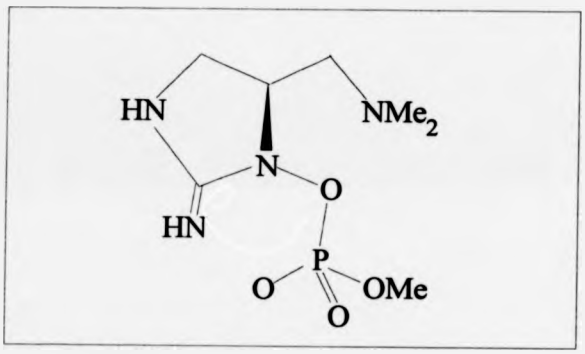


FIGURE 1.3 Structure of Anatoxin-A(S).

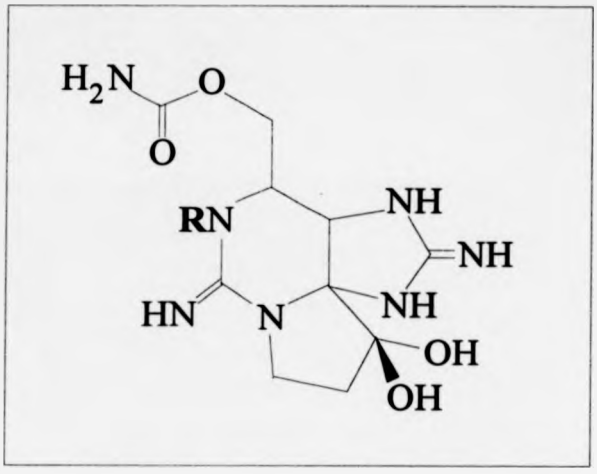


1.3.1.2 The saxitoxin groups (PSP)

Two neurotoxic alkaloids, neosaxitoxin and saxitoxin (Figure 1.4), have been reported to be produced by *Aphanizomenon flos-aquae*. The LD_{50} for intra-peritoneal administration to mice for both toxins is 10 $\mu g\ kg^{-1}$. These toxins have a fast-acting neuromuscular action inhibiting nerve conduction by blocking sodium channels without affecting the permeability of potassium, the transmembrane potential or membrane resistance (Utkilen, 1992).

This group of toxins is actually better known as paralytic shellfish poisons (PSP) which are generally produced by the marine dinoflagellate *Gonyaulax catenella*. These toxins have been studied for many years as they may be accumulated by shellfish and have been responsible for human fatalities.

FIGURE 1.4 Structure of saxitoxin and neosaxitoxin.



R = H IN SAXITOXIN

R = OH IN NEOSAXITOXIN

1.3.2 Hepatotoxins

The hepatotoxins, or liver toxins are produced by various cyanobacteria e.g. *Microcystis*, *Oscillatoria*, *Anabaena*, *Aphanizomenon* and *Nostoc* species. Although the greatest and most dramatic animal losses have been due to alkaloid neurotoxins, they are not ubiquitous as the hepatotoxins which have been implicated in incidents involving toxic freshwater blooms from all over the world. Neurotoxins have been reported mainly in North America (with some in Great Britain, Australia and Scandinavia) (Carmichael, 1994). Therefore, peptide hepatotoxins are of greater concern with regard to animals and human health. These toxins are contained within the cyanobacterial cells and are usually only released upon lysis or when changes in cell wall permeability occur (Fay & Van Baalen, 1987).

Toxic blooms of *Microcystis aeruginosa* are currently the most common bloom-forming cyanobacterium in freshwater ecosystems. Symptoms of animals ingesting toxic *Microcystis aeruginosa* include weakness, vomiting, pilorection, diarrhoea, cold extremities, pallor, heavy breathing and death due to circulatory shock induced by pooling of blood in the liver usually within 2 to 24 hours (Codd & Poon, 1988).

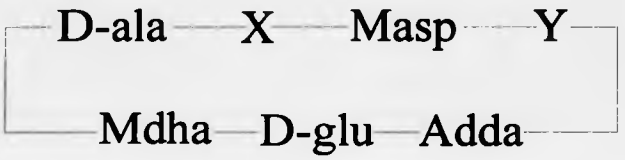
The first hepatotoxin isolated was from *Microcystis aeruginosa* NRC-1 and was shown to be a cyclic polypeptide consisting of seven amino acids. In addition, *Microcystis aeruginosa* strains produce a variety of peptide toxins, some strains produce a single toxin, others four, and some as many as six (Botes *et al.*, 1982; Poon *et al.*, 1987). However, in all cases only one or two toxins account for 90% of the total toxicity. Up to date, several related cyclic peptides with varying composition have been isolated and partially characterised (Gorham & Carmichael, 1979). Those consisting of seven amino acids are generically referred to as microcystins whereas those consisting of five amino acids are termed nodularins. Microcystins contain a common moiety composed of the novel amino acids, 3-

amino-9-methoxy-10-phenyl-2,3,8-trimethyl-deca-4,6-dienoic acid (Adda); N-methyldehydroalanine (Mdha); D-alanine; β-linked D-erythro-β-methylaspartic acid and γ-linked D-glutamic acid, plus two L-amino acids as variants. They have molecular weights of about 900-1100 Daltons and an LD_{50} in the range of 50-200 μg kg^{-1} mouse body weight (Botes *et al.*, 1982). Nodularin is a pentapeptide composed of Adda; β-linked D-erythro-β-methylaspartic acid; γ-linked D-glutamic acid; L-arginine and N-methyl-Z-dehydrobutyrine. The schematic structures of microcystins and nodularin are given in Figure 1.5. There are over 50 structural variations of the microcystin-like hepatotoxins reported from different cyanobacterial strains with several of these having been isolated from the same strain (Carmichael, 1992; Namikoshi *et al.*, 1992d). Some of the structural variations of microcystins are given in Table 1.4. These toxins show similar toxicities following intra-peritoneal injection into mice (50-100 μg kg^{-1}), thus it is possible that the invariable components are related to the toxicity of the molecule. Wieland & Faulstich (1978) have reported that the microcystins act similarly to another group of cyclic peptides, the hepatotoxic phallotoxins of the mushroom *Amanita*.

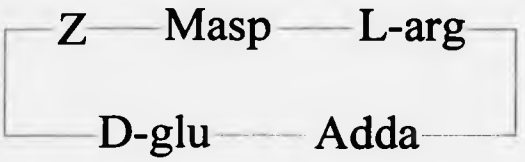
Microcystin-LR (Figure 1.6), in which the two variable amino acids are leucine and arginine, is the most commonly found microcystin. This toxin is produced by at least one strain of *Anabaena flos-aquae* and several strains of *Microcystis aeruginosa*. The acute LD_{50} of this toxin in mice following intra-peritoneal injection is about 50 μg kg^{-1}, but the toxicity is about 30-50 times higher by oral administration (Konst *et al.*, 1965). This toxin causes rapid and extensive centribular necrosis of the liver with loss of the characteristic architecture of hepatic cords. Both hepatocytes and endothelial cells are destroyed with vesiculation of the cell membranes (Ostensvik *et al.*, 1981). By determination of the distribution of radioisotope-labelled (^{125}I) microcystin between the major organs of rodents, Falconer *et al.* (1986) showed that the liver was the main target for accumulation and deposition of the toxin. Similar results have also been shown by Brooks & Codd

FIGURE 1.5 The schematic structures of microcystins and nodularin.

(a) Microcystins



(b) Nodularin



where Masp is D-erythro-β-methylaspartic acid

Adda is 3-amino-9-methoxy-10-phenyl-2,3,8-trimethyl-deca-4,6-dienoic acid

Mdha is N-methyldehydroalanine

X and Y are variable L-amino acids including leucine, alanine, arginine, tyrosine and methionine

Z is N-methyl-Z-dehydrobutyrine

TABLE 1.4 Some of the structural variations of microcystins (Carmichael, 1992)

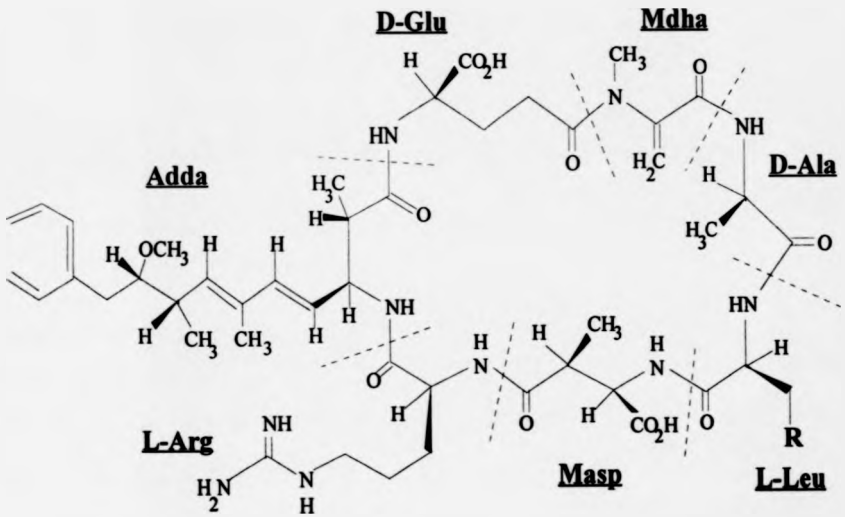
Microcystin[a]	MW	Formula	Organism
MCYST-LA	909	$C_{46}H_{67}N_7O_{12}$	*M. aeruginosa*[b]
MCYST-LAba	923	$C_{47}H_{69}N_7O_{12}$	*M. aeruginosa*[b]
MCYST-AR	952	$C_{46}H_{68}N_{10}O_{12}$	*Microcystis* spp.[c]
MCYST-YA	959	$C_{49}H_{65}N_7O_{13}$	*M. aeruginosa*[b]
[D-Asp^3,Dha^7]MCYST-LR	966	$C_{47}H_{70}N_{10}O_{12}$	*M. aeruginosa*[b]
[D-Asp^3]MCYST-LR	980	$C_{48}H_{72}N_{10}O_{12}$	*A. flos-aquae*[b], *M.viridis*[c]
[Dha^7]MCYST-LR	980	$C_{48}H_{72}N_{10}O_{12}$	*M. aeruginosa*[b]
[DMAdda^5]MCYST-LR	980	$C_{48}H_{72}N_{10}O_{12}$	*Microcystis* spp.[c], *Nostoc* sp.[b]
MCYST-LR	994	$C_{49}H_{74}N_{10}O_{12}$	*M. aeruginosa*[b], *A. flos-aquae*[b]
MCYST-LY	1,001	$C_{52}H_{71}N_7O_{13}$	*M. aeruginosa*[b]
[D-Asp^3,ADMAdda^5]MCYST-LR	1,008	$C_{49}H_{72}N_{10}O_{13}$	*Nostoc* sp.[b]
[D-Asp^3,Dha^7]MCYST-RR	1,009	$C_{47}H_{71}N_{13}O_{12}$	*O. agardhii*[c]
[Mser^7]MCYST-LR	1,012	$C_{49}H_{76}N_{10}O_{13}$	*Microcystis* spp.[c]
[ADMAdda^5]MCYST-LR	1,022	$C_{50}H_{74}N_{10}O_{13}$	*Nostoc* sp.[b]
[D-Asp^3,ADMAdda^5]MCYST-LHar	1,022	$C_{50}H_{74}N_{10}O_{13}$	*Nostoc* sp.[b]
[D-Asp^3]MCYST-RR	1,023	$C_{48}H_{73}N_{13}O_{12}$	*O. agardhii*[b]
[Dha^7]MCYST-RR	1,023	$C_{48}H_{73}N_{13}O_{12}$	*M. aeruginosa*[b]
MCYST-FR	1,028	$C_{52}H_{72}N_{10}O_{12}$	*Microcystis* spp.[c]
MCYST-M(O)R	1,028	$C_{48}H_{72}N_{10}O_{13}S$	*Microcystis* spp.[c]
MCYST-YM(O)	1,035	$C_{51}H_{69}N_7O_{14}S$	*M. aeruginosa*[c]
[ADMAdda^5]MCYST-LHar	1,036	$C_{51}H_{76}N_{10}O_{13}$	*Nostoc* sp.[b]
MCYST-RR	1,037	$C_{49}H_{75}N_{13}O_{12}$	*M. aeruginosa*[b], *M. viridis*[b]
[D-Ser^1,ADMAdda^5]MCYST-LR	1,038	$C_{50}H_{74}N_{10}O_{14}$	*Nostoc* sp.[b]
[ADMAdda^5,Mser^7]MCYST-LR	1,040	$C_{50}H_{76}N_{10}O_{14}$	*Nostoc* sp.[b]
MCYST-YR	1,044	$C_{42}H_{72}N_{10}O_{13}$	*M. aeruginosa*[b], *M. viridis*[b]
[D-Asp^3]MCYST-HtyR	1,044	$C_{52}H_{72}N_{10}O_{13}$	*A. flos-aquae*[b]
[D-Glu(OC$_3$H$_7$O)^6]MCYST-LR	1,052	$C_{52}H_{80}N_{10}O_{13}$	*Microcystis* spp.[c]
MCYST-HtyR	1,058	$C_{53}H_{74}N_{10}O_{13}$	*A. flos-aquae*[b]
MCYST-WR	1,067	$C_{54}H_{73}N_{11}O_{12}$	*Microcystis* spp.[c]

[a] Aba, aminoisobutyric acid; Dha, dehydroalanine; DMAdda, *O*-demethylAdda; ADMAdda, *O*-acetyl-*O*-demethylAdda; Mser, N-methylserine; Har, homoarginine; M(O), methionine S-oxide; Hty, homotyrosine.

[b] Toxins isolated from strain samples.

[c] Toxins isolated from bloom samples.

FIGURE 1.6 Structure of microcystin-LR.



R = $CH(CH_3)_2$

(1987) with (^{14}C) microcystin, and by Robinson *et al.* (1989) with (^{3}H) microcystin. Moreover, microcystin-LR is found to be an extremely specific and potent inhibitor of protein phosphatases 1 and 2A in eukaryotic cells from phyla as diverse as mammals, protozoa and plants (see more detail in chapter 5). Hepatotoxic peptides have also been isolated from strains of *Oscillatoria agardhii*, but their toxicities are apparently lower than those of the microcystins. Nodularin (Figure 1.7), the only cyclic pentapeptide known, is produced by a brackish water cyanobacterium, *Nodularia spumigena*.

Table 1.5 compares the toxicities of some hepatotoxins (nodularin, microcystin-LR and microcystin-RR) with neurotoxins (saxitoxin and anatoxin-a) as well as other biological toxins (NRA, 1990).

Although the toxins produced by *Anabaena flos-aquae*, *Microcystis aeruginosa* and *Oscillatoria agardhii* have been studied extensively since they are the most common bloom-forming species that have been linked to animal deaths, there are other cyanobacteria that produce peptide and alkaloid toxins e.g. *Anabaena hassalii*, *Anabaena variabilis*, *Calothrix* species and *Nostoc linkia* (Utkilen, 1992). Many toxins have not been successfully isolated, but they are recognised by their pathological effects and survival times after ingestion.

FIGURE 1.7 Structure of nodularin.

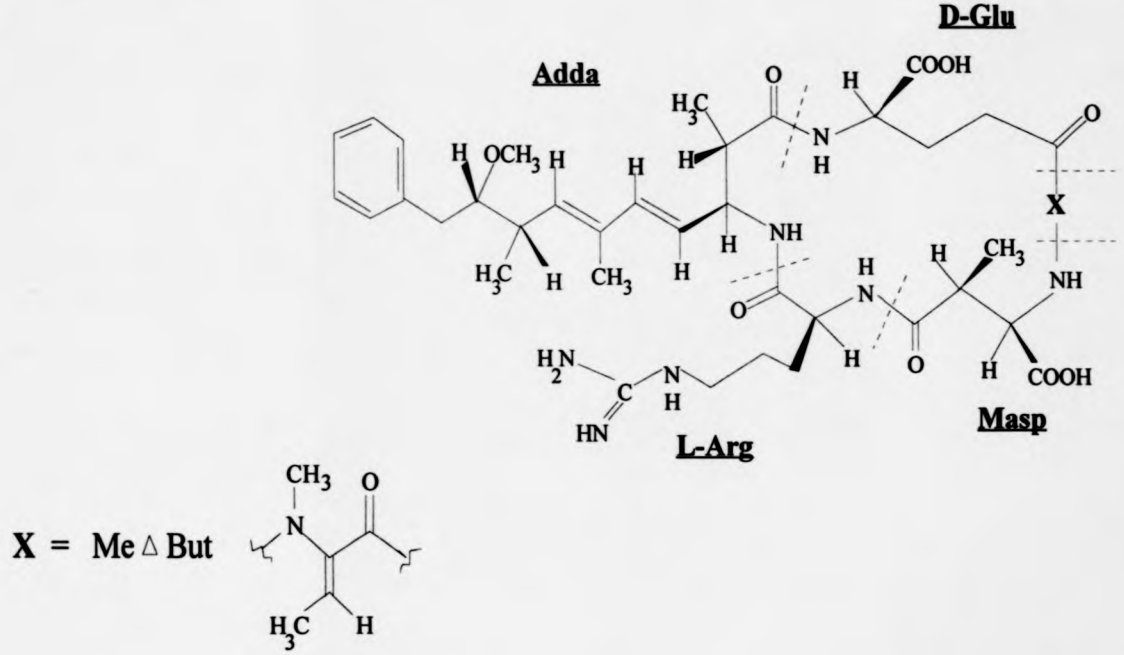


TABLE 1.5 Comparison of toxicities of biological toxins (NRA, 1990)

Toxin	Source	Common Name	Lethal Dose* (LD_{50})
BOTULINUM TOXIN-a	*Clostridium botulinum*	(BACTERIUM)	0.00003
TETANUS TOXIN	*Clostridium tetani*	(BACTERIUM)	0.0001
RICIN	*Ricinus communis*	(CASTOR BEAN PLANT)	0.02
DIPHTHERIA TOXIN	*Corynebacterium diphtheriae*	(BACTERIUM)	0.3
KOKOI TOXIN	*Phyllobates bicolor*	(POISON ARROW FROG)	2.7
TETRODOTOXIN	*Sphaeroides rubripes*	(PUFFER FISH)	8
SAXITOXIN	*Aphanizomenon flos-aquae*	(CYANOBACTERIUM)	9
COBRA TOXIN	*Naja naja*	(COBRA)	20
NODULARIN	*Nodularia spumigena*	(CYANOBACTERIUM)	30-50
MICROCYSTIN-LR	*Microcystis aeruginosa*	(CYANOBACTERIUM)	50
ANATOXIN-a	*Anabaena flos-aquae*	(CYANOBACTERIUM)	200
MICROCYSTIN-RR	*Microcystis aeruginosa*	(CYANOBACTERIUM)	300-600
CURARE	*Chrondodendron tomentosum*	(BRAZILAIN POISON ARROW PLANT)	500
STRYCHNINE	*Strychnos nux-vomica*	(PLANT)	500
AMATOXIN	*Amanita phalloides*	(DEATH CAP)	600
MUSCARIN	*Amanita muscaria*	(FLY AGARIC)	1100
PHALLATOXIN	*Amanita phalloides*	(FUNGUS)	1800
GLENODIN TOXIN	*Peridinium polonicum*	(DINOFLAGELLATE ALGA)	2500
SODIUM CYANIDE			10000

* The acute LD_{50} in μg per kg bodyweight: intra-peritoneal injection: some with mice, some with rats.

1.3.3 Lipopolysaccharides

Many cyanobacteria produce lipopolysaccharides (LPS) as a normal component of their outer layers. LPS are complex molecules consisting of variable fatty acids, phosphate and attached sugars. The LPS derived from cyanobacteria and from Gram-negative bacteria, although basically similar, differ in both chemical and biological characteristics (Keleti & Sykora, 1982). In the study of LPS from species of *Oscillatoria*, *Anabaena* and *Microcystis*, Utkilen *et al.* (1991) found that LPS in all cases contained 3-hydroxyoctanoate (3-OH-18:O) as a major constituent. This fatty acid probably can be used to distinguish cyanobacterial LPS from those of other bacteria. There are few data on the toxicity of cyanobacterial LPS. However it is much less toxic than the hepatotoxins and neurotoxins. The cyanobacterial LPS may be responsible for outbreaks of human gastroenteritis, diarrhoea, cramps, nausea and dizziness in the USA (Billings, 1981; Carmichael *et al.*, 1985), and for the irritation of skin observed in swimmers in contact with algal blooms in the UK (NRA, 1990).

1.4 Factors affecting the production of cyanobacterial toxins

Under natural conditions, not all blooms of toxin-producing cyanobacteria have the same toxicity, and even individual blooms can vary in toxicity with time (van der Westhuizen & Eloff, 1985). These variations may be due to the effects of environmental factors on toxicity and/or the alterations in the algae population itself, either in the toxicity of an individual strain or in the relative proportion of toxic and non-toxic strains within the population. Thus, an understanding of the regulation of toxin production is necessary in order to provide a rationale for the prediction and limitation of toxin production in aquatic environments, and also to advance further research on toxic cyanobacteria. Although a large number of studies have been carried out on the isolation and characterisation of the toxins as well as their mechanisms of action, only a very few studies have been carried out on the regulation of toxin production. Does the toxin content per cell vary in the population or is the toxicity of the environmental biomass a reflection of the toxic/non-toxic cells present?

Production of toxins appears not to be influenced by a single stimulus but rather by a complex set of environmental factors. The effects of temperature and light are the physical factors which have been investigated most frequently. Both have been reported to be important stimuli for toxin production in *Anabaena flos-aquae* NRC-44 (Peary & Gorham, 1966) as well as *Microcystis aeruginosa* NRC-1 (Gorham, 1964). Although the optimal growth temperature for *Microcystis aeruginosa* strain NRC-1 is between 25-32.5°C, toxin production is maximal at 25° C and decreases above 25°C. The optimal conditions for growth do not coincide with those for toxin production in *Microcystis aeruginosa* UV-006 (van der Westhuizen & Eloff, 1985). Watanabe & Oishi (1985) reported that a marked change in toxicity was observed in light intensity experiments on *Microcystis aeruginosa* strain M228, but only slight changes were observed to be caused by

temperature and phosphorus deficiency. Another study on *Microcystis* species revealed that four to fivefold decreases in toxicity of the cells were obtained when *Microcystis aeruginosa* 7813 was maintained at 10°C and 34°C, near the lower and upper temperature limits for this strain, the optimal growth temperature being 25°C (Codd & Poon, 1988).

The effects of pH and nutrients on toxicity have also been analysed. The highest growth rate of *Microcystis aeruginosa* in batch culture occurs around pH 9.0, but toxicity is greater at higher or lower pH values (van der Westhuizen & Eloff, 1983). The removal of phosphate from the media may affect the growth of this strain, but does not influence toxicity, however, the removal of nitrate and bicarbonate resulted in an approximately tenfold decrease in toxicity per unit biomass (Codd & Poon, 1988). Toxin production by *Oscillatoria agardhii* strains 97 and CYA 128, in contrast, was usually highest under conditions that also favoured growth (Sivonen, 1990). The highest toxin production for these strains is at the lowest light intensities which correspond with the preference of *Oscillatoria agardhii* to grow at low light intensities in nature. High toxin production also correlates with high nitrogen concentrations. Also, low levels of phosphorus in the range of 0.1 to 0.4 mg of P per litre enhance toxin production, however, higher concentrations have no additional effects. Furthermore, Carmichael (1986) reported that the toxic cyanobacterial strains that are reportedly subcultured in media enriched by nutrients were found to undergo a decrease or loss of toxin production over time.

The effect of trace metals on the growth of *Microcystis aeruginosa* PCC 7806 and its production of microcystin was studied by Lukac & Aegerter (1993). Only Zn and Fe were reported to significantly affect toxin yield. Zn was shown to be required for optimal growth as well as toxin production. However, in the absence of Fe the cells grew much more slowly than under standard conditions, but they produced more toxin. It is not yet clear how Fe deficiency modulates microcystin production.

The genetic basis of cyanobacterial toxin production has received very little attention. The plasmids of cyanobacteria have been considered as the possible location of genes involved in toxin production, however, plasmid-curing agents do not affect toxicity (Vakeria *et al.*, 1985), and there are some toxic strains that contain no plasmids (Schwabe *et al.*, 1988). Studies indicate that the genes responsible for the regulation of toxin production are not connected with extra-chromosomal DNA, although the possibility remains that plasmid DNA that has become integrated into the chromosome may be involved in some way. The presence of the uncommon amino acid residues suggest that the cyclic peptide is not a single gene product but that its synthesis is controlled by multiple genes (Lukac & Aegerter, 1993).

1.5 Toxin release

The release of toxins is variable between species of cyanobacteria, but this subject has not been extensively studied under different environmental conditions. According to laboratory studies of toxic *Microcystis* strains (Table 1.6), a substantial release of toxins from the cells into the water occurs when the cells die because of cell lysis (NRA, 1990). Although most of the microcystins produced by pure *Microcystis* cultures in the laboratory are retained within the cells, a substantial release of microcystins from healthy growing cultures of *Oscillatoria* has been observed (NRA, 1990). Generally microcystins remain with the cell during growth and "free" toxin is only detectable in raw water following leakage from, or lysis of, the cells upon death.

Since toxins are released when the cells die or become old and leaky, the fatal incidents involving animals happen as a consequence of ingesting these whole cells of cyanobacteria. However, a sudden release of toxins into the cell-free water

TABLE 1.6 Distribution of microcystins during laboratory culture of *Microcystis aeruginosa* (NRA report; Waters Quality Series 2, 1990)

Age of culture	% Distribution of toxins	
	Cells	**Water**
Young, slowly-growing cells	100	0
Young, rapidly-growing cells	75-90	10-25
Old, slowly-growing intact cells	70-80	20-30
Old, decaying cells (leaking cell contents)	30-40	60-70

can be achieved if the water is treated with copper sulphate, in an attempt to break up the cyanobacterial bloom.

Treatment with copper sulphate to give a concentration in the water of about 0.2 mg l^{-1} has been used extensively for the control of algae blooms without damaging higher plants or fish since 1905 (McKnight *et al.*, 1983). Low doses of copper sulphate can be effective in controlling cyanobacterial blooms (Whitaker *et al.*, 1978). The critical problem is that copper sulphate treatment may cause the release cyanobacterial toxins into the water as the cells die and lyse - copper sulphate does not reduce the toxicity of the released toxins. In laboratory batch experiments, samples of *Microcystis aeruginosa* cells treated with copper sulphate at a concentration of 0.5 mg l^{-1} demonstrated a loss of cell wall integrity, allowing intracellular toxins to be rapidly released. However, *Microcystis aeruginosa* cells treated with lime at a concentration of 100 mg l^{-1} in a similar manner, maintained intact cell walls and did not release microcystin-LR (Kenefick *et al.*, 1992). Thus, it has been suggested that copper sulphate should not be used to treat potentially toxic cyanobacterial blooms in waters that may be consumed by humans or animals within several weeks following treatment.

Selective algicides such as 2,3-dichloro-1,4-napthoquinone are an alternative way to control cyanobacterial growths in swimming pools and fish tanks, but are not applicable to lakes or reservoirs because of their high cost (Fogg *et al.*, 1973). It has also been proposed that ultra-sound as well as the pressures generated by detonating explosive devices might be used to collapse cyanobacterial gas vacuoles and so destroy their buoyancy and prevent scum formation (Walsby, 1968).

1.6 EFFECTS OF TOXINS ON WILDLIFE, DOMESTIC ANIMALS AND HUMANS

Toxic blooms of cyanobacteria have been reported in many waters worldwide. The majority of species have the tendency to form scums in which the concentration of toxin is enhanced many fold. These scums are usually observed to accumulate in thick layers along the margin of a water body when there is a gentle on-shore breeze. Alternatively, the scums can disperse thinly over the water surface in calm weather. Animal death most probably occurs because they consume toxic biomass from the shore and not as a consequence of ingesting water containing free toxin, although the latter cannot be completely ruled out.

Many drinking water supplies throughout the world also encounter problems from periodic massive surface accumulations of cyanobacteria. Although there are no confirmed reports of human deaths due to these toxins, dense populations of cyanobacteria are suspected of causing human illness, skin reactions and so on. Such research is attracting interest today, in part because of the worry over public health as cyanobacterial blooms become more common in reservoirs, rivers, lakes and ponds.

A combination of biological and physical conditions has usually led to the reported animal poisonings. The amount of water necessary to cause death/illness in animals depends on such factors as the type and amount of toxin produced by the cyanobacterial cells, the concentration of the cells, as well as the species, and the size, sex and age of the animal at risk (Carmichael, 1994).

1.6.1 Wildlife and domestic animals

The first report of the lethal effects of cyanobacteria on animals was published by Francis in 1878. That incident was concerned with a bloom of *Nodularia spumigena* in the Murrey River, Australia. Since then, a large number of poisoning incidents affecting agricultural livestock, wild animals, birds or fish have been widely reported.

Only a few studies have focussed on the toxicity of cyanobacteria to freshwater invertebrates, moreover, contradictory results have been obtained. For instance, three *Daphnia* species were able to grow and reproduce although they were fed by a diet consisting exclusively of *Microcystis aeruginosa* (De Barnardi *et al.*, 1981). However, acute toxic effects of this cyanobacterial species on *Daphnia* species were reported by Nizan *et al.* (1986) and Reinikainen *et al.* (1994). Caution must be exercised in interpreting these results, however, since other studies have compared several cyanobacterial strains and have shown that some strains are toxic, while others are not (Vasconcelos, 1990). Fulton & Paerl (1988) also demonstrated that blooms of *Microcystis aeruginosa* can affect interspecies competition in zooplankton. Moikeha & Chu (1971) extracted a toxin from material gathered from Hawaiian offshore blooms of *Lyngbya majuscula* which possessed antibacterial activity, and caused lysis of protozoa and death of rats when injected intravenously.

The occurrence of fish kills during periods of heavy cyanobacterial blooms has also been reported. However it was not clear whether the fish deaths were specifically due to cyanobacterial toxins (NRA, 1990). Nevertheless, a severe liver disease (NLD, netpen liver disease), characterised by severe necrosis and hepatic megalocytosis, has been observed in Atlantic salmon at several separate netpen sites in coastal British Columbia and Washington and it has been shown to be caused by microcystin-LR or a closely related cyclic peptide toxin (Anderson *et al.*, 1993). Atlantic salmon in this study also appeared to be resistant to much higher

concentrations of microcystin-LR than mice. Deaths of large numbers of roach, bream, orfe and pike have been ascribed to microcystin poisoning in Scandinavia, although again this is not proven (NRA, 1990). However, microcystins have been shown to cause hepatocellular damage in carp, *Cyprinus carpio* (Rabergh *et al.*, 1992) and rainbow trout, *Oncorhynchus mykiss* (Phillips *et al.*, 1985). Death due to deoxygenation of the water by decaying blooms cannot be overlooked and it has been postulated to be the cause of trout deaths (NRA, 1990).

Some marine cyanobacteria are also toxic. For instance, marine animals and fish may be killed, at least indirectly, because of clogging of the gills by a dense bloom of *Oscillatoria erythraea*, following by suffocation (Humm & Wicks, 1980). It is not known whether the toxins accumulate in fish. However, outbreaks of disease in fish-eating people, such as Haff disease around the Baltic and Ciguatera in the West Indies, are suspected to be a consequence of eating cyanobacterial poisonous fish.

There is evidence supporting the hypothesis that microcystins and Antx-a can be accumulated in the tissues of the freshwater Swan Mussel, *Anodonia cygnaea* (NRA, 1990). Deaths of mussel due to microcystins has not been reported, but small numbers of Swan Mussel fatalities due to exposure to neurotoxic *Anabaena* have occurred in laboratory trials (NRA, 1990).

The deaths of wild and domestic animals have usually been reported after consuming water heavily contaminated with toxic cells or toxins released from decaying cells. These toxic water blooms have been found particularly during mid to late summer and early fall in freshwater ponds, lakes and slow moving rivers throughout the world. Under certain conditions, due to their possession of buoyancy-conferring gas vacuoles, surface toxigenic blooms can accumulate and form thick scums containing concentrated algae which lead to most natural cases of poisoning. The outbreaks sometime also occur in recreation water supplies and/or water reservoirs serving as potable water supplies for domestic consumption.

The toxicity of hepatotoxins have been experimentally conducted by dosing whole cells or toxin extracts into laboratory and domestic animals. Histological examination has revealed extensive haemorrhage in mouse liver with initial perilobular distribution, necrosis of hepatocytes, cell detachment and loss of tissue architecture, accumulation of erythrocytes and breakdown of sinusoidal epithelial cells (Falconer *et al.*, 1981). Similar observations have been reported for sheep (Jackson *et al.*, 1984). Marked increases in serum activities of aspartate aminotransferase, lactate dehydrogenase, glutamate dehydrogenase and alkaline phosphatase, an increased amount of bilirubin and a decrease in serum glucose were also observed. Terminal clonic spasms and opisthotonous were observed in some rabbits (Konst *et al.*, 1965). On an equivalent weight basis it required three to five times the oral dosage to kill the large animals, i.e. calves and lamb, and birds as it did to kill the laboratory animals. The symptoms were less pronounced and the survival times were longer in the more resistant animals (Konst *et al.*, 1965). Although, most of the studies suggest that peptide toxins act primarily on the liver, an alternative hypothesis has been proposed that the toxins may primarily cause pulmonary congestion due to acclusion by thrombi-containing plates (Slatkin *et al.*, 1983). Multiple pulmonary thrombi were found in mice after acute poisoning by microcystin. This pulmonary thrombosis was not relieved by anticoagulants and was estimated to precede hepatomegaly (Slatkin *et al.*, 1983). However, in the administration of microcystins from Australian and Scottish *Microcystis aeruginosa* isolates, liver damage occurred before the appearance of the thrombi in the lungs, and measurements of jugular and hepatic portal venous blood pressures during the course of toxicity did not indicate congestion consistent with pulmonary thrombosis (Falconer *et al.*, 1981; Theiss *et al.*, 1988). Toxins other than hepatotoxins have been reported to cause animal deaths e.g. dog deaths at Loch Insh, Scotland had been affected by cyanobacterial neurotoxicosis, the clinical signs of which are convulsions, coma, rigors, cyanosis, limb twitching and hypersalivation (Edwards *et*

al., 1992); cattle poisonings in Finland at Lake Vesijarvi, Lake Saaskjarvi and at Lake Sayhteenjarvi were also caused by neurotoxic blooms (Sivonen *et al.*, 1990b). However, laboratory experiments on the effect of neurotoxins are very limited.

1.6.2 Humans

Effects on human health caused by cyanobacteria and their toxins have been reported from the USA, India, Canada, Zimbabwe, Norway, the Baltic coast, the USSR, Australia and the UK. Although much of the evidence implicating cyanobacterial toxins in human health problems is circumstantial and also no confirmed human death has yet been attributed to the toxins, research is becoming intensive because of the perceived public health risks. The extensive use of detergents and fertilisers is altering the chemistry of many municipal water supplies and swimming areas, primarily increasing the concentration of nitrogen and phosphorus, and these promote the growth of cyanobacteria often leading to the formation of dense blooms. These occasions are becoming more common in reservoirs, rivers, lakes and ponds, and consequently the likelihood grows that people will be exposed to increased doses of toxins.

It has been reported that the hepatotoxins are normally contained within the cyanobacterial cells, and released only when the cells die or are damaged. When cyanobacteria move down many kilometres through water pipes, and pass through pressure reduction values, it is likely that cell lysis may occur so freeing toxin into the water (Falconer *et al.*, 1983a). Ordinary water purification and filtration processes cannot remove or inactive the toxin (Hoffman, 1976). Some toxins are not destroyed by boiling because of their thermostability. Although Falconer *et al.* (1983b) reported that activated carbon filters can be used to remove the toxin, they are not, however, used routinely in conventional purification processes. Therefore

there is a high probability that the presence of microcystin in drinking water may be affecting human health.

The incidents of human health compromised by cyanobacterial toxins can be separated into two main groups as follows :-

1.6.2.1 Allergic reactions and skin irritations

Incidents of contact skin irritation or dermatitis have been reported after bathing and showering in marine and freshwaters containing toxic cyanobacteria. For instance, Moikeha & Chu (1971) reported that severe dermatitis in swimmers around the coasts of Hawaii was caused by a toxin from material gathered from Hawaiian offshore blooms of *Lyngbya majuscula*. Similar cases were also reported in Florida (Moikeha *et al.*, 1971). The characteristic symptoms were burning or itching of the skin, erythematous wheals, redness of the eyes and lips, sore throat, ear ache and dizziness. Bathing in water containing blooms of *Anabaena*, *Aphanizomenon*, *Gloeotrichia* and *Oscillatoria* may contribute to skin irritations in freshwaters, such as "swimmers' itch" (Schwimmer & Schwimmer, 1964) which is an unpleasant conduction of the skin caused by bathing in waters densely populated with *Aphanizomenon* species. Cases of skin rashes after sail-boarding in water containing toxic cyanobacterial blooms have also been reported in the UK (NRA, 1990).

1.6.2.2 Gastroenteritis and hepatoenteritis

Gastroenteritis, abdominal pain, diarrhoea, headache, nausea, vomiting, stomach cramps and dizziness are the symptoms which have been reported for swimmers in waters containing *Anabaena* species and *Schizothrix calcicola* in the USA (Billings 1981; Carmichael *et al.*, 1985). The cyanobacterial LPS may be

responsible, since enterobacteria and viruses were not implicated with these outbreaks. Gastroenteritis has also been reported in Zimbabwe, when children had been drinking water from a source with a *Microcystis* bloom (NRA, 1990).

An outbreak of hepatoenteritis in Palm Island off Australia's Queensland coast in 1979 was most probably a consequence of cyanobacterial toxicity (Bourke *et al.*, 1983). The symptoms included hepatitis, followed by lethargy and diarrhoea. The incident took place after a heavy bloom in the local reservoir had been treated with copper sulphate. This possibility was supported by the isolation of *Cylindrospermopsis raciborskii* from the reservoir which was established as being highly hepatotoxic for mice.

There is another route by which cyanobacterial toxins may lead to human disease. The toxins are suspected of causing "Haff disease", which affects large numbers of people eating fish along the Baltic coast. Symptoms are severe muscular pain, respiratory distress, vomiting and brownish-black urine and even a few cases of death resulted from eating fish, particularly the livers. These illnesses may not be directly due to cyanobacterial toxins. However, the possibility that the toxins may accumulate in some species of fish requires further study.

An epidemiological investigation carried out in Armidale, New South Wales, Australia (Falconer *et al.*, 1983a) showed significant links between toxic cyanobacteria and human liver illness in the population following a heavy *Microcystis aeruginosa* bloom in the water supply. The significant elevation of the levels of hepatic γ-glutamyltranspeptidase and a lesser elevation of alanine aminotransferase in the plasma, a pattern of liver damage, were observed in a population who obtained drinking water from the reservoir containing the *Microcystis* blooms. Such elevations did not occur in the adjacent population who did not use water from this source.

The expression of toxins either in aquatic organisms or their presence in municipal water supplies requires increased vigulance since exposure to low-levels of toxin over a long-time may lead to tumour promotion.

1.7 AIMS OF THE PROJECT

This project was initiated in response to the increasing frequency of reservoirs and lakes, which are used for recreation and as sources of drinking water, supporting the growth of toxic cyanobacteria. Knowledge of the chemical structure and activity of the toxins should help us to devise more sensitive ways to measure these compounds in water and to develop antidotes to lethal doses. Physiological studies will also facilitate an understanding of the long term effect of exposure to non-lethal doses, for example the promotion of liver tumours.

The aims of the research are as follows :

1) To detect and quantify the "presumptive" hepatotoxins produced by freshwater cyanobacteria by high performance liquid chromatography (HPLC), and also to develop an analytical method for toxins which is simple, rapid and reliable.

2) To study the spatial and temporal variation of presumptive toxins in dominant cyanobacterial species.

3) To identify these presumptive toxins from environmental cell biomass by characterisation of the structure using the technique of fast atom bombardment mass spectrometry (FAB-MS).

4) To study the environmental factors that promote the expression of peptide cyanotoxins in order to understand, and eventually control toxin production.

5) To study the inhibition of phosphorylase phosphatases by presumptive toxins, and to investigate this as a method for toxicity assessment.

6) To study the inhibition effect of these cyanotoxins on other liver enzymes as an alternative method of toxicity assessment.

CHAPTER 2

CHAPTER 2 : MATERIALS AND METHODS

2.1 MATERIALS

Most of the reagents used were purchased from BDH Chemicals Ltd. (analytical grade) and/or Fisons/FSA plc (HPLC grade). Fine chemicals were obtained mainly from Sigma Chemical Co. Ltd., BDH Chemicals Ltd., Aldrich Chemical Co. Ltd. or Fisons/FSA plc. The sources of other specific reagents and chemicals are detailed below.

BDH Chemicals Ltd.

Acetonitrile (HPLC grade); ammonium molybdate; potassium dihydrogen orthophosphate; tris (hydroxymethyl) methylamine; tris (hydroxymethyl) methylammoniumchloride.

Calbiochem-Novabiochem (UK) Ltd.

Microcystin-LR; microcystin-RR; nodularin.

Fisons/FSA plc.

β-mercaptoethanol; trichloroacetic acid (TCA); trifluoroacetic acid (TFA).

ICN Pharmaceuticals, Incorporated

(γ-^{32}P) Adenosin-5'-triphosphate (specific radioactivity 7,000 Ci mmol^{-1}).

Sartorius Filters, Inc.

Cellulose nitrate filter, 8 μm.

Sigma Chemical Co. Ltd.

Adenosine-5'-monophosphate (AMP), sodium salt type II, from yeast; adenosine-5'-triphosphate (ATP) from equine muscle; acid phosphatase assay-kit; alkaline phosphatase assay-kit; bovine serum albumin (BSA); dithiothreitol (DTT); Fiske & Subbarow reducer (1-amino-2-naphthol-4-sulfonic acid, 0.8% sodium sulfite and sodium bisulfite); γ-glutamyltransferase (GGTP) assay-kit; β-glycerophosphate disodium salt, hydrate; imidazole; β-nicotinamide adenine dinucleotide reduced form (NADH), disodium salt; Norit-A, activated charcoal; okadaic acid; phosphorylase *b* from rabbit muscle; phosphorylase kinase from rabbit muscle (specific activity, 480 units mg^{-1} protein); pyruvic acid, sodium salt; Sephadex G-25; tartrate, disodium salt; theophylline (1,3-dimethylxanthine).

Waters, Millipore (UK) Ltd.

Sep-pak octadesylsilyl (ODS) C_{18} cartridge.

Whatman Scientific Ltd.

Whatman 3MM chromatography paper.

2.2 HPLC COLUMNS

Two analytical C_{18} columns were used in two different HPLC systems as shown below.

(i) Nova-Pak C_{18} 4 μm, 3.9 x 300 mm, attached to a Nova-Pak C_{18} Guard-Pak insert, was supplied by Waters, Millipore (UK) Ltd. This column was used in a Varian HPLC system with a photo-diode array detector.

(ii) SuperPac™ Pep-S 5 μm, 4.0 x 250 mm, with the guard cartridge connected directly to the main column, was supplied by Pharmacia Biotech Ltd. This column was used in a Pharmacia LKB HPLC system with a UV detector.

2.3 ORGANISMS

2.3.1 Reference toxic cyanobacterium

The reference toxic cyanobacterium used in this study was *Microcystis aeruginosa* PCC 7806, a known hepatopeptide toxin producer, obtained from the Pasteur Culture Collection (PCC, Paris, France).

2.3.2 Environmental cyanobacteria

About 10 litres of water from freshwater reservoirs or lakes in the Midlands were sampled, from 1991 to 1993 (Table 2.1). Physical and chemical parameters (temperature, pH, dissolved oxygen, chlorophyll-a concentration, cyanobacterial cell counts, total nitrate concentration, ortho and total phosphate concentration) were determined by Severn Trent Water personnel. The cyanobacterial species present were observed by light microscopy.

TABLE 2.1 Reservoirs and lakes in the Midlands as sources of cyanobacteria during 1991 to 1993

Warwickshire District :	Draycote
	Stanford
	Lower and Upper Shustoke
	Wille's Meadow
Leicestershire District :	Foremark
	Cropston
	Blackbrook
	Nanpantan
	Staunton Harold
	Swithland
	Thornton

2.4 Preparation of standard toxin solution

Three standard hepatotoxins; microcystin-LR, microcystin-RR and nodularin, were obtained as freeze-dried samples (500 μg). 500 μl of 30% (v/v) methanol was added to give reference toxin solutions of 1 μg μl^{-1} concentration. These were stored at -20°C. Reference solutions were diluted in 10% (v/v) methanol as appropriate.

2.5 Preparation of *Microcystis aeruginosa* PCC 7806

The reference toxic cyanobacterium *Microcystis aeruginosa* PCC 7806 was maintained in medium BG-11 (Stanier *et al.*, 1971), as described in Table 2.2, with continuous illumination by fluorescent light. Medium was made using analytical grade chemicals and double distilled water, and sterilised by autoclaving at 15 lb $inch^{-2}$ for 15 minutes.

Cells were harvested by centrifugation at 20,000 x g, lyophilised and stored at -20°C.

TABLE 2.2 Composition of BG-11 medium (Stanier *et al.*, 1971)

	g l^{-1}
$NaNO_3$	1.5
$K_2HPO_4 \cdot 3H_2O$	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
$FeNH_4$ citrate	0.006
Na_2Mg·EDTA	0.001
Na_2CO_3	0.02
Trace metal mix A_5+Co*	1 ml l^{-1}
* Trace metal mix A_5+Co	
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$Na_2Mo \cdot O_4 \cdot 2H_2O$	0.390
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.049

2.6 TREATMENT OF WATER SAMPLES

2.6.1 Preparation of potable water

Water from local reservoirs, many serving as sources for potable water, was collected by Severn Trent Water personnel as required. 10 litres of reservoir water was passed through a Whatman 41 course filter. Biomass retained on the filter was collected in a small volume of reservoir water and allowed to float. The floating material, which consisted almost entirely of cyanobacteria, was collected, lyophilised and stored at -20°C prior to analysis. The filtrate was refiltered through an 8 μm cellulose nitrate Millipore filter to remove the remaining cells and other cell debris.

2.6.2 Lyophilisation

The floating material, cyanobacterial cell biomass, was collected, in as small a volume as possible and frozen at -70°C prior to lyophilisation. Watery samples were quick frozen by dropping into liquid N_2. Lyophilised samples were stored at -20°C.

2.7 PREPARATION OF ENVIRONMENTAL CYANOBACTERIAL BIOMASS

2.7.1 Toxin extraction procedure

2.7.1.1 Small scale preparation

Lyophilised cells (~ 100 mg) were extracted three times with 10 ml of 5% (v/v) acetic acid in a sonicating waterbath for 20 minutes. The mixture was stirred at room temperature for 30 minutes, centrifuged at 4,750 x g for 15 minutes in a MSE Mistral bench centrifuge and the supernatants pooled (total ~ 30 ml).

2.7.1.2 Large scale preparation

Lyophilised cells (~ 5 g) were extracted three times with 100 ml of 5% (v/v) acetic acid in a sonicating waterbath for 20 minutes. The mixture was then stirred at room temperature for an hour, centrifuged at 9,300 x g for 20 minutes and the supernatants pooled (total ~ 300 ml).

2.7.2 Purification and concentration procedure

2.7.2.1 Small scale preparation

The aqueous extract was partially purified by passing through a 500 mg Sep-pak octadecylsilyl (ODS) C_{18} cartridge which had been preconditioned by washing with 5 ml of methanol and 5 ml of distilled water. The cartridge containing toxin was washed with water (10 ml), followed by a water-methanol mix (8:2, 10 ml). The toxin containing fraction was finally eluted from the cartridge with 15 ml of methanol. This fraction was rotary evaporated (using a Gyrovapping procedure) and resuspended in 300 µl of 30% (v/v) methanol. The concentrate was stored at -20°C prior to further analysis *via* (i) reversed phase HPLC, (ii) mouse bioassay, (iii) phosphatase assay and (iv) mass spectrometry.

2.7.2.2 Large scale preparation

The aqueous extract was partially purified by passing through a 5 g Sep-pak octadecylsilyl (ODS) C_{18} column which had been preconditioned by washing with 70 ml of methanol and 70 ml of distilled water. The column containing toxin was washed with water (400 ml), followed by a water-methanol mix (8:2, 200 ml). The toxin containing fraction was finally eluted from the column with 200 ml of methanol. This fraction was rotary evaporated and resuspended in 600 µl of 30% (v/v) methanol. The concentrate was stored at -20°C prior to further analysis.

2.7.2.3 Gyrovapping procedure

Sample solutions were transferred into centrifuge tubes. The solvent was evaporated by centrifugation at > 1,000 rpm under vacuum in a Gyrovap attached to a refrigerated solvent trap (Howe). The sample pellets were resuspended in 30% (v/v) methanol and stored at -20°C.

2.8 PREPARATION OF RAW WATER SAMPLES

After filtration through an 8 µm filter, the pH of the "cell free" raw water (about 10 litres) was adjusted to between 3.5 and 6.5 using concentrated HCl and passed through a 5 g Sep-pak C_{18} column which had been conditioned with 70 ml of methanol and 70 ml of distilled water. The column was washed with 10% (v/v) methanol (70 ml) and toxin eluted with 40 ml of methanol. The eluate was rotary evaporated and resuspended in 300 µl of 30% (v/v) methanol. The concentrate was stored at -20°C.

2.9 High performance liquid chromatography (HPLC) analysis

2.9.1 HPLC system

Cell biomass or "cell free" water samples were analysed by reversed phase HPLC using either a Varian LC star 9010 solvent delivery system with a Polychrom 9065 photo-diode array detector or a Pharmacia LKB gradient pump 2249 solvent delivery system with a variable wavelength UV detector. The Varian photo-diode array detection system facilitated the spectral analysis of the peaks which was performed using the Varian software "PolyView" which gave the similarity/dissimilarity breakpoints and perceived matches. This software also permitted the evaluation of the purity of each eluted peak.

2.9.2 Mobile phase composition

The mobile phases used in this study were 30% and 60% acetonitrile containing 0.05% TFA. After preparation, all mobile phases were filtered through a 0.45 μm filter and degassed, especially when using the Pharmacia LKB system. All the other solvents used in the washing step such as methanol and distilled water were also filtered through a 0.45 μm filter.

2.9.3 HPLC conditions

The reversed phase C_{18} analytical columns used were either Nova-Pak C_{18} or SuperPac™ Pep-S. The toxin(s) was eluted in a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA within 30 minutes, at a flow rate of 1 ml min^{-1} and detection at 238 nm. Between each run, the column was washed with distilled water and methanol.

2.9.4 Fraction collection

Peaks were collected using a Jaytee 5512 fraction collector (Jaytee Biosciences Ltd.), lyophilised, resuspended in the appropriate known volume (as small as possible) of 30% (v/v) methanol, and stored at -20°C.

2.9.5 Calculation of toxin concentration

The amount of toxin in environmental samples was determined by reference to microcystin-LR. Quantification of each toxic peak, identified by spectral characteristics similar to those of reference microcystins or nodularin, was made by extrapolating peak areas to the standard curve for microcystin-LR.

2.10 Precision of extraction from cell biomass

This experiment was run in duplicate. An accurately known quantity of microcystin-LR (5-10 μg) was added to non-toxic lyophilised cells (100 mg) and mixed well. Spiked-toxin samples were extracted with 5% (v/v) acetic acid following the procedure as described in section 2.7.1.1 and 2.7.2.1. The spiked-toxin samples were analysed by HPLC and the amount of microcystin-LR added was calculated. % precision of the extraction procedure was assessed as follows:

$$\% \text{ precision} = \frac{\text{amount of toxin obtained from HPLC}}{\text{known amount of toxin added}} \times 100$$

2.11 TOXICITY TESTING BY MOUSE BIOASSAY

The toxicity of the cell biomass, water samples and HPLC peak fractions were assessed by mouse bioassay as described below.

2.11.1 Lyophilised cell biomass

100 mg (or as much as possible) of lyophilised cells were resuspended in 1 ml of phosphate buffered saline (PBS), pH 7.5 (or 600 µl if less than 100 mg of cells were used). Samples were kept on ice at all times. Cells were disrupted by sonication for 15 seconds (x 8 times). The sonicate was serially diluted and 200 µl aliquots of each dilution used to assay toxicity. Male and female Balb C mice with a body weight of approximately 20 g were injected intraperitoneally with 0.1-0.2 ml of the lysate. The control mice received saline buffer injections only. The mice were challenged before 10.00 am and examined hourly until 5.00 pm. Mice showing severe sings of toxicity were culled, if not they were further examined at 8.00 am of the following morning. Any signs of toxic symptoms were recorded and toxicity break points were scored by death of the injected mice.

2.11.2 Water samples

The rotary evaporated Sep-pak eluates of 10 litres of raw water were resuspended in 100 µl of 30% (v/v) methanol. Serial dilutions were subsequently prepared in PBS, pH 7.5. 200 µl aliquots were then injected into mice for toxicity assessments as described previously (see section 2.11.1).

2.11.3 HPLC peak fractions

The non-toxic and toxic peak fractions separated by HPLC were lyophilised, resuspended in buffer and injected into mice. Toxicity was assessed as described previously (see section 2.11.1).

2.12 PROTEIN PHOSPHATASE ASSAY

2.12.1 Preparation of stock solution

ATP, unlabelled (0.1 M)

0.1 M ATP stock was made up by dissolving 60 mg ATP in 0.8 ml of double distilled water. The pH was adjusted to 7.0 with 0.1 M NaOH and the volume of the solution was adjusted to 1.0 ml. The stock solution was then dispensed into small aliquots and stored at -70°C.

DTT (1 M)

1 M DTT stock was made up by dissolving 3.09 g of DTT in 20 ml of 0.01 M sodium acetate, pH 5.2. The solution was sterilised by filtration, dispensed into aliquots and stored at -20°C.

EDTA, pH 8.0 (500 mM)

500 mM EDTA stock was made up by adding 186.1 g of disodium ethylene diamine tetraacetate·$2H_2O$ to 800 ml of double distilled water. This was stirred vigorously on a magnetic stirrer while adjusting the pH to 8.0 by slow addition of sodium hydroxide pellets (approximately 20 g). The volume was then made up to 1 litre. The solution was dispensed into aliquots and sterilised by autoclaving.

β-glycerophosphate (1 M)

1 M β-glycerophosphate was made up by dissolving 2.16 g of β-glycerophosphate in 10 ml of double distilled water.

Imidazole (200 mM)

200 mM imidazole stock was made up by dissolving 2.7232 g of imidazole in 200 ml of double distilled water.

β-mercaptoethanol (1M)

β-mercaptoethanol was usually obtained as a 14.4 M solution. 1 M β-mercaptoethanol was prepared by diluting 3.49 ml of β-mercaptoethanol to a final volume of 50 ml with double distilled water.

$MgCl_2$ solution (1 M)

1 M $MgCl_2$ stock was made by dissolving 203.3 g of $MgCl_2 \cdot 6H_2O$ in 800 ml of double distilled water. The volume of the solution was adjusted to 1 litre. The solution was dispensed into aliquots and sterilised by autoclaving.

Theophylline (20 mM)

20 mM theophylline stock was made by dissolving 1.802 g of theophylline in 500 ml of double distilled water.

Tris buffer (1M)

1 M Tris buffer stock was made up by dissolving 121.1 g Tris base in 800 ml of double distilled water. The pH was adjusted to the desired value by adding concentrated HCl at room temperature. The volume of the solution was then made up to 1 litre. The solution was dispensed into aliquots and sterilised by autoclaving.

2.12.2 Preparation of major buffers used

Buffer A (Brautigan & Shriner, 1988)

Buffer A was made up by using the above stock solutions as follows:

1 M Tris base	1 ml
1 M β-glycerophosphate	5 ml
1 M β-mercaptoethanol	1.5 ml

made up to 50 ml with double distilled water, the pH being adjusted to 8.2 with 2.0 M Tris base.

Buffer B (Brautigan & Shriner, 1988)

Buffer B was made up by using the above stock solutions as follows:

1 M Tris base	5 ml
1 M β-mercaptoethanol	12.5 ml

made up to 250 ml with double distilled water, the pH being adjusted to 6.8 with 1 M acetic acid.

Buffer B containing 0.1 M NaCl (Brautigau & Shriner, 1988)

Buffer B containing 0.1 M NaCl was prepared as per buffer B with the addition of 1.461 g of NaCl.

Assay buffer (Killilea *et al.*, 1978)

Protein phosphatase assay buffer was prepared by using the above stock solutions as follows:

200 mM imidazole	12.5 ml
500 mM EDTA	0.50 ml
1 M DTT	0.025 ml
20 mM theophylline	12.5 ml

made up to 50 ml with double distilled water, the pH being adjusted to 7.2 with 1 M acetic acid.

2.12.3 Preparation of reference and sample solutions

Lyophilised reference toxins (nodularin, microcysin-LR and microcystin-RR) were dissolved in 30% (v/v) methanol to give a concentration of 1 μg μl^{-1}, whereas okadaic acid was dissolved in dimethyl sulphoxide (DMSO) to give a 20 nM solution. The presumptive toxins from cyanobacterial biomass and "cell free" water concentrates, including the peak fractions collected from HPLC analysis, were prepared as described earlier. All reference and sample solutions were diluted as a series of 1:10 dilutions in assay buffer before use.

2.12.4 Preparation of phosphorylase substrate

Serine (^{32}P) phosphorylase *a* was prepared as described by Brautigan & Shriner (1988).

The procedures were performed at 0-4°C unless otherwise stated. Approximately 15-20 μCi of (γ-^{32}P) ATP was diluted with 3 μl of 0.1 M unlabelled ATP solution, pH 7.0. 1.0 M $MgCl_2$ (1.0 μl) was added as a cofactor in the phosphorylation reaction. 50 units of rabbit muscle phosphorylase kinase, dissolved in buffer A, were added. The resulting mixture was added to 30 mg of crystallised phosphorylase *b* from rabbit muscle and the solution was made up to a final volume of 0.45 ml with freshly prepared buffer A. The solution was adjusted to pH 8.2 (an acceptable pH for activity of the kinase) by addition of aliquots of 2 M Tris base (2 μl). The pH was checked after each addition by means of narrow range pH strips. The reaction was left at 30°C for 1 hour. After that, the reaction was diluted with freshly prepared buffer B to a final volume of 0.9 ml and the pH was adjusted again

to 6.8 by addition of aliquots of 1 M acetic acid (2 µl). The reaction was then placed on ice for 15 minutes (if the pH is correct the phosphorylase will crystallise within 10 minutes). After crystals appeared, the reaction was chilled for another 30 minutes on ice. The crystals were collected by centrifugation for 2 minutes in a clinical centrifuge and were subsequently redissolved to the same volume (0.9 ml) in buffer B containing 0.1 M NaCl at 30°C. Any remaining free nucleotides were removed by column chromatography. A 0.7 cm × 5 cm disposable plastic column was set up containing a 0.5 ml layer of Norit A activated charcoal sandwiched between two 1 ml layers of Sephadex G-25 fine gel beads (charcoal and beads were slurried in buffer B). The column was equilibrated with buffer B at 30°C. The collected protein solution was transferred to 6 mm diameter dialysis tubing and dialysed against buffer B overnight at 4°C. Since the crystalline phosphorylase *a* cannot be frozen the dialysis tubing containing the crystals was transferred to a 100 ml cylinder containing glycerol. The cylinder was tumbled end over end for 30 minutes to concentrate the protein solution and dissolve the phosphorylase crystals. The sample volume was considerably reduced thus allowing the protein solution to be subsequently diluted with buffer B to a known volume. A sample of this solution was taken and the protein content determined. The phosphorylase *a* solution was diluted to a final concentration of 3.0 mg ml^{-1} and stored at 4°C.

2.12.5 Preparation of crude mouse liver homogenate

Balb C mice served as liver donors. The livers were minced and homogenised with a Potter-Elvehjem tissue homogeniser in either 2.5 or 3 ml buffer [4.0 mM EDTA, 250 mM sucrose, 0.1% (v/v) β-mercaptoethanol, pH 7.0 (Ingebritsen *et al.*, 1983b)] g^{-1} wet weight. The homogenate was centrifuged for 10 minutes at 12,000 × g. The supernatant was decanted and used as the source of protein phosphatases. The protein concentration of extracts was determined and

these were diluted to a final concentration of 15 mg protein ml^{-1} in 50 mM imidazole, 5 mM EDTA, 0.5 mM dithiothreitol, pH 7.45 containing 1 mg ml^{-1} bovine serum albumin.

2.12.6 Phosphorylase phosphatase assay procedure

The assay was carried out as described by Killilea *et al.* (1978). Protein phosphatase activity was determined by the liberation of ^{32}P from ^{32}P-labelled proteins. (^{32}P) phosphorylase *a* substrate was diluted to 0.2 mg protein ml^{-1} in assay buffer. All assays were conducted in a final volume of 100 µl (80 µl of assay buffer, 10 µl of phosphorylase *a* substrate and 10 µl of liver homogenate), and incubated at 30°C for 20 minutes. The reactions were terminated by the addition of 20 µl of 50% (v/v) TCA, kept on ice for 5 minutes and then centrifuged. The supernatant (50 µl) was spotted onto a 1 inch square of Whatman 3MM chromatography paper. The paper was placed in a scintillation vial, dried under a heat lamp and the amount of radioactivity present determined by liquid scintillation counting. The amount of radioactivity released was used to determine the amount of phosphorylase *a* converted into phosphorylase *b*. Two control tubes were prepared: one lacking enzyme to determine the basal level radiation due to background and/or non-specific dissociation of phosphate groups from the protein, and one in which water was added rather than TCA, to determine the total counts in the assay. One unit of activity (U) was that amount of enzyme which catalysed the dephosphorylation of 1.0 nmol of phosphorylase *a* min^{-1}. Inhibition of phosphatase activity by purified cyanotoxins, okadaic acid or environmental samples was determined by adding the indicated compound to the enzyme mixture and preincubating for 5 minutes prior to initiating the reaction with substrate.

2.12.7 Determination of protein concentration

The protein concentration was determined by the Lowry protein assay (Lowry *et al.*, 1951) using BSA as the standard.

2.12.8 Scintillation counting

The amount of ^{32}P released from ^{32}P-labelled phosphorylase *a* was determined by liquid scintillation counting. 4 ml of Optiphase Safe scintillation fluid was added to each sample vial and the cpm determined using an LKB 1216 RACKBETA liquid scintillation counter.

2.13 ENZYME ASSAY

2.13.1 Preparation of samples

Crude mouse liver homogenate, prepared as describes in section 2.12.5 and adjusted to a final concentration of 15 mg protein ml^{-1}, were used as the source of enzymes. In order to study the effect of hepatotoxin on the enzymes in liver homogenate, a portion of crude mouse liver homogenate was mixed thoroughly with the same volume of either distilled water (non-toxic sample) or a serial dilution of reference toxin (toxic samples) and left at room temperature for 15 minutes before use in the enzyme assays.

2.13.2 Preparation of solutions

The composition and procedures for the preparation of solutions referred to in the text are described below:

AMP pH 7.4, 0.05 M

0.05 M AMP solution was prepared by dissolving 1.736 g of AMP, sodium salt, in 100 ml of Tris-HCl buffer pH 7.4.

Disodium tartrate, 0.2 M

0.2 M disodium tartrate solution was prepared by dissolving 4.602 g of tartrate, crystallised disodium salt, in 100 ml of double distilled water.

H_2SO_4, 5N

5 N H_2SO_4 was prepared by dilution of concentrated H_2SO_4 (36 N) with double distilled water.

KCl, 2 M

2 M KCl solution was prepared by dissolving 14.912 g of KCl in 100 ml of double distilled water.

Molybdate-H_2SO_4 solution

Molybdate·H_2SO_4 solution was prepared by dissolving 2.5 g of ammonium molybdate with 100 ml of 5 N H_2SO_4.

Phosphorus standard solution

A standard solution for the determination of phosphate was made up by dissolving 1.3613 g of analytically pure KH_2PO_4 in 1000 ml of double distilled water. A few drops of chloroform were added as preservative and the solution was stored at 4°C. For use, this solution was diluted 10 times with double distilled water i.e. 1 ml contained 1 µmole of phosphorus.

Reducing agent

Reducing agent was prepared by dissolving 0.25 g of Fiske & SubbaRow reducer in 10 ml of double distilled water. This solution was not used after more than a week.

Tris-HCl buffer pH 7.4, 0.4 M

0.4 M Tris-HCl buffer was prepared by dissolving 15.76 g of Tris (hydroxymethyl) methylammoniumchloride in 250 ml of double distilled water. The pH was then adjusted to 7.4 with 2 M Tris base.

Tris/NaCl solution

This solution was made up by dissolving 4.92 g of Tris base and 5.95 g of NaCl in 400 ml of double distilled water. The pH was adjusted to 7.2 at 30°C with 5 M HCl and the volume made up to 500 ml. This solution was stored at 0-4°C.

Tris/NaCl/NADH solution

This solution was prepared by dissolving 0.0173 g of NADH, disodium salt, in 100 ml of Tris/NaCl solution. The solution was frozen and stored for up to 6 months.

Tris/NaCl/puruvate solution

This solution was prepared by dissolving 0.107 g of pyruvate, crystallised monosodium salt, in 100 ml of Tris/NaCl solution. This solution is stable at 4°C or -20°C for not more than 2 months.

2.13.3 Lactate dehydrogenase

Lactate dehydrogenase (LDH) was assayed according to the method of Vassault (1984). A 0.1 ml aliquot of sample (see section 2.13.1) was taken, mixed thoroughly with 2.0 ml of Tris/NaCl/NADH solution in a cuvette and allowed to equilibrate at 30°C for 1 minute before the reaction was commenced by the addition of 0.5 ml Tris/NaCl/pyruvate solution. After mixing, the absorbance was read at 340 nm at 60 second intervals for 5-10 minutes using a Hewlett Packard 8452A diode array spectrophotometer. The value $\Delta A / \Delta t$ should be < 0.16 for 60 second otherwise the crude mouse liver homogenate must be diluted in assay buffer to optimise the concentration. LDH activity was expressed as the rate of pyruvate consumed μmole per minute per ml sample using the following formula:

$$\text{Enzyme activity (U)} = \frac{2.6 \times \Delta A \times \text{Dilution factor}}{0.63}$$

2.13.4 5'-Nucleotidase

2.13.4.1 Assay procedure

5'-nucleotidase (5'-NT) was assayed according to the method of Pletsch & Coffey (1972). A 0.1 ml aliquot of sample (see section 2.13.1) was added into 1 ml of substrate mixture (Table 2.3). The reaction was allowed to proceed at 37°C for 1 hour, and then stopped by the addition of 1 ml of 15% (w/v) TCA. Precipitated protein was removed by centrifugation and 1 ml of the cleared supernatant was used for phosphate determination.

2.13.4.2 Calibration curve

A standard phosphorus solution (1 mM) and/or double distilled water were pipetted into labelled tubes as indicated in Table 2.4. 0.5 ml of the molybdate·H_2SO_4 solution and 0.2 ml of the reducing agent were subsequently added. The solution was then made up to 5 ml with double distilled water, mixed and the color allowed to develop for 20 minutes. Absorbance was read at 660 nm. A phosphorus calibration curve was prepared by plotting absorbance values vs the corresponding phosphorus values.

TABLE 2.3 Composition of the substrate mixture for 5'-nucleotidase assay

0.05 M AMP, pH 7.4	20 ml
2 M KCl	10 ml
0.2 M disodium tartrate	10 ml
0.1 M $MgCl_2$	20 ml
0.4 M Tris-HCl, pH 7.4	25 ml
distilled water	15 ml

TABLE 2.4 Calibration phosphorus solution preparation

Tube no.	Phosphorus standard solution (ml)	Distilled water (ml)	Phosphorus (μmole)
1	0	1.0	0
2	0.1	0.9	0.1
3	0.2	0.8	0.2
4	0.4	0.6	0.4
5	0.6	0.4	0.6
6	0.8	0.2	0.8
7	1.0	0	1.0

2.13.4.3 Phosphate determination

The inorganic phosphate (P_i) in the supernatant was measured by a method adapted from that of Fiske & SubbaRow (1925). 0.5 ml of the molybdate·H_2SO_4 solution was added into a sample tube containing 1 ml of cleared supernatant, followed by the addition of 0.2 ml of the reducing agent for color development. The solution was then made up to a final volume of 5 ml with double distilled water, mixed thoroughly and the color allowed to develop for 20 minutes. Absorbance was read at 660 nm against a blank prepared using 1 ml of double distilled water instead of sample. The concentration of P_i in µmoles was estimated from the calibration curve. Enzyme activity (U) was expressed in µmoles of the hydrolised substrate consumed per hour using the following formula:

$$\text{Enzyme activity (U)} = \frac{1.1 \times (b-a) \times \text{dilution factor}}{0.1}$$

a and b represented µmoles of P_i in the blank and each sample, respectively.

2.13.5 Alkaline and acid phosphatases

The activities of alkaline and acid phosphatases, in crude mouse liver homogenate incubated with and without hepatotoxins, were both assayed using commercial kits (Sigma Chemical Co. Ltd.). The procedures depended upon the hydrolysis of p-nitrophenol and inorganic phosphate. When the solution becomes alkaline, p-nitrophenol is converted to a yellow complex readily measured colorimetrically at 400-420 nm. The intensity of color formed is proportional to phosphatase activity.

2.13.6 γ-glutamyltransferase

The activity of γ-glutamyltransferase (GGTP) in the samples was measured colorimetrically using a commercial kit (Sigma Chemical Co. Ltd.). The assay is based on the transfer of the glutamyl group from γ-L-glutamyl-p-nitroanilide to glycylglycine catalysed by GGTP. The liberated p-nitroanilide is diazotised. The absorbance of the pink azo-dye product measured at 530-550 nm is proportional to GGTP activity.

2.14 FAST ATOM BOMBARDMENT MASS SPECTROMETRY

2.14.1 Purified cyclic peptide toxins

Purified toxins were analysed by fast atom bombardment (FAB) mass spectrometry using a Kratos MS 50 (Kratos Analytical) for molecular weight determinations and a Kratos CONCEPT II HH four sector instrument (Kratos Analytical) to characterise, identify and fingerprint toxins.

2.14.2 Hydrolysis of the cyclic peptide toxins with TFA

The cyclic peptide toxins were hydrolysed with TFA by the method of Krishnamurthy *et al.* (1989). An aliquot of each reference toxin (~ 100 μg) was taken and lyophilised. The lyophilised toxin was treated with 1 ml of TFA for derivatisation and allowed to stand overnight at room temperature. The solution was then lyophilised and the residue was redissolved in 100 μl of methanol.

2.14.3 Characterisation of the peptide toxins by static FAB mass spectrometry

A Kratos analytical MS-50 (double focussing forward geometry) was used for this study. The mass spectrometer was calibrated with triazine. After this, the probe was cleaned with water, methanol and then dried. A portion of the peptide solution (5 μl) was introduced to the probe tip and the solvent was evaporated using a hairdryer, followed by adding glycerol (2-3 μl), as the matrix, to the solid residue on the probe tip. Then, the solid residue and glycerol were mixed together using the glass capillary tube so as to get a homogeneous film.

2.14.4 Characterisation of the peptide toxins by off-line HPLC continuous flow fast atom bombardment mass spectrometry

The Kratos analytical MS-50 linked to an HPLC comprising of Brownlee Micrograd ient & Microflow pump system unit and reversed phase C_{18} microbore column (0.25 x 100 mm) was used for continuous flow fast atom bombardment (CF-FAB).

For the off-line CF-FAB, the injector head had to move from the pre-column to the post-column position. Methanol containing 5% (v/v) glycerol was used as the mobile phase and the flow rate was set at 7 μl min^{-1} and run through the column for 15 minutes for equilibration. The source heater was turned up to 70°C and the cold finger filled with dry ice/acetone. The capillary was then aligned to the frit face and checked for the distribution of liquid on the probe. The probe was inserted into the source and the extra ballast pump was opened to the system. The system was left for 15 minutes in order that the probe reached the correct temperature and for the pressure to stabilise.

2.14.5 Characterisation of the peptide toxins by a Kratos CONCEPT II HH four sector mass spectrometer

The peptides were prepared by adding 10 μl of thioglycerol to 100 μg of peptide. 1 μl of the resulting mixture was then ionised by FAB by Xe atoms having a kinetic energy of 8 kV and with the four sector mass spectrometer operating at 8 kV accelerating voltage.

2.15 MATRIX ASSISTED LASER DESORPTION IONISATION (MALDI) MASS SPECTROMETRY

The relative molecular masses of reference standards and samples were determined using a Kratos Analytical Kompact MALDI mass spectrometer (Kratos Analytical). Concentrations (pmole) of reference toxins and samples which had been rotary evaporated to dryness were dissolved in a few millilitres of methanol and then mixed with the matrix, sinapinic acid, before being spotted onto a sample slide and evaporated to dryness.

CHAPTER 3

CHAPTER 3 : APPLICATION OF HPLC TO THE DETECTION AND IDENTIFICATION OF PEPTIDE TOXINS

3.1 INTRODUCTION

3.1.1 Analytical procedures

Assessment of toxicity is generally provided by mouse bioassay (Elleman *et al.*, 1978), where sample is injected intraperitoneally into mice. Toxicity is designated as the dose that will cause either 50% or 100% mortality, (LD_{50}) and (LD_{100}) respectively. Although, the mouse bioassay is used as the primary assay for toxicity, it does not provide good quantitative results nor give confidence that it is only the compound of interest that is being detected or is effective. It is therefore necessary to pursue appropriate quantitative analytical methods in order to determine toxin concentrations more accurately. Furthermore, it is desirable to minimise the use of laboratory animals. Any analytical method must be simple, rapid and reliable, requiring the minimum of sample preparation so minimising analytical error. As toxins need to be separated from a matrix of other organic compounds extracted from cyanobacterial cells, chromatography is currently the most suitable technique.

Three physical/chemical approaches have previously been used for the detection of hepatotoxins: thin-layer chromatography (TLC) (Harada *et al.*, 1988a, 1991), high-performance thin-layer chromatography (HPTLC) (Poon *et al.*, 1987; Codd *et al.*, 1989), and high performance liquid chromatography (HPLC) (Brooks & Codd, 1986; Krishnamurthy *et al.*, 1986; Berg *et al.*, 1987). However, HPLC is the

most accurate and has been used extensively. Before analysis, the toxin(s) is extracted from the cells and/or free toxin recovered from raw water. Purification procedures have been commonly carried out using gel filtration and/or C_{18} columns followed by reversed-phase liquid chromatography (Harada *et al.*, 1988b; Kungsuwan *et al.*, 1988; Watanabe *et al.*, 1989a; Martin *et al.*, 1990).

3.1.2 Reversed-phase liquid chromatography

Reversed-phase chromatography (RP) is the most versatile and most widely used HPLC mode. The stationary phase is silica chemically bonded with an alkylsilyl compound to give a non-polar, hydrophobic surface. Solute retention is mainly due to hydrophobic interactions between the solutes and the hydrocarbonaceous stationary phase surface. Polar mobile phases are used for elution. Solutes are eluted in order of decreasing polarity (increasing hydrophobicity); increasing the polar (aqueous) component of the mobile phase increases the retention of the solutes.

RP was found to be particularly suited to the study of peptide toxins because of their slightly polar and acidic nature. Many studies have reported success using this technique to separate and quantify microcystins in an extract matrix (Siegelmann *et al.*, 1984; Dierstein *et al.*, 1988; Harada *et al.*, 1988b; Luukkainen *et al.*, 1993). Separation has been performed on 3 μm and 5 μm diameter columns in which octadecylsilyl (ODS) groups are bonded to the silica surface; the lengths of columns have also been variable. The most common elution mobile phase is a gradient of methanol (Harada *et al.*, 1988b; Watanabe *et al.*, 1989a; Harada *et al.*, 1990) and acetonitrile (Eriksson *et al.*, 1988b; Meriluoto *et al.*, 1989; Dierstein *et al.*, 1990; Kiviranta *et al.*, 1992) in water containing the appropriate reagents such as trifluoroacetic acid (TFA), acetic acid or phosphoric acid as the modifiers/additives. Mobile phase mixtures lacking modifiers/additives results in broad peaks and long,

unpredictable retention times, whereas the addition of modifiers/additives gives a marked improvement in both reproducibility and retention times of peptides. Examples of the HPLC conditions reported are given in Table 3.1.

In the traditional application of HPLC, three main types of detectors are currently in use: (i) selective property detectors such as the UV photometer; (ii) bulk property detectors such as the refractive index monitor; and (iii) solute/eluent separation systems such as the wire transport-FID detector. However, UV detection is the most extensively used detection system amongst these due to the low sensitivity of the refractive index monitor. There has been little application of FID detectors for peptides which is presumably because of the difficulties of detecting polar solutes in the presence of a large excess of an ionic modifier. UV detection at 238 or 240 nm has been used almost exclusively for the detection of microcystins isolated from cyanobacterial cells. The maximum absorption at this wavelength is reliant on the conjugated double bond system in the Adda side chain (Meriluoto *et al.*, 1990a). Nevertheless, the absorption at this wavelength is not specific for the microcystins (Martin *et al.*, 1990).

Since peptides containing arginine (and hence possessing a guanidino side-chain) form fluorescent derivatives, fluorescence detection (following either pre- or post-column derivatisation) was investigated using ninhydrin (Hiraga & Kinoshita, 1981; Rhodes & Boppana, 1988; Boppana & Rhodes, 1990) as a derivatising agent in a post-column mode, and benzoin (Kai *et al.*, 1983; Kai *et al.*, 1985; Ohno *et al.*, 1987) in both pre- and post-column modes. However, UV detection still provided to have the higher sensitivity.

TABLE 3.1 HPLC conditions used for the analysis of microcystins in cyanobacterial extracts (UV detection)

Column	Eluent	Reference
Nucleosil 5 C_{18}	MeOH/0.05% TFA	Harada *et al.*, 1988a
Nucleosil 5 C_{18}	MeOH/0.05 M phosphate buffer (pH 3)	Watanabe *et al.*, 1988
Nucleosil 7 C_{18}	ACN/0.0135 M ammonium acetate	Meriluoto *et al.*, 1989
Nucleosil 5 C_{18}	MeOH/0.05 M phosphate buffer (pH 3)	
Cosmosil 5 C_{18}	MeOH/0.05 M sodium sulphate	Harada *et al.*, 1990
	MeOH/0.05% TFA	
Hypersil 5 ODS	ACN/10 mM ammonium acetate	Brooks & Codd, 1986
μ Bondapak C_{18}	ACN/10 mM ammonium acetate	
μ Bondapak C_{18}	MeOH/10 mM phosphate buffer (pH 6.8)	Namikoshi *et al.*, 1992a
Alltech C_{18}	ACN/20 mM ammonium acetate (pH 5)	
Regis - ISRP	ACN/0.1 M phosphate buffer (pH 6.8)	
μ Bondapak C_{18}	ACN/10 mM ammonium acetate	Luukkainen *et al.*, 1993
Partisil-5	MeOH in AcOH/Chloroform in AcOH	Birk *et al.*, 1988
Novapak 5 C_{18}	MeOH/acetate buffer	Gathercole & Thiel, 1987
Alltech C_{18}	MeOH/acetate buffer	Zhang *et al.*, 1991

Other techniques have also been evaluated such as internal-surface reversed-phase (ISRP) columns to overcome the problem of protein denaturation (Meriluoto *et al.*, 1990a, b). Fast protein liquid chromatography (FPLC) systems have also been used to separate structurally similar microcystins (Cremer & Henning, 1991).

3.1.3 Aims

The main aim was to detect and quantify "presumptive" peptide hepatotoxins produced by cyanobacteria in the natural environment by reversed-phase liquid chromatography.

The HPLC conditions were modified to achieve optimum resolution and sensitivity for the characterisation of purified microcystins and nodularin, as well as the related peptide toxins from environmental cyanobacterial biomass. The performance of the method was assessed by adding known quantities of reference toxins and carrying out the standard analytical procedures.

The temporal distribution of the peptide toxins from environmental biomass was used to investigate which environmental factors affected cyanotoxin expression.

Mouse bioassay was used as the primary assay for toxicity assessment.

3.2 Materials and methods

The basic materials and methods utilised in this study have been given in section 2.9.

3.3 EQUIPMENT

3.3.1 HPLC instrumentation and columns

A schematic diagram of the HPLC system used with UV detection is illustrated in Figure 3.1.

The following reversed phase HPLC systems were used in this study:-

(a) Varian LC Star system :

This is a powerful and highly versatile liquid chromatography system which consists of :-

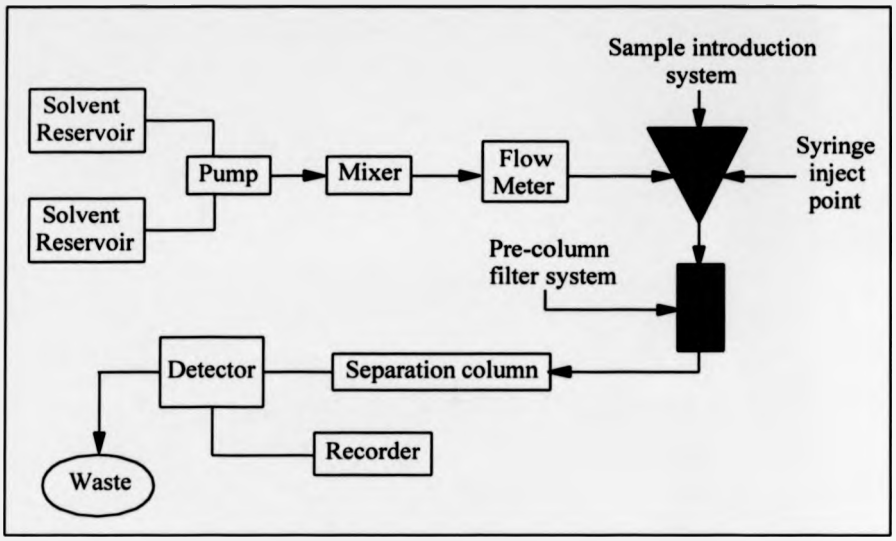
- Star 9010 solvent delivery system
  (a reciprocating single piston liquid chromatographic pump)
- Star 9050 variable wavelength UV-VIS detector
  (variable wavelength absorbance detector)
- Polychrom 9065 diode array detector
- Star 9095 autosampler
- Star PolyView™ spectral processing application software

(b) Pharmacia LKB system :

This model consists of the following components :-

- HPLC gradient pump 2249 / HPLC pump 2248
- Rapid spectral detector (RSD)
- Variable wavelength monitor (VWM)
- HeliFrac fraction collector, equipped with a flow diverter and drop counter
- Manual injection, with a position sensing switch (input of start signal)

FIGURE 3.1 Schematic diagram of HPLC system.



3.3.2 Mobile phases

The optimised mobile phase, a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA, was run at a flow rate of 1 ml min^{-1} within 30 minutes. The solvents were filtered through a 0.45 μm filter and degassed (exclusively for the Pharmacia LKB system) concurrently before use.

3.4 Results

3.4.1 Mobile phase

In this study acetonitrile in water was used as the mobile phase. The initial experiments were run in a linear gradient of acetonitrile/water (solvent A : 10% acetonitrile containing 0.05% TFA in water, v/v; solvent B : 90% acetonitrile containing 0.05% TFA in water, v/v), from 100% A to 100% B within 30 minutes, at a flow rate of 1 ml min^{-1}. Since the HPLC fractions were required for further study, it was an advantage to use a volatile solvent system. TFA was added to the mobile phase as an ion pair reagent to enhance retention. Acetonitrile was used without phosphate buffer since the latter would leave non-volatile salts after evaporation.

3.4.2 Optimum conditions

Separation of peptide toxins was achieved by using a linear gradient of increasing concentration of organic solvent. The acetonitrile concentration (the starting and final concentrations of mobile phase A and B) was then adjusted, this step resulted in the alteration of polarity of the mobile phase so giving more rapid and efficient separation. Preliminary studies were performed to achieve maximum resolution of a mixture of three reference peptide toxins; microcystin-LR, microcystin-RR and nodularin. A linear gradient of 30% acetonitrile containing 0.05% TFA in water, v/v (mobile phase A) to 60% acetonitrile containing 0.05% TFA in water, v/v (mobile phase B) within 30 minutes at a flow rate of 1 ml min^{-1} proved to be the most suitable mobile phase for good resolution. Figure 3.2 shows the typical HPLC elution profile of the mixture of reference toxins; nodularin, microcystin-LR and microcystin-RR [300 ng of each toxin dissolved in 30% (v/v) methanol]. These were clearly resolved when detected by UV using the Pharmacia

system and had retention times (tRs) of 24.347, 27.693 and 28.480 minutes, respectively (Figure 3.2.a). Photo-diode array detection - Varian LC Star system - gave tRs of 23.344, 26.490 and 31.044 minutes, respectively (Figure 3.2.b). Shorter retention times could be obtained by increasing the flow rate, however, this was accompanied by a loss of resolution.

FIGURE 3.2.a High performance liquid chromatogram of a mixture of reference toxins using UV detection. The amount of toxin standards nodularin, microcystin-LR and microcystin-RR, injected was 300 ng of each. Column: SuperPac™ Pep-S (5 µm, 4.0 x 250 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: 238 nm.

(tR 24.347 minutes is nodularin peak, tR 27.693 minutes is microcystin-LR peak and tR 28.480 minutes is microcystin-RR peak)

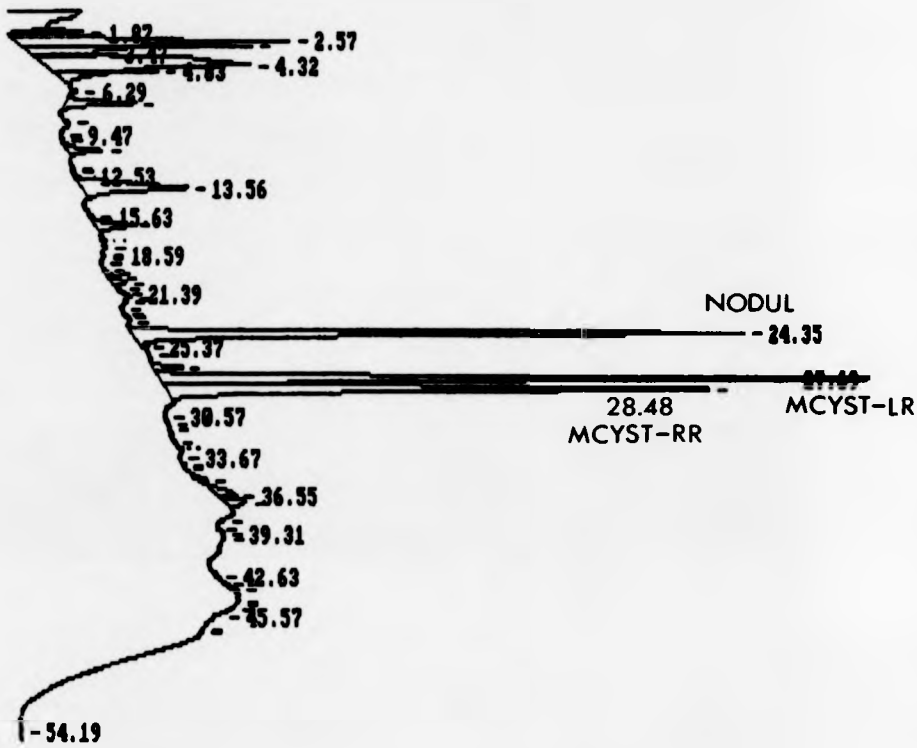
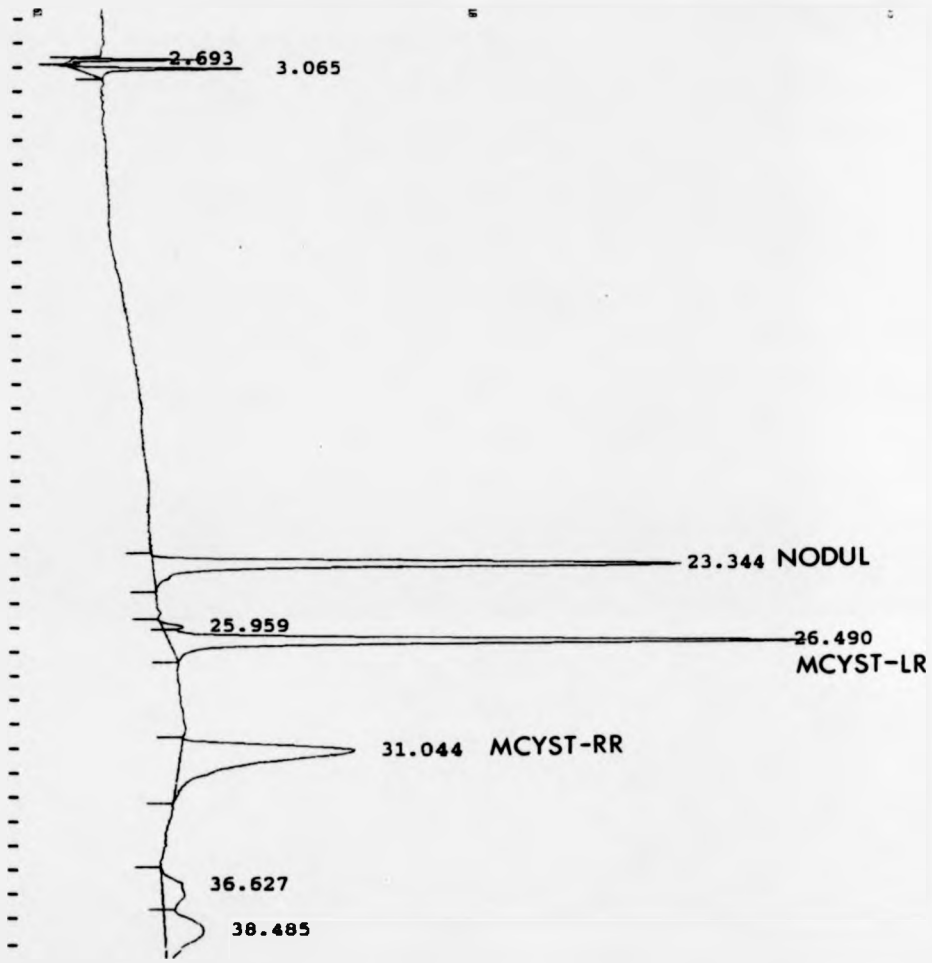


FIGURE 3.2.b High performance liquid chromatogram of a mixture of reference toxins using photo-diode array detection (attenuation = 23). The amount of toxin standards nodularin, microcystin-LR and microcystin-RR, injected was 300 ng of each. Column: Nova-Pak C_{18} (4 µm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: 238 nm.

(tR 23.344 minutes is nodularin peak, tR 26.490 minutes is microcystin-LR peak and tR 31.044 minutes is microcystin-RR peak)



3.4.3 Sensitivity

Using the derived optimum conditions, a linear relationships between peak area and amount of toxin injected was obtained. Typical sets of data obtained from reference toxins using UV detection and photo-diode array detection are shown in Tables 3.2 and 3.3, respectively. Likewise, the data were plotted giving calibration curves for the three reference toxins (Figure 3.3 and 3.4). The limit of detection by the HPLC procedure was established as 20 ng when using UV detection (Figure 3.3). Under the same conditions, photo-diode array detection had a detection limit of 50 ng for microcystin-LR and 100 ng for both microcystin-RR and nodularin (Figure 3.4).

However, further refinement of the elution conditions by increasing the concentration of acetonitrile in mobile phase B to 70% and decreasing the flow rate increased the sensitivity. The HPLC elution profile of the reference mixtures showed good resolution between microcystin-LR and nodularin when using an acetonitrile gradient of 30 to 70% containing 0.05% TFA with a flow rate of 0.6 ml min^{-1}. Table 3.4 shows the data obtained for microcystin-LR and nodularin using the optimised conditions with the calibration curve being shown in Figure 3.5. These optimised conditions extended the detection limit down to 30 ng for both microcystin-LR and nodularin, unfortunately, microcystin-RR could not be reliably resolved under these conditions.

A linear gradient of 30 to 60% ACN containing 0.05% TFA within 30 minutes at a flow rate of 1 ml min^{-1} was used as the optimised conditions for the detection of peptide hepatotoxins.

Although the UV detection system was shown to be more sensitive, photo-diode array detection presents the ability to (i) perform spectral analysis of the eluted material which enables comparison of unknowns with known reference toxins giving an enhanced probability of identification of peptide toxins other than by retention

times, and (ii) assessment of the purity of eluate peaks. For these reasons, photo-diode array detection was used for the separation and characterisation of "presumptive" peptide toxins from environmental samples. However, UV detection was still used in cases where the samples contained very low level of toxins and therefore required higher sensitivity for detection, and also in cases where large volumes of sample were injected for collecting of toxic-fractions.

TABLE 3.2 Peak area response of standard microcystin-LR, microcystin-RR and nodularin resolved by UV detection. Each datum was the average of 2-3 injections

Amount injected (ng)	Peak area mcyst-LR	mcyst-RR	nodularin
20	13,862	9,321	15,935
40	23,630	25,986	42,390
60	35,392	45,067	71,644
80	45,537	55,902	83,354
100	56,901	79,187	113,627

TABLE 3.3 Peak area response of standard microcystin-LR, microcystin-RR and nodularin resolved by photo-diode array detection. Each datum was the average of 2-3 injections

Amount injected (ng)	Peak area mcyst-LR	mcyst-RR	nodularin
50	8,965	-	-
100	14,800	10,395	14,989
250	47,945	34,848	38,969
400	71,552	-	-
500	89,133	65,998	77,797
1000	189,128	154,425	160,668

FIGURE 3.3 Calibration curves for nodularin, microcystin-LR and microcystin-RR by HPLC using UV detection. Column: SuperPac™ Pep-S (5 μm, 4.0 x 250 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: 238 nm.

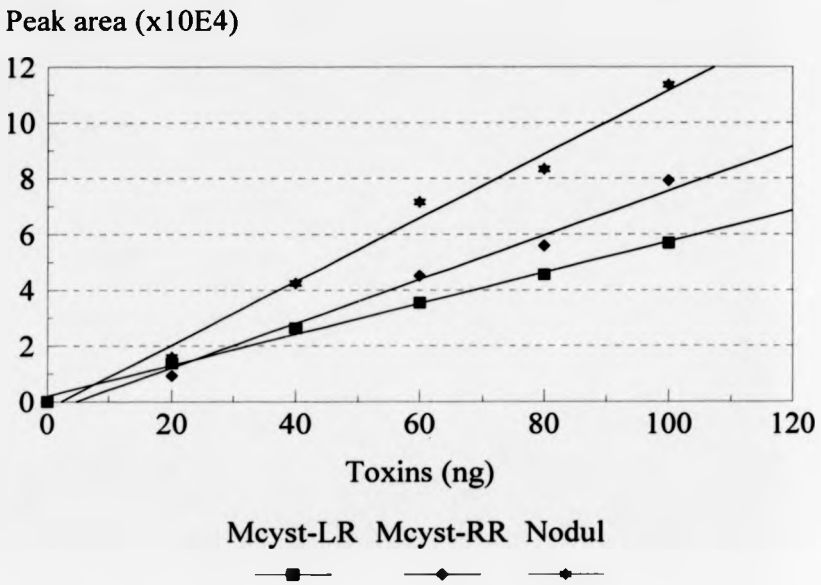


FIGURE 3.4 Calibration curves for nodularin, microcystin-LR and microcystin-RR by HPLC using photo-diode array detection. Column: Nova-Pak C_{18} (4 μm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: 238 nm.

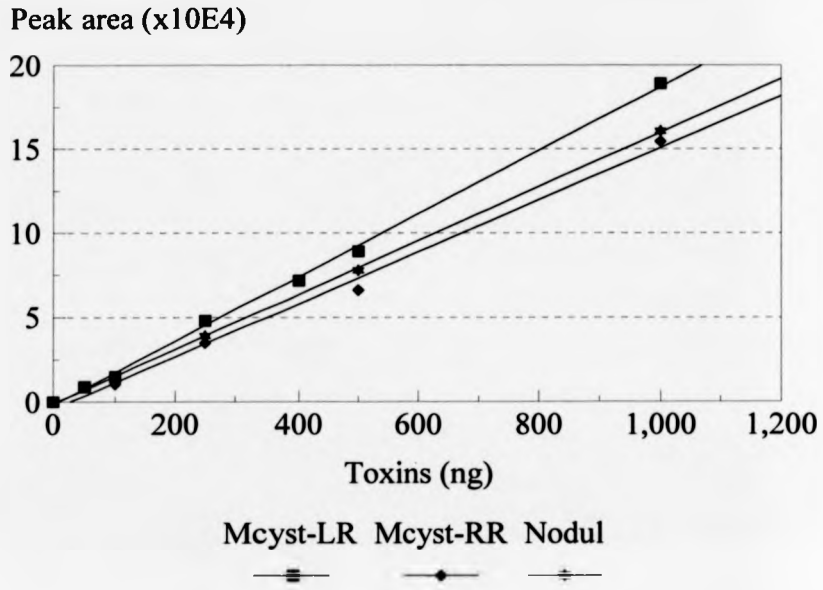
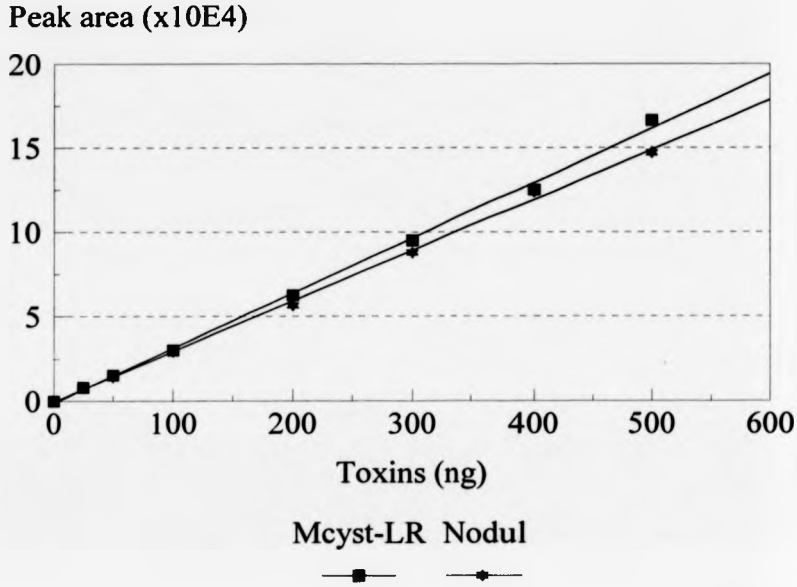


TABLE 3.4 Peak area response of standard microcystin-LR and nodularin resolved by photo-diode array detection (using the optimised condition). Each datum was the average of 2 injections

Amount injected (ng)	Peak area	
	mcyst-LR	nodularin
30	8,041	7,910
50	15,296	14,162
100	30,030	29,221
200	62,450	56,383
300	95,039	87,808
400	124,860	123,644
500	166,420	147,096

FIGURE 3.5 Calibration curves for nodularin and microcystin-LR by HPLC using photo-diode array detection. Column: Nova-Pak C_{18} (4 μm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 70% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 0.6 ml min^{-1}. Detection: 238 nm.



3.4.4 Peak identification

The spectral analysis facilities of the photo-diode array system were from 220-367 nm. Individual spectra of the three reference toxins were obtained and stored in a spectral data base. Figure 3.6 shows the spectral plot and the maximum absorbance of (a) nodularin, (b) microcystin-LR and (c) microcystin-RR. The maximum absorbances of all these three reference toxins were found between 234-239 nm. This provided valuable information for confirmation of peak identity. Spectral analysis was performed using the Varian software "PolyView" by comparison of the similarity/dissimilarity of spectral characteristics of each unknown spectrum from eluting peaks to those of the reference toxins. The spectral similarity indices provided during the library search, along with retention time information, were used to quantify the degree of confidence in identifying the unknown peak. The similarity/dissimilarity breakpoints and perceived match are given in Table 3.5. This software is also able to provide information on the purity of eluted peaks. Peak purity determinations are made by assessing the degree of difference in the UV spectra across the width of the peak. The Purity Parameter is plotted as a function of time and if the peak is free of co-eluting compounds the Purity Parameter will not change over the course of the peak and it will have a flat top e.g. as shown in Figure 3.7 (peaks number 2,3,6 and 7). However, if impurities are present, the top of the peak will slope, indicating that the Purity Parameter is not consistent across the width of the peak.

FIGURE 3.6.a Ultraviolet spectrum and the maximum absorbance of nodularin.

Absorbance Table

nm	mAU	nm	mAU	nm	mAU	nm	mAU	nm	mAU
190	19 087	229	18 577	268	0 320	306	0 041	344	0 131
195	23 409	234	19 152	273	0 175	311	0 066	348	0 135
200	19 379	239	17 841	278	0 093	316	0 066	353	0 152
205	16 934	244	13 824	283	0 068	321	0 074	358	0 156
210	15 709	249	9 028	287	0 047	325	0 102	362	0 152
215	15 342	254	3 569	292	0 045	330	0 118	367	0 148
220	15 718	259	1 205	297	0 049	335	0 124		
224	16 902	263	0 547	302	0 049	339	0 145		

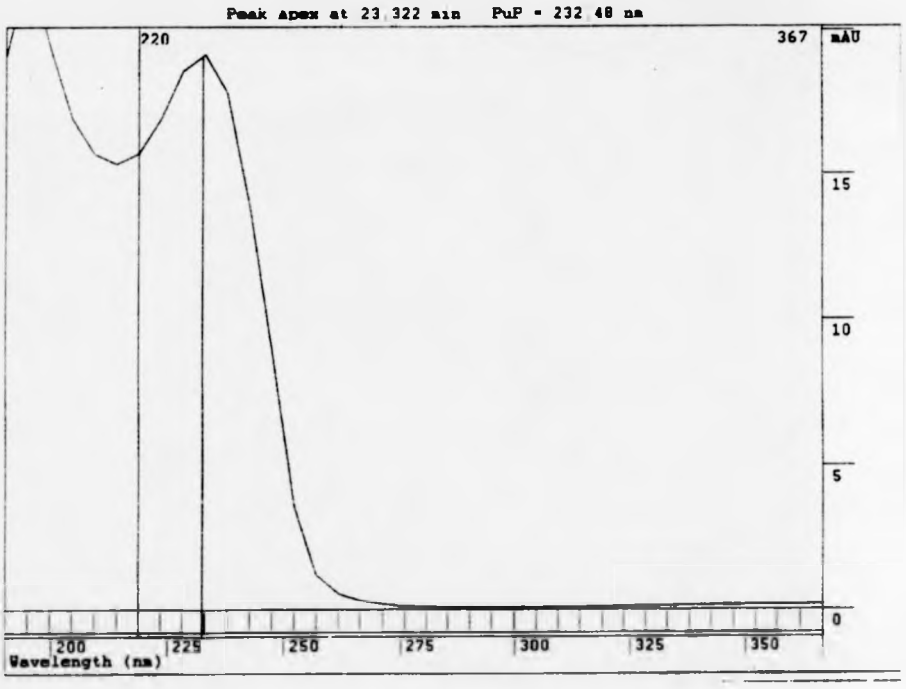


FIGURE 3.6.b Ultraviolet spectrum and the maximum absorbance of microcystin-LR.

Absorbance Table

nm	mAU	nm	mAU	nm	mAU	nm	mAU	nm	mAU
190	19.633	229	18.395	268	0.497	306	0.075	344	0.157
195	25.451	234	19.863	273	0.238	311	0.089	348	0.185
200	18.893	239	19.445	278	0.129	316	0.116	353	0.166
205	14.564	244	15.875	283	0.093	321	0.111	358	0.186
210	12.852	249	11.162	287	0.087	325	0.124	362	0.170
215	12.802	254	5.217	292	0.066	330	0.130	367	0.207
220	13.835	259	2.124	297	0.070	335	0.146		
224	15.833	263	0.998	302	0.072	339	0.137		

Channel Range: 220 to 367 nm Absorbance Range: 0.0657 to 19.863 mAU

Max Wavelength (nm): 235.56
Percent Max Abs. 100.4%

Within Peak at 26.517 min PuP = 234.16 nm

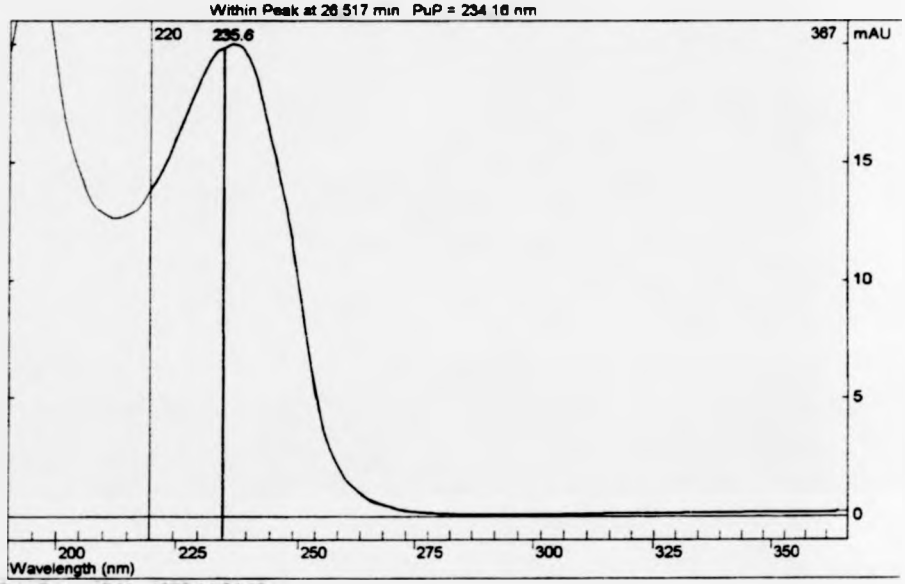


FIGURE 3.6.c Ultraviolet spectrum and the maximum absorbance of microcystin-RR.

Absorbance Table

nm	mAU	nm	mAU	nm	mAU	nm	mAU	nm	mAU
190	7 014	229	5 759	268	0 134	306	0 036	344	0 033
195	8 719	234	6 026	273	0 073	311	0 035	348	0 085
200	5 998	239	5 848	278	0 042	316	0 047	353	0 089
205	4 368	244	4 726	283	0 023	321	0 056	358	0 117
210	3 929	249	3 286	287	0 030	325	0 044	362	0 076
215	4 202	254	1 458	292	0 023	330	0 060	367	0 089
220	4 725	259	0 533	297	0 026	335	0 067		
224	5 171	263	0 244	302	0 016	339	0 068		

Peak Apex at 31 039 min PuP = 233 40 nm

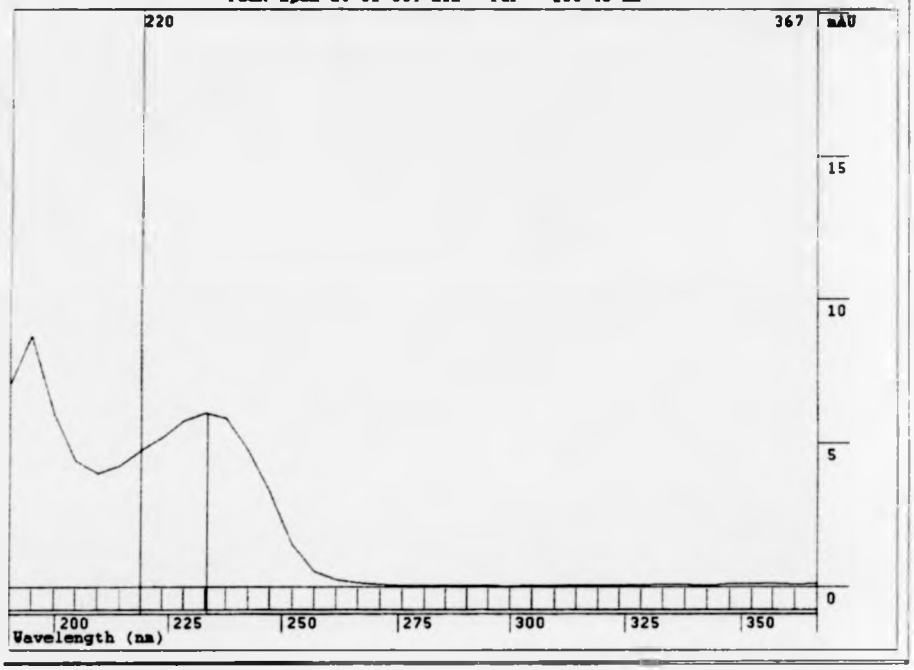
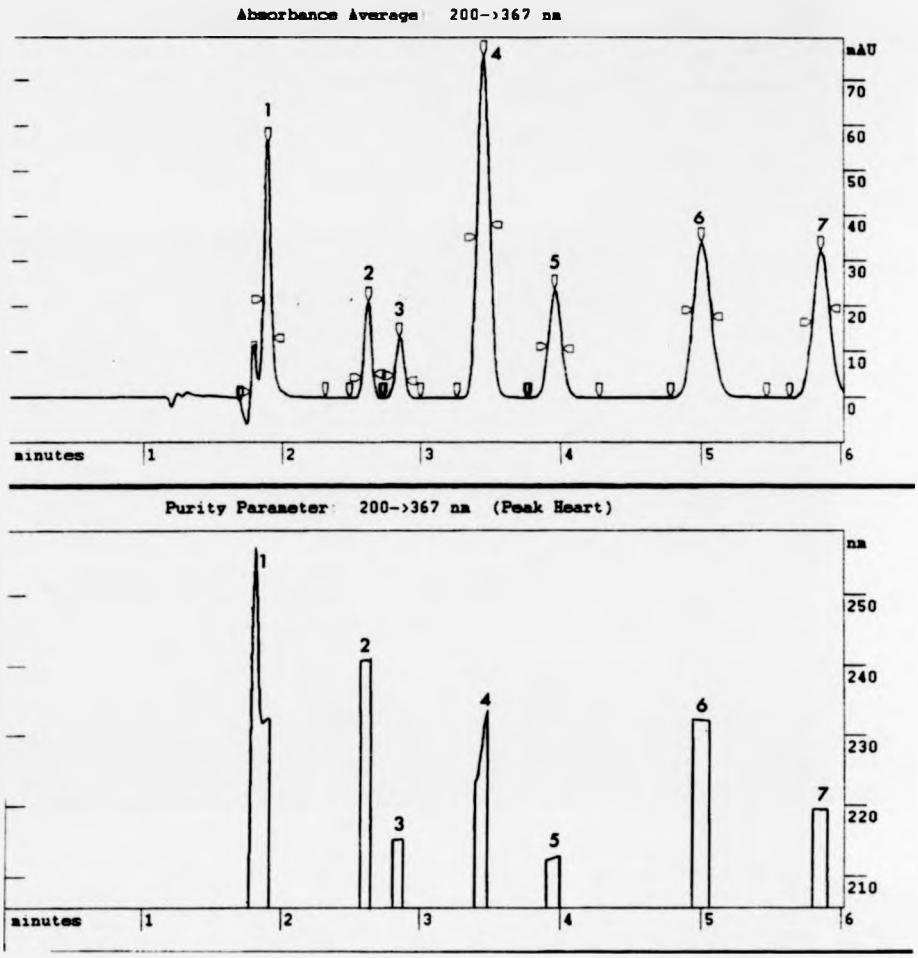


TABLE 3.5 Library matches in PolyView

Match quality	Similarity range	Dissimilarity range
Very good	1.0 - 0.998	0.0 - 0.06
Fair	0.998 - 0.980	0.06 - 0.20
Poor	< 0.980	0.20

FIGURE 3.7 Typical purity parameter plots of eluted peaks. Peaks number 2, 3, 6 and 7 are free of co-eluting compounds as the purity parameters do not change over the course of the peaks and they have flat tops.



3.4.5 HPLC analysis of *Microcystis aeruginosa* PCC 7806

Microcystis aeruginosa PCC 7806, a known hepato-peptide toxin producer, was used as the reference toxic cell biomass. Figure 3.8 shows (a) the HPLC elution profile of the biomass of an early stationary phase population, and (b) the spectral purity analysis of the eluted peaks. The HPLC analysis revealed the presence of two major pure toxic peaks, tRs 24.565 and 25.027 minutes, which had very good match of spectral similarity to that of microcystin-LR (similarity > 0.999). This indicated that these two toxic peaks might be microcystin-LR and/or microcystin-like derivatives. Another peak with tR 22.253 minutes had a relatively poor match (similarity 0.974), indicating that it was probably not a peptide toxin.

FIGURE 3.8.a High performance liquid chromatogram of *Microcystis aeruginosa* PCC 7806 cell biomass from the early stationary phase of growth (attenuation = 208). Column: Nova-Pak C_{18} (4 µm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

(tRs 24.565 and 25.027 minutes are the microcystin-like peaks)

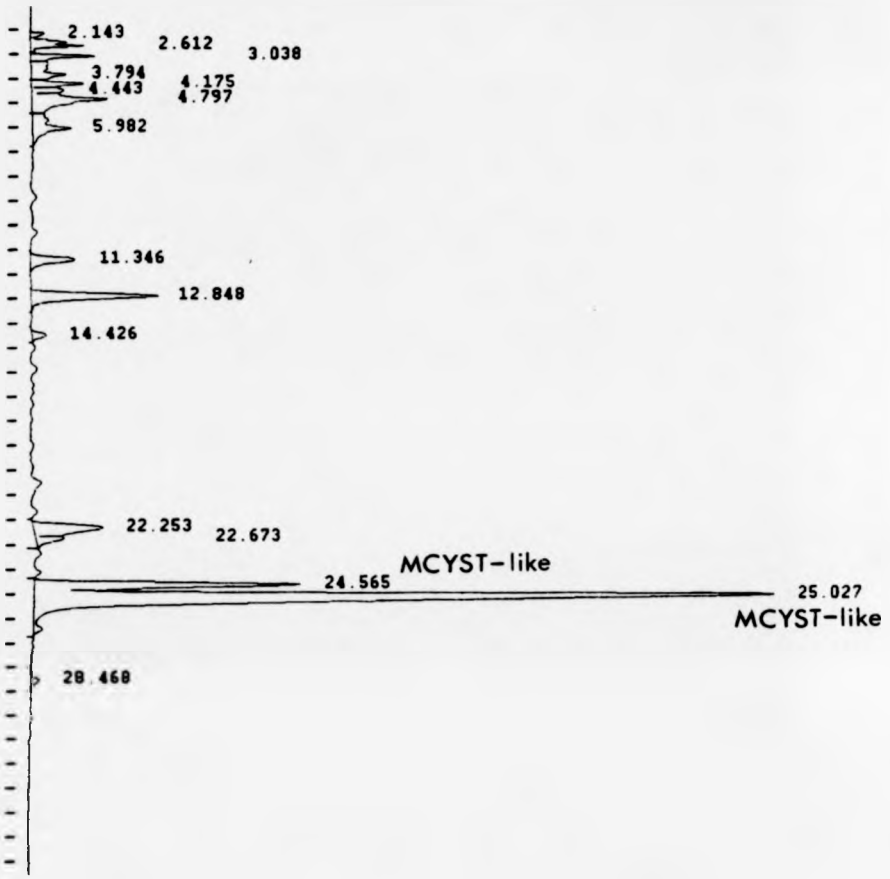
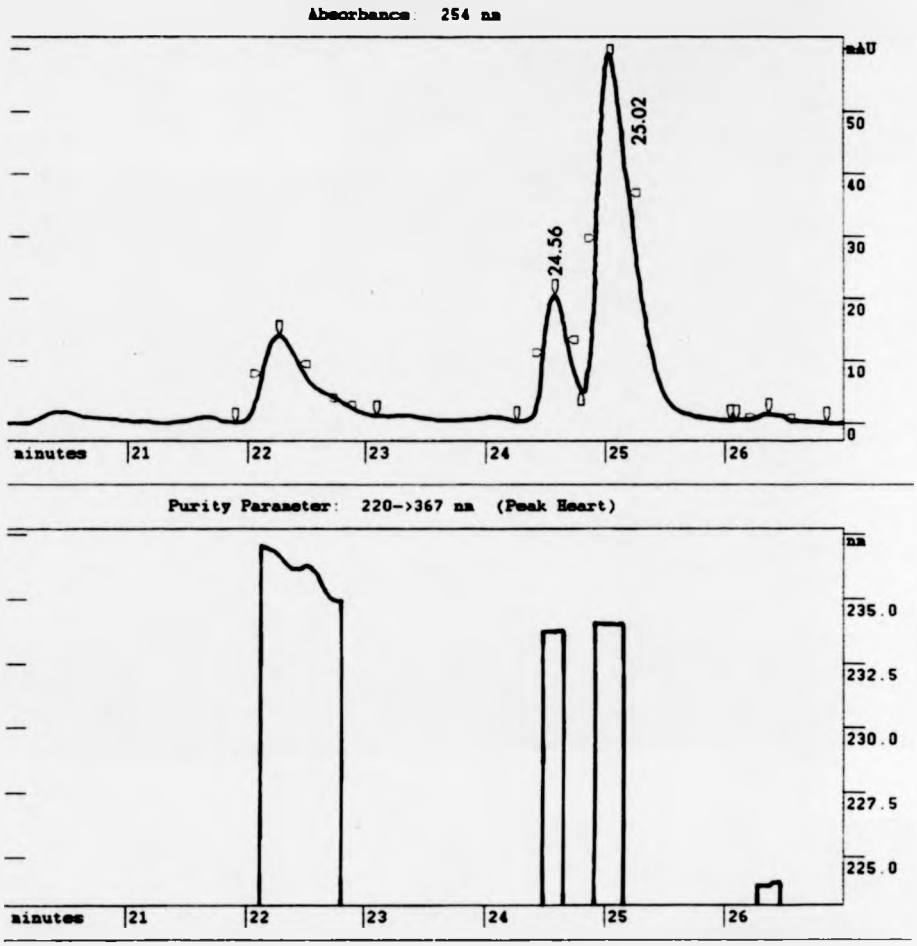


FIGURE 3.8.b Purity parameter plot of the eluted peaks of *Microcystis aeruginosa* PCC 7806 cell biomass from the early stationary phase of growth.
(tRs 24.565 and 25.027 minutes are microcystin-like peaks. they are pure compounds as assessed by spectral analysis)



3.4.6 Toxicity assessment by mouse bioassay

3.4.6.1 Symptoms of toxicity

Mouse bioassay was used as the primary means of detecting toxicity. Assay samples were introduced intraperitoneally (see section 2.11). The mice were observed frequently for toxic symptoms and the lethal doses were recorded. Table 3.6 shows the toxicity symptoms induced by the peptide toxins and how they differ from those of the alkaloid (neuro) toxins.

3.4.6.2 Toxicity of reference toxins

Toxicities were assayed by intraperitoneal injection into adult Balb C male and female mice. Table 3.7 summarises the toxicity breakpoint and lethal dose characteristics of the purified reference toxins; microcystin-LR, microcystin-RR and nodularin, and of the peptide toxins isolated from *Microcystis aeruginosa* PCC 7806. Lethal dose values show that administration of 3 μg, or greater, of microcystin-LR and nodularin caused death of the mice within 10 to 20 minutes. However, the toxicity of microcystin-RR was 10 times less than that of microcystin-LR as it was necessary to administer 30 μg of microcystin-RR to induce the same effects under the same conditions. Administration of > 1.1 mg (dry weight) of *Microcystis aeruginosa* PCC 7806 also caused rapid death of the mice and the toxic symptoms were characteristic of hepatotoxins. Autopsy of these mice showed engorged livers with internal bleeding, characteristic of hepatotoxins.

TABLE 3.6 Symptomatic responses to cyanobacterial toxins in mouse bioassays (Balb C mice)

Toxin	Signs of poisoning
Peptide hepatotoxin	- partial paralysis of hind limbs
	- fast heart beat / irregular pulse
	- eyes lack clarity
	- spiked fur - pilorection
	- arched spine
	- ears white
	- elevated (slightly) body temperature
	- heart beat slows - death
	- ataxia (loss of balance)
Alkaloid toxin	- marked drop in body temperature
	- diarrhoea
	- marked secretion from eyes

TABLE 3.7 Acute exposure mouse bioassays (Balb C mice) with purified reference toxins and the peptide toxins isolated from *Microcystis aeruginosa* PCC 7806

Toxin	Lethal dose	Toxicity
Microcystin-LR	3 μg	150 μg kg^{-1}
Microcystin-RR	30 μg	1500 μg kg^{-1}
Nodularin	3 μg	150 μg kg^{-1}
M. aeruginosa PCC 7806	1.1 mg	55 mg kg^{-1}

3.4.7 Toxin extraction and purification

Prior to analysis, it is necessary to extract the toxins from the cell biomass, to recover the "free toxin" from raw water, and to eliminate the contaminating materials from samples, as well as to prepare the sample in a form which is suitable for quantification and characterisation by HPLC.

3.4.7.1 Extraction procedure

In the extraction step, the focus has to be the efficiency of the procedure to recover the maximum quantity of toxin from the environmental sample. The lyophilised cell material must be weighed accurately and extracted using a known volume of solvent. Ultrasonic treatment using a sonicating water bath was used since it has proved to be an effective method for preparative extraction of toxic peptides from cyanobacteria (Eriksson *et al.*, 1988a, b) and it has major advantages over conventional extractions carried out by stirring e.g. it is a quick and practical method when dealing with small sample volumes (Meriluoto & Eriksson, 1988).
The extraction procedure used in this study was as follows :-

Lyophilised cells were weighed (100 mg or the available dry weight of sample) and extracted with 10 ml of 5% (v/v) aqueous acetic acid in a sonicating waterbath for 20 minutes. The samples were then stirred at room temperature for 30 minutes to ensure that the cells were completely disrupted. After that, the extracts were centrifuged at 4,750 x g for 15 minutes in a MSE Mistral bench centrifuge to remove cell debris and the supernatant was retained. The above extraction procedure was repeated two more times to recover any remaining toxins from the cell debris. The three supernatants were pooled.

3.4.7.2 Purification procedure

The extract was purified through a preconditioned octadecylsilanised (ODS) silica gel column (Harada *et al.*, 1988a; Kiviranta *et al.*, 1992) or a disposable C_{18} cartridge such as Bond Elute (Eriksson *et al.*, 1988b; Meriluoto *et al.*, 1989), Baker 10 (Watanabe *et al.*, 1988) or Sep-pak (Brooks & Codd, 1986; Dierstein *et al.*, 1988) depending on the original cell concentration. The extract was concentrated by eluting the toxins from the column/cartridge in a small volume of 100% methanol. This procedure was also used to concentrate the toxin from "cell free" raw water samples prior to HPLC analysis. Comparison of the three commercially available C_{18} cartridges (Bond Elute, Baker 10 and Sep-pak) has been undertaken by Harada *et al.* (1988b). All three C_{18} cartridges were successfully used to purify presumptive toxins.

In this study, the extraction of toxins was achieved by passage through a preconditioned Sep-pak C_{18} cartridge, washed with water and 10% (v/v) methanol, and finally eluted in 100% methanol (see section 2.7). The water and 10% (v/v) methanol washes did not contain any toxic peaks when analysed by HPLC (Figure 3.9.a and b, respectively).

FIGURE 3.9.a High performance liquid chromatogram of the water wash from the purification step of peptide toxin extraction (attenuation = 10). Column: Nova-Pak C_{18} (4 μm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

No toxic eluates were detected.

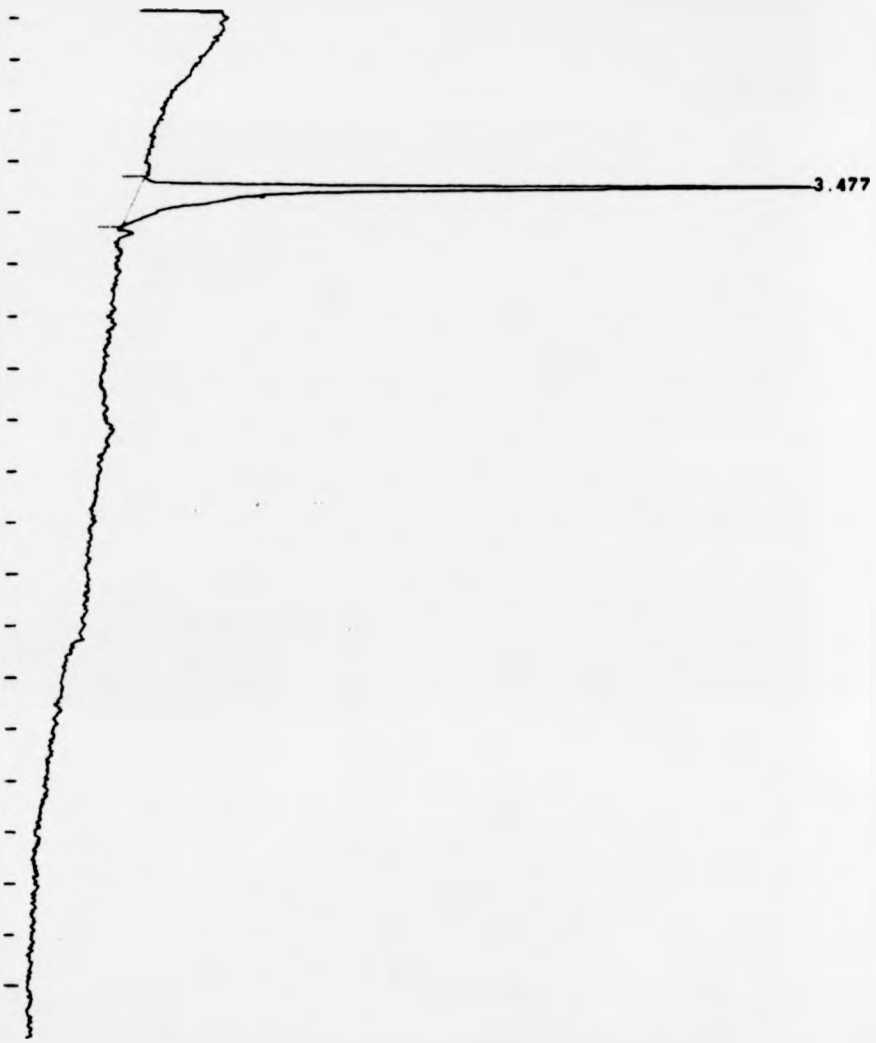
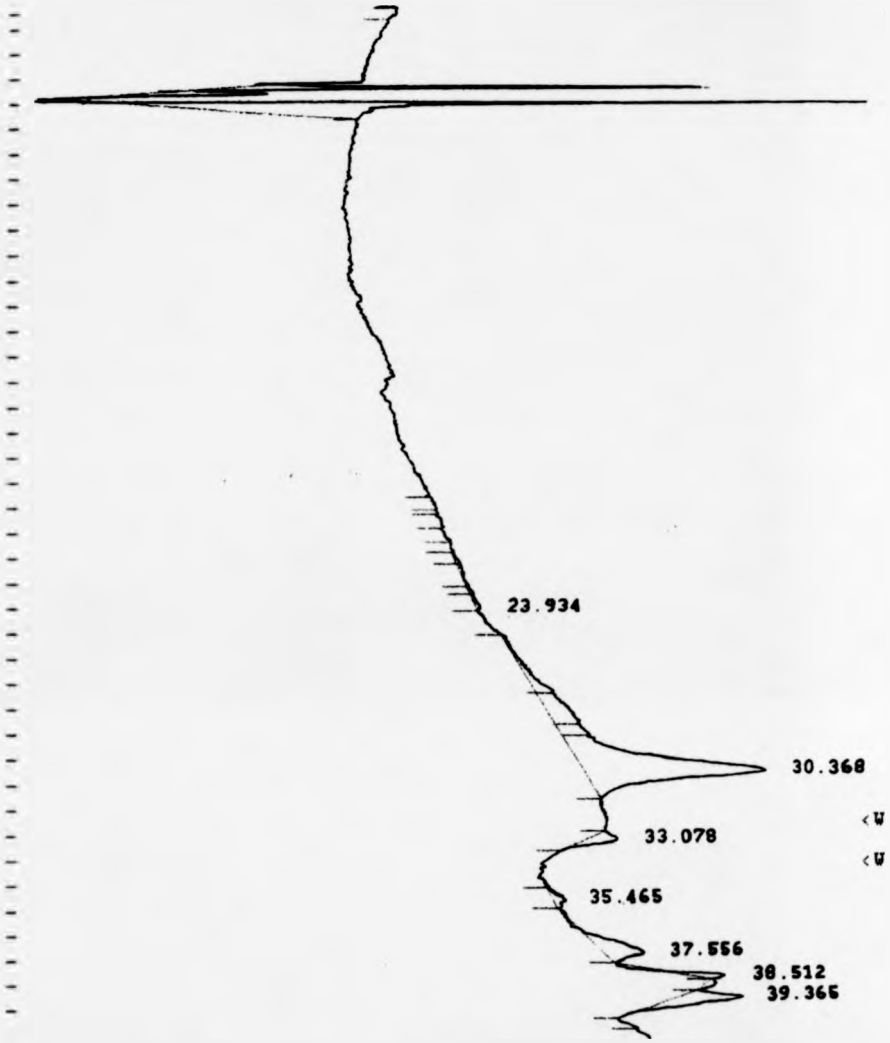


FIGURE 3.9.b High performance liquid chromatogram of the 10% (v/v) MeOH wash from the purification step of peptide toxin extraction (attenuation = 12). Column: Nova-Pak C_{18} (4 μm. 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

No toxic eluates were detected.



3.4.8 Efficiency and reproducibility of the extraction method

The extraction procedure was assessed for its efficiency and reproducibility in order to achieve the maximum amount of toxins from the cell biomass/raw water. The efficiency and reproducibility were examined by spiking samples with known quantities of purified toxins and carrying out the standard procedures. The "cell free" raw water (3 litres) was spiked with 1 μg of each reference toxin (nodularin, microcystin-LR and microcystin-RR), and the standard procedure followed. The extracts were examined by HPLC using photo-diode array detection. The area of each toxic peak was calculated and comparison made with the amount of toxins added initially. The % recoveries of the microcystins and nodularin are shown in Table 3.8.

TABLE 3.8 % recoveries of microcystins and nodularin from spiked raw water

Toxin	Recovery (%) *
Microcystin-LR	81
Microcystin-RR	69
Nodularin	75

* : Recovery value was the average of two experiments.

3.5 Discussion

The optimised HPLC conditions used in this study to separate the mixture of reference toxins were :-

A linear gradient of 30% aqueous acetonitrile(v/v) containing 0.05% TFA to 60% aqueous acetonitriles (v/v) containing 0.05% TFA within 30 minutes at a flow rate of 1 ml min^{-1}.

Detection was set at 238 nm since this was the maximum absorption wavelength. The retention time of reference nodularin was shorter than those for microcystin-LR and RR analysed on the reverse-phase C_{18} column under identical chromatographic conditions (see Figure 3.2.a and b). This indicated a higher polarity for the nodularin than for the microcystins.

The reliable detection limit of the purified toxins was approximately 20 ng using UV detection and about 50-100 ng using photo-diode array detection.

The later detection procedure facilitated spectral analysis and comparison of the eluate to the reference toxins which allowed identification of the toxic peaks other than by retention time. Since *Microcystis aeruginosa* has been the most studied, and is the most common toxin producing species worldwide, *Microcystis aeruginosa* PCC 7806 was used as the reference toxic cell biomass. The two toxins isolated from this strain were characterised as microcystin-LR and/or microcystin-like derivatives. The results compared with those from the study of Dierstein *et al.* (1990) who had also isolated and identified two toxic fractions from *Microcystis aeruginosa* PCC 7806 as microcystin-LR and a microcystin-LR derivative by using an alternative extraction and isolation procedure in conjunction with thin layer chromatography, mass spectrometry and nuclear magnetic resonance techniques. Thus, to confirm the identification of toxins produced by *Microcystis aeruginosa* PCC 7806 in this study, it was necessary to produce more detailed information requiring the use of mass spectrometry and ultimately, amino acid analysis.

Mouse bioassay was used to corroborate the toxicity of eluates. Mice, intraperitoneally injected with purified hepatotoxins, were observed, the symptoms recorded and the lethal dose recorded. The toxicity of microcystin-LR was shown to be 10 times greater than that for microcystin-RR. It should be noted that the amino acid difference between these two toxins is only 1 L-amino acid, leucine in microcystin-LR and arginine in microcystin-RR. This also indicates that the amino acid sequence is very important to the study of variation of toxicity. All mice injected with extracts of *Microcystis aeruginosa* PCC 7806 showed signs of toxicity that were characteristic of peptide cyanotoxins. Although mouse bioassay did not provided good quantitative information as to the toxicity of toxins, it was still used as the primary reference assay for all toxicity assessments.

The presumptive toxins from environmental samples were successfully extracted and purified with the % recovery of the extraction method being approximately 75%. Although C_{18} cartridges were utilised in the procedure, these do lack selectivity in respect to other organic compounds present in raw water samples, i.e. these are also extracted. Consequently to prevent destruction of the analytical column and also to maintain the efficiency of the column, a guard column was routinely used.

CHAPTER 4

CHAPTER 4 : CHARACTERISATION OF PEPTIDE TOXINS FROM FRESHWATER *Oscillatoria* SPECIES : VARIATION IN TOXICITY AND TEMPORAL EXPRESSION

4.1 INTRODUCTION

Blooms of cyanobacteria appear widely in eutrophic fresh, marine and brackish water throughout the world, which has been attributed to nutrient enrichment *via* agriculture, domestic and industrial effluents. Several of the common bloom forming genera, such as *Oscillatoria* and *Microcystis*, are known to produce toxins which have been responsible for the death of livestock and wildlife in many parts of the world (Gorham & Carmichael, 1979). These toxins have been implicated with illness in humans and it has been reported that standard water purification and filtration procedures do not remove or inactivate them (Hoffman, 1976). The cyanobacterial toxins are classified as either hepatotoxins or neurotoxins. Hepatotoxins are synthesised by species such as *Microcystis* (Jinno *et al.*, 1989; Sivonen *et al.*, 1992b), *Oscillatoria* (Bruno *et al.*, 1992; Swoboda *et al.*, 1992) and *Nodularia* (Carmichael, 1988; Sivonen *et al.*, 1989a; b), and are either cyclic hepta and pentapeptides with relative molecular masses of approximately 1,000 (Namikoshi *et al.*, 1992c).

It is now known that the toxicity of a particular cyanobacterial species varies between sites (and within a particular site), on a seasonal, weekly and even daily basis (Dow *et al.*, 1992; Swoboda *et al.*, 1992). This variability is probably due to

environmental factors and the presence of more than one strain of a cyanobacterial species (Wicks & Thiel, 1990).

A number of reports have been published on the influence of environmental factors on the toxicity of cyanobacterial species such as *Microcystis aeruginosa* in laboratory experiments (Eloff & van der Westhuizen, 1981; Runnegar *et al.*, 1983; van der Westhuizen & Eloff, 1985; Watanabe *et al.*, 1989b). These have included light intensity, temperature, pH, aeration, culture age and nutrients. Only a few studies have been done concerning the effect of environmental factors on the toxicity of *Microcystis aeruginosa* in lakes or reservoirs. Light, temperature, as well as nutrients, are the most important factors found to show an acceptable degree of correlation with toxin concentration (Wicks & Thiel, 1990).

In Hartbeespoort Dam, South Africa, dense populations of *Microcystis aeruginosa* often occur even during winter. However, toxins were not found to be present at high concentrations in these cells. This was most probably due to dominance by a non-toxic strain of *Microcystis aeruginosa* during the winter months, whereas toxic strains dominated in the summer (Wicks & Thiel, 1990).

Lukac & Aegerter (1993) reported the influence of trace metals on growth and toxicity of various *Microcystis aeruginosa* strains of which PCC 7806 was found to produce the most toxin per biomass. Within the same experiment, Zn and Fe were the only two trace metals that were shown to be required for optimal growth as well as for toxin production.

The growth of *Oscillatoria agardhii* is most successful under low light conditions (Post *et al.*, 1985), and also, *Oscillatoria* species are generally favoured by stratification and high TN/TP ratio (Harris, 1986).

With a lethal dose, death by hepatotoxin poisoning occurs within 1 to 2 hours as a consequence of haemorrhagic shock caused by pooling of blood in the liver resulting from the destruction of the endothelial lining of the liver sinusoids and the disintegration of hepatocyte membranes (Falconer *et al.*, 1981). At sublethal doses,

dermal irritation and gastrointestinal, neuromuscular, respiratory or cardiovascular problems may occur. For example, in 1989, ten army recruits were reported ill, two very seriously, from sublethal doses of hepatotoxin poisoning following a canoeing and swimming exercise in a reservoir in Central England which had developed a bloom of *Microcystis*. More seriously there is also some evidence of tumour promoting activity by microcystins (Falconer, 1991).

In this study, experiments were performed in order to obtain more information on the environmental and physiological conditions required for the induction of toxin expression in an *Oscillatoria* strain in Lower Shustoke reservoir, and also to elucidate the variation in toxicity under natural conditions.

4.1.1 Aims

Since there has been an increasing frequency of occurrence of blooms at many reservoirs in the English Midlands which serve for both recreational purposes and as sources of drinking water, the primary aims of this study were to monitor these reservoirs for the presence of toxic blooms, including the isolation and characterisation of the different toxins, and to elucidate a better understanding of the factors responsible for bloom formation and the induction of toxin expression. The study of toxins produced by naturally occurring cyanobacteria is particularly important both in terms of the presence of the toxins within the cells, which may be consumed accidentally, and the possible release of these toxins into the water.

Blooms of cyanobacteria in freshwater reservoirs in the English Midlands were regularly monitored over a three year period during March to November, with samples being collected on a regular bi-weekly basis. However, this study has concentrated on the investigation of the occurrence and toxicity of hepatotoxins produced by an *Oscillatoria* species which is the predominant cyanobacterial species in Lower Shustoke reservoir, since sufficient cell biomass was available throughout

the period of study and this species proved to be toxic by mouse bioassay, although toxicity was variable.

Environmental samples were analysed for the presence of presumptive toxins by HPLC analysis and for toxicity *per se* by mouse bioassay. Several prevailing environmental and chemical factors were recorded and compiled into a data base to assess the influence of these factors on toxin production and the overall toxicity of the cyanobacterial biomass.

4.2 MATERIALS AND METHODS

All pertinent materials and methods used in this chapter have been detailed in chapter 2.

4.3 Results

4.3.1 Cyanobacteria indigenous to Lower Shustoke

The predominant cyanobacterial species present in the reservoirs under study, as observed by light microscopy, are given in Table 4.1. Photomicrographs of the major cyanobacterial species expressed in the reservoirs are shown in Figure 4.1.

The primary cyanobacterial population in Lower Shustoke reservoir was always *Oscillatoria* species which accounted for in excess of 95% of the environmental cyanobacterial biomass over the period of study (3 years). Water samples from this reservoir were regularly monitored for species diversity and whether or not these were expressing toxins, emphasis being focused upon the toxic hepatopeptides.

TABLE 4.1 Primary cyanobacterial species observed in the English Midlands reservoirs during 1991 to 1993

Reservoir	**Predominant cyanobacterial genera**
Lower Shustoke	*Oscillatoria; Pseudoanabaena; Aphanizomenon*
Stanford	*Oscillatoria; Aphanizomenon*
Draycote	*Anabaena; Microcystis*
Wille's Meadow	*Aphanizomenon; Oscillatoria*
Foremark	*Anabaena; Nostoc; Microcystis*
Staunton Harold	*Anabaena; Microcystis; Oscillatoria*
Cropston	*Microcystis; Nostoc; Nodularia; Oscillatoria; Aphanizomenon; Anabaena*
Swithland	*Aphanizomenon*
Blackbrook	*Aphanizomenon; Microcystis*
Nanpanton	*Microcystis; Oscillatoria; Pseudoanabaena*

FIGURE 4.1.a Photomicrograph of *Oscillatoria* species present in the English Midlands reservoirs during 1991 to 1993. Bar marker represents 25 μm.

FIGURE 4.1.b Photomicrograph of *Microcystis* species present in the English Midlands reservoirs during 1991 to 1993. Bar marker represents 15 μm.

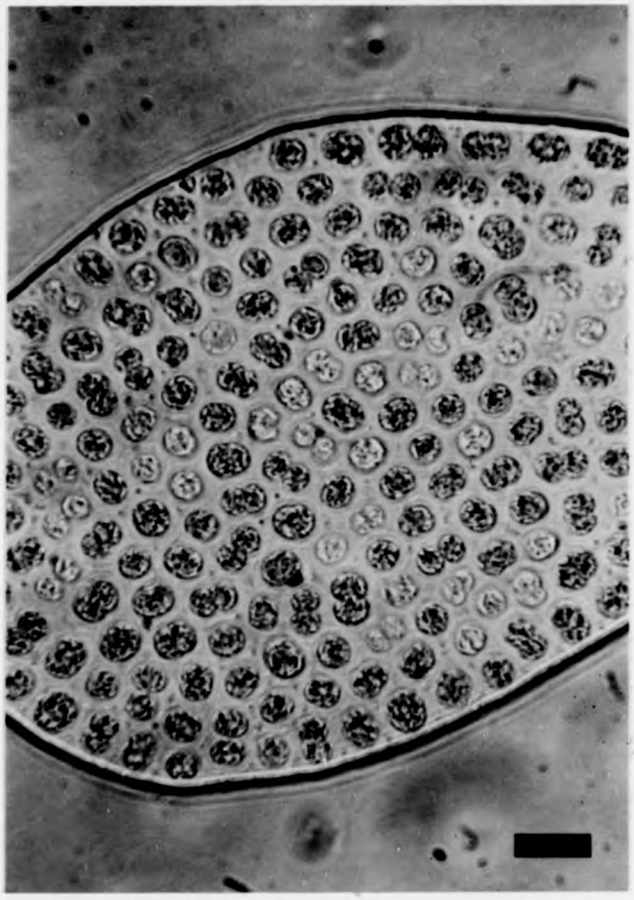
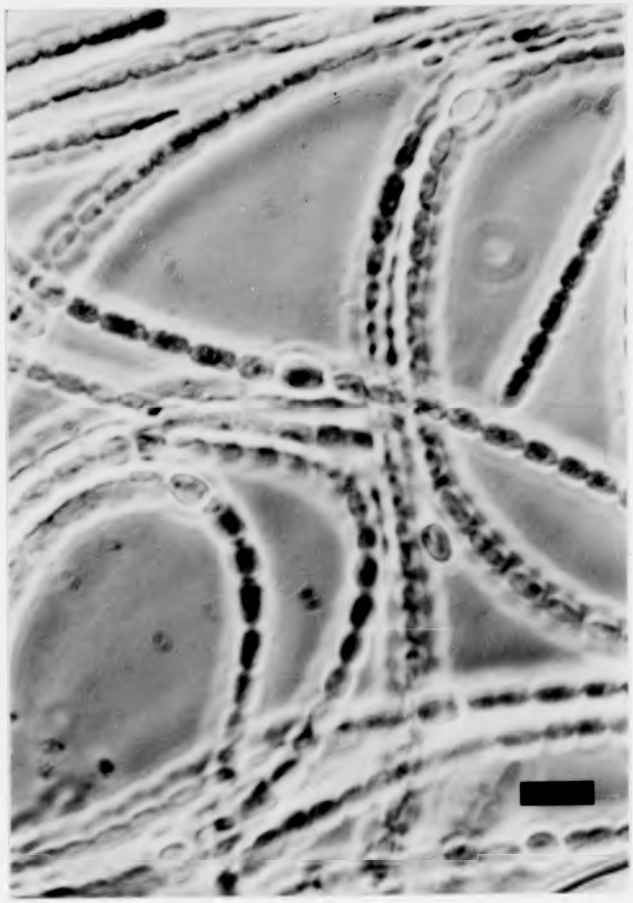


FIGURE 4.1.c Photomicrograph of *Anabaena* species present in the English Midlands reservoirs during 1991 to 1993. Bar marker represents 15 μm.



4.3.2 Mouse bioassay assessment of presumptive toxins in Lower Shustoke reservoir during 1991 to 1993

4.3.2.1 Toxicity of cyanobacterial biomass

A known weight (normally 100 mg) of lyophilised cyanobacterial cell biomass was resuspended in phosphate buffered saline, pH 7.5, and disrupted by sonication. The sonicate was serially diluted and 200 µl aliquots of each dilution were introduced intraperitoneally into adult Balb C mice, both male and female, with body weights of approximately 20 g (see section 2.11).

The predominant cyanobacterial species present in Lower Shustoke reservoir were found to be *Pseudoanabaena* at the beginning of the year, changing to be *Oscillatoria* as the major species in the summer months. It was quite noticeable that the samples of cell biomass which were shown to be non-toxic by mouse bioassay were when *Pseudoanabaena* was assigned as the major species in the reservoir. On the other hand, all samples of *Oscillatoria* biomass, when present as the predominant cyanobacterial species in Lower Shustoke reservoir, were shown to be toxic throughout the period of evaluation and the symptoms preceding death were similar to those reported earlier for the microcystins i.e. the mice died from intrahepatic haemorrhage and hypovolaemic shock. Intrahepatic haemorrhage resulted from an increase in the liver weight as a fraction of the body weight, hepatic haemoglobin and iron concentrations. There was therefore insufficient blood and irreversible shock was induced. Mice injected with buffered saline (controls) remained active and showed none of the toxic symptoms.

The mortality of the mice was shown to dependent on the concentration of the cyanobacterial biomass extracted and administered. The concentration of cell lysate required to cause the death of the mice varied over the environmental monitoring period (Table 4.2.a, b and c), with death resulting from within a few

minutes to several hours after intraperitoneal administration of equal concentrations of cell biomass. Generally, mice injected with cell biomass extracts from Lower Shustoke reservoir corresponding to more than 20 mg dry weight cells per kg body weight of mice, died. Levels of mortality were slightly different between male and female mice. It was noted that in most cases female mice showed signs of recovery more frequently than males when administered with the same concentration of biomass extract. As yet there is no apparent explanation for this observation.

Caution is required in interpreting some of these data (**"nd"**) as only limited cell material was available for analysis, consequently the toxin challenge to the mice may well have been below the lethal dose.

4.3.2.2 Toxicity of raw water from Lower Shustoke

To determine whether toxin(s) was detectable in "cell free" raw water, water was collected from Lower Shustoke reservoir and then prepared as described in section 2.8. Throughout the environmental monitoring study (during 1991-1993), "cell free" water concentrates from bloom situations were assayed for toxicity by mouse bioassay. After filtration and concentration, all raw water samples were found to be non-toxic although a concentrate, equivalent to 10 litres of water (concentrated by passage through a Sep-pak C_{18} cartridge), had been injected intraperitoneally into mice. No deaths occurred within 24 hours, and there were no signs of toxicity.

TABLE 4.2 Toxicity of cyanobacterial biomass present in Lower Shustoke reservoir as assessed by mouse bioassay (Balb C mice)

(a) 1991

Date	Cyanobacterial species	Toxicity	Lethal Dose (mg kg^{-1}) *
29/4	*Oscillatoria* (mainly) + *Pseudoanabaena*	toxic	70
12/5	*Oscillatoria* (mainly) + *Pseudoanabaena*	toxic	80
20/5	*Oscillatoria* (mainly) + *Pseudoanabaena*	toxic	20.25
10/6	*Oscillatoria* (mainly) + *Pseudoanabaena*	toxic	20.1
17/6	*Oscillatoria*	toxic	**nd**
15/7	*Oscillatoria*	toxic	**nd**
12/8	*Oscillatoria*	toxic	**nd**
10/10	*Oscillatoria*	toxic	**nd**
31/10	*Oscillatoria*	toxic	**nd**

nd : not determined - insufficient sample

* : mg dry weight of cells per kg mouse body weight

(b) 1992

Date	Cyanobacterial species	Toxicity	Lethal Dose (mg kg^{-1})
18/2	*Oscillatoria*	not tested	
8/4	*Pseudoanabaena* (mainly) + *Oscillatoria*	non-toxic	
29/4	*Oscillatoria*	toxic	< 22
13/5	*Oscillatoria*	toxic	< 44
26/5	*Oscillatoria*	toxic	36.50
10/6	*Oscillatoria*	not tested	
24/6	*Oscillatoria*	toxic	30.50
8/7	*Oscillatoria*	toxic	38.50
22/7	*Oscillatoria*	not tested	
5/8	*Oscillatoria*	toxic	23.50
19/8	*Oscillatoria* (mainly) + *Pseudoanabaena*	not tested	
2/9	*Oscillatoria*	not tested	
16/9	*Oscillatoria*	not tested	
30/9	*Oscillatoria*	not tested	
14/10	*Oscillatoria*	not tested	
27/10	*Oscillatoria* (mainly) + *Pseudoanabaena*	not tested	

(c) 1993

Date	Cyanobacterial Species	Toxicity	Lethal dose (mg kg^{-1})
19/4	none	not tested	
4/5	*Pseudoanabaena* (low)	not tested	
17/5	*Pseudoanabaena* (low)	not tested	
1/6	*Pseudoanabaena* (mainly) + *Oscillatoria* (few)	not tested	
9/6	*Pseudoanabaena* (mainly) + *Oscillatoria* (few)	not tested	
10/6	*Pseudoanabaena* (mainly) + *Oscillatoria* (few)	not tested	
14/6	none	not tested	
25/6	none	not tested	
12/7	*Oscillatoria* (mainly) + *Pseudoanabaena*	toxic	**nd**
26/7	*Oscillatoria*	toxic	**nd**
9/8	*Oscillatoria*	toxic	**nd**
19/10	*Oscillatoria*	toxic	**nd**

4.3.3 Characterisation of peptide hepatotoxins produced in Lower Shustoke reservoir

The presumptive toxins from environmental samples were isolated and characterised by applying reverse-phase high performance liquid chromatography (RP-HPLC). Peak identification was as described earlier (see section 3.4.4). All peaks with UV absorption spectra similar to those of the reference standards were tentatively identified as microcystin-like and/or nodularin-like. Each of the three toxic peaks from *Oscillatoria* cell biomass were separated and collected using analytical/preparative HPLC in order to identify/characterise them further by an enzyme assay for toxicity and mass spectrometry for the determination of molecular weight and structure.

Lower Shustoke, supported the growth of toxic cyanobacterial species, as assayed by mouse bioassay throughout the period of study. The cell biomass and "cell free" raw water concentrate were analysed by HPLC after the extraction of toxins by the method described in section 2.7. Quantitative data was obtained for the total toxin content of the individual samples together with a further quantitative characterisation of the individual toxins present in the analysed biomass.

4.3.3.1 Cyanobacterial biomass

A toxic strain of *Oscillatoria* species was found to be permanently resident in Lower Shustoke reservoir and it has been assumed that any toxin perceived in this reservoir was mainly produced by this species. This assumption is supported by the following:-

- in the vast majority of samples *Oscillatoria* accounted for > 95% of the cyanobacterial biomass.

- although a species of *Pseudoanabaena* was sometimes present this was found to be non-toxic.
- an *Aphanizomenon* species was encountered, however, all isolates of this cyanobacteria have been found to produce a neurotoxin rather than an hepatotoxin.
- the *Oscillatoria* species from Lower Shustoke is in axenic culture and the hepatotoxins characterised from laboratory culture are as per those identified for the environmental samples.

4.3.3.1.1 *Samples collected in 1991*

Figure 4.2.a shows a typical HPLC chromatogram of the *Oscillatoria* biomass present during the summer of 1991. The chromatogram revealed three major toxic peaks which had spectral characteristics similar to the reference hepatotoxins and the samples were found to be toxic to mice. The primary toxic peaks were those with tRs of (1) 24.946 minutes which was nodularin-like; with a similarity of 0.99811, (2) 26.428 minutes and (3) 28.470 minutes which were microcystin-like; with similarities of 0.99950 and 0.99959, respectively. The purity of these three chromatographic peaks are shown in Figure 4.2.b. Both of the microcystin-like peaks (tRs 26.428 and 28.470 minutes) had nearly flat tops indicating that they were high purity compounds while the nodularin-like peak (tR 24.946 minutes) contained impurities.

The three eluted toxic peaks had tRs very close to that for the reference microcystin-LR as judged from the cell biomass collected from Lower Shustoke on 15 July 1991 (Table 4.3). Figure 4.2.c indicated that all peaks were potential peptide toxins since they were spectrally very similar to microcystin-LR.

FIGURE 4.2.a High performance liquid chromatogram of *Oscillatoria* biomass present in Lower Shustoke reservoir on 15 July 1991 (attenuation = 29). Column: Nova-Pak C_{18} (4 µm. 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

(tR 24.946 minutes is nodularin-like peak. tRs 26.428 and 28.470 minutes are microcystin-like peaks)

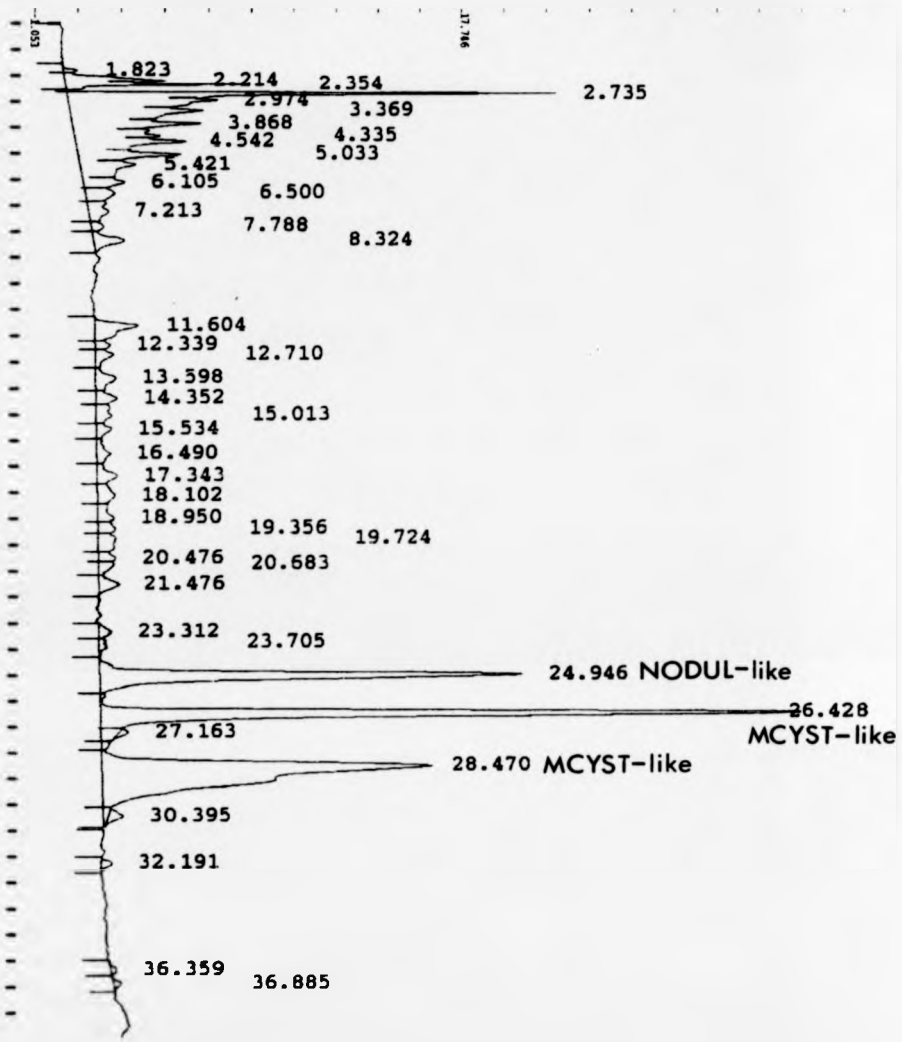


FIGURE 4.2.b Purity parameter plot of presumptive hepatotoxins from *Oscillatoria* biomass present in Lower Shustoke reservoir on 15 July 1991.
(tR 24.946 minutes is nodularin-like. However, peak contains impurities; tRs 26.428 and 28.470 minutes are microcystin-like)

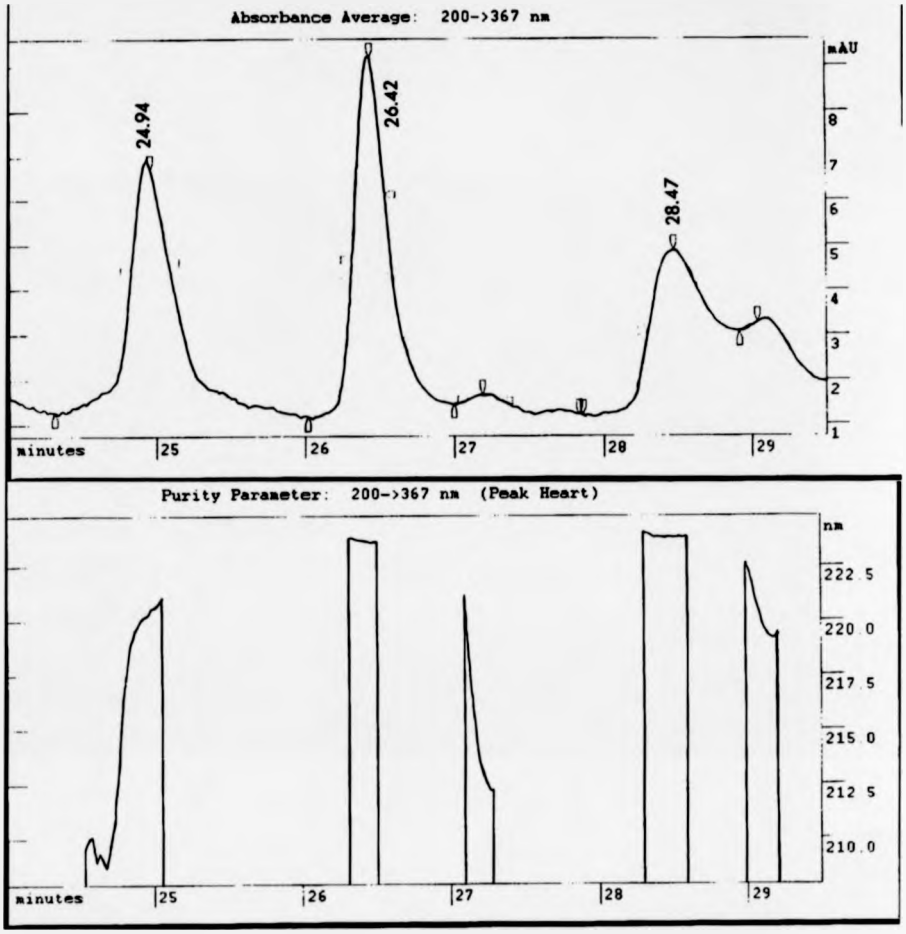


FIGURE 4.2.c The similarity between ultraviolet spectra of the toxic eluate peaks from *Oscillatoria* biomass present in Lower Shustoke reservoir on 15 July 1991 compared to that of reference microcystin-LR.

(a) reference microcystin-LR; (b) nodularin-like peak at tR 24.946 minutes; (c) microcystin-like peak at tR 26.428 minutes and (d) microcystin-like peak at tR 28.470 minutes.

Large arrow indicates the impurities present in the nodularin-like peak at tR 24.946 minutes.

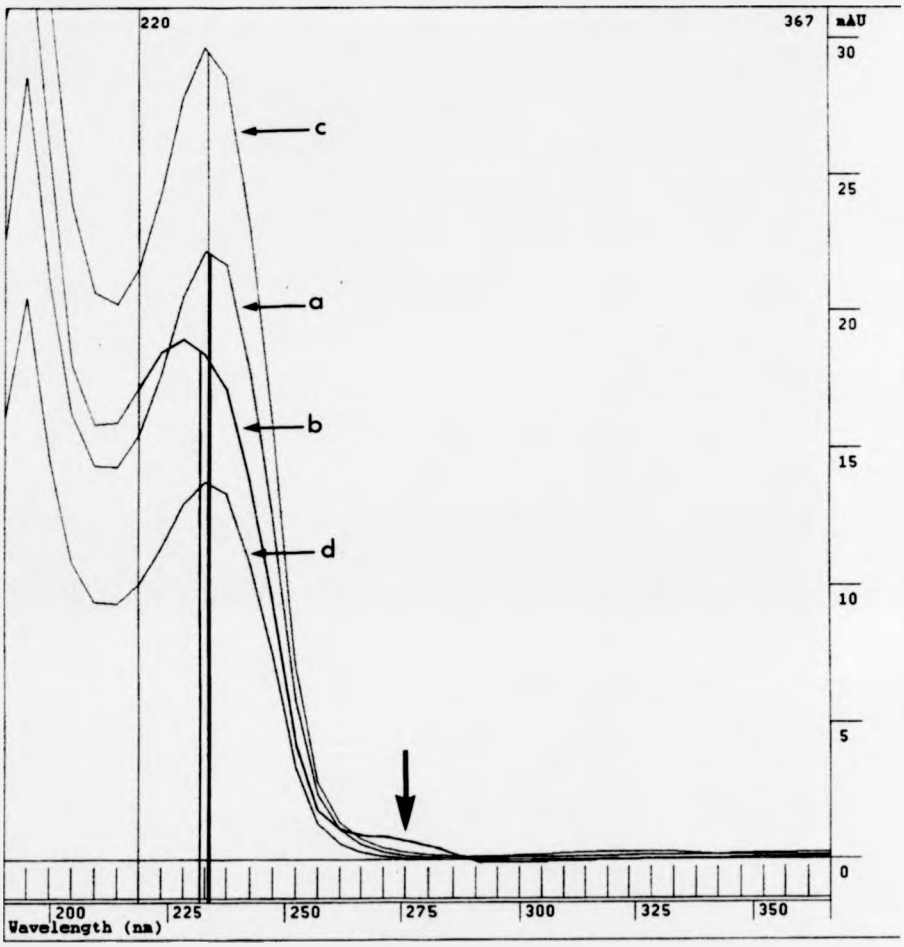


TABLE 4.3 Elution times for presumptive hepatotoxin peaks from *Oscillatoria* biomass present in Lower Shustoke reservoir on 15 July 1991, compared to microcystin-LR. The integrated area is indicative of the relative concentrations of each of the presumptive hepatotoxins

Sample	tR (min)	Area
Standard microcystin-LR	26.490	-
LS-cell peak 1	24.946	42,628
LS-cell peak 2	26.428	56,922
LS-cell peak 3	28.470	78,868

4.3.3.1.2 *Samples collected in 1992*

From HPLC analysis of the cell biomass collected in 1992, the presumptive hepatotoxins produced by the indigenous *Oscillatoria* species remained the same as for 1991. The HPLC analysis showed the same typical chromatogram with three toxic peaks which had spectral similarity close to nodularin and the microcystins (Figure 4.3.a). However, the retention times of these peaks were shorter than those for the peaks in 1991. This was almost certainly in consequence of a reduction in the retention activity of the column. The purity of the chromatographic peaks is shown in Figure 4.3.b. All of the samples containing presumptive toxic peaks proved to be toxic when injected intraperitoneally into mice.

FIGURE 4.3.a High performance liquid chromatogram of *Oscillatoria* biomass present in Lower Shustoke reservoir on 22 July 1992 (attenuation = 40). Column: Nova-Pak C_{18} (4 μm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

(tR 19.478 minutes is a nodularin-like peak, tRs 20.646 and 23.025 minutes are microcystin-like peaks)

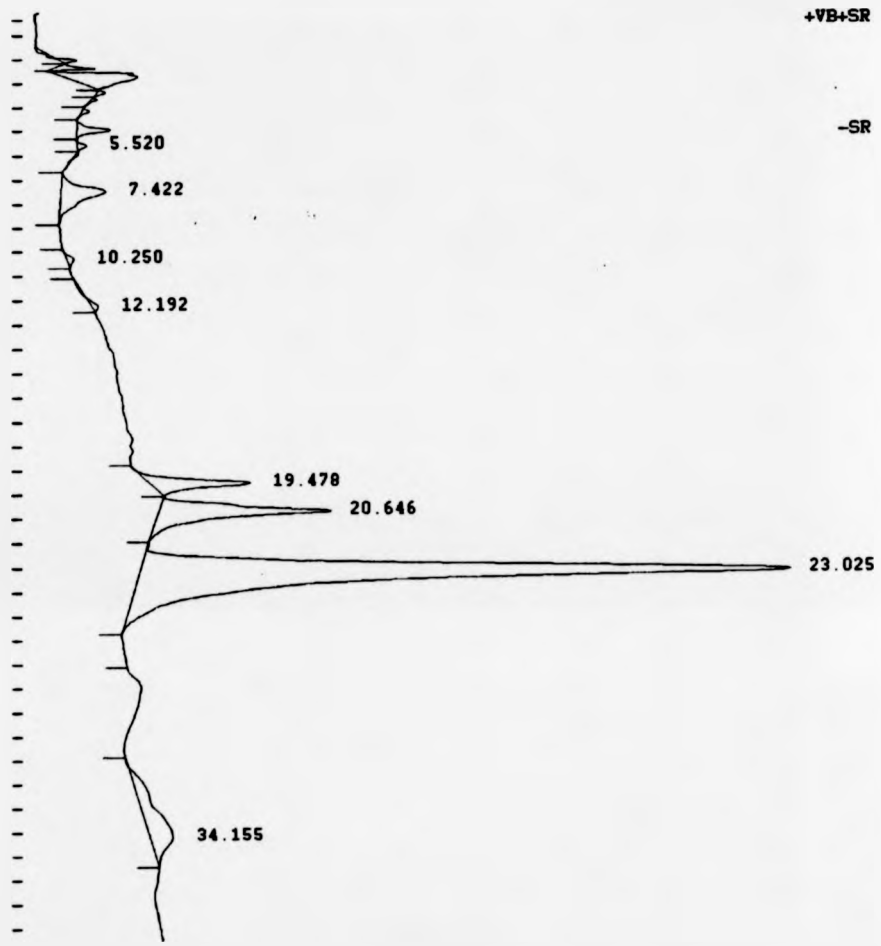
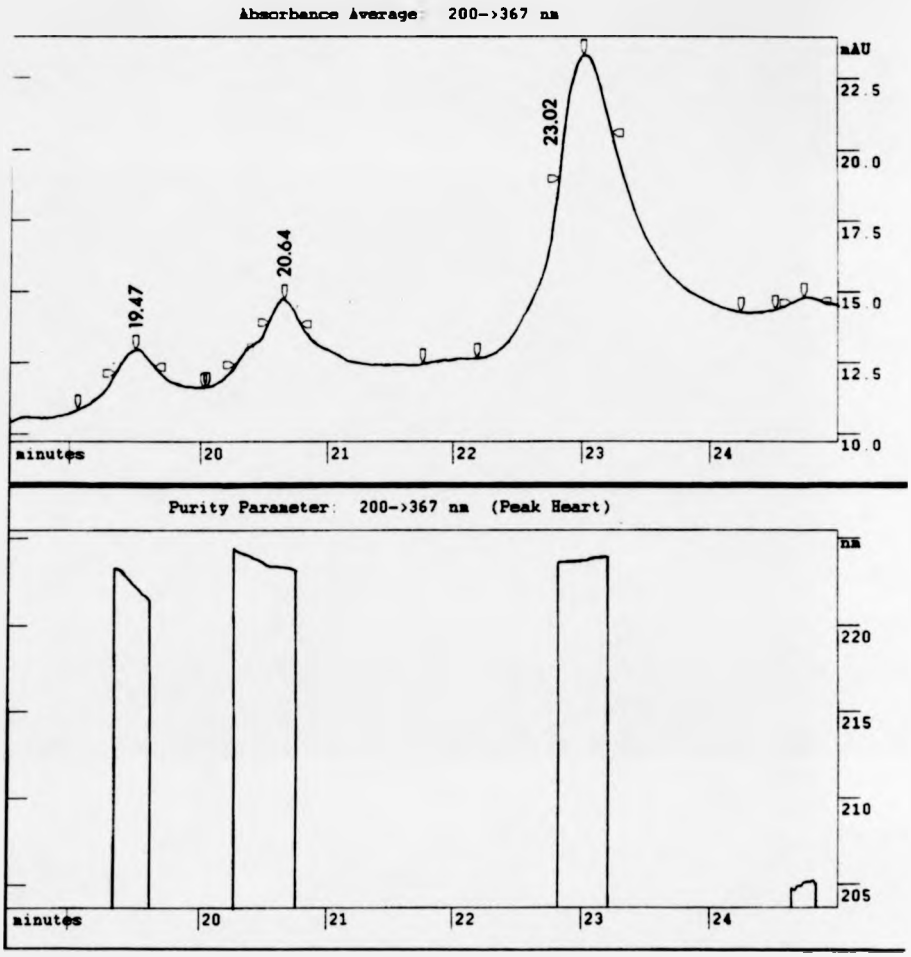


FIGURE 4.3.b Purity parameter plot of presumptive hepatotoxins from *Oscillatoria* biomass present in Lower Shustoke reservoir on 22 July 1992.

(tR 19.478 minutes is nodularin-like, tRs 20.646 and 23.025 minutes are microcystin-like)



4.3.3.1.3 *Samples collected in 1993*

At the beginning of 1993 (prior to the initiation of the monitoring program) a new Nova-Pak C_{18} column was installed as a consequence of a loss in column efficiency as indicated in section 4.3.3.1.2. Although the predominant cyanobacterial species was still *Oscillatoria* in 1993, especially in the summer months, HPLC chromatograms of the cell biomass were rather different from those obtained in 1991 and 1992. The elution profiles revealed four major toxic peaks three of which had the same characteristics as the presumptive hepatotoxins found in 1991 and 1992, however, the additional peak also had spectral similarity with the reference microcystins. The difference between these data was assumed to be a consequence of the higher efficiency of the new column. This was proved by repeat injection of the cell extract samples from 1992 which gave HPLC chromatograms with four toxic peaks similar to the 1993 cyanobacterial biomass.

Figure 4.4.a shows the HPLC chromatogram of the cell extract sample from a heavy bloom collected on 19 October 1993. The peaks at 14.982 minutes and 15.491 minutes had spectral similarities close to nodularin (similarity : 0.99858) and microcystin (similarity : 0.99997), respectively. The two peaks at 18.712 minutes and 19.685 minutes also had spectral characteristics similar to microcystin (similarity : 0.99997 and 0.99985, respectively). These two peaks were derived from the major third peak identified as a single microcystin-like peak in samples during 1991 and 1992. The purities of these four chromatographic peaks are shown in Figure 4.4.b.

FIGURE 4.4.a High performance liquid chromatogram of *Oscillatoria* biomass present in Lower Shustoke reservoir on 19 October 1993 (attenuation = 58). Column: Nova-Pak C_{18} (4 µm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

(tR 14.982 minutes is a nodularin-like peak, tR 15.491 minutes is a microcystin-like peak, tRs 18.712 and 19.685 minutes are microcystin-like peaks)

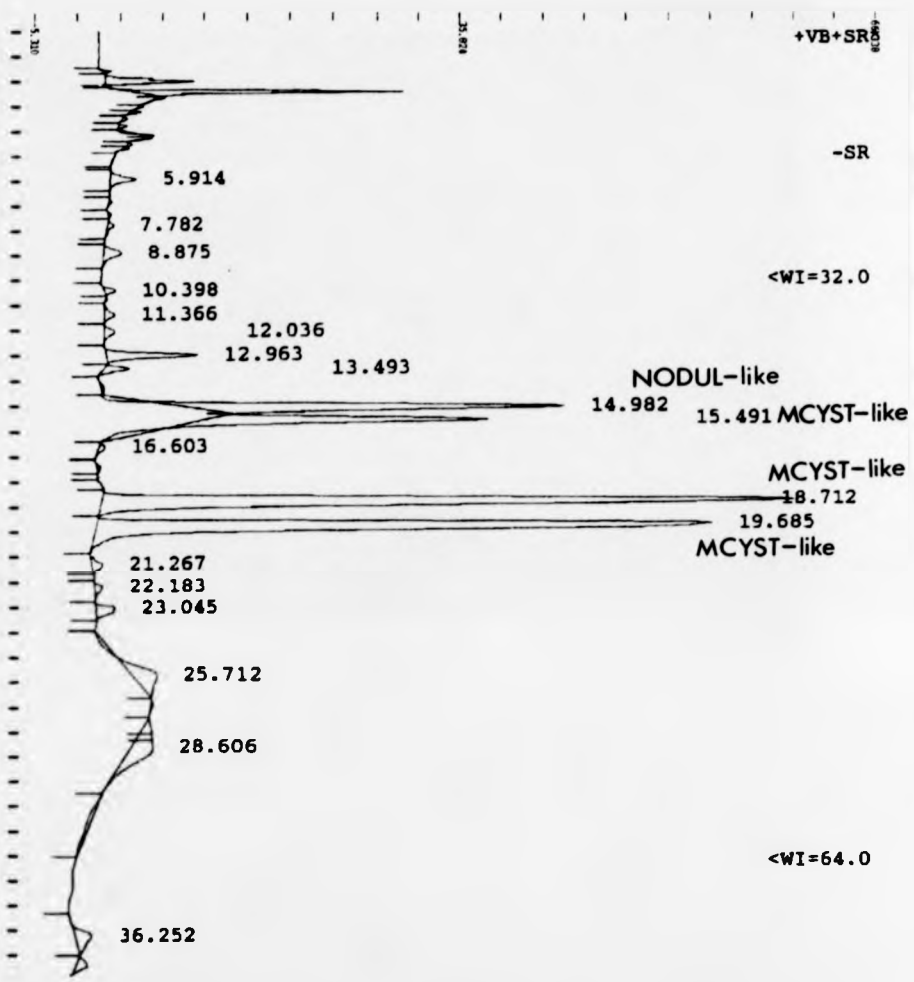
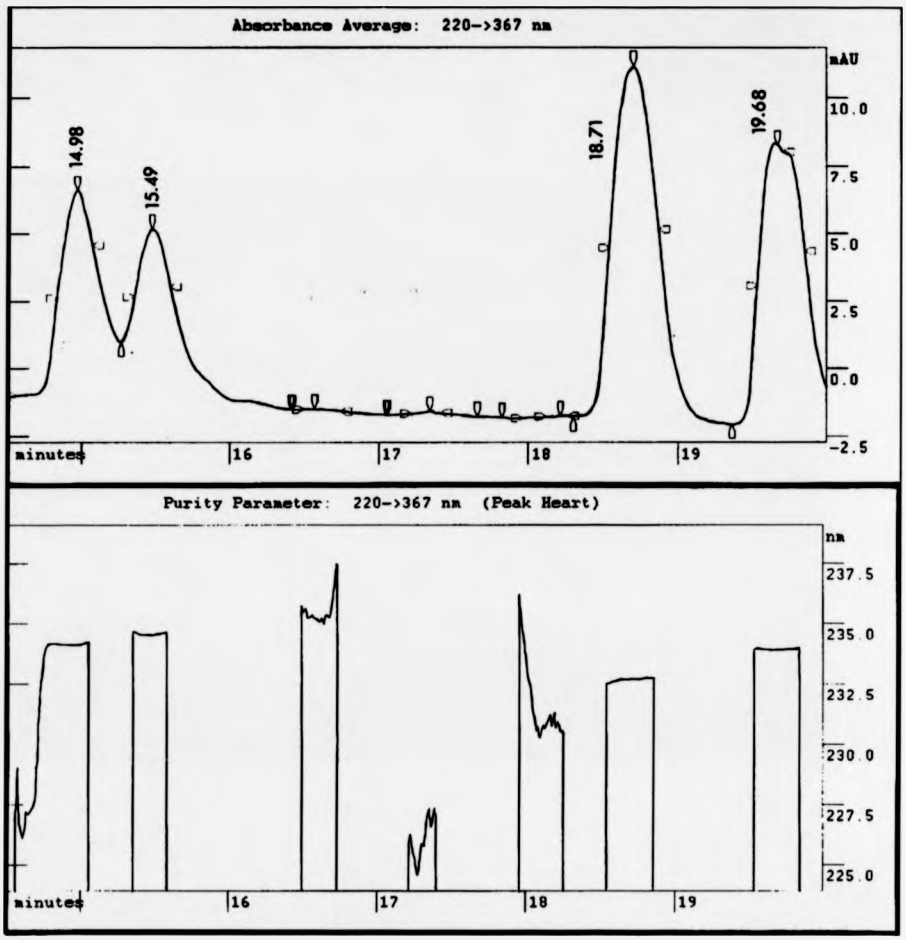


FIGURE 4.4.b Purity parameter plot of presumptive hepatotoxins from *Oscillatoria* biomass present in Lower Shustoke reservoir on 19 October 1993.
(tR 14.982 minutes is a nodularin-like, tR 15.491 minutes is a microcystin-like, tRs 18.712 and 19.685 minutes are microcystin-like)



4.3.3.2 "Cell free" raw water

Throughout 1991 and 1992, the "cell free" water concentrates from bloom situations were analysed for the presence of hepatotoxins by HPLC analysis but no toxic peak was identified in any of these samples. Figure 4.5 shows an HPLC chromatogram of the "cell free" water concentrate collected on 22 July 1992 with (a) attenuation set to give a full scale profile (att = 243) and (b) the same attenuation as the profiles derived from the cell biomass collected at the same time (att = 32). Although this chromatogram gave resolvable eluate peaks, none of these had spectral characteristic similar to the hepatotoxins. All water samples were non-toxic when assayed for toxicity by mouse bioassay (injection of the equivalent of 10 litres of water).

Unlike the above analysis, there were two water concentrates that contained toxic peaks identified as hepatotoxins since they had spectral similarities close to the microcystins and which were toxic by mouse bioassay. Figure 4.6 shows an HPLC chromatogram of a water concentrate sample collected on 23 August 1993 containing a microcystin-like peak at 19.224 minutes (similarity : 0.99982). Another HPLC chromatogram of water concentrate collected during a heavy bloom in the reservoir on 19 October 1993 indicated two toxic peaks; tRs 18.257 minutes and 19.247 minutes, both of which were identified as being nodularin-like with similarities of 0.99820 and 0.99423, respectively (Figure 4.7).

FIGURE 4.5.a High performance liquid chromatogram of "cell free" water concentrate (10 litre to 300 µl) collected from Lower Shustoke reservoir on 22 July 1992 at a full scale profile (attenuation = 243). Column: Nova-Pak C_{18} (4 µm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

No toxic eluates were detected.

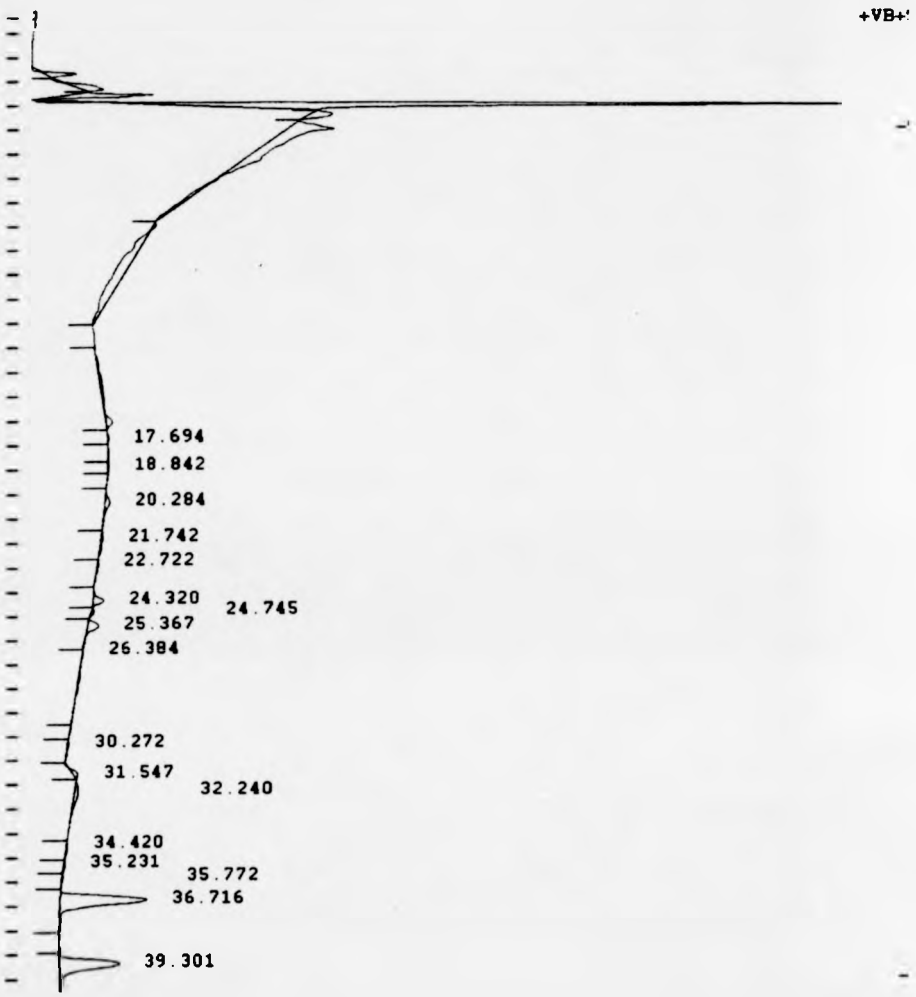


FIGURE 4.5.b High performance liquid chromatogram of "cell free" water concentrate (10 litre to 300 µl) collected from Lower Shustoke reservoir on 22 July 1992 at the same attenuation as the profiles derived from the cell biomass (attenuation = 32). Column: Nova-Pak C_{18} (4 µm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.
No toxic eluates were detected.

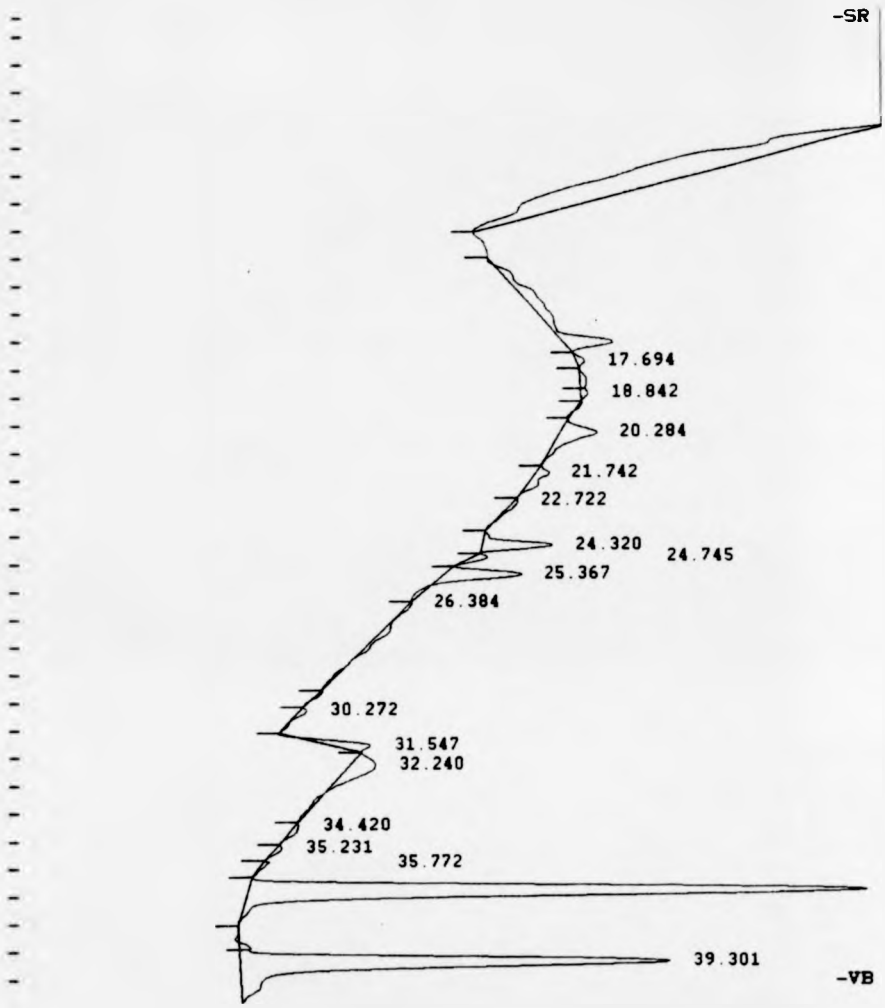


FIGURE 4.6 High performance liquid chromatogram of "cell free" water concentrate (10 litres to 300 µl) collected from Lower Shustoke reservoir on 23 August 1993 (attenuation = 32). Column: Nova-Pak C_{18} (4 µm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

(tR 19.224 minutes is a microcystin-like peak)

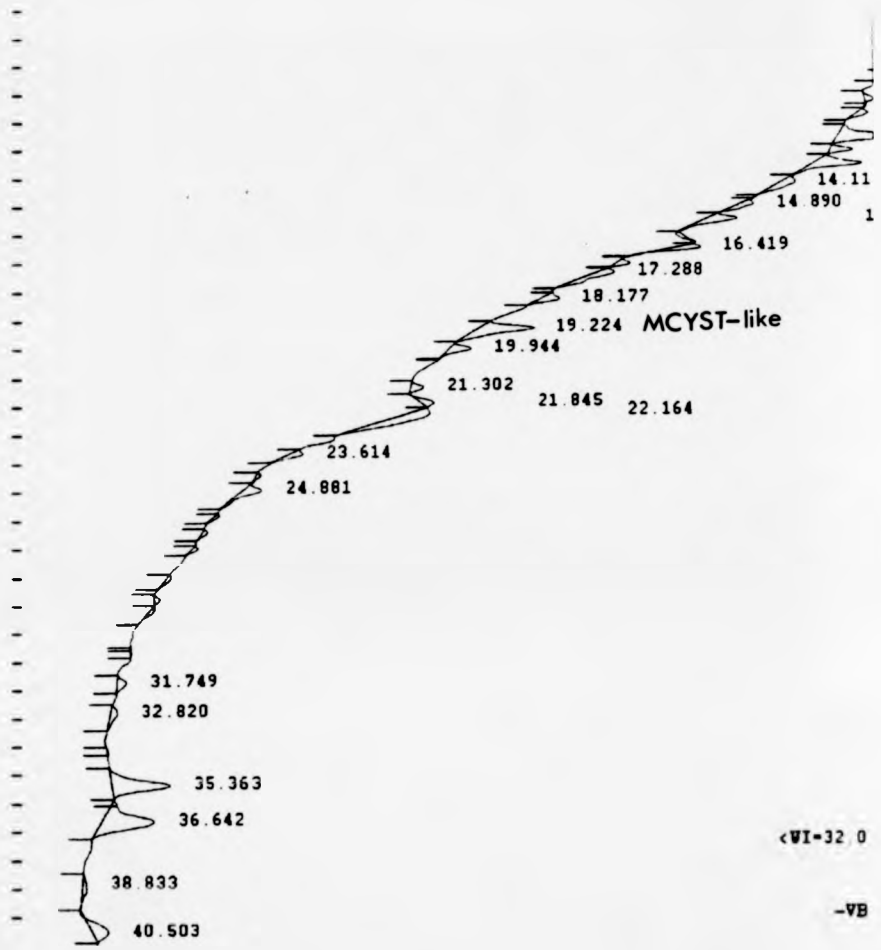
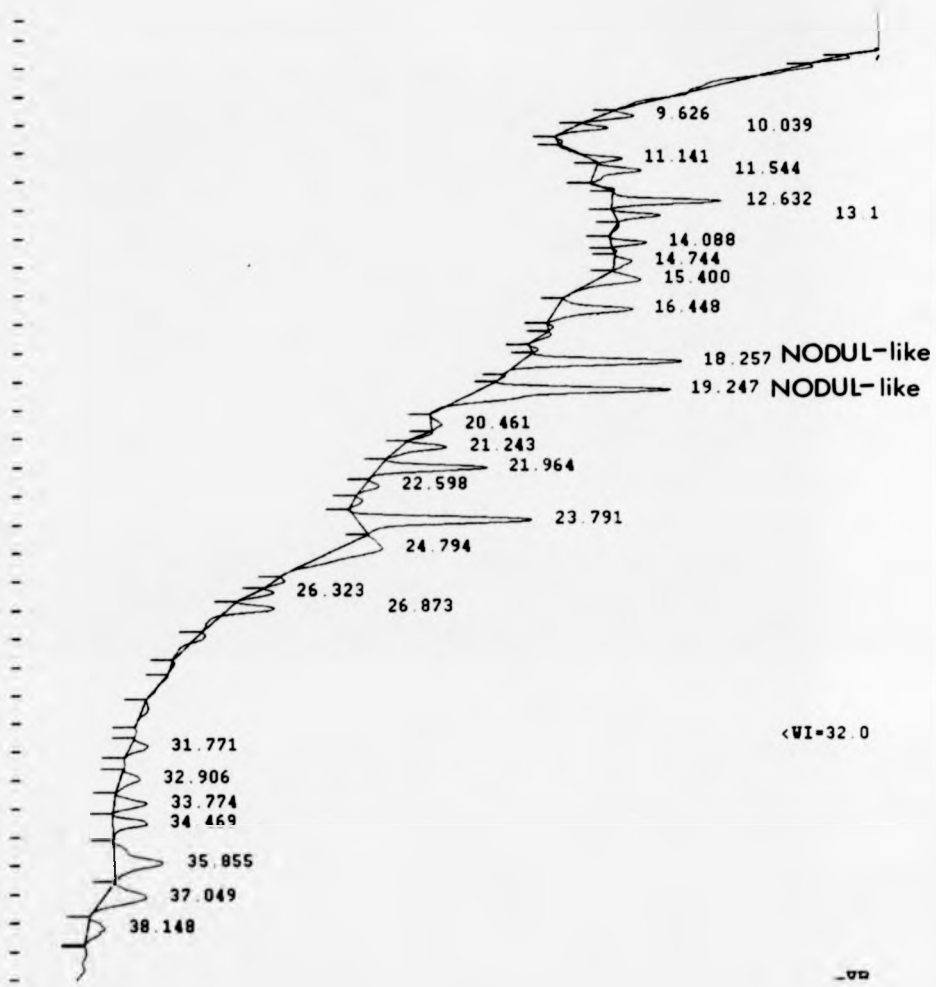


FIGURE 4.7 High performance liquid chromatogram of "cell free" water concentrate (10 litre to 300 µl) collected from Lower Shustoke reservoir on 19 October 1993 (attenuation = 32). Column: Nova-Pak C_{18} (4 µm, 3.9 x 300 mm); mobile phase: a linear gradient of 30% to 60% acetonitrile containing 0.05% TFA run within 30 minutes; flow rate: 1 ml min^{-1}. Detection: photo-diode array at 238 nm.

(tRs 18.257 and 19.247 minutes are nodularin-like peaks)



4.3.4 Quantitative analysis of toxin(s) from the cyanobacterial biomass from Lower Shustoke reservoir during 1991 to 1993

The concentration of either nodularin and/or microcystin-like toxins in any sample was determined by comparison to the reference microcystin-LR. This procedure was considered to be valid because of the very similar structures of the microcystins and the similarity of the absorption spectra of the toxins determined in the samples.

Since a linear relationship between peak area and concentration of microcystin-LR was already established (Figure 3.4, see section 3.4.3), the concentration of microcystin-like toxins in a sample was calculated by comparing the area of each toxic peak with that of reference microcystin-LR. Replicate injections of reference toxin and sample gave an assessment of the accuracy and precision of the technique.

The concentration of hepatotoxins in cell biomass from each environmental sample was calculated from the total area of the toxic peaks which had spectral similarity ($\geq$ 0.998) close to nodularin and/or the microcystins. The toxin concentrations expressed per gramme of lyophilised cell biomass collected at different times during 1991, 1992 and 1993 are given in Tables 4.4, 4.5 and 4.6, respectively. Figure 4.8 shows the comparison of the total amount of toxins in the *Oscillatoria* cell biomass during the three years (1991-1993). It can be observed that the toxins increased significantly only during the summer months when cyanobacterial blooms occurred. The total concentration of toxins was shown to be approximately at the same level except for those samples collected during August 1993 and for a heavy bloom in October 1993, which were about two times higher than the "regular" level.

TABLE 4.4 Total concentration of toxins in whole cell cyanobacterial biomass from Lower Shustoke during 1991 as detected by HPLC analysis

The concentration of toxins are shown as the "mean" values of the duplicate experiments. The data in the brackets are the minimum and maximum values. The total concentration of toxins are variable with time. The detected levels are in the ranges of 72 to 423 μg of toxins per g dry weight of cells.

Sample date	μg of toxins per g dry weight of cells
15/7	279 (268, 290)
22/7	310 (303, 317)
29/7	101 (91, 111)
5/8	206 (204, 208)
12/8	72 (68, 76)
19/8	423 (417, 429)
2/9	388 (368, 408)
9/9	375 (372, 378)
26/9	349 (339, 359)
3/10	387 (371, 403)
10/10	327 (312, 342)
17/10	241 (233, 249)
31/10	not detected
7/11	not detected
14/11	not detected

TABLE 4.5 Total concentration of toxins in whole cell cyanobacterial biomass from Lower Shustoke during1992 as detected by HPLC analysis

The concentration of toxins are shown as the "mean" values of the duplicate experiments. The data in the brackets are the minimum and maximum values. The total concentration of toxins are variable with time. The detected levels are in the ranges of 166 to 617 μg of toxins per g dry weight of cells.

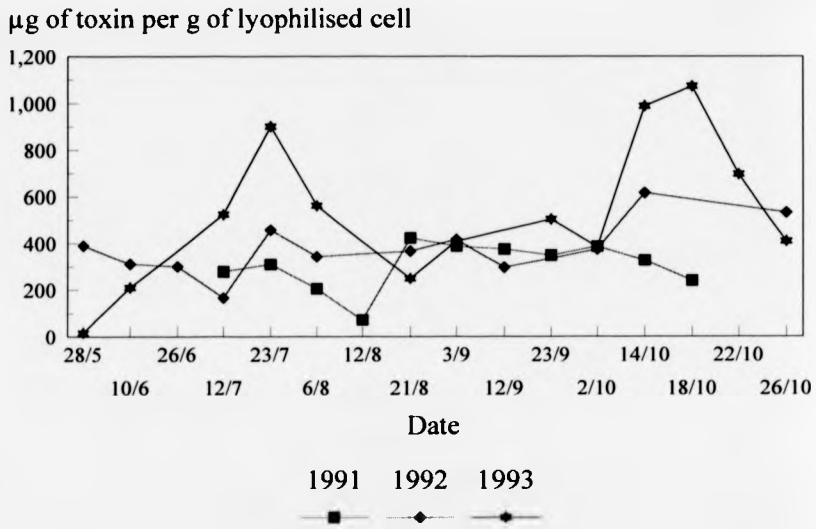
Sample date	μg of toxins per g dry weight of cells
26/5	390 (371, 409)
10/6	312 (308, 316)
24/6	301 (297, 305)
8/7	166 (154, 178)
22/7	457 (441, 473)
5/8	343 (329, 357)
19/8	367 (349, 385)
2/9	416 (402, 430)
16/9	297 (275, 319)
30/9	374 (372, 376)
14/10	617 (603, 631)
27/10	533 (518, 548)

TABLE 4.6 Total concentration of toxins in whole cell cyanobacterial biomass from Lower Shustoke during 1993 as detected by HPLC analysis

The concentration of toxins are shown as the "mean" values of the duplicate experiments. The data in the brackets are the minimum and maximum values. The total concentration of toxins are variable with time. During the beginning of the year the toxins are detected at very level (16 μg of toxins per g dry weight of cells) or not detected. However, during summer months the toxins are detected within the ranges of 162 to 1074 μg of toxins per g dry weight of cells.

Sample date	μg of toxins per g dry weight of cells
4/5	not detected
17/5	not detected
1/6	16 (15, 17)
9/6	162 (153, 171)
10/6	211 (203, 219)
14/6	insufficient cells
28/6	insufficient cells
12/7	524 (501, 547)
26/7	902 (899, 905)
9/8	562 (557, 567)
23/8	250 (237, 263)
6/9	410 (402, 418)
20/9	504 (497, 511)
4/10	384 (369, 399)
18/10	989 (963, 1015)
19/10	1074 (1041, 1107)
21/10	360 (341, 379)
22/10	697 (672, 722)
25/10	410 (399, 421)

FIGURE 4.8 Comparison of total concentration of toxins in whole cell biomass between 1991-1993 as detected by HPLC analysis.



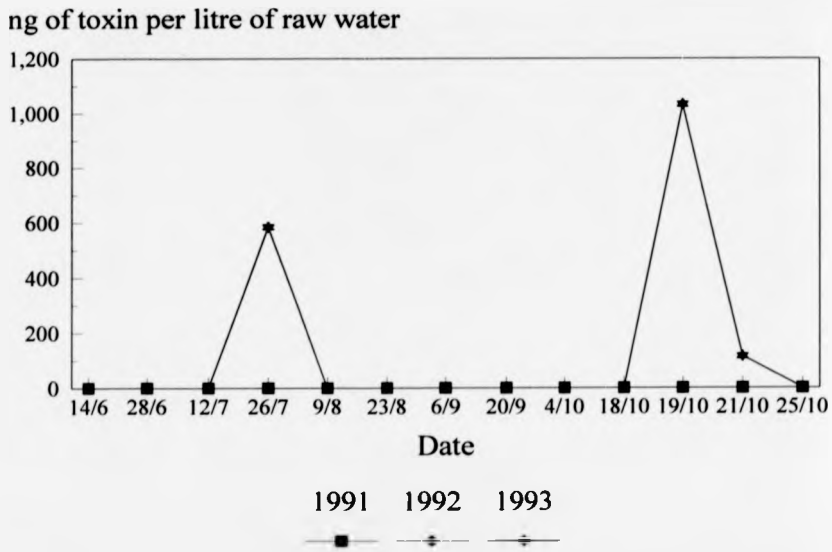
There were only two "cell free" water concentrates that contained microcystins at detectable levels (Table 4.7) both of which were collected during heavy blooms of *Oscillatoria* in the reservoir. These two samples were the same samples as the significantly high toxic level found for whole cell biomass in 1993 (Figure 4.9).

TABLE 4.7 Total concentration of toxins in "cell free" water concentrates from Lower Shustoke during 1993 as detected by HPLC analysis

The concentration of toxins are shown as the "mean" values of the duplicate experiments. The data in the brackets are the minimum and maximum values.

Sample date	**ng of toxins per litre of raw water**
23/8	484 (469, 499)
19/10	1032 (1015, 1049)

FIGURE 4.9 Comparison of total concentration of toxins in raw water concentrates between 1991-1993 as detected by HPLC analysis.



4.3.5 Temporal expression of toxins in Lower Shustoke reservoir during 1991 to 1993

Water samples and cyanobacterial scum (identified as *Oscillatoria* species) were collected from Lower Shustoke reservoir during 1991 to 1993. The amount of cyanobacterial scum varied over the sampling period peaking in the summer months. In order to explain the observed variation in toxicity within blooms of the same cyanobacterial species over time, the temporal expression of the "presumptive" toxins over the monitoring period was investigated.

During 1991 the toxic *Oscillatoria* species from Lower Shustoke was shown to express three distinct peptide toxins. Analysis of the temporal expression of the individual "presumptive" cyanotoxins showed that the ratio of one toxin to the other was found to vary considerably with time and was presumably dictated by either environmental conditions or the growth state of the biomass (Figure 4.10). Toxin expression was found to increase markedly during the summer months. Similar data were generated through 1992 (Figure 4.11). The predominant toxin expressed in both 1991 and 1992 was the third peak which had spectral similarity close to the microcystins. The temporal expression of the "presumptive" toxins was different in 1993 as there were four peptide toxins resolved by HPLC analysis (Figure 4.12). It is most probable that the third major peak shown in 1991 and 1992 data might be resolved into two toxic peaks as per the third and fourth peaks in 1993. Consequently the significantly high concentration of the third peak shown only in samples during 1991 and 1992, was an aggregate of peaks 3 and 4 found for the 1993 samples i.e. the concentration of toxin in peak 3 for 1991/1992 was found to be equivalent to the sum of peaks 3 and 4 for 1993.

During the three year monitoring period there was considerable variation of toxin expression on a per cell basis i.e. there was not one but several structural variants of the peptide toxin (it remains to be established whether or not these were

from the same "peptide toxin family"). It was also apparent that the toxicity of these cells varied and was dependent upon the ratio of the toxic peptide components present in the cells since each reference peptide toxin had a distinct toxicity in mouse bioassay (see Table 3.7).

The variation in cell toxin content and biomass toxicity was presumably dictated by intrinsic and environmental factors such as gene expression, the physiological state of the cell, nutrient concentration, water temperature and light intensity.

FIGURE 4.10 Temporal variation in concentration of toxins in whole cell biomass in 1991. 100 mg of biomass was extracted and analysed. Each eluate peak was referenced to microcystin-LR to facilitate determination of concentration.

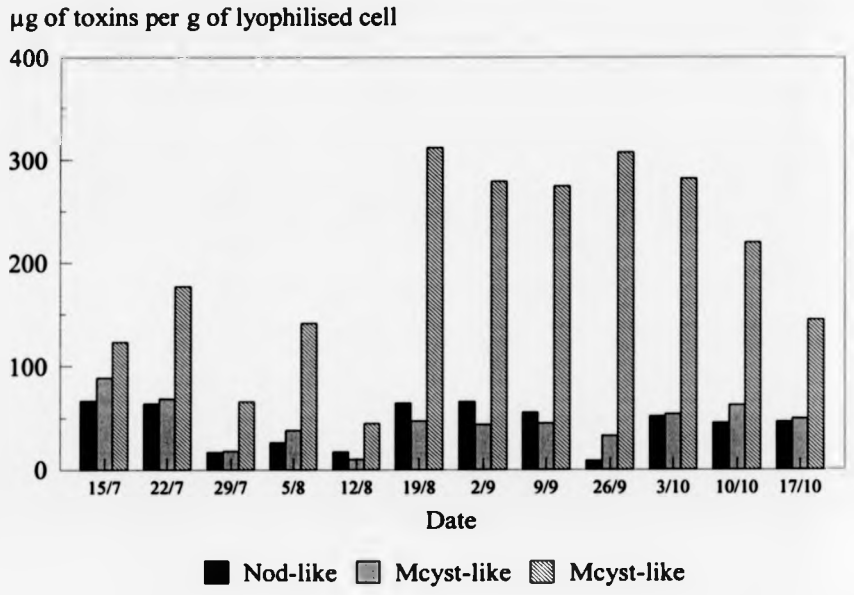


FIGURE 4.11 Temporal variation in concentration of toxins in whole cell biomass in 1992. 100 mg of biomass was extracted and analysed. Each eluate peak was referenced to microcystin-LR to facilitate determination of concentration.

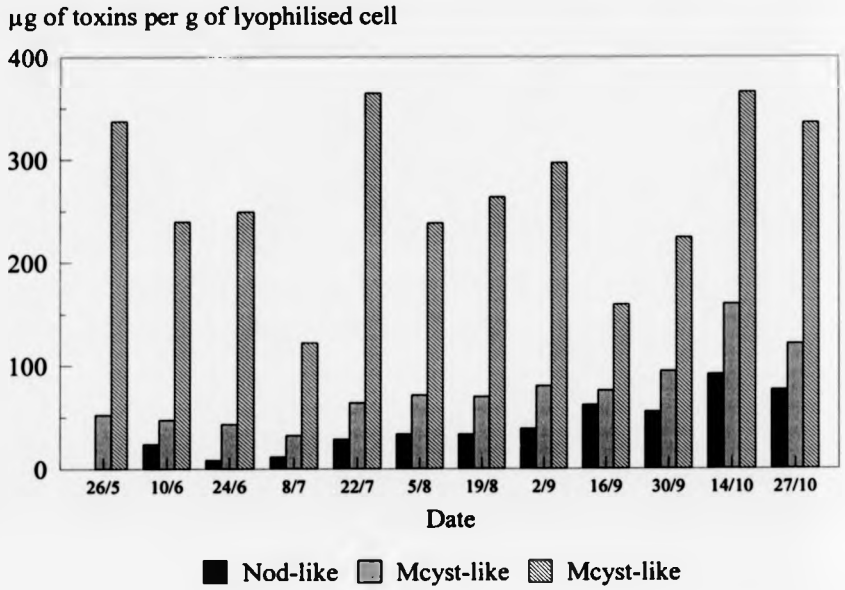
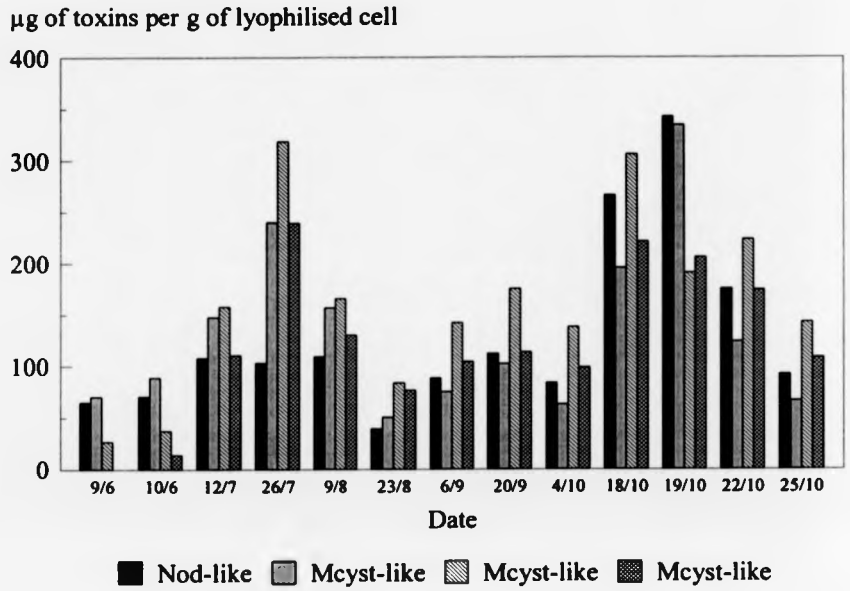


FIGURE 4.12 Temporal variation in concentration of toxins in whole cell biomass in 1993. 100 mg of biomass was extracted and analysed. Each eluate peak was referenced to microcystin-LR to facilitate determination of concentration.



4.3.6 Effects of environmental factors on toxin expression

Since toxin concentration and toxicity varied significantly with time, several measurable environmental factors (both physical and chemical data) were compiled for 1992 and 1993 in order to elucidate whether or not these influenced toxin expression.

In parallel with the cyanobacterial monitoring programme the physical and chemical data i.e. cyanobacterial cell count, pH, temperature, dissolved oxygen, chlorophyll-a, total nitrate, ortho-phosphate and total phosphate from Lower Shustoke reservoir in 1992 and 1993 were accumulated and are given in Tables 4.8 and 4.9, respectively.

TABLE 4.8.a The physical and chemical data from Lower Shustoke reservoir during 1992 : water temperature, dissolved O_2, pH and chlorophyll-a concentration

Date	Temp (°C)	Dissolved O_2 (%)	pH	Chlorophyll-a (mg m^{-3})
29/4	13	-	-	54
20/5	19.4	124.7	9.2	35
26/5	-	-	9.04	51
10/6	18.8	11.6	-	44
24/6	18.7	10.9	-	67
5/8	18.9	93.0	8.35	46
19/8	18.7	9.2	8.8	640
2/9	15.4	9.8	-	2
16/9	14.5	9.5	-	34
30/9	-	-	-	32
14/10	10.7	10.95	-	35
27/10	-	-	-	26
11/11	7.7	10.8	8.35	10
18/11	5.3	13.0	-	10
25/11	6.4	-	-	11
2/12	-	-	-	15
9/12	5.0	-	-	17
16/2	-	-	-	34

TABLE 4.8.b The physical and chemical data from Lower Shustoke reservoir during 1992 : total nitrogen, orthophosphate, total phosphate and cyanobacterial cell count

Date	Total-N ($mg\ l^{-1}$)	Ortho-P ($\mu g\ l^{-1}$)	Total-P ($\mu g\ l^{-1}$)	Cyanobacterial cell count (ml^{-1})
29/4	0.7	190	290	1,000,000
20/5	-	200	300	2,200,000
26/5	-	-	-	1,500,600
10/6	1.2	340	460	1,200,000
24/6	< 0.5	550	620	1,500,000
5/8	0.6	980	980	1,000,000
19/8	< 0.5	930	1060	-
2/9	< 0.5	830	760	870,000
16/9	0.8	640	630	770,000
30/9	< 0.5	560	570	600,000
14/10	< 0.5	370	430	737,000
27/10	< 0.5	250	320	493,000
11/11	2.8	240	260	-
18/11	12.5	140	220	111,000
25/11	21.5	60	170	146,000
2/12	19.2	< 50	100	150,000
9/12	17.5	< 50	70	224,000
16/2	14.8	< 50	70	222,000

TABLE 4.9.a The physical and chemical data from Lower Shustoke reservoir during 1993 : water temperature, dissolved O_2, pH and chlorophyll-a concentration

Date	Temp (°C)	Dissolved O_2 (%)	pH	Chlorophyll-a (mg m^{-3})
1/4	-	-	-	31
5/4	-	-	-	67
19/4	12.1	-	-	75
4/5	-	-	-	64
17/5	12.9	-	8.4	91
1/6	15.5	-	-	90
14/6	-	-	8.1	-
28/6	21.6	-	8.8	58
12/7	18.8	-	8.6	51
26/7	17.0	-	9.0	136
9/8	7.1	-	-	124
23/8	18.3	-	9.4	178
6/9	16.0	-	-	162
20/9	14.8	-	9.2	129
4/10	13.9	-	9.0	78
18/10	9.4	-	-	66

TABLE 4.9.b The physical and chemical data from Lower Shustoke reservoir during 1993 : total nitrogen, orthophosphate, total phosphate and cyanobacterial cell count

Date	Total-N ($mg\ l^{-1}$)	Ortho-P ($\mu g\ l^{-1}$)	Total-P ($\mu g\ l^{-1}$)	Cyanobacterial cell count (ml^{-1})
1/4	41.3	30	60	120,000
5/4	36.8	40	130	81,000
19/4	38.9	30	120	250,000
4/5	38.1	< 20	40	350,000
17/5	31.3	30	60	922,680
1/6	23.1	40	< 30	820,000
14/6	19.4	< 20	-	32,000
28/6	15.2	20	40	500,000
12/7	13.0	30	80	400,000
26/7	5.8	40	50	1,220,000
9/8	< 0.5	90	130	1,200,000
23/8	< 0.5	40	200	1,550,000
6/9	0.8	360	430	590,000
20/9	2.5	150	270	825,000
4/10	< 0.5	170	-	1,150,000
18/10	2.1	210	-	760,000

4.3.6.1 Temperature and pH (Figure 4.13.a and b)

Increased temperature was shown to parallel an increase in toxin production in both years. Higher temperature as in the summer (15-25°C) supported cyanobacterial blooms and toxin production in the reservoir. The toxin concentration dropped dramatically when the temperature was low (5-10°C). On the other hand, pH values were rather constant between the range of 8-9 which may or may not be the appropriate pH values for toxin production.

4.3.6.2 Dissolved oxygen (Figure 4.14)

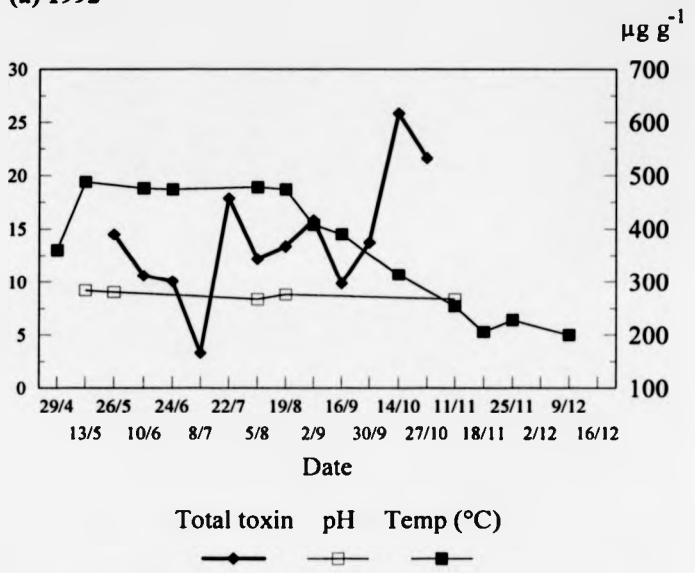
Data for this parameter were only available for 1992 and from the data it is difficult to show any correlation with toxin expression.

4.3.6.3 Chlorophyll-a (Figure 4.15.a and b)

The chlorophyll-a concentration was shown to correlate with the cyanobacterial biomass.

FIGURE 4.13 Temperature, pH and total toxin concentration in Lower Shustoke reservoir.

(a) 1992



(b) 1993

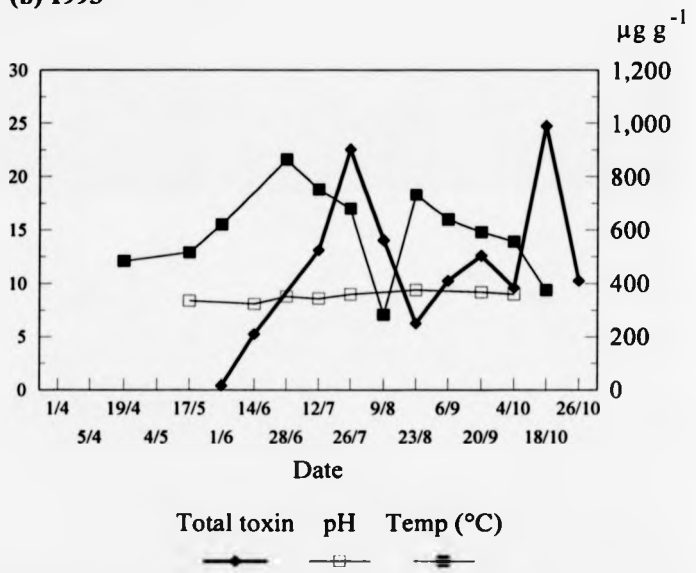


FIGURE 4.14 Dissolved O_2 and total toxin concentration in Lower Shustoke in 1992.

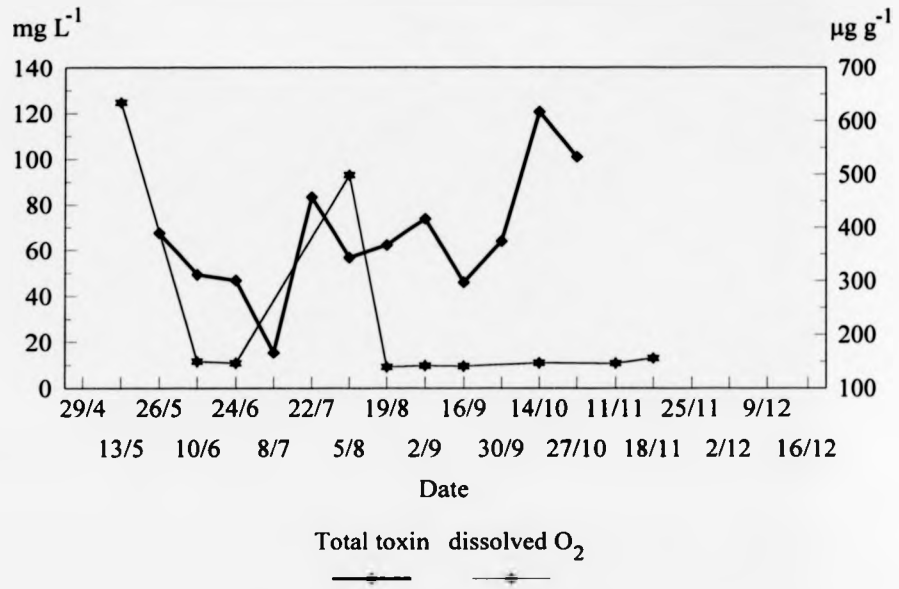
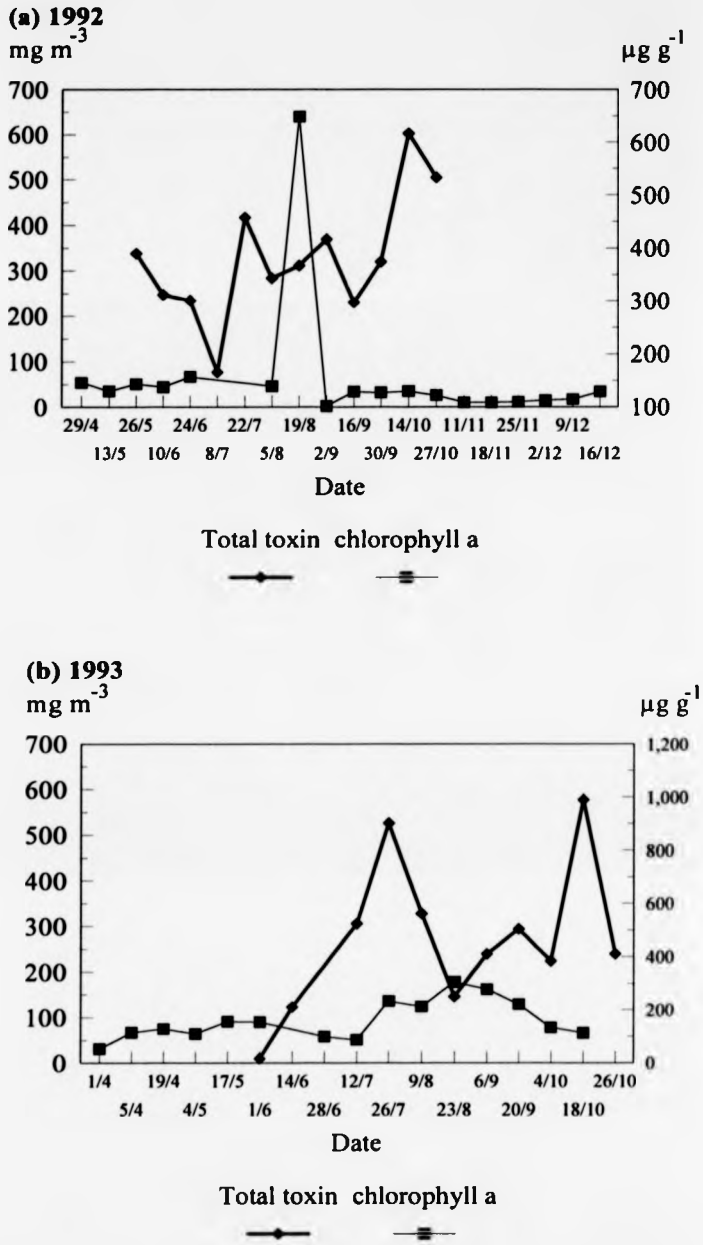


FIGURE 4.15 Chlorophyll a and total toxin concentration in Lower Shustoke reservoir.



4.3.6.4 Orthophosphate and total phosphate (Figure 4.16.a and b)

Orthophosphate and total phosphate concentration paralleled one another. The total phosphate concentration was not significantly higher than the ortho phosphate concentration at the same sampling time. In 1992, both total and orthophosphate values increased dramatically during the summer months (July-September) which was similar to the toxin concentration, although it is not possible to state that they are related to the other. The toxin concentration was very low at the beginning of 1993 (see Figure 4.8) as was the concentration of total and ortho-phosphate in the reservoir. This may have been the result of phosphate stripping from the reservoir which was carried out towards the end of 1992. However, these two parameters have increased gradually since August 1993 with a corresponding increase in the concentration of toxins in the cyanobacterial biomass.

4.3.6.5 Total nitrate (Figure 4.17.a and b)

From April until October 1992 the nitrate concentration in the reservoir was very low there after the nitrate level increased continuously until May 1993 and then started to decline with very low concentrations being reached in August, 1993. These data show that higher concentrations of toxins were apparent with low nitrate concentrations.

4.3.6.6 Cyanobacterial cell count (Figure 4.18.a and b)

Cyanobacterial cell count, as expected, had a direct relationship with the toxin concentration, although the 1993 data showed marked fluctuations.

FIGURE 4.16 Ortho and total phosphate and total toxin concentration in Lower Shustoke reservoir.

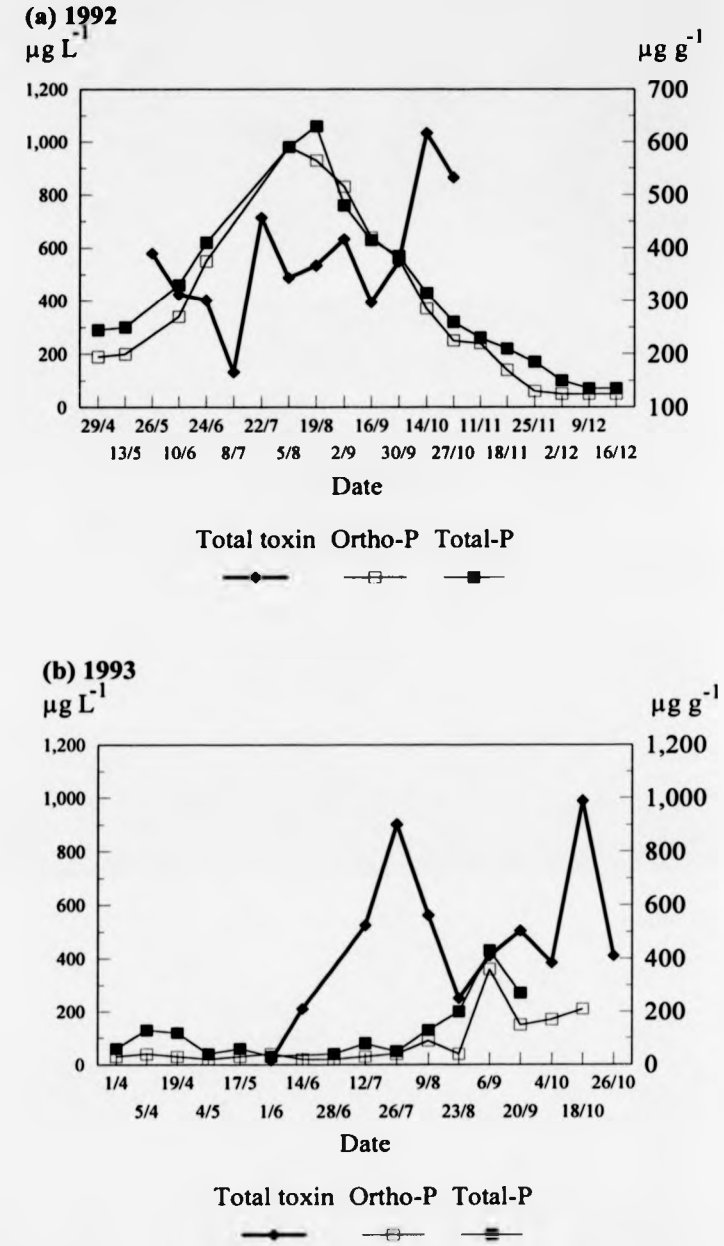


FIGURE 4.17 Total nitrate and total toxin concentration in Lower Shustoke reservoir.

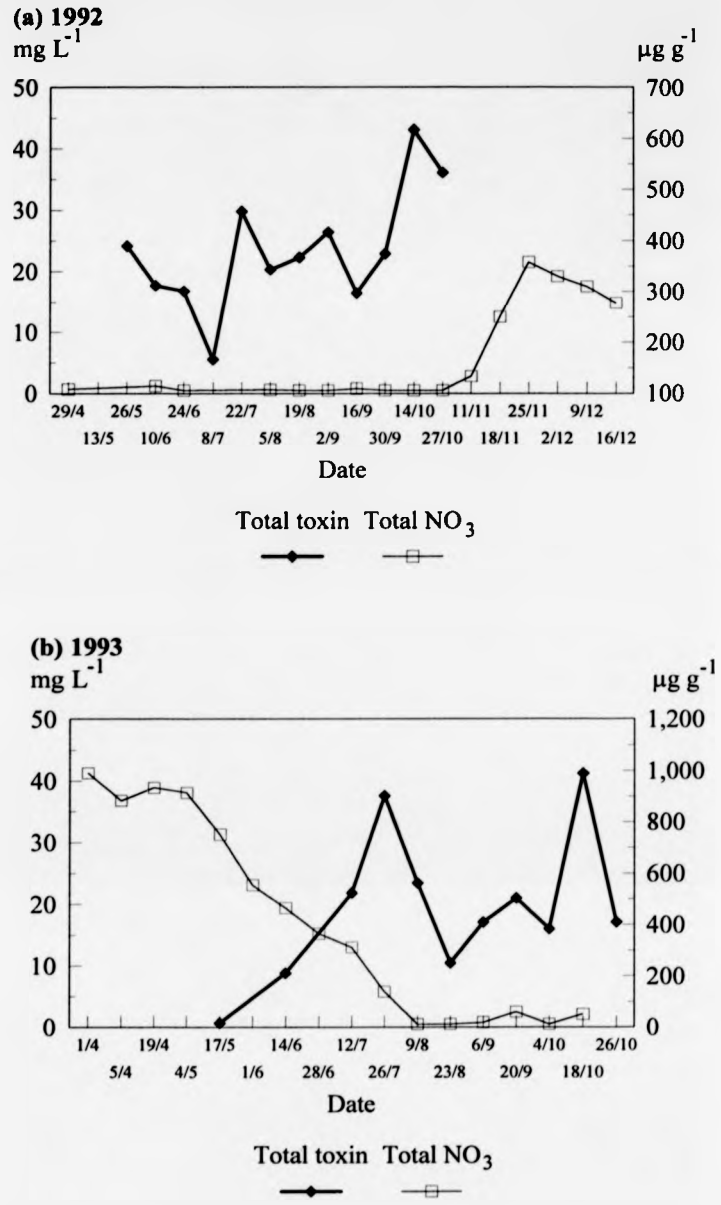
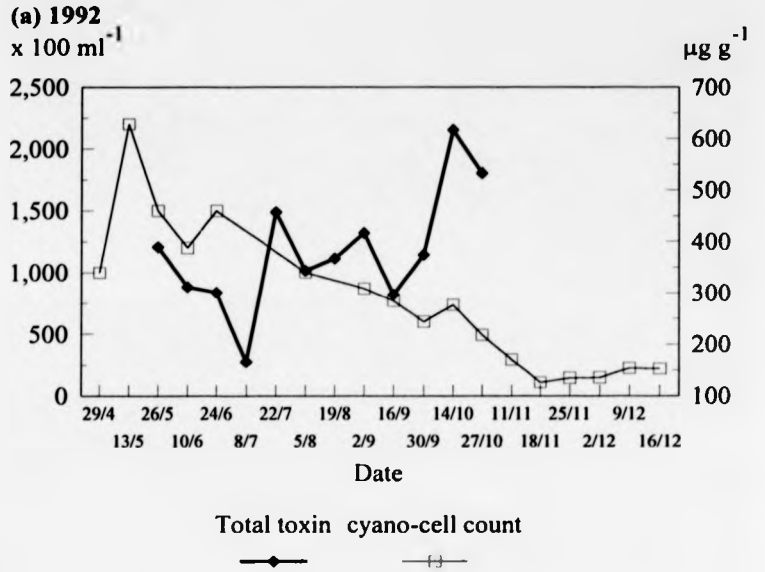
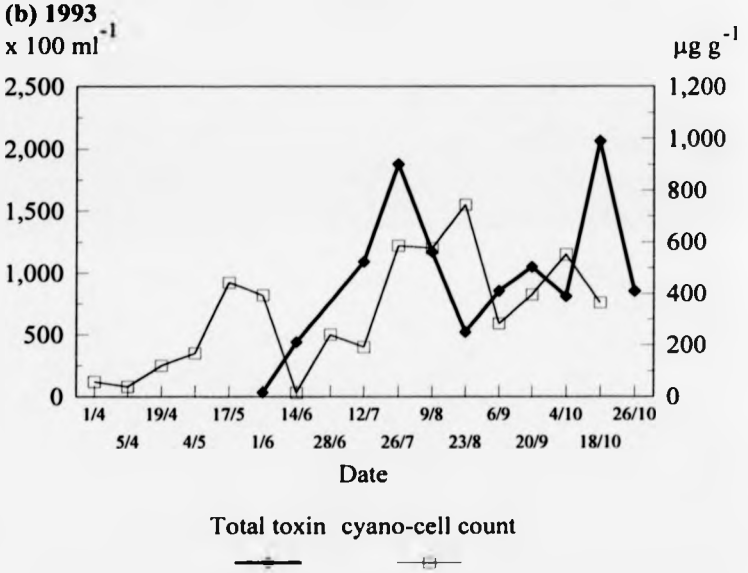


FIGURE 4.18 Cyanobacterial cell count and total toxin concentration in Lower Shustoke reservoir.





4.4 Discussion

Since the first report concerning cyanobacterial poisoning in aquatic environments (Francis, 1878), poisoning and death of wild and domestic animals, including human intoxication, due to toxic blooms of freshwater cyanobacteria have been reported worldwide and would appear to be occurring more frequently. Progressive eutrophication of lakes through changes in land use result in elevated nutrient loading of the aquatic environment a consequence of which is an increase in bloom frequency (Kotak *et al.*, 1993). During the 3-4 years monitoring period, there was an increasing frequency of reservoirs and lakes in the Midlands area supporting the growth of toxic cyanobacteria. The presence of these toxic cyanobacterial species is not just an ecological problem (Eriksson *et al.*, 1986; Lindholm *et al.*, 1989), but has become a human health problem since these reservoirs and lakes are used for recreation and as sources of drinking water.

Two predominant cyanobacterial species, *Pseudoanabaena* and *Oscillatoria*, were present at different times in Lower Shustoke reservoir, one of the freshwater reservoirs in the Midlands which we had chosen for more detailed study of the expression and temporal variation in the concentration of hepatotoxin(s) present in cyanobacterial bloom material. The *Pseudoanabaena* species proved to be non-toxic following intraperitoneal injections of the cell biomass into mice and also no toxic-peak could not be detected by HPLC where the detection limit was down to 20-40 ng toxin. However, these non-toxic samples may have contained very low amounts of toxin(s) in the algae cells or the toxic substance itself had too low a toxicity for detection by mouse bioassay.

On the other hand, the *Oscillatoria* species was considered to be responsible for the toxicity of blooms in Lower Shustoke reservoir since it was the primary species in all of the toxic samples. This was not surprising since *Oscillatoria agardhii* has been reported to be toxic in many studies (Ostensvik *et al.*, 1981;

Leeuwangh *et al.*, 1983; Skulberg *et al.*, 1984; Berg & Soli, 1985; Berg *et al.*, 1986; Eriksson *et al.*, 1988a) and it may also cause severe taste and odour problems (Persson, 1982). Intra-peritoneal injection of this cell biomass into mice gave toxic symptoms compatible with hepatotoxin poisoning, and the toxic peaks detected and analysed by HPLC had spectral characteristics similar to those of nodularin and/or the microcystins supporting the conclusion that this *Oscillatoria* species produced peptide hepatotoxins.

HPLC chromatograms of cell biomass extracts indicated that there were three or four peptide toxins being expressed by the predominant species of *Oscillatoria* from Lower Shustoke. The isolated toxins were not pure compounds since the purity check of these peaks indicated the presence of impurities. Repeated purification should be performed by HPLC or by other chromatographic methods, if the pure toxin(s) is necessary for further analysis.

It was most probable that the toxins discussed above were all heptapeptides with close structural similarity to microcystin and/or nodularin. The amounts of toxins present were estimated by comparison with a calibration curve of reference microcystin-LR. The ratio of the eluted peptides altered significantly with time and the concentration of peptide toxins also varied in the bloom material. It was apparent that the temporal variation in biomass toxicity was dependent upon the ratio of the toxic peptide components present in the cells since each peptide had a distinct toxicity level in mouse bioassay. Alternatively it could be attributed to the difference in the proportions of toxic and non-toxic species within the blooms. However, for the reasons outlined previously (see section 4.3.3.1) this was not the case.

Mouse bioassay was used to confirm the toxicity of the peptide toxins. All mice injected with extracts of *Oscillatoria* biomass showed toxic symptoms that were characteristic of hepatotoxins. The death of the mice occurred as a consequence of irreversible shock resulting from haemorrhage and the increase in

liver weight by pooling of blood. Adams *et al.* (1988) using ^{51}Cr-labelled erythrocytes, found that as much as 44% of the total blood volume of the body could end up pooling in the liver, as compared to 4% in control mice.

Although the *Oscillatoria* cell extracts proved to be toxic, the HPLC profiles of the "cell free" reservoir water concentrates did not show detectable levels of free toxin. The sensitivity of the HPLC analysis was such that samples spiked with concentrations as low as 200 ng litre^{-1} of microcystin-LR and nodularin could be readily detected (Dow *et al.*, 1994). These samples, concentrated to be equivalent to 10 litres of water, were also non-toxic when injected intraperitoneally into mice. Possible explanations are: (i) the toxin(s) is released from degraded cells into the water column but the amount of toxin is diluted by the lake water to be too low for detection by HPLC or (ii) they are rapidly degraded or (iii) they are not readily released from the cyanobacterial biomass.

However, there were only two water samples that contained toxins at detectable levels and which were confirmed to be toxic by mouse bioassay. It was quite noticeable that these two samples were the samples collected at the same time as the significantly high toxic level of the cyanobacterial cell biomass in 1993. This may have been a consequence of the release of toxins into the raw water or contamination of the concentrate by residual cell biomass during the filtration step.

The results obtained from both cell biomass extracts and "cell free" water concentrates indicated substantial temporal variation in the concentration of hepatotoxins present in Lower Shustoke reservoir during 1991-1993. The variability in toxicity as well as the component toxins, might be due to small structural changes presumably dictated by cellular physiology and/or a combination of physical and chemical factors. The environmental factors implicated as being important in the formation of cyanobacterial blooms (e.g. nutrient concentrations, water temperature, light, wind condition, etc.) may also be important in toxin production, although the exact mechanisms are not known (Carmichael, 1986). In this study, the physical and

chemical data from Lower Shustoke reservoir during 1992 to 1993 have been compiled and compared with toxicity and toxin concentration. Analysis of these data indicate that the concentration of hepatotoxins produced by *Oscillatoria* positively correlates with surface water temperature, cyanobacterial cell count, as well as ortho and total phosphate concentration while there is a negative correlation with total nitrate concentration. The correlation between the concentration of toxins and chlorophyll-a concentration had a direct relationship where as there was insufficient data to firmly establish the relationship between the concentration of toxins and dissolved oxygen in the reservoir. The pH values of the reservoir water were constant in the range of 8-9. The pH values of between 8-9, higher phosphate concentration and high temperature promote the growth of cyanobacteria and/or toxin production. It was difficult to conclude whether nitrate deficiency had any effect on toxin production.

Further studies, therefore, need to be undertaken to determine the biological and environmental factors which effect toxin production.

A further objective of this study was to fully characterise these peptides by mass spectrometry.

CHAPTER 5

CHAPTER 5 : INHIBITORY EFFECT OF PEPTIDE TOXINS ON HEPATIC PHOSPHORYLASE PHOSPHATASES

5.1 INTRODUCTION

Microcystis aeruginosa, the most common toxic cyanobacteria so far studied, produces potent hepatotoxins which have been implicated in the deaths of birds, fish, wild and domestic animals (Skulberg *et al.*, 1984; Carmichael, 1992) including human illness, and have most frequently been associated with the proliferation of cyanobacteria in reservoirs (Bourke & Hawes, 1983; Falconer *et al.*, 1983a; Codd & Poon, 1988). At the lethal dose, death by hepatotoxin poisoning occurs within 1 to 2 hours as a result of haemorrhagic shock caused by pooling of blood in the liver from the destruction of the endothelial lining of the liver sinusoids and the disintegration of hepatocyte membranes (Falconer *et al.*, 1981). As the liver is the major target organ of the microcystins, Runnegar *et al.* (1981) suggested that the specificity of the toxin for the liver may be due to transport of the toxin into the hepatocyte by the bile acid transport system.

Protein phosphatases, the enzymes involved in the regulation of glycogen metabolism, are a heterogeneous group of enzymes of almost ubiquitous occurrence that are involved in reversing the action of protein kinases (Figure 5.1). The interconversion between the two forms of muscle phosphorylase (phosphorylase *a* and *b*) by the converter enzymes phosphorylase kinase and phosphorylase phosphatase constitutes a major mode of control of energy metabolism.

FIGURE 5.1 Schematic diagram of the control of glycogen phosphorylase in skeletal muscle.

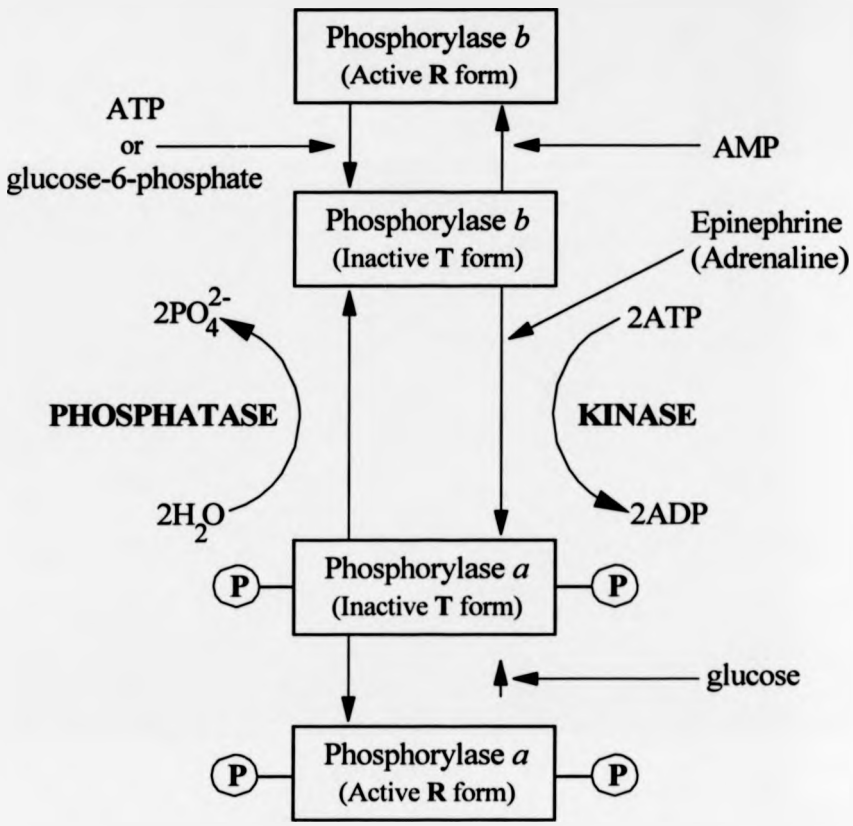


Figure 5.1 shows that phosphorylase *b* is active only in the presence of high concentrations of AMP which binds to the nucleotide binding site and alters the conformation of phosphorylase *b*. The active form of phosphorylase *b* can phosphorylate glycogen to produce glucose-6-phosphate. The end product inhibition and/or accumulation of ATP will downregulate the activity of this enzyme. However, when acted upon by epinephrine (adrenaline), phosphorylase *b* undergoes phosphorylation to phosphorylase *a* which can also phosphorylate glycogen. Phosphorylase *a* is deactivated by a specific phosphatase that hydrolyses the phosphoryl group attached to serine 14 (the basis of the main assay in this project).

Four major classes of serine/threonine-specific protein phosphatase catalytic subunits have been identified in the cytoplasmic compartment of mammalian cells (Cohen *et al.*, 1988). These enzymes have been classified into two types : protein phosphatase type 1, which dephosphorylates the β-subunit of phosphorylase kinase and is inactivated by two thermostable proteins, inhibitor 1 and inhibitor 2; protein phosphatase type 2, which dephosphorylates the α-subunit of phosphorylase kinase and is insensitive to inhibitors 1 and 2. The type 2 phosphatase comprises 3 distinct groups of enzymes, termed 2A, 2B and 2C depending on their requirements for divalent cations. The type 2A phosphatase is active in the absence of divalent cations, where as types 2B and 2C have absolute requirements for Ca^{2+} and Mg^{2+}, respectively.

Recently, many studies have reported that okadaic acid, a non-phorbol ester tumour promoter produced by *Prorocentrum lima* (Bialojan & Takai, 1988; Suganuma *et al.*, 1989; Holmes *et al.*, 1990) and microcystin-LR (Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990; Matsushima *et al.*, 1990; Nishiwaki-Matsushima *et al.*, 1992) are both potent and specific inhibitors of protein phosphatases type 1 and 2A, although the structure of microcystin-LR is not related to that of okadaic acid.

5.1.1 Aims

Since the primary target of the peptide toxins is the liver and the hepatotoxicity of the toxins may result from the inhibition of the liver protein phosphatases, this inhibition can be used as an assay for cyanobacterial hepatic peptide toxins. Furthermore, the microcystins have a particularly high affinity for protein phosphatases (MacKintosh *et al.*, 1990) enabling very low levels of toxin to be detected.

The aim of this project is to examine the inhibition of phosphorylase phosphatases by purified microcystins, nodularin, okadaic acid as well as the presumptive toxins from environmental samples. The method will also be examined as an alternative assay for toxicity assessment, since the mouse bioassay, which has been used as the primary means of detecting cyanotoxicity, poses ethical problems and excludes possible accumulative effects of subtoxic levels of toxins remaining undetected. Crude mouse liver homogenate has been used as the source of protein phosphatases 1 and 2A as the purification of protein phosphatases is rather complicated and time-consuming and therefore not suitable for routine use.

5.2 Materials and methods

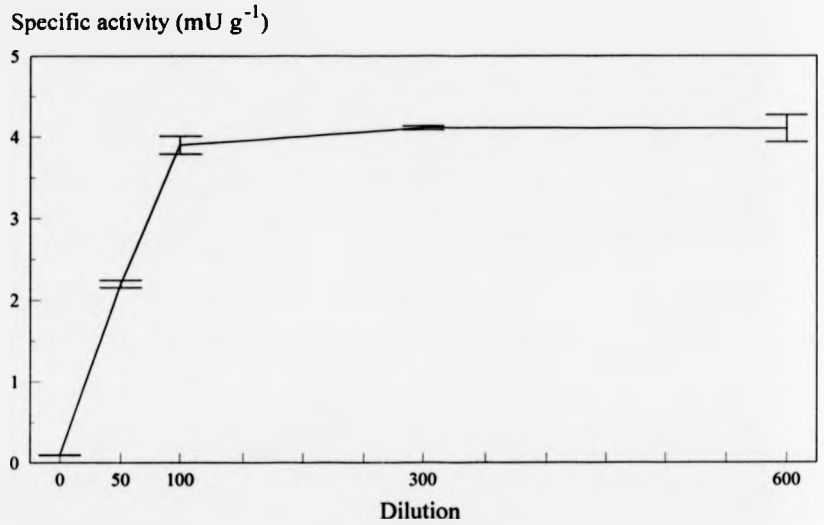
The basic materials and methods utilised in this study have been given in section 2.12.

5.3 Results

5.3.1 Effect of dilution on the inhibition of phosphorylase phosphatase activity

It was necessary to elucidate the optimum dilution to be employed when measuring protein phosphatase activities in crude tissue extracts of muscle and liver since as high a dilution as possible (Ingebritsen *et al.*, 1983b) is required in order to avoid underestimation of activity. Experiments were carried out to measure the phosphatase activities of liver extract (15 mg ml^{-1}) and a dilution series of 1:50, 1:100, 1:300 and 1:600 (or 300 $\mu g\ ml^{-1}$, 150 $\mu g\ ml^{-1}$, 50 $\mu g\ ml^{-1}$ and 25 $\mu g\ ml^{-1}$, respectively) in assay buffer. The effect of dilution on crude liver homogenate prepared as per the above method (see section 2.12) is shown in Figure 5.2. The phosphatase activity was strongly dependent on the dilution up to 100-fold, whereas at greater dilutions e.g. 100, 300 and 600-fold of crude mouse liver homogenate, the activity was approximately the same. Therefore, the assays should be performed at a final dilution of at least 100-fold, within the above experimental conditions, to eliminate dilution effects.

FIGURE 5.2 Effect of dilution on phosphorylase phosphatase activity in crude mouse liver homogenate. The reaction time was 20 minutes, at 30°C. Each point is the mean of triplicate assays ± S.D. at the indicated final dilution of the crude mouse liver homogenate.

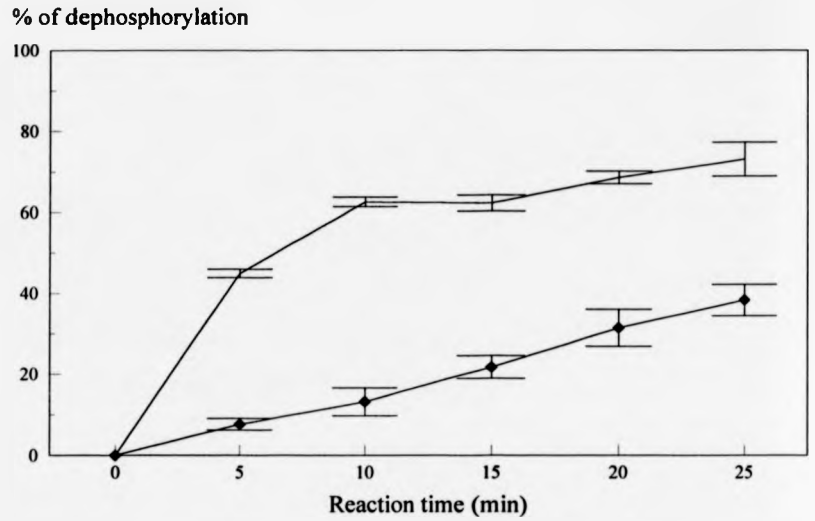


5.3.2 Time course for the inhibition of phosphorylase phosphatase activity

The phosphorylase assays frequently become non-linear when more than 30% of the (^{32}P) phosphorylase *a* is dephosphorylated (Cohen *et al.*, 1988). To ensure that rates of dephosphorylation were linear with respect to time, the % dephosphorylation was determined with variation in reaction time up to 25 minutes at 30°C (Figure 5.3). A linear-gradient between % dephosphorylation and reaction time was obtained when crude mouse liver homogenate at a dilution 100-fold was used in the assay. In contrast, the reaction was seen to reach its steady-state phase within 10 minutes for the undiluted crude mouse liver homogenate as a consequence of substrate limitation.

Hence, assays of protein phosphatase activity in this study were performed using crude mouse liver homogenate at a dilution of 100-fold (150 μg protein ml^{-1}) with an incubation time of 20 minutes, then following the procedure given in section 2.12.6.

FIGURE 5.3 Rate of dephosphorylation in crude mouse liver homogenate. The undiluted crude mouse liver homogenate and the 1:100 dilution in assay buffer were incubated for the indicated time, at 30°C. Each time point is the mean of triplicate assays ± S.D.



5.3.3 Microcystins and nodularin as inhibitors of protein phosphatase activity in an *in vitro* system

The phosphorylase activity was determined from the amount of ^{32}P released during a 20 minute reaction time. The inhibition was measured by comparing the phosphatase activity of the assays to the non-toxic control which was expressed as 100% phosphatase activity. As shown in Figure 5.4 and 5.5, the *in vitro* activity of phosphorylase phosphatase was strongly inhibited by the three reference toxins in a concentration dependent manner. The necessity for dilution of the crude mouse liver homogenate was confirmed when the enzymes were inhibited by microcystin-LR (Figure 5.4.a), microcystin-RR (Figure 5.4.b) and nodularin (Figure 5.4.c). The IC_{50}, or the concentration giving 50% of maximum inhibition of phosphorylase phosphatase activity was shown to vary approximately 10 times between the undiluted and the 100-300 fold dilution of crud e mouse liver homogenate. However, the IC_{50}s of microcystin-LR, microcystin-RR and nodularin obtained from the standard inhibition curves (Figure 5.5) were 2.4 nM, 2.8 nM and 2.3 nM, respectively, when the crude mouse liver homogenate was diluted below the end point (the dilution after which no change in IC_{50} was observed with subsequent dilution). The IC_{50} concentration of microcystin-LR (IC_{50} = 2.4 nM) was used as the standard value for the calculation of the concentration of toxins contained in the environmental cyanobacterial samples.

FIGURE 5.4 Influence of enzyme concentration on inhibition of phosphorylase phosphatase activity by (a) microcystin-LR, (b) microcystin-RR and (c) nodularin. Assays were carried out with phosphorylase *a* as substrate. The undiluted, 100- and 300-fold dilutions of crude mouse liver homogenate served as the source of enzymes. The assay mixture (100 µl) was incubated for 20 minutes at 30°C (n=4).

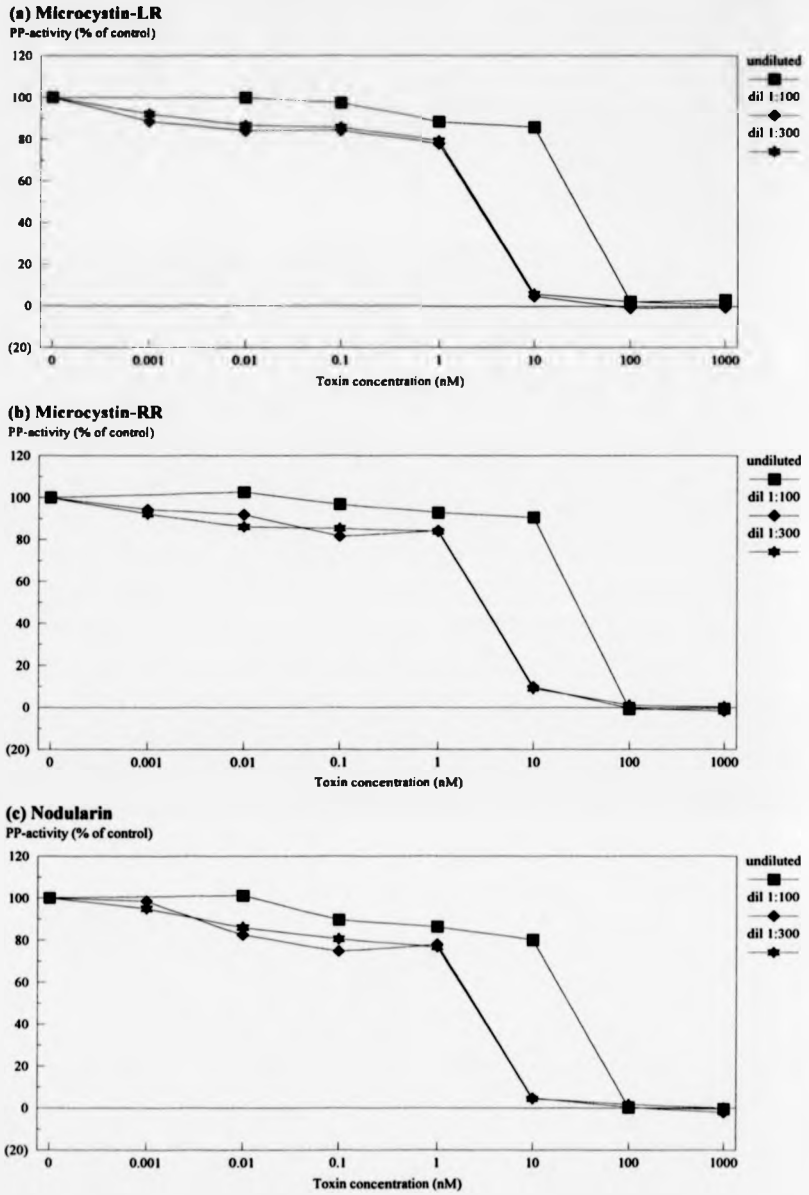
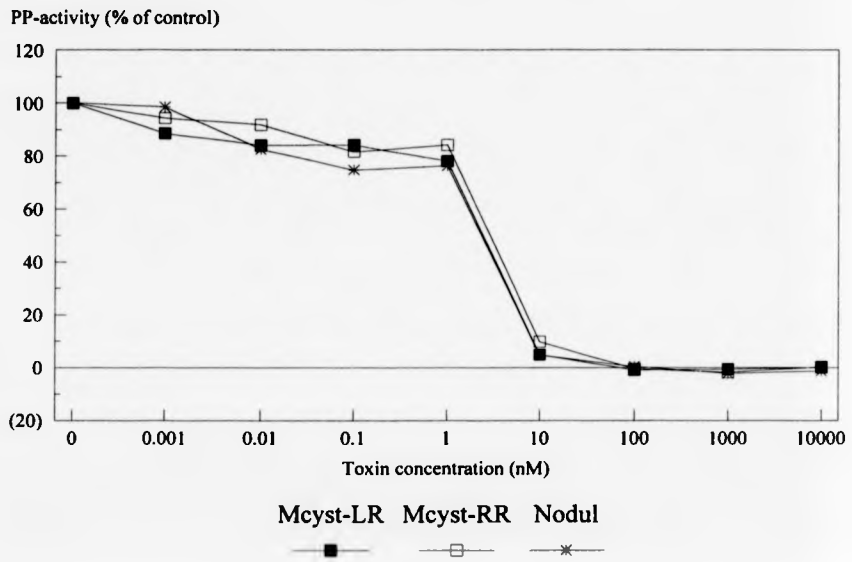


FIGURE 5.5 Standard inhibition curves of the purified peptide toxins; microcystin-LR, microcystin-RR and nodularin on the activity of protein phosphatase. The crude mouse liver homogenate which served as the source of enzymes was assayed at a 100-fold dilution. The assay mixture (100 µl) was incubated for 20 minutes at 30°C (n=3).



5.3.4 Okadaic acid as an inhibitor of phosphorylase phosphatase activity in an *in vitro* system

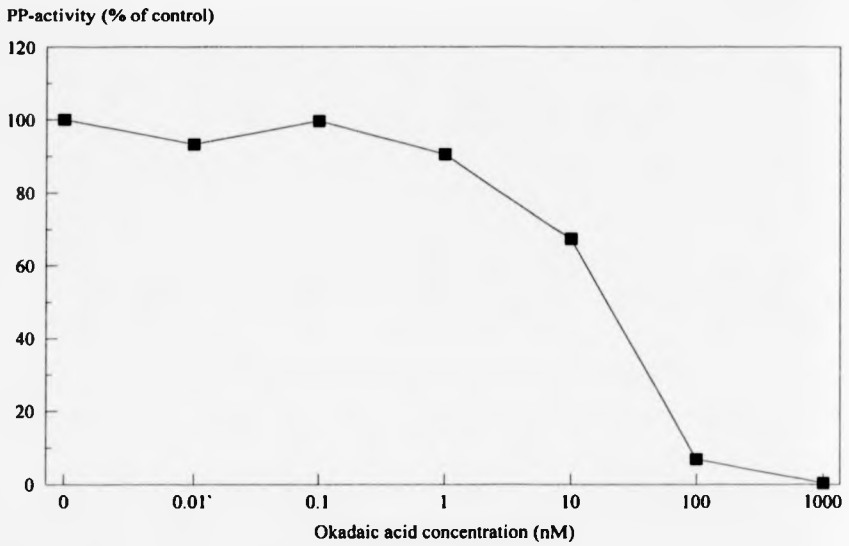
Since okadaic acid, a potent tumour promoter, has been shown to have an inhibitory effect on protein phosphatase activity (see section 5.1), a toxicity comparison between this toxin and the cyanobacterial hepatotoxins was undertaken. Using the same assay conditions, Figure 5.6 shows that the inhibition curve for okadaic acid was similarly to those for the microcystins and nodularin. Although the IC_{50} of okadaic acid was also in the nanomolar region, it was 10 times less toxic than the cyanotoxins (Table 5.1).

TABLE 5.1 IC_{50} of the microcystins and nodularin compared with that of okadaic acid

Toxin	* IC_{50} (nM)
Microcystin-LR	2.4
Microcystin-RR	2.8
Nodularin	2.3
Okadaic acid	24.5

* The concentration giving 50% of maximum inhibition of phosphorylase phosphatase activity

FIGURE 5.6 Inhibition effect of okadaic acid on the activity of protein phosphatase. The crude mouse liver homogenate which served as the source of enzymes was assayed at a 100-fold dilution. The assay mixture (100 μl) was incubated for 20 minutes at 30°C (n=3).



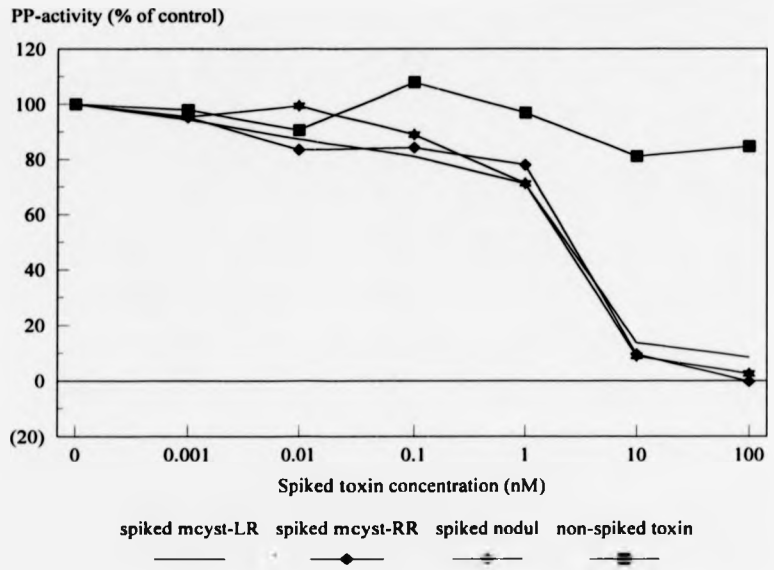
5.3.5 Detection of purified toxins in the presence of non-toxic cell biomass

From the previous studies on the purified toxins, the inhibition of phosphorylase phosphatase appeared to be a promising procedure for toxicity assessment. However, an important prerequisite before using the method to determine the amount of toxins in environmental samples was to undertake evaluation of the efficiency and precision of the assay to confirm the sensitivity of the method. Non-toxic biomass was spiked with purified microcystin-LR, microcystin-RR and nodularin at a concentration of 100 nM toxin per 1000 μg ml^{-1} cell biomass and analysed by the phosphatase assay (Figure 5.7). The inhibition curves, as shown in Figure 5.7, indicated that the IC_{50}s of the non-toxic cell biomass spiked with microcystin-LR, microcystin-RR and nodularin were 2.32 nM, 2.43 nM and 2.67 nM, respectively. Table 5.2 shows the % detection of the three purified toxins in the presence of non-toxic cell biomass. The % detection was determined by comparing the IC_{50} of each purified toxin to that of the spiked toxin cell-biomass. % recovery of microcystin-LR, microcystin-RR and nodularin were 96.7%, 86.8% and 116.1%, respectively.

TABLE 5.2 Detection of purified toxin in the presence of non-toxic biomass expressed as % recovery

Purified toxin	%Recovery
Microcystin-LR	96.7 %
Microcystin-RR	86.8 %
Nodularin	116.1 %

FIGURE 5.7 Inhibition effects of whole cell biomass spiked with purified toxic cell biomass; spiked microcystin-LR (—), spiked microcystin-RR (♦—♦) and spiked nodularin (✱—✱), compared to non-toxic cell biomass (■—■). The crude mouse liver homogenate which served as the source of enzymes was assayed at a 100-fold dilution. The assay mixture (100 µl) was incubated for 20 minutes at 30°C (n=3).



5.3.6 Use of okadaic acid as an inhibitor of specific phosphorylase phosphatase in tissue extracts

The total inhibition of protein phosphatase type 2A by okadaic acid is about 100 times more toxic than type 1 i.e. the type 2A protein phosphatase is inhibited completely by 1-2 nM okadaic acid while type 1 protein phosphatase requires at least 1 μM okadaic acid for inactivation (Hardie *et al.*, 1991). Since protein phosphatases type 1 and type 2A are the only significant phosphorylase phosphatase activities in tissue extracts (Ingebritsen & Cohen 1983; Ingebritsen *et al.*, 1983a), okadaic acid can be used as a specific inhibitor of type 2A protein phosphatase in order to measure the type 1 phosphatase in crude mouse liver homogenate.

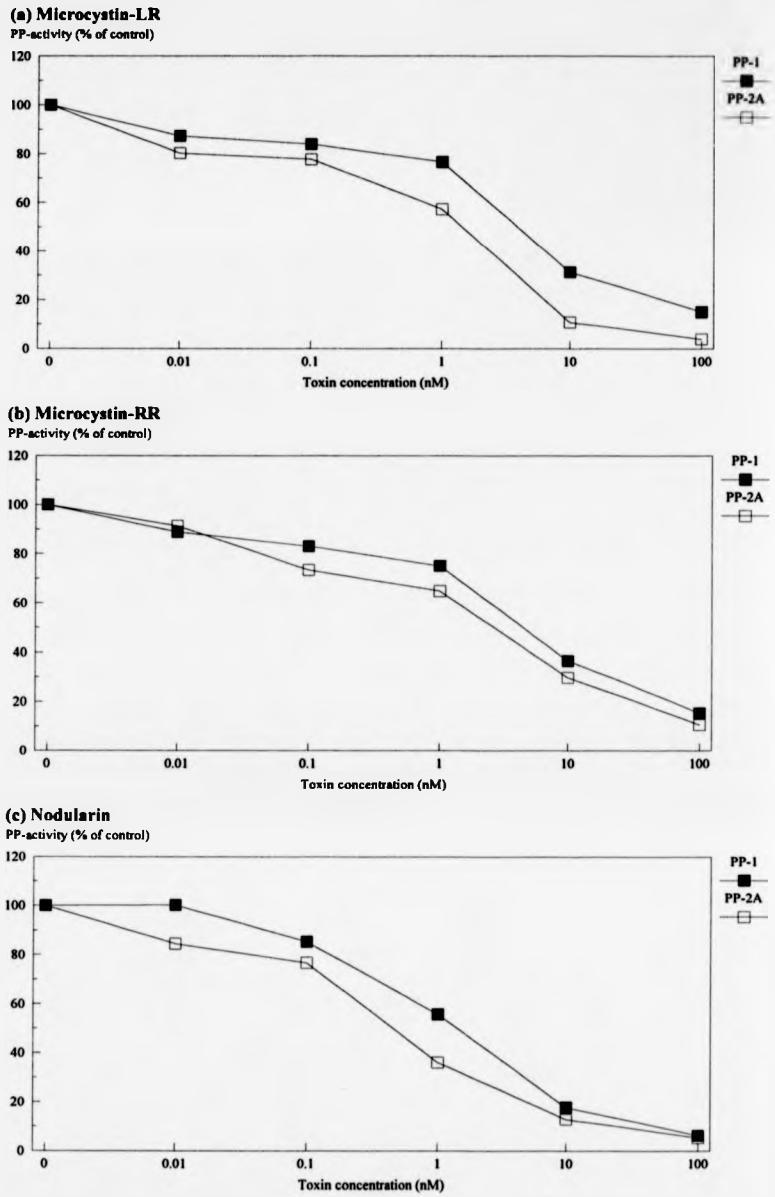
In this study, 1 nM okadaic acid was added to the assays which were preincubated for 15 minutes, followed by the addition of the microcystins or nodularin and the procedure described earlier carried out. This step allowed okadaic acid to interact with protein phosphatase type 2A without having any effect on protein phosphatase type 1. The IC_{50} from this experiment was therefore only derived from the effect of the hepatotoxins on the protein phosphatase type 1. For each assay of different concentrations of microcystins/nodularin, an assay without the addition of okadaic acid was performed under the same conditions to obtain the IC_{50} of protein phosphatases type 1 and type 2A. Thus, the IC_{50} of microcystins/nodularin on type 2A protein phosphatase was obtained by deduction of the IC_{50} obtained for type 1 from the total IC_{50} value. Table 5.3 shows the IC_{50}s of the three purified toxins on protein phosphatases type 1 and type 2A in crude mouse liver homogenate. These IC_{50}s were determined from the inhibition curves of microcystin-LR (Figure 5.8.a), microcystin-RR (Figure 5.8.b) and nodularin (Figure 5.8.c). The other types of protein phosphatases that may be contained in the crude mouse liver homogenate were assumed to account for only a minor proportion of the phosphatase activity.

TABLE 5.3 Effects of microcystins and nodularin on type 1 and 2A protein phosphatase. Okadaic acid was used as the specific inhibitor of type 2A protein phosphatase

Toxin	* IC_{50}	
	PP-1	**PP-2A**
Microcystin-LR	3.9 nM	1.44 nM
Microcystin-RR	4.35 nM	2.55 nM
Nodularin	1.7 nM	0.56 nM

* The concentration giving 50% of maximum inhibition of phosphorylase phosphatase activity

FIGURE 5.8 Inhibitory effects of (a) microcystin-LR, (b) microcystin-RR and (c) nodularin on type 1 phosphatases in crude mouse liver homogenates. Assays were preincubated with 1 nM okadaic acid at 30°C for 15 minutes to inhibit type 2A protein phosphatases before carrying out the assay procedure (see section 5.3.6) (n=3).



5.3.7 Toxicity assessment of peptide toxins from *Oscillatoria* blooms by phosphatase assays.

From the previous data, protein phosphatase assay was shown to be an effective method for assessing the toxicity of the cyanobacterial hepatotoxins with high sensitivity and reproducibility. This assay was therefore used to assess the toxicity level of the peptide toxins in/and from environmental samples. The *Oscillatoria* species studied was the predominant cyanobacterial species present in Lower Shustoke during the three year monitoring peroid. The toxicity of all of the cell-biomass extracts, raw water concentrates and the eluate fractions from HPLC analysis were determined. Since protein phosphatases are a heterogeneous group of enzymes of almost ubiquitous occurrence (see section 5.1), it is important to assay for the presence of endogenous protein phosphatase activity in cyanobacterial samples since this endogenous value might affect the toxicity assessment.

5.3.7.1 Calculation of toxin concentrations

In this study, all toxin concentrations were calculated by comparison with microcystin-LR as the reference toxin. The IC_{50} of microcystin-LR was determined from the standard inhibition curve as shown in Figure 5.5 (see section 5.3.3). Microcystin-equivalent concentrations present in cyanobacterial samples were determined by comparing the concentration of cell-extracts or raw water concentrates or the toxic eluate fractions which produced 50% inhibition of protein phosphatase activity with the IC_{50} of microcystin-LR (IC_{50} = 2.4 nM).

5.3.7.2 The presence of endogenous protein phosphatase activity

The endogenous protein phosphatase activity of cyanobacterial cell biomass was determined in assays carried out as described in "Materials and Methods" (see section 5.2), with the exception that crude mouse liver homogenate was absent. In this assay, the cyanobacteria served as the source of protein phosphatase enzymes. The freeze-dried cells (see section 2.7) were resuspended in assay buffer at a concentration of 10-25 mg ml^{-1}. The suspensions were then sonicated in a sonicating waterbath for 10 minutes. This step was repeated 3 times, the extracts were then serially diluted in assay buffer and assessed for the activity of protein phosphatases. Comparison with the control assay (the basal activity) showed no activity could be detected in any sample. This result shows that the *Oscillatoria* from Lower Shustoke reservoir does not contain the same types of protein phosphatases present in crude mouse liver homogenate which are inhibited by the hepatotoxins.

5.3.7.3 Toxicity assessment of environmental samples

Using crude mouse liver homogenate as the source of the enzymes, under the same assay conditions as described previously, the environmental toxins inhibited the activity of protein phosphatase giving similar inhibitory curves to those of the reference toxins. The IC_{50} inhibition concentrations obtained were shown to differ between the cell-biomass extracts, raw water concentrates and the toxic eluate fractions from HPLC analysis over the study times. The concentration of toxins was calculated as described earlier (see section 5.3.7.1).

5.3.7.3.1 *Whole cell-biomass extracts*

As shown in Table 5.4, the IC_{50}s of the whole cell-biomass extracts on the activity of phosphorylase phosphatase varied over the 3 years of this study. Although only a few samples gave sufficient cell biomass in 1992, toxicity assessments in 1991 were shown to be approximately the same as those for 1992. Toxicity assessments of the cell extracts in 1993 were on average 2 times higher than those for 1991 and 1992 with significantly higher toxic levels in samples collected in July and October (Figure 5.9). The samples collected during the beginning or the end of the year inhibited phosphatase activity only at very high concentrations of cell-biomass (>2,500 μg dry wt cell in 1 ml assay volume) and also did not show any effect when injected intraperitoneally into mice.

5.3.7.3.2 *Raw water concentrates*

Raw water samples from Lower Shustoke reservoir collected (and concentrated) during cyanobacterial blooms and at other times throughout the year were also tested for their effects on phosphatase activity. Table 5.5 shows the IC_{50}s of the raw water samples. The variations were rather similar to the whole cell extracts (see section 5.3.7.3.1). The 1993 samples were shown to be more toxic than those in 1991 and 1992. Temporal expression of the toxins from assays of raw water collected in 1991, 1992 and 1993 are shown in Figure 5.10.a, 5.10.b and 5.10.c, respectively. Most of the samples had high IC_{50}s (concentrates equivalent to 150 ml or more of "cell free" filtrate in 1 ml assay volume) and were also shown to have no toxic effect by mouse bioassay. The two samples in July and October 1993, which contained particularly high toxic levels, caused the death of mice when after intraperitoneal injection.

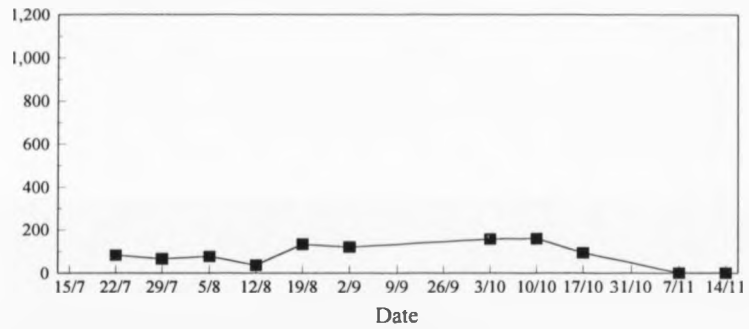
TABLE 5.4 The IC_{50} values of protein phosphatase activity for *Oscillatoria* biomass extracted from Lower Shostoke reservoir during 1991 to 1993. Each IC_{50} was calculated in 1 ml assay volumes

The IC_{50} values are shown as the "mean" values of the triplicate experiments. The data in the brackets are the minimum and maximum values. The toxicities are at the high levels during summer months (as low levels of IC_{50} values). However, during the beginning and the ends of years the toxicities are at the low levels (as high levels of IC_{50} values).

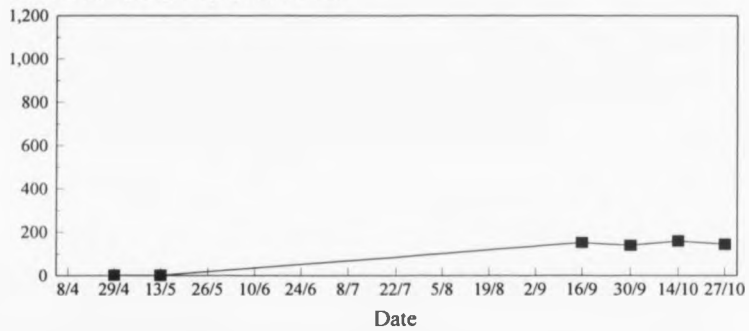
Date	IC_{50} (μg)	Date	IC_{50} (μg)
22/7/91	28.8 (26.5, 31.7)	4/5/93	347 (343, 349)
29/7/91	36.3 (32.4, 40.2)	17/5/93	190 (173, 210)
5/8/91	31.0 (29.6, 32.2)	1/6/93	200 (184, 214)
12/8/91	66.0 (64.8, 67.9)	9/6/93	26.2 (24.2, 28.3)
19/8/91	17.9 (16.9, 19.2)	12/7/93	7.6 (6.9, 8.3)
2/9/91	19.8 (16.1, 24.4)	26/7/93	3.5 (3.2, 3.8)
3/10/91	15.2 (14.7, 15.8)	9/8/93	6.6 (5.3, 7.8)
10/10/91	15.0 (13.7, 16.5)	23/8/93	11.7 (10.2, 13.4)
17/10/91	25.5 (24.2, 26.8)	20/9/93	6.6 (5.9, 7.6)
7/11/91	6,100 (6057, 6150)	4/10/93	6.8 (6.2, 7.3)
2/11/91	5,500 (5427, 5549)	18/10/93	3.1 (2.9, 3.2)
		19/10/93	2.2 (2.1, 2.4)
29/4/92	2,550 (2493, 2605)	21/10/93	9.6 (8.7, 10.4)
13/5/92	4,850 (4823, 4875)	22/10/93	9.0 (8.5, 9.6)
16/9/92	15.8 (13.9, 17.4)	25/10/93	5.9 (5.2, 6.4)
30/9/92	17.2 (15.8, 18.3)		
14/10/92	15.0 (13.3, 17.0)		
27/10/92	16.6 (14.9, 18.2)		

FIGURE 5.9 Temporal expression of toxin concentration in *Oscillatoria* cell biomass determined by phosphatase assay, during 1991-1993. The toxin concentration was calculated by comparison with the reference microcystin-LR.

(a) Toxin concentration in cell biomass (1991)



(b) Toxin concentration in cell biomass (1992)



(c) Toxin concentration in cell biomass (1993)

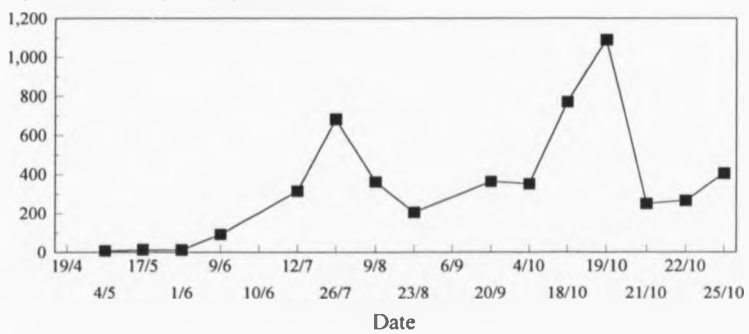


TABLE 5.5 The IC_{50} values of protein phosphatase activity for "cell free" water concentrates from Lower Shustoke reservoir during 1991 to 1993. Each IC_{50} was calculated in 1 ml assay volume (n=3)

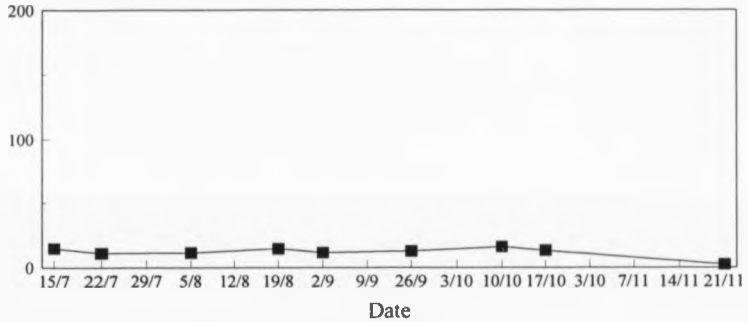
The IC_{50} values are shown as the "mean" values of the triplicate experiments. The data in the brackets are the minimum and maximum values. The toxicities are at the low levels for most of the sampling times. However, the toxicities of some samples in 1993 are at the high levels (as low levels of IC_{50} values).

Date	IC_{50} (ml)	Date	IC_{50} (ml)
15/7/91	160 (155, 163)	4/5/93	351 (338, 366)
22/7/91	213 (199, 228)	17/5/93	20.1 (19.3, 20.9)
5/8/91	207 (195, 215)	1/6/93	58.8 (58.2, 59.7)
19/8/91	163 (154, 171)	9/6/93	22.2 (21.7, 22.5)
2/9/91	205 (189, 222)	14/6/93	14.8 (13.5, 16.0)
26/9/91	188 (182, 198)	28/6/93	67.1 (66.2, 67.9)
10/10/91	149 (147, 151)	12/7/93	30.0 (28.0, 32.5)
17/10/91	180 (177, 185)	26/7/93	4.1 (3.7, 4.4)
21/11/91	> 1,000	9/8/93	12.5 (12.2, 12.7)
		23/8/93	39.5 (38.6, 40.1)
26/5/92	270 (259, 280)	20/9/93	10.4 (10.2, 10.7)
24/6/92	365 (355, 371)	4/10/93	82.3 (80.7, 83.0)
8/7/92	366 (362, 373)	18/10/93	26.2 (25.4, 26.9)
5/8/92	278 (276, 279)	19/10/93	1.8 (1.4, 2.1)
19/8/92	100 (97, 102)	21/10/93	6.7 (6.4, 7.0)
30/9/92	62.6 (61.9, 63.1)	22/10/93	6.3 (6.2, 6.4)
14/10/92	73.5 (70.2, 77.1)	25/10/93	3.8 (3.1, 4.2)
27/10/92	80.0 (78.6, 81.4)		
18/11/92	89.0 (83.2, 92.0)		
25/11/92	459 (457, 462)		

FIGURE 5.10 Temporal expression of toxin concentration in water samples from Lower Shustoke reservoir determined by phosphatase assay, during 1991-1993. The toxin concentration was calculated by comparison to the reference microcystin-LR.

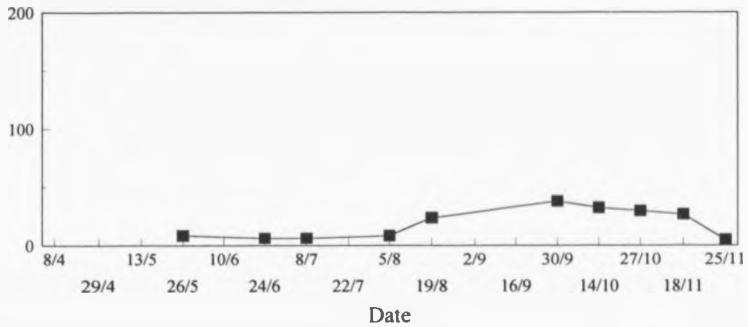
(a) Toxin concentration in raw water (1991)

ng of toxins per litre of raw water



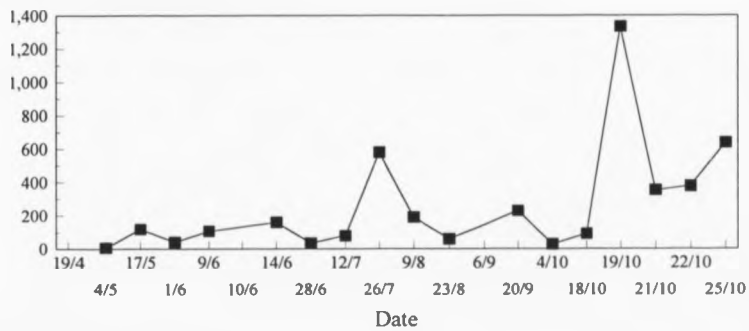
(b) Toxin concentration in raw water (1992)

ng of toxins per litre of raw water



(c) Toxin concentration in raw water (1993)

ng of toxins per litre of raw water



5.3.7.3.3 *Toxic eluate fractions from HPLC analysis*

The cyanobacterial biomass from Lower Shustoke reservoir were extracted and analysed for the presence of hepatotoxins by HPLC (see chapter 4). The peaks identified as toxic and non-toxic, as determined by comparison with the spectral similarity of those samples to that of the reference toxins, were also analysed by phosphatase assay. Table 5.6 shows that the toxic fractions, with a good match to the microcystins and nodularin, had IC_{50} values in the range of 2-4 nM which were not much different from those of the reference toxins. However, the non-toxic fractions had little or no effect on phosphatase activity.

TABLE 5.6 The IC_{50} values for toxic eluate fractions from HPLC analysis. The calculation was done using the IC_{50} of microcystin-LR as the reference

Date	**peak 1** (mcyst-like)	**peak 2** (nodul-like)	**peak 3** (mcyst-like)	**peak 4** (mcyst-like)
1991				
5/8	3.75 nM	3.1 nM	3.12 nM	
19/8	3.3 nM	3.28 nM	3.25 nM	
9/9	3.68 nM	2.6 nM	3.35 nM	
10/10	3.6 nM	3.1 nM	3.3 nM	
1992				
16/9	3.0 nM	4.0 nM	3.15 nM	
30/9	4.05 nM	3.85 nM	3.25 nM	
14/10	NT	3.7 nM	3.8 nM	
27/10	4.05 nM	3.42 nM	3.3 nM	
1993				
9/8	3.63 nM	2.07 nM	3.9 nM	2.57 nM
23/8	3.2 nM	2.7 nM	4.3 nM	2.63 nM
4/10	4.06 nM	3.8 nM	4.4 nM	3.0 nM
19/10	3.07 nM	3.45 nM	4.9 nM	2.85 nM
21/10	3.92 nM	2.91 nM	3.65 nM	NT
25/10	3.45 nM	3.45 nM	4.0 nM	3.92 nM

Mcyst : microcystins

Nodul : nodularin

NT : not determined

5.4 Discussion

The protein phosphatase assay is one of the most promising procedures by which to determine hepatotoxicity of cyanobacterial blooms. The assay involves the conversion of ^{32}P-labelled serine phosphorylase *a* to phosphorylase *b* using crude mouse liver homogenate as the source of phosphatase enzymes. During this reaction, ^{32}P released is measured and the potency of toxins on the enzymes is determined by its IC_{50}. Although crude mouse liver homogenate was used in this study, other extracts of mammalian tissues such as skeletal muscle, brain and heart have proved to be applicable sources of enzymes. However, the effect of dilution of the tissue extracts still requires to be addressed. Furthermore, preliminary experiments must be done to verify whether the rate of dephosphorylation is linear with respect to time under the assay conditions used.

From the results, the *in vitro* activity of phosphorylase phosphatases was strongly inhibited by nanomolar concentrations of the reference toxins microcystin-LR, microcystin-RR and nodularin (see section 5.3.3). The IC_{50}s of these three toxins were shown to be approximately the same. However, microcystin-LR and nodularin were 10 times more toxic than microcystin-RR when injected intraperitoneally into mice (see section 3.4.6). The difference between the *in vitro* phosphatase assay and the corresponding mouse bioassay may be due to either permeability effects of the toxin and/or toxin modification and differential activation of the two microcystins *in vivo*.

Okadaic acid, a polyether derivative of a 38-carbon fatty acid which has been implicated as the causative agent of diarrhetic shellfish poisoning (Haystead *et al.*, 1989), is a potent tumour promoter. Although the IC_{50} of okadaic acid was also in the nanomolar region under the same conditions, it was 10 times less inhibiting than the hepatotoxins. This result implies that the hepatotoxins are more active in inhibiting on liver protein phosphatases than okadaic acid. However, the activity

depends on the types of protein phosphatases contained in the tissue extracts, since the inhibitory action of okadaic acid appears to be specific for some restricted types of protein phosphatase (Bialojan & Takai, 1988). The IC_{50}s of protein phosphatases type 1 and type 2A in crude mouse liver homogenate were measured in the presence of okadaic acid as it inhibits type 2A phosphatase much more than type 1. This method permitted us to measure the specific phosphatases in tissue extracts and was most accurate when extracts contained similar amounts of each phosphatase. Type 1 and type 2A phosphatases are the major phosphatases in tissue extracts which have specific activities towards phosphorylase *a* substrate. It is therefore possible to ignore the phosphatase activity of the other phosphatases which account for only a very minor proportion.

The inhibition effect of the hepatotoxins on protein phosphatase activity has been established as an effective and sensitive assay to detect these toxins in environmental samples. The sensitivity and speed of the assay may facilitate screening analysis for cyanobacterial hepatotoxins.

The concentration of hepatotoxins produced by *Oscillatoria* confirmed by HPLC analysis and the hepatotoxic-symptoms in mice were calculated by comparison with reference microcystin-LR. However, caution must be exercised with this analysis as the inhibition effect may be due to any toxin which inhibits these enzymes. On the other hand, this assay permits the detection of any unidentified toxin if the toxin identification is not required (Sim & Mudge, 1993).

Toxicity assessments of presumptive toxins in *Oscillatoria* cell-biomass and in raw water concentrates from Lower Shustoke reservoir were shown to be variable with time. Non-toxic samples, injected intraperitoneally into mice, had little or no effect on the phosphatase activity with high IC_{50} values ($IC_{50} \geq 2{,}500$ μg dry weight of the cell-biomass extracts and $IC_{50} \geq$ concentrates equivalent to 150 ml of the raw water samples). The possibility cannot be excluded that these samples may still contain the toxins at very low concentrations.

The toxins expressed by the *Oscillatoria* species were separated by collecting the eluate fractions from HPLC and the toxicity of each toxin was ascertained by phosphatase assay. The IC_{50}s of the toxic peaks were variable within the range of 2-4 nM. This variation may be because of the differences in the purity of the toxins which may have been lower than that of the reference toxins and only microcystin-LR was used as a reference toxin to calculate the IC_{50}s for the 3 or 4 different toxic peptides. However, the IC_{50} values of these toxic eluate fractions were not much different from the reference toxins supporting the conclusion that they might be the same group of toxins.

The temporal variations of toxin concentration of both cell-biomass extracts and raw water will be compared with the results from the other detection methods and are discussed in chapter 6.

CHAPTER 6

CHAPTER 6 : ALTERNATIVES TO THE MOUSE BIOASSAY FOR CYANOBACTERIAL TOXICITY ASSESSMENT

6.1 INTRODUCTION

Mouse bioassay has been used as the primary assay for toxicity assessment of cyanobacterial toxins because:- (i) tests can be carried out with minimum delay, (ii) it is relatively economic to use, (iii) it requires minimal sample preparation and (iv) it provides maximum information with respect to dose response to the toxin, clinical symptoms, distribution of toxins in the body, clinical histopathology of the affected tissues, lesion characteristics and specificity of the toxin (Swoboda *et al.*, 1994). Reference toxins and experimental samples are screened for toxicity and recorded as positive or negative results, toxicity break points being scored by death and expressed in mg per kg mouse body weight for LD_{100} tests. Although the mouse bioassay has been extensively used in a number of laboratories worldwide, it presents problems which are of concern. For example, it requires the use of live animals therefore poses ethical problems. It also requires several hours to complete when the sample is non-toxic, and sometimes confirmation by another method is necessary. Thus, an alternative method to determine cyanobacterial toxicity would be advantageous.

In chapter 5, a phosphatase assay has been shown to be one of the promising methods as a means of determining the hepatotoxicity of cyanobacterial toxins. However, the major hindrance of this method is the requirement for the use of radioactive material which has limitations as regards routine commercial work.

6.1.1 Aims

The aim of this chapter is to examine non-radioactive methods as alternatives to the mouse bioassay in determining the toxicity of peptide cyanobacterial toxins. These alternatives must be fast, reproducible, inexpensive, sensitive and avoid the use of animals.

6.2 THE STUDY OF *in vitro* TOXIC EFFECTS OF PEPTIDE TOXINS ON LIVER ENZYMES

6.2.1 Introduction

The liver is the primary target of the cyanobacterial hepatotoxins. Studies using radiolabelled microcystins have shown that these toxins are rapidly taken up by the liver (Falconer *et al.*, 1986; Brooks & Codd, 1987; Robinson *et al.*, 1989). The repeated administration of sublethal doses of toxin to mice affect only the liver (Elleman *et al.*, 1978). When the toxin is administered to mice intraperitoneally, it causes death within 1-3 hours due to extensive liver lobular haemorrhage (Runnegar *et al.*, 1981). Both isolated hepatocytes (Runnegar *et al.*, 1981; Aune & Berg, 1986) and isolated perfused livers (Berg & Soli, 1985; Berg *et al.*, 1988; Theiss *et al.*, 1988) have been used to study the effects of hepatotoxins and found to be suited for screening purposes. However, the preparation of isolated hepatocytes, or isolated perfused livers, are rather complicated and demand skilled individuals with the necessary Home Office Animal Licence.

Since the activity of the protein phosphatase enzymes have been found to be inhibited by the hepatotoxins in crude mouse liver homogenate, this assay represents a useful screening system for cyanobacterial toxins.

The liver enzymes lactate dehydrogenase, γ-glutamyltransferase, 5'-nucleotidase, acid and alkaline phosphatases were studied since these enzymes have been shown to be indicative of particular liver diseases.

6.2.2 The studied enzymes

Crude mouse liver homogenate prepared as described in section 2.12.5 was used as the source of the following enzymes. The procedure for the study of the effects of hepatotoxins on each enzyme has been explained earlier (see section 2.13).

6.2.2.1 Lactate dehydrogenase

The enzyme lactate dehydrogenase (EC 1.1.1.27, LDH) catalyses the reversible reduction of pyruvate (equation 6.1).

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+ \quad \text{.....(eq. 6.1)}$$

This enzyme is located in the cytoplasm and can be liberated into solution when cells are broken by simple homogenisation in a *Potter-Elvehjem* homogeniser (Holbrook *et al.*, 1972). It has been demonstrated that abnormalities of serum lactate dehydrogenase isoenzyme pattern are useful in the characterisation of certain types of pathological disorders which including liver diseases (Moss 1982; Zondag 1982).

6.2.2.2 γ-glutamyltransferase

γ-glutamyltransferase (GGTP) is a sensitive indicator of liver disease, bile duct and pancreas abnormalities (Rutenburg *et al.*, 1963; Szasz 1969), particularly when due to alcoholism or malignant infiltration (Zein & Discombe, 1970).

The enzyme GGTP catalyses the transfer of the glutamyl group from γ-L-glutamyl-p-nitroanilide to glycylglycine which liberates p-nitroaniline as a product (see equation 6.2).

L-glutamyl-p-nitroanilide + glycylglycine $\longrightarrow$
p-nitroaniline + glutamyl-glycylglycine .....(eq. 6.2)

6.2.2.3 5'-nucleotidase

The enzyme 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) is considered to be a convenient enzymatic marker for the presence of plasma membranes from homogenate of rat liver (Pletsch & Coffey, 1972). 5'-nucleotidase catalyses the dephosphorylation of nucleoside phosphates to the corresponding nucleoside and orthophosphate (see equation 6.3) (van Husen & Gerlach, 1984).

AMP $\longrightarrow$ P_i + adenosine .....(eq. 6.3)

6.2.2.4 Acid and alkaline phosphatases

Alkaline phosphatase is one of the diagnostic enzymes for the assessment of liver disease. Its activity is found to correlate closely with the activities of GGTP and 5'-nucleotidase. Acid and alkaline phosphatases (AcP and ALP) catalyse the hydrolysis of p-nitrophenyl phosphate, yielding p-nitrophenol and inorganic phosphate (see equation 6.4).

p-nitrophenyl phosphate + H_2O $\longrightarrow$ p-nitrophenyl + P_i .....(eq. 6.4)

6.2.3 Results

In this study, the *in vitro* toxic effect of peptide toxins on five enzymes lactate dehydrogenase, 5'-nucleotidase, γ-glutamyltransferase, acid and alkaline phosphatases was studied by using crude mouse liver homogenate as the source of enzymes. The crude mouse liver homogenate was treated with three reference peptide toxins; microcystin-LR, microcystin-RR and nodularin up to μM concentrations.

6.2.3.1 Lactate dehydrogenase

The lactate dehydrogenase assay showed no significant difference between crude mouse liver homogenate treated with toxins up to 5 μM and the negative controls (Figure 6.1).

6.2.3.2 5'-nucleotidase

The 5'-nucleotidase assay also showed no significant difference between crude mouse liver homogenate treated with toxins up to 5 μM and the negative controls (Figure 6.2).

6.2.3.3 Acid and alkaline phosphatases

Similar results to the above enzymes were obtained for assays of acid and alkaline phosphatase. The activities of toxin-treated crude mouse liver homogenate were not different from those of the negative control (Figure 6.3 and 6.4).

FIGURE 6.1 Lactate dehydrogenase assay of crude mouse liver homogenate treated with reference toxins (microcystin-LR, microcystin-RR and nodularin). Assay procedures were carried out as described under "Materials and Methods" (see section 2.13.3).

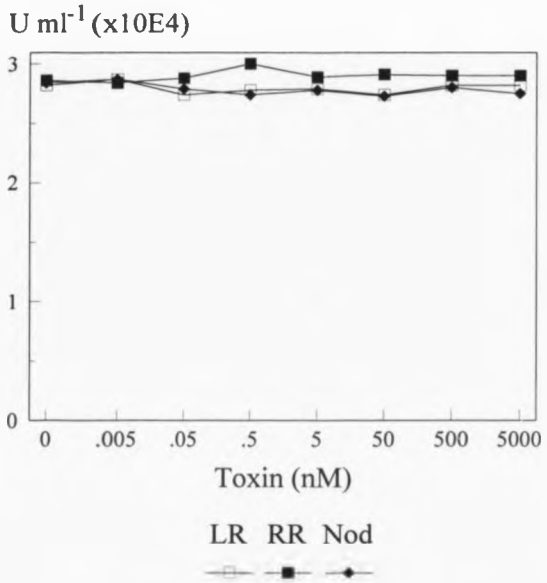


FIGURE 6.2 5'-nucleotidase assay of crude mouse liver homogenate treated with reference toxins (microcystin-LR, microcystin-RR and nodularin). Assay procedures were carried out as described under "Materials and Methods" (see section 2.13.4).

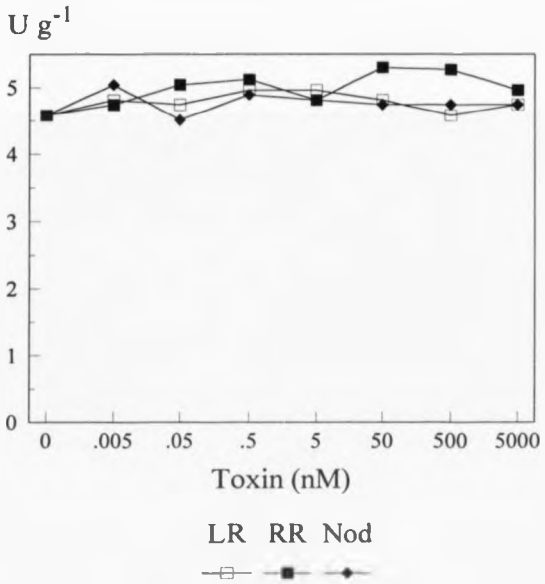


FIGURE 6.3 Acid phosphatase assay with crude mouse liver homogenate treated with reference toxins (microcystin-LR, microcystin-RR and nodularin). Assay procedures were carried out as described under "Materials and Methods" (see section 2.13.5).

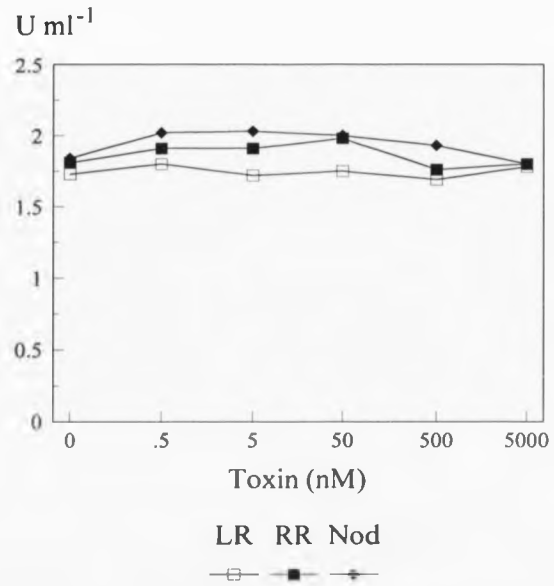
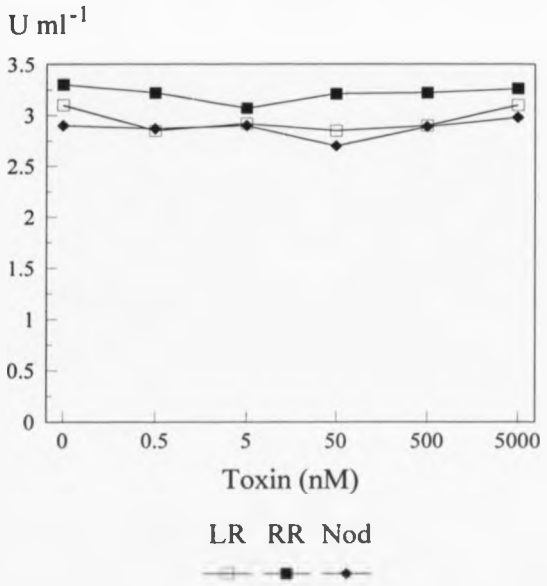


FIGURE 6.4 Alkaline phosphatase assay with crude mouse liver homogenate treated with reference toxins (microcystin-LR, microcystin-RR and nodularin). Assay procedures were carried out as described under "Materials and Methods" (see section 2.13.5).



6.2.3.4 γ-glutamyltransferase

The γ-glutamyltransferase assays of untreated and toxin-treated crude mouse liver homogenate showed that the enzyme activity was very low, consequently this enzyme was not assayed routinely.

6.2.4 Discussion

During the past few years, the toxicity of peptide hepatotoxins has been mainly tested in bioassays using large numbers of rodent animals. I have examined several alternatives for the *in vitro* assessment of toxicity.

Phosphorylase phosphatase activity has been shown to be inhibited by purified hepatopeptide toxins i.e. microcystin-LR, microcystin-RR and nodularin, as well as the environmental toxins extracted from cyanobacterial biomass in the range of nM concentrations (see chapter 5). However, since use was made of crude mouse liver homogenate as the source of the enzymes, the inhibitory effects of the peptide hepatotoxins were studied on five other enzymes; lactate dehydrogenase, 5'-nucleotidase, γ-glutamyltransferase, acid and alkaline phosphatases. The activity of these enzymes was shown to be unaffected by the three reference toxins although the crude mouse liver homogenate was treated with the toxins up to μM concentrations. The activity of γ-glutamyltransferase when assayed in control samples (crude mouse liver homogenate) was also very low compared with the other enzymes. Possible explanations for the results of these experiments are :-

(i) the hepatotoxins have no inhibitory effects on the activities of these enzymes in the chosen *in vitro* conditions, or

(ii) the *in vitro* conditions did not support the cascade of events that precede the toxic effects on these enzymes, or

(iii) with this preparation of crude mouse liver homogenate, some enzymes remained bound to the membrane resulting in very low activity in the assays.

The lactate dehydrogenase leakage assay from fresh rat hepatocytes incubated with hepatotoxins gave good dose- and time-dependent responses to the toxic cyanobacteria extracts and might be well suited for screening purposes (Aune & Berg, 1986). A number of reports also indicated elevation of the level of serum γ-glutamyltranspeptidase in various forms of liver disease which correlated closely with the activities of alkaline phosphatase and 5'-nucleotidase (Zein & Discombe, 1970; Phelan *et al.*, 1971; Whitefield *et al.*, 1972). However, the study of the inhibition effects of the hepatotoxins on the activity of these enzymes within the employed conditions did not seem to be an appropriate method to apply as an alternative for toxicity assessment. Freshly prepared hepatocytes would be more suitable as the source of the enzymes rather than crude mouse liver homogenate and the assay conditions require optimisation to align them with the *in vivo* situation.

Other alternatives which have been investigated in our group and the data correlated with mouse bioassay and protein phosphatase assay were:-

(a) *Drosophila melanogaster* bioassay (carried out by myself, Mr Peter Firth and Dr Uthaya Swoboda).

(b) a bioluminescence assay - Microtox (carried out by myself, Ms Nicola Smith and Dr Uthaya Swoboda).

6.3 *Drosophila melanogaster* BIOASSAY

A *Drosophila melanogaster* bioassay has yielded very favourable data, and has been shown to be a useful indicator of toxicity of cyanobacterial hepatotoxins. This study has shown that the reference hepatotoxins (microcystin-LR, microcystin-RR and nodularin), cyanobacterial toxins extracted from environmental biomass and okadaic acid (a non-phorbol ester tumour promoter, produced by *Porocentrum lima*) are all potent inhibitors of type 1 (PP1) and type 2A (PP2A) protein phosphatases which are present in organisms as diverse as mammals, fruit flies, starfish, yeast and higher plants (Cohen & Cohen, 1989). It was anticipated therefore that the sensitivity of these enzymes in the fruit fly to cyanobacterial hepatotoxins would be similar.

Known concentrations of reference toxins (microcystin-LR and nodularin) or 60 µl of disrupted cell biomass, as prepared for the mouse bioassay (see section 2.11), were mixed with sucrose to a final concentration of 1% (w/v) and spotted onto Whatman no. 1 filter discs and these were placed at the bottom of plastic vials. Subsequently 15-30 fruit flies, which had been starved overnight, were transferred to each vial. The number of flies per experiment was kept constant +/- 2 flies. Control tubes contained 1% (w/v) sucrose in phosphate buffered saline. Death of the flies was monitored regularly.

The results show that microcystin-LR and nodularin were toxic to the flies with the death rate being linked to toxin concentration and exposure time (Figure 6.5). The environmental cyanobacterial toxins were shown to be toxic to the flies except those which produced neurotoxins. There was a very good correlation between the *Drosophila melanogaster* and mouse bioassay as well as the phosphatase assay with toxic cyanobacterial biomass (Table 6.1). On the other hand, a neurotoxic *Aphanizomenon* bloom from Swithland reservoir, as assessed by mouse bioassay, had no toxic effect on the fruit flies as was to be expected.

The advantages of the *Drosophila melanogaster* assay are that it is cheap, relatively quick and easy to do. This assay also requires little sample preparation, and moreover, it avoids the use of mice and rats. The toxic effects of the hepatotoxins compared to the neurotoxins on the fruit flies required confirmation and further study. The *Drosophila melanogaster* assay is only useful as an indicator of toxicity since any detailed information such as dose response, would require the use of mouse bioassay.

FIGURE 6.5 *Drosophila* bioassay as an alternative method of toxicity assessment for (a) microcystin-LR and (b) nodularin. The legend indicates the amount of toxin added per filter disc.

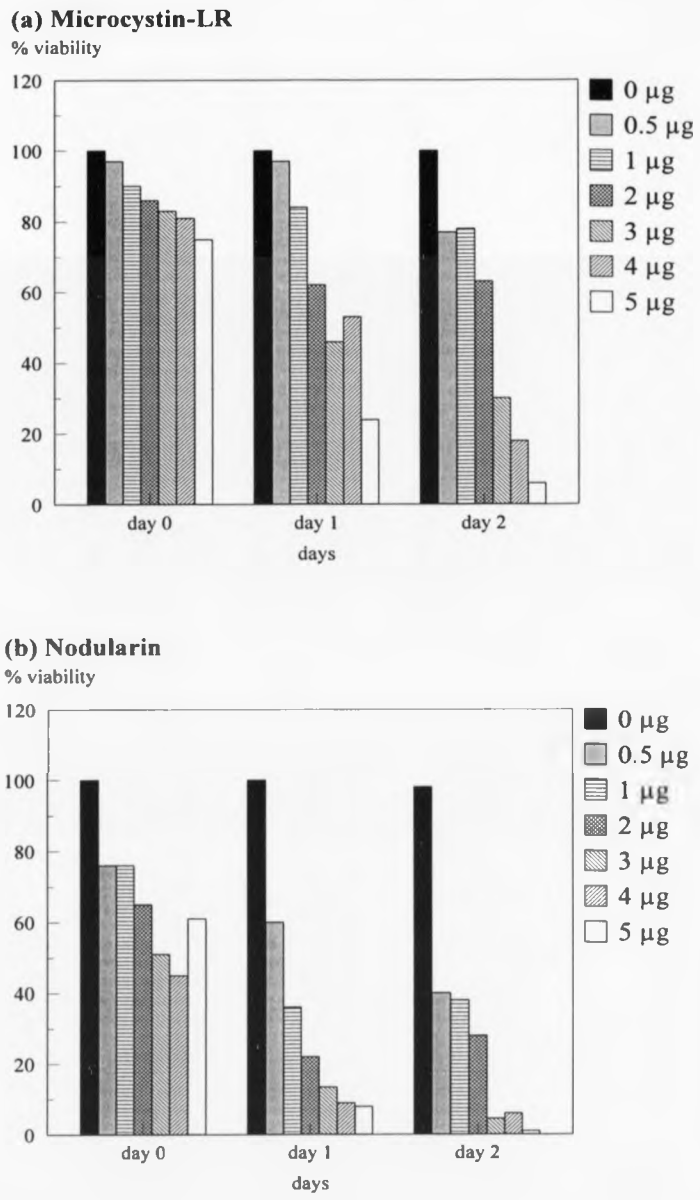


TABLE 6.1 Comparison of toxicity assessment of cyanobacterial toxins from different species as determined by mouse and *Drosophila* bioassays

Reservoir	Date	Species	Toxicity		Symptoms*
			Drosophila	**Mouse**	
Lower Shustoke	10/6	*Oscillatoria*	toxic	toxic	H
Upper Shustoke	2/9	*Oscillatoria*	toxic	toxic	H
Cropston	19/8	*Microcystis*	toxic	toxic	H
Swithland	26/6	*Aphanizomenon*	non-toxic	non-toxic	
Swithland	14/10	*Aphanizomenon*	non-toxic	toxic	N
		Microcystis PCC 7806	toxic	toxic	H

* H : hepatotoxin N : neurotoxin

6.4 Bioluminescence assay

The bacterial bioluminescence based Microtox assay involving *Photobacterium phosphoreum* was also investigated for toxicity assessment of the cyanobacterial toxins since this assay has been used to assess the toxicity of a wide range of aquatic pollutants (Ribo & Kaiser, 1987; Kaiser & Ribo, 1988; Lawton *et al.*, 1990). Three reference toxins (microcystin-LR, microcystin-RR and nodularin), 1 laboratory isolate and 9 natural blooms of cyanobacteria were tested and the toxicity data compared with mouse bioassay results.

The assay was carried out using a Microtox Toxicity Analyser Model 500 (Microbics Corporation, USA) according to the manufacturer's operating instructions (Microbics, 1982). Samples were prepared for the assay as follows:- (Lawton *et al.*, 1990). The reference toxins were prepared in Microtox diluent at a concentration of 0.5 mg dry weight per ml. Cyanobacterial biomass samples were lyophilised and then prepared at 5 mg dry weight per ml of Microtox diluent. Samples were sonicated (3 x 10 second bursts at maximum power in an MSE Soniprep ultrasonicator) and microfuged for 5 minutes to give supernatant fluids for bioassay.

All three reference toxins caused a dose-dependent inhibition of light emission in the Microtox assay. The effective concentration causing a 50% decrease in light emission 5 minutes after toxin addition, EC_{50}, was then calculated. The EC_{50} of microcystin-LR, microcystin-RR, nodularin and anatoxin-a were 0.102, 0.097, 0.147 and 0.164 mg ml^{-1} respectively (Table 6.2).

If the toxicity of a sample is inferred by taking an $EC_{50} < 0.5$ mg per ml as being toxic, then only 1 out of the 7 hepatotoxin producing strains (as determined by mouse bioassay and HPLC) would be classified as toxic, whereas 2 of the 4 non-toxic strains would be deduced to be toxic (Table 6.3). If, however, higher values of EC_{50} i.e. $EC_{50} < 1$ mg ml^{-1} were taken as inferring toxicity, then 5 out of the 7 hepatotoxin producing strains could be deduced as being toxic. Another contrary

result is that the same strain of *Oscillatoria* species from Lower Shustoke reservoir collected at different times, shown to be toxic by mouse bioassay, had lethal doses of 83.5 and 400 mg dry weight cell lysate per kg body weight, respectively. The former more potent sample was deduced as being non-toxic while the latter was toxic.

These results show a lack of correlation between different samples and a high risk of false toxic/non-toxic data indicates which that the Microtox assay is not a reliable procedure for assessing the toxicity of cyanobacterial biomass. Another disadvantage of this assay is the sensitivity of the Microtox system to other chemicals such as chlorine, which make the system unsuitable for assessing toxicity in treated water.

TABLE 6.2 Comparison of mouse bioassay and bioluminescence assay for the reference toxins

Sample	Mouse bioassay		Bioluminescence		
	LD(1)	Tox(2)	EC_{50}(3)	Tox(4)	Tox(5)
microcystin-LR	0.15	toxic	0.102	toxic	toxic
microcystin-RR	1.5	toxic	0.097	toxic	toxic
nodularin	0.15	toxic	0.147	toxic	toxic
anatoxin-a	0.25	toxic	0.164	toxic	toxic

(1) : mg kg^{-1} mouse body weight

(2) : death within 24 hours after intraperitoneal injection

(3) : mg ml^{-1}

(4) : inferred from $EC_{50} < 0.5$ mg ml^{-1}

(5) : inferred from $EC_{50} < 1.0$ mg ml^{-1}

TABLE 6.3 Toxicity assessment of cyanobacterial toxins from different cyanobacterial species as determined by bioluminescence assay

Sample	Source	Mouse bioassay		Bioluminescence		
		LD(1)	Toxic(2)	EC_{50}(3)	Tox(4)	Tox(5)
A. cylindrica	laboratory	> 1500	non-toxic	> 5	non-toxic	non-toxic
M. aeruginosa	PCC 7806	55	toxic	0.495	toxic	toxic
M. aeruginosa	Cropston	< 100	toxic	0.98	non-toxic	toxic
Oscillatoria	L. Shustoke	83.5	toxic	1.39	non-toxic	non-toxic
Oscillatoria	L. Shustoke	400	toxic	0.93	non-toxic	toxic
Oscillatoria	Linacre	50	toxic	0.82	non-toxic	toxic
Oscillatoria	Earlswood	< 125	toxic	1.08	non-toxic	toxic
Oscillatoria	Thornton	195	toxic	1.325	non-toxic	non-toxic
O. erythrea	Australia	> 1500	non-toxic	0.215	toxic	toxic
Aphanizomenon	Swithland	> 1000	non-toxic	0.525	non-toxic	toxic
Aphanizomenon	Swithland	> 1000	non-toxic	0.485	toxic	toxic

(1) : mg dry wt per kg body weight

(2) : death within 24 hours of intraperitoneal injection

(3) : mg dry wt per ml

(4) : inferred from $EC_{50} < 0.5$ mg ml^{-1}

(5) : inferred from $EC_{50} < 1$ mg ml^{-1}

6.5 Comparison of toxicity assessments between HPLC analysis and phosphatase assay

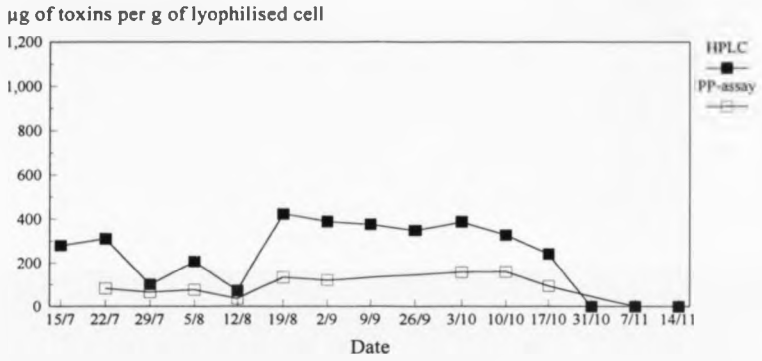
From the several alternatives that have been studied, phosphorylase phosphatase assay is the most promising assay to determine hepatotoxicity of cyanobacterial biomass although it involves using radioactive material (^{32}P). The temporal expression of peptide toxins in whole cell biomass of *Oscillatoria* and raw water concentrates from Lower Shustoke reservoir during 1991 to 1993 were obtained from both HPLC analysis and phosphorylase phosphatase assays. The aim of this part of the study was to compare the toxicity assessments using HPLC analysis and phosphorylase phosphatase assays.

Figure 6.6.a, b and c show the comparisons between the amount of toxins in whole cell biomass determined by HPLC and phosphorylase phosphatase assays in 1991, 1992 and 1993, respectively. The data from 1991 and 1992 show that the toxin concentration determined by HPLC analysis was about twice that determined by phosphorylase phosphatase assays. In 1993, however, the data shows close correlation between the amount of toxins in whole cells determined by HPLC analysis and phosphorylase phosphatase assays. An explanation for the difference in these results may be because all samples were determined by HPLC analysis from time to time, but those from 1991 and 1992 were stored at -20°C for about 1-2 years prior to the determination of toxins by the phosphorylase phosphatase assays in 1993.

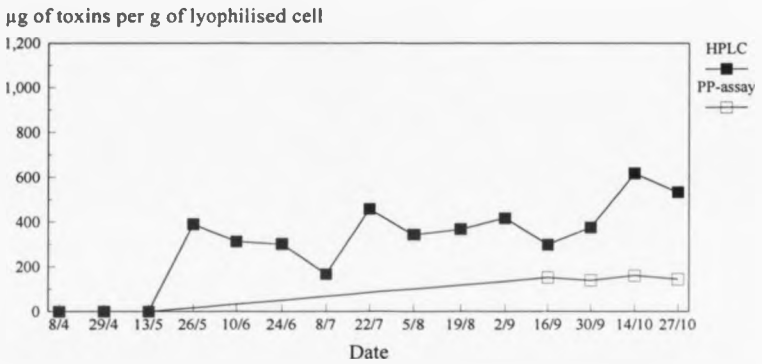
Phosphorylase phasphatase assays appear to be more sensitive than HPLC analysis. The comparison between the concentration of toxins in raw water concentrates determined by HPLC and phosphorylase phosphatase assays in 1991, 1992 and 1993 are shown in Figure 6.7.a, b and c, respectively. The raw water concentrate samples referred to as non-toxic by HPLC analysis still have little effect on the phosphorylase phosphatase activity. However, there remains the question as

FIGURE 6.6 Comparison between toxicity assessment of cyanobacterial biomass from Lower Shustoke reservoir during 1991 to 1993 as detected by HPLC analysis and by protein phosphatase assay.

(a) 1991



(b) 1992



(c) 1993

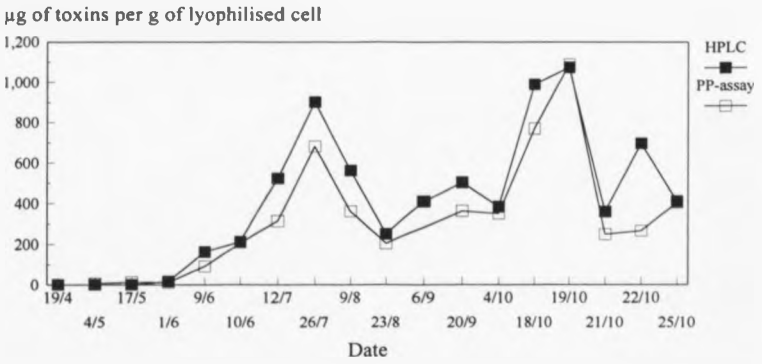
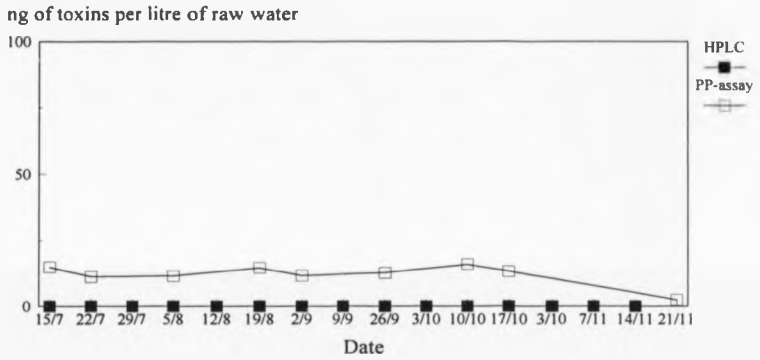
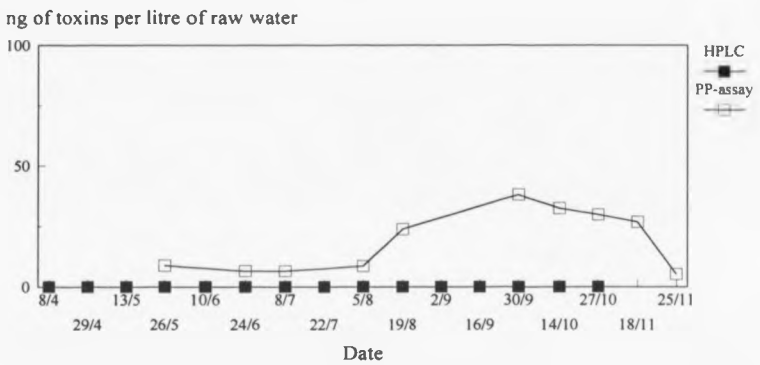


FIGURE 6.7 Comparison between toxicity assessment of "cell free" water concentrates from Lower Shustoke reservoir during 1991 to 1993 as detected by HPLC analysis and by protein phosphatase assay.

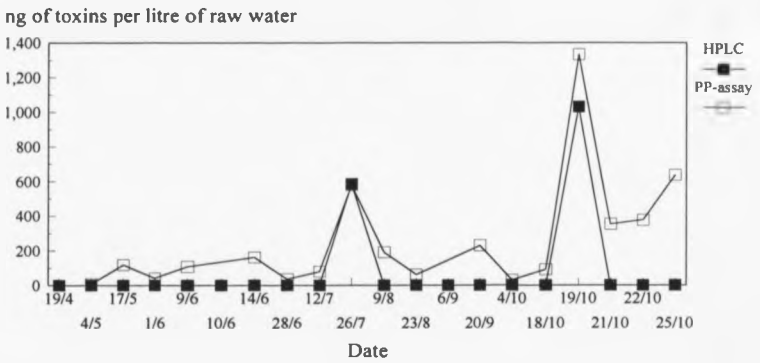
(a) 1991



(b) 1992



(c) 1993



to whether these samples contain low levels of toxins. This is supported by the low concentration of toxins in raw water concentrate samples determined by phosphorylase phosphatase assay from 1991 and 1992.

In 1993, only 2 raw water concentrate samples contained toxins at concentration detectable by HPLC However, the toxins could be detected in most raw water concentrate samples using phosphorylase phosphatase assays. Thus it can be concluded that samples which contain high concentrations of toxins can be detected by either HPLC and/or phosphorylase phosphatase assay with approximately similar results. On the other hand, in samples which contain low concentrations of toxins, only the phosphorylase phosphatase assay is sufficiently sensitive. The phosphorylase phosphatase assay is, therefore, more sensitive and more suitable to the assessment of low concentrations of toxins.

The high background of impurities displayed by certain HPLC chromatograms may impact on the degree of confidence in relation to the spectral analysis i.e. only the dominant toxic peak can be detected by HPLC analysis with a high degree of confidence. However, both whole cell biomass and raw water concentrates that contain high amount of toxin can be detected by either HPLC analysis or phosphorylase phosphatase assay with approximately the same sensitivity.

CHAPTER 7

CHAPTER 7 : STRUCTURAL CHARACTERISATION OF THE PEPTIDE TOXINS BY MASS SPECTROMETRY

7.1 INTRODUCTION

Many groups have attempted to characterise the structure of the toxins from *Microcystis aeruginosa* (Bishop *et al.*, 1959; Toerien *et al.*, 1976; Elleman *et al.*, 1978; Botes *et al.*, 1982; Santikarn *et al.*, 1983). However, the structure remained rather confusing until 1984, when Botes *et al.* (1984) published a cyclic heptapeptide structure for a toxin isolated from a natural bloom in Witbank Dam, South Africa. At present, about 40 variants of microcystins have been isolated so far from many strains of cyanobacteria (Sivonen *et al.*, 1989 b, 1990 a, 1992 a, b, c, d; Namikoshi *et al.*, 1992 a, b, c, d). Their toxicities are also found to be variant. However, the general structure of the microcystins is cyclo (—D-Ala—L-X—D-*erythro*-β-methyl-Asp—L-Z—Adda—D-Glu—*N*-methyldehydro-Ala—); where X and Z represent two variable L-amino acids. Adda denotes the β-amino acid (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, the unique C_{20} amino acid found only in these cyanobacterial peptide toxins (Botes *et al.*, 1984; Rinehart *et al.*, 1988). This unusual amino acid appears to be important for the toxicity of these compounds (Harada *et al.*, 1990). The study by Namikoshi *et al.* (1989) found that chemical modification of the alkyl chain of Adda from microcystin-LR resulted in a marked reduction of toxicity.

Blooms of cyanobacteria in freshwater reservoirs in the English Midlands area have been regularly monitored during 1991 to 1993 and the primary

cyanobacterial species isolated characterised. *Microcystis*, *Oscillatoria* and *Anabaena* species produced hepatotoxins, whereas *Aphanizomenon* species were found to produce alkaloid neurotoxins. Both types of toxin were found to be extremely potent when injected intraperitoneally into mice. However, the toxicity has been shown to vary in both qualitative and quantitative terms for all of the environmental isolates, there being both *in vivo* (mouse bioassay) and *in vitro* (protein phosphatase assay) variation. To further understand this, it is necessary to elucidate the detailed structure of these toxins.

In this study, the technique of fast atom bombardment mass spectrometry was applied to elucidate the structural information of the presumptive toxins in natural water. A new high performance matrix assisted laser desorption time-of-flight mass spectrometer (MALDI) was also used for mass determination.

7.1.1 Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectrometry (FAB-MS) was introduced by Barber *et al.* (1982). This technique is a desorption ionisation method that has been used widely as it allows the investigator to obtain mass spectra from polar and involatile compounds for molecular weights up to approximately 20 kDa without the use of derivatisation techniques.

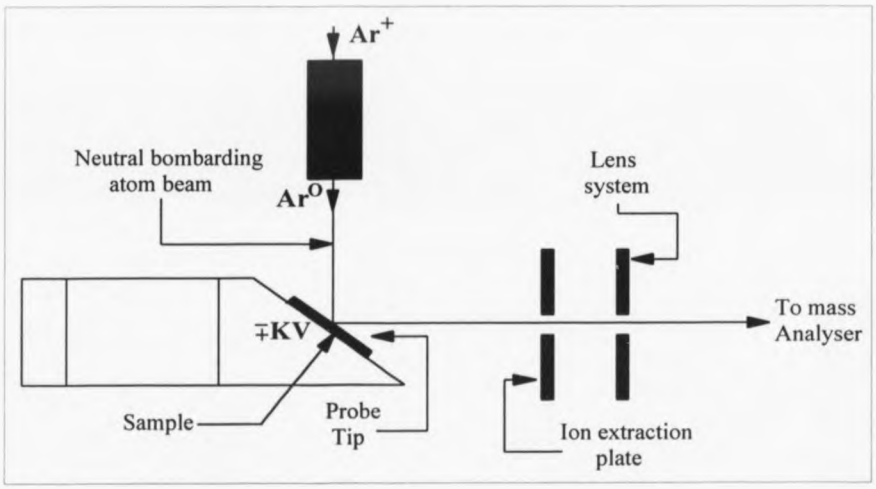
The principle of the FAB method is illustrated in Figure 7.1. A beam of fast moving neutral atoms is directed to strike a sample film carried on a clean metal support and much of the high kinetic energy of the atoms is transferred to the sample molecules on impact (Chapman, 1985; David & Frearson, 1987). The energy of this spike is instantaneously dissipated through the surface layers of the sample causing molecules to be detached from these surface layers as a dense gas comprising of neutral, negative and positive ions. Any neutral species remaining are eventually ionised by the plasma just above the sample surface. If the plate on which the

sample is placed is held at a suitable potential with respect to an ion exit plate, either positive or negative ions can be directed through an ion slit towards the analyser.

The bombarding atoms for FAB ionisation are usually rare gases, either xenon or argon. To achieve a high kinetic energy, atoms of the gas are first ionised and then passed through a very high potential difference, normally in the region of 4-8 keV. After acceleration, the fast moving ions pass into a chamber and collide with the corresponding neutral gaseous atoms which leads to charge-exchange. An exchange of charge in this process occurs with little or no change in momentum.

$$Xe^{+}\ (fast) + Xe\ (thermal) \longrightarrow Xe\ (fast) + Xe^{+}\ (thermal) \quad .....(eq.\ 7.1)$$

FIGURE 7.1 Schematic diagram of fast atom bombardment ion source.



Structural information (sequence/modifications) can be deduced from the characteristic species formed by fragmentation of the protonated molecular ion, $(M+H)^+$, either spontaneously or upon collision with a neutral gas. The fragment ions appear as peaks at the corresponding mass in the conventional FAB spectrum. If collision is required to induce enhanced fragmentation, a tandem mass spectrometer has to be used, which allows the mass analysis of the fragment ions in the second of the two mass spectrometers.

It is important to note that the FAB process is basically a surface analysis technique for liquid samples, analysing molecules which reside in the molecular layers at the liquid/vacuum interface. However, the advantage of the liquid nature of the samples is that the surface is continually renewed by molecules from deeper within the sample droplet (Caprioli, 1990).

The form of monolayer at the surface of the matrix material is important to obtain efficient ionisation. It is also important to keep the sample liquid inside the high-vacuum environment of the mass spectrometer by adding a viscous organic liquid such as glycerol to the sample in relatively high concentrations. The viscosity of this matrix ensures that there is suitable diffusion, rapid enough to provide continuous surface renewal so that sample beams may be prolonged for 20 minutes or more, sufficient to observe an acceptable intensity. Typically, a sample is prepared for FAB analysis by applying 1-2 μl of sample solution, of approximately 1-15 $\mu g\ \mu l^{-1}$, to the probe tip. The solvent is evaporated leaving the solid sample only and this is mixed with 2-4 μl of glycerol. A wide variety of molecules such as intact and underivatised peptides, sugars, antibodies, organophosphate, nucleotides and drug metabolites, can be analysed by mass spectrometry in this manner (Caprioli, 1990).

This process, however, has several drawbacks. Firstly, the matrix liquid gives rise to a very intense background chemical noise in the mass spectrum. Secondly, the sample is contaminated and diluted by the matrix liquid producing

poor sensitivity. Thus, high-sensitivity analyses are extremely difficult in the low mass range below approximately m/z 400. Thirdly, the matrix liquid enhances ion suppression in which the more hydrophobic ions or molecules reside at the surface suppressing any other ions or molecules beneath them (Caprioli, 1990).

Continuous flow fast atom bombardment mass spectrometry (CF-FAB MS) is the developed technique which has been devised to enhance the advantages of the analytical FAB and, at the same time, alleviate the negative aspects of the technique. An HPLC may be linked to the mass spectrometer giving a multi-dimensional analyser for the identification of the masses of specific compounds as they elute from the chromatograph. However, mixtures of similar compounds cannot be resolved although individual compounds are easily identified as long as they have different molecular weights (Caprioli & Tomer, 1990).

The use of CF-FAB MS offers several advantages, relative to the static FAB technique. These include :-

(i) the direct introduction of aqueous sample solutions; small amounts of an aqueous solution can be injected directly into the mass spectrometer *via* the carrier solution. Thus, the sample is concentrated on the target tip over a relatively small period of time (30 to 60 seconds) giving maximum signal intensities.

(ii) the tendency to decrease the ion suppression. Since the composition of the liquid surface layers in CF-FAB are in motion and constantly being renewed as new eluent passes through the column, there is no time interval for the more dominant hydrophobic ions or molecules to occupy the surface region.

(iii) the reduction in the intensity of the background noise and matrix cluster ions. The matrix is able to mix within the eluting solution and thus lower the overall concentration of the matrix. This increases ion production from the sample and consequently lowers the limit of detection.

In order to obtain optimal performance of the CF-FAB a thin film on the target surface must be achieved and maintained. Stable operation and the highest

sensitivity occurs when a thin liquid sample film is bombarded, and several dynamic parameters maintained; the temperature of the probe tip, the pumping capacity of the instrument and the rate of flow of liquid onto the surface. The mobile phase is necessary to contain a small concentration of matrix to ensure the formation of a non-volatile hydrophilic coating on the mesh and thus create a smooth flow of solution onto the surface (Takeuchi *et al.*, 1988). Since the temperature of the probe could drop due to the evaporation of liquid from the sample, the temperature of the probe must be maintained to prevent the blockage of the capillary by frozen solution. All of these are specific operating disadvantages of the CF-FAB. The application of the technique and/or modifications are varied depending on each instrument, the complexity of the sample as well as the data required by the analyst.

7.1.2 The matrix assisted laser desorption time-of-flight mass spectrometer (MALDI)

The matrix assisted laser desorption time-of-flight mass spectrometer, or the KOMPACT MALDI, is a new technique of mass spectrometry which has been developed by Kratos Analytical and Johns Hopkins University. It provides high mass range analysis utilising the latest in time-of-flight technologies. The applications of this system include unrivalled sample handling facilities, high sample throughput, ease of use and fast on-line data processing based on workstation technology. With the matrix assisted laser desorption technique, only picomole quantities of sample are required. KOMPACT MALDI has a mass range of more than 200 kDa with low picomole sensitivity. One of the major benefits of this system is fast, accurate molecular weight determination.

7.1.3 Aims

The aim of this study was to elucidate the detailed structures of cyanobacterial toxic peptides from environmental samples. This will enhance understanding of variation of the toxins re toxicity and the quantity of each variant present in different species and strains of cyanobacteria. This information will also facilitate the development of the method in order to detect and identify toxins.

The relative molecular mass of reference and presumptive toxins eluted from HPLC were determined using a MALDI mass spectrometer.

In conjunction with the Department of Chemistry, the peptide toxins were characterised by static-FAB and HPLC CF-FAB mass spectrometry.

A Kratos CONCEPT II HH four sector mass spectrometer was utilised to characterise, identify and fingerprint the reference toxins for both cyclic and linearised forms.

7.2 Materials and Methods

The basic materials and methods used in this study have been given in section 2.14 and 2.15.

7.3 RESULTS

The reference and environmental samples of the available presumptive peptide toxins were analysed using MALDI mass spectrometry for molecular weight determination. HPLC CF-FAB MS and the Concept II HH four sector mass spectrometer were also used to study the reference toxins.

Positive ion spectra were obtained for each sample. For a molecular weight determination, this was calculated for each fraction from the dominant positive ion in the spectrum, which was usually $(M+H)^+$, although on occasions it was $(M+Na)^+$. Thus, the molecular weight was obtained from the dominant ion -1 for $(M+H)^+$ and -23 for $(M+Na)^+$.

7.3.1 The KOMPACT MALDI mass spectrometry

As determined by MALDI mass spectrometry, the $(M+H)^+$ ions of reference microcystin-LR, microcystin-RR and nodularin were shown at m/z 995.0, 1037.4 and 824.3, respectively (Figure 7.2, 7.3 and 7.4). These results represent the correct or close molecular weight of microcystin-LR (MW 994 Da), microcystin-RR (MW 1037 Da) and nodularin (MW 824 Da). The other two major $(M+H)^+$ ions at m/z 207 and 225 were the matrix and were used to calibrate the instrument (Figure 7.5).

Environmental peptide toxins produced by *Oscillatoria* species from Lower Shustoke reservoir during the period of study, were prepared for mass spectrometry (section 2.7.2). Fractions corresponding to chromatographic "peaks" were obtained by repeated collection of the eluent from HPLC separations. Those fractions with a characteristic microcystin/nodularin spectrum were prepared as described (section 2.9.4). Whenever the concentration was sufficient, the presumptive toxic peak was analysed by MALDI mass spectrometry under the same conditions as the reference toxins. The results of mass spectral analysis obtained with the *Oscillatoria* isolates

are outlined in Table 7.1. There was no microcystin-LR (MW 994 Da) identified in any sample throughout the three years.

TABLE 7.1 The mass spectral analysis of environmental peptide toxins produced by *Oscillatoria* species in Lower Shustoke reservoir during 1991 to 1993 as separated by HPLC analysis and determined by MALDI analysis

	Mass (Dalton)			
Date	**Peak-1 (nodul-like)**	**Peak-2 (mcyst-like)**	**Peak-3 (mcyst-like)**	**Peak-4 (mcyst-like)**
1991				
5/8/91	nd	1021.6	nd	
19/8/91	nd	1022.6	1043.5	
9/9/91	982.6	1022.6	1043.5	
10/10/91	982.6	1022.6	1043.5	
1992				
30/9/92	982.0	1023.2	nd	
14/10/92	981.8	1023.2	1044.5	
1993				
9/8/93	982.1	1024.7	1022.1	1046.6
4/10/93	981.1	1024.2	1023.7	1048.1
19/10/93	981.6	1024.7	nd	1044.0
21/10/93	982.1	1024.2	1022.1	1044.0

nd : not detected due to insufficient sample

FIGURE 7.2 MALDI mass spectrum of the reference toxin microcystin-LR (m/z 995.0). Sinapinic acid, 50 ng μl^{-1}, was added as the matrix.

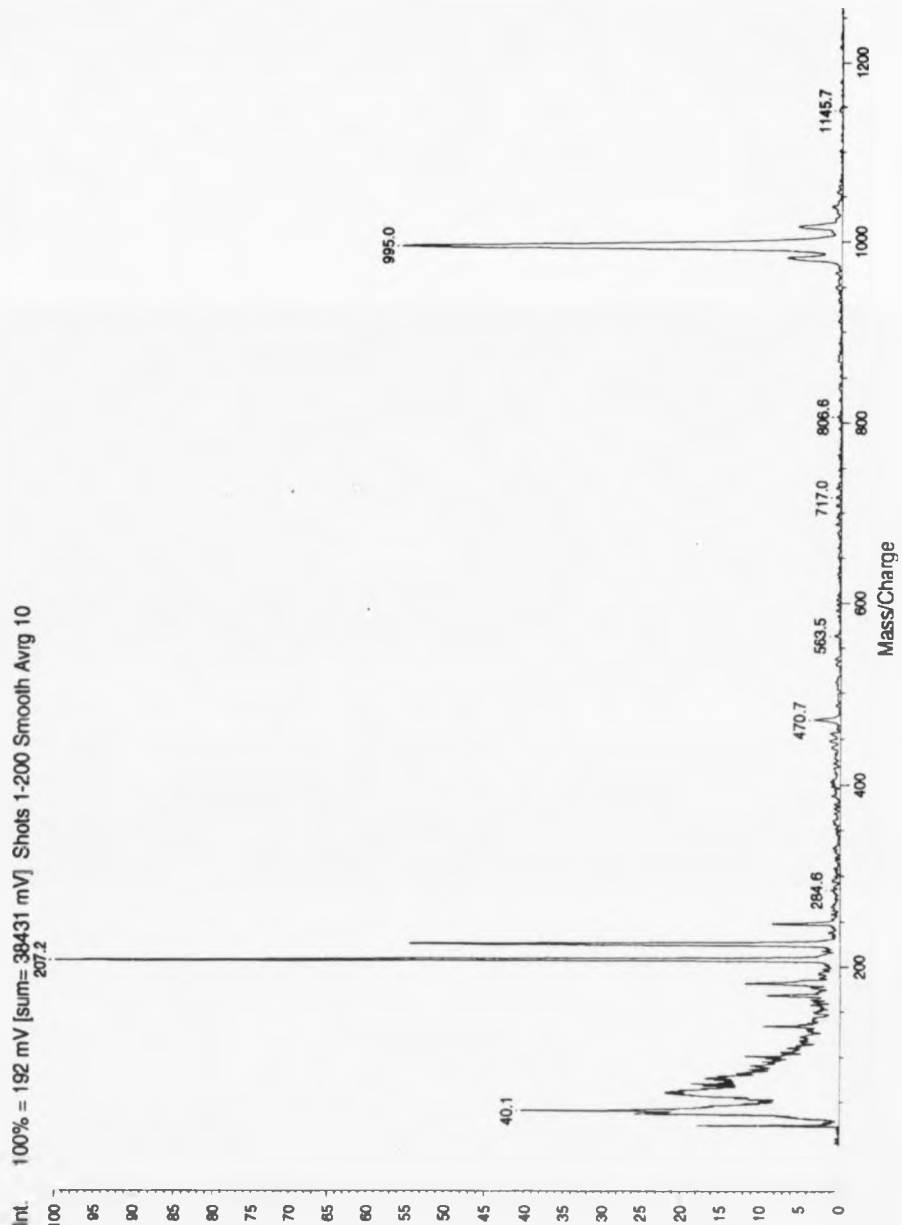


FIGURE 7.3 MALDI mass spectrum of the reference toxin microcystin-RR (m/z 1037.4). Sinapinic acid, 50 ng μl^{-1}, was added as the matrix.

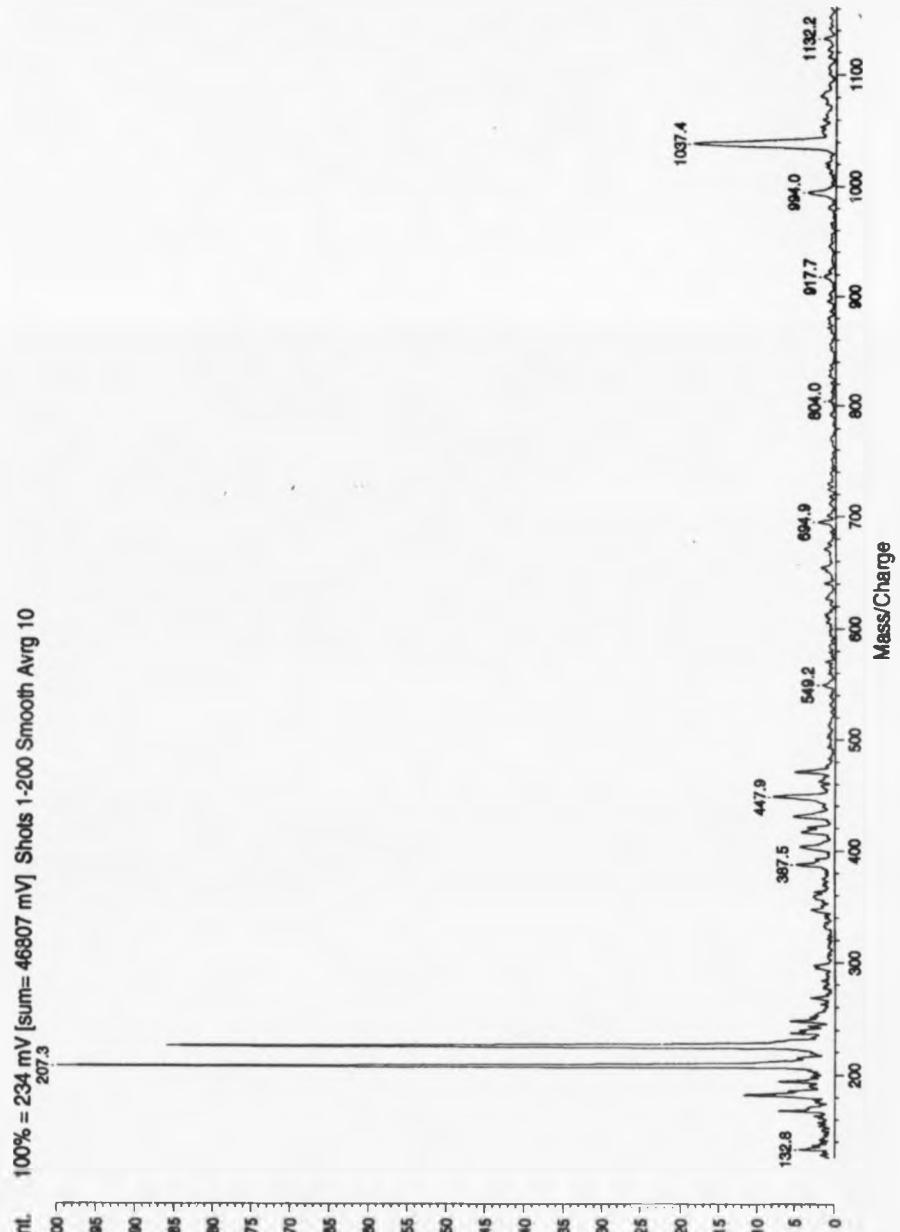


FIGURE 7.4 MALDI mass spectrum of the reference toxin nodularin (m/z 824.3). Sinapinic acid, 50 ng μl^{-1}, was added as the matrix.

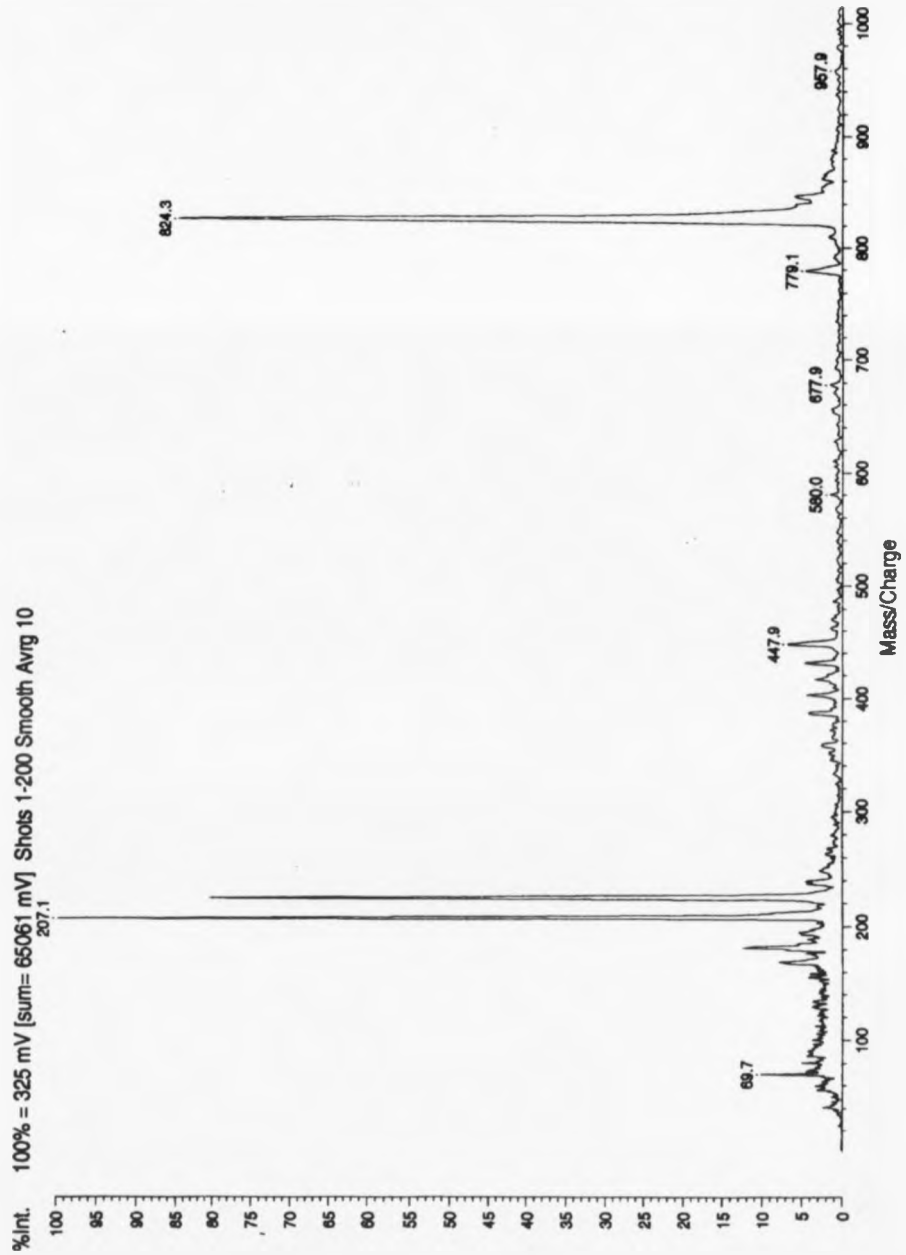
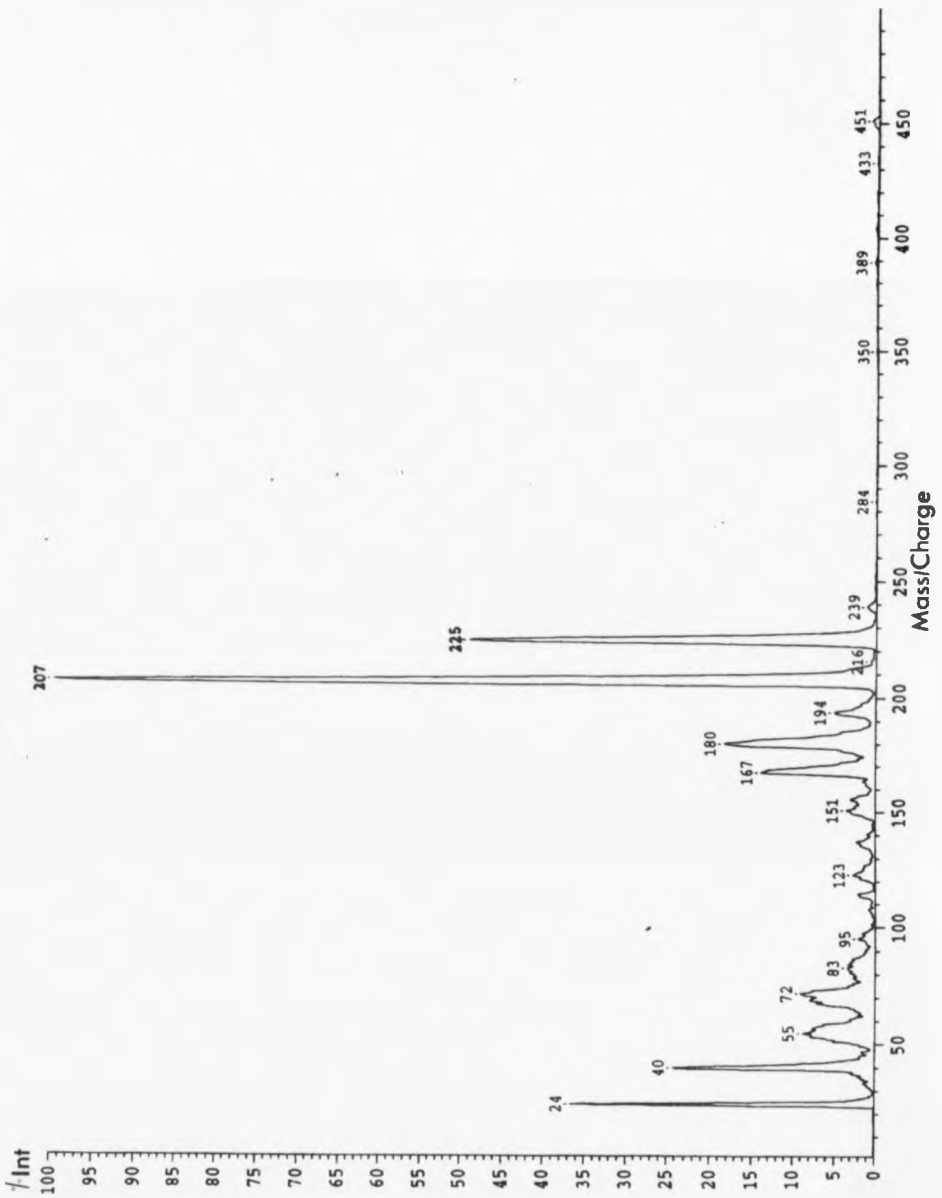


FIGURE 7.5 MALDI mass spectrum of the matrix. Peaks at m/z 207 and 225 were used as the calibration peaks for the instrument.



7.3.2 The Kratos MS-50 mass spectrometer

The Kratos MS-50 double focusing mass spectrometer was connected to a micro high performance liquid chromatography unit to facilitate CF-FAB-HPLC/MS analysis. The installation and the modification of the FAB probes was done by the Department of Chemistry. The pneumatic splitter was connected between the column and the probe in order to split the eluent from the column outlet and the resulting smaller portion was introduced into the CF-FAB probe *via* a fine capillary tube with an accurate flow rate.

Figure 7.6 shows the off-line CF-FAB mass spectrum of 5 µl of a reference microcystin-LR solution (1 µg μl^{-1}). The spectrum showed the peak intensity at m/z 995.6 which was the $(M+H)^+$ peak of microcystin-LR, MW 994.0 Da. The static FAB mass spectrum of the same reference toxin was also studied in order to compare the sensitivity of each technique (Figure 7.7). Off-line CF-FAB mass spectrum of linearised microcystin-LR is illustrated in Figure 7.8.a and b.

The standard microcystin-LR was treated directly with TFA to convert the cyclic peptide to a linear structure. From the study of Krishnamurthy *et al.* (1989), two products were formed. The peak of $(M+H)^+$ ions for product-I was shown at m/z 1013, which is to be expected for a linear structure resulting from the addition of water to a cyclic peptide of molecular mass 994 Da. For product-II, the $(M+H)^+$ ions was shown at m/z 930. This m/z value is to be expected for a peptide formed by the loss of the Mdha residue (83 Da) from the linear peptide.

FIGURE 7.6 The off-line continuous flow fast atom bombardment mass spectrum of the reference toxin, microcystin-LR (m/z 995.9).

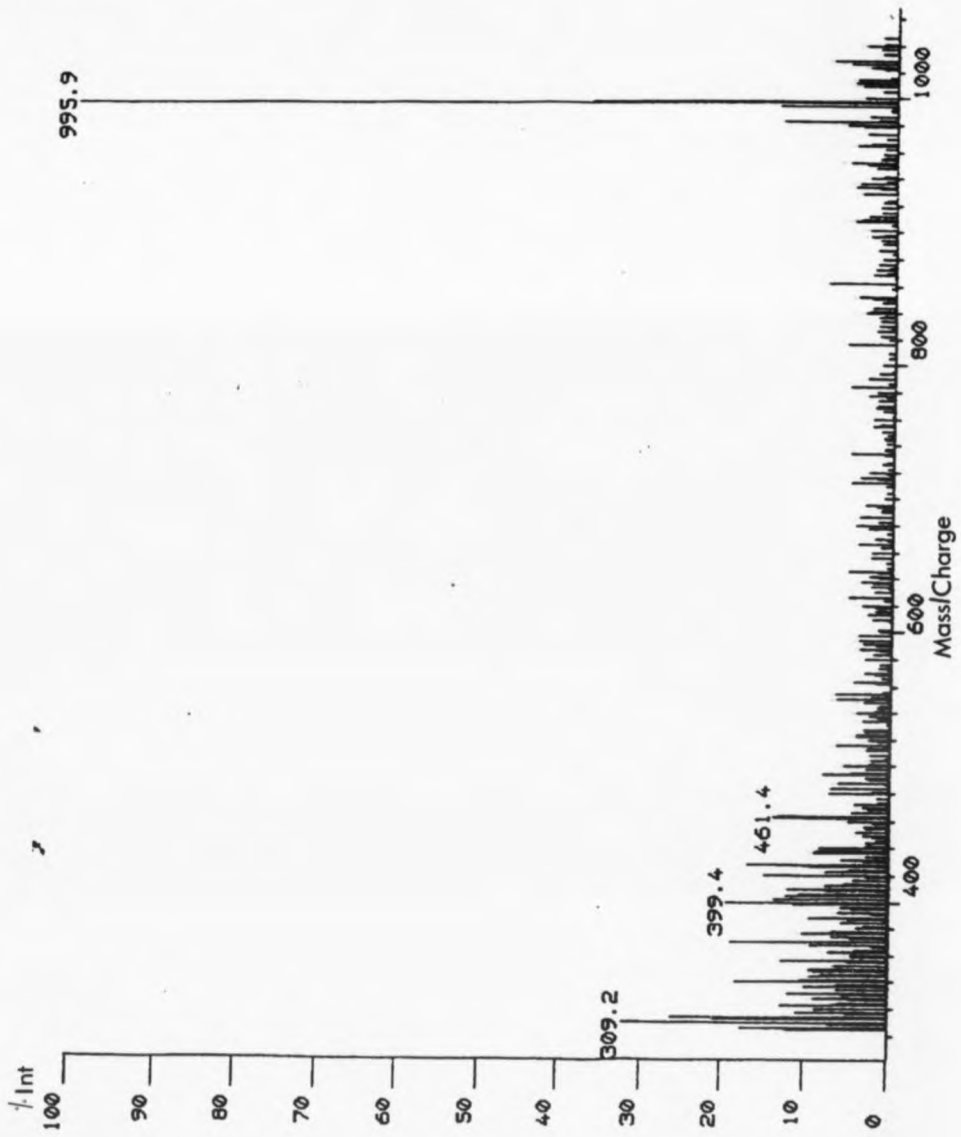


FIGURE 7.7 The static fast atom bombardment mass spectrum of the reference toxin, microcystin-LR (m/z 995.6).

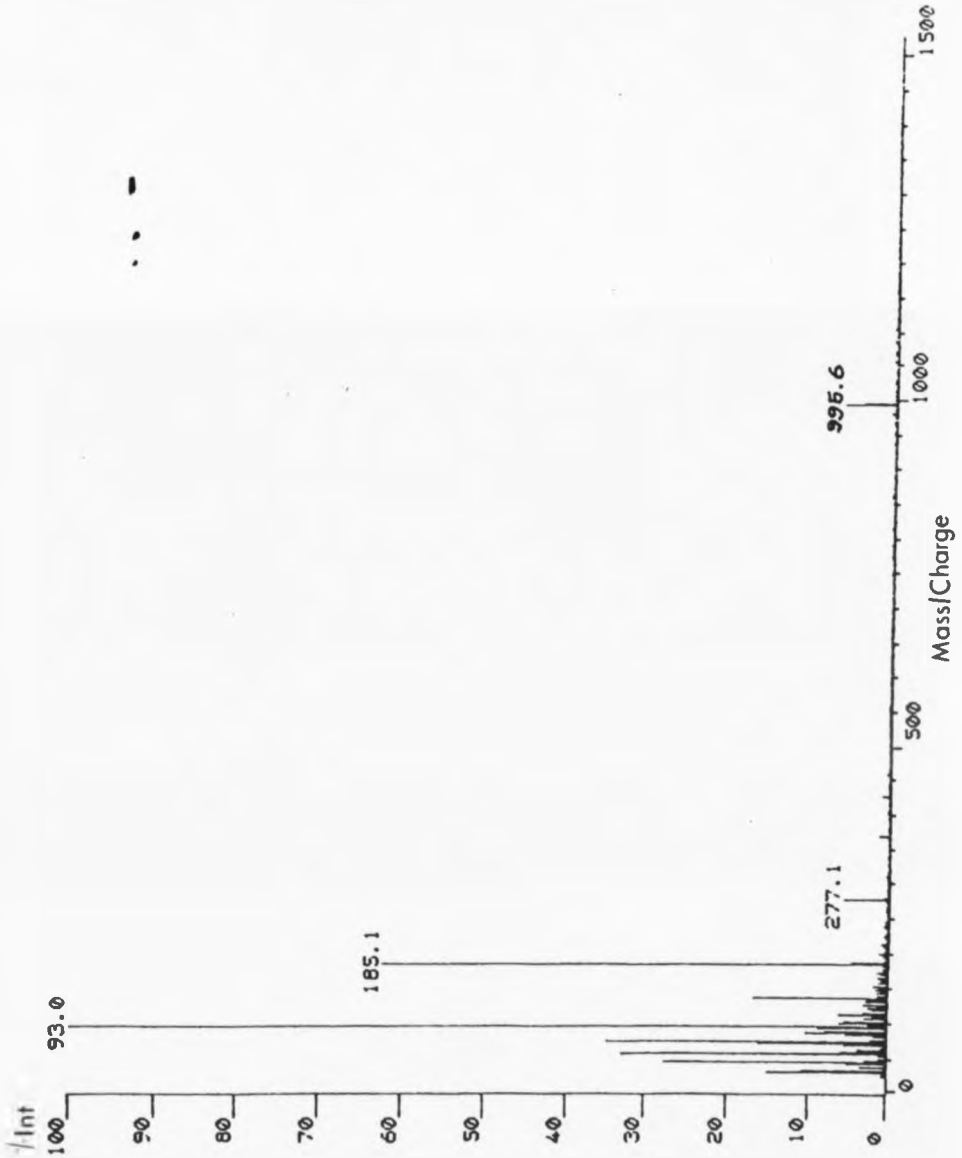


FIGURE 7.8.a The off-line continuous flow fast atom bombardment mass spectrum of linearised microcystin-LR indicated $(M+H)^+$ ion product-I at m/z 1013.2.

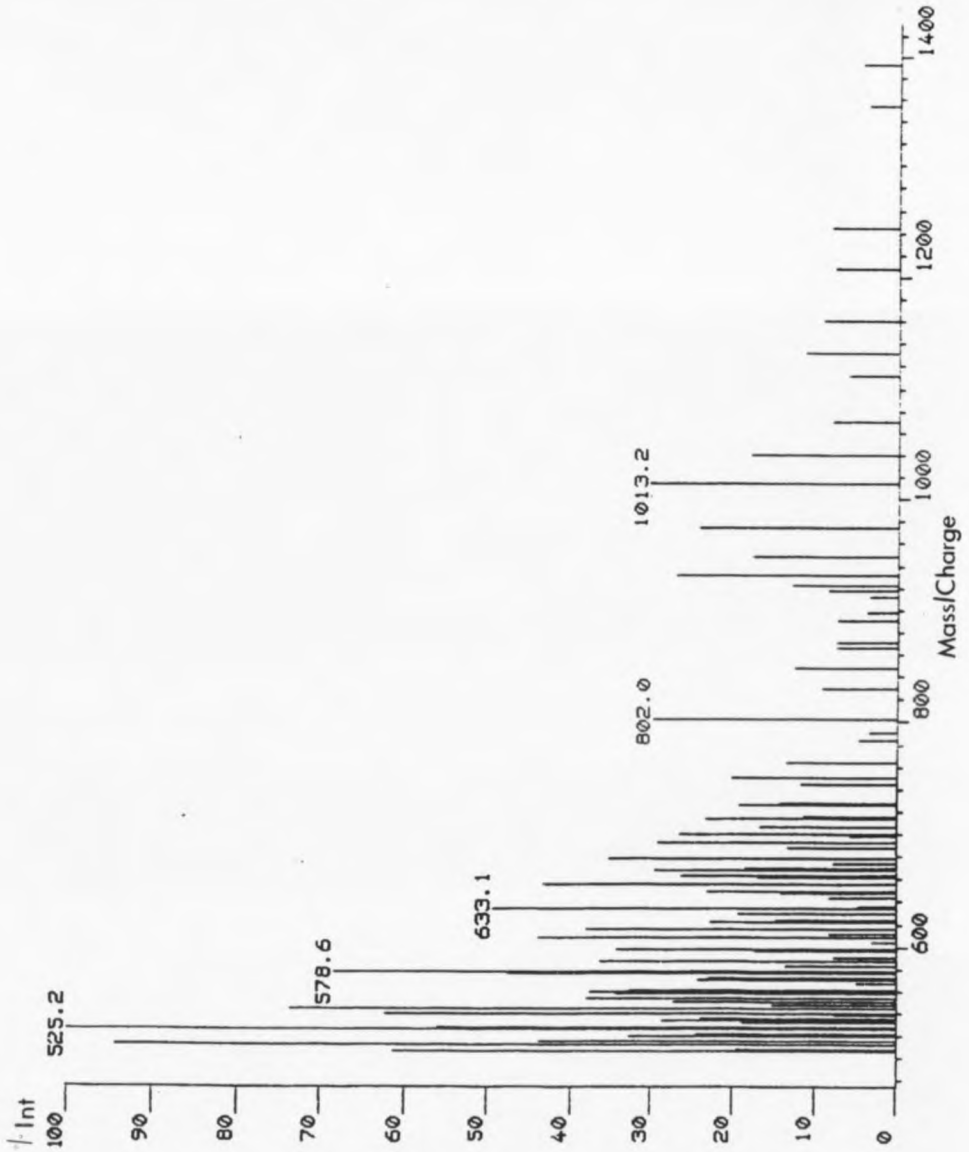
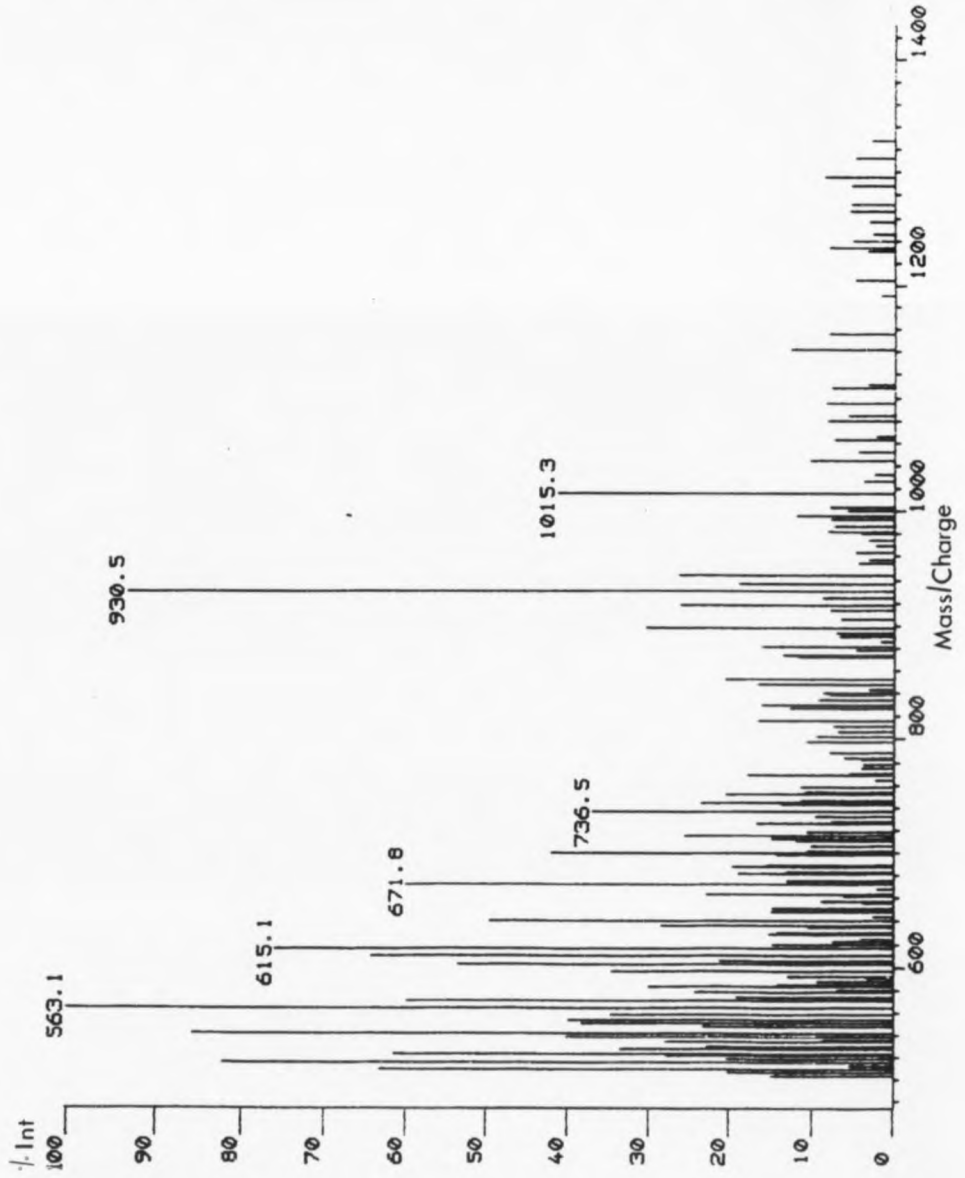


FIGURE 7.8.b The off-line continuous flow fast atom bombardment mass spectrum of linearised microcystin-LR indicated $(M+H)^+$ ion product-II at m/z 930.5.



7.3.3 The Kratos CONCEPT II HH four sector mass spectrometer

The Kratos CONCEPT II HH four sector mass spectrometer was operated by Dr. Dominique Despeyroux, Department of Chemistry. The mass spectra of the cyclic reference toxins showed the $(M+H)^+$ ion at m/z 995, 1038 and 825, which indicated the correct molecular masses of microcystin-LR, microcystin-RR and nodularin respectively (Figure 7.9). To convert the cyclic peptide to a linear structure, toxin was treated with neat trifluoroacetic acid (TFA). The linearised fingerprints of the three reference toxins are shown in Figure 7.10. The $(M+H)^+$ ion at m/z 1013 for linearised microcystin-LR and at m/z 1058 for linearised microcystin-RR were as expected for a linear structure resulting from the addition of water. The $(M+H)^+$ ion at 930 for linearised microcystin-LR and 973 for linearised microcystin-RR were the expected protonated molecules for a peptide formed by the loss of methyldehydroalanine residue from the linear peptides. However, the mass spectra of nodularin has shown that this toxin is not easily linearised as it was still shown to be the complete cyclic peptide.

FIGURE 7.9 Mass spectra of the cyclic reference toxins (microcystin-LR, microcystin-RR and nodularin) as obtained using a Kratos CONCEPT II HH four sector mass spectrometer.

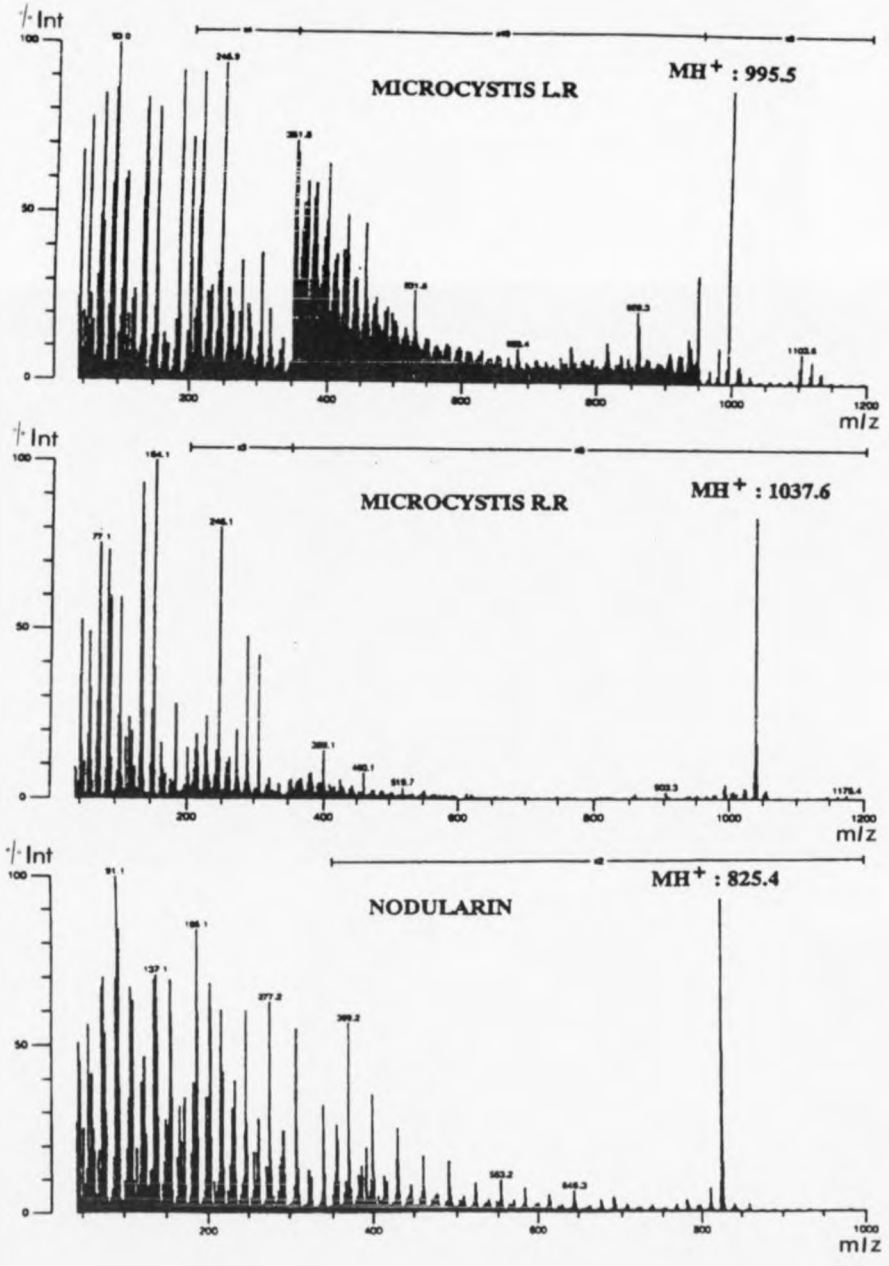
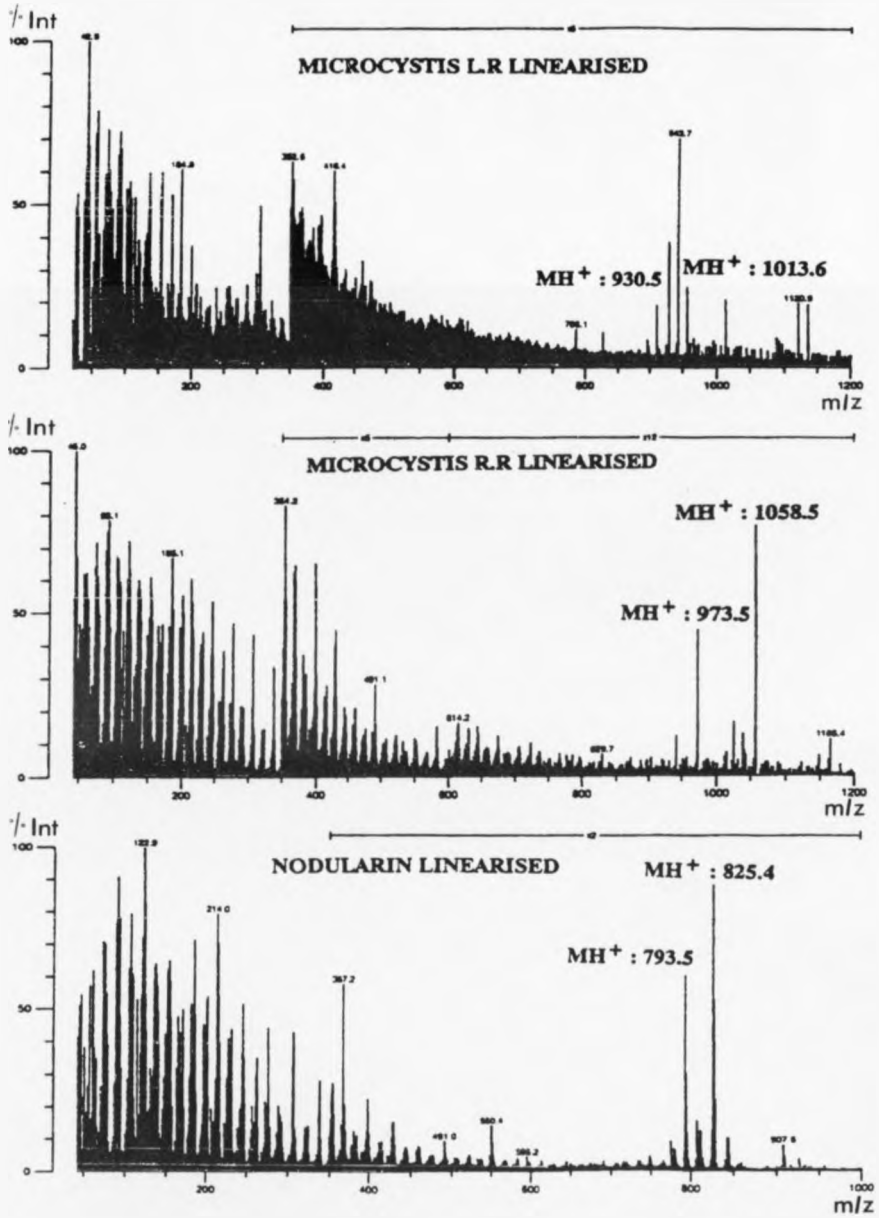


FIGURE 7.10 Mass spectra of the linearised reference toxins (microcystin-LR, microcystin-RR and nodularin) as obtained using a Kratos CONCEPT II HH four sector mass spectrometer.



7.4 Discussion

MALDI mass spectrometry was shown to be a fast and convenient technique for molecular mass determination of the peptide toxins from cyanobacteria. From the results illustrated (see Table 7.1), the relative molecular mass of the three peptide toxins produced by the *Oscillatoria* species from Lower Shustoke reservoir during 1991 to 1992, were 1022, 1043 and 982 Da, respectively. However, there were four peptide toxins produced in 1993, the relative molecular mass being 1022, 1023, 1043 and 981 Da respectively. According to the structural variation of microcystins in the scientific literature (see Table 1.4), it is possible to predict these three/four peptide toxins by comparison with published data. Nevertheless, the structural characterisation is still necessary to obtain the definite structure of each toxin.

The HPLC-CF-FAB-MS was a practical method for analysing the peptide toxins. The conditions operated in this study were the "off-line" system which was suitable when only the presence of the toxin and/or its molecular weight was required. There was no separation of the sample within this system since the injector was placed after the column which was used as a means of attaining a constant back pressure for the pump and flow into the probe. The HPLC-CF-FAB-MS was more sensitive than static FAB due to the higher intensity of the same sample. The "on-line" system was, however, studied by Jonathan Haywood, a third year student of the Chemistry Department. It was found that HPLC-CF-FAB-MS with the "on-line" system could be used quickly and efficiently to separate the toxic peaks for the molecular mass determination. However, the "off-line" system would be the better choice if the individual toxins were to be studied and structural fragmentation information was not necessary. The latter reduces the analysis time when compared with the "on-line" analysis, and also the procedure was more simple to use.

The concept II HH four sector mass spectrometer is the most powerful technique since picomole amounts of sample can be detected. The cyclic peptides

were treated with neat trifluoroacetic acid to convert them to the linear structure and then, they were analysed by mass spectrometry. Each linearised fingerprint can be used as the reference for comparison with those of the linearised unknown toxins. This mass spectrometer also provides the necessary structural information to deduce the sequence of amino acids in the derivatised linear peptides. In this study, nodularin was not successfully linearised by the employed method.

Due to the time limitations, the identification and characterisation of the structures of the presumptive peptide toxins produced by *Oscillatoria* species have not been achieved.

CHAPTER 8

CHAPTER 8 : GENERAL DISCUSSION AND CONCLUSIONS

Toxins produced by cyanobacteria (blue-green algae) have, over the past decade, achieved heightened importance and have been reported in virtually all parts of the world. The increased frequency of cyanobacterial blooms is almost certainly a consequence of nutrient enrichment of lakes and/or reservoirs. Indeed, it is noticeable that lakes and/or reservoirs in agricultural areas have problems with cyanobacterial blooms more frequently than those in less agriculturally active regions. A combination of long periods of calm, warm weather, sunlight in summer and stable, near-surface stratification is also found to accelerate the high incidence of cyanobacterial blooms. However, cold and windy conditions that lead to weaker stratification and/or a deeper mixed layer, do not promote the development of large populations. In suitable weather conditions these excessive growths of cyanobacteria can lead to surface scums which may produce toxins that are potentially poisonous to fish and mammals.

The discussion of the study of peptide toxins has been divided into 4 subjects as following:

(1) The requirement for an acceptable threshold of concentration of toxic substances.

Illnesses and death of wildlife, domestic animals, bird and fish have frequently been reported as a consequence of ingesting cyanobacterial biomass. Till now, no human fatalities as a consequence of cyanobacterial toxins have been recorded. However, it is essential that the potential threat to human and animal health be adequately investigated and assessed primarily as a consequence of the

increased demands for both sources of drinking water and recreational activities in the future. Also of paramount importance is the finding that hepatotoxins are potent tumour promoters at low concentrations and might contribute to the development of cancer (Carmichael, 1994). Most of the cases of sickness of large numbers of people which have been attributed to cyanobacteria, have followed the lysis of cyanobacterial blooms in water supply reservoirs. The fate of toxins once released from lysed cells is of considerable importance from a water supply perspective. This lysis may occur naturally when the cells die, or may occur if the cyanobacterial population is challenged by copper sulphate addition to the reservoir or after chlorination of water for drinking. Many drinking water supplies, particularly overseas, are simply chlorinated water from rivers or lakes, with no water treatment plant to filter off organisms or absorb toxins. However, preliminary experiments with highly toxic cyanobacterial biomass in raw water indicate that upon cell lysis very little "free" toxin is detected presumably as a consequence of bacterial activity. The hazard of recreational exposure to cyanobacteria depends upon the level of contact. The ingestion of concentrated cyanobacterial cells in the form of scum constitutes the greatest hazard. However, oral consumption of toxic cyanobacteria during recreation is relatively rare.

As a consequence of these potential health problems associated with toxic cyanobacteria, it is important to ensure that the public are informed to avoid direct contact with, and ingestion of, the scums and blooms of live and decaying cyanobacteria on the surface and shores of water bodies. The requirement for an acceptable threshold concentration of cyanobacterial toxins present in drinking water supplies and recreational waters must be addressed. The best established toxicity data is, at present, for the cyclic peptide hepatotoxins since these toxins have been subject to extensive animal testing. Water safety guidelines have been recently derived by Falconer (1994), using data for intra-peritoneal toxicity in mice, for chronic oral toxicity in mice, and particularly using new data for sub-chronic oral

toxicity in pigs. By application of a series of safety factors used in occupational health and drug evaluation, a reasonable safety guideline level for sub-chronic exposure to peptide hepatotoxins in drinking water has been calculated (Table 8.1). From these data 1 μg litre^{-1} cyanobacterial peptide hepatotoxins can be regarded as the maximum safe concentration in drinking water. This threshold concentration can be applied to peptide hepatotoxins produced by *Microcystis*, *Nodularia* and *Oscillatoria*. However, in situations involving neurotoxins, further work on toxicity is required to derive similar safety guideline concentrations.

TABLE 8.1 Safety factors in the determination of water guidelines for the maximum acceptable microcystin concentrations (Falconer, 1994)

Subchronic data to lifetime risk	-	10
Pig data to human risk	-	10
Intra-human population variation	-	10
Tumour promotion risk	-	10
Overall safety factor		10,000

Basic data - subchronic pig exposure. Lowest observable effect level 280 μg toxins per kg bodyweight per day.

Intake assumption 2 litres water per day by 60 kg adult, therefore 16.8 mg per day in 2 litres = 8.4 mg litre^{-1}.

÷ 10,000 = 0.84 μg litre^{-1} — approximately 1 μg litre^{-1} can be regarded as the maximum safe concentration.

It is extremely difficult to define a safety guideline concentration for a toxic substance in recreational water. This is because of the very wide variation in cell numbers of cyanobacteria per volume of water. Lake size, shape and circulation are important factors in determining cyanobacterial species succession and abundance in an individual lake or reservoir. According to water safety guidelines proposed by Falconer (1994), a cyanobacterial cell concentration of 20,000 cell ml^{-1} is the maximum for safe use of recreational waters and is based on sampling the top metre of open water. At this cell concentration, scums of concentrated cyanobacteria can be formed with suitable weather conditions and will present a significant hazard if these scums are toxic.

(2) The measurements of toxins.

To assess the toxicity of these toxins, mouse bioassay has been used as the primary procedure for a long time. However, the use of live animals, the associated ethical and the inherent experimental limitations have prompted studies of alternative methods for toxicity evaluation. The application of HPLC to detect and quantify the peptide toxins produced by cyanobacteria has been shown to be successful in this study. A gradient of ACN, with the addition of TFA as the modifier, was used as the mobile phase. Within the employed conditions, the detection limits were of the order of 50-100 ng for the reference toxins. The use diode array detection permitted spectral characterisation of the unknowns which could be compared with those of the reference toxins. A purity parameter was also generated. These facilities increased the degree of confidence in identifying unknown toxins rather than simply relying upon the retention time.

Oscillatoria species blooms in Lower Shustoke reservoir consistently produced sufficient cell biomass during the three years of study to allow study of the temporal expression of the toxicity over this period. This species gives expression up to four peptide toxins, with close structural similarity to either the microcystins or

nodularin. The ratio of the eluted peptides from the environmental biomass altered significantly with time. Variation in biomass toxicity was shown to be dependent upon the ratio of the toxic peptide components present in the cells with each peptide having a distinct toxicity level in mouse bioassays.

Some 60-70 raw water concentrate samples were analysed. However, it was not possible to detect the presence of free toxins with any acceptable degree of confidence in any sample except those associated with two heavy bloom samples in July and October 1993. This finding can be explained by either the toxins remaining associated with the cell debris or that free toxin is not in the raw water at detectable levels. The two raw water concentrate samples in 1993 that were positive for peptide toxins may have been a consequence of the release of toxins into raw water or contamination from the high level of cell biomass during the filtration step.

The phosphorylase phosphatase assay has been shown to be promising as a means of determining the hepatotoxicity of cyanobacterial toxins. Since hepatotoxicity results from the inhibition of the liver protein phosphatases, this inhibition is able to be used as an assay for cyanobacterial hepatic peptide toxins. Very low concentrations of toxin can be detected since the microcystins have a particularly high affinity for protein phosphatases. The concentration of toxin causing 50% inhibition of the maximum phosphorylase phosphatase activity (IC_{50}) was obtained from calibration curves for each reference toxin. Although microcystin-LR (LD_{100} = 150 μg kg^{-1}) was 10 times more toxic than microcystin-RR (LD_{100} = 1500 μg kg^{-1}) when injected intraperitoneally into mice, they both gave approximately the same IC_{50} (microcystin-LR = 2.4 nM, microcystin-RR = 2.8 nM). This difference may be due to either permeability effects or differential activation of the two microcystins *in vivo*. Okadaic acid, a potent tumour promoter, is also a potent protein phosphatase inhibitor. It is quite interesting that, under the same conditions of study, okadaic acid was shown to be about 10 times less toxic than the microcystins (IC_{50} = 24.5 nM).

Comparison of the HPLC and phosphatase assays, have shown that they have approximately the same detection limits for toxins in whole cell biomass as well as for high levels of "free" toxins in raw water concentrates. However, in the case of raw water concentrates which were normally non-toxic by both HPLC (detection limit = 200 ng litre^{-1}) and mouse bioassay the phosphatase activity appeared to be more sensitive (detection limit = 20 ng litre^{-1}).

The peptide hepatotoxins were shown to have no inhibitory effects on the other liver enzymes studied; lactate dehydrogenase, 5'-nucleotidase, γ-glutamyltransferase, acid and alkaline phosphatases, when the assays were carried out under the same *in vitro* conditions of study as the phosphatase assays. These enzymes were challenged with reference peptide toxins up to 5 μM.

Several alternative bioassays were assessed of which, the *Drosophila* bioassay was shown to be a promising alternative since there was high correlation between it and mouse bioassays.

Since the risks from cyanobacterial contamination of water supplies are important, the use of appropriate analytical and biological methods for the detection of cyanobacterial toxin concentrations can help establish regular monitoring for toxins in water supplies and recreational sites.

Of the "cell free" water concentrate samples over a three year period of study, only two samples contained cyanobacterial peptide hepatotoxins at detectable levels. One of these contained toxin at a concentration that was higher than the threshold value for peptide hepatotoxins proposed by Falconer (1994). The majority of toxic cell biomass samples contained peptide hepatotoxins at detectable concentrations. These ranged from 16 to 1074 μg of toxins per g dry weight of cell biomass. These results correlated strongly with the cell concentration of cyanobacteria present in the reservoirs always being greater than 20,000 cells ml^{-1}. Similar data for toxin concentrations were obtained as measured by the phosphorylase phosphatase inhibition assay with toxin concentrations ranging from 10 to 1350 ng of toxins per

litre of "cell free" raw water samples, and from 15 to 1100 μg of toxins per g dry weight of cell biomass. Thus, the toxin concentration measured by HPLC and the concentration of toxin estimated by phosphorylase phosphatase inhibition assay showed good correlation, particularly with the samples that contained high concentrations of toxin.

The *Drosophila melanogaster* bioassay gave good correlation of toxicity data between it and the mouse bioassay for both standard reference peptide hepatotoxins and toxic cyanobacterial biomass. However, further study is required to ascertain the dose response in order to accurately calculate the concentration of toxin in assayed samples.

The peptide hepatotoxins produced by the *Oscillatoria* species present in Lower Shustoke reservoir during the study period were readily detected in the cell biomass but rarely from "cell free" water concentrates. From comparison of the toxin concentrations with the proposed acceptable threshold of concentration for peptide hepatotoxins, it is possible to say that water in Lower Shustoke reservoir is toxic throughout the Summer months. However, "cell free" raw water (equivalent to 10 litres of water) has been shown to be non-toxic.

HPLC and phosphorylase phosphatase inhibition assays have been shown to be sufficiently sensitive methods of analysis to determine the concentration of peptide hepatotoxins in both raw and potable water.

Matrix assisted laser desorption time-of-flight (MALDI) mass spectrometer was used for molecular mass determination of the presumptive peptide toxins produced by *Oscillatoria* from Lower Shustoke reservoir. The four peptide toxins had molecular weights of 1023, 1022, 1043 and 981 dalton. Linearised fingerprints of the reference toxins; microcystin-LR and microcystin-RR were obtained using a Kratos CONCEPT II HH four sector mass spectrometer. Unfortunately, the structures of the four presumptive *Oscillatoria* peptide toxins were not elucidated in this study because of the limitation of time and the availability of the

instrumentation. It is not possible, therefore, to correlate the difference in structure of the toxins with the perceived toxicities from both mouse and phosphatase assays.

Physico-chemical quantitative analytical alternatives to mouse bioassays for determining the toxicity of cyanobacterial blooms have been developed. Biochemical methods for toxicity assessment have also been assessed. The choice of the most suitable method to detect cyanotoxins is governed by many factors i.e. the type of toxin, the toxin concentration. However, it is suggested that the method of choice should be complemented by high quality chemical analytical techniques to confirm the identify and concentration of the toxins.

(3) Natural function(s) of toxins ?

The factors leading to the production of cyanobacterial blooms require investigation. In this study, most common species of scum-forming cyanobacteria such as *Microcystis*, *Oscillatoria* and *Anabaena* were found to be capable of producing toxins. During the three years of study (1991-1993), the development and composition of algae communities in Lower Shustoke reservoir showed little variation. The predominant cyanobacterial species was *Oscillatoria* although during the early part of the year, *Pseudoanabaena* was present in significant numbers. However, its toxicity was very low or undetectable. Light and temperature were the most significant environmental factors that appeared to effect the growth of the *Oscillatoria* species and showed significant correlation with toxin production. Another environmental factor that promoted the growth of *Oscillatoria* species and toxin production was the phosphate concentration. It is difficult to conclude whether nitrate deficiency had an effect on toxin production during the course of this study. The pH of the reservoirs under study was shown to be consistently between 8-9 i.e. close to the optimum for cyanobacterial growth. Physical factors such as wind, site and stable weather may also have played significant roles in influencing cyanobacterial growth rate. The heavy blooms of *Oscillatoria* in July and October

1993 were almost certainly the result of long, stable and warm weather conditions.

The toxin-forming cyanobacteria are all naturally occurring members of the phytoplankton of freshwater. The increase in planktonic cyanobacterial populations may be dramatic with the occurrence of surface scum (water-blooms) due to dense accumulations of cells of usually one or two species which develop when ecological conditions are favourable. Toxicity of cyanobacteria varies spatially and temporally through a bloom and with geographical location. The toxins of cyanobacteria are a relatively new group of natural products and their function(s), in general, remain unclear. The published literature presents conflicting views as to the possible roles of the cyanobacterial hepatotoxins.

It seems unlikely that the toxins play any role in the growth and development of the organisms or perform an antibiotic or allelopathic function which would account for the ecological dominance of a particular strain (Fay & Van Baalen, 1987). However, there is some early evidence to support a possible allelopathic function. There are documented examples of algal allelopathy showing that toxic substances from cyanobacterial blooms are specifically toxic to other algae isolated from the same lake (Keating, 1977). Metabolites excreted by cyanobacteria are also inhibitory to diatom species which may be dominating the water body (Keating, 1978).

The cyanobacterial toxins can be extremely harmful not only to bird, fish, cattle and so on, but also to the zooplankton living in lakes and reservoirs. Some *Microcystis* hepatotoxins have been shown to be toxic to filter-feeding water fleas (*Daphnia*) which are potential consumers of the algae, and to some protozoan ciliates (Reinikainen *et al.*, 1994). The toxins may be directly lethal, or they may reduce the number and size of offspring produced by the creatures that feed on cyanobacteria. Thus, it is likely that cyanobacteria synthesise toxins to ward off/minimise attack by predators. However, zooplankton species generally do not

eat cyanobacteria unless there is no other food available as a consequence, for example, of an enhanced ciliate or rhizopod protozoa populations.

Another suggestion on the function of cyanobacterial toxins is that these substances serve to maintain basic metabolism in circumstances when its products (phosphates, reduced coenzymes and substrates for synthesis) can not, due to nutritional imbalances, be used for replication, and to do this in a way which can safely be turned off when environmental circumstances improve, or when a new program of activity can be activated. Since most common toxic cyanobacteria can also be the main phytoplankton organism for many herbivore zooplankton, their toxins may serve both as anti-herbivore devices and as stores of needed nutrients (Carmichael, 1982).

It also has been proposed that the toxin-producing *Microcystis aeruginosa* strain has a more efficient iron uptake system than the strain that does not produce toxin. The relationship between light intensity and iron uptake has been examined and it is found that iron uptake increased as light intensity increased (Utkilen & Gjolme, 1995). The biological function of the peptide toxin could be as an intracellular chelator which keeps the cellular level of free Fe^{2+} low, since it also has been revealed that the toxin binds iron. The non toxin-producing Microcystis strain does not have this intracellular chelator (toxin) and must therefore have a lower cellular Fe^{2+} concentration.

The production of the peptide toxin appears to be uncoupled from general protein synthesis. This finding together with the amino acids present in the toxin indicates that in *Microcystis* its synthesis is mediated by a nonribisomal mechanism. Since the peptide toxin produced by *Microcystis* has a structure similar to bacitracin, a peptide produced by *Bacillus* strains through a nonribosomal mechanism, the production of the cyanobacterial peptide could be *via* a similar mechanism and that the activity of the enzyme involved in the production could be effected by Fe^{2+}.

Moreover, the cyanobacterial toxins may once have had some function which is suggested by the fact that microcystins and nodularin act on the protein phosphatases that regulate the proliferation of eukaryotic cells. The peptide hepatotoxins do not seem to participate in cell function and cell division in cyanobacteria, but they may have played such a role early in the evolution of these organisms (Carmichael, 1994).

(4) The statistical analysis of the results.

In this project, each experiment has been done at least in duplicate or triplicate. Thus, it is important to apply statistical analysis to the results of replicate experiments. The data from each experiment are calculated and presented as arithmetic "mean" values. The mean value shows less fluctuation than the median (the middle value of the data after they have been ranked in size order) or the mode (the most frequently occurring value). Therefore, it gives the best estimate of the location of the groups of data.

For example, from table 4.4 (page 149), the total concentration of peptide toxins in whole cell cyanobacterial biomass from Lower Shustoke reservoir during 1991 as detected by HPLC analysis are shown in μg of toxins per g dry weight of cells. The concentration of toxins shown in the table at each sample date are the "mean" values calculated from duplicate experiments. The data in the brackets are the minimum and maximum values. For example, on 15 June 1991, the mean value of total concentration of toxin is 279 μg of toxins per g dry weight of cells. The minimum total concentration of toxin is 268 μg of toxins per g dry weight of cells and the maximum total concentration of toxin is 290 μg of toxins per g dry weight of cells.

The data from the experiments relating to the total concentration of toxins and the concentration giving 50% of maximum inhibition of phosphorylase phosphatase activity, IC_{50} have been used to show the variation of toxin

concentration at different sampling times for the year, the "mean" value being plotted. The standard deviation has not been calculated and shown for these data. However, some experiments in this project such as the effect of dilution on the inhibition of phosphorylase phosphatase activity (section 5.3.1) and the time course for the inhibition of phosphorylase phosphatase activity (section 5.3.2), the standard deviation values have been calculated and are shown on the graphs.

With hind-sight it would have been better if the data from all experiments had been treated similarly with respect to calculation of the standard deviation.

The incidence of cyanobacterial poisoning will decline if suitable monitoring and subsequent precautions are taken. The long term effects of the peptide toxins require further study, since the peptide toxins act similarly to known potent tumour promoters. It is also necessary to continue the study of the biological factors which affect toxin production in the laboratory which will hopefully facilitate the control of toxin production in the environment. The biochemical pathways leading to the production of toxins and their regulation have yet to be elucidated and will most certainly be the phase of greatest activity in the next few years.

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