ALGAL TOXIN IN DRINKING WATER SUPPLIES

This report consists of 2 sections

Section I

The occurrence of algal toxins in the
Umgeni catchment and an investigation into their remediation

by B Rae and R W Moollan

and

Section II

Use of protein phosphatase enzyme inhibition
as a detection and quantification method for the
presence of *Microcystis* toxins in drinking water

by R C Clark

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Section I

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SECTION I

THE OCCURRENCE OF ALGAL TOXINS IN THE UMGENI CATCHMENT AND AN INVESTIGATION INTO THEIR REMEDIATION

Report to the

WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

Cyanobacterial (blue-green algae) blooms are common to many South African freshwater impoundments that supply bulk water to water works. Certain genera are known to produce a range of toxins that are potentially harmful to humans and animals if consumed. The most prevalent group is the hepatotoxic microcystin toxins and nodularin, that have caused numerous animal deaths throughout the world, including South Africa. In humans, these toxins are suspected tumor promoters and more recent reports (1996) have linked the death of 47 kidney dialysis patients in Brazil to microcystin toxin poisoning.

Previous investigations conducted in South Africa have found that microcystin toxins can persist even after conventional water treatment processes, and the concern that these toxins may enter drinking water supplies prompted this investigation.

The objective of this project was threefold:
(i) to investigate sensitive and reliable methods for the detection of algal toxins,
(ii) to monitor critical impoundments (such as Inanda and Vaal dams) for at least a year, and
(iii) to research various remedial actions for the safe and effective removal of these toxins from water supplies.

The project was a joint venture between the CSIR and Umgeni Water. Based on the available equipment and expertise, the CSIR was assigned to develop an immunoassay method, and Umgeni Water, a chemical method for the determination of algal toxins in water supplies. The reports have been separated into Section I and II. (Please refer to the Executive Summary in Section II for details of the immunoassay work).

Section I reports on the methods investigated for the determination of microcystin toxins in aqueous media, the detailed monitoring of major impoundments in the Umgeni catchment, and the investigated treatment options for the removal of these toxins. Also included, are aspects concerning the isolation of microcystin toxins from algal material and the laboratory culture of toxic *Microcystis* to serve as a possible source of the toxins that are not available commercially.

1. Fluorescence Detection Methods

Fluorescence detection methods were investigated for the determination of microcystin toxins in an attempt to obtain the lowest possible detection limits that can be provided by High Performance Liquid Chromatography (HPLC). The research was modeled on the relative success obtained by the derivatisation of various amino acids and reduced nitrosoamines (reported in the literature), which involved the attachment of a fluorescent reagent to the guanidino moiety of the analyte molecules. It was reasoned that these particular fluorescence reactions may be applicable to microcystin toxins containing a guanidino moiety.

The reaction mixture was separated using C_{18} reversed-phase HPLC and fluorescent compounds were detected using a Scanning Fluorescence detector.

The results from this investigation were inconclusive. Fluorescent peaks were observed in the chromatograms of the reacted toxin solutions; but no reliable evidence of the derivatised toxin product was obtained because of the difficulty in assigning particular chromatographic peaks to the derivatised products. The derivatisation reaction with 4-(2-phthalimidyl)benzoyl chloride (PIB-Cl) appeared to be promising, as one of the chromatographic peaks was observed to increase with increased toxin concentrations. The peak in question, however, was later established to be that of the methyl ester of the fluorescent reagent - produced by the reaction of the reagent with the methanol from which the toxins were crystallised.

2. Ultra-violet (UV) Detection Methods

The derivatisation reactions of microcystin-LR were also briefly investigated using reversed-phase HPLC with UV detection. The results provided no evidence of reaction, since no new peaks were and both the microcystin toxin and reagent peaks were observed unchanged in the reaction mixture

A literature method for the determination of microcystin toxins in raw and treated waters by Lawton *et al.*[36] (Analyst, July 1994) was evaluated. The method utilises C_{18} solid-phase extraction of the toxins from aqueous media, followed by reversed-phase HPLC with photodiode array detection. A novel feature of this particular method is the use of characteristic UV spectra to identify possible microcystin toxins. All the microcystin toxins reported have one of two characteristic UV spectra; one having a local absorption maximum around 238nm and the other having two local maxima, at 238 and 222 nm. Chromatographic peaks in a sample may be tentatively assigned as microcystin toxins on this basis.

It was concluded that the principles upon which the method is based are reliable; in that all the microcystin toxins studied gave very similar UV spectra with absorption maxima at 238 nm; the toxins were well resolved using reversed-phase HPLC and could be reliably concentrated from aqueous media using C_{18} solid-phase extraction; but that the method needed modification if more consistent and accurate results were to be obtained.

Microcystin toxin standards prepared in methanol were found to give HPLC-UV detection limits between 0.2 and 0.4 $\mu g.m\ell^{-1}$. Using the Umgeni Water method of analysis, these detection limits would correspond to sample detection limits between 0.04 and 0.08 $\mu g.\ell^{-1}$. Assuming 100% toxin recovery, the toxins could be concentrated 5000 times.

Microcystin-LR recoveries from ultra-pure water (at concentrations of 0.4 $\mu g.\ell^{1}$) ranged from 66 to 107%, with an average recovery of 86% for 78 test samples with a relative standard deviation 8.7. Mixed toxin recoveries (microcystin -LR, -RR, -YR and nodularin) gave similar recoveries but replicate analyses produced variable percentage recoveries, implying that the exact toxin recovery from a sample cannot truly be determined accurately, but only estimated using spiked recoveries from which 100% recovery can be calculated.

Consistently low nodularin recoveries (between 24 and 57%) were recorded from tap (treated) water when residual chlorine was not removed prior to the addition of the toxins. Addition of sodium thiosulphate (and not sodium sulphite) is recommended for the removal of residual chlorine as this procedure improved nodularin recoveries to between 68 and 87%. The recovery of the other toxins was not so markedly affected by the presence of residual chlorine.

Microcystin toxin recoveries from raw waters varied depending on the sample matrix. Clear waters produced higher recoveries than more turbid samples.
The following limitations were observed:

(i) toxins could not be determined at very low concentrations (0.4 $\mu g.\ell^{1}$) from all types of water matrices. Due to interferences, the more turbid the samples the lower the percentage toxin recoveries and the more complex the resultant chromatogram.

(ii) Co-elution of other organic compounds made the determination of the toxins difficult especially when toxins were present at low concentrations. Sometimes the interferences completely masked the spiked toxins.

(iii) The use of the UV spectra to identify possible microcystin toxins was not possible at low toxin concentrations. In addition, other peaks in the sample chromatograms sometimes had very similar spectral characteristics to those of the microcystin

toxins although the local UV maximum is either slightly greater or lower than the specified 238 nm.

Despite the above limitations, reversed-phase HPLC with photodiode array detection is a convenient method for the identification and quantification of the microcystin toxins and it has been successfully applied in many cases. However, other possible techniques, including capillary zone electrophoresis would have been evaluated had time permitted.

3. Monitoring

Samples were taken from the 8 major dams in the Umgeni catchment (Midmar, Albert Falls, Henley, Shongweni, Nagle, Inanda, Hazelmere and Nungwane dams) on a routine basis from February 1995 to March 1997. Samples were taken from sites located near the abstraction points as these were considered to be representative of the water entering the water treatment works. Surface samples were taken instead of integrated samples since the *Microcystis* and *Anabaena* cells are buoyant and there would thus be an increased chance of finding the toxins near the surface.

To date, no algal toxins have been detected in the water samples taken from the abstraction sites, implying that no toxins were entering the water works. However, algal scum samples taken where *Microcystis* cells had accumulated in Nagle, Inanda, Albert Falls and Hazelmere dams were shown to contain microcystin toxins. Raw water samples, submitted from the routine sampling points during the same period or on the same day as the toxic scums were noted, did not contain microcystin toxins. This may be attributed either to the low cell numbers in the water samples or to toxin concentrations being too low to be detected. None of the toxic scums were of a persistent nature, and were rapidly dispersed by wind and water currents. Other sites from which toxic *Microcystis* algae were collected included : Camp's Drift weir on the Umsindusi river and the kwaMakutha Wastewater Works. Algal scums taken from Nagle and Inanda dams were found to contain microcystin toxins on each occasion. Other *Microcystis* scums from Nungwane and Shongweni dams however, did not contain microcystin toxins.

Microcystin-LR was the most common toxin found but other toxins were also observed – as indicated by the characteristic UV spectra of the microcystin toxins. No toxin standards of the other microcystin toxins were available to make an identification/confirmation and quantification possible. The highest extracellular microcystin-LR concentration found was 2.8 $\mu g.\ell^{-1}$, obtained from one of the Inanda Dam scum samples. The highest intracellular microcystin-LR concentration recorded was 979 $\mu g.g^{-1}$ of freeze-dried algae, found in a *Microcystis* scum taken from the kwaMakutha Wastewater Works.

The other dams (Henley and Midmar) did not have elevated numbers of blue-green algae and no observed accumulation of cells was encountered during the period of this investigation.

In conclusion, the results indicate that microcystin toxins are not currently entering the potable water supply in the Umgeni Catchment. The absence of microcystin toxins may be attributed to the low incidence of cyanobacterial blooms near the abstraction sites during the period of this investigation. The algal cell counts from the samples taken near the abstraction points did reach relatively high numbers from a hydrobiological perspective; but these cell concentrations did not warrant concern from a microcystin toxin perspective, as very high cell numbers are usually required before toxins are detected in the surrounding water. Massive *Microcystis* blooms in Inanda, Nagle, Albert Falls or Hazelmere dam, however, should be considered as a potential health risk, as it is likely that they could contain microcystin toxins. The sampling sites chosen for monitoring purposes, however,

were biased in the sense that the study was designed to indicate microcystin toxin levels entering the treatment works, and did not necessarily account for possible human exposure to these toxins. The monitoring of rural areas and smallholdings, where people consume water directly from raw waters, is strongly recommended as the risk of consumption would be greater in these areas and a study of this nature has not been conducted in South Africa.

4. Remediation Processes

The three remediation processes investigated were ozonation, chlorination and adsorption byactivated carbon. All three treatment processes have been reported in the literature for the removal of microcystin toxins. Laboratory bench-scale experiments were performed using various water matrices and two types of toxin standards, namely, pure microcystin toxins and a toxic *Microcystis* extract containing microcystin-LR. While the use of the pure toxins would provide data for the removal of the toxins *per se*, the use of the toxic *Microcystis* extract, as a toxin standard, provided information on the removal of microcystin toxin in the presence of associated organic compounds that are normally found with a toxic algal bloom.

Ozonation:

Ozone was found to be very effective for the removal of microcystin toxins. Pure microcystin-LR at concentrations up to 16 $\mu g.\ell^{-1}$ (in ultra-pure water) was removed to below the method detection limit (of 0.04 to 0.08 $\mu g.\ell^{-1}$) using an ozone dose as low as 0.2 $mg.\ell^{-1}$. However for pure microcystin-LR with a concentration of 9.6 $\mu g.\ell^{-1}$ in Camp's Drift raw water, a pH trend was observed: using an ozone dose of 0.2 $mg.\ell^{-1}$, the toxin was completely removed at pH 3.2, whereas at pH 7.7 and pH 10.0, the toxin was reduced by 18% and 10-28%, respectively. The DOCs (dissolved organic carbon) of the samples used in these experiments were all similar, as they were prepared from one batch of raw water and were spiked with the same amount of toxin standard. Other experiments using ozone doses of 1.2 -1.4 $mg.\ell^{-1}$, also showed higher toxin removal at lower pHs. Using a higher ozone dose of 2.3-2.4 $mg.\ell^{-1}$, the microcystin-LR at a concentration of 9.0 $\mu g.\ell^{-1}$ was reduced by 91-100% at the natural raw water pH of 7.7, whereas at the elevated pH of 10.0, the microcystin-LR from the extract-containing samples was reduced by 85-97%. At the same ozone dose at pH 7.7, pure microcystin-LR at a concentration of 156 $\mu g.\ell^{-1}$ was reduced by 98%. A mixture of pure microcystins-RR, -YR, -LR and nodularin having concentrations of 8.8, 6.2, 7.6 and 7.6 $\mu g.\ell^{-1}$, respectively, in Camp's Drift water, was reduced by 89-100% at an ozone dose of 2.2-2.4 $mg.\ell^{-1}$. A recommended ozone dose of between 2.5 to 3.0 $mg.\ell^{-1}$ would therefore be considered suitable for the treatment of microcystin toxins in raw waters having pHs of between 7 and 8.

Chlorination:

The removal of microcystin toxins by chlorination was also found to be pH dependent. Using the toxic *Microcystis* extract in Camp's Drift water, microcystin-LR at a concentration of 7.4 $\mu g.\ell^{-1}$ was completely removed after 30 minutes at pH 5.5 with a chlorine dose of 2.0 $mg.\ell^{-1}$. Under the same conditions, the toxin was reduced by 81-97% at pH 7.7, and by 12-21% at pH 10, despite the chlorine residuals remaining above 0.5 $mg.\ell^{-1}$. At the natural raw test water pH of 7.7, a chlorine dose of 4.0 $mg.\ell^{-1}$ produced complete removal of the toxin, whereas at pH 10.1 the toxin was reduced by 43-56%. Trihalomethane (THM) formation at this chlorine dose was between 10-18 $\mu g.\ell^{-1}$, which is below the guideline limit of 460 $\mu g.\ell^{-1}$. At pH 10.2, a chlorine dose of 12 $mg.\ell^{-1}$ only reduced the microcystin toxin concentration by 42-70%, implying that even higher dose must be applied to obtain complete removal of the toxins at this pH.

Powdered Activated Carbon (PAC):

The removal of microcystin toxins using PAC differed according to the type and dose of carbon applied, as well as the type of toxin standard used. In most of the tests the toxin removal was higher from samples spiked with the pure toxin standards than those spiked with the toxic *Microcystis* extract containing microcystin-LR. Three carbons derived from wood (Sutcliffe WF, Zetachem and Aktacarb PWT) and one derived from coal (Sutcliffe 207CP) were tested. The Sutcliffe WF PAC was the most effective carbon tested, and produced 93-98% removal of the microcystin-LR at a PAC dose of 10 $mg.\ell^{-1}$ from the extract-containing samples with an initial microcystin-LR concentration of 6.9 $\mu g.\ell^{-1}$. At a PAC dose of 20 $mg.\ell^{-1}$, 96-100% removal of the toxin was obtained. The Zetachem PAC was also effective producing 46-50% microcystin-LR removal at 10 $mg.\ell^{-1}$ and complete removal at 25 $mg.\ell^{-1}$. The other two carbons (Sutcliffe 207CP and Aktacarb PWT) failed to produce complete removal of the toxin at the highest PAC dose of 25 $mg.\ell^{-1}$.

5. HPLC-MS Analysis and Toxin Isolation

In an attempt to determine the unknown microcystin toxin found in a *Microcystis* scum sample taken from Nagle dam, the toxins were extracted from the freeze-dried cells with methanol and submitted for High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis. The aim was to determine whether the molecular ion or fragmentation pattern of the "unknown" toxin could be established and referenced in the literature. The use of methanol for the extraction of the toxins was not selective, however, and many other components (lipids, proteins, pigments, etc.) were also extracted as indicated by the numerous peaks in the total ion chromatogram (TIC), many of which were not well resolved. A sample clean-up procedure or partial purification of the raw toxin extract is therefore necessary prior to LC-mass spectral analysis.

The isolation procedure investigated involved a combination of silica gel and C_{18} flash chromatography which adequately purified the toxin for HPLC-MS analysis.

6. Culture of Toxic *Microcystis*

This exercise was not in-depth, but was done to ascertain whether toxic scums could be cultured in the laboratory to serve as a constant supply of the toxins, most of which are not commercially available. *Microcystis* grows very slowly in batch culture in the laboratory and thrives best in BG-11 culture medium. Non-axenic cultures are sensitive to light conditions. Higher light intensities favour the green algae which can completely dominate an almost pure *Microcystis* culture within 1-2 weeks. Sterile culture conditions are preferred, but this is difficult with non-axenic strains. Toxic cultures, which were predominantly *Microcystis,* were kept in culture for 5 months and maintained their toxicity. Methods to increase the cell numbers would require further study.

7. Recommendations

7.1 The investigation of other analysis techniques such as capillary zone electrophoresis, to obtain better peak resolution of the microcystin toxin peaks. This would provide an alternate method for samples in which co-elution is a problem.

7.2 The isolation and identification of microcystin toxins from natural or cultured agal scums to increase the availability of toxin standards, especially those that are not commercially available.

7.3 A comparative cost analysis for the treatment of microcystin toxins using ozone, chlorination and various powdered activated carbons.

7.4 Investigation of the chlorination dose required and trihalomethane formation at which complete removal of the microcystin toxins can be achieved at pH10 (and higher).

7.5 Determination of the nature and toxicity of the oxidation products of the chlorinated microcystin toxins.

7.6 Investigation of the efficacy of chloramination, relative to chlorination, for the removal of microcystin toxins in water.

7.7 Investigation into human exposure to cyanobacterial toxins in South Africa.

TABLE OF CONTENTS

Section 1

LIST OF FIGURES

LIST OF TABLES

Abbreviations

A	= Alanine
Aba	= Aminoisobutyric acid
Adda	= 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid
ADMAdda	= O-Acetyl-O-demethylAdda
CF	= conversion factor
Dha	= Dehydroalanine
DMAdda	= O-demethylAdda
DOC	= dissolved organic carbon
DNS-Cl	= 5-dimethylaminonaphthalene-1sulphonyl chloride
ECD	= electron capture detector
F	= Phenylalanine
GC	= gas chromatography
$(H_4)Y$	= 1,2,3,4-tetrahydrotyrosine
Har	= Homoarginine
Hil	= Homoisoleucine
Hph	= Homophenylalanine
HPLC	= High Performance Liquid Chromatography
Hty	= Homotyrosine
KI	= potassium iodide
L	= Leucine
LC	= liquid chromatography
MeLan	= N-Methyl-lanthionine
M(O)	= Methionine-S-oxide
MeSer	= N-Methylserine
MS	= mass spectrometry
NBD-Cl	= 4-chloro-7-nitro-benzofuran
NOM	= natural organic matter
OPA	= othophthaldialdehyde
PAC	= powdered activated carbon
PIB-Cl	= 4-(2-phthalimidyl)benzoyl chloride
R	= Arginine
SPE	= solid phase extraction
TFA	= trifluoroacetic acid
THM	= trihalomethane
TIC	= total ion chromatogram
UV	= ultra-violet
W	= Tryptophan
WISP	= Waters Intelligence Sample Procedure
Y	= Tyrosine
(6Z)Adda	= Stereoisomer of Adda at the δ^6 double bond

CHAPTER ONE
Literature Survey

1.1 Cyanobacteria

Cyanobacteria are micro-organisms that have the combined features of both bacteria and algae. Physiologically, they resemble bacteria and are classed as prokaryotes since they do not have intracellular membrane-bound organelles such as a true nucleus or mitochondrion, etc., and their cell walls are made up of peptidoglycan and lipopolysaccharide layers instead of cellulose.[1] However, because of their size and oxygen-evolving photosynthetic mode of nutrition, which is characteristic of all nucleate algae, they may also be associated with eukaryotes. The ambiguity of their classification has led to the introduction of several terms that can be used synonymously with *'cyanobacteria'*, namely, *'blue-green algae'*; *'blue-green bacteria'*; *'Cyanophyta'*; *'Myxophyta'* and *'Cyanochloronta'*. An evolutionary scheme would describe this group of micro-organisms as the link between bacteria and green plants (algae).[2]

Both marine and freshwater cyanobacteria are globally ubiquitous, and occur in nature in unicellular, colonial, filamentous and branched filamentous forms. With some practice, they can be microscopically distinguished from green algae by their pigmentation and the relatively homogenous appearance of their cytoplasm. Pigmentation is caused by the phycobiliproteins, phycocyanin and phycoerythrin, the ratio of which, produces the bluish-green and reddish-brown appearance which is characteristic of most cyanobacteria.[2] The colouration is usually more noticeable when the cells are aged and dry out, leaving the pigments exposed - hence the common reference to "blue-green algae".[1]

Individual cells can only be observed under considerable magnification, but mass accumulation or algal "waterblooms" within an impoundment make them visible from a distance. Some cyanobacteria have gas vacuoles which enable them to regulate their buoyancy according to the light conditions within the water column and under calm conditions, or at night, the cells may rise to the surface where gentle wind and wave action causes them to aggregate at the water edges. Blooms can reach such proportions that a thick sludge may form which may be visually unappealing and usually accompanied by a repulsive odour.

Although the presence of cyanobacteria in surface waters is considered a natural phenomenon, massive bloom formation is considered to be promoted by several factors. The most commonly cited cause is the eutrophication of water bodies, i.e., the increased availability of algal nutrients such as phosphates and nitrates, primarily introduced by the pollution of the main water body from discharged sewage and run-off from fertilised fields. Other factors may include drought, reduced water turbulence, atmospheric conditions and the lack of zooplankton feeders.[3]

Extensive cyanobacterial growth poses severe implications on the general water quality as well as the maintenance of water treatment standards set for potable water. Massive blooms can deplete the dissolved oxygen content resulting in fish kills, and the surrounding water may be discoloured by the release of pigments from the cells.[1] Because of their relatively small cell size these blue-greens easily penetrate and clog both coarse and fine sand filtration processes, which are fundamental stages in the purification of water for drinking purposes. Biodegradation of cyanobacterial blooms also contributes to the organic loading of bulk waters, resulting in increased treatment costs.[2] Compounds such as geosmin (trans-1,10-dimethyl-trans-9-decalol); MIB (2-methylisoborneol); β-cyclocitral, IPMP (2-isopropylmethoxypyrazine) and IBMP (2-isobutylmethoxypyrazine) that cause taste and odour problems in final waters have been either directly or indirectly associated with

cyanobacteria.[4] All these "nuisance" compounds are considered non-toxic; however, but certain cyanobacteria are also capable of producing super-toxic compounds, the consumption of which, has caused numerous animal deaths world-wide, including South Africa.[5,6,7]

1.2 Cyanobacterial Toxins

Only certain genera of cyanobacteria are known to produce secondary toxic metabolites. These metabolites can be divided into three groups, namely, hepatotoxins, neurotoxins and lipopolysaccharide endotoxins. The toxins are released into the water during cell lysis, and ingestion of either the algal cells or the contaminated water can be harmful to both human and animal health.[5,8] Acute hepatotoxin and neurotoxin poisoning can be fatal, and the occurrence of massive cyanobacterial blooms increases the risk of concentrated amounts of toxin being ingested by watering animals or of entering the potable water supply.

1.2.1 The Hepatotoxins

The hepatotoxins are the largest group of cyanobacterial toxins, consisting of 47 microcystin variants and nodularin.

Structurally, the microcystin toxins are cyclic heptapeptides, i.e., the molecules contain seven amino acid residues linked in a cyclic arrangement. Five of these residues are common to all the microcystin toxins but two vary giving rise to a range microcystin variants.
Nodularin consists of five amino acid residues and is therefore a cyclic pentapeptide. There are also modifications of the common amino residues on the toxin molecules of both the microcystins and nodularin. (See Appendix B)

These toxins are protein phosphatase enzyme inhibitors that destroy the cytoskeletal arrangement (cell structure) and sinusoids (capillaries) of the liver and death usually results from hypovolemic shock caused by pooling of blood in the hepatocytes. Symptoms of hepatotoxin poisoning in animals include: necrosis of the liver, vomiting, diarrhoea, weakness, photosensitivity, and cold extremities. Apparent symptoms encountered in humans include: acute hepatitis, fever, pains in the muscles and joints, vomiting, diarrhoea, skin rashes and eye irritation.[3,7,9,10] It is not certain whether the latter symptoms are caused solely by the contact or ingestion of these hepatotoxins or in combination with other biotoxins produced by the algal cells, since the probability that only the toxin was ingested is remote.

Epidemiological investigations have found that chronic sub-lethal doses may lead to liver damage as indicated by increased levels of gammaglutamyl transpetidase in blood serum of the patients tested, but damaged livers can apparently recover once the intake of these toxins is terminated.[3] It has also been inferred in the literature that these toxins may act as skin tumour promoters and that the increased incidence of liver cancer in sections of the Chinese population is due to the long term consumption of microcystin-contaminated waters. Fairly recent research has also found nodularin to be carcinogenic to rat livers, more so than microcystin-LR.[11,12] An article in the May 1996 issue of *New Scientist* reported that 43 patients in a Brazilian hospital died from suspected microcystin-LR poisoning introduced from the water used in kidney dialysis - microcystin-LR was found in the water and the filters of the dialysis machines.[13,14]

Lethal dosage (LD_{50}) values reported differ from animal to animal and those recorded for oral toxicity tests were higher than those for intraperitoneal injections. (See Appendix C) Other factors, however, such as age, sex and amount of food in the animal's gut may influence the toxicity results and there is no reliable extrapolation by which these lethal dosage concentrations may be applied to humans.[1,15]

As yet there is no treatment available to remedy hepatotoxin poisoning, but activated charcoal and cholestrymine are suspected to provide some relief. Research is being conducted on the use of cylcosporin-A, rifampin and silymarin to reduce the uptake of the toxin by the liver.[7]

Cyanobacterial genera that produce hepatotoxins include : *Microcystis* (microcystins), *Anabaena* (microcystins), *Oscillatoria* (microcystins), *Nostoc* (microcystins) and *Nodularia* (nodularin).

Other hepatotoxins from *Cylindrospermopsis*, *Aphanizomenon*, *Gloeotrichia* and *Coelosphaerium* are currently undefined and are also being studied.[10]

1.2.2 The Neurotoxins

The neurotoxins are a fast acting group of alkaloid toxins that block the neuro-muscular transmission mechanism in the body.[7]

Five neurotoxins have thus far been isolated: anatoxin-a, anatoxin-a(s), homoanatoxin-a, saxitoxin and neosaxitoxin. Saxitoxin and neosaxitoxin are the paralytic shellfish poisons which are more commonly associated with the marine dinoflagellates (red tides) but have also been isolated from freshwater *Aphanizomenon* and *Anabaena* spp.

Acute poisoning causes paralysis of the peripheral muscles, and death usually results from cardiac arrest caused by respiratory failure. Symptoms of neurotoxin poisoning in animals include: staggering, muscular tremor, gasping and convulsions.
No treatment for neurotoxin poisoning has been established but activated charcoal and administration of saline cathartic are recommended.[7]

Neurotoxin producing genera include *Anabaena*, *Aphanizomenon*, *Oscillatoria* and *Trichodesmium* and *Lyngbya*.[10]

1.2.3 Lipopolysaccharide Endotoxins

Lipopolysaccharide endotoxins are the least studied of the algal toxins and are composed of phosphate-containing fats and sugar molecules that are contained in the outer cell covering. Although considered to be non-lethal, they have been linked to gastro-enteritis and contact dermatitis.[3,4]
It is not known whether these toxins are produced in association with the other toxins or whether these toxins are always present in the cells.

Some genera are capable of producing more than one type of toxin simultaneously and as a precautionary measure, cyanobacteria that are capable of producing toxins should be treated as toxic until relevant toxicity tests are done to verify its safety. Confirmed toxin-producing strains are given in Appendix A.

1.3 Toxin Production

The causes of toxin production are not fully understood since both toxic and non-toxic strains of the same species are known to exist. Several physico-chemical (pH, temperature, nitrogen to phosphorus ratio, etc.) and biological (predation) conditions within the impoundment are cited as possible factors, but there is no direct relationship by which toxicity can be determined or predicted.

Toxicity of a particular species also cannot be deduced by microscopic inspection of the cyanobacterial cells or by the presence of taste and odour compounds such as geosmin or 2-MIB.[7,16] A chemical or biological assay is therefore essential to determine the toxicity of cyanobacterial cells.

1.4 General Methods to Determine Algal Toxicity

Biological, immunological and chemical techniques can provide some indication of the toxicity of a cyanobacterial sample. A combination of all three techniques would provide a complete toxicological assessment but, for analytical purposes, a chemical assay can provide a quantitative determination of the individual toxins present. The capital costs for a chemical analysis, however, are relatively very high and the other techniques developed may become useful for screening algal samples submitted for toxicity testing. The limitation of biochemical methods would be non-specificity to the toxins present in a sample, but they have the ability to detect overall toxicity. With chemical methods, only the compounds being determined can be quantified and other possible toxins (that may require different chemical detection techniques) may be overlooked. Part of this project is to supply the "current best" screening and quantitative analysis methods for the determination of algal toxins.
The method of L. Lawton, C. Edwards and G. Codd, published in the *Analyst*, 1994, Vol. 119[36] was chosen for investigation because it was the most recent of the chemical methods published that could detect microcystin toxin concentrations as low as 250 $ng.\ell^{-1}$ within 24 hours. The method proposed in this paper had been specifically designed for the determination of intracellular and extracellular microcystin toxins in raw and treated waters using High Performance Liquid Chromatography and photodiode array detection. The UV absorption spectra of the toxins were shown to have similar characteristics and can be used to detect other possible microcystin toxins in a sample. This method closely suited our requirements and investigations began in February 1994. This was prior to the paper actually being published but earlier contact was made with Professor Codd who supplied the relevant information. A critique of the literature can be found in Appendix D, and some of the other chemical and biochemical techniques that have been used for the detection of algal toxins are also summarised in Appendix D to provide an indication of the scope of the techniques that can be applied for the determination of microcystin toxins, nodularin and anatoxin-a. Many of these methods appeared to have lengthy sample preparation steps and some of the equipment was not readily available.

1.5 Algal Toxicity in South Africa

The earliest reports of aminal deaths due to algal toxins date back to 1927. Since then South African researchers have made significant contributions in this field by determining the chemical structure of the microcystin toxin molecule.

Microcystis is the predominant blue-green genus found in South African freshwaters and most of the research was conducted on the hepatotoxins, microcystin toxins and nodularin, since they would most likely pose a threat to the environment.[6] *Anabaena* is the second most common cyanobacterium and is also capable of producing microcystin toxins. Usually some *Anabaena* cells are found where *Microcystis* is predominant, and vice versa.

Animal deaths have only been reported for toxic *Microcystis* and *Nodularia*. Toxic *Anabaena* has also been recorded; but so far no reported animal deaths have been linked to this genus.[6] The more recent stock deaths conclusively caused by algal toxicosis occurred in the Western Cape region in 1994 and 1996 (reported by the Cape Town Municipality to be toxic *Nodularia* and *Microcystis*).

Other possibly toxic genera such as *Oscillatoria* and *Nostoc* have not been frequently encountered in South African freshwaters and, as yet, there has been no report of any massive bloom formations having occurred for these genera.

A National Surveillance Programme for toxic blue-green algae is being introduced by the Department of Water Affairs and Forestry to monitor the occurrence of toxic cyanobacteria. Several testing centres around the country, namely CSIR (Pretoria), Rand Water (Vereeniging), the Cape Town Municipality and Umgeni Water (Pietermaritzburg), are adequately equipped to determine the toxicity of an algal sample.

CHAPTER TWO
Fluorescence Derivatisation

2.1 Introduction

Fluorescence is one of the ways in which energy absorbed by a molecule is released as it returns to its ground state, radiating light energy (or fluorescing) in the process. Most molecules reach their ground states by non-radiative methods and only a small percentage of compounds are known to fluoresce naturally. Usually molecules containing aromatic rings are capable of fluorescent behaviour; their fluorescent intensity increasing with the number of aromatic rings, their degree of condensation and the rigidity of the molecule. Certain highly conjugated aliphatics and alicyclic carbonyl compounds are also known to fluoresce, but simple heterocyclics, such as pyridine etc., are not capable of fluorescing.[17]

Generally, detection limits of 10^{-9} g.ℓ^{-1} can be achieved with fluorescence techniques whereas detection limits in the 10^{-6} g.ℓ^{-1} range can be obtained with UV detection.[18]
The development of a fluorescent detection method for the determination of algal toxins would possibly have the advantages of specificity as well as enhanced detection at picogram levels (after solid-phase extraction and concentration). However, fluorescence would have to be induced into the toxin molecules by means of a reaction with a fluorescent reagent, the choice of which, would depend on the available reactive sites on the analyte molecules. The literature contains many methods involving fluorescent reactions that are reportedly used with success.[19-23]

Since the microcystins and nodularin are peptide structures composed of amino acid residues, research describing the fluorescent detection of amino acids and peptides was used for modelling purposes.

There are two types of HPLC derivatising techniques, namely pre- and post-column derivatisation, i.e., the fluorescent reaction occurs either before or after HPLC separation. Both procedures have their respective advantages and can be automated with specialised HPLC equipment.[19] Here, only pre-column derivatisation techniques were attempted since the reaction was to be done qualitatively at first. By reacting the molecules before injection onto the HPLC column, the reaction conditions could be controlled more easily.

2.2 Derivatisation Reactions

Two fluorescent derivatisation procedures have been used in an attempt to attach fluorescent moieties to the guanidine (NH_2 containing) group of the microcystin toxins. Figs. 2.1, 2.2 & 2.3 show typical microcystin toxins containing a primary amine group necessary for the fluorescent reactions to occur.

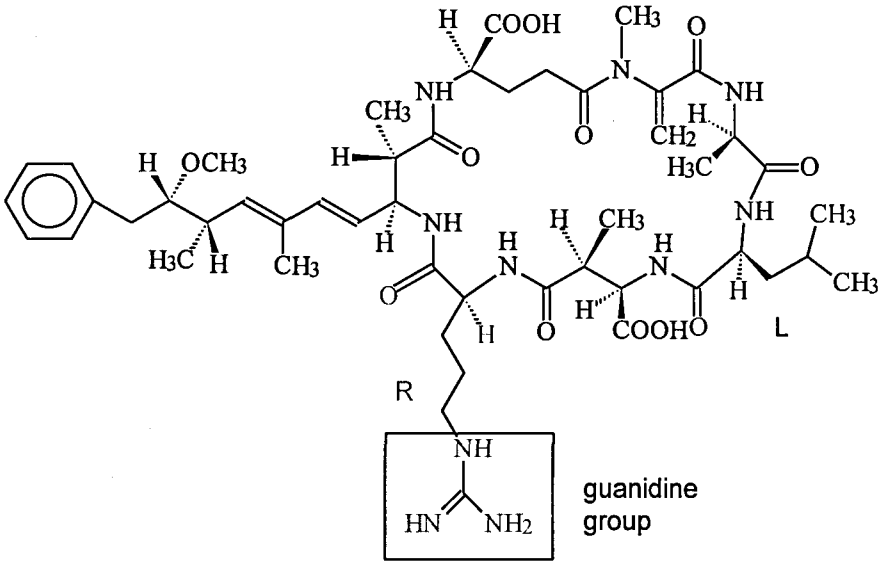


Fig. 2.1 Chemical Structure of Microcystin-LR[34]
D-Ala-L-Arg-erythro-β-Me-D-isoAsp-L-Arg-Adda-D-isoGlu-N-Me-dehydroAla
M.wt. = 994.54

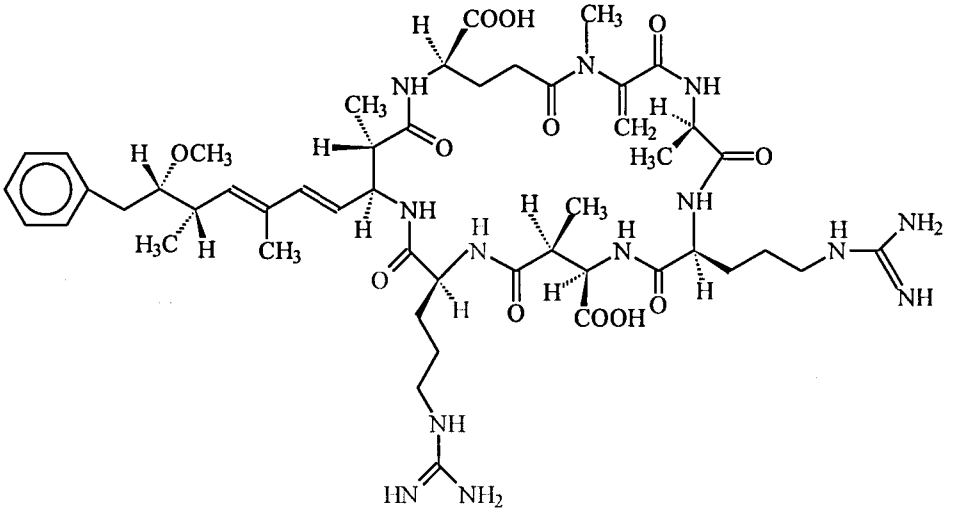


Fig. 2.2 Chemical Structure of Microcystin-RR[34]
D-Ala-L-Arg-erythro-β-Me-D-isoAsp-L-Arg-Adda-D-isoGlu-N-Me-dehydroAla
M.wt. = 1037.57

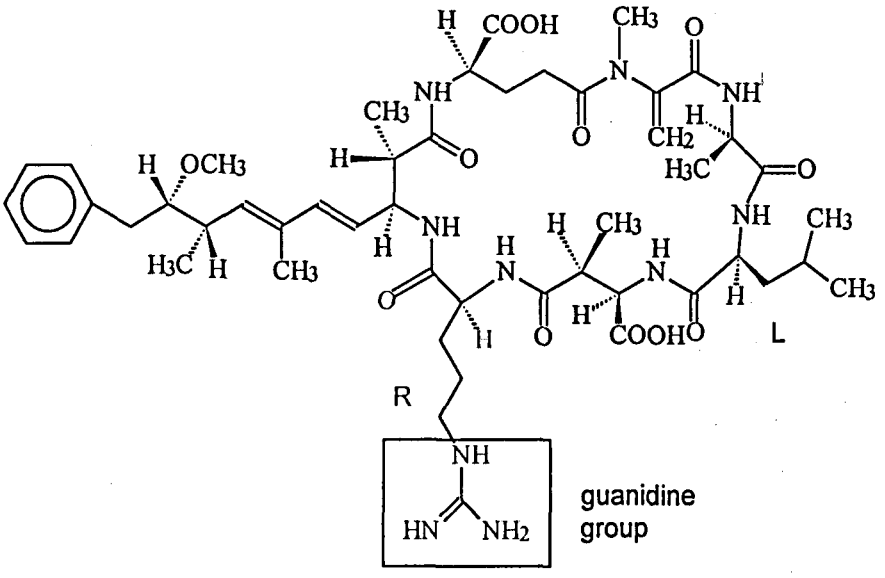


Fig. 2.1 *Chemical Structure of Microcystin-LR*[34]
D-Ala-L-Arg-erythro-β-Me-D-isoAsp-L-Arg-Adda-D-isoGlu-N-Me-dehydroAla
M.wt. = 994.54

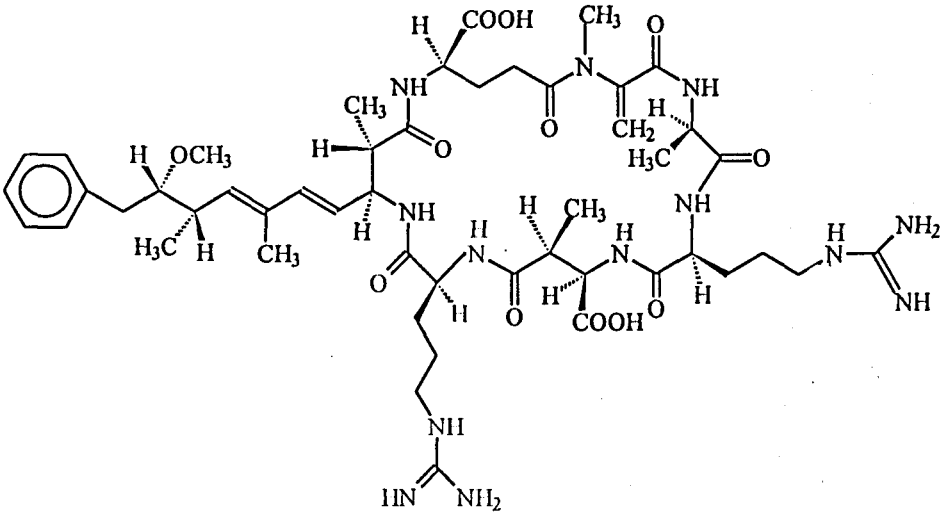


Fig. 2.2 *Chemical Structure of Microcystin-RR*[34]
D-Ala-L-Arg-erythro-β-Me-D-isoAsp-L-Arg-Adda-D-isoGlu-N-Me-dehydroAla
M.wt. = 1037.57

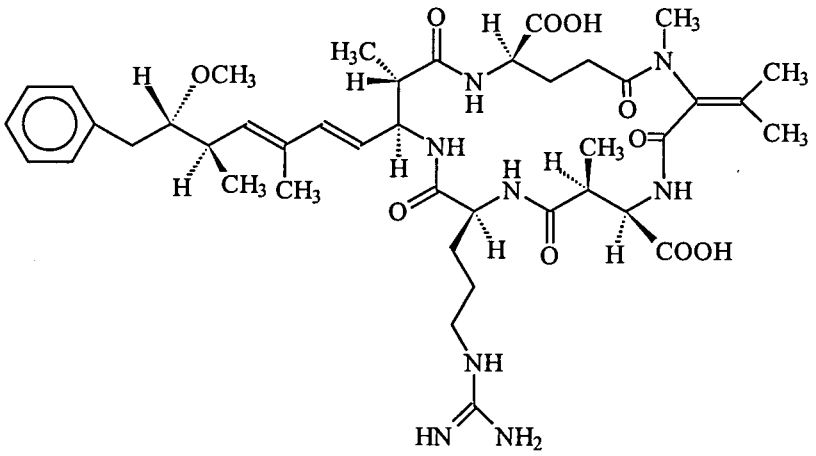


Fig. 2.3 Chemical Structure of Nodularin[34]
erythro-B-Me-D-isoAsp-L-Arg-Adda-D-isoGlu-N-Me-dehydroVal
M.wt. = 824.44

Although not all the microcystin toxins have guanidine functional groups, microcystin-LR is the most commonly encountered hepatotoxin while many others have either a primary or secondary amine group. It was anticipated that if the fluorescent reaction would work for microcystin-LR it would be likely to be applicable to other microcystin toxins containing a primary amine functional group. Other fluorescent reactions could then be sought for the rest of the microcystin toxins.

Reaction 1 was based on the fluorescent properties of ortho-phthaldialdehyde (OPA) derivatives of amino acids. OPA has largely been used in analytical procedures where high sensitivity is required, and it is reportedly more sensitive than fluorescamine for amino acid analyses. It reacts rapidly with primary amino acids at room temperature in the presence of ethyl mercaptan or ethanethiol to form hydrophobic derivatives that can easily be chromatographed using reversed-phase HPLC. OPA has the added advantage that it is soluble in water, but an unattractive feature of this reaction is the requirement of the thiol that is toxic, extremely pungent and may also lead to side reactions. However, the thiol is necessary for the reason that it forms part of the fluorescent product and has been shown to reduce the instability of the derivatised product formed.[22] The proposed derivatisation reaction is shown in Fig. 2.4.

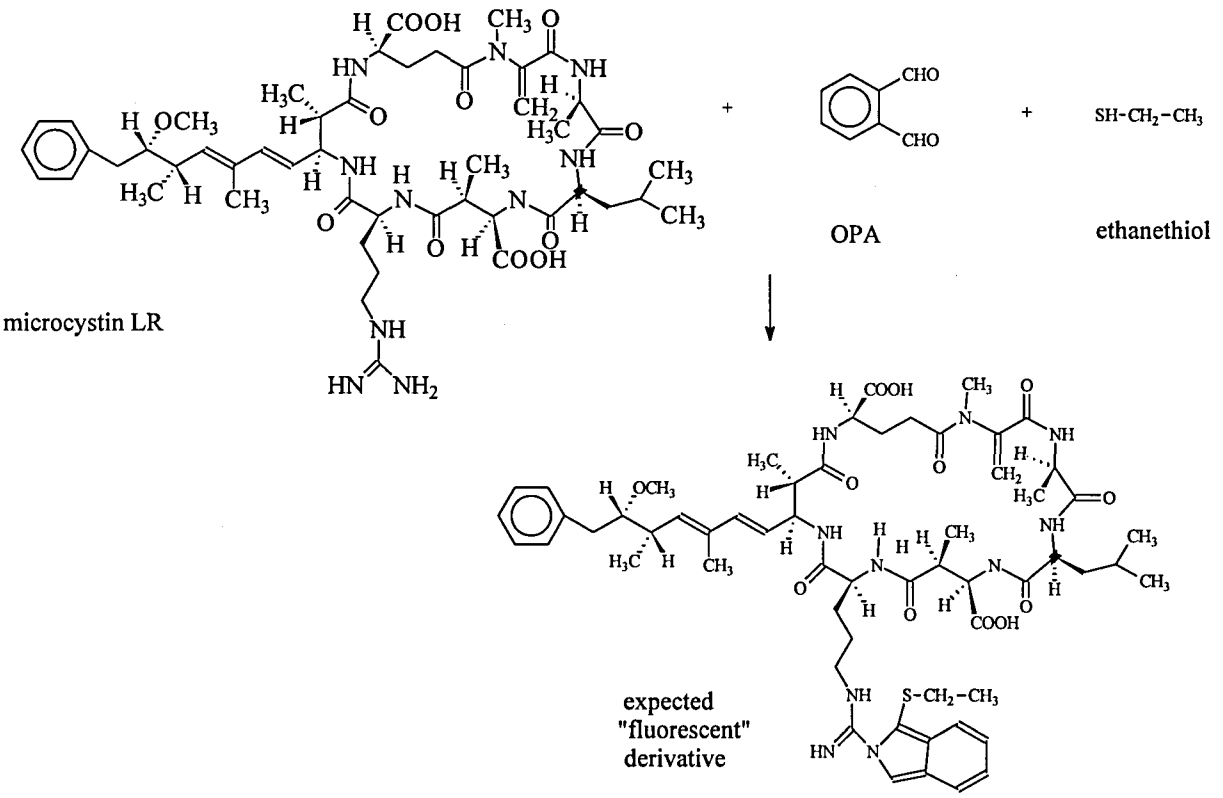
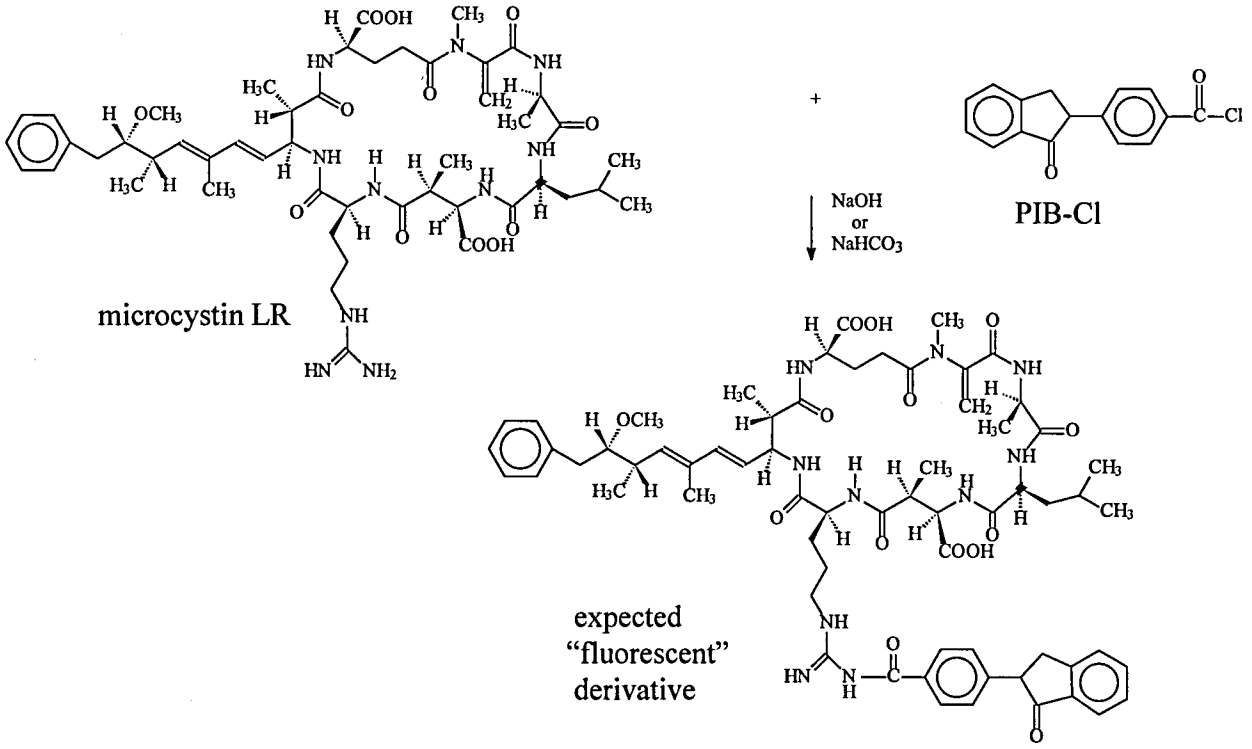


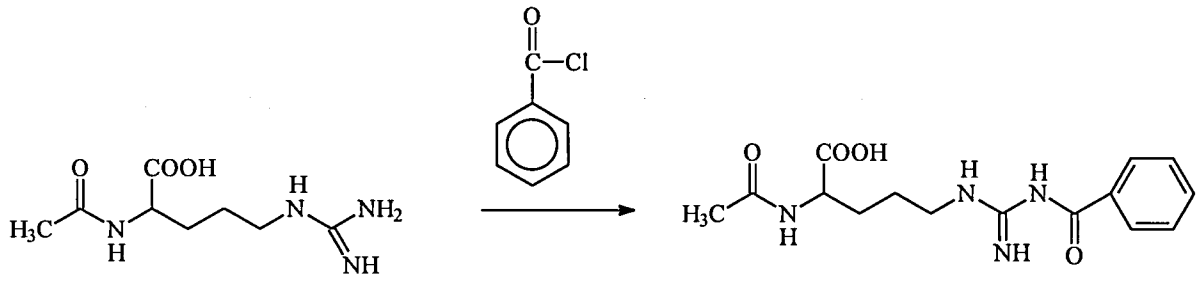
Fig. 2.4 Proposed Fluorescent Reaction of OPA and microcystin-LR

Reaction 2 involved the reaction of the primary amine group with the acid chloride of various derivatisation reagents: 4-(2-phthalimidyl)benzoyl chloride (PIB-Cl); 4-chloro-7-nitro-benzofuran (NBD-Cl); and 5-dimethylaminonaphthalene-1-sulphonyl chloride (DNS-Cl).[25]
These acid chlorides were expected to react quantitatively with amines or alcohols under alkaline conditions to produce stable fluorescent derivatives. The representative reaction is given in Fig. 2.5.



2.5 Proposed Fluorescent of microcystin-LR and PIB-Cl

Since toxin standards are very expensive, these reactions were initially attempted with model compounds, such as N-α-acetylarginine to check whether reaction would be likely to occur with the toxin molecule (See 2.6). Similar model reactions were also carried out with benzoyl chloride. All the reaction solutions were tested for fluorescence.

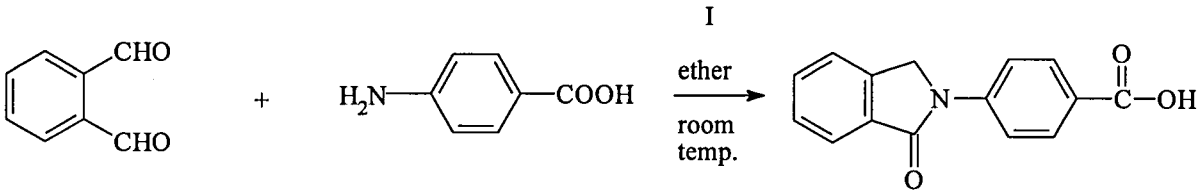


N-α-acetylarginine

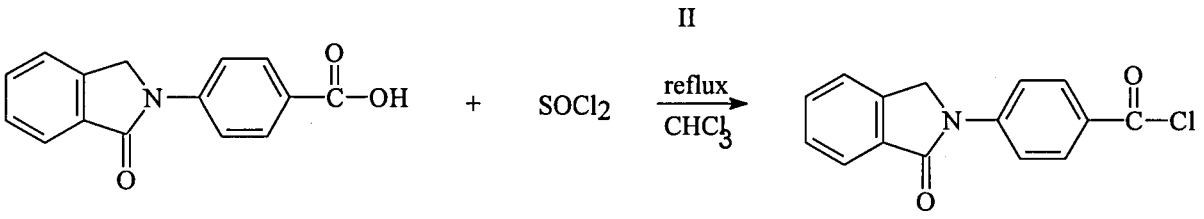
N-ω-benzoylated-N-α-acetyl arginine

2.6 Proposed Fluorescent of N-α-acetylarginine and benzoyl chloride

PIB-Cl reagent is not commercially available and had to be prepared in the laboratory as represented by Fig.2.7.



OPA 4-aminobenzoic acid 4-(2-phthalimidyl)benzoic acid



4-(2-phthalimidyl)benzoic acid thionyl chloride 4-(2-phthalimidyl)benzoyl chloride

2.7 Reactions for the preparation of PIB-Cl

2.3 EXPERIMENTAL

Equipment and Apparatus:
Waters HPLC including: 712 WISP, 600E System Controller, 470 Scanning Fluorescence Detector; Nova-pak C_{18} analytical column (3,9 × 150 mm); μBondapak C_{18} analytical column (3,9 × 300 mm); Millennium 2010 software for data acquisition.

Chemicals:
All solvents were HPLC grade; ultra-pure water (Millipore); microcystins LR, RR and nodularin (Sigma); OPA solution (Sigma); 4-aminobenzoic acid (Merck); 5-dimethylaminonaphthalene-1-sulphonyl chloride (DNS-Cl) (Sigma); 4-chloro-7-nitro-benzofuran (NBD-Cl) (Sigma); N-α-acetyl arginine (Merck); 4-(2-phthalimidyl)benzoyl chloride (PIB-Cl) was synthesised.

Preparation of 4-(2-Phthalymidyl)benzoyl chloride (PIB-Cl) :
Ortho-phthaldialdehyde (OPA) (≈1,34 g) and 4-aminobenzoic acid (≈1,37g) were dissolved separately in 100 ml and 150 ml of diethyl ether respectively. The two solutions were combined and allowed to stir overnight. A pale yellow precipitate formed that was filtered off and dried. The precipitate, 4-(2-phthalimidyl)benzoic acid, was dissolved in chloroform (100mℓ) and refluxed with thionyl chloride (6mℓ) for an hour. A dark brown/black precipitate formed which was filtered off and washed twice with cold chloroform. The retained solid was recrystallised from benzene to produce white PIB-Cl crystals.

2.4 METHODS :

2.4.1 Fluorescence Chromatographic Conditions :

Reaction 1 :

Mobile phase : methanol-water
Gradient (linear) : 50% (v/v) methanol to 100%
Run Time : 20 minutes
Flow Rate : 1.2 $m\ell.min^{-1}$
Injection Volume : 20 $\mu\ell$
Column Temperature : ambient

Reaction 2 :

Mobile Phase : acetonitrile-water
Isocratic elution : 58% (v/v) acetonitrile + 5ml PIC-A per litre
Run Time : 10 minutes
Flow Rate : 1 $m\ell.min^{-1}$
Injection Volume : 20 $\mu\ell$
Column Temperature : ambient

Fluorescence Optimisation :

With fluorescence detection, two wavelengths have to be optimised, namely, the excitation and emission wavelengths. The excitation wavelength is the incident energy applied to the molecule (to reach its excited state) which releases the absorbed energy by non-radiative means and by fluorescence to its ground state. The emission wavelengths therefore have slightly lower energy and hence longer wavelengths. These wavelengths have to be determined by experimentation and can be found by keeping one of the wavelengths constant while scanning a range of the other and then reversing the process.

2.4.2 UV Chromatographic Conditions

Mobile Phase : A = 0.05 % TFA in ultra-pure water
B = 0.05 % TFA in acetonitrile

Gradient Elution : (linear)

TIME /min	*0*	*10*	*40*	*42*	*44*	*46*	*50*
% Solvent A	70	65	30	0	0`	70	70
% Solvent B	30	35	70	100	100	30	30

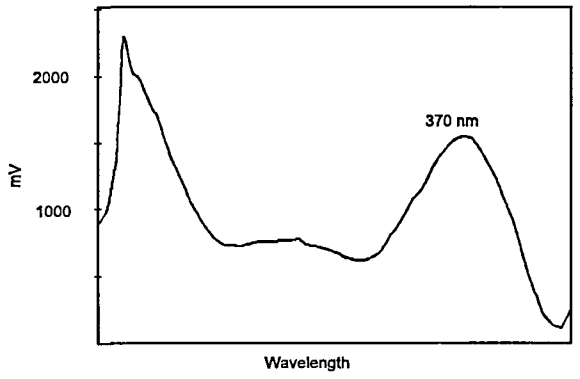
Run Time : 45 minutes
Flow Rate : 1 $m\ell.min^{-1}$
Injection Volume : 25 $\mu\ell$
Column Temperature : 40°C

2.5 RESULTS

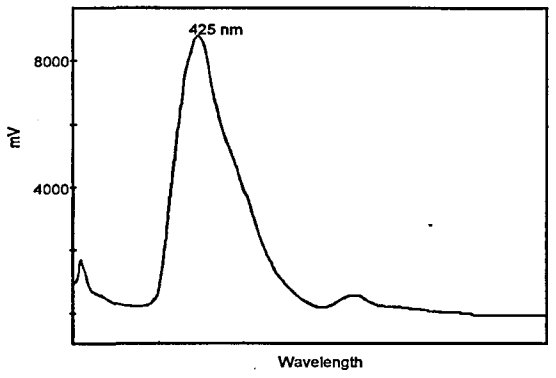
Reaction 1

Optimal wavelengths for the derivatised solutions were found to be 370 nm (excitation) and 425 nm (emission). (See Figs. 2.8 & 2.9)

The same excitation wavelengths were observed for OPA and ethanethiol.



2.8 Scanned Fluorescence excitation wavelengths



2.9 Scanned Fluorescence emission wavelengths

The derivatisation reaction using OPA and ethanethiol was initially carried out using two amino compounds, namely, glycine and N-α-acetylarginine. The reaction with glycine produced an extremely broad fluorescent peak whilst the N-α-acetylarginine reaction with OPA produced several chromatographic peaks as shown in Fig . 2.10

The derivative peak could not be conclusively identified even though various chromatographic parameters were adjusted, (including injection volumes, mobile phases, delay times.)

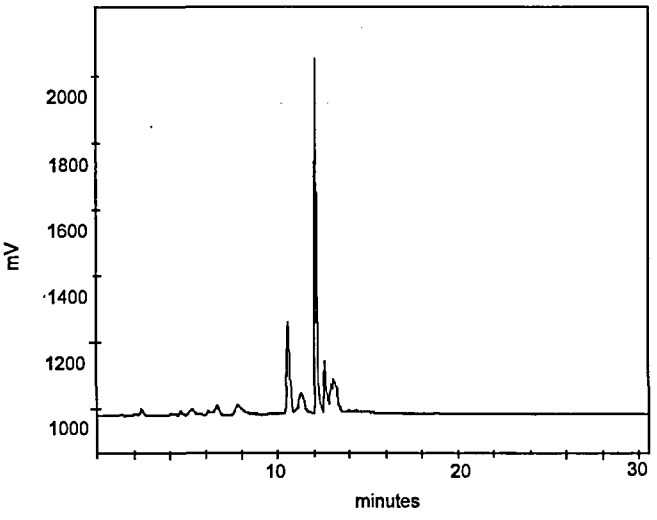


Fig. 2.10 Fluorescence chromatogram of N-α-acetyl arginine and OPA

The fluorescence chromatogram of the "derivatised" Microcystin-LR solution, taken immediately after the reaction with OPA produced a single peak, but the chromatogram of the same solution taken approximately 48 hours after reaction, contained three peaks; one being the same as before while the other two were relatively larger. It was not possible to determine which of the peaks belonged to the derivatised product but it was evident that some reaction had taken place. See Figs 2.11 (a) & 2.11(b).
These results were reproducible.

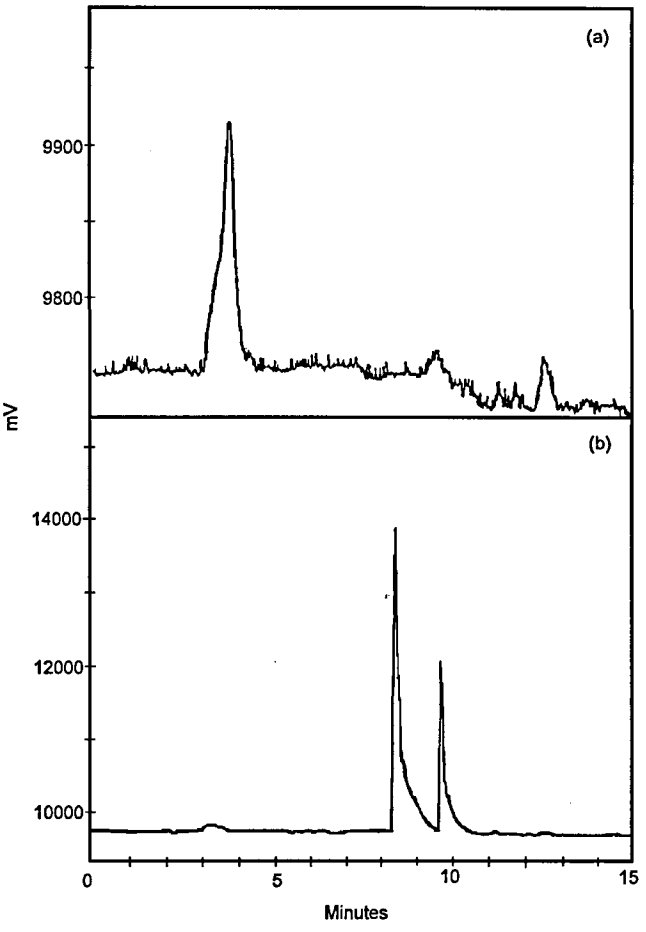


Fig. 2.11 "Derivatised" microcystin-LR with OPA:
(a) immediately after the reaction (enlarged)
(b) 48 hours after the reaction

Another attempt at the derivatisation reaction using another batch of OPA produced the following results, Figs. 2.12 & 2.13

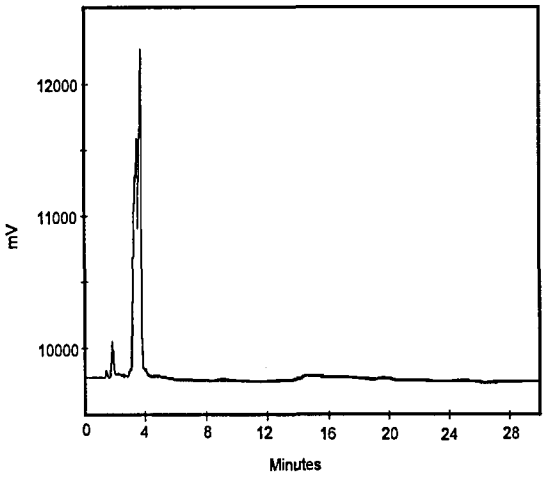


Fig. 2.12 Fluorescence chromatogram of reactants

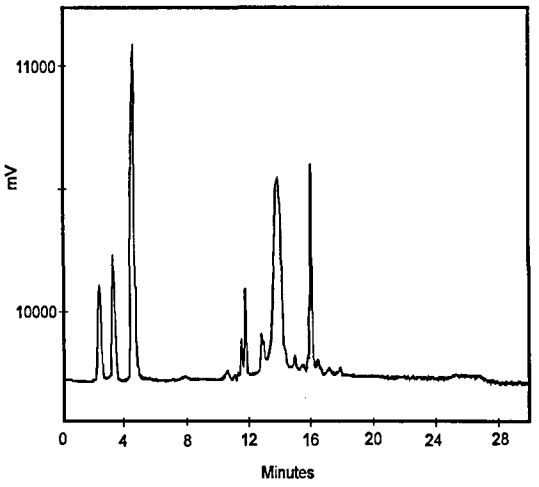


Fig. 2.13 "Derivatised" microcystin-LR and OPA

Once again the toxin derivative peak could not be distinguished from other possible reaction products in the solutions analysed.

Reaction 2

As a precursor to the toxin derivatisation reaction with PIB-Cl, a model reaction was attempted using N-α-acetyl arginine and benzoyl chloride. There was no indication of an increase or decrease in any of the peak areas at different concentrations.

Some of the chromatographic peaks of the PIB-Cl reaction with the microcystin toxins were distorted and not well resolved. However, a fluorescent peak at approximately 7.6 minutes was observed to increase with the increased toxin concentrations in the "derivatised" solutions as shown in Figs 2.14 to 2.19.

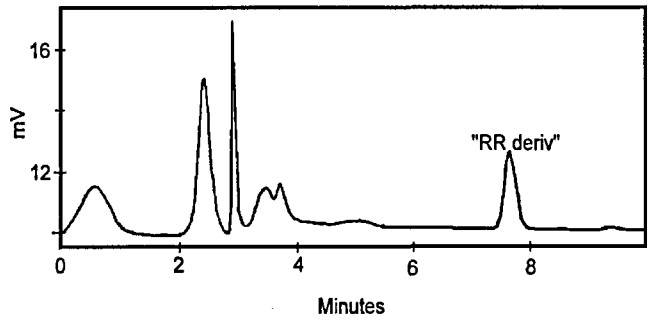


Fig. 2.14 "Derivatised" microcystin-RR (10 ng per litre) with PIB-Cl

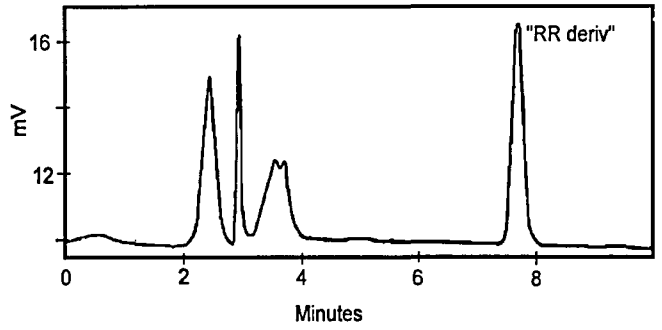


Fig. 2.15 "Derivatised" microcystin-RR (50 ng per litre) with PIB-Cl

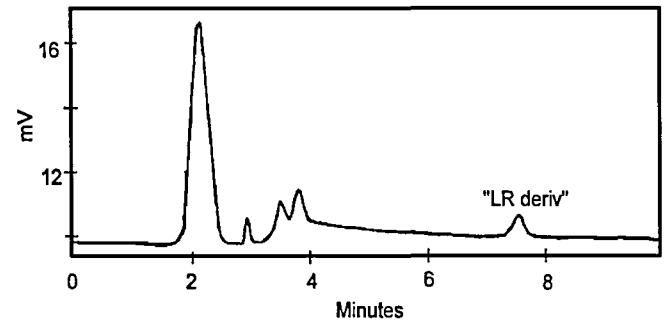


Fig. 2.16 "Derivatised" microcystin-LR (300ng per litre) with PIB-Cl

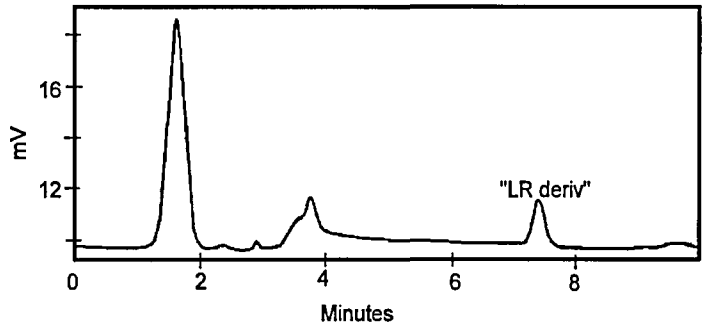


Fig. 2.17 "Derivatised" microcystin-LR (700ng per litre) with PIB-CI

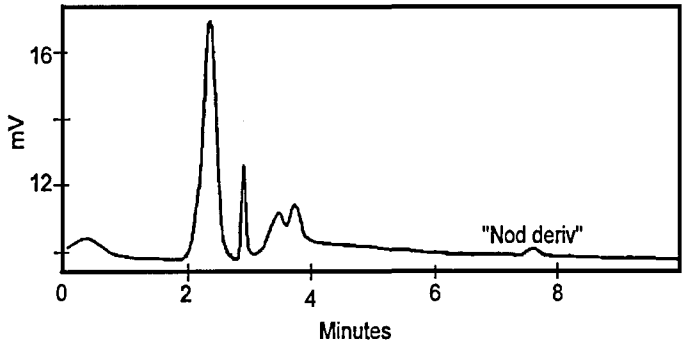


Fig. 2.18 "Derivatised" nodularin (300ng per litre) with PIB-CI

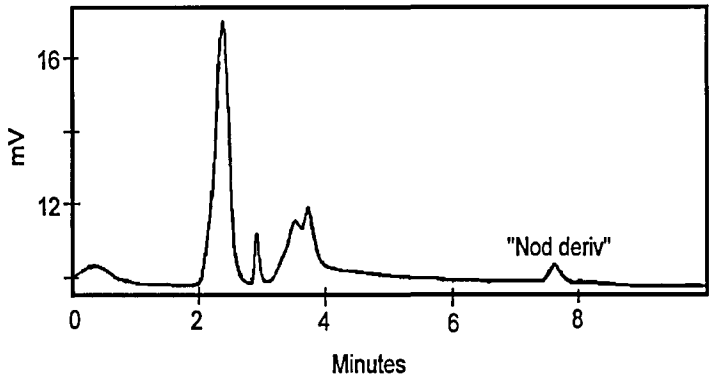


Fig. **2.19** "Derivatised" nodularin (500ng per litre) with PIB-CI

The peak data were plotted to produce the following calibration graphs:

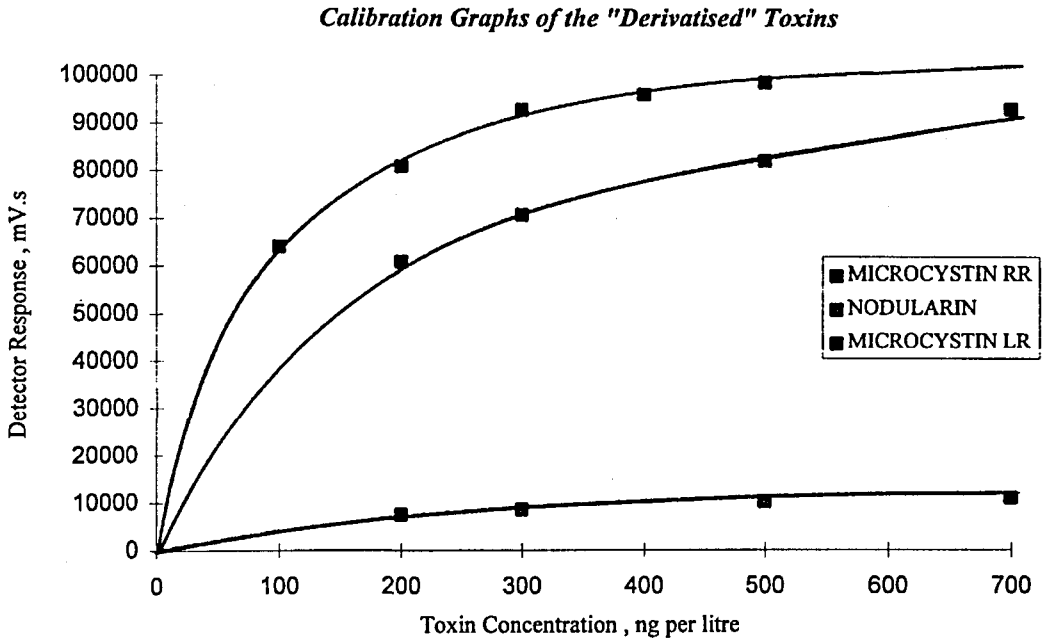


Fig. 2.20 Calibration graphs of the "derivatised" toxins

The three "derivatised" toxins all had the same retention time and had similar peak characteristics which would imply that a derivatised solution containing a mixture of toxins would not be resolved using these chromatographic conditions.

The chosen "derivative" peak in the fluorescence chromatogram was later found to be of no value because:

(i) the same retention times were observed for all "derivatised" toxins,

(ii) PIB-Cl dissolved in methanol containing only Triton B produced a peak having the same retention time as that of the "derivatised" toxin (≈7.8 minutes), and

(iii) the peak height of the "derivative" was observed to increase when an aliquot of PIB-Cl in methanol solution was added to a solution containing the "derivatised" toxin.

It therefore cannot be assumed that the increased peak area in the chromatograms was caused by an increase in the amount of the "derivatised" toxins.

It was suggested that the peak at approximately 7.8 minutes was produced by the methyl ester of PIB-Cl, since the PIB-Cl dissolved in methanol produced the same peak in the fluorescence chromatogram, and the GC/MS spectrum of PIB-Cl dissolved in methanol produced a mass spectrum that was consistent with that of the methyl ester of PIB-Cl. See Figs 2.21 & 2.22

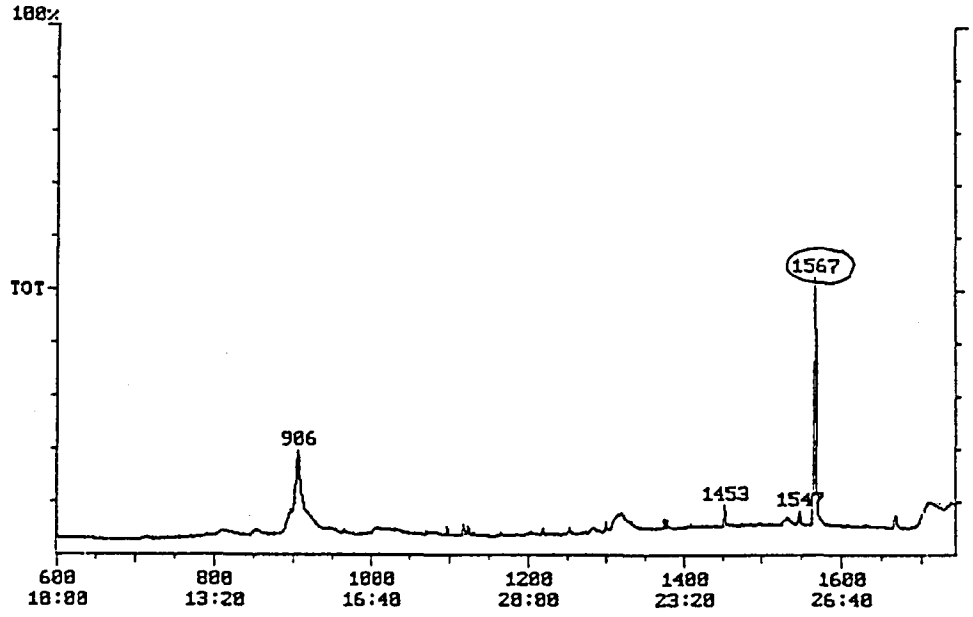


Fig. 2.21 Gas chromatogram of the PIB-Cl dissolved in methanol

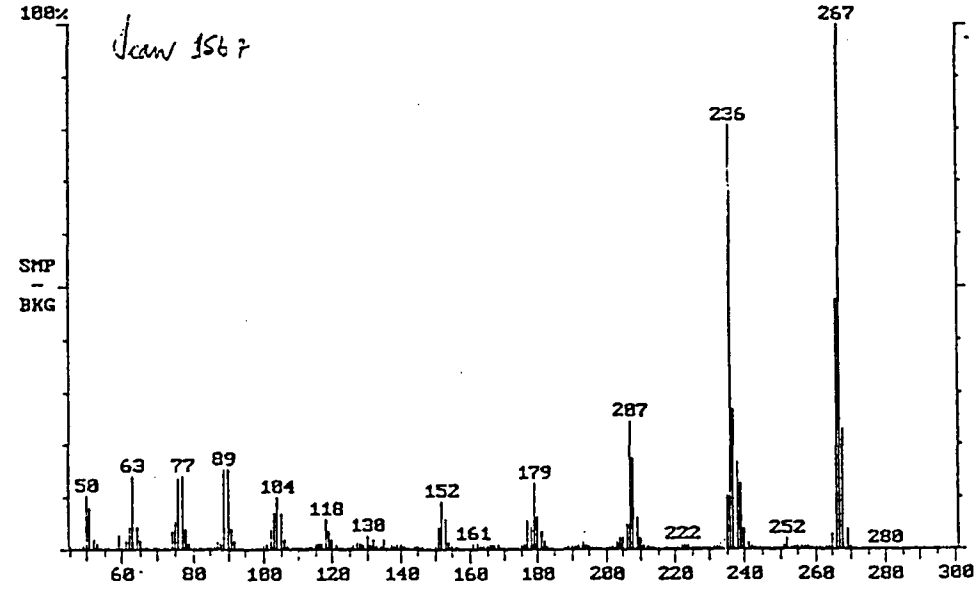


Fig. 2.22 Mass spectrum of the peak at 1567 in the GC chromatogram (in Fig. 2.21)

The PIB-Cl acid precursor, 4-(2-phthalimidyl)benzoic acid, dissolved in methanol did not produce a peak at approximately 7.8 minutes, so the possibility that it could have been due to unreacted acid precursor reacting with the methanol was excluded.

The overall approach was unsuccessful mainly because the actual toxin peak could not be identified. The microcystin toxins are known to absorb in the UV region at 240nm, and it was thought that the derivatised solutions could be analysed using a UV detector instead of the fluorescence detector, since at least the toxin peak could be identified. It was considered

wasteful to repeat these reactions using the model peptide compounds because of the time already spent and the possibility that the assumption that these model compounds react similarly to the toxins might not hold. The reaction was therefore attempted directly with microcystin-LR only. The purity of the derivatising reagent PIB-Cl was also a factor influencing the identification of the "derivatised" peak in the chromatograms and new chemicals were thus used to prepare pure PIB-Cl before the derivatisation reaction was re-attempted. The reagents and the toxins were analysed individually at 240 nm.

Pure PIB-Cl was prepared (see Fig. 2.23) and as a precautionary measure, methanol was not used as a solvent in order to avoid the reaction between the acid chloride and the primary amine group on the toxin molecule.

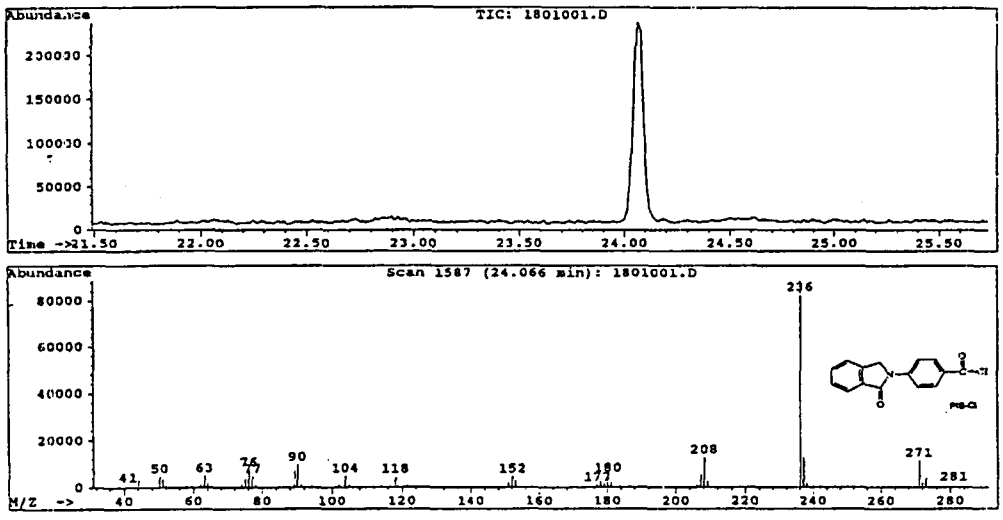


Fig. 2.23 GC/MS spectrum of PIB-Cl

A major problem experienced was incompatible solubility of the toxins and the reagents. The toxins are soluble in methanol, ethanol, acetone, water and acetonitrile, but appear to be insoluble in pyridine, methylene chloride, hexane and benzene which were ideal solvents for the reagents (PIB-Cl, DNS-Cl, NBD-Cl). The toxin solubility in the various solvents was established by evaporating an aliquot of microcystin-LR under a nitrogen flow and re-suspending it in the various solvents. Whilst the microcystin-LR peak was observed when the dried solutions were re-suspended in methanol, the disappearance of the chromatographic peak when dissolved in the other solvents was used to indicate its insolubility. (See Figs. 2.24 & 2.25)

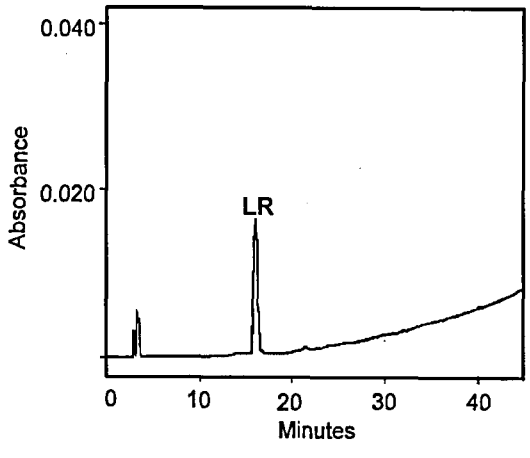


Fig. 2.24 Solubility of Microcystin-LR in acetonitrile

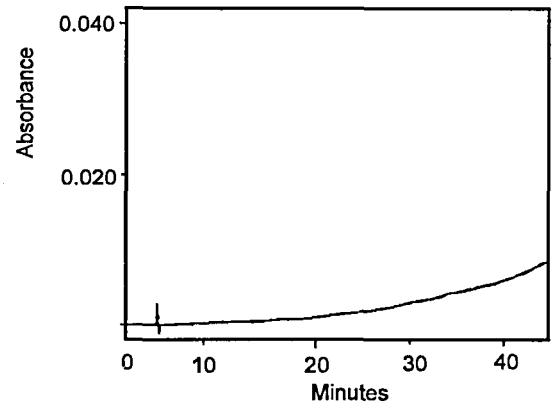


Fig. 2.25 Insolubility of Microcystin-LR in methylene chloride

Because of the stet of the solvents, the microcystin toxins were dried under a flow of nitrogen and re-suspended in the appropriate reagent solution in an attempt to initialise the reaction from the dried toxins.

The reagent and the toxins had been analysed separately by HPLC, using the same chromatographic conditions, and the peaks identified before the reaction was attempted. The advantage here was that the toxin peak, which had a known retention time, could be monitored with confidence. If a reaction product formed, it could be monitored by two means, namely, the additional peak in the chromatogram and by the disappearance of the toxin peak or an alteration of its characteristic retention time. The photodiode array detector allowed all the wavelengths to be investigated without re-analysing the sample. The chromatograph was inspected for the presence of a possible third chromatographic peak that formed at another wavelength in the UV region between 200 to 400 nm.

The results showed that the reagent solutions were visible in the UV at 240 nm except for sodium hydrogen carbonate. The results were consistent with the fact that no reaction product was being formed, i.e., the reagent peaks were visible and there was no sign of a third (or possible product peak) being formed. (See Figs. 2.26 - 2.29) The solutions were also subjected to longer reaction times and higher reaction temperatures (40-60°C) but similar results were obtained.

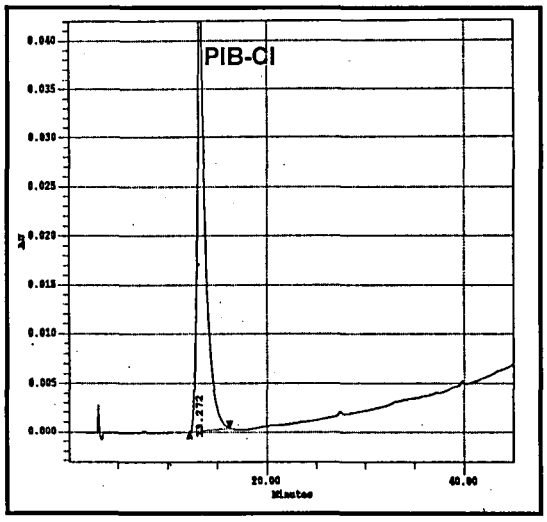


Fig. 2.26 UV chromatogram of PIB-Cl

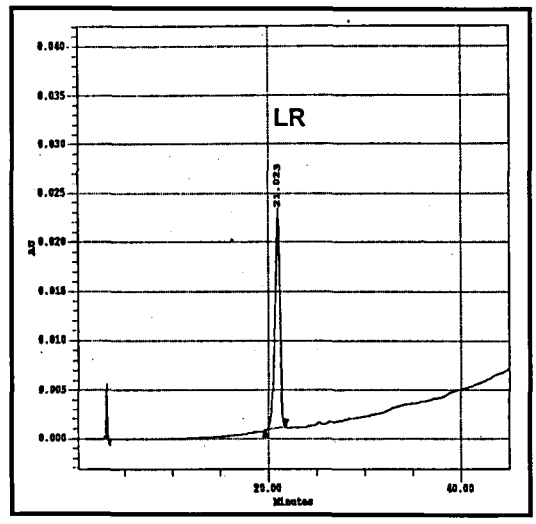


Fig. 2.27 UV chromatogram of microcystin-LR

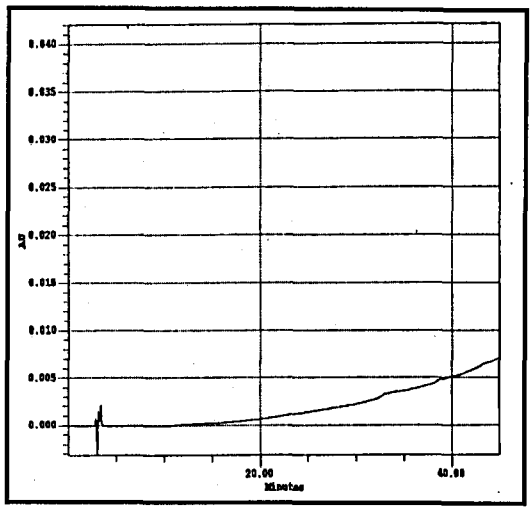


Fig. 2.28 UV chromatogram of sodium hydrogen carbonate in water

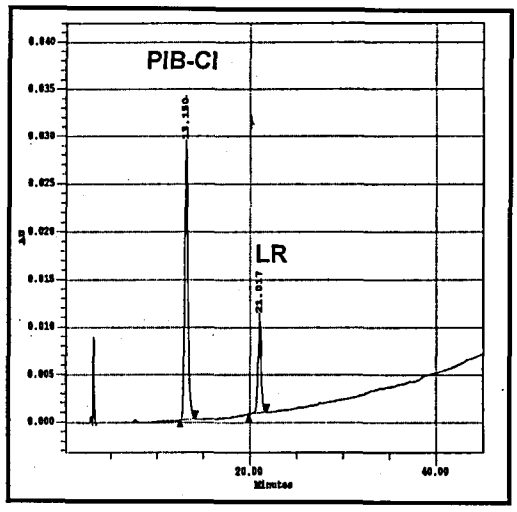


Fig. 2.29 UV chromatogram of the reaction mixture of PIB-Cl and microcystin-LR

2.6 DISCUSSION

The assigned "derivative" peaks could not be confirmed using any simple procedure and were merely chosen on the basis of an increased peak height or area that corresponded with increased concentrations of the standard solutions added to the reaction solutions. It may also be possible that the concentrations of the standard toxin solutions were too low to fluoresce.
For OPA, it has been reported that the reaction products are sometimes not stable with certain amino acids[22] and it is possible that the reaction product may have decomposed to form the two fluorescent peaks observed after 48 hours of reaction. Alternatively, it is possible that the reaction has a low kinetic rate and that two products could have resulted from two separate reactions. Another observation was that a fluorescent peak was observed for OPA solutions containing ethanethiol and buffer, although OPA has been reported in the literature to be non-fluorescent.
The PIB-Cl used in the reactions was initially not confirmed to be pure and side reactions with impurities could possibly have resulted in the fluorescent peaks in these chromatograms. The PIB-Cl reactions were expected to react with the toxins more easily than the OPA because the reaction of amine groups with acid chlorides is usually instantaneous. The results were under suspicion because the peak chosen as the derivative peak in each case had the same elution time. Since the peaks were observed to increase with increased toxin concentrations it was most likely that the peak was produced either from the derivatised toxin or some other compound within the standard toxin solution. The possibility that the PIB-Cl preferentially reacted with the solvent instead of the toxins was plausible and was therefore investigated further. The GC/MS results from the methanolic solution of toxin indicated that some reaction had taken place with the PIB-Cl. The increased amount of methanol from the standard toxin solution added could have resulted in the increased peak area of the "derivative" peaks. This was confirmed when PIB-Cl dissolved in methanol was spiked in to the derivatised solutions - and the peak in question was observed to increase.

From the UV chromatograms it was evident that neither of the derivative reactions with microcystin LR and PIB-Cl, NBD-Cl or DNS-Cl appeared to be successful as indicated by the presence of the reagent peaks but no additional peaks to suggest the formation of a product.

Factors that could possibly have a negative effect on the results of a fluorescent derivatisation method include :

(i) the possibility that fluorescent reactions can occur with other components in the water or algal scum sample being analysed,
(ii) the optimum reaction conditions may differ from sample to sample,
(iii) more than one reaction product may form,
(iv) the reaction products may not be stable, and
(v) complete reactions may not be instantaneous.

More drastic derivatisation procedures involving several reaction steps involving refluxing, etc., were thought to be impractical and likely to result in toxin "losses" and increased variability in the results obtained.

2.7 CONCLUSION

The results from the derivatisation exercise were inconclusive. Fluorescence was observed in the reaction solutions analysed but no clear evidence of any toxin derivatisation was obtained. The microcystin-LR derivatisation with OPA resulted in two major peaks in the fluorescence chromatogram taken approximately 48 hours after the reaction. It was uncertain whether either could be used as the "derivative" peak. The second attempt also produced several chromatographic peaks, none of which could be conclusively identified as that of the derivatised toxin.

In the fluorescent reaction with PIB-Cl, one of the peaks was observed to increase with increasing concentrations of the toxin, but this peak was later established to be from the methyl ester of the reagent, which was selectively reacting with the methanol in which the microcystin-LR was originally dissolved or with the methanol of crystallisation in the toxin crystals.

The reactions attempted with the toxins using a UV detector revealed that no reaction had occurred when microcystin-LR was mixed with PIB-CL, DNS-Cl and NBD-Cl.

CHAPTER THREE
Evaluation of a Literature Method for the Determination of Algal Toxins[36]

3.1 Introduction

For the purpose of monitoring the algal toxins in drinking water supplies and for the study of remedial actions, the method developed should be flexible enough to quantify both the extracellular and intracellular toxin concentrations, i.e., the toxins that have been released into the surrounding water by cell lysis as well as the amount of toxins contained in the cyanobacterial cells (that have the potential to be released). Ideally, the method should produce the lowest possible detection limits and should be reliable for all types of water matrices. The results produced should be reproducible with a relatively simple sample preparation procedure so that the results can be obtained rapidly.

C_{18} solid-phase extraction has certain advantages over liquid-liquid extraction in that operator attention and the volume of organic solvents required are reduced. Even if more sensitive detection methods are developed, it is unlikely that the use of solid-phase extraction for the concentration of algal toxins from water would be omitted from the analysis procedure.

To evaluate the solid-phase extraction (SPE) technique, test samples were prepared by spiking toxins into various water matrices and analysed. A known amount of toxin was added to the matrix (ultra-pure water, tap water or raw water) and recovered before analysis by HPLC. The percentage recoveries were used as an indication of the reliability of the technique and to establish any limitations with regard to detection limits and interferences.

Preliminary work

The preliminary work included:

(i) determining the identifying retention times of the microcystin toxins available,
(ii) establishing the HPLC resolution, and
(iii) the preparation of calibration graphs.

The recommended spike of only one toxin (microcystin-LR), as per Lawton *et al.* was not used to adjust the recoveries of the other toxins analysed. Instead the recovery of each toxin was calculated individually.

Initial work was done using three toxins namely microcystin-RR, microcystin-LR and nodularin. Later work was carried out using five toxins: Microcystin-RR, microcystin-YR, microcystin-LR, microcystin-YA and nodularin.

The stable retention times of the toxins changed during the course of the entire investigation due to degeneration of the analytical column. Thus it is crucial that a set of standards is injected on each run in order to revalidate the retention times.

3.2 Preparation of Toxin Standards and Calibration Graphs

The toxins were dissolved in methanol and quantitatively transferred to a volumetric flask and made up to the appropriate volume with methanol. Since the toxins could not be weighed out accurately with the available balances, the toxin concentrations were calculated using the masses taken from the supplier container labels.

Stock standard solutions were prepared as follows:

Toxin	*Mass* (μg)	*Volume* (mℓ)	*Concentration* ($\mu g.m\ell^{-1}$)
nodularin	500	50	10
microcystin-RR	500	50	10
microcystin-LR	5000	500	10

A mixed toxin standard solution containing 3.3 μg.mℓ$^{-1}$ of each toxin was prepared by adding together equal volumes of the stock solutions. These solutions (25 μℓ) were injected onto the HPLC column to establish the toxin retention times and their elution order.

The chromatographic conditions were the same as those given by Lawton *et al.*, except the chromatogram wavelength was set at 240nm which is near the UV absorption maximum.

Chromatographic Conditions

Mobile Phase : Solvent A = 0,05% TFA in ultra-pure water
Solvent B = 0,05% TFA in acetonitrile

Gradient Elution Profile :

TIME / min.	*0*	*10*	*40*	*42*	*44*	*46*	*50*
% Solvent A	70	65	30	0	0	70	70
% Solvent B	30	35	70	100	100	30	30

Analytical column : μBondapak C18 (300 x 3.9mm) (Waters)
Column Temperature : 40°C
Injection Volume : 25 μℓ
Flow Rate : 1 mℓ.min^{-1}
Run Time : 45 minutes
Equilibration Delay : 15 minutes
Sparge rate : 25 mℓ.min^{-1}
Detector : 996 Photo diode Array Detector (Waters)
Detection Wavelengths : 200 nm to 400nm
Wavelength Monitored : 238 nm
HPLC : 710 B WISP (Waters); 600E System Controller (Waters), column heater (Waters)
Data Acquisition : Millennium 2010 Chromatography Manager (Waters)

The following standards were prepared from the stock solutions, the data from which was used to prepare the respective calibration graphs:

Table 3.1 Toxin Standard Solutions prepared from Stock Solutions

Std	***Volume of Toxins Added (mℓ)***			***Total Volume (mℓ)***	***Concentration (μg.mℓ$^{-1}$)***		
	RR	***NOD***	***LR***		***RR***	***NOD***	***LR***
1	4,00	0,50	0,50	5,00	8,0	1,0	1,0
2	0,50	4,00	0,50	5,00	1,0	8,0	1,0
3	0,50	0,50	4,00	5,00	1,0	1,0	8,0
4	3,00	1,00	1,00	5,00	6,0	2,0	2,0
5	1,00	3,00	1,00	5,00	2,0	6,0	2,0
6	1,00	1,00	3,00	5,00	2,0	2,0	6,0
7	2,50	1,25	1,25	5,00	5,0	2,5	2,5
8	1,25	2,50	1,25	5,00	2,5	5,0	2,5
9	1,25	1,25	2,50	5,00	2,5	2,5	5,0
10	2,00	1,50	1,50	5,00	4,0	3,0	3,0
11	1,50	2,00	1,50	5,00	3,0	4,0	3,0
12	1,50	1,50	2,00	5,00	3,0	3,0	4,0

Table 3.2 Standard Toxin Solutions prepared from the Mixed Standard Solution 3,3 μg.mℓ$^{-1}$

Std	*Volume of Mixed Toxin Standard*	*Volume of Methanol Added*	*Total Volume*	*Final Toxin Concentrations (μg.mℓ$^{-1}$)*		
	(mℓ)	(mℓ)	(mℓ)	*RR*	*NOD*	*LR*
13	2,42	1,58	4,00	2,0	2,0	2,0
14	1,82	2,18	4,00	1,5	1,5	1,5
15	1,22	2,78	4,00	1,0	1,0	1,0
16	2,42	7,58	10,00	0,8	0,8	0,8
17	1,81	8,19	10,00	0,6	0,6	0,6
18	1,21	8,79	10,00	0,4	0,4	0,4
19	0,61	9,39	10,00	0,2	0,2	0,2

Graduated glass pipettes were used to transfer the required volumes of standard solutions since the plastic autopipette tips used were found to deliver inaccurate volumes for organic solutions.

The 4 mℓ solutions were contained in HPLC (4 mℓ) vials.

Some of the standards (4, 5, 6, 10, 12, 15 and 16) were re-prepared from the same stock solutions and compared against the original results to determine the repeatability of the exercise and HPLC performance. The results were plotted onto the calibration graphs.

3.3 Results

The chromatograms in Figs. 3.1 to 3.4 show the identifying retention times of the microcystin-RR, nodularin and microcystin-LR and their order of elution. The HPLC column temperature was not at the specified 40°C for these determinations as no column heater was available; instead it was left at ambient temperature 22 - 25°C.

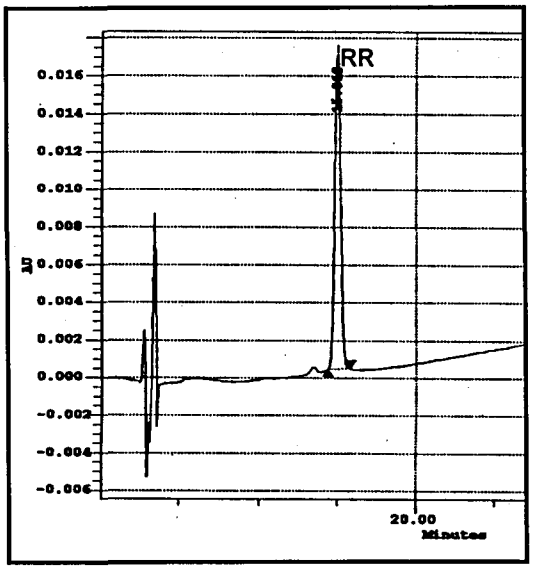


Fig. 3.1 Identifying retention time of microcystin-RR (≈ 15 min.)

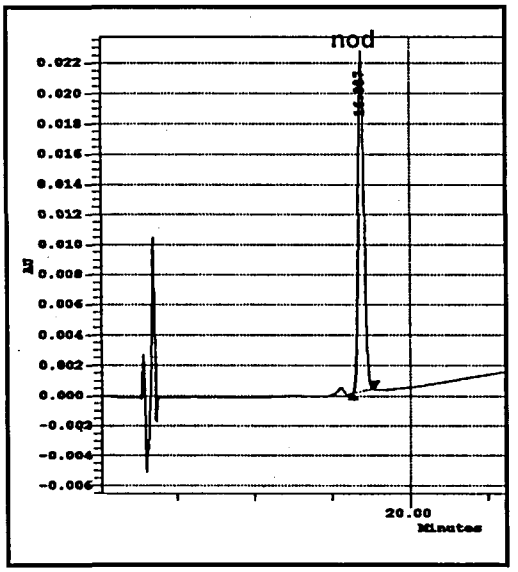


Fig. 3.2 Identifying Retention time of nodularin (≈ 17 min.)

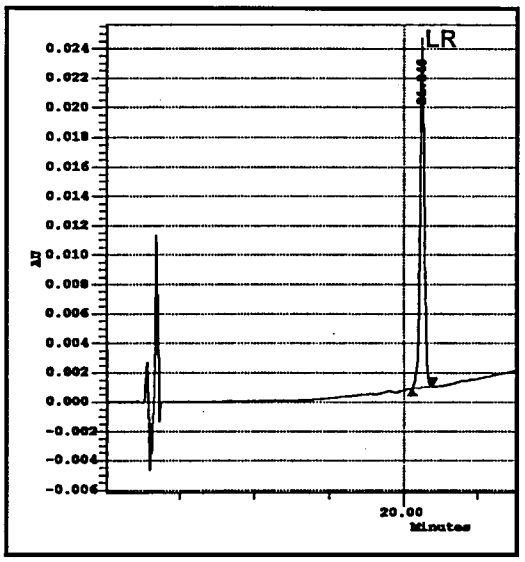


Fig. 3.3 Identifying retention time of microcystin-LR (≈ 21 min.)

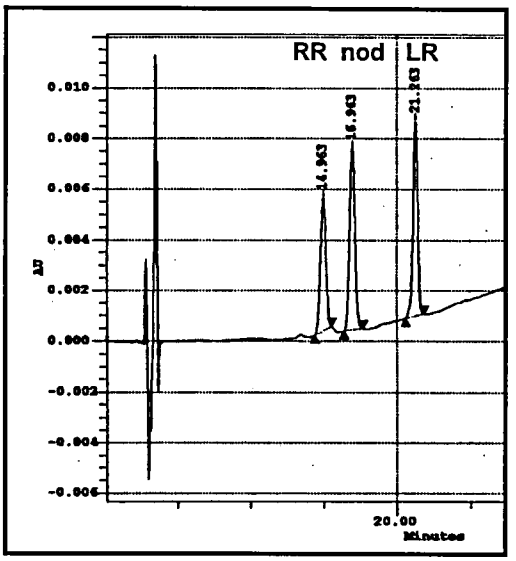


Fig. 3.4 Elution order of the mixed toxins (RR < NOD < LR)

Several mixed toxin standards were prepared and analysed as illustrated in Figs 3.5 to 3.10

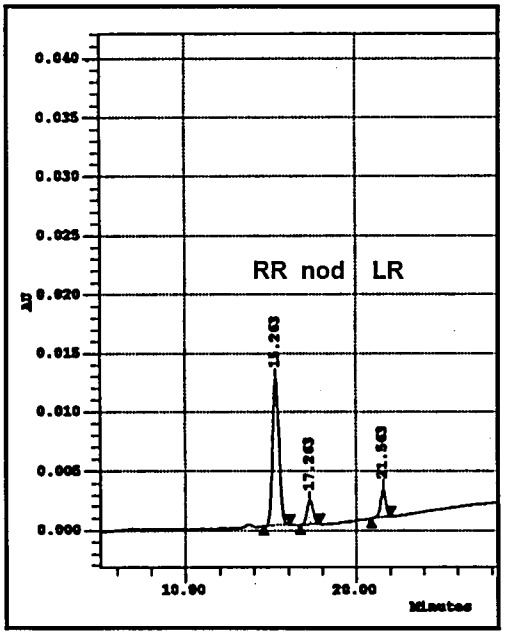


Fig. 3.5 Calibration standard with concentrations of 8:1:1 $\mu g.m\ell^{-1}$ for RR, LR & Nod respectively

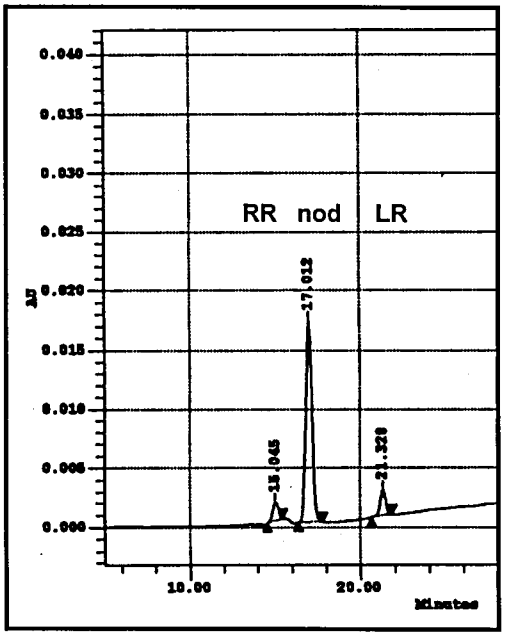


Fig. 3.6 Calibration standard with concentrations of 1:8:1 $\mu g.m\ell^{-1}$ for RR, LR & Nod respectively

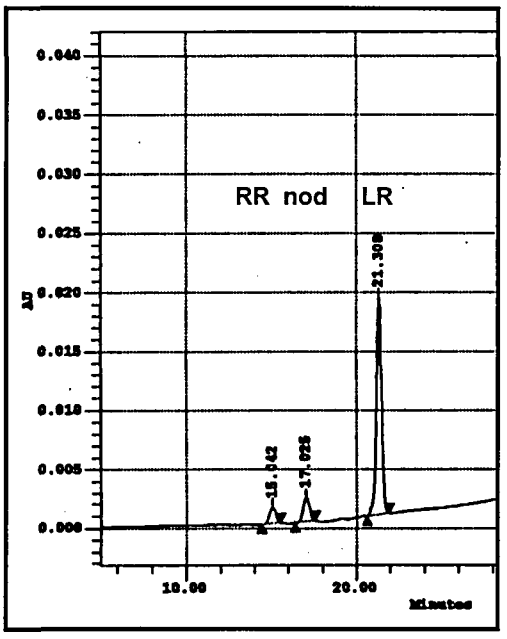


Fig. 3.7 Calibration standard with concentrations of 1:1:8 $\mu g.m\ell^{-1}$ for RR, LR & Nod respectively

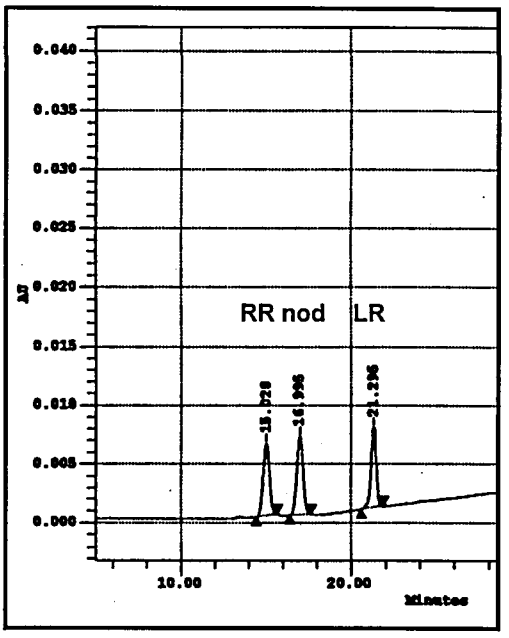


Fig. 3.8 Calibration standard with concentrations of 4:3:3 $\mu g.m\ell^{-1}$ for RR, LR & Nod respectively

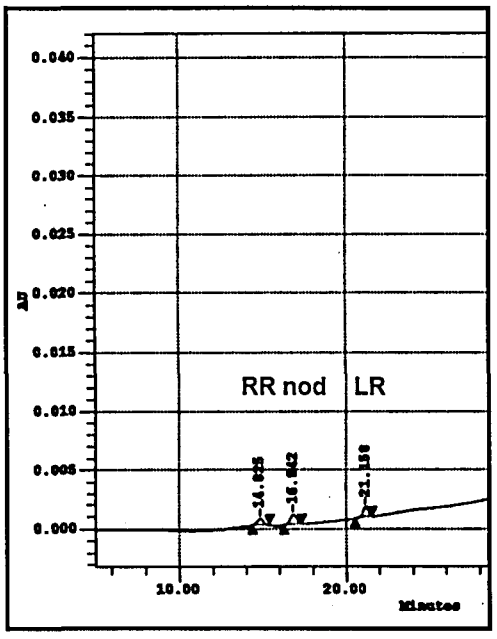


Fig. 3.9 Calibration standard with concentrations of 0.4 $\mu g.m\ell^{-1}$ for RR, LR & Nod

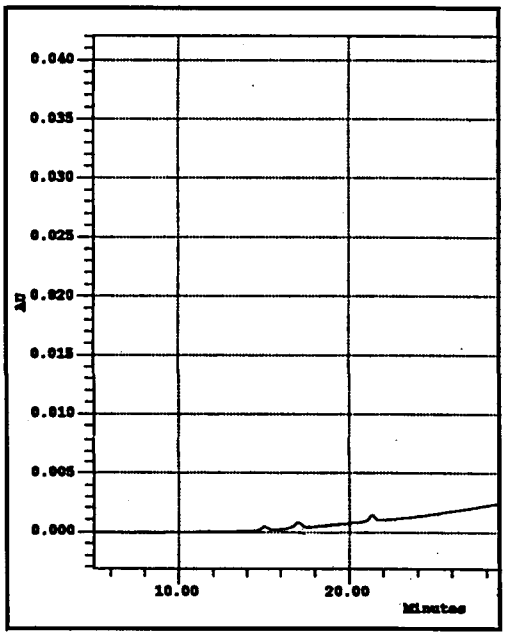


Fig. 3.10 Calibration standard with concentrations of 0.2 $\mu g.m\ell^{-1}$ for RR, LR & Nod

The peak areas of the various standards were tabulated as follows:

Table 3.3 Calibration graph data

Concentration ($\mu g.m\ell^{-1}$)	*Microcystin RR Peak Area*	*Nodularin Peak Area*	*Microcystin LR Peak Area*
8,0	326747	437758	390402
6,0	244489	238655	294178
5,0	198866	281223	251232
4,0	161012	228508	195761
3,0	119736	172328	145188
3,0	118624	170791	148318
2,5	100490	132986	123600
2,5	99040	138779	127256
2,0	78888	104137	97283
2,0	73189	109182	96833
2,0	74275	113519	100745
1,5	60537	87128	76667
1,0	38355	51378	48017
1,0	34292	51411	44486
1,0	37737	55781	46971
0,8	30370	42409	36519
0,6	21575	32546	28120
0,4	13190	20623	18155
0,2	no peak data	no peak data	no peak data

The above data were used to construct the calibration graphs, from which linear equations were established using computer-assisted regression analysis.

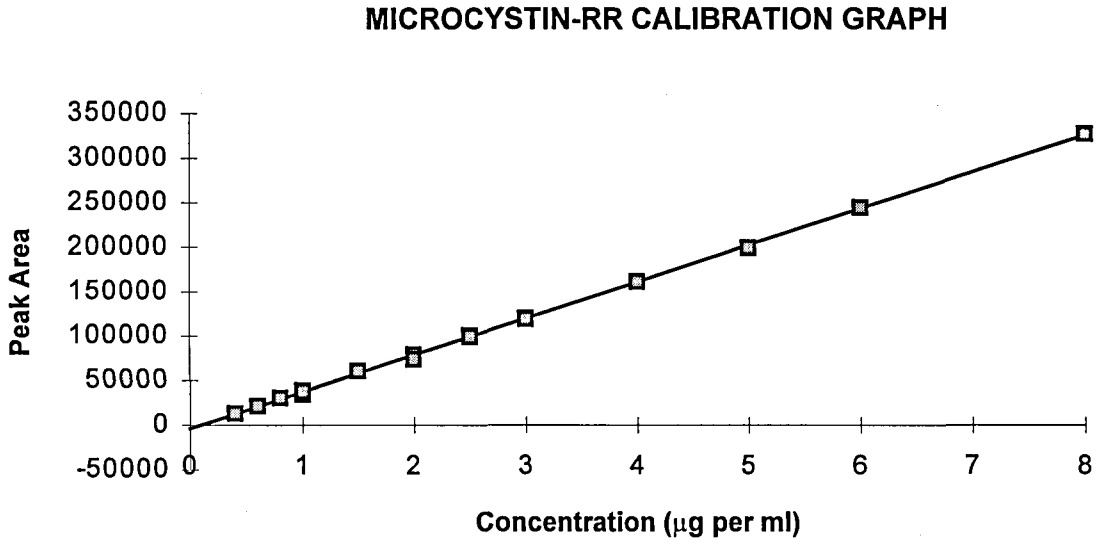


Fig. 3.11 Linear calibration of microcystin-RR standards

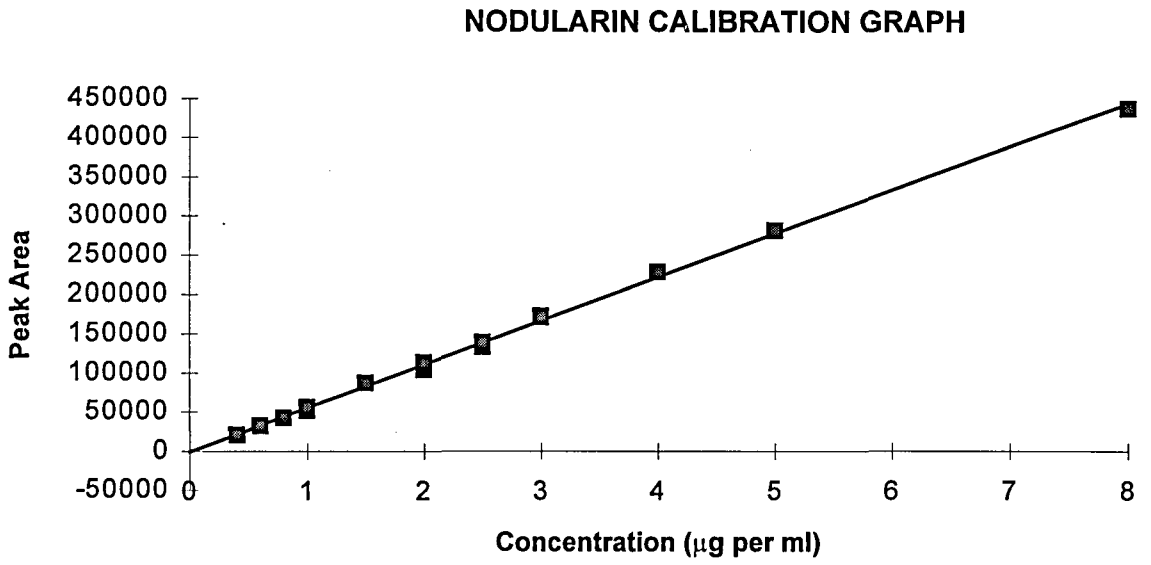


Fig. 3.12 Linear calibration of nodularin standards

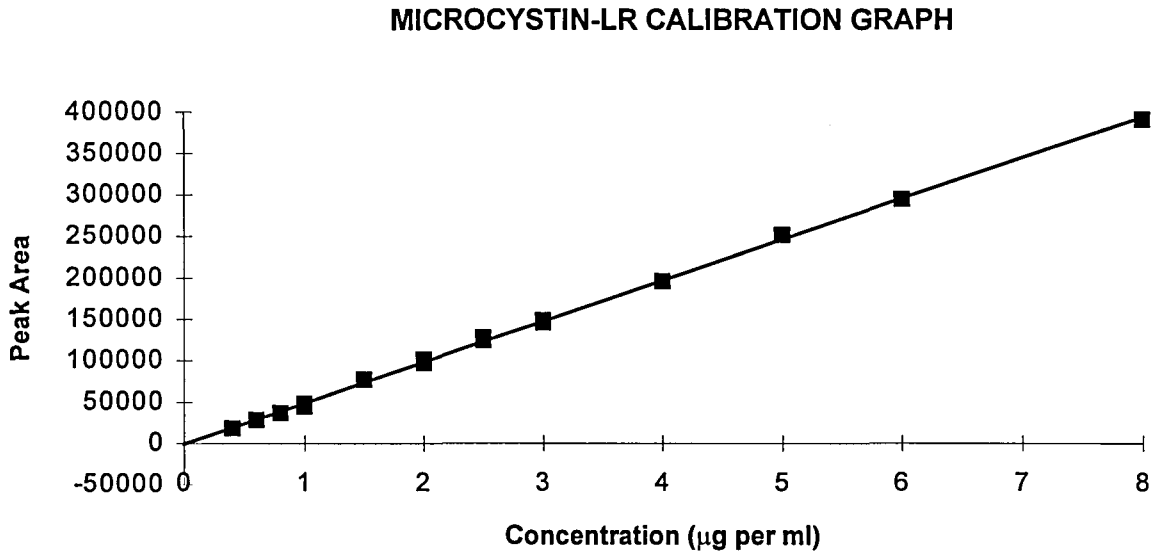


Fig. 3.13 Linear calibration of microcystin-LR standards

The calibration equations were verified as shown by the plotted data from the duplicate standards analysed.

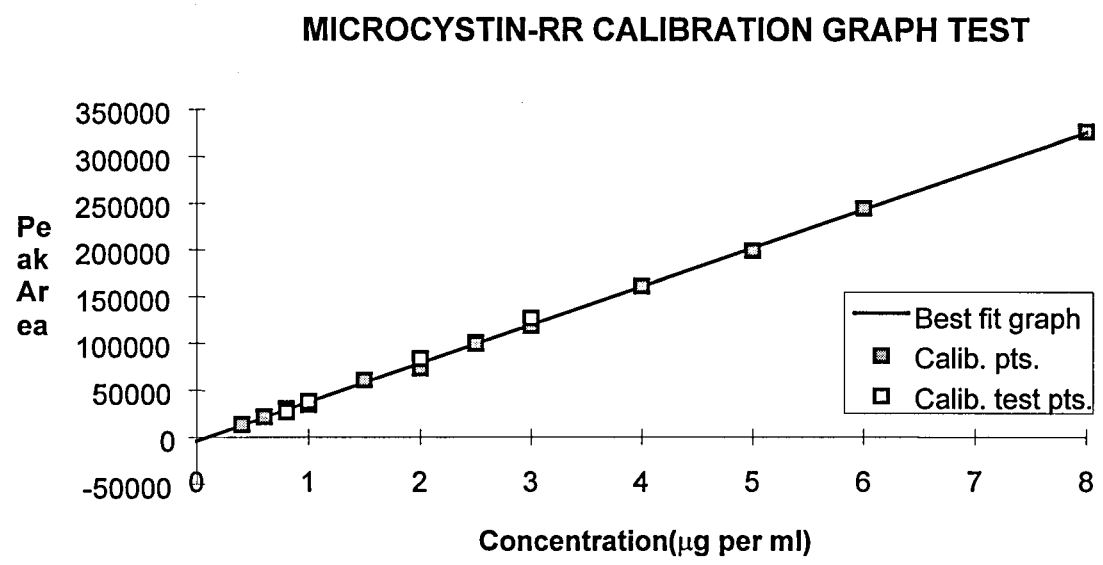


Fig. 3.14 Separate set of microcystin-RR calibration standards

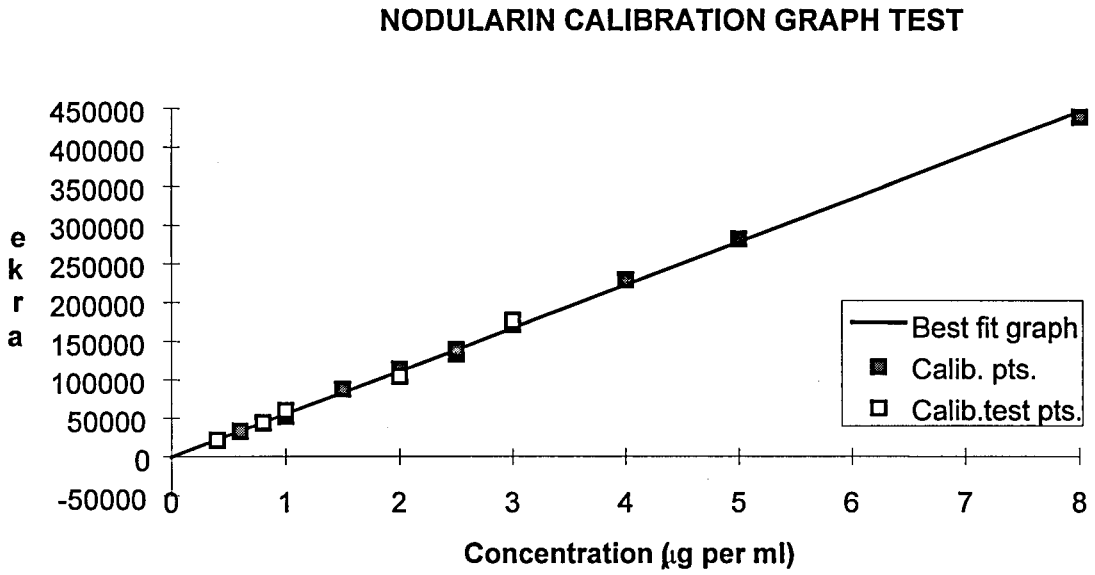


Fig. 3.15 Separate set of nodularin calibration standards

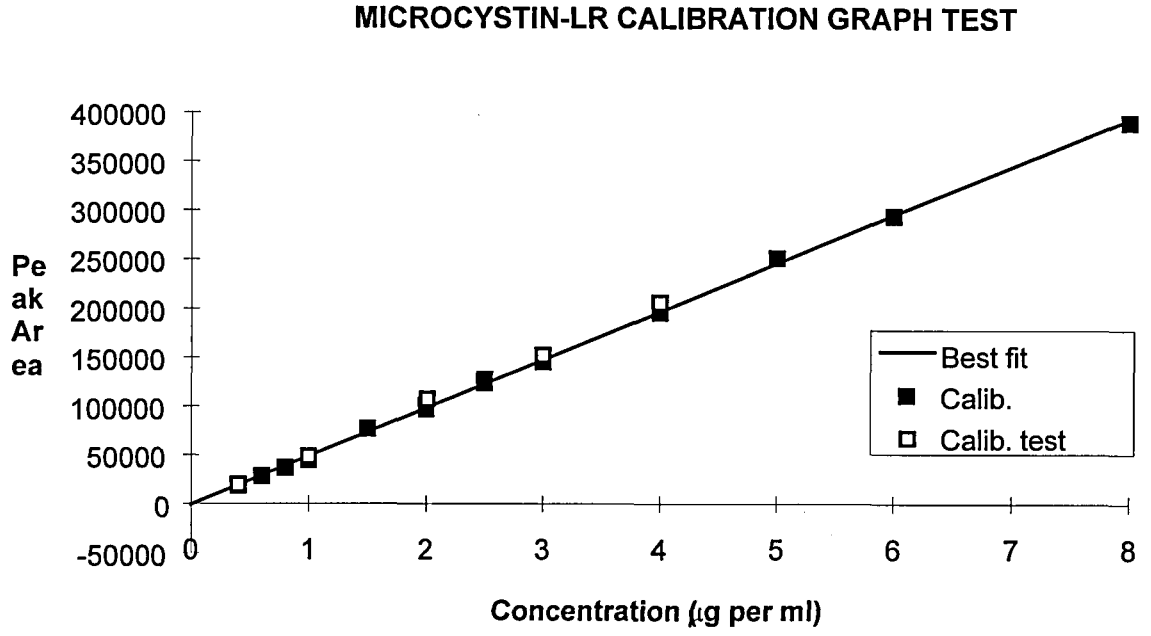


Fig. 3.16 Separate set of microcystin-LR Calibration standards

A calibration graph for microcystin-YR was also constructed later in the year and all the calibration equations were verified regularly as validation of their prolonged use. These standards were all prepared from fresh stock solutions. The calibration equations were effectively used throughout the report period since they did not vary significantly during the course of the recovery tests.

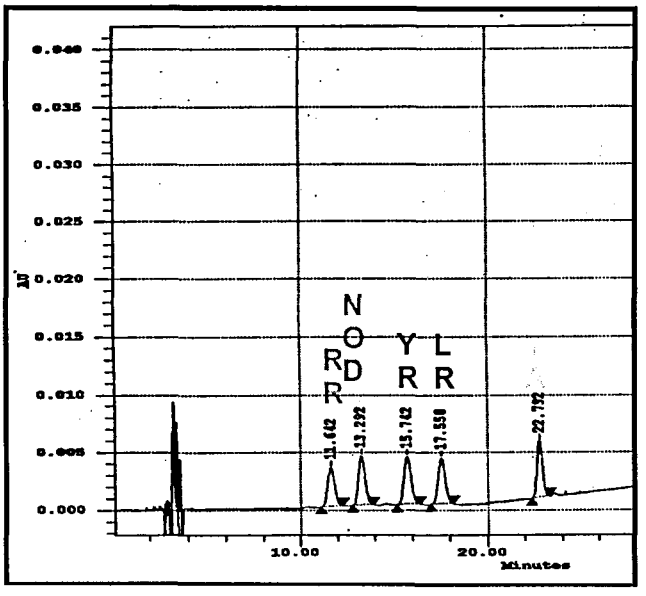


Fig. 3.17 Identifying retention time of mixed microcystin standards and nodularin

Calibration graphs were constructed as shown before using the mixed toxin standard solutions, which simultaneously determined whether the previous calibration equations were still valid.

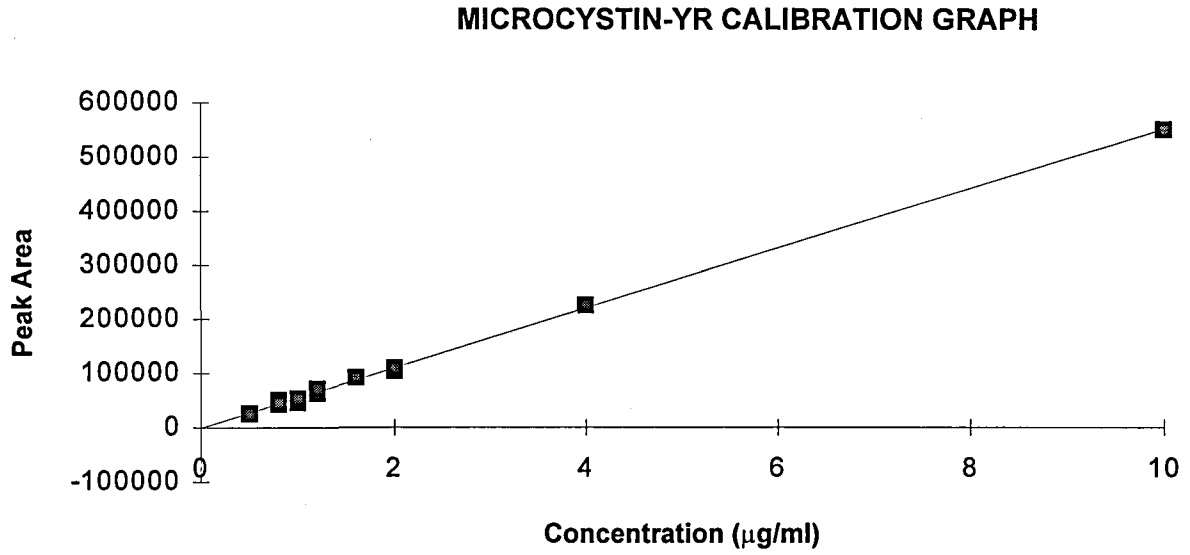


Fig. 3.18 Linear calibration of microcystin-YR standards

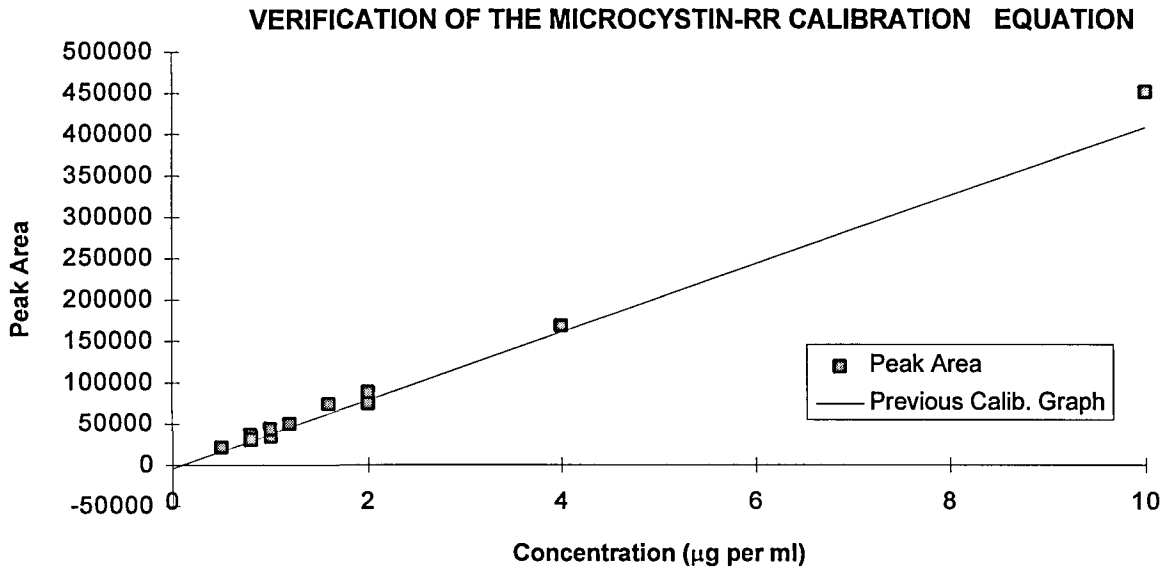


Fig. 3.19 Calibration standards of microcystin-RR, validating the use of the previous calibration equations

VERIFICATION OF THE MICROCYSTIN-LR CALIBRATION EQUATION

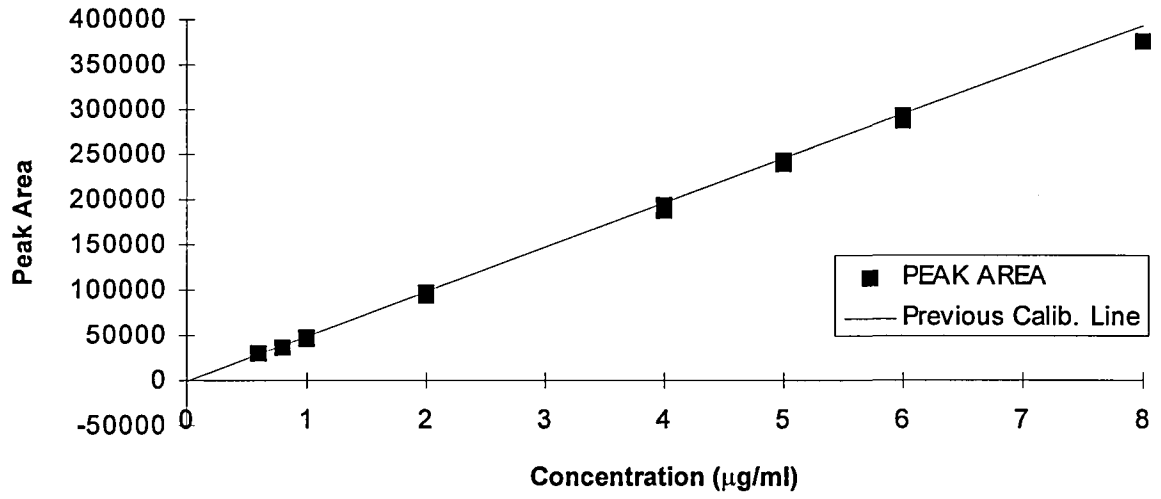


Fig. 3.20 Calibration standards of microcystin LR, validating the use of the previous calibration equations

VERIFICATION OF THE NODULARIN CALIBRATION EQUATION

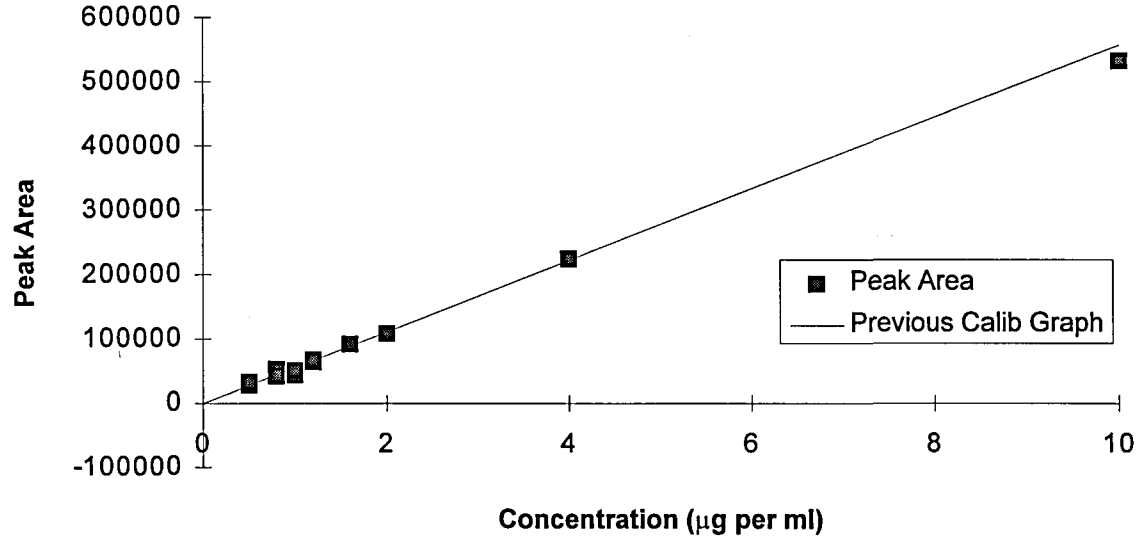


Fig. 3.21 Calibration standards of nodularin, validating the use of the previous calibration equations

The following linear equations were derived from the respective calibration graphs and were used to determine the toxin concentrations of solutions injected onto the HPLC :

$$\text{microcystin-RR concentration } (\mu g.m\ell^{-1}) = \frac{\text{Peak Area} + 3960}{41200}$$

$$\text{nodularin concentration } (\mu g.m\ell^{-1}) = \frac{\text{Peak Area} + 700}{55600}$$

$$\text{microcystin-YR concentration } (\mu g.m\ell^{-1}) = \frac{\text{Peak Area} + 1100}{55300}$$

$$\text{microcystin-LR concentration } (\mu g.m\ell^{-1}) = \frac{\text{Peak Area} + 830}{49300}$$

[x = (y-c)/m]

3.4 Methodology of Determining the Percentage Toxin Recoveries

Toxin concentrations of the standard solutions (spiked into the test samples) were determined from the respective calibration equations, and using the concentration determined and the volume spiked, the *expected* concentration of the recovered solution (in 0.1 mℓ of methanol) was calculated, assuming 100% recovery. After recovery and HPLC analysis, the toxin concentrations in the recovered solutions were again calculated using the peak areas and the calibration equations. The percentage toxin recovery was calculated on the basis of this value and the *expected* concentration as follows:

$$\% \text{ toxin recovered} = \frac{\textit{Recovered} \text{ concentration } (\mu g/m\ell)}{\textit{Expected} \text{ concentration } (\mu g/m\ell)} \times 100\%$$

For raw waters, two sets of recoveries were analysed one unspiked, and the other after spiking with toxin. When a non-microcystin peak co-eluted with a toxin peak, the averaged peak area of the co-elutant was subtracted from the peak area in the spiked solution. If any toxin was present, the percentage recovery of the spiked toxin was used to adjust the calculated toxin concentration recovered from the sample.

An illustration of this methodology for the determination of the percentage toxin recovery is given for the recovery of microcystin-LR from ultra-pure water See page 41.

3.5 DISCUSSION

The elution order of the toxins RR (M=1037) < NOD (M=824) < YR (M=1044) < LR (M=994) < YA (M=959) is expected for reversed-phase HPLC - the more polar the compound the shorter its retention on the column. Nodularin, however, is less polar than microcystin-LR, but has a much lower molecular mass and therefore has a lower retention time.

Considering the cost of the standard toxins, 5 mg of microcystin-LR costing ≈ R25 000 (1996), it was not possible to establish calibration graphs every time a set of recovery experiments was conducted. The stock solutions can be stored in the refrigerator with negligible variation in the toxin concentrations provided the stopper on the volumetric flask was adequately secured. When a standard of a particular concentration was required, it was made up and injected onto the HPLC column to verify its exact concentration.
A new calibration graph was not essential for the determination of the percentage recoveries if the concentration of the standard solution was within approximately 10% of the value from the respective calibration graphs.

Usually, three HPLC injections of the standard solutions were made just prior to the set of recovered solutions and one standard just after the set of recovered samples were analysed. The averaged peak areas of the standard solutions were used to determine the toxin concentration of the standard solution added to the test samples. The placement of the standards along the sample tray (of the HPLC) also served to indicate the stability of the analytical column. By

observing the toxin retention times before and after the test samples, it could be established whether any retention time drift had occurred.

3.6 Conclusion

The microcystin toxins studied were well resolved by reversed-phase HPLC and the calibration graphs were found to be linear in the concentration range up to 10 $\mu g.\ell^{-1}$. The determination of the percentage microcystin toxins is best determined from the expected concentrations of the spiked toxins as the toxin recovery may vary for the various toxins. The concentration of the stock microcystin toxin solutions did not vary considerably on storage and the calibration equations can be used successfully provided they are validated occasionally.

CHAPTER FOUR
Microcystin Toxin Recovery from Ultra-pure Water

4.1 Introduction

It was necessary to establish the reliability of the extraction and recovery technique without the added interference or recovery losses that are expected from "real" samples. By adding the toxins to ultra-pure water, it was thought that the optimum recovery could be determined as well as the variation in the percentage recoveries that could be expected for other samples.
Some setbacks were encountered in the initial stages and have been detailed in the preliminary work. It was found that certain unidentified peaks (as shown in the blank recoveries) had apparently co-eluted with microcystin-LR and nodularin causing their percentage recovery to exceed the amount added to the test water. Experiments were carried out to investigate this phenomenon further. Once the contamination problem was addressed toxin recovery experiments were initially carried out with microcystin-LR and then with the other toxins available.

4.2 Preliminary work

4.2.1 Procedure

The C_{18} cartridge was conditioned using 10 mℓ of methanol followed by 10 mℓ of ultra-pure water.
An aliquot of toxin standard solution was added to 500 mℓ of the ultra-pure water and, using the vacuum manifold, the sample was passed through the cartridge at a flow rate of less than 10 mℓ.min^{-1}. Two types of tubing (Rehau and Teflon) were used to transport the sample to the cartridge.
The cartridges were washed with 10 mℓ of 10%, 20% and 30% (v/v) aqueous methanol and the toxins eluted into LC vials with 4 mℓ of methanol containing 0.1% TFA. A polyethylene 10 mℓ syringe was used to contain the eluting solvent.
The eluates were dried under a nitrogen flow and re-suspended in 0.1 mℓ of methanol.
25 μℓ of the solution was injected onto the column.
HPLC conditions were as given before (page 24).

4.3 Results

The toxin peaks having elution times of 14.6, 16.6 and 21.0 for microcystin-RR, nodularin and microcystin-LR respectively were found in the chromatograms of the recovered solutions as illustrated in Figs. 4.1 & 4.2.

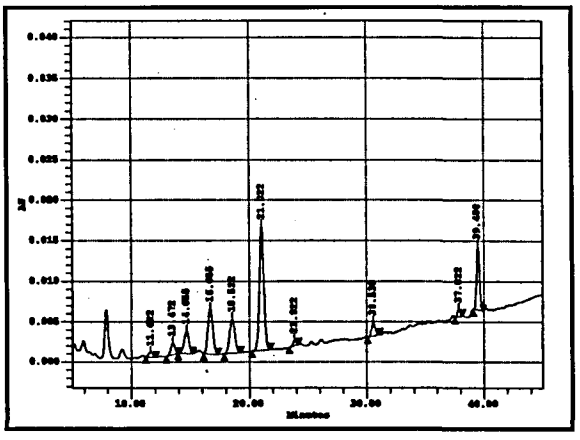


Fig. 4.1 Chromatogram of recovered microcystin-RR, nodularin & microcystin-LR from ultra-pure water

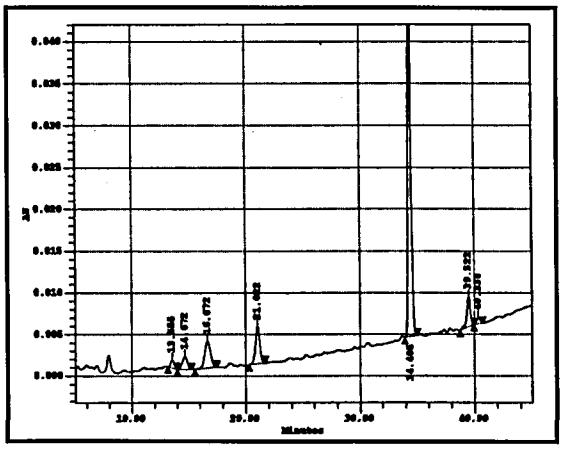


Fig. 4.2 Chromatogram of recovered microcystin-RR, nodularin & microcystin-LR from ultra-pure water

The calculated recovery values indicated considerable error for microcystin-LR and nodularin, as shown in Table 4.1.

Table 4.1 Recovery of microcystin LR, RR and nodularin from ultra-pure water

"Expected" Conc. ($\mu g.m\ell^{-1}$)	Observed Concentration ($\mu g.m\ell^{-1}$)			Percentage Recovery		
	RR	LR	NOD	RR	LR	NOD
2.5	2.3	10.7	3.4	93	427	134
2.5	2.1	8.3	3.0	84	331	119
2.0	1.9	9.2	2.5	94	461	124
2.0	1.7	7.4	1.6	84	360	78
1.5	1.5	2.3	2.0	100	156	136
1.5	1.3	2.4	2.2	88	161	145
1.0	1.0	2.0	1.4	98	198	142
1.0	0.9	2.7	1.5	94	267	155
0.50	0.6	1.4	1.0	118	273	195
0.50	0.6	1.2	0.8	111	243	159
0.25	-	0.4	-	-	175	-
0.25	-	1.0	-	-	414	-
0.15	-	0.7	0.5	-	441	338
0.15	-	1.0	0.4	-	680	255

While the recoveries of microcystin-RR are quite acceptable, those of microcystin-LR and nodularin are clearly anomalous. Chromatograms of blank recoveries (consisting of 500 $m\ell$ of ultra-pure water without the toxins) that were analysed with the test recoveries show that certain compounds that absorb at 240 nm had co-eluted with microcystin-LR and nodularin, resulting in the apparent toxin over-recoveries, as shown in Figs. 4.3 & 4.4.

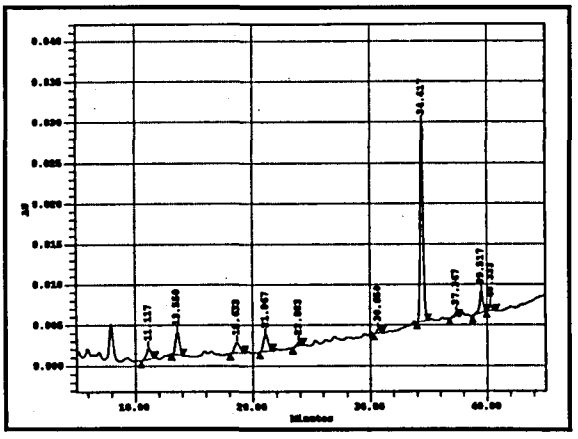


Fig. 4.3 Blank recovery from ultra-pure water

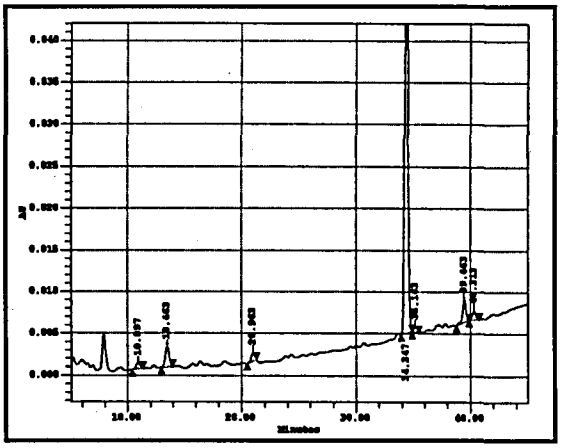


Fig. 4.4 Blank recovery from ultra-pure water

4.4 Investigation into the possible Source(s) of the Contamination

The source of the contamination that had caused the toxin over-recoveries had to be eliminated if the solid-phase extraction technique was to be successful. It was therefore necessary to determine the origin of the contaminant peaks so corrective measures could be taken. Neither sodium sulphite nor TFA were added to the recovery test samples and were excluded as the possible cause of the co-eluting peaks.

Several tests using only ultra-pure water and the relevant solvents were done to determine the cause of the contaminant peaks. Ideally, a smooth chromatographic baseline should be produced, and any chromatogram having peaks would imply that there had been some contamination.
A contaminated HPLC column was ruled out as a possible cause of the peaks in the blank recoveries since these peaks were absent when the standard solutions were injected.

The following apparatus used in the recovery of the toxins were all considered possible sources of the contamination:

1. glass bottles in which contained the ultra-pure water
2. the ultra-pure water
3. the tubing used to transport the sample to the cartridge
4. the C18(t) solid-phase extraction cartridge
5. the plastic syringe used to hold the eluting solvent
6. trifluoroacetic acid
7. methanol (HPLC grade)
8. HPLC vials into which the eluate was collected
9. taps on the vacuum manifold
10. nitrogen gas (high purity)

The following diagrams represent some of the finding that leads to the conclusion that the most likely source of the contaminant peaks was the solid-phase extraction cartridges:

The evidence for linking the Sep-Pak cartridges to the source of the contamination, comes partly by testing and excluding the other possible sources as given above, and partly from the fact that it was the only common apparatus in the tests where contamination was observed.
However, the major indication that it was indeed the Sep-Pak C_{18} trifunctional cartridges, was from the drastic difference in the amount of contamination which was found when comparative tests were carried out on other C_{18} cartridges (MegaBond Elut, Isolute), and when most of these contaminants apparently disappeared once the conditioning steps were altered.

The solvent portions collected were dried under nitrogen and re-suspended in 0.1 mℓ of methanol before analysis by HPLC

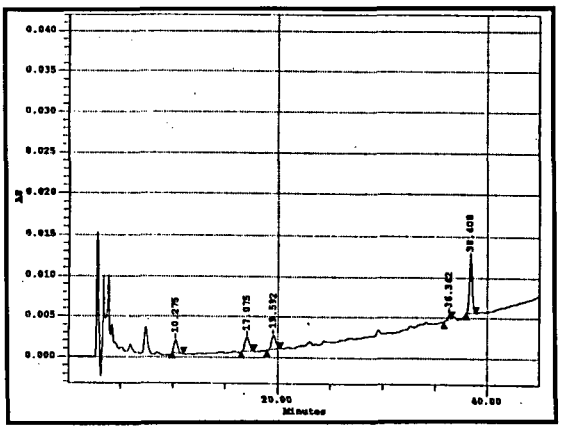


Fig. 4.5 30% methanol (30 mℓ) passed through a C18(t) cartridge using Teflon tubing ; 3.5 mℓ methanol (with 0.1%TFA) passed through and collected

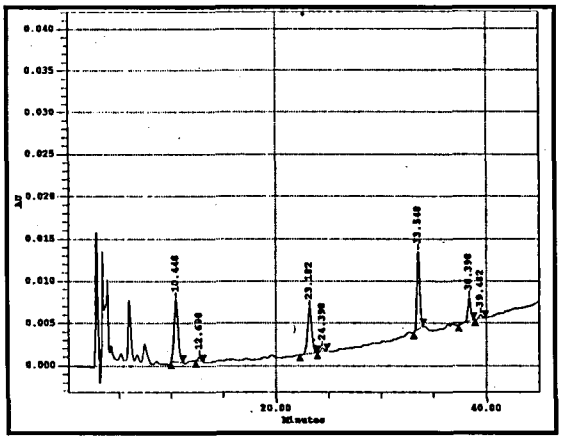


Fig. 4.6 30% methanol (30 mℓ) passed through a C18(t)cartridge using Rehau tubing ; 3.5 mℓ methanol (with 0.1%TFA) passed through and collected

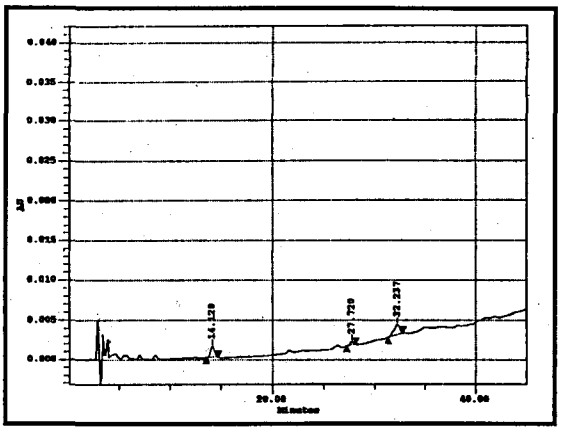


Fig. 4.7 Methanol (200 mℓ) passed through a C18(t) cartridge using glass tubing ; 10 mℓ of methanol passed through and collected

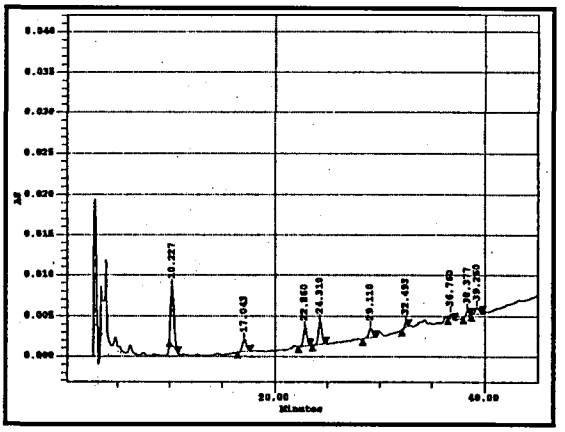


Fig. 4.8 Methanol (640 mℓ) passed through the same C18(t) cartridge using glass tubing ; 10mℓ methanol passed through and collected

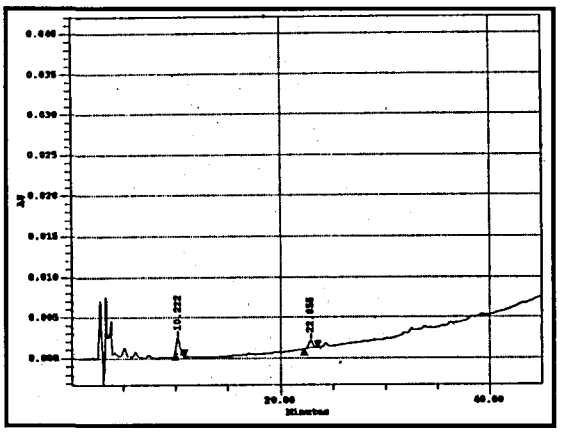


Fig. 4.9 Methanol (740 mℓ) passed through the same C18(t) cartridge using glass tubing ; 10 mℓ of methanol passed through and collected

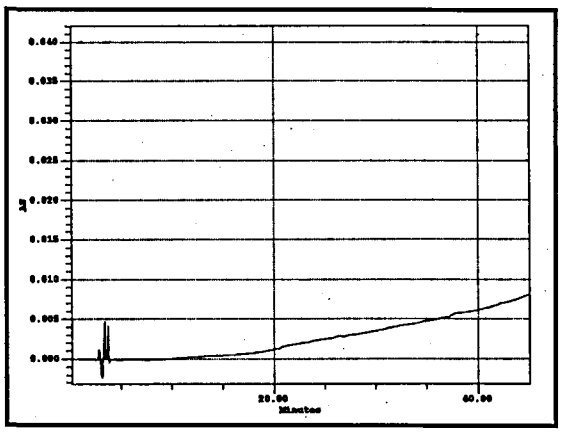


Fig. 4.10 A blank recovery using ultra-pure water; using the modified cartridge conditioning procedure of ethyl acetate (15 mℓ), methanol with 0.1% TFA (5mℓ), methanol (20mℓ) and ultra-pure water (20mℓ)

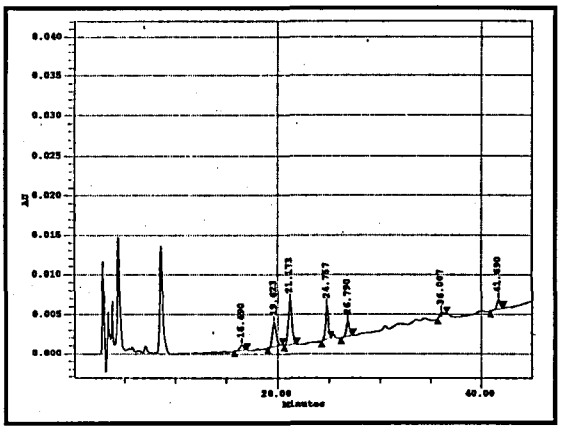


Fig. 4.11 Methanol (10mℓ) passed through an Isolute C18 cartridge and collected

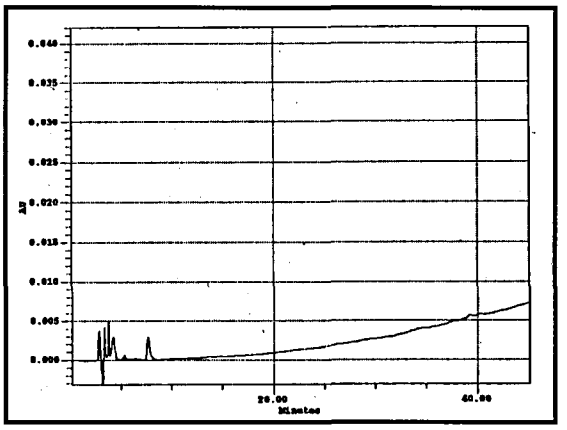


Fig. 4.12 Methanol (10mℓ) passed through the same Isolute cartridge and collected

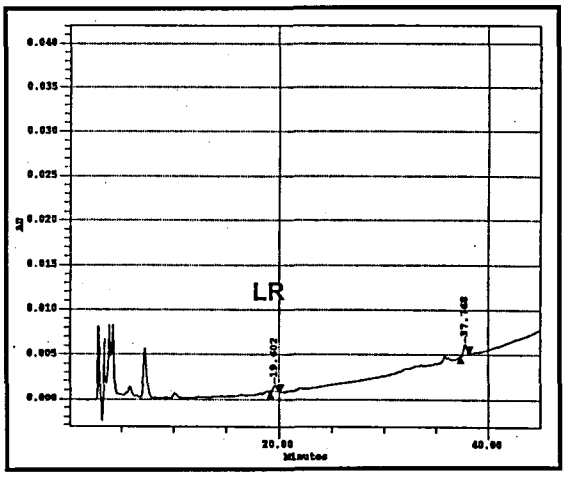


Fig. 4.13 Microcystin-LR recovery from ultra-pure water using the new cartridge-conditioning procedure

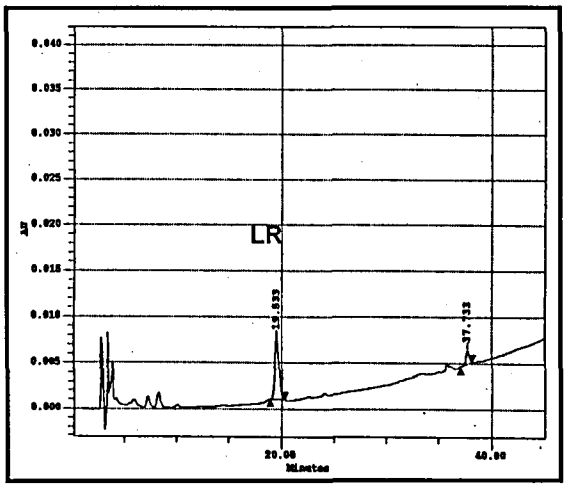


Fig. 4.14 Microcystin-LR recovery from ultra-pure water using the new cartridge-conditioning procedure

It appears that these contaminants can be washed out of the cartridges as indicated in Figs 4.7 to 4.12 The corrective measures taken to minimise or eliminate the contamination included the introduction of a more rigorous conditioning procedure and the replacement of all plastic apparatus with glass alternatives. The contaminant peaks did not appear to interfere with the determination of microcystin-LR when the new cartridge conditioning procedure was applied as shown in Figs. 4.13 & 4.14.

4.5 RECOVERIES

The detection limits found for the toxins were between 0.2 and 0.4 $\mu g.m\ell^{-1}$ which would correspond to detection limits in water samples of between 0.04 to 0.08 $\mu g\ \ell^{-1}$, assuming that the extracted residues were re-suspended in 0.1 mℓ of methanol and the toxins were completely recovered.

4.5.1 Procedure

The recovery procedure from ultra-pure water was the same as given earlier (page 36) except for the modification of the cartridge conditioning procedure, namely: ethyl acetate (15 mℓ), 0.1% TFA in methanol (5 mℓ), methanol (20 mℓ) and ultra-pure water (20 mℓ).

The toxin recoveries were initially attempted with microcystin-LR then with a mixture of the available toxins.

4.6 Results

The following diagrams indicate the methodology of determining the percentage toxin recoveries using the peak area of the standards and recovered solutions and the calibration equations:

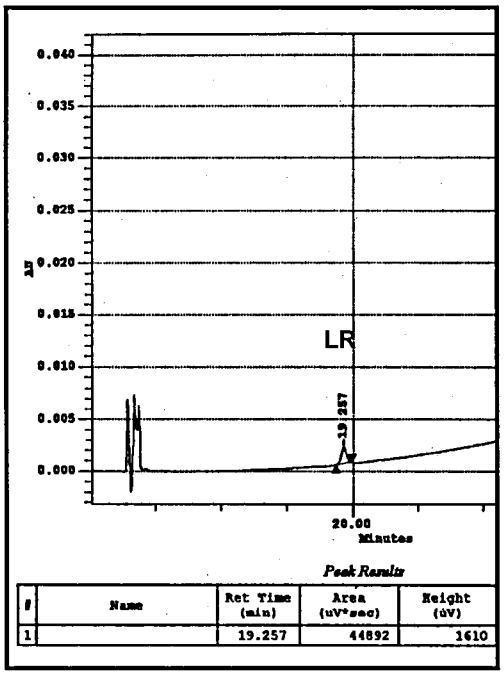


Fig. 4.15 Microcystin-LR standard added to the test samples (500mℓ)
LR Conc. = (44892 +830)/49300
= 0.93 $\mu g.m\ell^{-1}$

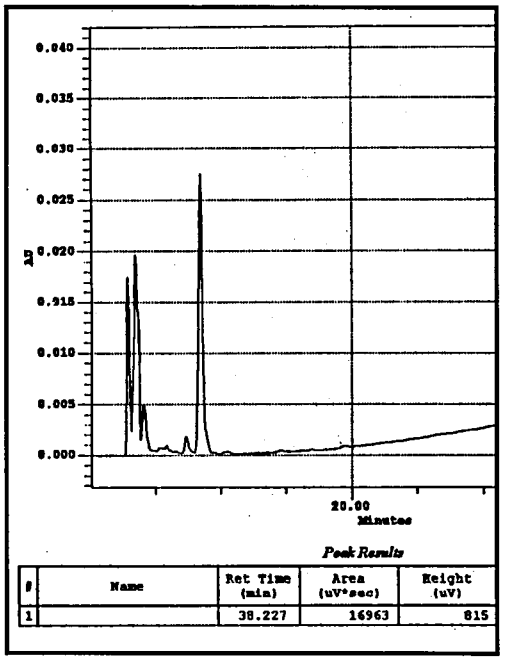
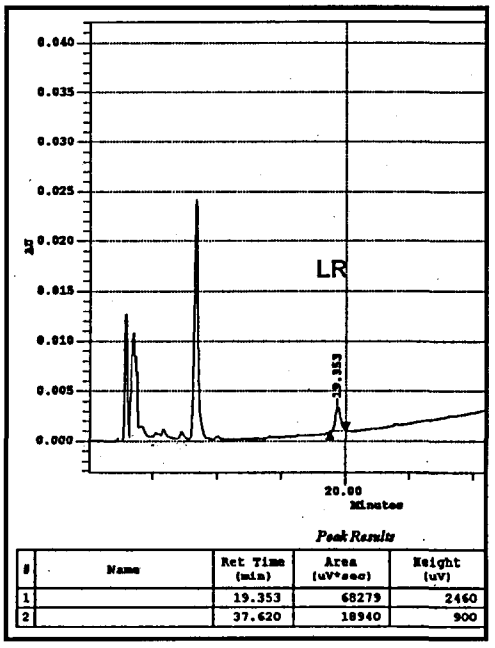
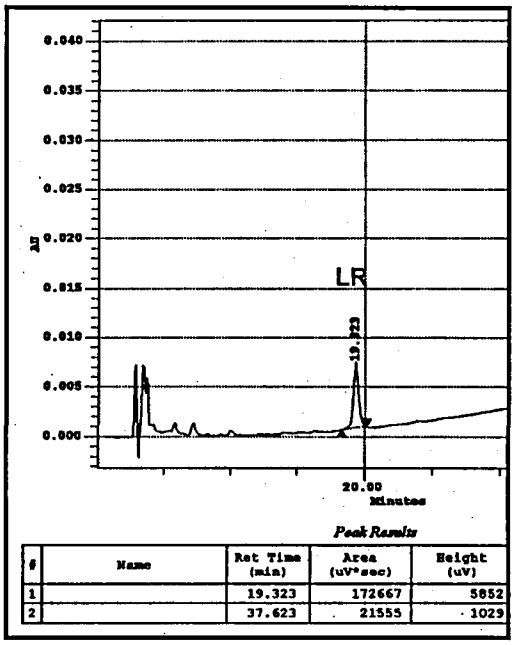


Fig. 4.16 Blank recovery from ultra-pure water



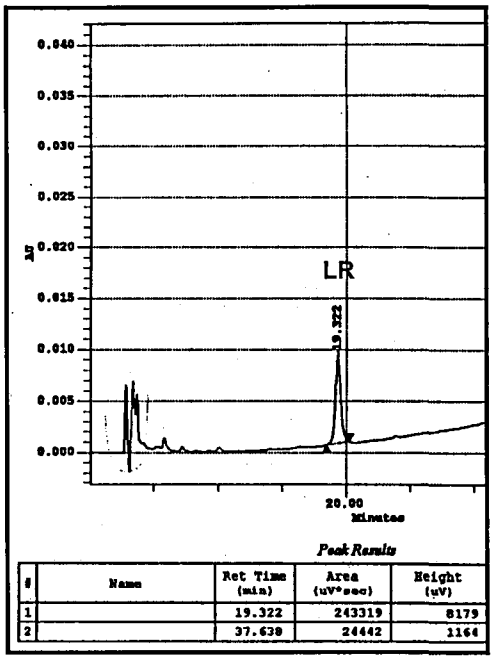
#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)
1		19.353	68279	2460
2		37.620	18940	900

Fig. 4.17 75% microcystin-LR recovery



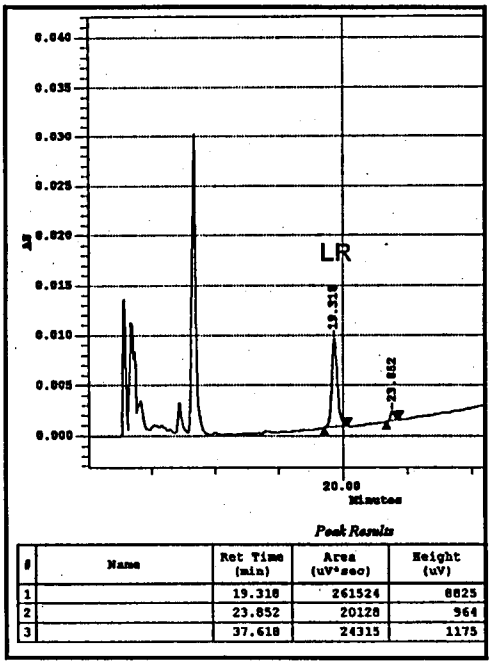
#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)
1		19.323	172667	5852
2		37.623	21555	1029

Fig. 4.18 95% microcystin-LR recovery



#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)
1		19.322	243319	8179
2		37.638	24442	1164

Fig. 4.19 89% microcystin-LR recovery



#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)
1		19.318	261524	8825
2		23.852	20128	964
3		37.618	24315	1175

Fig. 4.20 94% microcystin-LR recovery

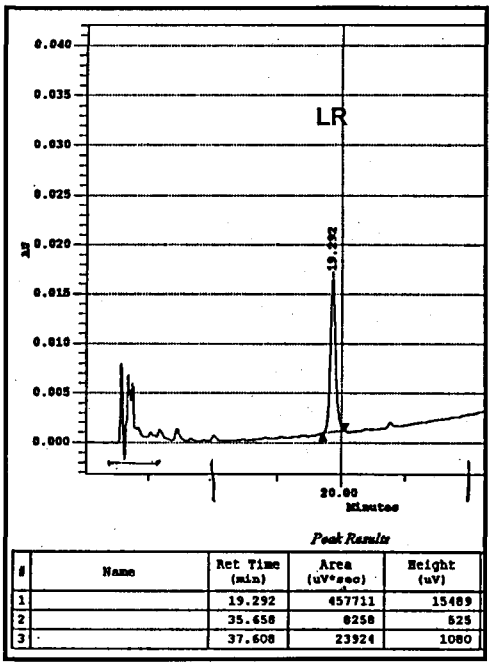


Fig. 4.21 100% microcystin-LR recovery

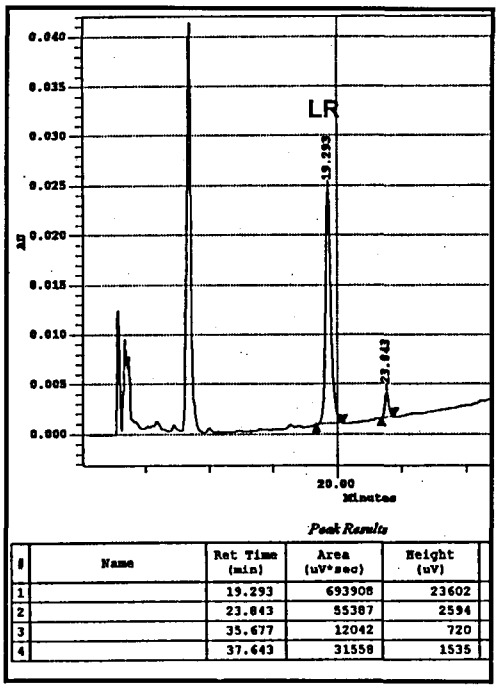


Fig. 4.22 101% microcystin-LR recovery

The following table details the calculation of the Microcystin-LR Recovery:

Table 4.2 Calculation of microcystin-LR recoveries from ultra-pure water

Fig	Vol. of Std LR Soln. added to 500 mℓ of water (mℓ)	Expected Recovery μg mℓ$^{-1}$	LR Peak Area Recovered Soln.	Obtained Recovery μg mℓ$^{-1}$	Percentage Recovery
3	0.2	1.86	68279	1.40	75
4	0.4	3.72	172667	3.52	95
5	0.6	5.58	243319	4.95	89
6	0.6	5.58	261524	5.32	94
7	1.0	9.30	457711	9.30	100
8	1.5	13.95	693908	14.09	101

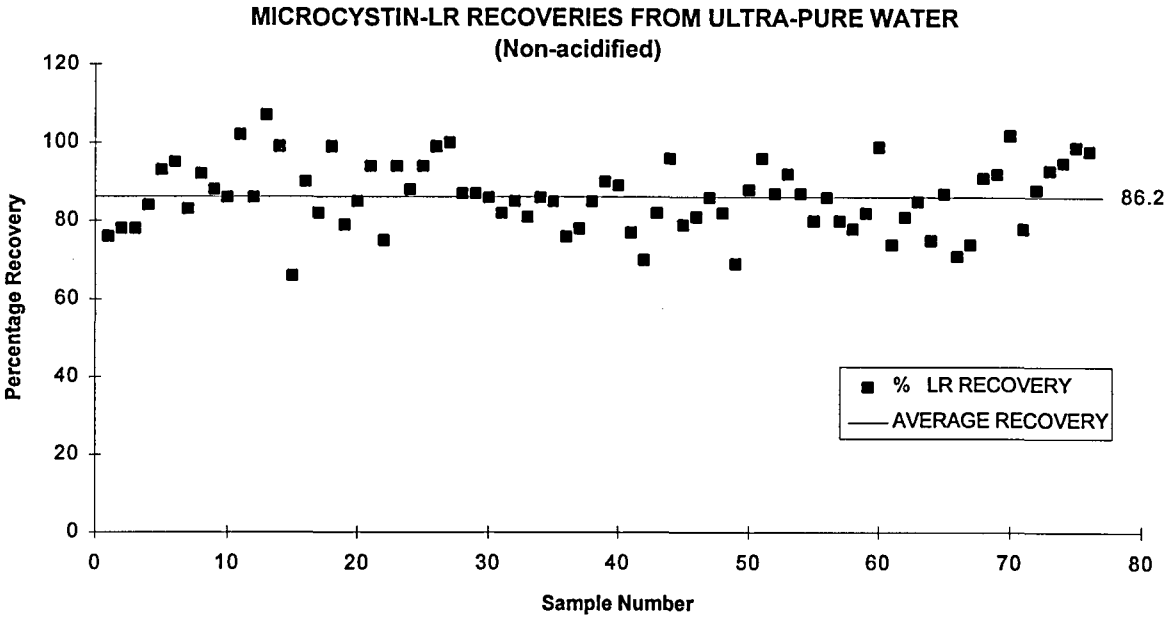


Fig. 4.23 Microcystin-LR recoveries from ultra-pure water

Relative Standard deviation = 8.7, *n*=78
Recovery Range = 66 to 108%

The recovery of mixed toxin standards produced comparable recoveries to those above. Toxin elution times have shifted during the course of the investigations due to column degeneration. Recorded elution times were 11.7; 13.3; 15.7; 17.6 & 22.7 for microcystin-RR, nodularin, microcystin-YR, microcystin-LR and microcystin-YA respectively.

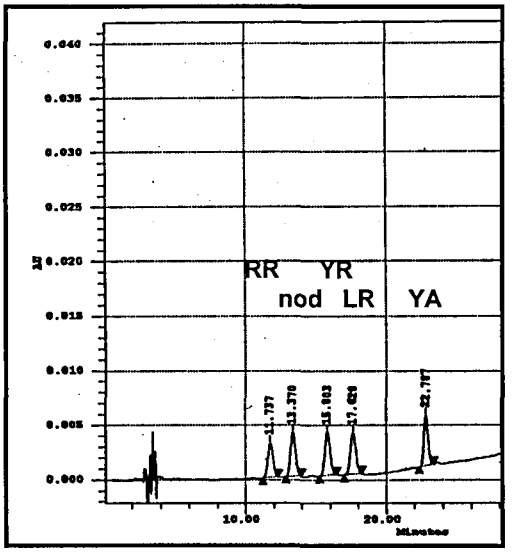


Fig. 4.24 Mixed toxin standard solution added to ultra-pure water

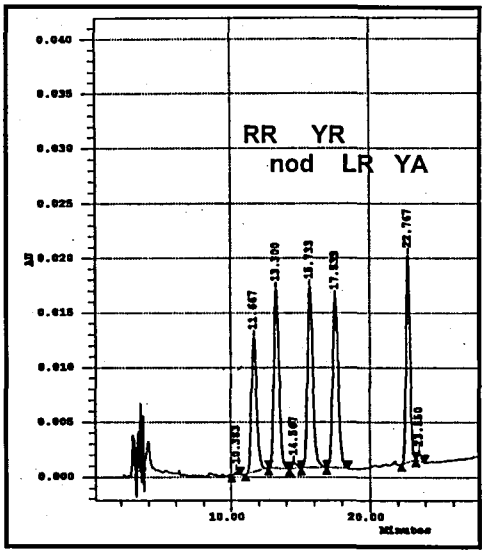


Fig. 4.25 Recovered mixed toxins from ultra-pure water

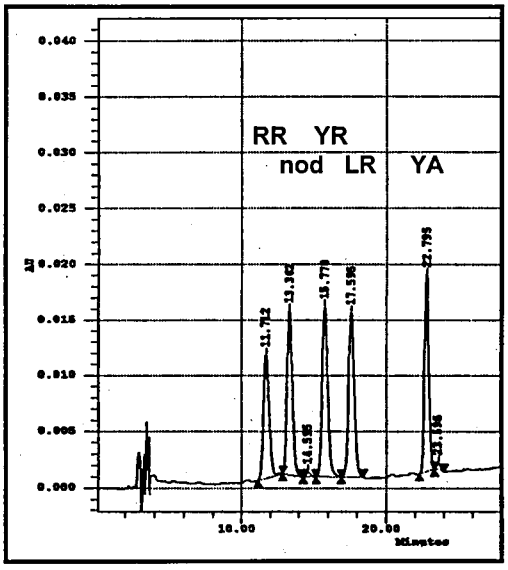


Fig. 4.26 Recovered mixed toxins from ultra-pure water

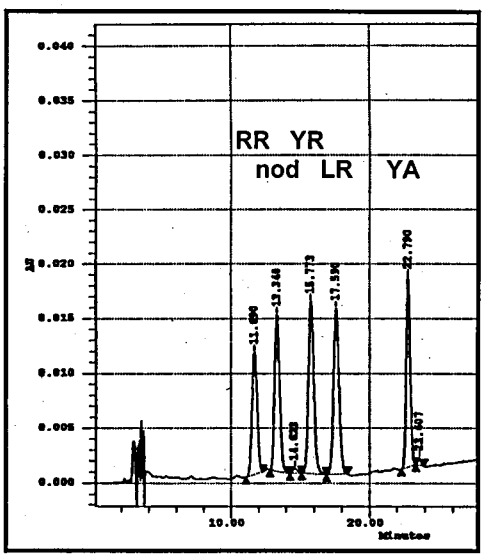


Fig. 4.27 Recovered mixed toxins from ultra-pure water

4.7 Discussion

Even though it is likely that other compounds may interfere or co-elute with the toxins in raw water samples, the peaks that are observed in the blank extraction of ultra-pure water (Fig. 4.3) imply that the apparatus itself cannot be used with confidence and may unnecessarily cause erroneous recoveries. These peaks may also be falsely labelled as sample peaks. The contaminant peaks varied randomly from cartridge to cartridge in both the number of peaks and peak size. The peak areas of the co-elutant therefore could not be subtracted from the peak area of the toxin as a possible corrective measure.

Other peaks observed to arise from the apparatus that did not co-elute with the toxins being studied may potentially interfere with the recovery of other microcystin toxins, of which 47 have been reported to date.

4.8 Conclusion

The modified cartridge conditioning procedure of ethyl acetate (15 mℓ), methanol containing 0.1% TFA (5 mℓ) , methanol (20 mℓ) and ultra-pure water (20 mℓ), reduced the contamination to an acceptable extent (Fig. 4.10) that the co-elution did not interfere with the determination of the percentage recovery of the toxins. Microcystin-LR recoveries using the modified cartridge conditioning procedure averaged 86.2% for 78 test samples, with a standard deviation of 8.7.

CHAPTER FIVE
Recoveries from Tap Water

5.1 Introduction

In the event of a toxic cyanobacterial bloom developing in the storage supplies, it would become necessary to analyse water samples after the water purification works. The laboratory tap water with pH values between 8 to 9 and total chlorine values of 1.5 - 2 ppm was used as the test water for these recovery experiments. The recommended procedure (by Lawton *et al.*[36]) required that the filtered samples be treated with sodium sulphite (Na_2SO_3) and trifluoroacetic acid (TFA), spiked with microcystin-LR (5μg) and methanol (5 mℓ) prior to solid-phase extraction of the toxins. The addition of the sodium sulphite was to remove the residual chlorine from the samples, but no explanation was provided for the addition of the trifluoroacetic acid or the methanol. While the advantage of adding the methanol may be to improve the extraction efficiency by wetting the sorbent layer of the SPE cartridge, the reason for the addition of the trifluoroacetic acid was not obvious. At the outset, the possibilities included the reduction of the pH of the sample to be more suitable for extraction by the trifunctional cartridges, or to keep certain compounds from precipitating out and clogging the cartridges or probably, to remove the excess sodium sulphite.

5.2 Procedure

Tap water was filtered through Whatman GF/C and 500 mℓ placed into Pyrex bottles and spiked with an aliquot of toxin standard solution. Sodium sulphite solution (50μℓ ;1 g/100 mℓ) was added and the solution shaken before filtering. Aqueous TFA (5 mℓ ;10%, v/v) was added and the solution was passed through the extraction cartridge at a flow rate of 4-8 mℓ.min^{-1}. (using the modified cartridge conditioning procedure). The cartridge was washed with 10 ml of 10%, 20% and 5 mℓ of 30% (v/v) aqueous methanol and the toxins eluted with 3.5 mℓ of 0.1% (v/v) TFA in methanol. The eluate was dried under a flow of nitrogen and the residue re-suspended in 0.1 mℓ of methanol. 25μℓ was injected onto the HPLC column. HPLC conditions were as described on page 24.

5.3 Results

The chromatograms in Figs. 5.1 and 5.2 are of recovered microcystin-LR (0.4 μg.ℓ$^{-1}$) using the procedure as described above. (Microcystin-LR elution time: 19.3 minutes)

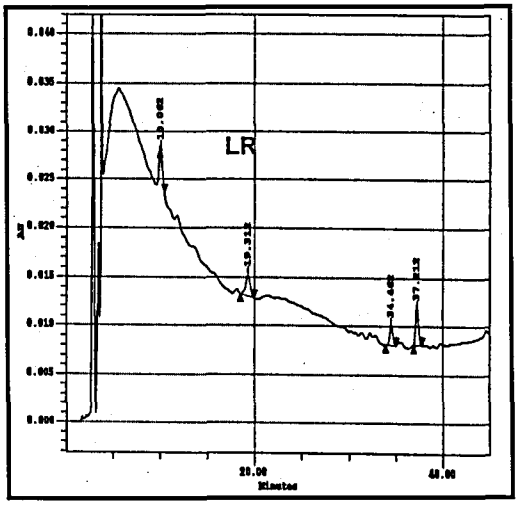


Fig. 5.1 Microcystin-LR recovery using the C18 (t) cartridge and the procedure described by Lawton *et al.* (with Na_2SO_3 & TFA)

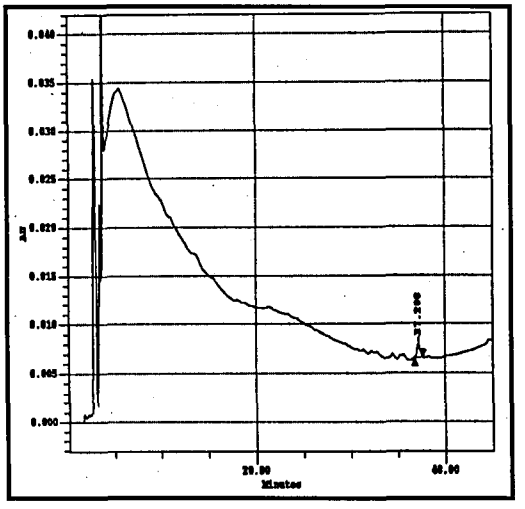


Fig. 5.2 Zero Microcystin-LR "recovery" using the C18 (t) cartridge and the procedure described by Lawton *et al.* (with Na_2SO_3 & TFA)

The microcystin-LR solutions having concentrations of (0.4 $\mu g.\ell^{-1}$) were not recovered consistently when the procedure (as described above) was applied even when the recommended Isolute C18(t) cartridges were used, see Figs 5.3 & 5.4. In most cases a zero percent recovery was obtained as shown in Table 6.

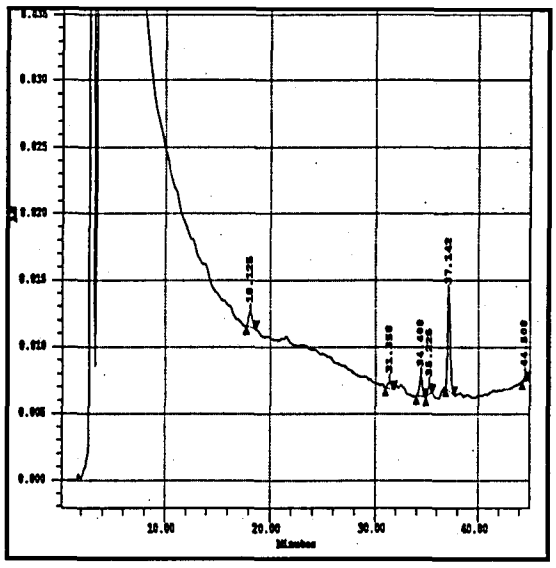


Fig. 5.3 Zero Microcystin-LR recovery using an Isolute cartridge and the procedure described by Lawton *et al.* (with Na_2SO_3 & TFA)

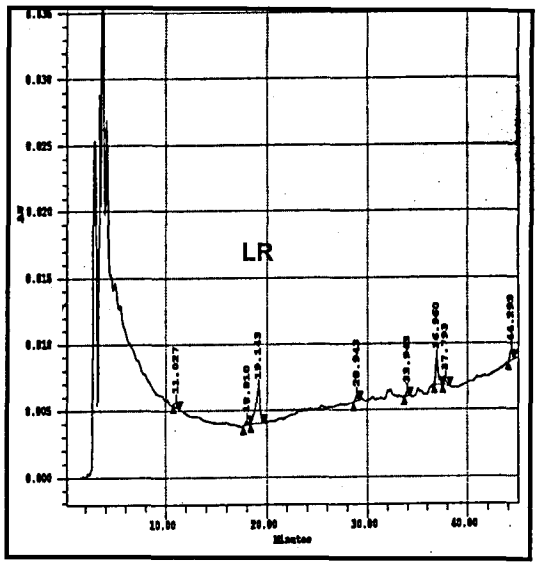


Fig. 5.4 Microcystin-LR recovery using Isolute cartridge and the procedure described by Lawton *et al.* (with Na_2SO_3 & TFA)

Table 5.1 Acidified Recoveries from tap water

Cartridge	*No. of Tests*	*Percent Recoveries Recorded*	*No. of Zero Percent Recoveries*
Sep Pak	22	88, 103, 88, 93, 78, 77. 90	17
Isolute	13	17 , 18	11
Megabond	5	0	5

Omission of both the acidification step (with TFA) and the addition of sodium sulphite produced consistent non-zero recoveries as follows:

Table 5.2 Non-acidified Recoveries from tap water (without addition of sodium sulphite)

Cartridge	*No. of Tests*	*Percent Recoveries Recorded*
Sep- Pak C18 (t)	6	100, 86, 78, 63, 74, 74
Isolute	7	90, 85, 83, 80, 50, 63, 53

Figs. 5.5 & 5.6 show the chromatograms of microcystin-LR recoveries from tap water samples without the addition of sodium sulphite or TFA. Also noticeable was the lower background absorbance of the non-acidified tap water samples compared to those of the acidified samples (in Figs. 5.1 & 5.4)

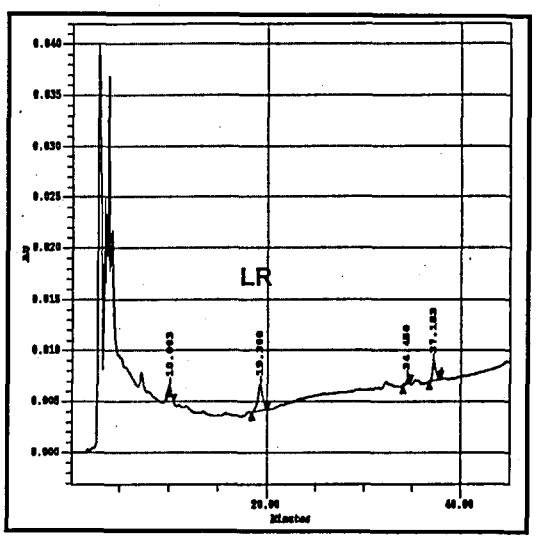


Fig. 5.5 Microcystin-LR recovery using the C18 (t) cartridge without Na_2SO_3 & TFA

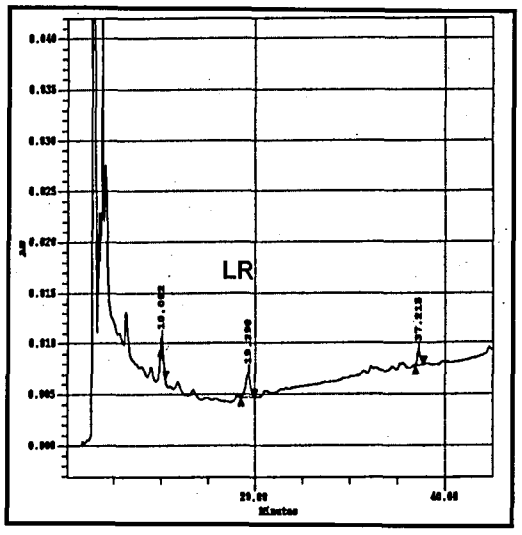


Fig. 5.6 Microcystin-LR recovery using the C18(t) cartridge without Na_2SO_3 & TFA

The non-recoveries did not appear to be a result of the acidification *per se*, since on occasion good recoveries were obtained, (Table 5.1). The toxins were also found to be stable in acidic solutions of TFA.

Recoveries of microcystin-LR from non-acidified tap water without the removal of residual chlorine ranged from 57% to 102% with a standard deviation of 14,5 (n=18). (See Fig. 5.7)

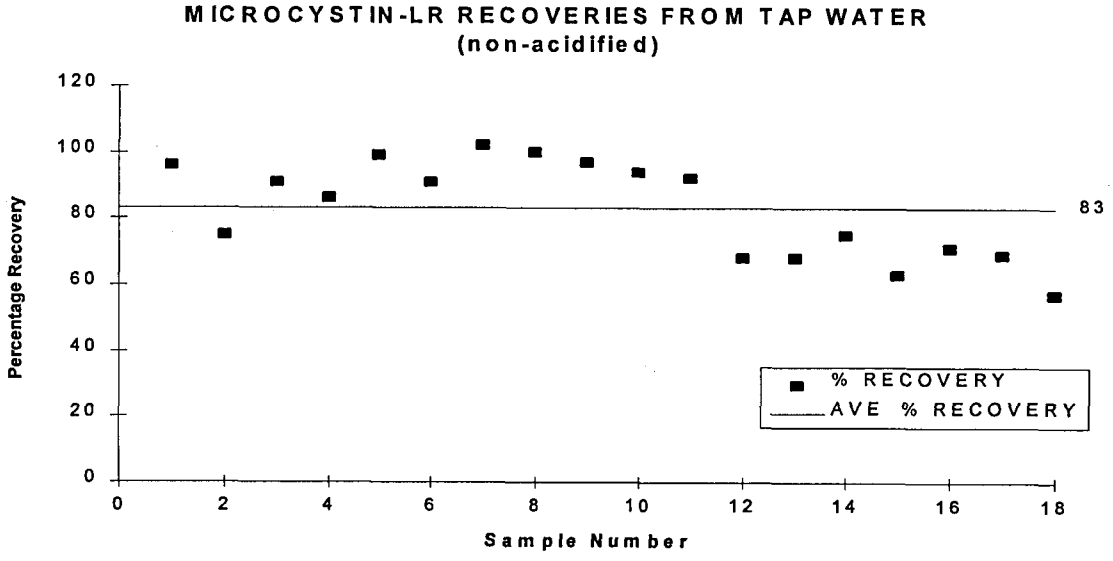


Fig. 5.7 Microcystin-LR recoveries from laboratory tap water

It was initially thought unnecessary to remove the residual chlorine from the water (using sodium sulphite) since the microcystin-LR was recovered from each of the test samples. However, when mixed toxin standards were recovered from tap water without the prior removal of the residual chlorine,(i.e., when neither sodium sulphite nor TFA was added), it was observed that the nodularin recoveries were consistently low. The addition of sodium sulphite (without the TFA) improved the average recovery as shown in Table 5.3 . The addition of sodium thiosulphate improved the recoveries even further and was adopted into the procedure instead of the sodium

sulphite. It was clearly necessary to remove chlorine prior to the addition of toxin standards to avoid poor nodularin recovery.

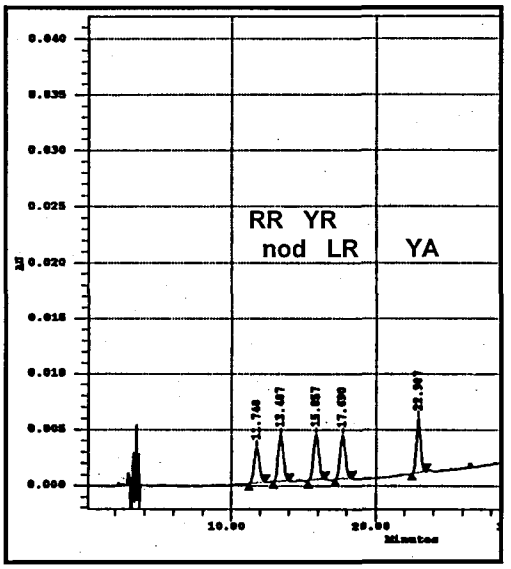


Fig. 5.8 Mixed toxin standard added to the test samples (RR < Nod < YR < LR < YA)

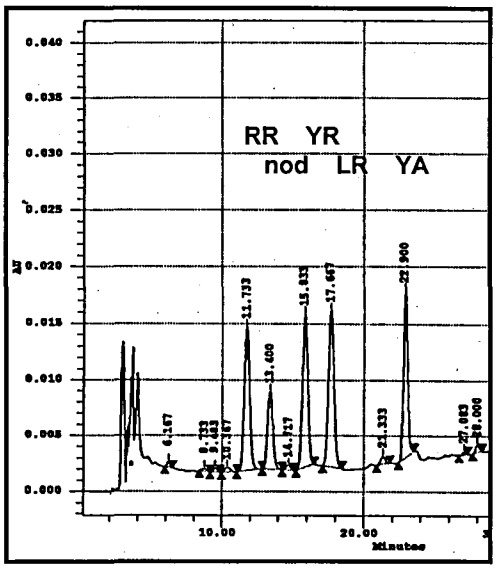


Fig. 5.9 Mixed toxins recovered using the C18(t) cartridge without removal of residual chlorine

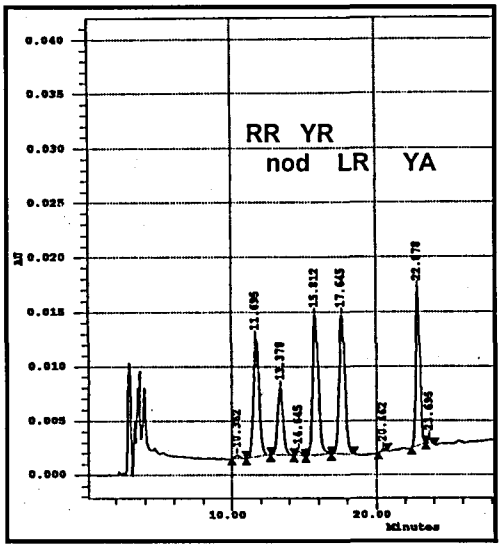


Fig. 5.10 Mixed toxins recovered using the C18 (t) cartridge without removal of residual chlorine

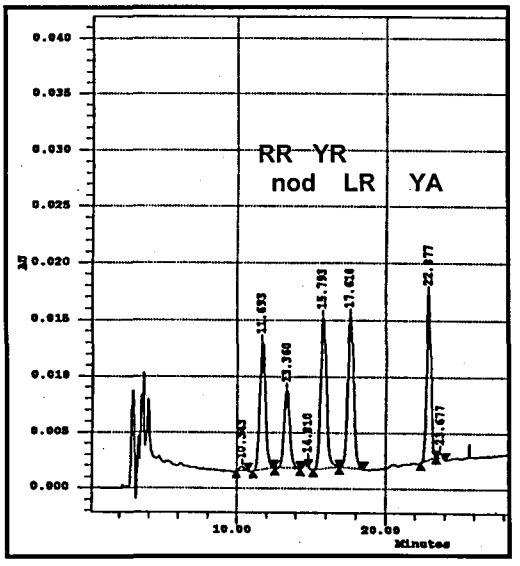


Fig. 5.11 Mixed toxins recovered using the C18 (t) cartridge without removal of residual chlorine

Table 5.3 Mixed Toxin Recoveries from treated water (standard deviations in parentheses)

Water & removal of Cl_2	Sep-Pak	Evap.	n	*MICYST RR*		*NODULARIN*		*MICYST YR*		*MICYST LR*	
				% Range	Ave %	% Range	Ave %	% Range	Ave %	% Range	Ave %
Tap *	C18	Air	6	85-94	89 (3.0)	22-33	29 (4.7)	54-64	58 (3.6)	63-75	69 (4.3)
Tap *	C18	N_2	6	65-103	86 (13.5)	28-48	38 (6.5)	59-82	72 (8.2)	70-88	78 (6.1)
Tap *	C18 (t)	Air	6	76-96	85 (5.7)	24-46	35 (11.8)	50-89	79 (13.5)	61-89	82 (9.7)
Tap Na_2SO_3	C18	N_2	6	80-102	86 (13.5)	27-48	38 (6.5)	59-82	73 (8.2)	70-88	78 (6.1)
Tap Na_2SO_3	C18	N_2	7	91-105	98 (4.6)	33-81	72 (6.0)	60-77	79 (11.8)	73-90	83 (6.3)
Tap $Na_2S_2O_3$	C18 (t)	N_2	4	92-112	108 (6.8)	79-87	78 (6.8)	73-96	84 (9.4)	71-94	83 (10)

*water samples not dosed with sodium sulphite or sodium thiosulphate

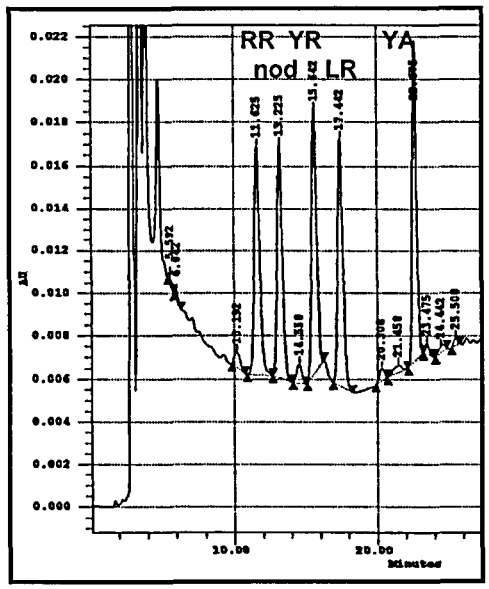


Fig. 5.12 Mixed toxins recovered using the C18 (t) cartridge with removal of residual chlorine (addition of $Na_2S_2O_3$)

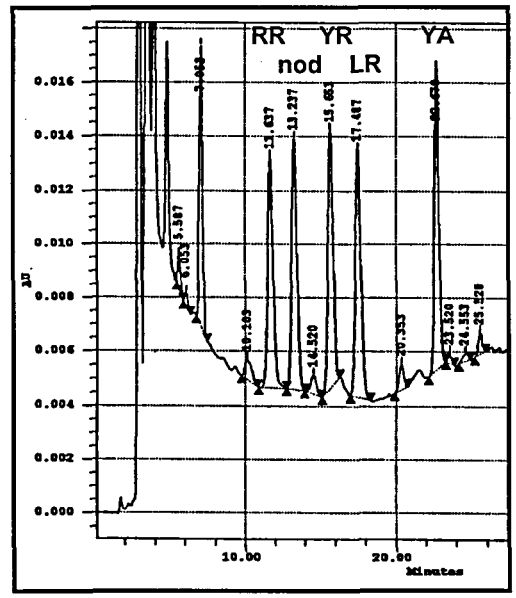


Fig. 5.13 Mixed toxins recovered using the C18 (t) cartridge with removal of residual chlorine (addition of $Na_2S_2O_3$)

The use of air instead of nitrogen for the evaporation of the eluate was investigated because of the financial implications of using excessive amounts of nitrogen. Student's t Tests (at 95% confidence) showed that the difference in the recoveries were insignificant for all the toxins tested except microcystin-RR.

C_{18} cartridges are also less expensive than the C_{18} trifunctional cartridges and since the samples were no longer being acidified, the normal C_{18} cartridges could also be used or the recovery of the toxins. However, Student's t-tests (at 95% confidence) indicate a difference in the recoveries of nodularin, microcystin-YR and microcystin-LR when the two cartridges were compared, whereas there was no significant difference for the recovery of microcystin-RR.

5.4 Discussion

Possible causes for the non-recoveries of the toxins when tap (treated) water was acidified with TFA are that either the toxins were destroyed in the process or that they were not being retained on the cartridges. The latter is more likely, considering that in some instances the toxins were recovered in relatively high percentage. A published article also reported that chlorine at pH<8 causes destruction of the microcystin toxins and nodularin provided a free chlorine residual of 0.5 mg.ℓ^{1} was present.[26] It was also indicated that nodularin was more susceptible to decomposition by chlorine than the microcystins,.

The inconsistent performance in the recoveries as a result of the addition of TFA when different cartridges (Isolutes) were used, supports the omission of the acidification step from the analysis procedure. Upon communication with Prof. Codd (co-author of the published paper), it was established that the samples were acidified with trifluoroacetic acid to eliminate the excess sodium sulphite. It was decided that the acidification of the samples with TFA should be omitted from the procedure altogether since it was shown to have an adverse effect on the toxin recovery.

Although the Student's t-tests indicate that there is a significant difference between the recoveries of nodularin, microcystin-YR and microcystin-LR, the range of percentage recoveries overlapped, implying that the variability in the percentage recoveries are to an extent dependent on the performance of the particular SPE cartridge. The extraction conditions such as flow rate through the cartridge, the type and quantity of solvent used for conditioning and washing of the extraction cartridge are important if the method is to be applied routinely and successfully.

5.5 Conclusion

Based on the results obtained, the acidification of treated water samples with trifluoroacetic acid is not recommended. The residual chlorine should be removed from the sample by the addition of sodium sulphite or, preferably, sodium thiosulphate since the presence of chlorine reduces the recovery of the spiked toxins, particularly nodularin. Sodium thiosulphate (50 $\mu\ell$; 1g/100mℓ) is preferred for the removal of residual chlorine since the average recovery of nodularin was slightly better than when sodium sulphite was used.

Although the normal C_{18} cartridges produce similar percentage toxin recoveries, the average percentage was slightly better when the C_{18} trifunctionals were used. The use of air to evaporate the toxin samples eluted from the solid-phase extraction cartridges did not appear to oxidise the toxins and can be used as an alternative to high purity nitrogen gas.

CHAPTER SIX
Recoveries from Dam water

6.1 Introduction

Toxin recovery tests from raw waters were expected to give an indication of the recoveries that would be obtained from samples to be submitted for toxin analyses.
The non-acidified recovery procedure (without the addition of trifluoroacetic acid) was applied for raw water samples. Spiked and unspiked dam water samples were extracted and analysed.

6.2 Procedure

Raw sample water (4ℓ) was filtered through glass fibre filters (GF/C, Whatman). If any algal material was retained, it was kept for intracellular toxin determinations. The filtrate was divided into 500 mℓ portions and methanol (5 mℓ) was added to sample filtrates with high turbidities. Half the number of samples were spiked with toxin standard and the other half analysed as samples. The C_{18}(t) cartridges were conditioned using the modified procedure and the samples extracted at a flow rate of 4 - 8 mℓ.min^{-1}. After washing the cartridges with 10%, 20% and 30% (v/v) aqueous methanol (10 mℓ, 10 mℓ and 5 mℓ respectively), the samples were eluted with 4.0 mℓ of 0.1% TFA in methanol, dried and suspended in 0.1 mℓ of methanol. An aliquot (25μℓ) was injected onto the HPLC column.
Acidification was included in some of the tests using dam waters to check any difference in the percentage recoveries.

6.3 Results

Microcystin-LR was recovered from both acidified and non-acidified raw water, but lower recoveries were obtained from the acidified samples as shown in Figs. 6.1 to 6.4.

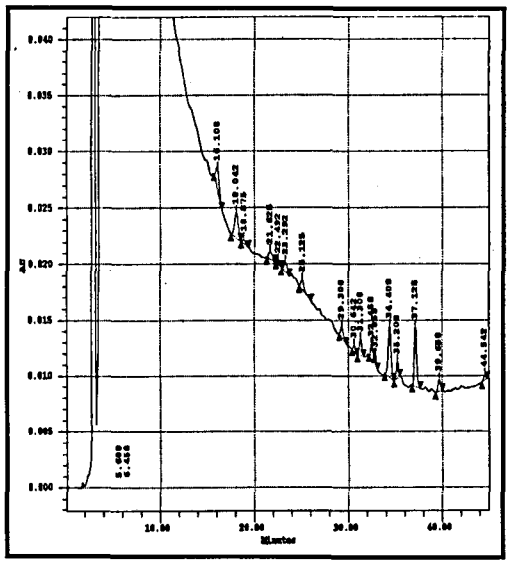


Fig. 6.1 Blank recovery from Albert Falls dam using Na_2SO_3 and TFA

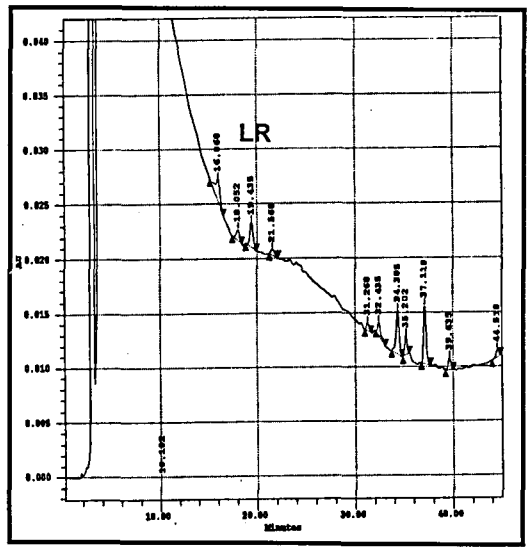


Fig. 6.2 Microcystin-LR recovery from Albert Falls dam using Na_2SO_3 and TFA

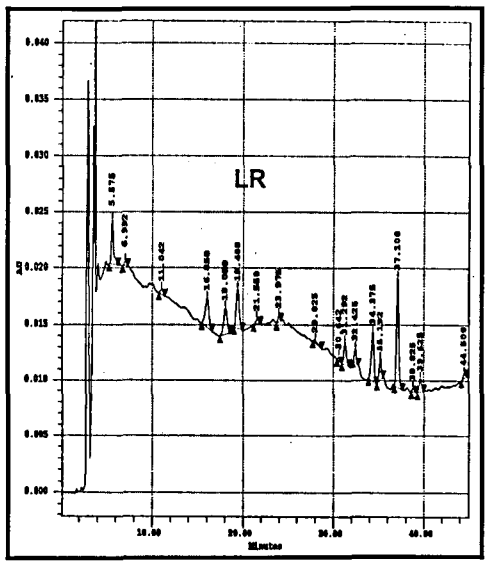


Fig. 6.3 Microcystin-LR recovery from Albert Falls dam without the addition of Na_2SO_3 and TFA (microcystin-LR at 19.4 min.)

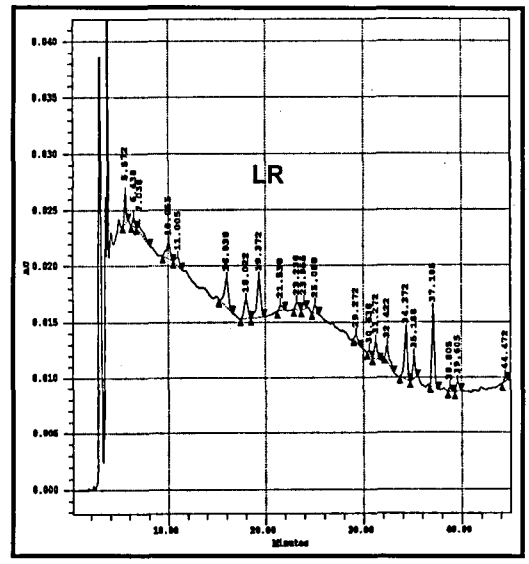


Fig. 6.4 Microcystin-LR recovery from Albert Falls dam without the addition of Na_2SO_3 and TFA

The percentage toxin recovery from dam waters tends to vary depending on the physical and/or chemical conditions of the particular samples. The more turbid/brown waters tend to produce lower recoveries due to clogging of the extraction cartridges with a resultant decrease in the extraction efficiency. An aliquot of methanol (5 mℓ) was added to these solutions.

The absorbance scale of the HPLC chromatograms had to be adjusted to accommodate the peak sizes and higher background absorbances as shown in Fig. 6.5:

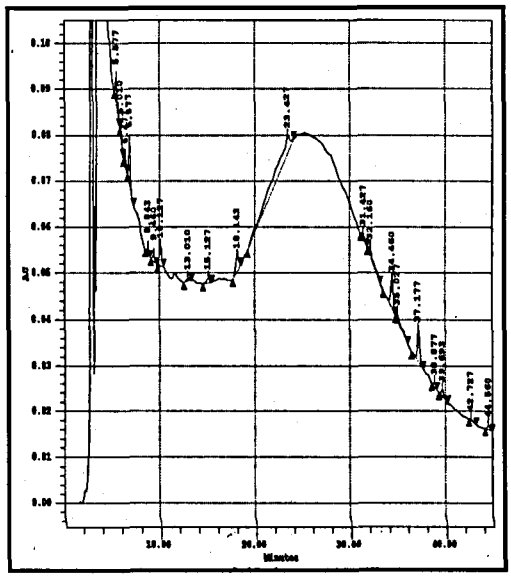


Fig. 6.5 Microcystin-LR "recovery" from turbid Shongweni dam water (the absorbance scale was adjusted to 0,1)

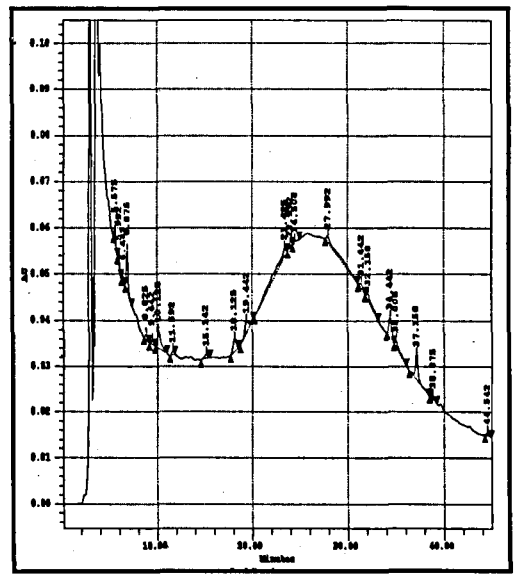


Fig. 6.6 Microcystin-LR (19 min.)recovery from turbid Shongweni dam water

It was also noted that the chromatograms of recovered dam waters of the turbid solutions were much more complex than that of the clearer samples.

In Fig. 6.7 none of the peaks had the characteristic UV spectrum of the microcystin toxins, but, because of the numerous peaks, co-elution would be inevitable if any toxins were present or if any of the toxin standards were spiked into the water and recovered.

Again the absorbance scale has been adjusted to accommodate the increased peak heights.

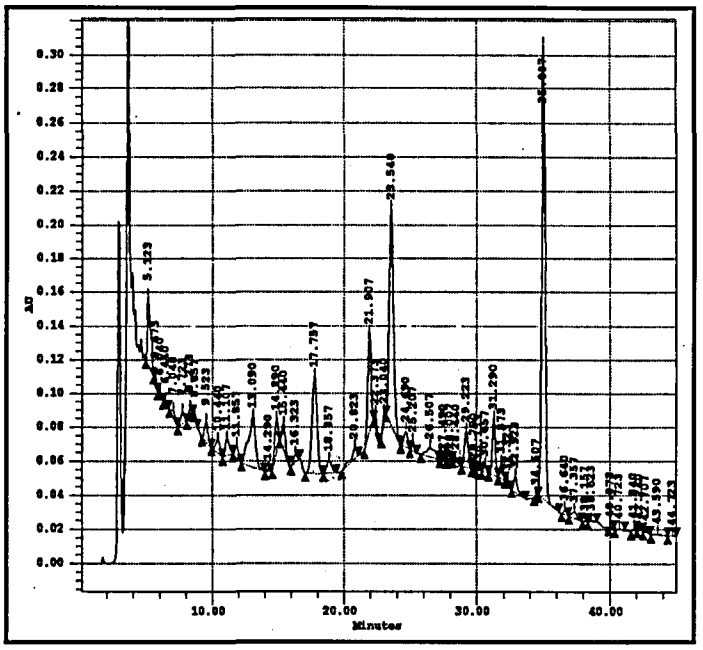


Fig. 6.7 An unspiked sample recovery of Shongweni raw water surrounding a non-toxic cyanobacterial mass

The toxins were also not recovered to the same extent from the dam water samples (see Figs. 6.8 to 6.11), supporting the earlier statement that the "internal standard method" of adjusting the actual recoveries based only on a spike of microcystin-LR only is not reliable. See Table 6.1

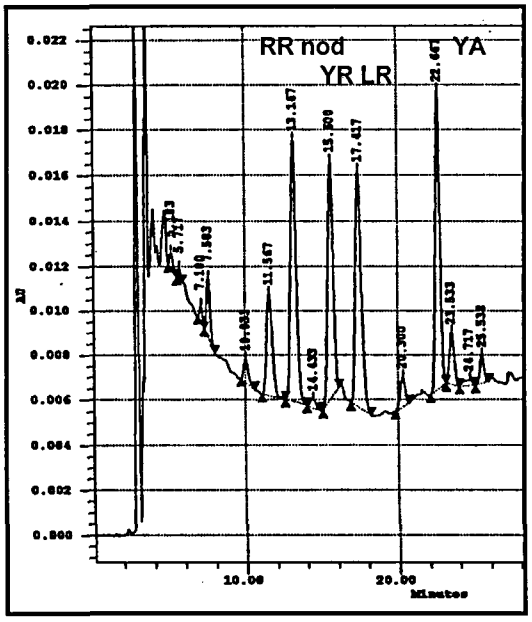


Fig. 6.8 Mixed toxin recovery from clear spiked Midmar dam water

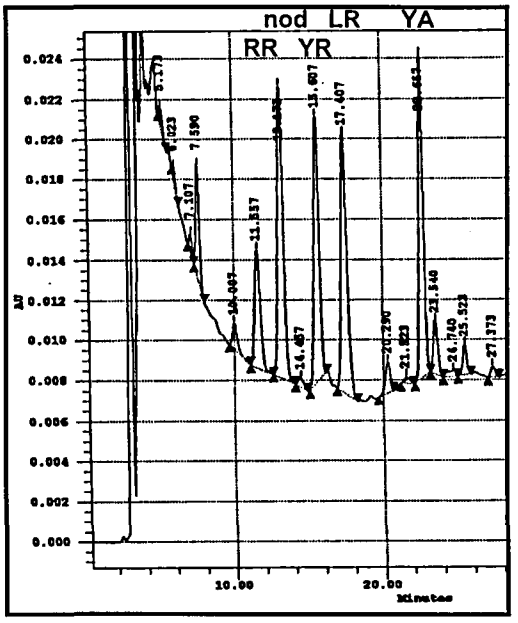


Fig. 6.9 Mixed toxin recovery from clear spiked Midmar dam water

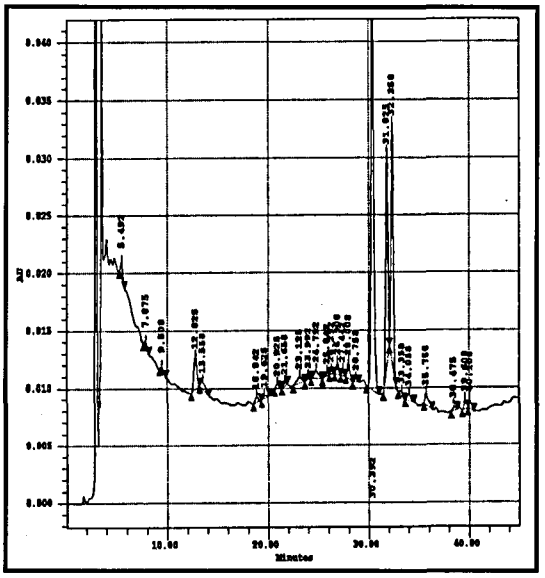


Fig. 6.10 Unspiked Inanda dam water

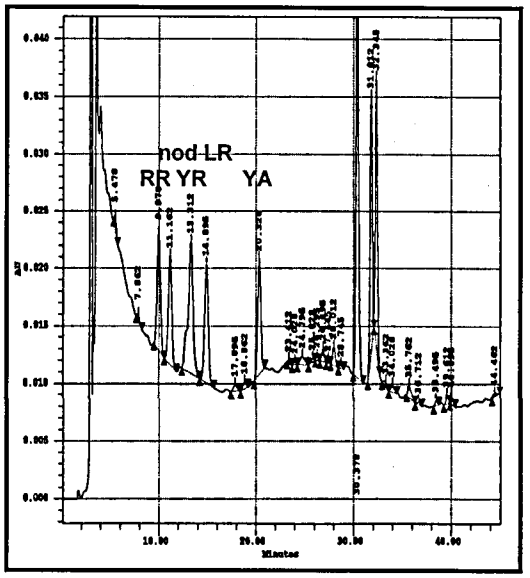


Fig. 6.11 Mixed toxin recovery from clear spiked Inanda dam water

Table 6.1 Mixed Toxin Recoveries from relatively clear dam waters (n=1)

Dam water	Spiked Concentrations $\mu g.\ell^{-1}$				Percentage Recovery			
	RR	*YR*	*LR*	*NOD*	*RR*	*YR*	*LR*	*NOD*
Midmar	1.2	1.2	1.2	1.2	55	97	87	95
Inanda	1.4	1.4	1.4	1.4	87	72	88	88

In the Inanda sample analysed above, a non-microcystin (≈12 min.) peak co-eluted with microcystin-YR. The averaged peak area from the unspiked chromatogram was subtracted from that of the spiked peak area to obtain the percentage recovery of the spike.

In some instances, chromatograms of dam water samples contained relatively large peaks that had the same retention times as those of certain toxins. The UV spectra indicated that they were not, however, toxins. It is not possible to establish whether or not there may be some toxin that had co-eluted with the unknown compound, since the UV spectrum was dominated by that of the unknown.

For example, a Nungwane dam water sample had *Microcystis* counts of approximately 11000 cells.$m\ell^{-1}$. A peak in the chromatogram Fig 6.12 contained a peak that co-eluted with nodularin.

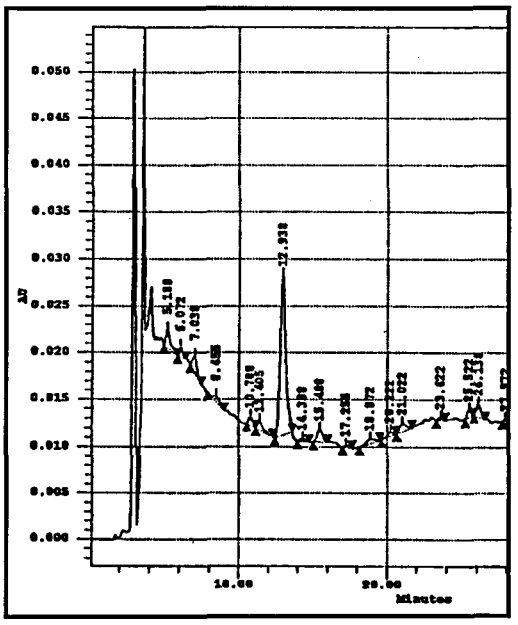


Fig. 6.12 Sample recovery of a relatively clear Nungwane dam water sample

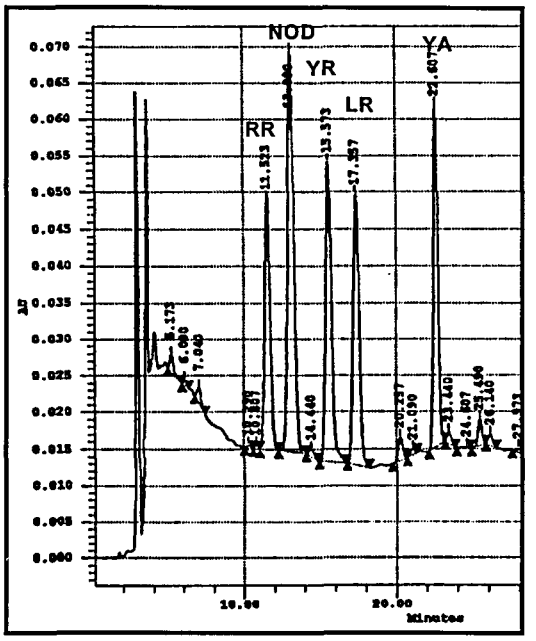


Fig. 6.13 Spiked recovery from Nungwane dam water

The UV spectrum of the co-eluting compound does not have the characteristic UV spectrum of nodularin as shown in Fig. 6.14, and therefore not considered to be a toxin.

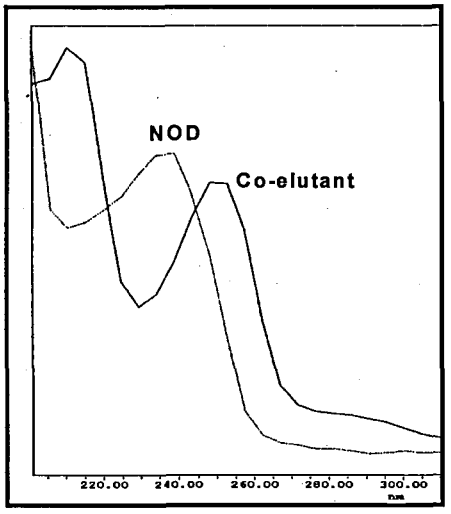


Fig. 6.14 The UV spectrum of the peak that co-eluted with nodularin compared with the nodularin standard

However, an enlargement of the chromatogram peak at approximately 13 minutes in Fig. 6.12 shows a peak shoulder, implying that the peak may be a composite and that there remains the possibility that some nodularin may be present in the sample (see Fig. 6.15). The UV spectrum of the peak shoulder shows that it is different to that of the main peak, See Fig. 6.16.

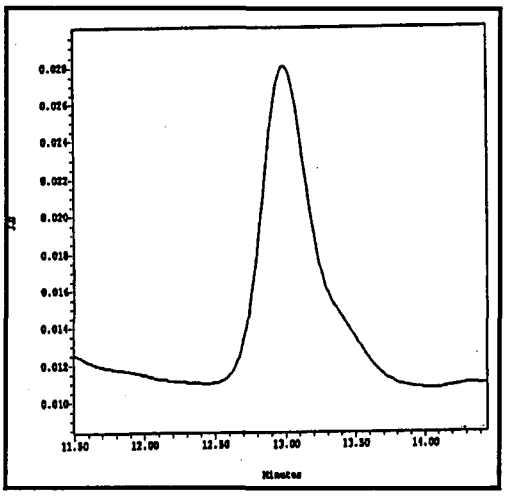


Fig.6.15 Enlargement of the chromatographic peak at approximately 13 min. (in Fig. 6.12)

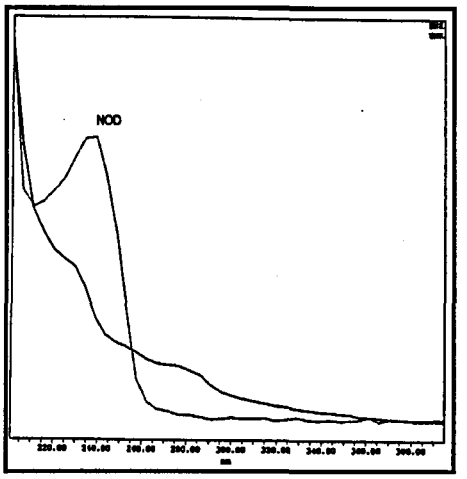


Fig. 6.16 UV spectrum of the peak shoulder in Fig. 6.15

The evidence presented cannot conclusively prove or disprove the absence of nodularin in the sample. Since the UV spectrum of the peak in question does not have the true characteristics of those described for microcystin toxins, it may be that of a non-toxin or a modified toxin. On the other hand, it is possible that a low concentration of nodularin is present in the sample but the typical microcystin UV spectrum is masked by that of the co-elutant even when a partial separation may have occurred.

Some other samples that have been analysed have also posed similar problems, in that the UV spectrum cannot be relied upon to conclusively indicate the presence of a microcystin toxin especially at low toxin concentrations. Sometimes there are similarities in the shape and region of the absorptions observed in the UV, but the local absorption wavelength is shifted from that of the specified 238 nm as illustrated in another Nungwane dam sample in Fig 6.17 and 6.18.

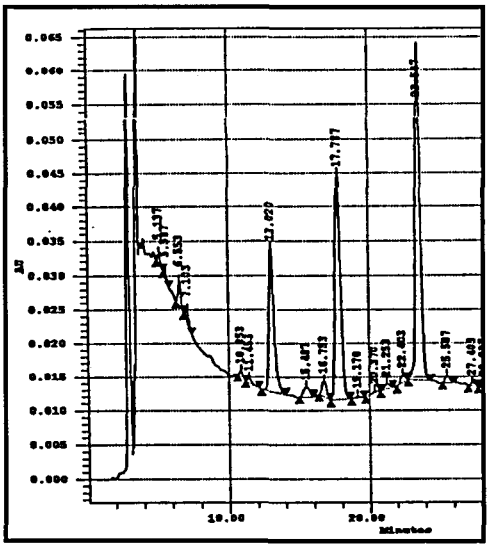


Fig. 6.17 Blank recovery of Nungwane dam water sample

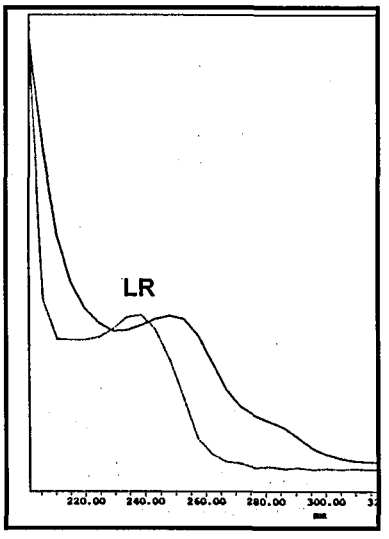


Fig. 6.18 UV spectrum of the peak at 17 min. in 6.17 , which co-elutes with microcystin-LR

A shift in the UV maximum does occur as indicated in Figs 6.19 and 6.20 where microcystin-LR was found in an Inanda sample. The peak in question had the same retention time as microcystin-LR and its UV spectrum was characteristic of the microcystins, but its UV maximum was not exactly 238 nm. A diluted microcystin-LR solution (≈ 0.3 μg.mℓ$^{-1}$) also does not have its local absorption maximum at 238 nm and may have been considered a non-toxin if it was found in a sample.

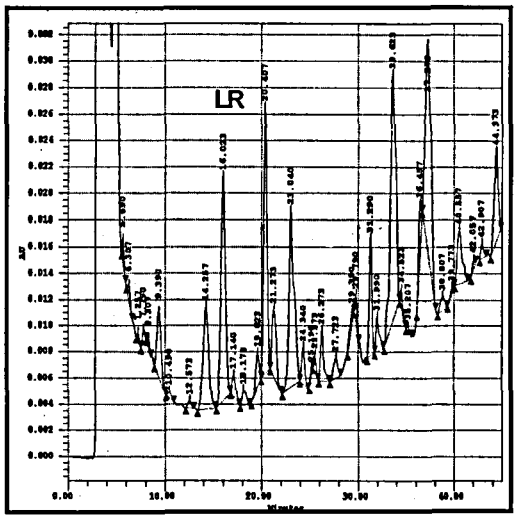


Fig. 6.19 A chromatogram of a *Microcystis* extract containing microcystin-LR

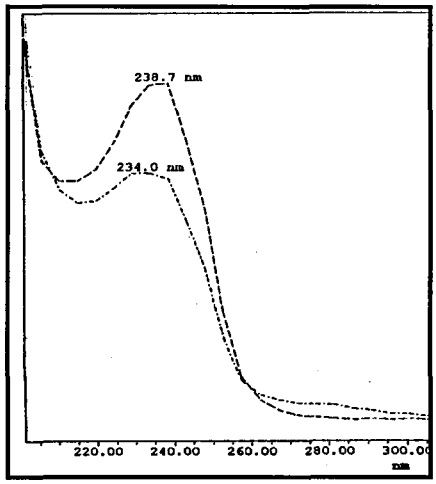


Fig. 6.20 A UV spectrum of microcystin-LR found in an Inanda scum sample compared with that of the microcystin-LR standard

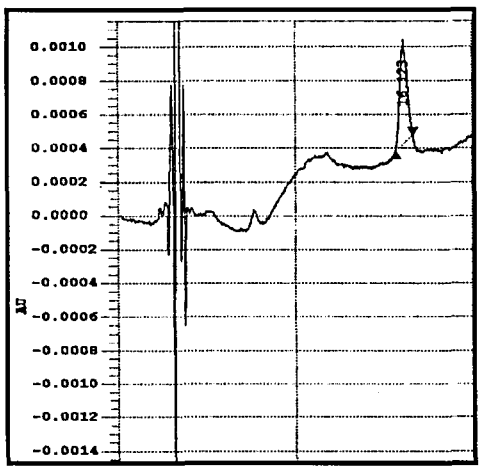


Fig. 6.21 A dilute microcystin-LR standard (0.3 μg.mℓ$^{-1}$)

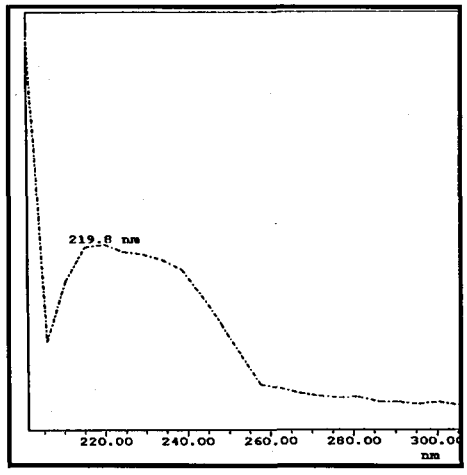


Fig. 6.22 The UV spectrum of the diluted microcystin-LR standard

6.4 Discussion

HPLC separations can be improved by altering the chromatographic parameters such as mobile phase polarity or flow rates, etc., but the peak widths for some of the toxins range between 1 and 1.5 minutes, under the present HPLC conditions. It is unlikely that these adjustments would completely resolve the peaks if the same C_{18} analytical column is used.

The required adjustments would also depend on the sample itself, i.e., the region of the gradient elution profile in which the co-elution occurs.

The purity of non-toxin peaks is difficult to ascertain based on the UV spectra alone. However, recent Millennium PDA software (Waters, 1995) has been improved to the extent that peak purity can be determined, but it is still not possible to distinguish whether the impurity is a microcystin toxin or not.

Some of the microcystin toxin standards, especially at low concentrations, did not give a local maximum exactly at 238 nm, implying that at some point of lower toxin concentration, the characteristic UV spectrum cannot be relied upon for identification of microcystin toxins. See Fig. 6.22

6.5 Conclusion

Improved HPLC separation may be required for certain samples where the recovered toxins co-elute with non-toxin compounds. The identification of possible toxins in blank raw water recoveries using the UV spectra becomes increasingly difficult at low toxin concentrations especially where chromatograms are complex.

Clear raw waters produced recoveries comparable to those from ultra-pure water while relatively poorer recoveries were obtained from waters having high turbidities.

The detection limits possible with raw waters would depend on the sample water itself since the detection of the toxins would be associated with the percentage recoveries obtained and these vary from sample to sample.

The use of microcystin-LR to determine the percentage recoveries of the other microcystin toxins is unsound. The recoveries of various spiked toxins from raw waters varied even when recovered from the same sample. The percentage recoveries of the toxins are better determined individually by spiking with all the relevant toxins. The toxin spike should also be related to the amount of toxin present in the sample if maximum accuracy is to be achieved. The unavailability of a wide range of toxin standards to identify unknown toxins is a problem. The use of High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) may be advantageous in certain circumstances where the use of the characteristic UV spectra and the molecular ion and fragmentation patterns could be used to identify toxins that have previously been isolated. The compilation of mass spectral libraries would be advantageous where toxin standards are not available. The establishment of an international network is another possibility of obtaining mass spectral data on certain toxins that are rare and it would also provide a means of gleaning current knowledge on the subject from prominent researchers.

CHAPTER SEVEN
Intracellular Toxin Determination

7.1 Introduction

Intracellular toxin concentrations can be analysed in two ways: either from the algal cells that are retained on the filter paper after filtration of the aqueous sample, or from freeze-dried algae.

7.2 Procedure

(a) The filter papers containing the retained algal cells were soaked in a suitable aliquot of methanol for 1 - 2 hours. The solution was filtered under gravity and the filtrate evaporated to dryness. Depending on the amount of algal material, an aliquot of methanol (usually 0.1 mℓ) was added to the residue and 25μℓ was injected onto the column.

(b) Freeze-dried algae (≈1g) was soaked overnight in methanol (50 mℓ) and centrifuged (10 000 rpm). The pellet was re-extracted in methanol for another hour and the combined supernatants were evaporated under a nitrogen flow (or air). The residue was suspended in methanol (5 mℓ) and filtered through Millex 0.45 μm filter. 25 μℓ of the filtrate was injected onto the column.
The extract was treated follows :

unspiked toxin sample : 0.1 mℓ of extract + 0.1 mℓ of methanol

spiked toxin sample : 0.1 mℓ of extract + 0.1 mℓ of mixed toxin standard

Concentrations of the identified toxins (based on the retention times and UV spectra) were calculated from the peak areas and the calibration equations.

7.3 Results

Both procedures have been applied successfully (as shown in Chapter 8). Some microcystin toxins were identified by their UV spectrum and the increased peak height of the spiked extract relative to the unspiked extract. Several possible microcystin toxins could not be identified.
One freeze-dried *Microcystis* sample (supplied by the Cape Town City Council) analysed was alleged to have caused stock deaths in the Paarl area. The sample contained microcystin-LR and another microcystin toxin that appeared to have the characteristic UV spectrum of a microcystin toxin containing tryptophan. See Figs. 7.1 and 7.2.

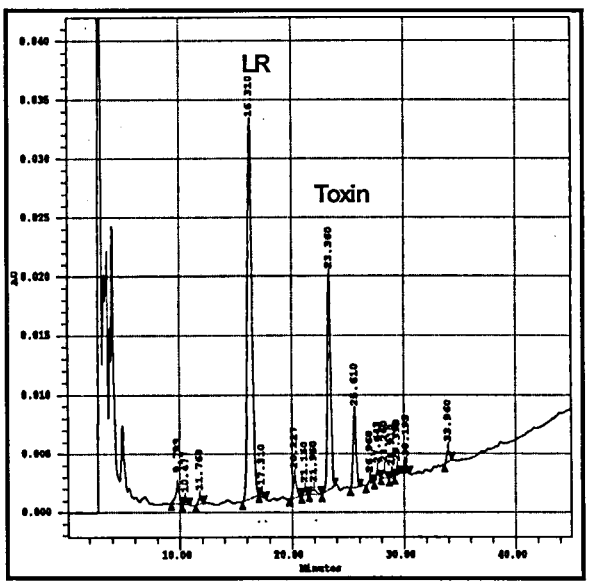


Fig. 7.1 A methanol extract of a freeze-dried *Microcystis* sample from Cape Town containing Microcystin-LR and a microcystin toxin

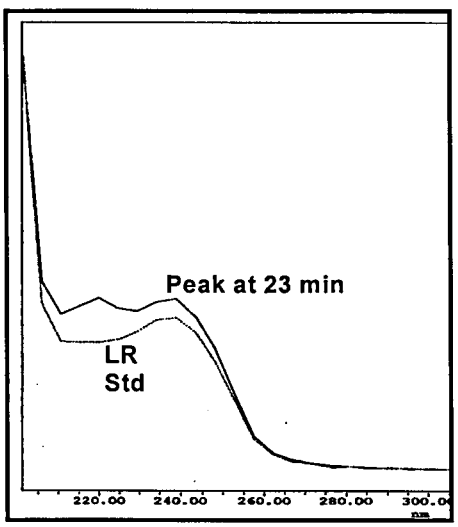


Fig. 7.2 The UV spectrum of the microcystin toxin at 23 min. that has the typical tryptophan absorptions

An investigation was carried out with the algal extract to determine whether all the contents from the algal cells would be extracted onto the Sep-Pak C_{18} trifunctional cartridge. An aliquot of the algal extract (Cape extract) was then added to 500 mℓ of water and analysed using the procedure for aqueous samples.

Notwithstanding the difference in concentration of the two extracts injected onto the column, the results showed that all the organic compounds contained within the algal cells were retained on the cartridge. See Figs. 7.1 and 7.2.
However, an interesting observation was that the UV spectrum of the toxin peak at about 23 minutes in the methanol extract in Fig. 7.1 which was typical of a "microcystin containing tryptophan" had, in the chromatogram of the spiked water extract, lost the tryptophan absorptions at approximately 222 nm and resembled the UV spectrum that is characteristic of the rest of the microcystin toxins. See Figs 7.3 and 7.4

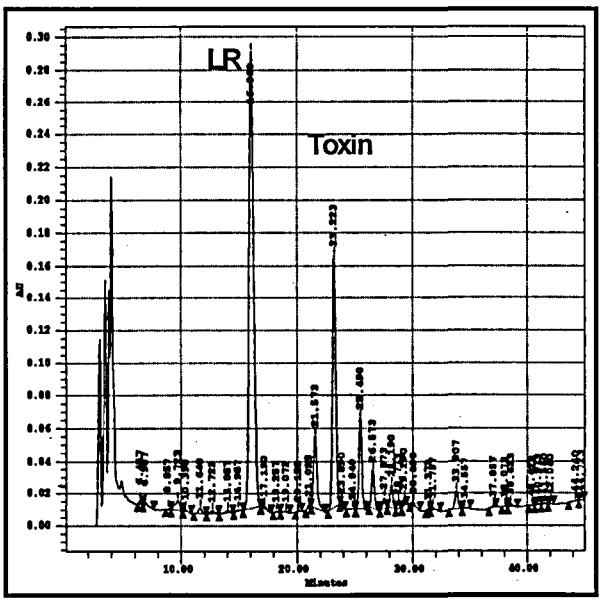


Fig. 7.3 The *Microcystis* extract recovered from water

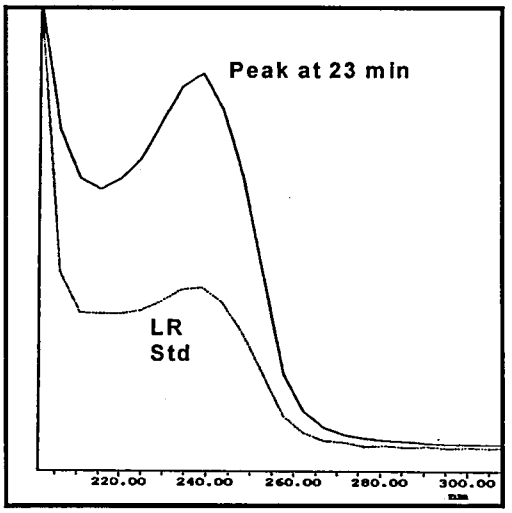


Fig. 7.4 The UV spectrum of the microcystin toxin at 23 min. which has lost the typical tryptophan absorptions

7.4 Discussion

The reason for the loss of the tryptophan-type absorption in the UV spectrum is not known. It is not likely that the UV spectrum of a particular compound will change except when dissolved in another solvent or some structural change had occurred in the molecule itself. At the time when the analysis was performed, it was not possible to establish the purity of the peak in question using the facilities of the PDA software. It is possible that the toxin peak may have been a composite, with one of the co-elutants being removed during the C_{18} solid-phase extraction process. However, no evidence to suggest this (such as, for example, abnormal peak shape or the presence of peak shoulders) could be found.
The implications this might have on the analysis are that certain toxins may be incorrectly thought to contain tryptophan. In the literature method, the UV spectrum given for microcystin-LW and WR were given for the methanol extract and the toxin standard, but not of the recovered toxins spiked into tap water. It therefore has to be assumed that the characteristic UV spectrum did not alter. Unfortunately we did not have standards available to carry out similar experiments.

7.5 Conclusion

The intracellular toxin content is better analysed from the freeze-dried algal material than from the algal cells retained on the filter paper (after filtration of the sample). The toxin concentration would be given on a dry algal mass basis rather than on the volume filtered. Also, the amount of retained cells from a filtered sample may be small and the chromatographic peaks thus not large enough to obtain clear UV spectra. Caution should be advised when labelling certain toxins as having a tryptophan residue, based solely on the UV spectrum.

CHAPTER EIGHT
Algal Toxin Monitoring

8.1 Introduction

Phase two of the proposed project included the monitoring of a critical water supplies using the HPLC method developed. The following dams in the Umgeni Water Operational area were monitored on a routine basis :

DAM	*RIVER SYSTEM*	*TREATMENT WORKS*
Midmar	Umgeni	D V Harris
"	"	Mill Falls
"	"	Howick
"	"	H D Hill
Albert Falls	Umgeni	none
Nagle	Umgeni	Durban Heights
Inanda	Umgeni	Wiggins
Henley	Umsinduzi	H D Hill
Hazelmere	Mdloti	Hazelmere
Shongweni	Umlaas	Shongweni (domestic)
Nungwane	Nungwane	Amanzimtoti

A diagram of the Umgeni water catchment is attached in the Appendix E.

The sample sites within the impoundments were chosen so that the samples would be representative of the water entering the respective treatment works. Surface samples were taken because the cyanobacteria are buoyant and were most likely to be present near the water surface. If any toxins were found in the raw water, then samples from the various treatment works would also be monitored.

8.2 Procedure

Two 2ℓ samples were taken from surface water near the abstraction site of the dam. A 2ℓ surface scum sample was collected whenever possible.
The water samples were filtered and analysed using the analytical method used for raw waters.
A preliminary qualitative analysis was made on each sample as a screening procedure to reduce the number of samples which required a full set of recovery analyses.
If toxins were observed in the preliminary analysis four sets of samples were analysed, two spiked with toxins and the others unspiked. A summary can be found in Appendix G.

Algal counts were determined by filtering an aliquot of the sample through cellulose nitrate filters (0.45 μm) under vacuum. After the filters were dried and spotted with immersion oil, several randomly selected microscope fields were viewed and the algal cells identified to genus level and counted. The number of fields counted (usually between 15 and 35) were determined statistically depending on the amount of algae counted per field and the amount of sample filtered.

$$\text{Algal number} = \frac{\text{CF} \times \text{no. of individual cells counted}}{\text{No. of fields} \times \text{volume filtered (m}\ell\text{)}}$$

$$\text{Conversion Factor (CF)} = \frac{\text{Area of filter}}{\text{Area of view under microscope}}$$

8.3 Results

Examples of typical chromatograms of the extracted water samples taken from some of the impoundments are shown in Figs. 8.1 to 8.7. The UV spectra of each peak in the chromatograms were reviewed to identify possible microcystin toxins or nodularin.

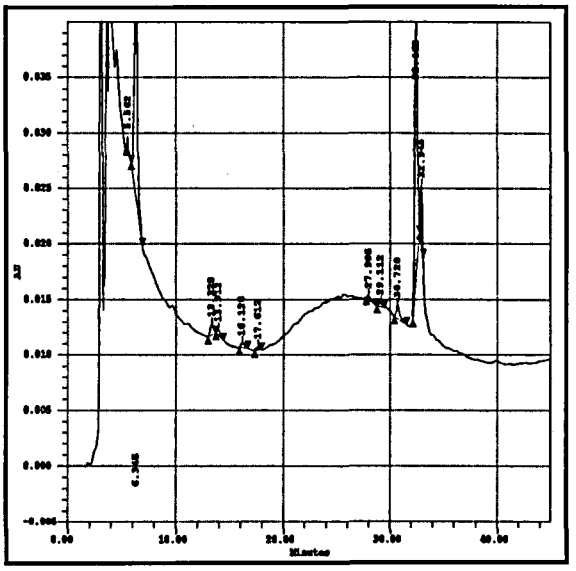


Fig. 8.1 Typical chromatogram of an Albert Falls dam sample

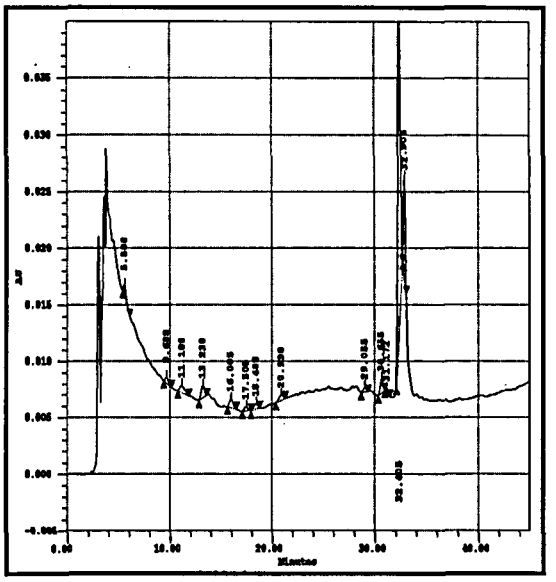


Fig. 8.2 Typical chromatogram of a Henley dam sample

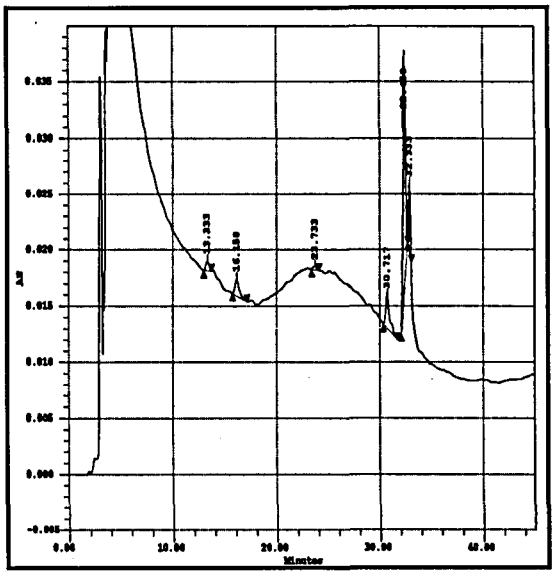


Fig. 8.3 Typical chromatogram of a Midmar dam sample

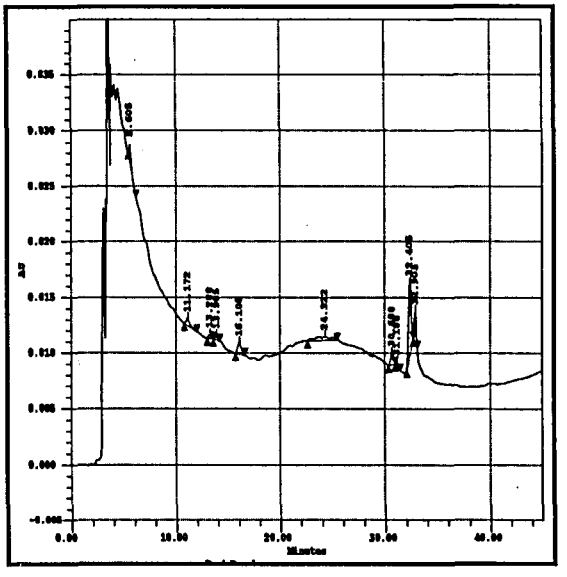


Fig. 8.4 Typical chromatogram of a Nagle dam sample

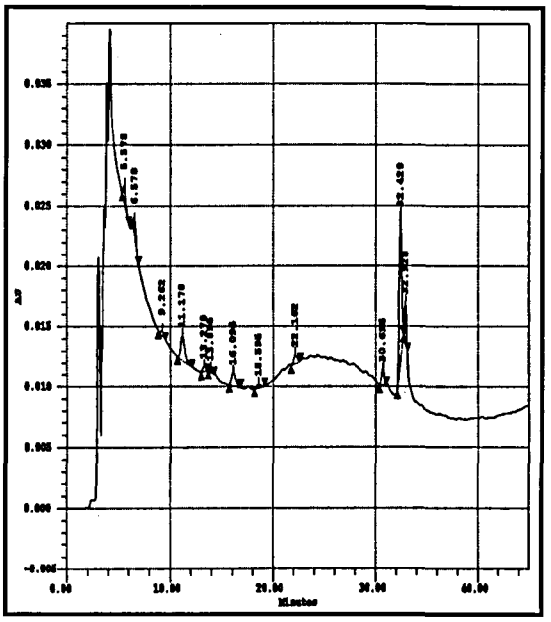


Fig. 8.5 Typical chromatogram of a Nungwane dam sample

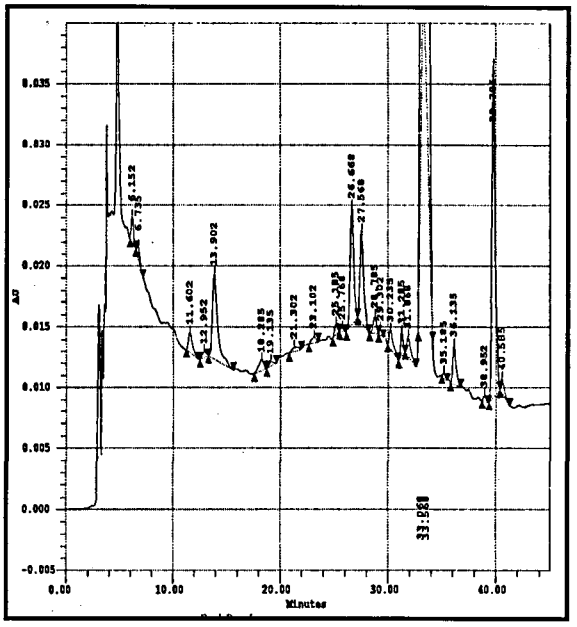


Fig. 8.6 Typical chromatogram of a Inanda dam sample

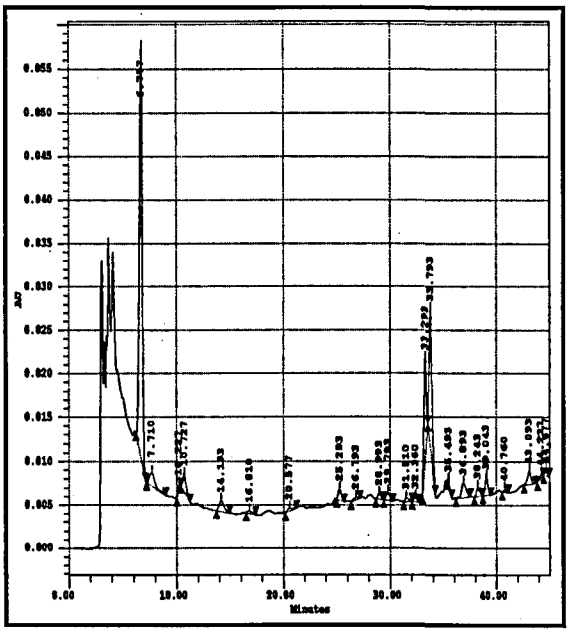


Fig. 8.7 Typical chromatogram of a Hazelmere dam sample

Results from the algal toxin monitoring are given in Table 8.1 together with the algal counts taken from the same sample points:

Table 8.1 Results of the Algal Toxin Monitoring

Date	Dam sampled	Sample Type	Blue-green Algae (genus)	Cell Count per mℓ	Toxins μg. ℓ^{-1}					
					RR	*NOD*	*YR*	*LR*	*YA*	*Other*
20/02/95	Shongweni	surface scum	-	-	-	-	-	-	-	-
22/02/95	Nagle	surface water	Microcystis Anabaena	1258 570	-	-	-	-	-	-
22/02/95	*Nagle	surface scum	Microcystis	ND	-	-	-	+	-	+
22/02/95	Nungwane	surface water	Microcystis	719	-	-	-	-	-	-
23/02/95	Hazelmere	surface water	-	-	-	-	-	-	-	-
02/03/95	Midmar	surface water	-	-	-	-	-	-	-	-
02/03/95	Inanda	surface water	Microcystis Anabaena	590 197	-	-	-	-	-	-
06/03/95	Shongweni	surface water	-	-	-	-	-	-	-	-
07/03/95	Hazelmere	surface water	Microcystis	177	-	-	-	-	-	-
07/03/95	Nungwane	surface water	Microcystis	7415	-	-	-	-	-	-
08/03/95	Nagle	surface water	Microcystis Anabaena	2811 98	-	-	-	-	-	-
13/03/95	Inanda	surface water	Microcystis Anabaena	721 8552	-	-	-	-	-	-
13/03/95	Shongweni	surface water	-	-	-	-	-	-	-	-
14/.3/95	Midmar	surface water	-	-	-	-	-	-	-	-
15/03/95	Albert Falls	surface water	Anabaena	688	-	-	-	-	-	-
20/03/95	Shongweni	surface water	Microcystis	98	-	-	-	-	-	-
22/03/95	Nagle	surface water	Microcystis Anabaena	1651 334	-	-	-	-	-	-
23/03/95	Midmar	surface water	Merismopedia	62	-	-	-	-	-	-
23/03/95	Nungwane	surface water	Microcystis	419	-	-	-	-	-	-
29/03/95	Albert Falls	surface water	Microcystis	39	-	-	-	-	-	-
30/03/95	Inanda	surface water	Microcystis Anabaena Merismopedia	2392 24444 131						
03/04/95	Shongweni	surface water	-	-	-	-	-	-	-	-
04/04/95	Nungwane	surface water	Microcystis Oscillatoria	4707 623	-	-	-	-	-	-
05/04/95	Nagle	surface water	Microcystis Anabaena Merismopedia	25 447 34	-	-	-	-	-	-

Date	Dam sampled	Sample Type	Blue-green Algae (genus)	Cell Count per mℓ	Toxins μg. ℓ^{-1}					
					RR	*NOD*	*YR*	*LR*	*YA*	*Other*
10/04/95	Shongweni	surface water	-	-	-	-	-	-	-	-
12/04/95	Albert Falls	surface water	-	-	-	-	-	-	-	-
13/04/05	Midmar	surface water	-	-	-	-	-	-	-	-
13/04/95	Inanda	surface water	Microcystis Anabaena Merismopedia	2673 34144 184	-	-	-	-	-	-
18/04/95	Nungwane	surface water	Microcystis	20	-	-	-	-	-	-
20/04/95	Shongweni	surface water	Microcystis Anabaena	4620 2556	-	-	-	-	-	-
24/04/95	Shongweni	surface water	Microcystis	1573	-	-	-	-	-	-
26/04/95	Albert Falls	surface water	Anabaena	511	-	-	-	-	-	-
26/04/95	Inanda	surface water	Microcystis Anabaena	147 20741	-	-	-	-	-	-
28/04/95	Midmar	surface water	-	-	-	-	-	-	-	-
13/07/95	Henley	Surface water	-	-	-	-	-	-	-	-
20/07/95	Midmar	Surface water	-	-	-	-	-	-	-	-
20/07/95	Henley	Surface water	-	-	-	-	-	-	-	-
26/07/95	Albert Falls	Surface water	Microcystis Anabaena	16 573	-	-	-	-	-	-
27/07/95	Inanda	surface water	Microcystis	934	-	-	-	-	-	-
31/07/95	Shongweni	surface water	-	-	-	-	-	-	-	-
04/08/95	Hazelmere	surface water	-	-	-	-	-	-	-	-
08/08/95	Nungwane	surface water	-	-	-	-	-	-	-	-
10/08/95	Nagle	surface water	-	-	-	-	-	-	-	-
14/08/95	Henley	surface water	-	-	-	-	-	-	-	-
15/08/95	Midmar	surface water	-	-	-	-	-	-	-	-
24/08/95	Inanda	surface water	Microcystis Anabaena	721 164	-	-	-	-	-	-
25/08/95	Albert Falls	surface water	Microcystis Anabaena	1298 278	-	-	-	-	-	-
29/08/95	Nungwane	surface water	Microcystis	87	-	-	-	-	-	-
30/08/95	Nagle	surface water	Microcystis	413	-	-	-	-	-	-
08/09/95	Hazelmere	surface water	Microcystis	135	-	-	-	-	-	-

Date	Dam sampled	Sample Type	Blue-green Algae (genus)	Cell Count per mℓ	Toxins μg. ℓ^{-1}					
					RR	*NOD*	*YR*	*LR*	*YA*	*Other*
14/09/95	Henley	surface water	-	-	-	-	-	-	-	-
20/09/95	Albert Falls	surface water	Microcystis	1258	-	-	-	-	-	-
21/09/95	Inanda	surface water	Microcystis	388	-	-	-	-	-	-
26/09/95	Nungwane	surface water	Microcystis	3834	-	-	-	-	-	-
27/09/95	Nagle	surface water	Microcystis	1671	-	-	-	-	-	-
02/10/95	Shongweni	surface water	Microcystis Anabaena	52492 1671	-	-	-	-	-	-
06/10/95	Hazelmere	surface water	-	-	-	-	-	-	-	-
12/10/95	Midmar	surface water	Microcystis	39	-	-	-	-	-	-
12/08/95	Henley	surface water	-	-	-	-	-	-	-	-
18/10/95	Albert Falls	surface water	Microcystis	1264	-	-	-	-	-	-
19/10/95	Inanda	surface water	Microcystis Anabaena	295 295	-	-	-	-	-	-
24/10/95	Nungwane	surface water	Microcystis	197	-	-	-	-	-	-
25/10/95	Nagle	surface water	Microcystis	246	-	-	-	-	-	-
03/11/95	Hazelmere	surface water	-	-	-	-	-	-	-	-
09/11/95	Midmar	surface water	Microcystis	2595	-	-	-	-	-	-
09/11/95	Henley	surface water	-	-	-	-	-	-	-	-
15/11/95	Albert Falls	surface water	Microcystis Anabaena	42466 1769-	-	-	-	-	-	-
16/11/95	Inanda	surface water	Microcystis	79	-	-	-	-	-	-
21/11/95	Nungwane	surface water	Microcystis Oscillatoria	18 194	-	-	-	-	-	-
22/11/95	Nagle	surface water	Microcystis	570	-	-	-	-	-	-
22/11/95	*Nagle	surface scum	Microcystis	ND	-	-	-	+	-	+
01/12/95	Hazelmere	surface water	-	-	-	-	-	-	-	-
07/12/95	Midmar	surface water	Microcystis Merismopedia	1514 7	-	-	-		-	-
07/12/95	Henley	surface water	-	-	-	-	-	-	-	-
13/12/95	Albert Falls	surface water	-	-	-	-	-	-	-	-
14/12/95	Inanda	surface water	-	-	-	-	-	-	-	-

Date	Dam sampled	Sample Type	Blue-green Algae (genus)	Cell Count per mℓ	Toxins µg. ℓ^{-1}					
					RR	*NOD*	*YR*	*LR*	*YA*	*Other*
19/12/95	Nungwane	surface water	-	-	-	-	-	-	-	-
20/12/95	Nagle	surface water	Microcystis	84	-	-	-	-	-	-
29/12/95	Hazelmere	surface water	-	-	-	-	-	-	-	-
08/01/96	Shongweni	surface water	-	-	-	-	-	-	-	-
11/01/96	*Inanda	surface scum	Microcystis Anabaena	40303 7274	-	-	-	**2.8 ext 2.3 intr**		
22/01/96	*Inanda	surface scum	Microcystis Anabaena	3138719 52099	-	-	-	**11 intr**		
05/02/96	Shongweni	surface water	-	-	-	-	-	-	-	-
14/02/96	Albert Falls	surface water	Microcystis Anabaena	29539 6193	-	-	-	-	-	-
15/02/96	Inanda	surface water	Microcystis Anabaena	15040 2556	-	-	-	-	-	-
16/02/96	Hazelmere	surface water	-	-	-	-	-	-	-	-
20/02/96	Nungwane	surface water	-	-	-	-	-	-	-	-
21/02/96	Nagle	surface water	Microcystis Anabaena	5819 2519	-	-	-	-	-	-
22/02/96	Midmar	surface water	Microcystis	550	-	-	-	-	-	-
22/02/96	Henley	surface water	-	-	-	-	-	-	-	-
29/02/96	Inanda	surface water	Microcystis	3735	-	-	-	-	-	-
29/02/96	Inanda	surface scum	-	-	-	-	-	-	-	-
01/03/96	Hazelmere	surface water	Microcystis	35	-	-	-	-	-	-
05/03/96	Nungwane	surface water	-	-	-	-	-	-	-	-
06/03/96	Nagle	surface water	Microcystis Anabaena	231 795	-	-	-	-	-	-
06/03/96	*Inanda	surface scum	Microcystis	ND	-	-	-	+	-	-
06/03/96	Albert Falls	surface water	Microcystis Anabaena	6144 4915	-	-	-	-	-	-
07/03/96	Midmar	surface water	Microcystis	361	-	-	-	-	-	-
07/03/96	Henley	surface water	Microcystis	21	-	-	-	-	-	-
14/03/96	Inanda	surface water	Microcystis Anabaena	22019 885	-	-	-	-	-	-
14/03/96	Inanda	surface scum	-	-	-	-	-	-	-	-
15/03/96	Hazelmere	surface water	-	-	-	-	-	-	-	-

Date	Dam sampled	Sample Type	Blue-green Algae (genus)	Cell Count per mℓ	Toxins µg. ℓ^{-1}					
					RR	*NOD*	*YR*	*LR*	*YA*	*Other*
20/03/96	Albert Falls	surface water	Microcystis Anabaena	1475 12845	-	-	-	-	-	-
20/03/96	Nagle	surface water	Microcystis Anabaena	138 786	-	-	-	-	-	-
22/03/96	Midmar	surface water	-	-	-	-	-	-	-	-
22/03/96	Henley	surface water	Microcystis	10	-	-	-	-	-	-
28/03/96	Inanda	surface water	Microcystis Anabaena Oscillatoria	5865 557 183	-	-	-	-	-	-
29/03/96	Hazelmere	surface water	-	-	-	-	-	-	-	-
01/04/96	Nungwane	surface water	Microcystis	186	-	-	-	-	-	-
03/04/96	Nagle	surface water	Microcystis Anabaena	1278 5131	-	-	-	-	-	-
03/04/96	Albert Falls	surface water	Microcystis Anabaena	885 18893	-	-	-	-	-	-
04/04/96	Midmar	surface water	-	-	-	-	-	-	-	-
04/04/96	Henley	surface water	-	-	-	-	-	-	-	-
11/04/96	Inanda	surface water	Microcystis Anabaena	4129 2772	-	-	-	-	-	-

\+ = present
\- = not detected
ND = not determined
* = not from abstraction site

intr = intracellular toxins
extr = extracellular toxins

8.3.1 Algae Count Profiles

Of a total number of 923 routine samples analysed from the period of 20/02/95 to 30/03/97, no microcystin toxins were detected in the surface water samples. The following graphs (Figs. 8.8 to 8.15) give an algae profile for this period for each of the dams, except for Nagle and Inanda dams where the profile is given for the entire monitoring period.

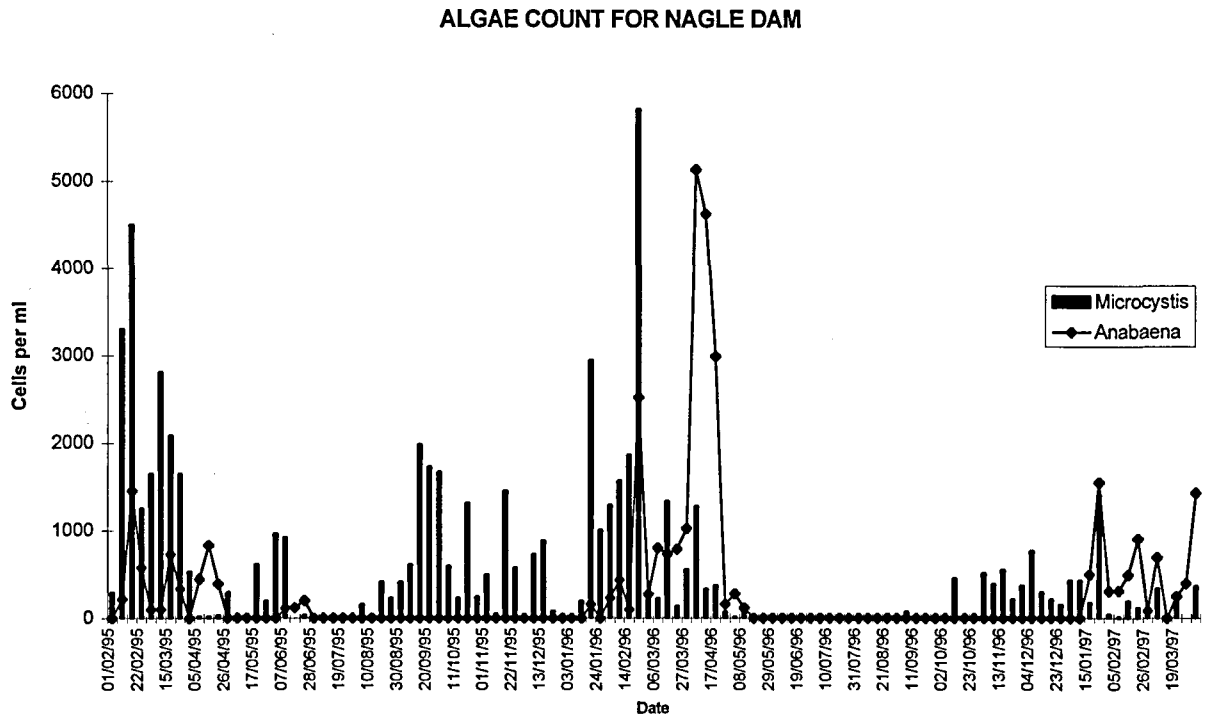


Fig. 8.8 Algae count profile for Nagle dam from 20-02-95 to 30-03-97

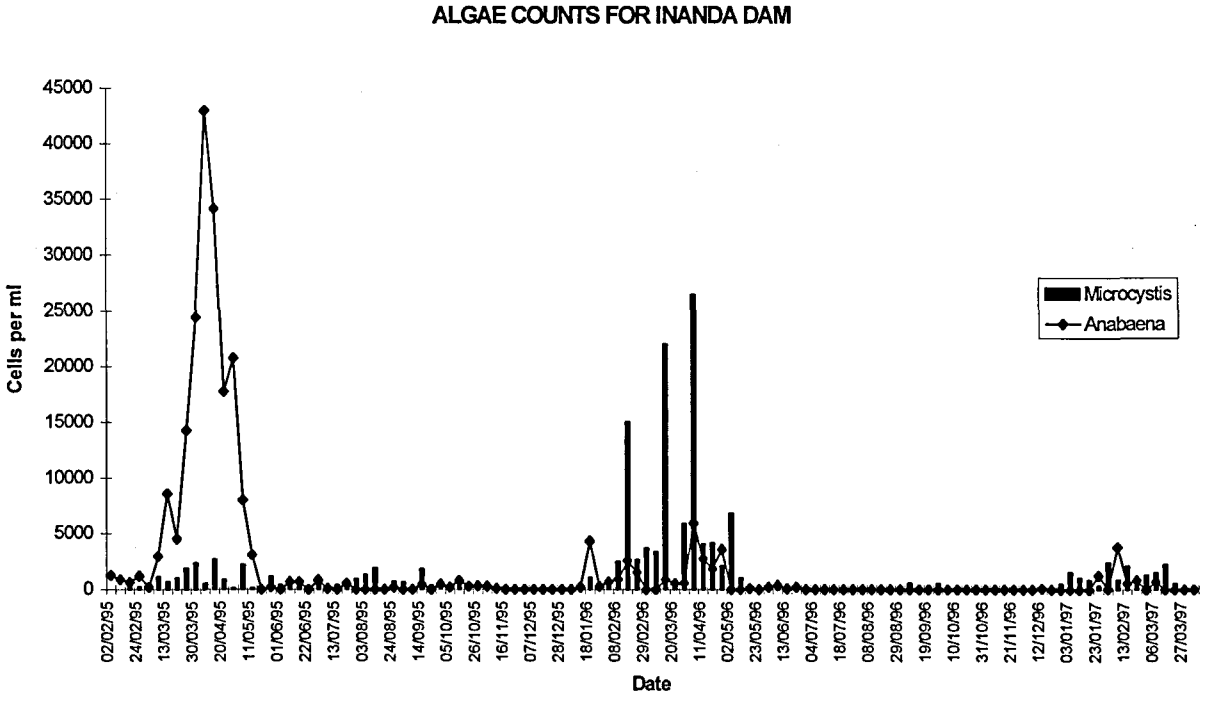


Fig. 8.9 Algae count profile for Inanda dam from 02-02-95 to 30-03-97

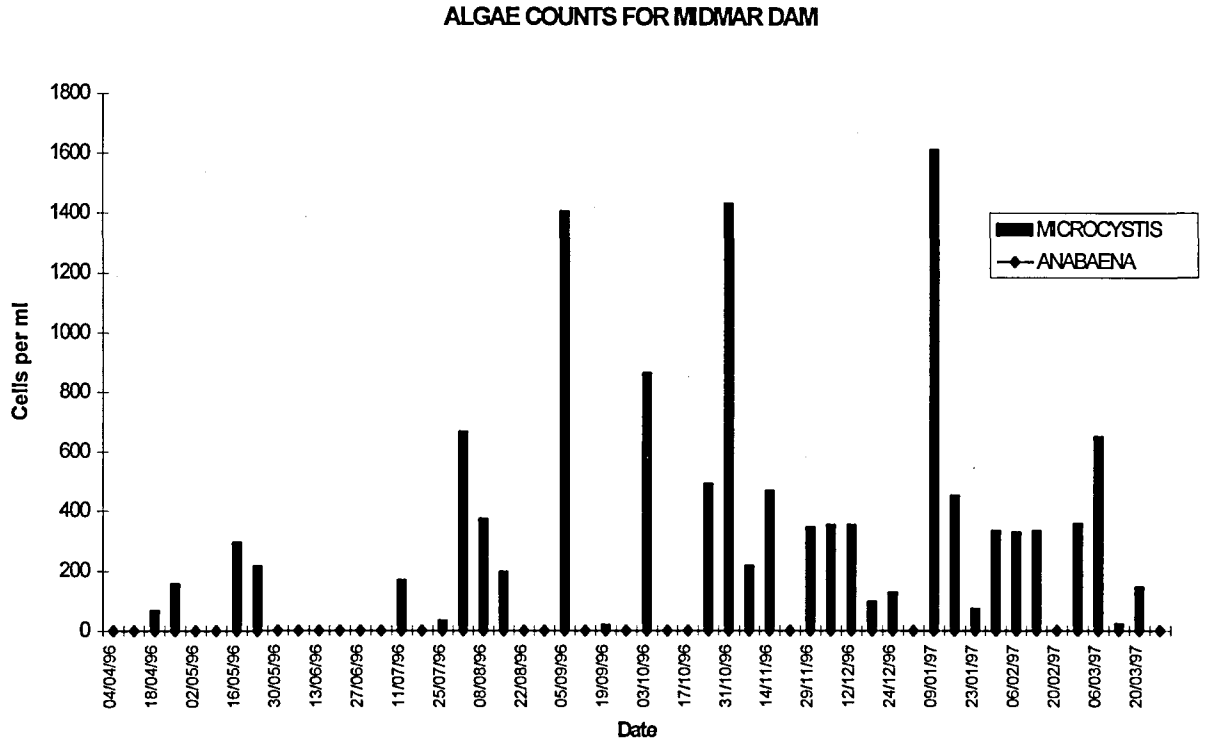


Fig. 8.10 Algae count profile for Midmar dam from 04-04-96 to 29-03-97

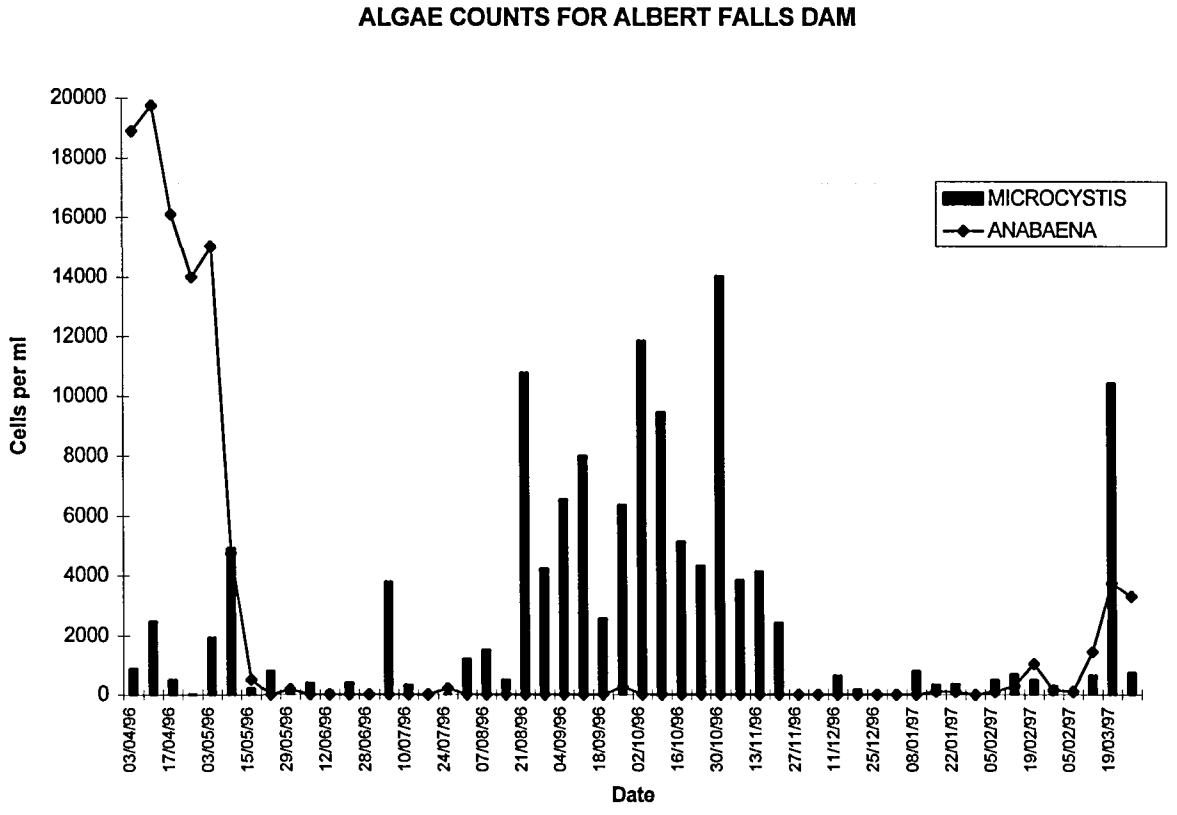


Fig. 8.11 Algae count profile for Albert Falls dam from 03-04-96 to 25-03-97

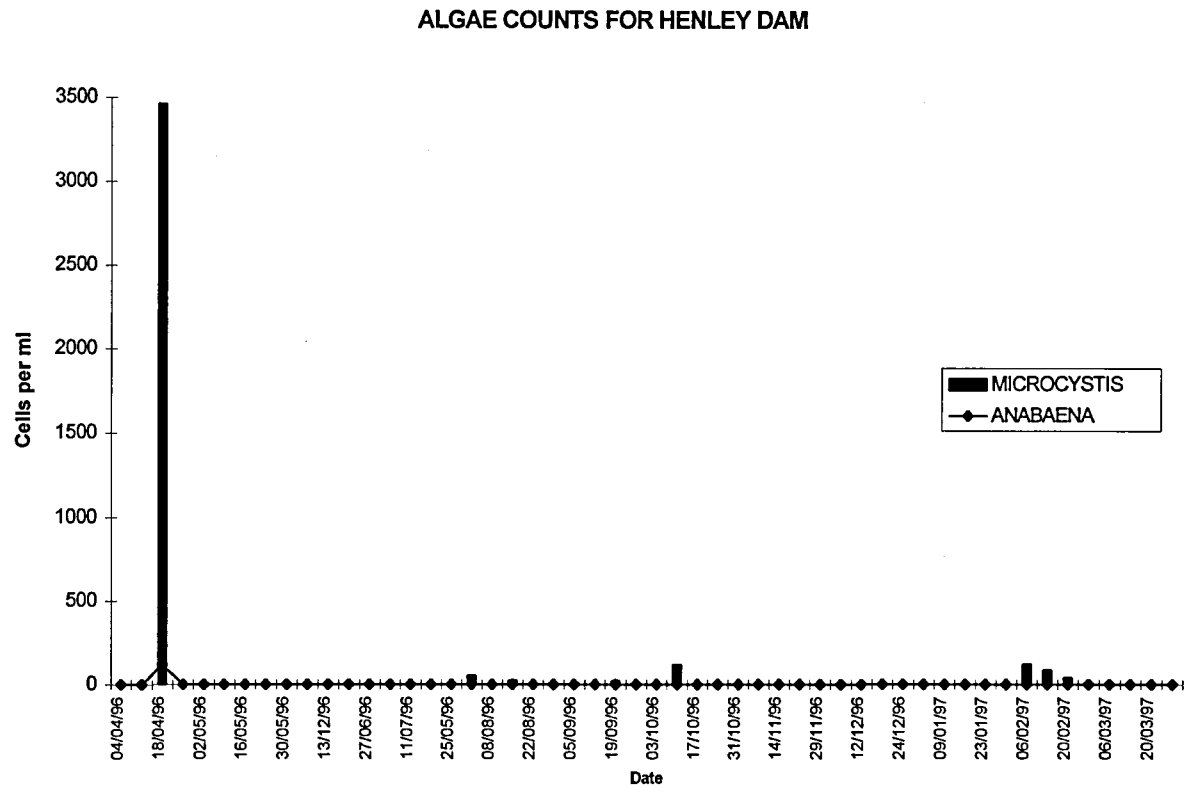


Fig. 8.12 Algae count profile for Henley dam from 04-04-96 to 29-03-97

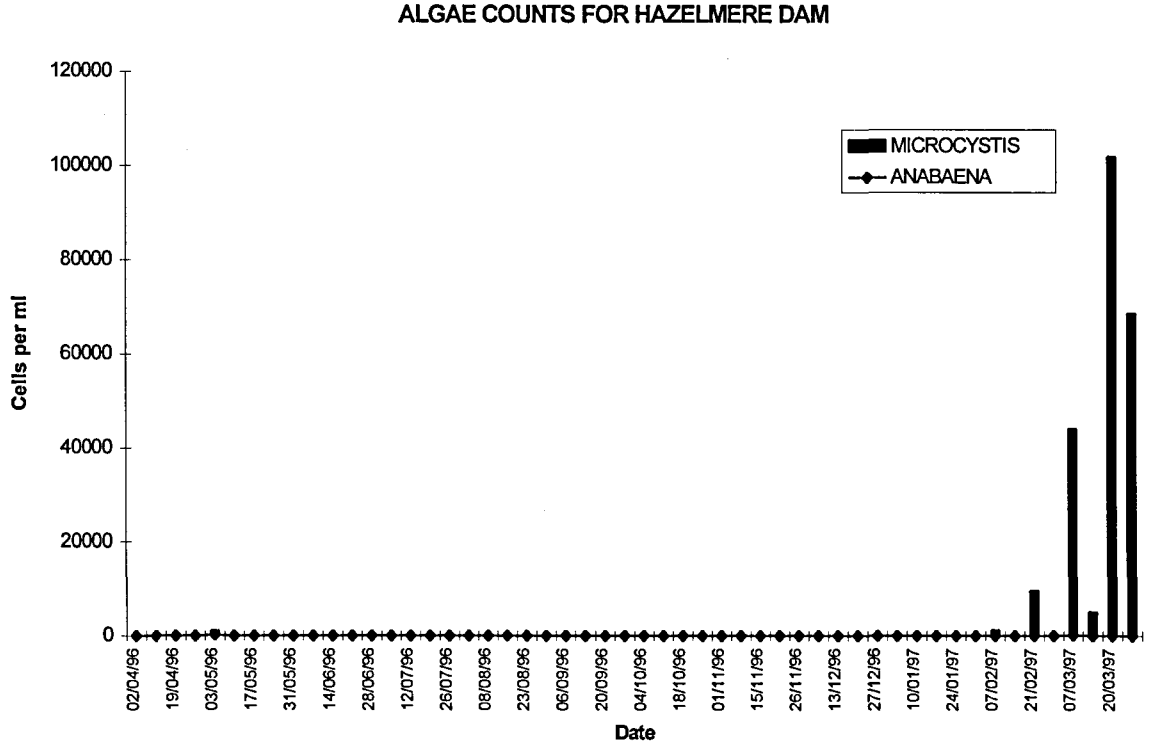


Fig. 8.13 Algae count profile for Hazelmere dam from 02-04-96 to 29-03-97

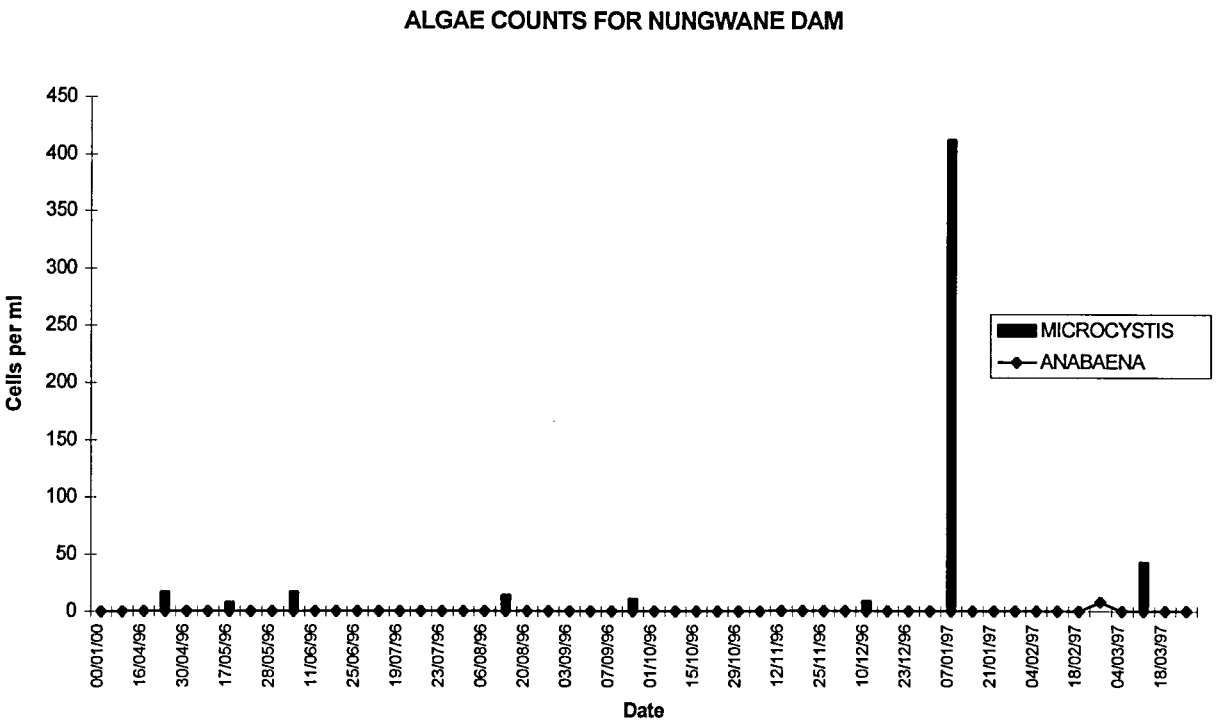


Fig. 8.14 Algae count profile for Nungwane dam from 10-04-96 to20-03-97

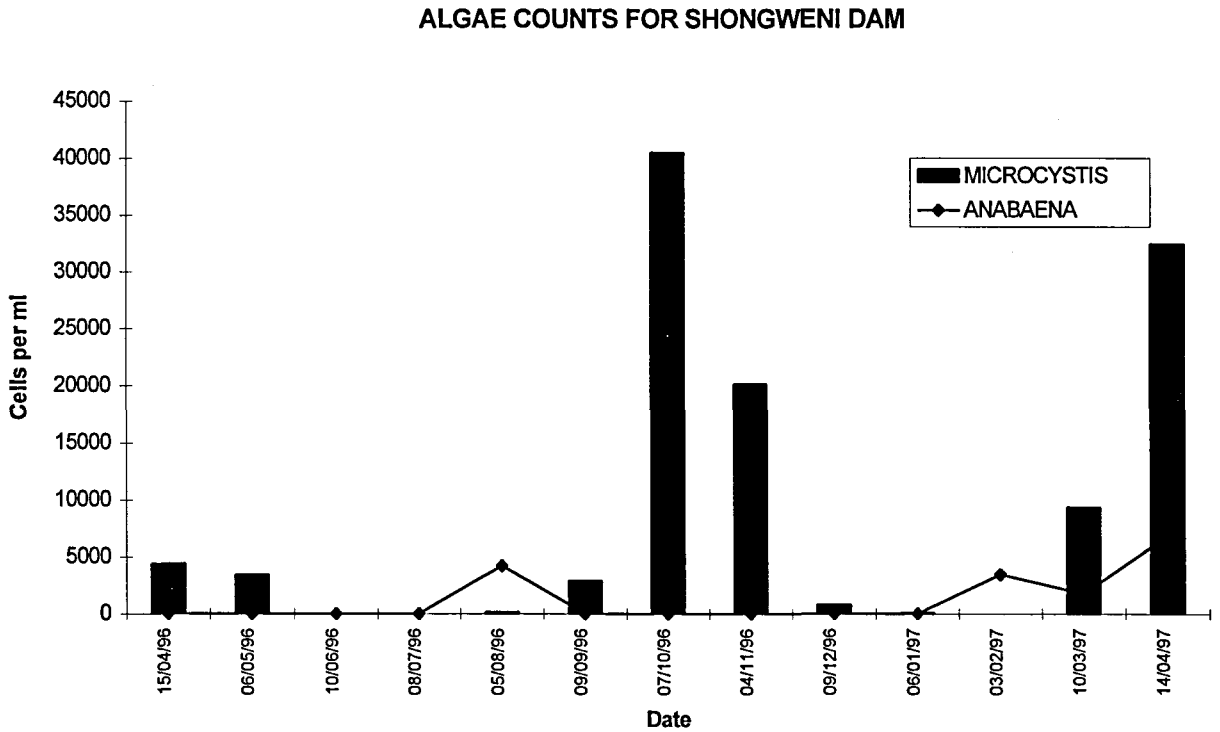


Fig. 8.15 Algae count profile for Shongweni dam from 15-04-96 to 14-03-97

8.3.2 Toxic Scums Analysed

Although no toxins were found in the samples from the abstraction sites, microcystin toxins were found in non-routine samples taken from other areas located within the catchment. The dams and other sampling sites were surveyed regularly and algal scum samples were taken whenever algal accumulations were found. Several of these were determined to be toxic:

A toxic *Microcystis* scum sampled from Nagle dam (22/02/95) contained microcystin-LR (16 min.) and another suspected microcystin toxin (23 min.) for which there was no suitable standard available to make an identification. (See Fig. 8.16)

The intracellular microcystin-LR concentration was determined to be 45.8 $\mu g.g^{-1}$ of freeze-dried algae.

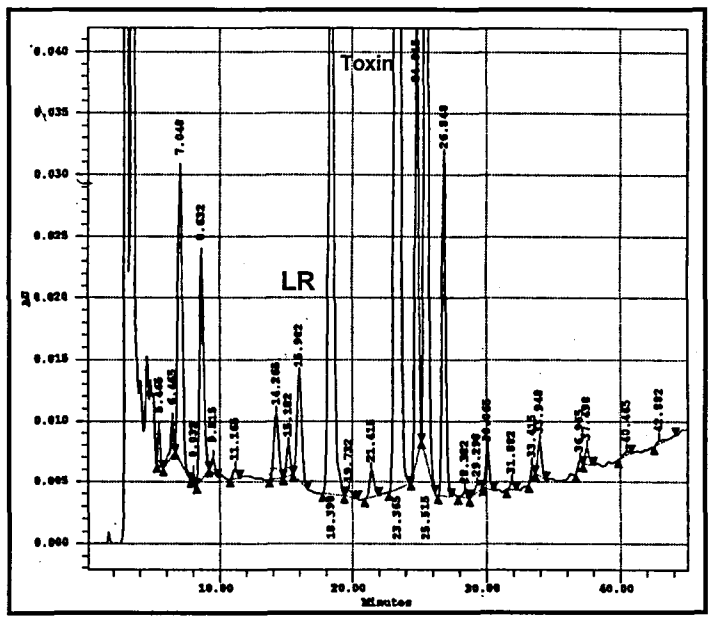


Fig. 8.16 Nagle *Microcystis* extract

Two other *Microcystis* scums that were sampled from Nagle dam in Nov. '95 and Jan '96 were found to contain microcystin toxins. Microcystin-LR was identified in each, but other unidentified microcystin toxins were also present at relatively higher concentrations, based on peak height.

Microcystin-LR was found in a *Microcystis* scum from the kwaMakutha Wastewater treatment area (23/03/96). (See Fig. 8.17). Four samples were taken on a weekly basis and freeze-dried and stored for use in the investigation of remedial actions. Both the natural sample and freeze-dried sample had a repulsive odour.
This scum would also be a good source of the toxin for isolation purposes.
Intracellular toxin concentration of this particular freeze-dried sample was determined to be 979.4 $\mu g.g^{-1}$ of dried algae.

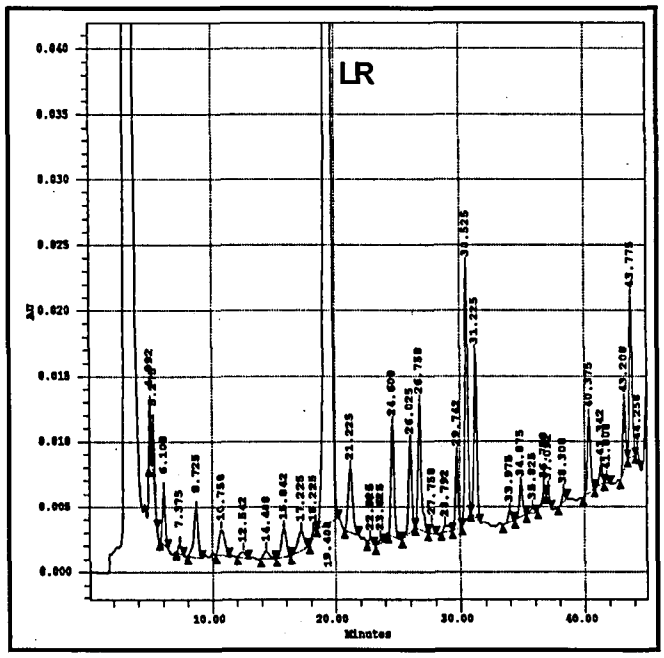


Fig. 8.17 Toxic *Microcystis* extract from the kwaMakutha wastewater treatment works

Four algal scum samples taken from Inanda dam (May '95, Dec. '95, Jan '96, May 96) were found to contain microcystin toxins. In one of the samples the toxins were found mainly in the filtered water. Microscopic inspection of the cells indicated that most of the cells were distorted or were ruptured from age. The intracellular toxin concentration was low in comparison to that in the surrounding water. Intracellular concentration was found to be 0.2 μg per liter of filtered cells whereas the microcystin-LR concentration (15.3 min. in Fig. 8.18) in the water was found to be 1.0 μg.ℓ^{-1}. On this occasion another suspected microcystin toxin was also found (23 min.).

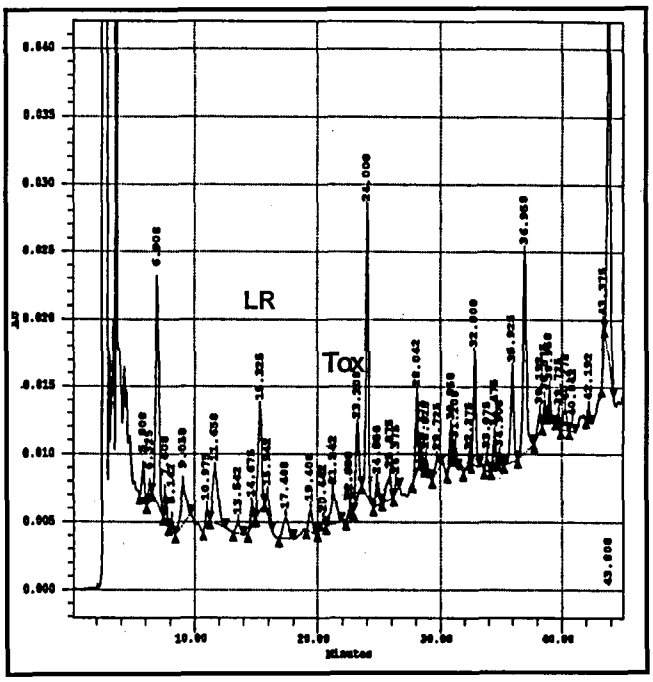


Fig. 8.18 Inanda water sample containing microcystin-LR

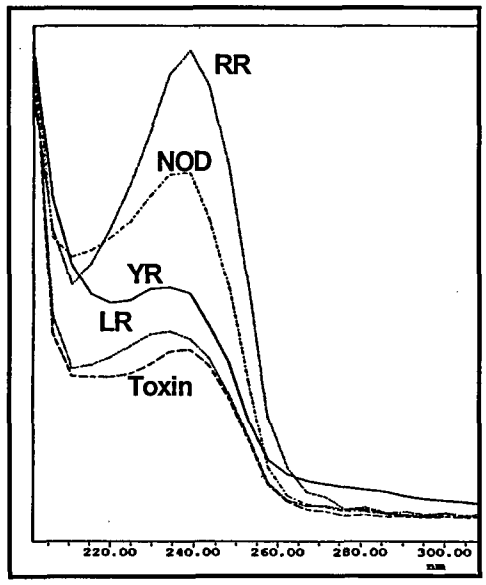


Fig. 8.19 UV spectrum of the peak at 23 minutes

A toxic *Microcystis* sample was obtained from the Camp's Drift weir site. The cells were old and most were decomposing. Microcystin-LR was found in the water but none was found in the methanol extract from the filtered cells. Camp's Drift is a recreational site and popular among canoeists. The localised scum was dispersed quickly.
This particular sample site was not on the routine monitoring programme.

The microcystin-LR concentration in the water (17.6 min., Fig. 8.20) was determined to be 2.0 μg ℓ^{-1}.

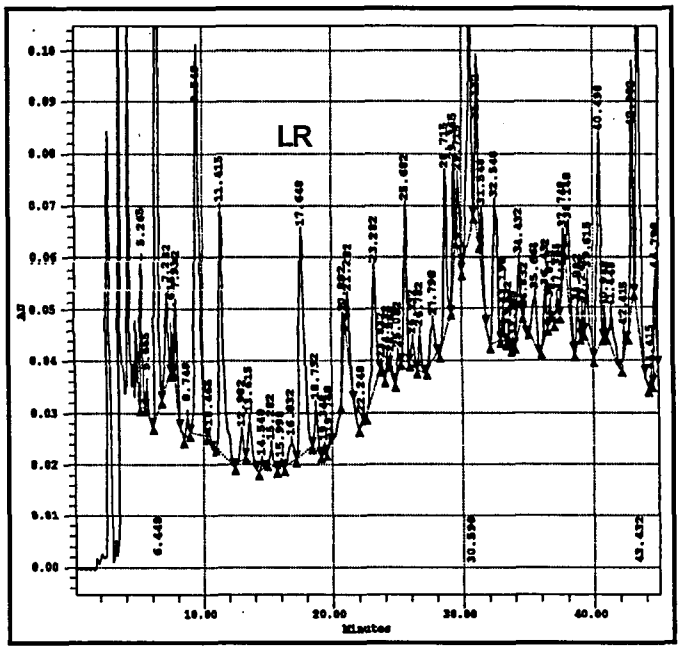


Fig. 8.20 A toxic *Microcystis* water sample from the Camp's Drift Weir

An unusually yellow-brown *Microcystis* bloom developed (Sept '96) near the Albert Falls dam wall. The dried cells at the scum surface were dark brown and covered with a mucous sheath and from a distance it resembled mud. Under the microscope, however, the cells were the same as the normal blue-green type. Microcystin-LR (145 $\mu g.g^{-1}$ of dried cells) was detected in the methanol extract (as shown in Figs 8.21 and 8.22) as well as possibly two other toxins at 24 and 29 minutes, based on their UV spectra.

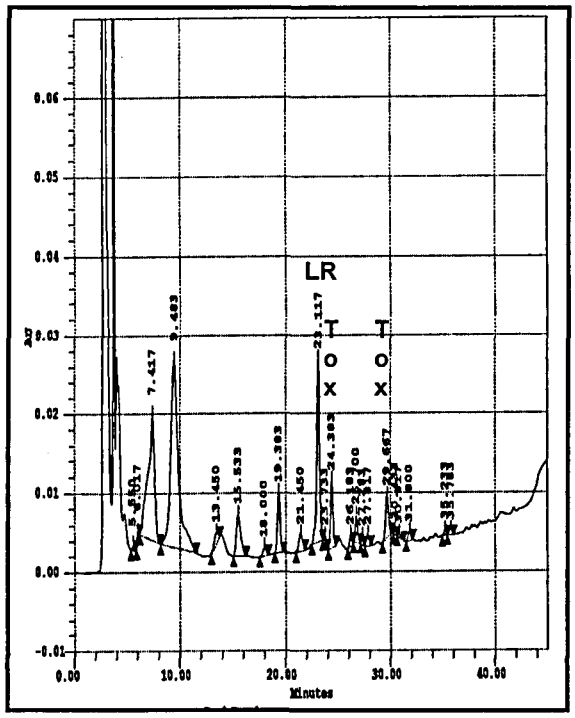


Fig. 8.21 A toxic *Microcystis* water sample from the Albert Falls dam

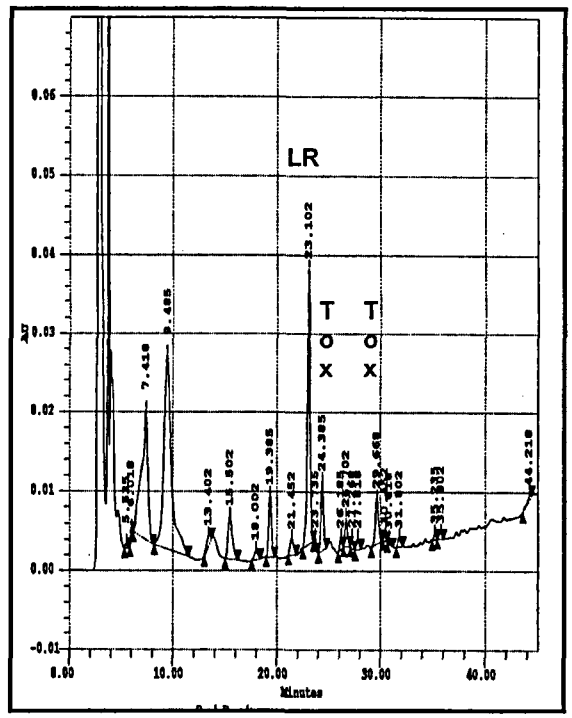


Fig. 8.22 A toxic *Microcystis* water sample from the Albert Falls dam, spiked with microcystin-LR

A summary of the toxic scum samples analysed are given in Table 8.2:

Table 8.2 Microcystin-LR concentrations found in *Microcystis* scums collected

Date	Dam	Blue-green	Cell Count cells.mℓ^{-1}	Microcystin-LR Concentration Intracellular	Extracellular
Feb '95	Nagle*	*Microcystis*	nd	45.8 $\mu g.g^{-1}$	
Nov '95	Nagle	*Microcystis*	nd	180 $\mu g.\ell^{-1}$ (filt. cells)	
Mar '95	kwaMakutha	*Microcystis*	nd	979 $\mu g.g^{-1}$	
May '95	Camp's Drift	*Microcystis*	nd	0	2.0 $\mu g.\ell^{-1}$
May '95	Inanda	*Microcystis*	nd	0.2 $\mu g.\ell^{-1}$ (filt. cells)	1.0 $\mu g.\ell^{-1}$
Dec '95	Inanda	*Microcystis* *Anabaena*	40 303 72 744	2.3 $\mu g.\ell^{-1}$ (filt. cells)	2.8 $\mu g.\ell^{-1}$
Jan '96	Inanda	*Microcystis* *Anabaena*	3 138 719 52 099	11 $\mu g.\ell^{-1}$ (filt. cells)	0
Mar '96	Inanda*	*Microcystis*	nd	1.1 $\mu g.\ell^{-1}$ (filt. cells)	2.1 $\mu g.\ell^{-1}$
Mar '96	Inanda*	*Microcystis*	nd	2.8 $\mu g.\ell^{-1}$ (filt. cells)	0.4 $\mu g.\ell^{-1}$
May '96	Inanda	*Microcystis*	693 998	3.4 $\mu g.\ell^{-1}$ (filt. cells)	2.5 $\mu g.\ell^{-1}$
Sep '96	Albert Falls*	*Microcystis*	>99 999 999	145 $\mu g.g^{-1}$	
Mar '97	Hazelmere	*Microcystis*	16 596 040	1.7 $\mu g.\ell^{-1}$ (filt. cells)	2.8 $\mu g.\ell^{-1}$

nd = not determined
* = other microcystin toxins were present

8.4 Discussion

Of the 923 routine surface water samples tested, none were found to contain toxins. The absence of microcystin toxins in these samples, even when *Microcystis* or *Anabaena* cells were sometimes present, suggests that either the toxin concentrations were too low or that none was present. The results were therefore recorded as "not detected" instead of zero on these occasions. On the other occasions were no *Microcystis* or *Anabaena* cells were found in the samples submitted, the amount of toxins can be taken as zero.

No massive algal blooms occurred during the monitoring period but it appears possible that the dams which contained toxic cyanobacteria will probably produce toxic scums in the future. Scums from Inanda, Nagle, Albert Falls and Hazelmere dams, taken at different times of the year were found to contain toxins. Several scums were also taken from Shongweni dam, but did not contain any toxins. Microcystin-LR was found in all the toxic algal scums analysed, implying that this toxin is the most commonly encountered microcystin variant in this region. However, sometimes other suspected toxins were also found at comparatively much higher concentrations than microcystin-LR, e.g., the *Microcystis* scum from Nagle dam (Feb '95).

The monitoring of routine algal toxin samples needs to be rationalised for South African conditions. Some researchers have suggested that algal counts can be used as a screening technique and toxin samples taken only when cells counts are between 500 and 2000 cells $m\ell^{-1}$. Inspection of the data obtained in this investigation indicate that these numbers appear to be far too low; but factors such as the variability of toxin production (as shown in Table 8.2) may make it difficult to determine the level of cell concentration above which samples should be taken until more data on toxic samples is available. Another way in which the routine monitoring can be rationalised is by determining the probability of a particular dam producing toxic cyanobacteria, based on its history of toxic algal blooms and their potential to enter the potable water treatment plant.

It should be emphasised, however, that the actual human exposure to cyanobacterial toxins may not be accounted for in this investigation as samples were taken from the abstraction sites of treatment works within the Umgeni catchment which have been designed to take in the least amount of cyanobacterial cells. People in rural areas who consume water directly from raw water supplies would be at greater risk than those receiving treated waters.

8.5 Conclusion

Microcystis and *Anabaena* were the most frequently encountered cyanobacterial genera in the Umgeni catchment during the period monitored. *Merismopedia* and *Oscillatoria* were also identified in a few of the samples. Although some of the scum samples were found to be toxic, the surface water samples submitted from nearby the abstraction sites on the sample day were toxin-free. This would indicate that no detectable amount of toxins was entering the potable water supply. The lack of massive cyanobacterial blooms and the relatively low cell counts recorded, are most likely reasons for the low incidence of toxins in the water samples.

Toxic *Microcystis* scums were recorded at Nagle, Inanda, Albert Falls and Hazelmere dams, the kwa Makutha wastewater treatment works and the Camp's Drift weir on the Umsindusi river. The algal scums taken from Shongweni dam were found to be non-toxic. Microcystin-LR was found in all the toxic scums, but other microcystin toxins (based on the characteristic UV spectra of the chromatographic peaks) could not be identified due to the lack of suitable standards. The highest extracellular microcystin-LR concentration was 2.8 $\mu g.\ell^{-1}$, found in an Inanda scum sample containing 40 303 *Microcystis* and 72 744 Anabaena cells per $m\ell$ of water, and a scum sample from Hazelmere dam containing greater than 16 million cells per $m\ell$.

There does not appear to be any threat of microcystin toxins entering the potable treatment works because the occurrence of microcystin toxins appears to be sparse, and there may only be real concern when thick cyanobacterial blooms occur. However, as a precautionary measure samples should be taken whenever the algal cell counts appear to increase rapidly.

CHAPTER NINE
Toxin Isolation and Culture of *Microcystis*

9.1 Microcystin toxin Isolation

Certain Microcystis scums analysed contained microcystin toxins (based on their UV spectra) that could not be identified because appropriate standards were not available. Only four of the 47 known microcystin toxins (see Appendix B) are available commercially, and other microcystin toxins are not easily obtained from other researchers.

Certain toxins that may be common to freshwater impoundments in this region may be left unidentified unless attempts are made to isolate and characterise them using the necessary research techniques such as various NMR analyses and mass spectrometry. Purified toxins could also serve as standards for future analyses.

One of the *Microcystis* scums collected from Nagle dam, contained a comparatively large concentration of an "unknown" toxin. A portion of the raw methanol extract was analysed by HPLC-MS and the rest was used to establish a reliable isolation method.

9.1.1 Procedure

The freeze-dried algae (1.3g) was allowed to extract in methanol for 20 hours, and the solution filtered under gravity (GF/C, 0.45μm). The filtrate was evaporated under nitrogen to approximately 1.5 mℓ before being applied to a silica column (Kieselgel, 9385, Merck) (3 × 15 cm) under low-medium pressure. Fractions (50 mℓ) were collected and evaporated on a steam bath.

Hexane-ethyl acetate (3:2) mobile phase was used to separate the various coloured (green/yellow) bands. The slow eluting bands were eluted using 100% ethyl acetate followed by EtOAc:CH_3OH (7:3). The toxic fractions were determined by HPLC and combined before being re-applied to the silica column. After elution as before, the toxic fractions were again combined, but further purification was done on a C_{18} column (3 × 5 cm) using acetonitrile as the mobile phase and taking 20 mℓ fractions.

Some of the original extract was sent for HPLC-MS analysis to determine if the molecular ion and ion fragmentation (m/z) could reveal the identity of the unknown toxin. A list of the molecular masses of the microcystin toxins are given in Appendix B.

9.1.2 Results

The toxin fractions separated early from most of the other components, e.g., pigments, lipids etc., The chromatograms of the initial fractions containing the toxins did not differ significantly from the original extract but many coloured bands from the extract were separated from the toxin-containing portion.

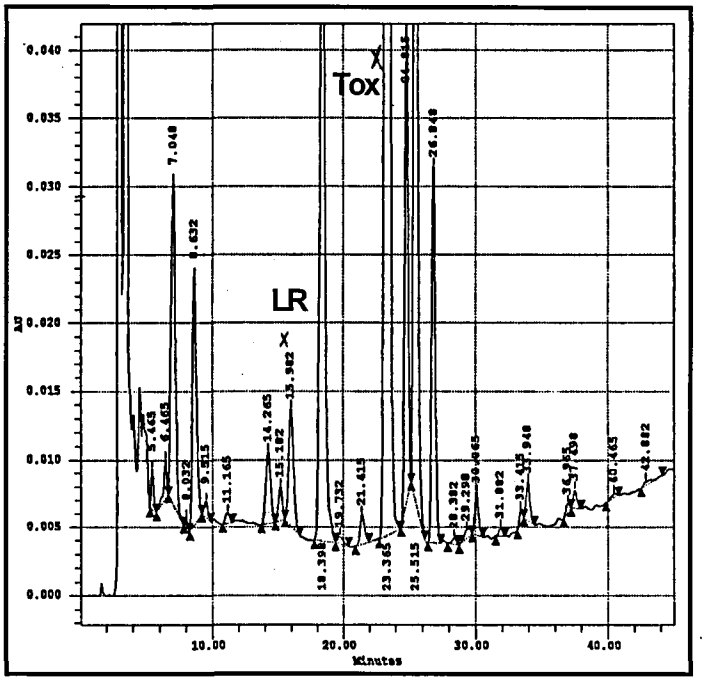


Fig. 9.1 Methanol extract of freeze-dried Microcystis sampled from Nagle dam, containing microcystin-LR and an unknown microcystin toxin

The fractions taken from the C_{18} column were colourless and the most pure fraction was sent for solid-probe mass spectrometric analysis. This was intended to establish a molecular ion mass of the toxin that could be checked against that of the other toxins cited in the literature. It was considered necessary to find out whether the toxin could be identified by its molecular ion before the toxin was purified further. If the toxin could not be identified based on its mass spectrum, the pure fraction would be sent for NMR analysis.

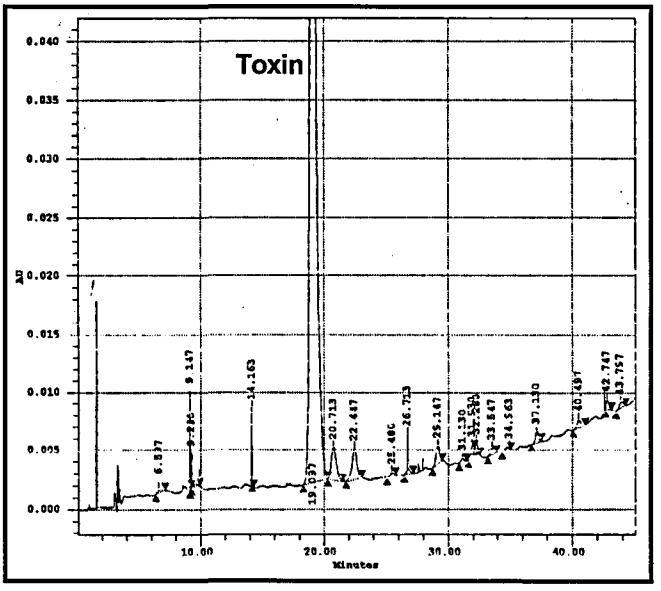


Fig. 9.2 After silica gel and C18 isolation the unknown toxin (elution time had shifted down to 19 minutes after HPLC column regeneration)

The following mass spectra were obtained from the solid-probe mass spectral analysis:

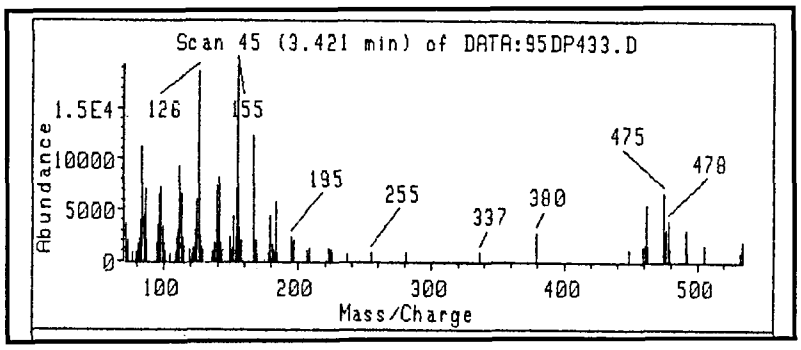


Fig. 9.3 Solid-probe mass spectrum of the purified fraction

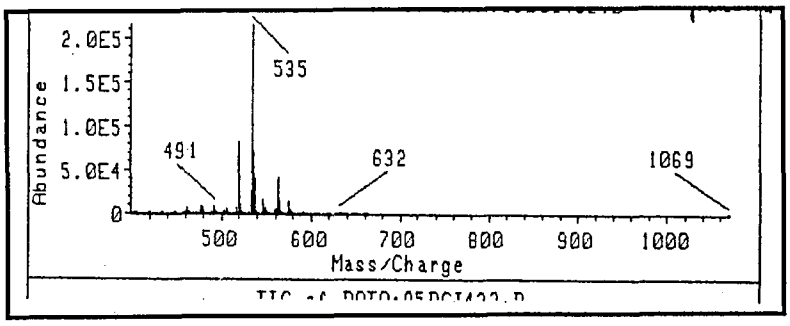


Fig. 9.4 Chemical ionisation mass spectrum of the purified fraction

None of the microcystin toxins could be successfully matched with the mass spectra obtained.

The HPLC-MS results could not be used to determine the molecular ion of the toxin due to interferences from other components in the methanol extract. After C_{18} separation and PDA-UV detection, the eluate was passed into the mass selective detector. Even though the microcystin toxins could be recognised in the UV chromatogram, the total ion chromatogram was too complex for evaluation because all components in the extract were detected. See Figs. 9.5 to 9.7. The molecular ions and corresponding ion fragmentation patterns could therefore not be linked to the peaks in the UV chromatograms. It is likely that the mass spectral results were not of the pure compounds. These results indicate that a preliminary clean-up procedure of the methanol extract of freeze-dried algae is required prior its analysis by HPLC-MS.

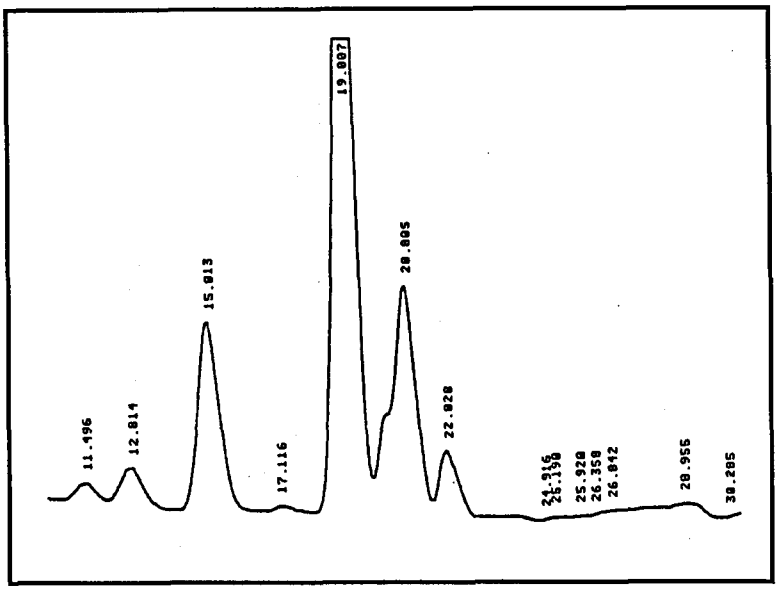


Fig. 9.5 The UV chromatogram of the Nagle *Microcystis* extract prior to mass spectral analysis
HPLC-MS analysis was done by the Jockey club (Jhb)

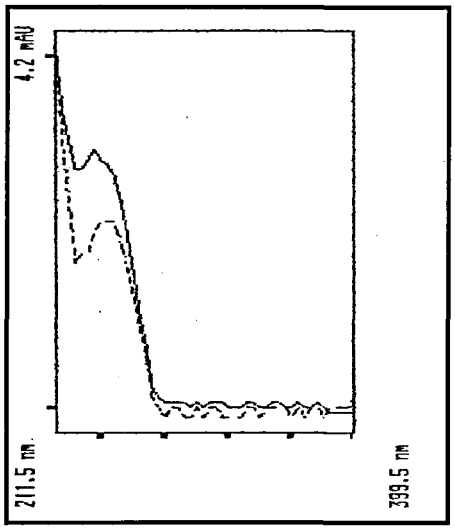


Fig. 9.6 The UV spectra of the peaks at 12.8 and 20.8 minutes in Fig. 9.5 correlate with the of toxins shown in Fig. 9.1

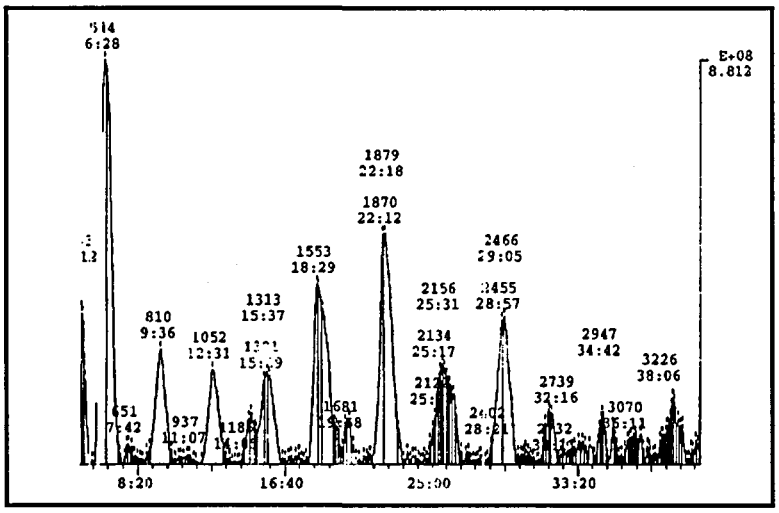


Fig. 9.7 The total ion chromatogram of the raw Nagle *Microcystis* extract

9.1.3 Discussion

Since there are 47 microcystin toxins that are known, it is likely that the "unknown" toxin may have been characterised previously from the list available (See Appendix B). It would have been unnecessary to carry out NMR and other analyses to determine its structure if the mass spectrum of the compound could reveal the identity. None of the common fragmentation patterns given in the literature could be matched to our HPLC-MS results. The molecular ion alone, however, would sometimes not reveal the identity of the toxin since there are several of the microcystin toxin variants that have the same molecular ion, but different chemical formulae. Therefore, differences in the fragmentation patterns will have to be sought to provide additional information on the identity of the particular compound.

Methanol has proven to be an efficient solvent for the extraction of the toxins from the algal cells. However, it appears that many other compounds (including the pigments, etc.), are also extracted. This is indicated by the numerous poorly resolved peaks in the total ion chromatogram (TIC), implying that the compounds entering the mass detector were not pure. It

is also likely that the other components in the extract are more concentrated than the toxins and would distort the ion fragmentation pattern of the toxin if they co-eluted. More selective forms of extracting the toxins (such as supercritical carbon dioxide or other solvents) should be investigated as an alternative.

9.1.4 Conclusion

Methanol does not selectively extract the toxins from the freeze-dried cells. A clean-up procedure is essential prior to HPLC-MS analysis of the crude algal extract, and a combination of silica and C_{18} flash chromatography can be used for this purpose.
The solid probe MS investigation of the purified toxin was not successful, mostly because of the small quantity isolated, producing molecular ion peaks that were not very intense while reliable fragmentation pattern could not be established.

9.2 Culture of *Microcystis*

Since *Microcystis* scums were not frequent and were usually rapidly dispersed, the culture of toxic *Microcystis* was one way of obtaining a constant supply of the microcystin toxins, especially those that are not presently known.

9.2.1 Procedure

Details of the blue-green algae culture media used (BG-11) are detailed in Appendix F. To obtain an axenic culture of *Microcystis* cells, a dilute solution of the scum sample was homogenised and 100 μℓ added to 100 mℓ of autoclaved water. After filtration of the solution through cellulose nitrate filters (0.45 μm), the filter paper was placed onto absorption pads soaked in culture media. The cultures were then placed under a light bench containing 4 fluorescent tubes.
After two weeks the filters were inspected under a light microscope to check for *Microcystis* colonies that could be removed and placed into the sterilised culture media solution.
Other non-axenic cultures were placed directly from the sample to an aliquot of the sterilised blue-green media. All the cultures were kept at laboratory temperature (22 -28°C) which is within the reported optimum range. The pH of the culture medium was 7.8, which is also in the optimum growth region.

9.2.2 Observations:

The algae plates were almost completely dominated by *Scenedesmus* cells. The non-axenic cultures were also bleached after a few weeks, possibly due to the high light intensity. It was apparent that the conditions favoured the proliferation of the green algae even though a culture medium more suitable for blue-green algae was used. One observation was that the original sample not under the light bench had maintained a *Microcystis* dominance and very few *Scendesmus* cells were found. The lighting conditions were therefore not suited for *Microcystis* growth and the number of fluorescent lights used was reduced to two. The bleached cells in the non-axenic cultures regained their green appearance but still resembled *Microcystis* cells. The colony formations had dispersed, lost their cell vacuoles and the cell size had diminished. Subsequent chemical analysis of the previously toxic *Microcystis* algae found them now to be non-toxic.

Thereafter, other toxic samples from Inanda and Nagle dams were also being kept in culture under one fluorescent light bulb approximately 40 cm above the culture flasks. The cultures prefer the BG-11 media, but cell numbers increase very slowly. These cultures maintained their cell size and buoyancy. With the reduced light intensity, however, a Nagle *Microcystis* sample

was kept in culture from November 1995 to May 1996, and an HPLC analysis on one of the cultures in March 1996 indicated that the culture still contained several microcystin toxins (identified by their UV spectra).

9.2.3 Conclusion

Microcystis grows very slowly in culture and appears to be most affected by light intensities when cultured in small quantities in conical flasks. Higher light intensities were found to cause a shift in algal genus from predominantly blue-green to a green algae. Other features included the diminished cell size and the loss of colony formation. Under relatively lower light intensity, the *Microcystis* cells retain their original characteristics such as buoyancy, colony formation and toxicity. The latter observation indicates that toxic *Microcystis* can be cultured and this should therefore be explored more intensively. The major problem was the infiltration of bacteria into the cultures.

CHAPTER TEN
Remedial Actions: Ozonation

10.1 Introduction

A laboratory study by I.T. Cousins *et al.*, [28] showed that microcystin toxins that are released into natural raw water biodegrade within 5 to 10 days. Although this study gives some idea of the lifetime of the toxins, it does not necessarily account for the toxins being replenished by a thriving cyanobacterial bloom. If a massive toxic bloom happens to occur near an abstraction point of a water treatment works, the toxins can possibly persist long enough to be consumed if they are not removed during the purification process. A reliable treatment method should, therefore, be available in the event of a cyanobacterial bloom becoming a potential threat to human health.

Ozone is a powerful oxidant that is very effective for the removal of taste, odour and colour in water treatment. The negative aspects of its use include its low residual qualities and its expense of generation. Ozone has been reported to reduce the microcystin concentrations by more than 90%. Studies by Finnish researchers indicated that an ozone dosage of 1 $mg.\ell^{1}$ is adequate for the removal of microcystin toxins.[7] This has been confirmed by an Australian study where 99% removal of microcystin-LR was accomplished with an ozone dose of 0.07 $mg.\ell^{1}$ in 15 seconds.[30] With water containing natural organic matter (NOM) with a DOC level of 8.5 $mg.\ell^{1}$, a dose of 1 $mg.\ell^{1}$ of ozone was required. Other researchers such as Bernazeau[7] (Lyonnaise des Eaux) produced 50% removal of microcystin-LR (500 $\mu g.\ell^{-1}$) from micro-filtered Seine river water with an ozone dose of 0.5 $mg.\ell^{-1}$. Deionised water having the same toxin concentration was reduced by 99% with and ozone dose of 0.2 $mg.\ell^{-1}$.
According to Carmichael (1992), the ADDA residue on the microcystin toxin molecule imparts toxicity to the molecule and ozonolysis of the toxins produces the free ADDA and the cyclic peptide. Oxidised microcystin-LR also did not result in the formation of other toxic products.[7]

The investigations carried out in this study indicate the feasibility of the various treatment options mentioned for the removal of microcystin toxins and would also provide some indication of the doses that should be applied in a treatment works.

10.2 Experimental

Ozone was produced on a laboratory scale by passing high purity oxygen gas through a Sorbios ozone generator. Alteration of the applied potential, ozonation period or flow of oxygen to the generator was used to adjust the amount of ozone produced .
The ozone generated was passed through to a 3-way tap that directed the flow of ozone either to the sample ozonating vessel or to the standby chamber, in which an excess amount of potassium iodide (KI) solution was kept to neutralise the ozone, before the flue gas was vented to the atmosphere.

The diagram that follows describes the design of the ozonation process.

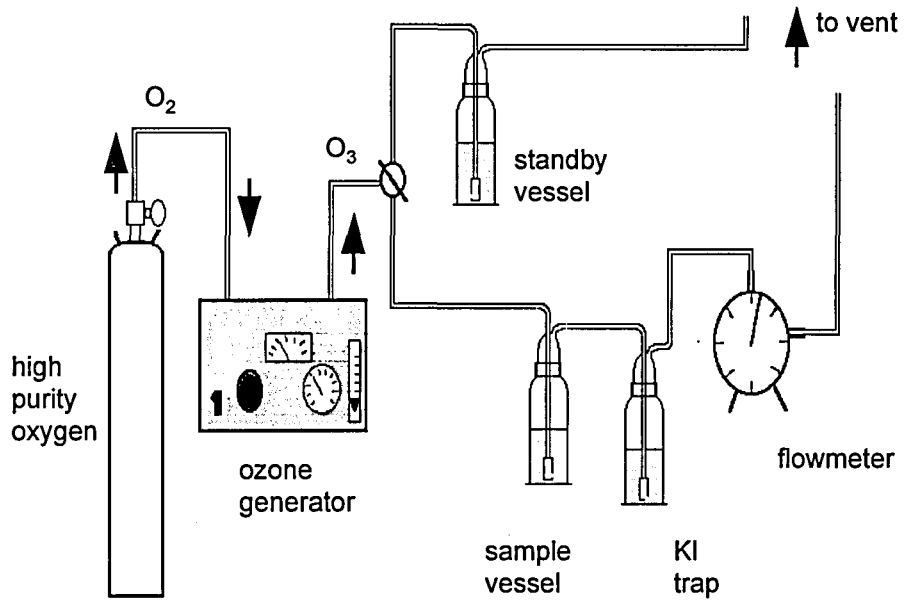


Fig.10 Instrumentation used in the ozonation of the test samples

The ozone gas flow was directed to the standby vessel during the generator stabilisation period and between sample changes.

10.2.1 Determination of Ozone Dose:

Ozone dosages were determined by the iodometric titration method Potassium iodide (200 mℓ) was placed in the sample vessel and dosed with ozone for a specified time period. The iodide was immediately oxidised to iodine which caused the colourless solution to turn yellow.
The iodine liberated was titrated against a standardised sodium thiosulphate solution to a starch endpoint. The second vessel, trapped any ozone that escaped the first vessel.
Usually the second vessel did not become yellow, except when test samples were ozonated, indicating that some of the applied dose was escaping. The gas liberated from the second vessel passed through a flowmeter that was used to check the volume of gas flow.

The following reactions were used in the determination of the ozone dose:

$$O_3 + 2KI\ (aq) + 2H^+ \rightarrow O_2 + I_2 + H_2O + 2K^+$$

$$I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-}$$

The mass of ozone produced (mg) = $V \times N \times 24$

where V = volume of titrant sodium thiosulphate ($Na_2S_2O_3$) consumed in millilitres
N = normality of $Na_2S_2O_3$ (usually 0.05 or 0.1N, which is the same for Molarity)
24 = equivalent mass of ozone

The sodium thiosulphate was standardised using a primary standard solution of potassium dichromate ($K_2Cr_2O_7$).

The ozone generator was allowed to stabilise for at least an hour at the set voltage and gas pressure, before several consecutive dosage tests were performed, each over a specified time period. Once the dosage was consistent, the set of samples (500 mℓ) spiked with toxins were ozonated for the same time period, followed by more dosage tests using KI solution. The samples were reported to be dosed within this range because the ozone dose usually decreased slightly over time due to the slight decrease in gas flow from the cylinder.
As the labscale design allowed part of the ozone applied to escape the sample ozonation vessel, the KI solution in the second trap was titrated with sodium thiosulphate to determine the amount escaping. The effective dose was obtained by subtraction of the amount escaped from the applied dose.

The following three types of samples (prepared from the same matrix) were analysed for microcystin toxins :
(i) Blanks: i.e. non-ozonated samples without a toxin spike
(iii) Tests: i.e. samples that had been spiked with toxin and then ozonated
(ii) Controls: i.e. non-ozonated samples having the same pH and toxin concentration as the test sample.

10.2.2 Toxin Standards
Two types of toxin standards were used: (i) pure toxins prepared in ultra-pure water and (ii) algal extract containing microcystin-LR. The particular extract used was obtained from freeze-dried *Microcystis* cells taken from the kwaMakutha wastewater works, and is referred to as the "kwaMakutha standard". The standard toxin concentrations were determined by HPLC each time a set of tests was conducted.

10.2.3 Determination of Recoveries and Percent Toxin Reduction:
The volume of toxin standard added to the test samples was used to calculate *expected* toxin concentration of the control sample. The percent toxin recovered from the control samples were used to adjust the concentrations found for the test samples.
The percent reduction was calculated as follows:

$$\text{Percent toxin removal} = 100 - \frac{\textit{Adjusted}\text{ toxin Conc. of Test sample} \times 100}{\textit{expected}\text{ Concentration}}$$

10.2.4 Ozone Residuals:
Ozone residuals were determined using the Indigo Colorimetric Method. The principle of the method involves the rapid decolourisation of indigo in acidic media by trace quantities of ozone. The decrease in colour intensity is linear with increasing concentrations of ozone, and the residual ozone concentration was deduced from the following equation:

$$\text{mg } O_3 \text{ per litre}^{35} = \frac{100 \times \Delta A}{f \times b \times V}$$

where

ΔA = difference in absorbance between sample and blank
b = path length of cell , cm
V = volume of sample, mℓ (90 mℓ)
f = 0.42 based on a sensitivity factor for the change in absorbance (at 600 nm) per mole of added ozone per litre

Unspiked samples and spiked toxin samples were analysed for ozone residuals. The non-ozonated samples were used as blanks.

Reagent Solutions:
Indigo stock reagent : potassium indigo trisulphonate (770 mg) (Sigma) was dissolved in ultra-pure water (500 mℓ) containing 1 mℓ of concentrated phosphoric acid and diluted to 1ℓ with ultra-pure water.
The indigo reagent used for the residual tests was prepared by adding indigo stock solution (20 ml), sodium dihydrogen phosphate (10 g), and conc. phosphoric acid (7 mℓ) to a 1ℓ volumetric flask and diluting to the mark with ultra-pure water.

Spectrophotometric procedure:
Indigo reagent (10 mℓ) was added to a volumetric flask (100 mℓ) and made up to the mark with the non-ozonated sample (blank). The same was done with the ozonated test samples. Absorbance readings of the samples were taken at 600 nm in a 1 cm pathlength cell. A Phamacia - Ultraspec 3 UV visible spectrophotometer was used.

10.3 Results

The following chromatograms (Figs 10.1 to 10.4) show the removal of microcystin-LR from ultra-pure water.

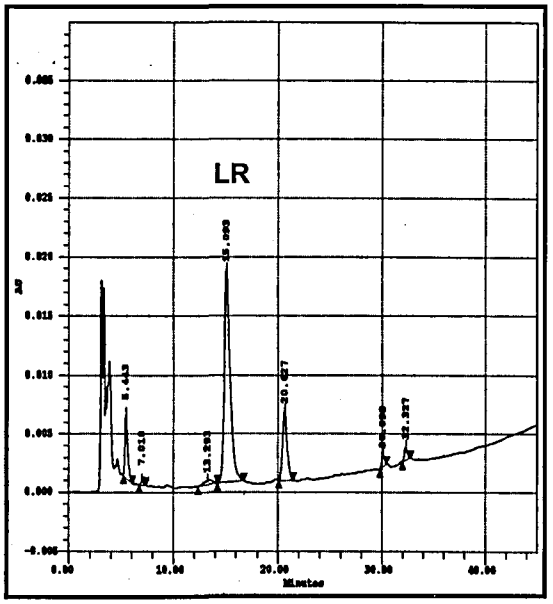


Fig. 10.1 *Control sample:* ultra-pure water containing microcystin-LR (4 $\mu g.\ell^{-1}$)

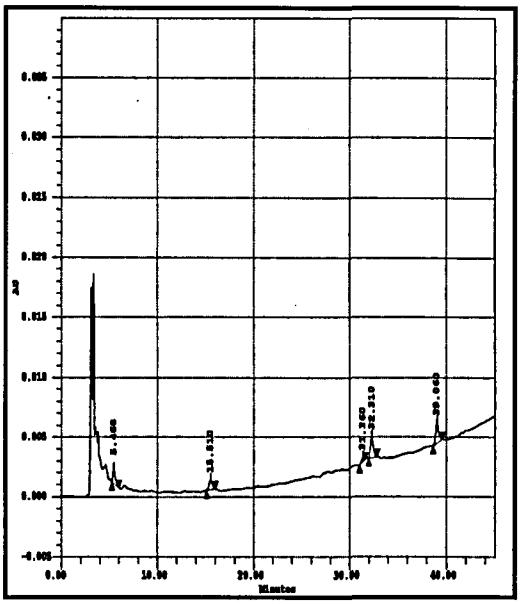


Fig. 10.2 *Ozonated sample*: showing 100% removal of microcystin-LR (4 $\mu g.\ell^{-1}$)(O_3 dose : 0.2 - 0.3 $mg.\ell^{-1}$)

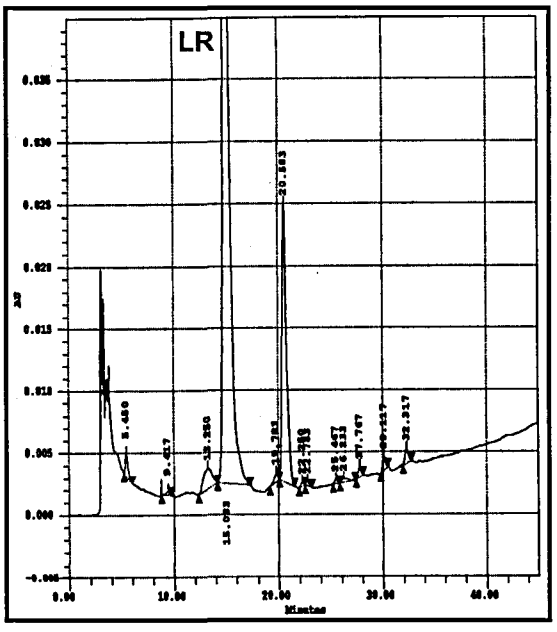


Fig. 10.3 *Control sample*: ultra-pure water containing microcystin-LR (16 $\mu g.\ell^{-1}$)

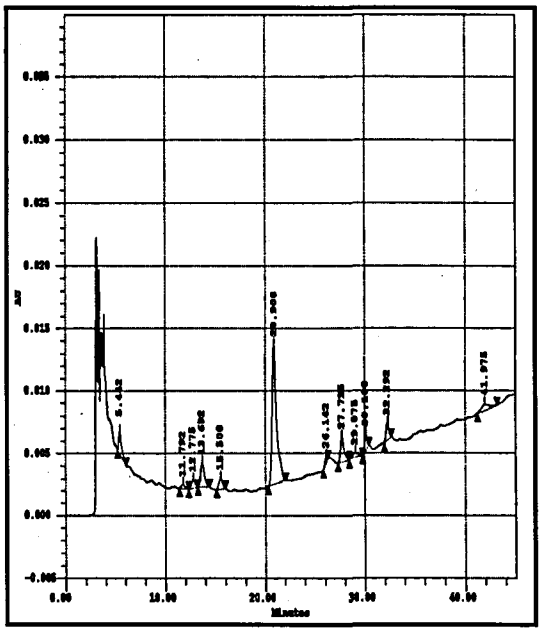


Fig. 10.4 *Ozonated sample*: showing 100% removal of microcystin-LR (16 $\mu g.\ell^{-1}$)(O_3 dose : 0.2 - 0.3 $mg.\ell^{-1}$)

The chromatograms in Figs. 10.6 to 10.11 show the removal of microcystin-LR from Camp's Drift raw water at various pHs, using samples spiked with the *Microcystis* extract taken from the kwaMakutha wastewater works.

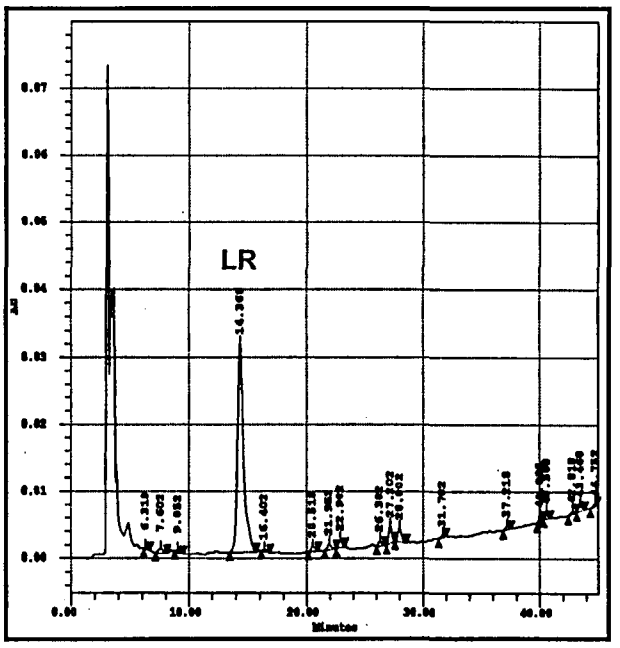


Fig. 10.5 The standard kwaMakutha extract containing microcystin-LR (22.6 μg.ℓ^{-1})

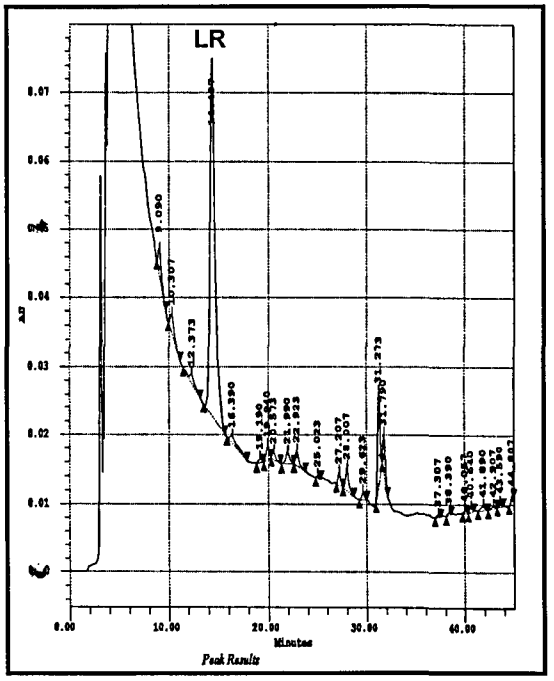


Fig. 10.6 *Control sample*: Camp's Drift water containing microcystin-LR (9 μg.ℓ^{-1}) at pH 3.2

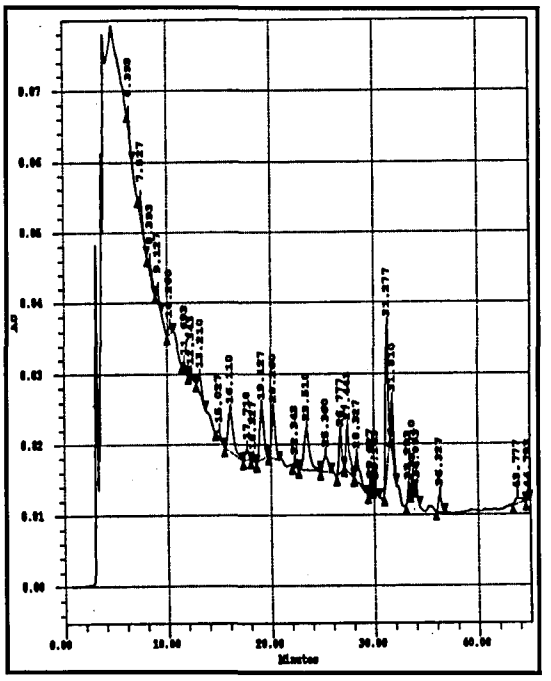


Fig.10.7 *Ozonated sample* : showing 100% removal of microcystin-LR (9 μg.ℓ^{-1}) at pH 3.2 O_3 dose : 1.2-1.4 mg.ℓ^{-1})

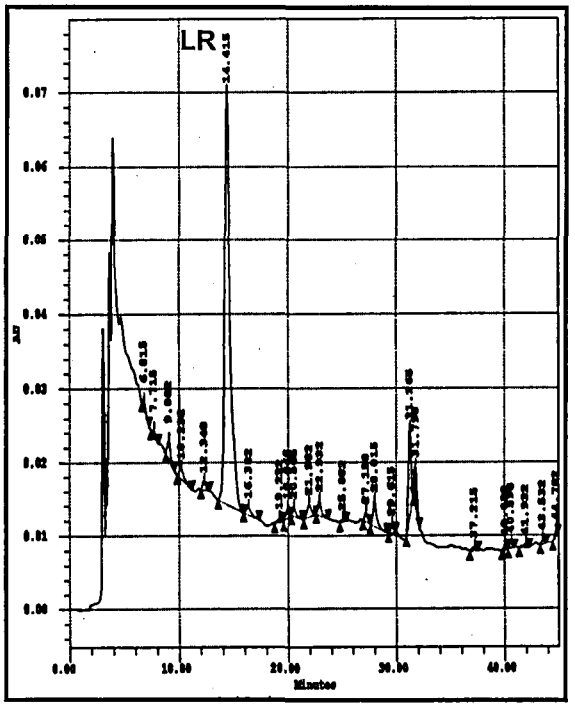


Fig. 10.8 *Control sample*: Camp's Drift water containing microcystin-LR (9 μg.ℓ^{-1}) at pH 5.5

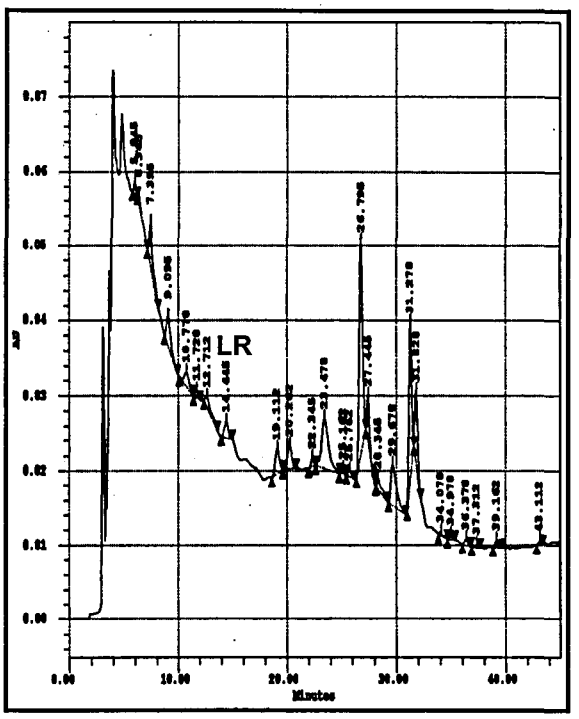


Fig. 10.9 *Ozonated sample* : showing 98% removal of microcystin-LR (9 μg.ℓ^{-1}) at pH 5.5 (O_3 dose : 1.2-1.4 mg.ℓ^{-1})

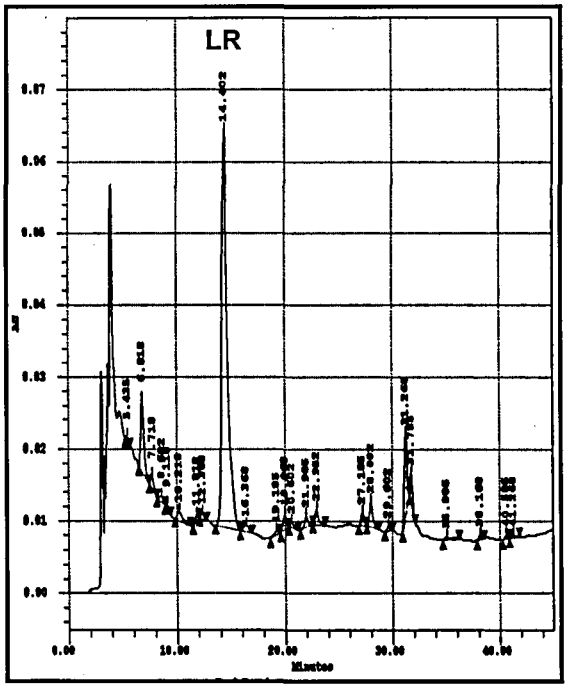


Fig. 10.10 *Control sample*: Camp's Drift water containing microcystin-LR (9 μg.ℓ^{-1}) at pH 9.0

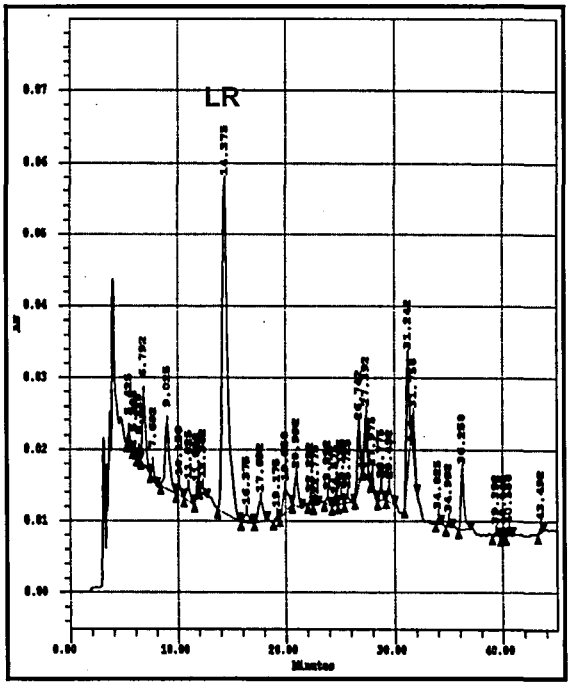


Fig. 10.11 *Ozonated sample* : showing 17% removal of microcystin-LR (9 μg.ℓ^{-1}) at pH 9.0 (O_3 dose : 1.2-1.4 mg.ℓ^{-1})

The removal of microcystin toxins are given in Table 10.1

Table 10.1 Microcystin-LR removal by Ozone

Test	*Matrix*	*Toxin Std Spiked*	*Initial Toxin Conc.* ($\mu g.\ell^{-1}$)	*pH*	*Ozone Dose (Effective)* ($mg.\ell^{-1}$)	*Dosage Time* min./sec	*Final Toxin Conc.* ($\mu g.\ell^{-1}$)	*Percent Reduction*
1	Ultra-pure	LR Std	5.4	6.8	0.09- 1.0	1 / 30	0	100
		"	5.4	"	"	"	0	100
		"	5.4	"	"	"	0	100
		"	21.4	"	"	"	0	100
		"	21.4	"	"	"	0	100
			21.4	"	"	"	0	100
2	Ultra-pure	LR Std	4	6.7	0.17 - 0.29	0 / 30	0	100
		"	4	"	"	"	0	100
		"	8	"	"	"	0	100
		"	8	"	"	"	0	100
		"	8	"	"	"	0	100
		"	16	"	"	"	0	100
		"	16	"	"	"	0	100
		"	16	"	"	"	0	100
3	Camp's Drift Raw	LR Std	9.6	3.2	0.23-0.24	0 / 30	0	100
		"	9.6	3.2	"	"	0.7	92
		"	9.6	3.2	"	"	0.8	92
		"	9.6	7.7	"	"	7.8	18
		"	9.6	7.7	"	"	7.8	18
		"	9.6	10.0	"	"	6.8	29
		"	9.6	10.0	"	"	6.8	28
		"	9.6	10.0	"	"	8.6	10
4	Camp's Drift Raw	Kwa Makutha	9.0	3.2	0.33 - 0.44	0 / 30	3.0	67
		"	9.0	3.2	"	"	3.7	59
		"	9.0	7.7	"	"	6.8	24
		"	9.0	7.7	"	"	9.0	0
		"	9.0	7.7	"	"	8.3	8
		"	9.0	10.0	"	"	8.3	8
		"	9.0	10.0	"	"	7.7	15
5	Ultra-pure	Kwa Makutha	9.0	3.2	0.32	0 / 30	2.1	77
		"	9.0	3.2	"	"	3.5	61
		"	9.0	3.2	"	"	2.7	70
		"	9.0	5.5	"	"	0	100
		"	9.0	5.5	"	"	1.8	80
		"	9.0	5.5	"	"	1.8	80
		"	9.0	5.5	"	"	0.9	90
		"	9.0	9.0	"	"	4.8	47
		"	9.0	9.0	"	"	7.7	14
		"	9.0	9.0	"	"	5.9	35

Table 10.1 cont. Microcystin-LR removal using ozone

Test	*Matrix*	*Toxin Std Spiked*	*Initial Toxin Conc.* ($\mu g.\ell^{-1}$)	*pH*	*Ozone Dose* ($mg.\ell^{-1}$)	*Dosage Time* min./sec	*Final Toxin Conc.* ($\mu g.\ell^{-1}$)	*Percent Reduction*
6	Camp's Drift Raw	kwa Makutha	9.0	3.2	1.2 - 1.4	1 / 30	0	100
		"	9.0	3.2	"	"	0	100
		"	9.0	3.2	"	"	0	100
		"	9.0	3.2	"	"	0	100
		"	9.0	5.5	"	"	0.3	97
		"	9.0	5.5	"	"	0.2	98
		"	9.0	5.5	"	"	0.5	95
		"	9.0	9.0	"	"	7.5	17
		"	9.0	9.0	"	"	7.1	21
7	Inanda	kwa Makutha	9.0	3.2	1.3 - 1.4	1 / 30	0	100
		"	9.0	3.2	"	"	0	100
		"	9.0	3.2	"	"	0.4	96
		"	9.0	3.2	"	"	1.0	89
		"	9.0	5.5	"	"	0.6	93
		"	9.0	5.5	"	"	1.3	86
		"	9.0	5.5	"	"	1.9	79
		"	9.0	7.7	"	"	6.5	28
		"	9.0	7.7	"	"	3.5	61
		"	9.0	7.7	"	"	6.1	32
8	Nagle	kwa Makutha	9.0	3.2	1.2 - 1.3	1 / 30	2.0	78
		"	9.0	3.2	"	"	2.0	78
		"	9.0	5.5	"	"	0.6	93
		"	9.0	5.5	"	"	0.8	91
		"	9.0	7.7	"	"	4.8	47
		"	9.0	7.7	"	"	3.6	60
		"	9.0	7.7	"	"	5.3	41
9	Camp's Drift Raw	kwa Makutha	9.0	5.5	2.3 - 2.4	4 / 30	0	100
		"	9.0	5.5	"	"	0	100
		"	9.0	5.5	"	"	0.1	99
		"	9.0	7.7	"	"	0.8	91
		"	9.0	7.7	"	"	0.5	94
		"	9.0	7.7	"	"	0	100
		"	9.0	9.0	"	"	1.4	85
		"	9.0	9.0	"	"	0.3	97
		"	9.0	9.0	"	"	0.6	93
		LR Std	156	7.7	2.3 - 2.4	4 / 30	4.7	98
		"	156	7.7	"	"	4.7	98

The pH trends observed for the removal of microcystin-LR in Tests 6, 7 and 9 (Table 10.1) are shown in Figs. 10.12, 10.13 and 10.14 respectively :

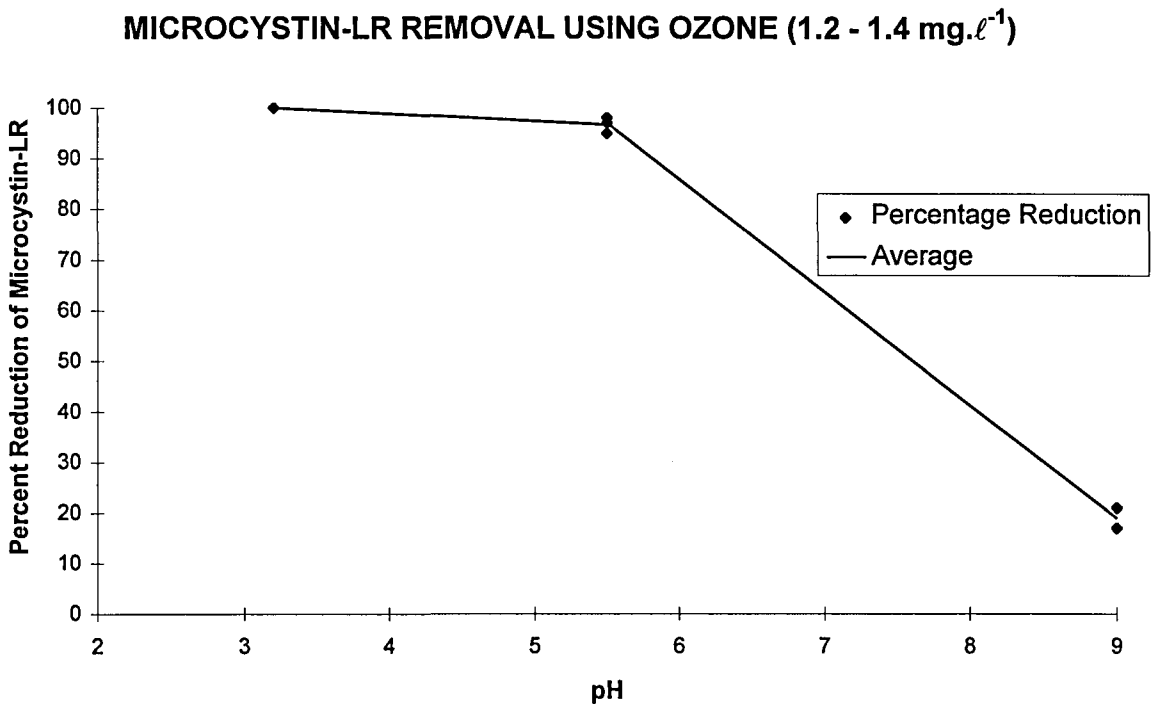


Fig. 10.12 Removal of Microcystin-LR from Camp's Drift raw water, showing higher removal at lower pHs

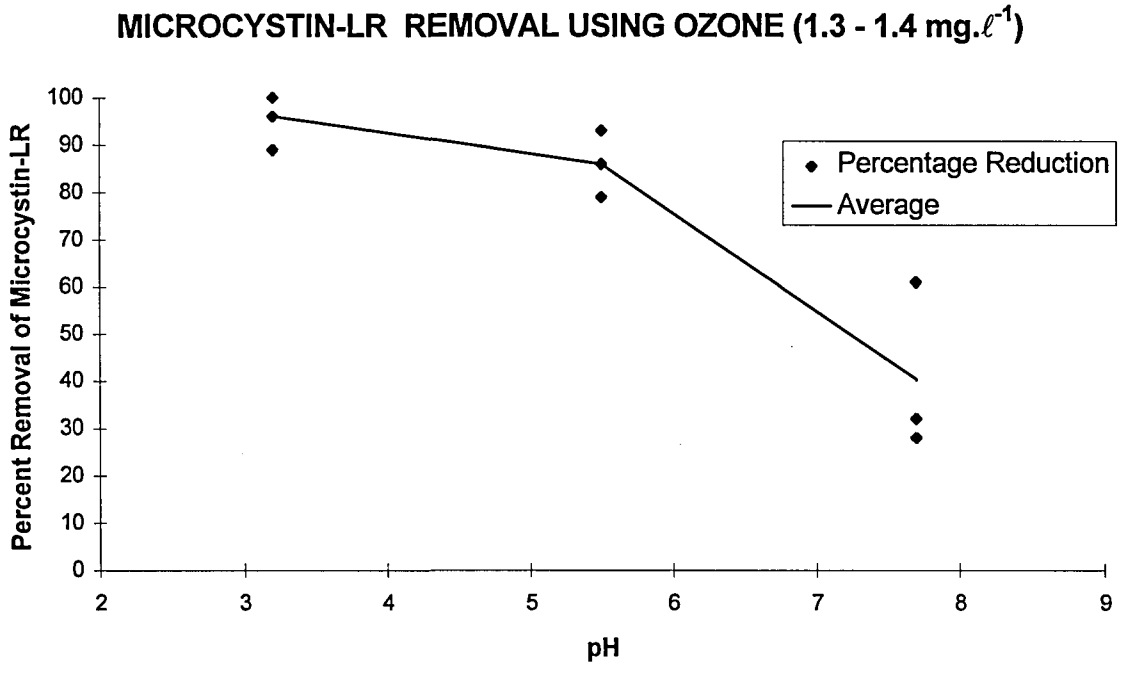


Fig. 10.13 Removal of Microcystin-LR from Inanda water

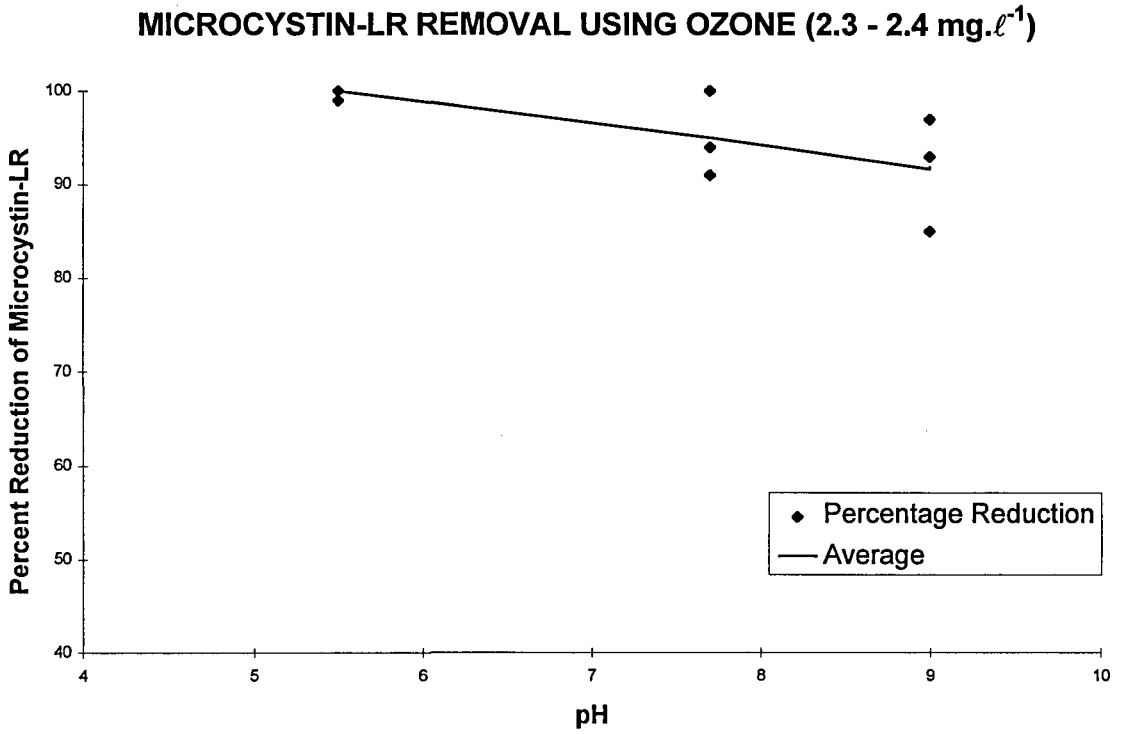


Fig. 10.14 Removal of Microcystin-LR from Camp's Drift raw water, showing higher removal at lower pHs

Removal of other Microcystin Toxins

Toxin reduction from Camp's Drift raw water spiked with a mixed toxin standard are shown in Table 10.2:

Table 10.2 Removal of Microcystin toxins using ozone

Toxin	*Initial Toxin Conc.* $\mu g.\ell^{1}$	*pH*	*Ozone Dose (Effective)* $mg.\ell^{-1}$	*Dose Time* min. / sec	*Final Toxin Conc.* $\mu g.\ell^{1}$	*Percent Reduction*
Microcystin-RR	8.8	7.6	2.2 - 2.4	3 / 10	1.0	89
"	8.8	"	"	"	0.8	91
"	8.8	"	"	"	0	100
"	8.8	"	"	"	0	100
Nodularin	9.6	7.6	2.2 - 2.4	3 / 10	1.1	89
"	9.6	"	"	"	1.0	90
"	9.6	"	"	"	0.2	98
"	9.6	"	"	"	0	100
Microcystin-YR	6.2	7.6	2.2 - 2.4	3 / 10	0	100
"	6.2	"	"	"	0	100
"	6.2	"	"	"	0	100
"	6.2	"	"	"	0	100
Microcystin-LR	8.0	7.6	2.2 - 2.4	3 / 10	0.7	91
"	8.0	"	"	"	0.6	92
"	8.0	"	"	"	0.2	98
"	8.0	"	"	"	0	100

Ozone Residuals

The ozone residual results for the kwaMakutha extract spiked into ultra-pure water and Camp's Drift raw water were as follows:

Table 10.3 Ozone Residuals

Test Matrix	pH	Toxin Spike	Toxin Conc. $\mu g.\ell^{-1}$	Ozone Dose $mg.\ell^{-1}$	Abs	Residual Ozone Conc. $mg.\ell^{-1}$
Ultra-pure	6.7	kwaMakutha	0	0	0.229	-
"	"	"	0	2.2 - 2.4	0.176	0.14
Ultra-pure	6.7	kwaMakutha	7.8	0	0.227	-
"	"	"	7.8	2.2 - 2.4	0.201	0.07
"	"	"	7.7	2.2 - 2.4	0.201	0.07
Camp's Drift	7.7	kwaMakutha	0	0	0.223	-
"	"	"	0	2.2 -2.4	0.174	0.12
Camp's Drift	7.7	kwaMakutha	7.8	0	0.227	-
"	"	"	7.8	2.2 -2.4	0.210	0.05
"	"	"	7.8	2.2 - 2.4	0.212	0.04

10.3.1 Intracellular toxin removal

An Inanda sample was received (4/96) with a *Microcystis* cell concentration of 693 998 cells.$m\ell^{-1}$. Microcystin-LR was detected in the water at a concentration of 2.5 $\mu g.\ell^{-1}$, and the intracellular concentration was determined to be 3.4 $\mu g.\ell^{-1}$ of filtered cells.

Samples containing *Microcystis* cells were spiked with an aliquot of Microcystin-LR standard to produce a total extracellular toxin concentration of 6.8 $\mu g.\ell^{-1}$ and ozonated at 0.4 to 0.42 $mg.\ell^{-1}$. The total extracellular toxin concentration decreased as shown in Table 10.4.

Table 10.4 Ozonation of a toxin containing *Microcystis* sample from Inanda dam

Matrix	*Toxin Std Spiked*	*Total Toxin Conc.*	*pH*	*Ozone Dose*	*Dosage Time*	*Percent Reduction*
		($\mu g.\ell^{-1}$)		*($mg.\ell^{-1}$)*	*sec*	
Inanda	LR Std	6.8	7.7	0.4 - 0.42	30	29
	"	6.8	7.7	"	"	21
	"	6.8	7.7	"	"	1

The intracellular Microcystin-LR concentration was not reduced as shown in Figs. 10.15 and 10.16.

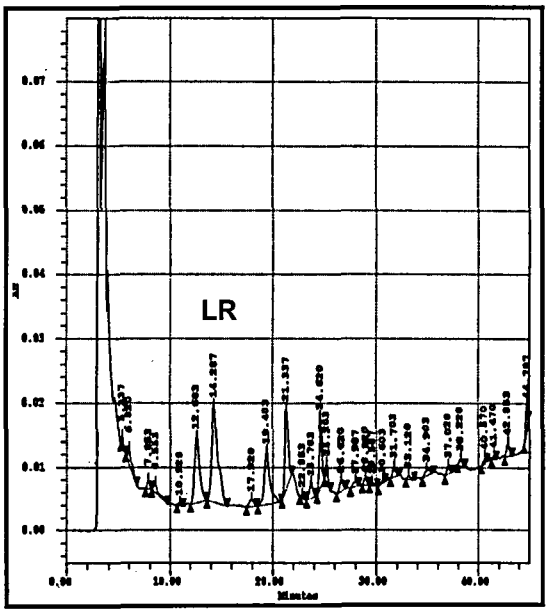


Fig. 10.15 Intracellular toxin analysis of *Microcystis* cells before ozonation

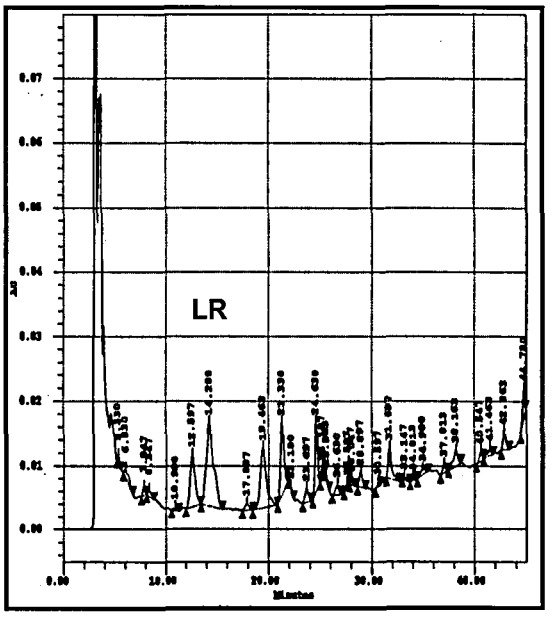


Fig. 10.16 Intracellular toxin analysis *Microcystis* cells after ozonation (O_3 dose : 0.3 mg.ℓ^{-1})

10.4 Discussion

The vast difference in scale between the laboratory ozonation procedure and an actual treatment plant, required that the ozone dose applied be adjusted to account for the amount of ozone escaping the sample ozonating vessel. In an actual treatment plant normally less than 5% of the ozone escapes in the flue gas, whereas with the laboratory scale design approximately 40% was escaping. The results were therefore reported as the effective dose (i.e. the difference between the applied dose and the amount escaping) rather than the applied dose.

The use of the algal extract containing microcystin-LR has been preferred in this investigation as it contains other water soluble organic components that would be associated with a natural *Microcystis* bloom after cell lysis. Based on the extracellular microcystin toxins found during the monitoring period, a toxin concentration of 9μg.ℓ^{-1} was considered a reasonable extreme.

From the results obtained in Tests 1 & 2, it is evident that microcystin toxins spiked into ultra-pure water are effectively removed by ozone. Pure microcystin-LR at concentrations of 16μg.ℓ^{-1} was completely removed at ozone doses of as low as 0.17 mg.ℓ^{-1}. However, with pure toxin spiked into raw water having a pre-spiked DOC of between 2.5 and 3.0 mg.ℓ^{-1}, the toxin was poorly removed (18%) at the normal raw water pH of 7.7. Two factors may have caused the low removal; either the increased DOC of the raw water or the difference in pH between the raw and ultra-pure waters. The actual DOC was not considered critical in these experiments as the test samples were prepared from the same batch of raw water and all were spiked with an equal amount of toxin standard.

In Tests 3, 4 & 5, pHs were adjusted and a trend was observed for the removal of both the pure microcystin-LR as well as the microcystin-LR from samples containing the toxic extract. The removal was more effective at acidic pHs (3.2 and 5.5) than at the normal raw water pH (7.7) or above (9.0 and 10). This trend was confirmed at various ozone doses, as indicated in Tests 6 to 9. A possible explanation for this trend could be that either the ozone is more efficiently used, i.e.

at a faster reaction rate at lower pHs or that a different oxidation mechanism operates and produces more effective oxidation at lower pHs. The literature does report two mechanisms; one involves free radical formation and the other involves an ozonide intermediate at the double bonds.[32,33] However, the cutoff pH at which these two mechanisms operate was not established. Although there is a lack pH data in the literature that could account for any variation in the results obtained, some of the results are similar.

From an economic point of view a compromise between the ozone dose applied and the lowering of pH may be considered as lower ozone doses (1.2mg.ℓ^{-1}) can be used, provided the pH is lowered. At normal raw water pHs (approximately 7.7) an ozone dose of 2.3-2.4 reduced the microcystin-LR concentration (9.0 $\mu g.\ell^{-1}$) from the kwaMakutha extract by 91 to 100%. At the same ozone dose pure microcystin-LR at a concentration of 156 $\mu g.\ell^{-1}$ in Camp's Drift water was reduced by 98%. There was also very little variation in ozone residuals at a similar ozone dose of 2.2 - 2.4 mg.ℓ^{-1}, as shown in Table 14. The recommended microcystin toxin guideline in potable water is 1.0 $\mu g.\ell^{-1}$ in microcystin-LR equivalents. Therefore any remedial method capable of reducing the total toxins concentration below this limit would be considered effective. A mixture of microcystins-RR,-YR,-LR and nodularin having a total concentration of 32 $\mu g.\ell^{-1}$ in Camp's Drift water was reduced to 0 to 2.8 $\mu g.\ell^{-1}$ with an ozone dose of 2.2 to 2.4 mg.ℓ^{-1}.

10.5 Conclusion

pH appears to be an important variable in the treatment of microcystin toxins using ozone. For the same toxin concentrations and DOC, it has been found that higher toxin removal can be achieved at lower pHs.

Pure microcystin-LR, at concentrations as high as 16 $\mu g.\ell^{-1}$, in ultra-pure water was completely removed at an ozone dose of 0.2 mg.ℓ^{-1}. In raw water samples spiked with a toxic *Microcystis* extract, the microcystin-LR concentration of 9.0 $\mu g.\ell^{-1}$ was reduced by 91 to 100 % with an ozone dose of 2.3-2.4 mg.ℓ^{-1}, whereas pure microcystin-LR at a concentration of 156 $\mu g.\ell^{-1}$ was reduced by 98%. A mixture of pure microcystins-RR,-YR,-LR and nodularin having concentrations of 8.8, 6.2, 7.6 and 7.6 $\mu g.\ell^{-1}$, respectively, in Camp's Drift water were reduced by 89-100% at an ozone dose of 2.2-2.4 mg.ℓ^{-1}. At this ozone dose residuals of 0.05 mg.ℓ^{-1} and lower were recorded for the ozonated raw water samples containing the toxic *Microcystis* extract.

For the treatment of microcystin toxins in typical raw water having a pHs of between 7 and 8, an ozone dose of 2.5 to 3 mg.ℓ^{-1} is recommended.

CHAPTER ELEVEN
Remedial Actions: Chlorination

11.1 Introduction

Chlorination is one of the oldest and most widely used disinfection procedures used in the production of potable water. Its long use in the water industry is due to its convenience of use and its ability to maintain a residual which helps the prevent re-growth of algae and other micro-organisms. The negative aspect of the chlorination process, however, is the production of objectionable compounds such as trihalomethanes (THMs) most of which are considered to be carcinogens.

For the removal of microcystin toxins, conventional water treatment processes such as alum coagulation, sand filtration, lime pre-treatment and chlorination have previously been reported to have no effect on the microcystin toxins.[7] However, more recent publications (1994)[30] have indicated that chlorine contact times and pH were factors responsible for the negative results obtained previously, and maintaining a chlorine residual of 0.5mg.ℓ^{-1} for 30 minutes at pH<8 removes microcystin toxins at 99% efficiency. Chlorination is a widely used purification process in South Africa and if the above mentioned statement is verified then there may be minimum risk of consuming microcystin toxins from water treated in this way provided the specific treatment conditions are met.

11.2 Experimental

Chorine in the form of sodium hypochlorite was used in this investigation. A stock solution was prepared by diluting an appropriate volume (usually 1 mℓ) of a concentrated aqueous hypochlorite solution with a free chlorine content of 10-14% (v/w) to one litre. After pH adjustment, the free chlorine concentration was determined by iodometric titration of the stock solution. Potassium iodide (1mg) was added to an aliquot of the stock hypochlorite solution (25.00 mℓ) and titrated against a standardised sodium thiosulphate solution (≅0.005N) to a starch endpoint.

The stock chlorine concentration was determined from the following equation:

$$\text{Conc. } Cl_2 \ (\text{mg}.\ell^{-1}) = \frac{\text{Vol}_{Na_2S_2O_3} \times 35450 \times N_{Na_2S_2O_3}}{\text{Vol}_{NaOCl\ soln}}$$

(volumes in millilitres)

This was done in triplicate and the average value was used as the concentration of the stock solution. A Merck Dital II burette was used for the titration.

The following samples were analysed for microcystin toxins:

Test samples: Filtered raw water (500 mℓ) was spiked with toxin standard and the appropriate volume of the hypochlorite stock solution was then added to produce the desired chlorine concentration. The samples were shaken and allowed to stand for the required contact time, after which an aliquot of sodium thiosulphate solution (2 g/100 mℓ) was added (in excess) to neutralise the chlorine.

Controls: Unspiked raw water was dosed with the same amount of chlorine and allowed to stand for the required contact time. The sample was then dosed with sodium thiosulphate, and an aliquot of toxin standard, equivalent to the test sample, was added.

Blanks: Unspiked raw water samples that were not dosed with chlorine.

The determination of the percentage removal was as described earlier in Chapter 10.

11.2.1 pH Adjustment

The temperature and pH were always recorded. Sodium hydroxide (0.05M) and hydrochloric acid (0.05M) were used to adjust the pH of the test samples. pH was measured using a Beckman pH meter.

11.2.2 Chlorine Residuals

Chlorine residuals were determined using the DPD method. This is a colorimetric method that required the use of a Lovibond 2000 DPD comparator. The procedure involved crushing two DPD tablets into separate 10 mℓ cuvettes; one was made up to the mark with test sample containing chlorine and the other with sample not containing chlorine (blank). Both cuvettes were placed into a comparator and a measurement was taken one minute after mixing. The residual concentration was determined visually by matching the intensity of the pink colouration of the test solution to that of the comparator.

11.2.3 Trihalomethane Analysis

Trihalomethanes (THM) were analysed by gas chromatography using a Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector (ECD). Direct aqueous injections were made onto a DB-624 column with an injector port temperature of 80°C. The oven temperature was programmed to remain at 80°C for 3 minutes then increased by 2°C.min^{-1} until 100°C, which was maintained for a further 3 minutes.

Tests were done on chlorinated and non-chlorinated samples that were spiked with either the pure toxin or the toxin-containing extract. Controls were also done using unspiked samples. Excess sodium thiosulphate was added after the specified contact time to prevent further THM formation whilst awaiting analysis. Four major THMs were analysed, namely, chloroform, bromoform, dichlorobromomethane and dibromochloromethane. The individual THM concentrations were added and submitted as a total value.

11.3 Results

Examples of the chromatographic results showing the removal of the microcystin toxins from Camp's Drift raw water are shown in Figs 11.1 to 11.8.

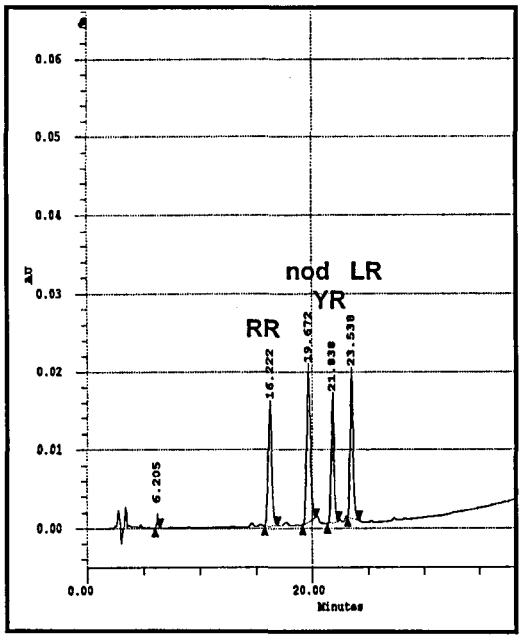


Fig. 11.1 Mixed Toxin Standard

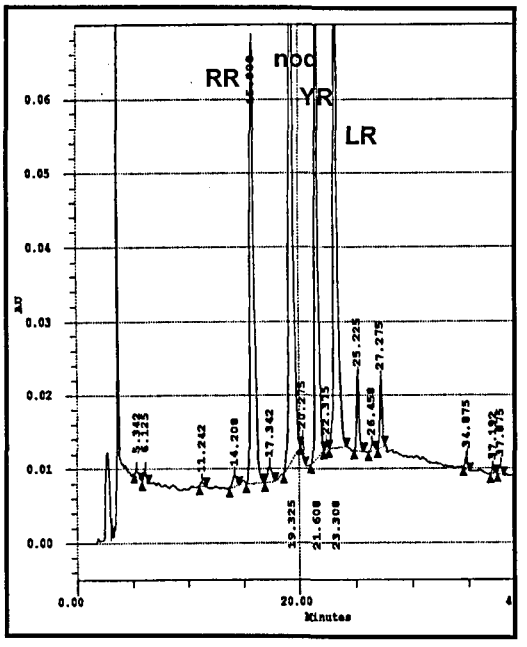


Fig. 11.2 Control at pH 5.5

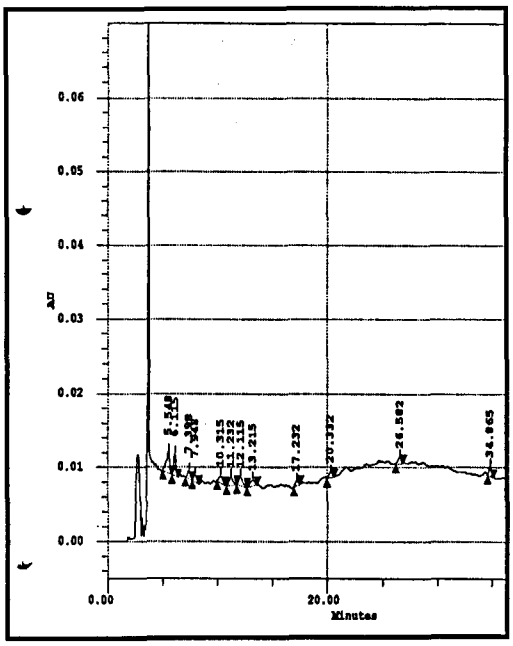


Fig. 11.3 Mixed Toxin Sample at pH 5.5, after chlorination at (2 mg.ℓ^{-1}), Contact time: 30 min.

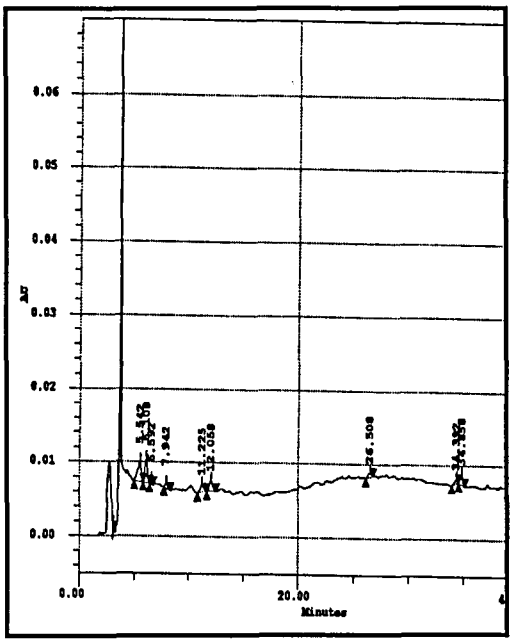


Fig. 11.4 Mixed Toxin Sample at pH 5.5, after chlorination (4 mg.ℓ^{-1}), Contact time: 30 min.

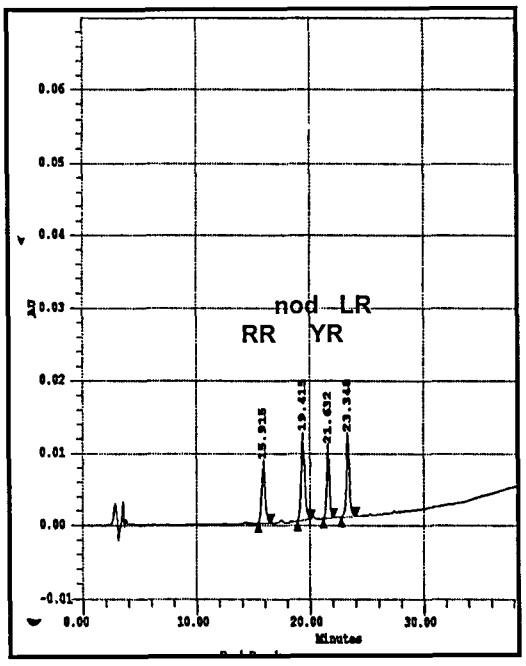


Fig. 11.5 Mixed Toxin Standard

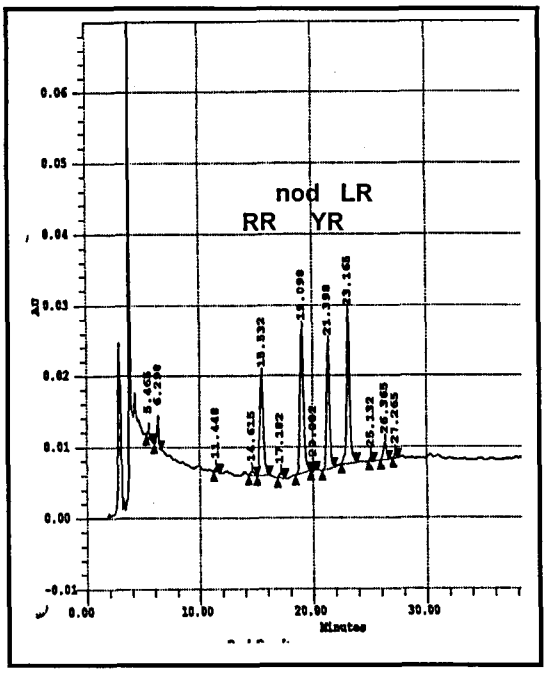


Fig. 11.6 Control at pH 7.7

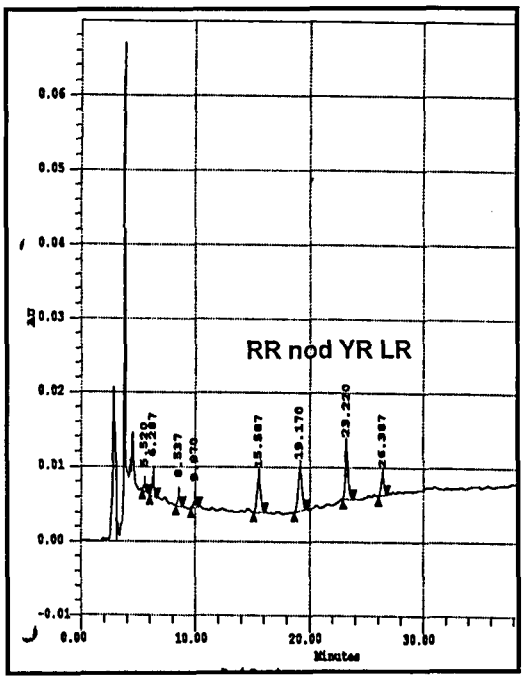


Fig. 11.7 Mixed Toxin Sample at pH 7.7, after chlorination at (3 mg.ℓ^{-1}), Contact time: 10 min.

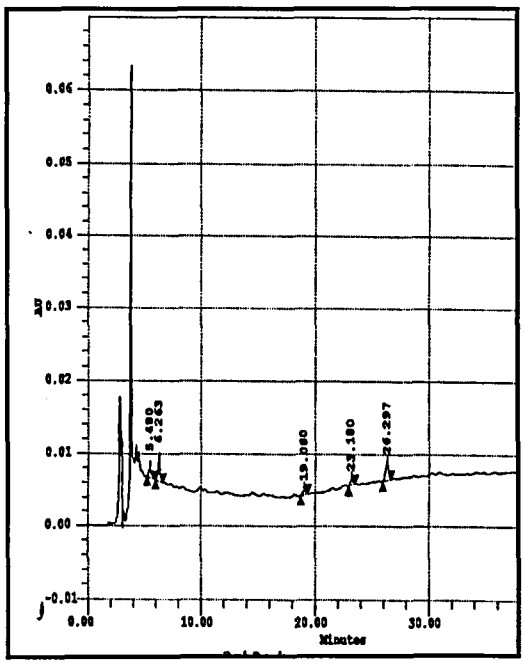


Fig. 11.8 Mixed Toxin Sample at pH 7.7, after chlorination (3 mg.ℓ^{-1}), Contact time: 20 min.

11.3.1 Microcystin toxin removal

The chlorinated removal of the microcystin toxins from Camp's Drift raw water are given in Table 11.1

Table 11.1 Microcystin toxin removal by chlorination

Test	Toxin Spike	Initial Toxin Conc. µg.ℓ^{-1}	pH	Temp °C	Cl_2 Dose mg.ℓ^{-1}	Contact Time min.	Cl_2 Residual mg.ℓ^{-1}	Final Toxin Conc. µg.ℓ^{-1}	Percent Removal
1	LR Std	3.3	5.5	21.0	1.1	30	0.4	0	100
	"	3.3	5.5	"	"	"		0.1	97
	"	3.3	5.5	"	"	"		0.1	98
2	kwa Makutha	7.4(LR)	5.5	20.0	1.0	30	0.4	5.5	26
	"	7.4(LR)	5.5	"	"	"		2.5	62
	"	7.4(LR)	5.5	"	"	"		2.8	66
	"	7.4(LR)	5.5	"	2.0	"	1.6	0	100
	"	7.4(LR)	5.5	"	"	"		0	100

Table 11.1 cont. Microcystin toxin removal by chlorination

Test	Toxin Spike	Toxin Conc. $\mu g.\ell^{-1}$	pH	Temp °C	Cl_2 Dose $mg.\ell^{-1}$	Contact Time min.	Cl_2 Residual $mg.\ell^{-1}$	Final Toxin Conc. $\mu g.\ell^{-1}$	Percent Reduction
3	Mix Std	2.6 (LR)	5.5	20.5	2.0	30	1.7	0	100
	"	2.6 (LR)	5.5	"	"	"		0	100
	"	3.2 (RR)	5.5	"	2.0	"		0	100
	"	3.2 (RR)	5.5	"	"	"		0	100
	"	3.1 (nod)	5.5	"	2.0	"		0	100
	"	3.1 (nod)	5.5	"	"	"		0	100
	"	1.9 (YR)	5.5	"	2.0	"		0	100
	"	1.9 (YR)	5.5	"	"	"		0	100
	"	2.6 (LR)	5.5	"	4.0	"	3-4	0	100
	"	2.6 (LR)	5.5	"	"	"		0	100
	"	3.2 (RR)	5.5	"	4.0	"		0	100
	"	3.2(RR)	5.5	"	"	"		0	100
	"	3.1 (nod)	5.5	"	4.0	"		0	100
	"	3.1 (nod)	5.5	"	"	"		0	100
	"	1.9 (YR)	5.5	"	4.0	"		0	100
	"	1.9 (YR)	5.5	"	"	"		0	100
4	kwa Makutha	7.1(LR)	7.7	19.6	2.0	30	1.0	1.3	81
	"	7.1(LR)	7.7	"	"	"		0.6	92
	"	7.1(LR)	7.7	"	"	"		0.5	93
	"	7.1(LR)	7.7	"	"	"		0.2	97
	"	7.1(LR)	7.7	"	4.0	"	3-4	0	100
	"	7.1(LR)	7.7	"	"	"		0	100
	"	7.1(LR)	7.7	"	"	"		0	100
	"	7.1(LR)	7.7	"	"	"		0	100
5	Mix Std	1.6 (LR)	7.7	20.5	3.0	10	2-3	0.5	70
	"	1.6 (LR)	7.7	"	"	"		0.7	58
	"	1.6 (LR)	7.7	"	"	"		0.7	59
	"	1.6 (RR)	7.7	"	"	10	2-3	0.5	69
	"	1.6 (RR)	7.7	"	"	"		0.7	59
	"	1.6 (RR)	7.7	"	"	"		0.7	56
	"	1.8 (nod)	7.7	"	"	10	2-3	0.5	71
	"	1.8 (nod)	7.7	"	"	"		0.7	63
	"	1.8 (nod)	7.7	"	"	"		0.8	54
	"	1.1 (YR)	7.7	"	"	10		0	100
	"	1.1 (YR)	7.7	"	"	"		0	100
	"	1.1 (YR)	7.7	"	"	"		0	100

Test	Toxin Spike	Toxin Conc. $\mu g.\ell^{-1}$	pH	Temp °C	Cl_2 Dose $mg.\ell^{-1}$	Contact Time min.	Cl_2 Residual $mg.\ell^{-1}$	Final Toxin Conc. $\mu g.\ell^{-1}$	Percent Reduction
5	Mix Std	1.6 (LR)	7.7	20.5	3.0	20	2-3	0.1	97
	"	1.6 (LR)	7.7	"	"	"		0.1	97
	"	1.6 (LR)	7.7	"	"	"		0.1	96
	"	1.6 (RR)	7.7	"	"	20	1.8	0	100
	"	1.6 (RR)	7.7	"	"	"		0	100
	"	1.6 (RR)	7.7	"	"	"		0	100
	"	1.8 (nod)	7.7	"	"	20	1.8	0.1	97
	"	1.8 (nod)	7.7	"	"	"		0.1	97
	"	1.8 (nod)	7.7	"	"	"		0.1	97
	"	1.1 (YR)	7.7	"	"	20		0	100
	"	1.1 (YR)	7.7	"	"	"		0	100
	"	1.1 (YR)	7.7	"	"	"		0	100
6	LR Std								
		7.2	10.2	21.3	1.0	30		7.4	0
	"	7.2	10.2	"	"	"		7.0	3
	"	7.2	10.2	"	"	"		7.9	0
	"	7.2	10.2	"	2.0	"		6.7	7
	"	7.2	10.2	"	"	"		6.4	10
	"	7.2	10.2	"	"	"		6.9	3
	"	7.2	10.2	"	"	"		6.4	10
7	kwa Makutha	6.8(LR)	10.1	20.4	2.0	30	1.0	6.0	12
	"	6.8(LR)	10.1	"	"	"		5.5	20
	"	6.8(LR)	10.1	"	"	"		5.4	21
	"	6.8(LR)	10.1	"	4.0	"	3-4	3.9	43
	"	6.8(LR)	10.1	"		"		3.5	49
	"	6.8(LR)	10.1	"	"	"		3.0	56
8	Mixed Std	1.3 (LR)	10.2	21.0	8.0	30	>4	0.5	63
	"	1.3 (LR)	10.2	"	"	"		0.1	94
	"	1.3 (LR)	10.2	"	"	"		0.1	94
	"	1.3 (LR)	10.2	"	"	"		0.1	95
	"	1.1 (RR)	10.2	"	"	"		0.3	70
	"	1.1 (RR)	10.2	"	"	"		0.1	95
	"	1.1 (RR)	10.2	"	"	"		0.1	93
	"	1.1 (RR)	10.2	"	"	"		0.1	94
	"	1.1 (nod)	10.2	"	"	"		0.1	92
	"	1.1 (nod)	10.2	"	"	"		0.2	85
	"	1.1 (nod)	10.2	"	"	"		0.2	63
	"	1.1 (nod)	10.2	"	"	"		0.6	84
	kwa Makutha	6.5(LR)	10.2	21.0	12	30		1.8	72
	"	6.5(LR)	10.2	"	"	"		2.3	64

"	6.5(LR)	10.2	"	"	"	2.3	64
"	6.5(LR)	10.2	"	"	"	2.8	42

11.3.2 pH Trend

The graph below shows the removal of the microcystin-LR from the samples containing the kwaMakutha extract at various pHs.

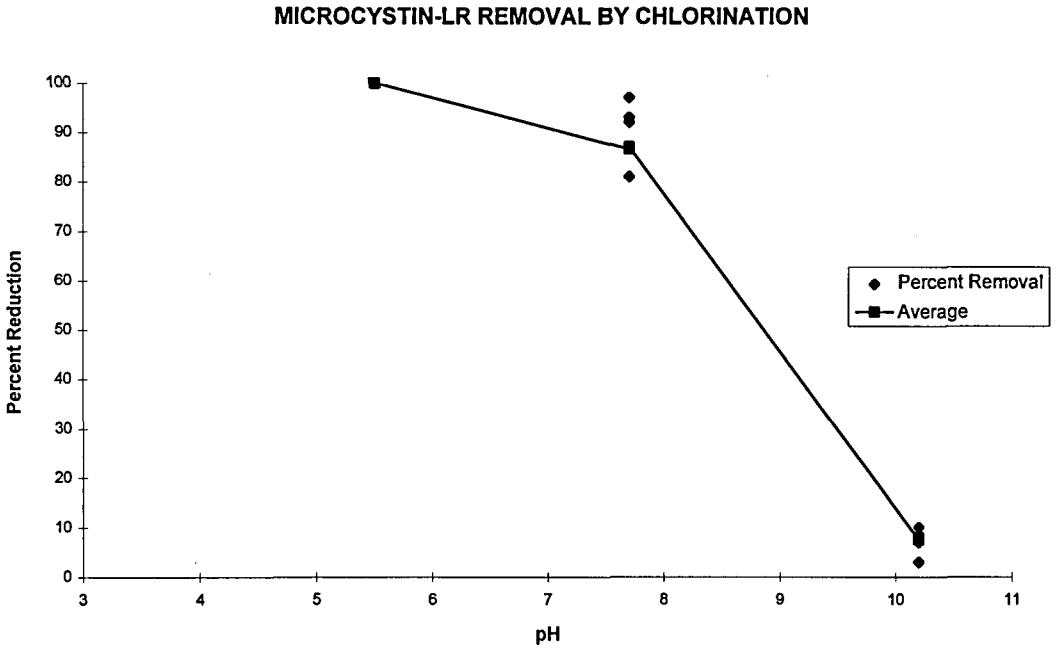


Fig. 11.9. pH trend for the removal of microcystin-LR by chlorination (Cl_2 dose : 2 mg.ℓ^{-1})

11.3.3 Trihalomethane Formation

The following table indicates the level of THM formed at various chlorine doses and pHs :

Table 11.2 THM formation after 30 minutes

Test Water	*Temp* °C	*pH*	*Toxin Spike*	*Toxin Conc.* $\mu g.l^{-1}$	*Chlorine Dose* $mg.l^{-1}$	*Total THM* $\mu g.l^{-1}$
Camp's Drift	20.0	5.5	kwa Makutha	0	0	1.2
"	"	"	"	0	2.0	4.8
"	"	"	"	7.4(LR)	0	<0.8
"	"	"	"	7.4(LR)	2.0	4.8
				7.4(LR)	2.0	5.4
Camp's Drift	20.5	5.5	Mixed Std	0	0	<0.8
"	"	"		0	2.0	<0.8
"	"	"		3.2(RR), 3.1(nod), 1.9(YR), 2.6(LR)	0	<0.8
"	"	"		3.2(RR), 3.1(nod), 1.9(YR), 2.6(LR)	2.0	<0.8
"	"	"		3.2(RR), 3.1(nod), 1.9(YR), 2.6(LR)	2.0	<0.8

Table 11.2 cont. THM formation after 30 minutes

Test Water	*Temp* °C	*pH*	*Toxin Spike*	*Toxin Conc.* $\mu g.\ell^{-1}$	*Chlorine Dose* $mg.\ell^{-1}$	*Total THM* $\mu g.\ell^{-1}$
Camp's Drift	19.6	7.7	kwa Makutha	0	0	<0.8
"	"	"	"	0	4.0	13.8
"	"	"	"	7.1(LR)	0	<0.8
"	"	"	"	7.1(LR)	4.0	17.6
"	"	"	"	7.1(LR)	4.0	10
Camp's Drift	20.4	10.0	kwa Makutha	0	0	<0.8
"	"	"	"	0	2.0	10.4
"	"	"	"	6.8(LR)	0	<0.8
"	"	"	"	6.8(LR)	2.0	14.3
Camp's Drift	21.9	10.2	Mixed Std	0	0	0.1
"	"	"	"	0	8.0	31.8
"	"	"	"	1.1(nod), 1.5(YR), 1.3(LR)	0	<0.8
"	"	"	"	1.1(nod), 1.5(YR), 1.3(LR)	8.0	36.1
"	"	"	"	1.1(nod), 1.5(YR), 1.3(LR)	6.0	31.5
Camp's Drift	21.0	10.2	Mixed Std	0	0	1.3
				0	8	18.2
				1.6(RR), 1.8(nod), 1.1(YR), 1.6(LR)	0	1.7
				1.6(RR), 1.8(nod), 1.1(YR), 1.6(LR)	8	27
				1.6(RR), 1.8(nod), 1.1(YR), 1.6(LR)	8	29
Camp's Drift	21.9	10.2	kwa Makutha	0	0	<0.8
"	"	"	"	0	8.0	135
"	"	"	"	7.0(LR)	0	<0.8
"	"	"	"	7.0(LR)	6.0	124
"	"	"	"	7.0(LR)	8.0	139

11.4 Discussion

In Test 1, a chlorine dose of 1.1 $mg.\ell^{-1}$ reduced pure microcystin-LR at a concentration of 3.3 $\mu g.\ell^{-1}$ in raw water, at pH 5.5, by 97 to 100%, showing that chlorine can effectively remove the toxin in raw water. However, a similar chlorine dose reduced the microcystin-LR (7.4 $\mu g.\ell^{-1}$) from the samples containing the kwaMakutha extract by less than 66%. Possible explanations for the

lower removal could be the higher toxin concentration in the extract-containing sample or that the presence of the other organics in the extract competed for the oxidant.
Using the microcystin-LR containing extract in raw water and an increased chlorine dose of 2.0 $mg.\ell^{-1}$, complete removal was achieved at pH 5.5. At pH 7.7 the toxin was removed by 81 to 97%, and 12 to 21% removal at pH 10.7, indicating that the removal of the toxin varied according to pH, as shown in Fig 95 .
A chlorine dose of 4.0 $mg.\ell^{-1}$ completely removed the microcystin-LR at pH 7.7 showing that this dose was adequate at the natural raw test water pH. Various contact times were investigated using an intermediate chlorine dose of 3.0 $mg.\ell^{-1}$ and the mixed toxin standard at pH 7.7. Microcystin-YR was completely removed after 10 minutes, while the rest of the toxins were reduced by 54 to 70%. After 20 minutes, microcystins-LR,-RR and nodularin were all reduced by 96 to 100%, indicating that a contact time of 30 minutes would be adequate.
At the elevated pH of 10.2, toxin removal was relatively poor (43 to 56%) at a chlorine dose of 4 $mg.\ell^{-1}$ despite having a chlorine residual of 3-4 $mg.\ell^{-1}$ after 30 minutes. A higher chlorine dose of 8.0 $mg.\ell^{-1}$ produced 63 to 94 % removal of the pure mixed toxins, indicating that even higher doses may be required for the removal of the microcystin-LR from the extract-containing samples. Chlorine concentrations as high as 12.0 $mg.\ell^{-1}$ only produced 42 to 72 % removal of the microcystin-LR, indicating that chlorine concentrations in excess of 12.0 $mg.\ell^{-1}$ will have to be dosed to achieve complete removal.
At a chlorine dose of 8.0 $mg.\ell^{-1}$ and samples spiked with mixed toxins, the total THM formation reached 36 $mg.\ell^{-1}$, which is extremely high in any treatment works. Because of the serious health risk associated with these compounds, many water authorities have strict regulations. According to Umgeni Water guidelines, the maximum permissible THM level is 460 $mg.\ell^{-1}$. At the natural pH of 7.7, and a chlorine dose of 4.0 $mg.\ell^{-1}$ produced a total THM concentration of 10 to 17 $\mu g.\ell^{-1}$, which is well below the maximum permissible concentration.
From the results in Table 11.2 , there was no trend to establish whether the THMs formed were produced from the reactions with the toxins themselves or from reactions with organics contained in the raw water. However, the increase in total THM formation was consistent with an increase in chlorine dose. It is also likely that chlorinated compounds, other than those determined in this exercise, were formed by the reaction of the toxins with chlorine. The nature and toxicity of the chlorinated toxin products at this stage is yet unknown as no reference has been found in the literature.

11.5 Conclusion

The removal of microcystin toxins from raw water by chlorination was found to be pH dependant. For the same chlorine dose, higher removal was obtained from test samples with slightly acidic pHs than from samples with alkaline pHs. At pH 5, a chlorine dose of 2.0 $mg.\ell^{-1}$ was required for complete removal of microcystin toxins having concentrations of 1.9 to 3.2 $\mu g.\ell^{-1}$, for the pure toxins, and 7.4 $\mu g.\ell^{-1}$ for the *Microcystis* extract-containing samples. At the natural test water pH of 7.7 and a contact time of 30 minutes, a chlorine dose of 4.0 $mg.\ell^{-1}$ produced complete removal of microcystin-LR at a concentration of 7.4 $\mu g.\ell^{-1}$, from the samples dosed with a toxic algal extract. The total THM formation under these conditions were between 10 and 18 $\mu g.\ell^{-1}$. At pH 10, a chlorine dose of 12 $mg.\ell^{-1}$ produced 42 to 72 % removal of microcystin-LR at a concentration of 6.5 $\mu g.\ell^{-1}$. Chlorine doses of greater than 12.0 $mg.\ell^{-1}$ would therefore be required for complete toxin removal, but the THM formation would need to be monitored at this dose.

CHAPTER TWELVE
Remedial Actions: Activated Carbon

12.1 Introduction

Powdered activated carbons (PACs) are generally usually used in water treatment to remove taste and odour compounds such as geosmin, 2-methylisoborneol and other potentially harmful organic compounds. Removal is accomplished by the adsorption of the troublesome compounds onto the carbon surface. For the removal of microcystin toxins, earlier investigations (1976)[7] showed that PAC doses of 8 and 80 mg.ℓ^{-1} failed to remove toxicity, but toxin concentrations of 10 mg.ℓ^{-1}, used in these experiments, was extremely high, implying that these PAC doses were not adequate. Using toxin concentrations of 30 to 58 μg.ℓ^{-1}, Himberg, *et al.* (1989) found that a PAC dose of 5 mg.ℓ^{-1} in combination with alum coagulation, sand filtration and chlorination, produced 12 to 34 % removal of the toxins. Finnish pilot plant trials produced 99% toxin removal of the toxins whereas an Australian study using frozen toxic *Microcystis* showed that PAC doses of 1 to 10 g.ℓ^{-1} were required to completely remove acute toxicity.[7]

Donati (1994), indicated that the wood based carbons are the most effective for the removal of microcystin toxins, and with the best carbon, a dose of 25 mg.ℓ^{-1} was found to reduce microcystin-LR (50 μg.ℓ^{-1}) from raw water by more than 98%.[7] In this study four different types of carbons were tested namely , Sutcliffe WF (wood source) , Sutcliffe 207CP (coal source), Zetachem (wood source) and Aktacarb PWT (wood source). Additional specifications on the carbons used are found in Appendix H. Toxin removal was tested from two different raw water matrices; one was of generally poor quality (Camp's Drift) and the other was relatively clear and of good quality (DV Harris).

12.2 Experimental

The powdered activated carbon was kept at 150°C and placed into a dessicator at least 30 minutes prior to its use. The activated carbon (approximately 1 gram) was weighed out accurately and added to ultra-pure water (1ℓ) whilst stirring to allow for thorough mixing. All the samples (500 mℓ) were spiked with the same amount of toxin standard, either the mixed toxins or the extract. Two samples were mixed into a 1ℓ beaker and placed on a multiple jar stirrer (Phipps & Bird). and stirred at 124 to 130 rpm. The samples were dosed with the appropriate quantity of carbon slurry and allowed to mix for 30 minutes. The samples were then separated into two 500 mℓ portions after which, they were filtered through Whatmann GF/C filters and analysed for toxins.

Blanks consisted of unspiked raw water that was not dosed with activated carbon, and controls were spiked with the same amount of toxin as the test sample but not dosed with carbon.

Toxin recoveries and percent reduction were determined as described earlier in Chapter 10.

12.3 Results

The chromatograms shown below (Figs. 12.1 to 12.4) depict the removal of microcystin toxins from DV Harris raw water using the Sutcliffe WF PAC

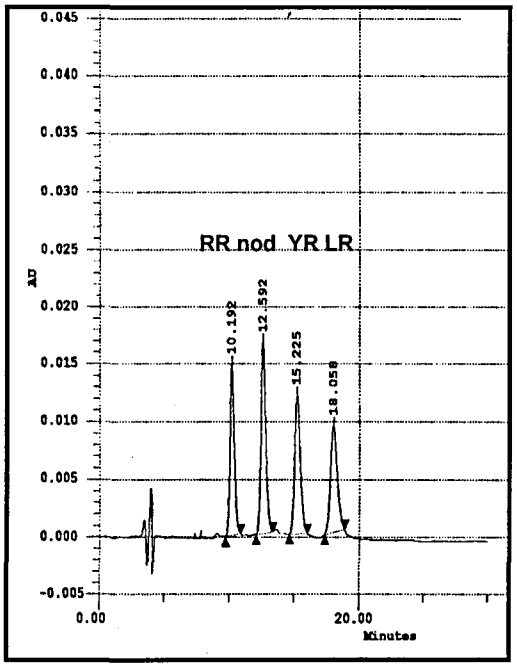


Fig. 12.1 Mixed Toxin Standard

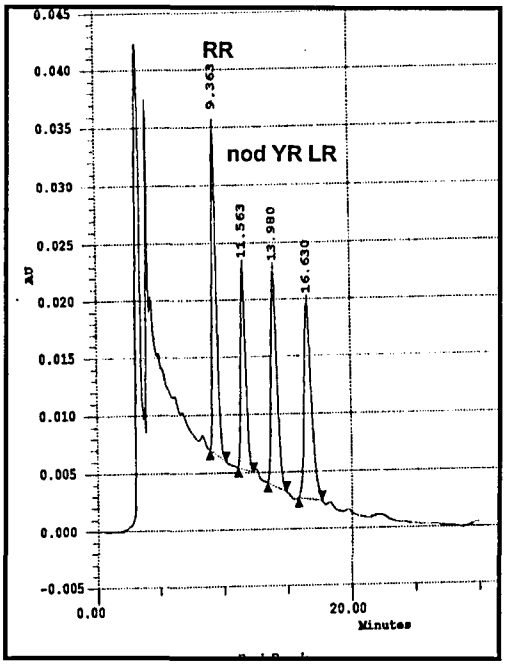


Fig. 12.2 Control : DV Harris water

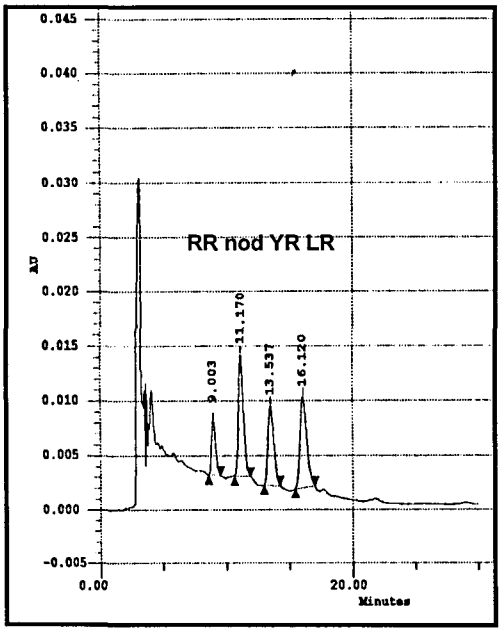


Fig. 12.3 Toxin removal using Sutcliffe WF PAC (Dose 10 mg.ℓ^{-1})

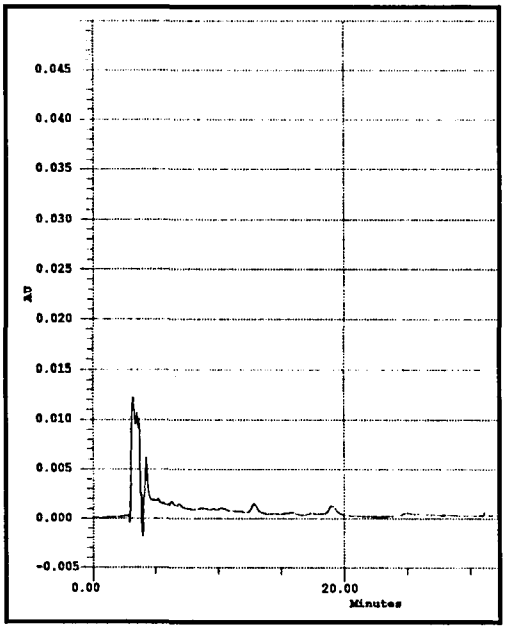


Fig. 12.4 Toxin Removal using Sutcliffe WF PAC (Dose 20 mg.ℓ^{-1})

The removal of microcystin toxins using various types of PACs are given in Table 12.1.

Table 12.1 Microcystin toxin removal by PAC

Test	*Test Water*	*Temp* °C	*PAC*	*Toxin Spike*	*Initial Toxin Conc.* $\mu g.\ell^{-1}$	*PAC Dose* $mg.\ell^{-1}$	*Final Toxin Conc.* $\mu g.\ell^{-1}$	*Percent Removal*
1	Camp's Drift	20.0	**Sutcliffe WF**	kwa Makutha	6.9	5	4.1	41
	"	"	"	"	6.9	"	3.7	46
	"	"	"	"	6.9	"	3.8	43
	"	"	"	"	6.9	6	2.8	60
	"	"	"	"	6.9	"	3.0	56
	"	"	"	"	6.9	10	0.2	98
	"	"	"	"	6.9	"	0.2	98
	"	"	"	"	6.9	"	0.5	93
	"	"	"	"	6.9	"	0.3	95
	"	"	"	"	6.9	20	0	100
	"	"	"	"	6.9	"	0	100
	"	"	"	"	6.9	"	0.3	96
	"	"	"	"	6.9	"	0.1	99
2	Camp's Drift	21.2	**Zetachem**	Mix Std	3.2 (LR)	5	1.6	51
	"	"	"	"	3.2 LR)	"	1.9	40
	"	"	"	"	3.0 (nod)	"	1.4	53
	"	"	"	"	3.0 (nod)	"	1.8	41
	"	"	"	"	2.4 (YR)	"	0.9	61
	"	"	"	"	2.4 (YR)	"	1.2	55
	"	"	"	"	3.2 (LR)	10	0.6	81
	"	"	"	"	3.2 (LR)	"	0.8	76
	"	"	"	"	3.0 (nod)	"	0.7	77
	"	"	"	"	3.0 (nod)	"	0.8	73
	"	"	"	"	2.4 (YR)	"	0.3	89
	"	"	"	"	2.4 (YR)	"	0.3	86
	"	"	"	"	3.2 (LR)	20	0.3	91
	"	"	"	"	3.2 (LR)	"	0	100
	"	"	"	"	3.0 (nod)	"	0.5	83
	"	"	"	"	3.0 (nod)	"	0	100
	"	"	"	"	2.4 (YR)	"	0.1	95
	"	"	"	"	2.4 (YR)	"	0	100
	"	"	"	Kwa Makutha	7.0	5	6.3	9
	"	"	"	"	7.0	"	6.7	4
	"	"	"	"	7.0	10	3.5	50
	"	"	"	"	7.0	"	3.8	46
	"	"	"	"	7.0	20	0.5	92
	"	"	"	"	7.0	"	0.5	93

Table 12.1 cont. Microcystin toxin removal by PAC

Test	*Test Water*	*Temp* ℃	*PAC*	*Toxin Spike*	*Initial Toxin Conc.* $\mu g.\ell^{-1}$	*PAC Dose* $mg.\ell^{-1}$	*Final Toxin Conc.* $\mu g.\ell^{-1}$	*Percent Removal*
3	Camp's Drift	23.6	**Actacarb PWT**	Mixed Std	3.3(LR)	5	2.9	12
	"	"	"	"	3.3 (LR)	"	2.9	11
	"	"	"	"	3.2 (nod)	"	2.1	35
	"	"	"	"	3.2 (nod)	"	1.9	42
	"	"	"	"	2.9 (YR)	"	2.6	16
	"	"	"	"	2.9 (YR)	"	2.4	12
	"	"		"	3.3 (LR)	10	2.8	14
	"	"	"	"	3.3 (LR)	"	2.9	11
	"	"	"	"	3.2 (nod)	"	1.8	45
	"	"	"	"	3.2 (nod)	"	2.5	24
	"	"	"	"	2.9 (YR)	"	2.5	14
	"	"	"	"	2.9 (YR)	"	2.5	15
	"	"	"	"	3.3 (LR)	20	1.5	55
	"	"	"	"	3.3 (LR)	"	1.6	50
	"	"	"	"	3.2 (nod)	"	1.5	54
	"	"	"	"	3.2 (nod)	"	1.8	45
	"	"	"	"	2.9 (YR)	"	0.9	70
	"	"	"	"	2.9 (YR)	"	1.0	65
	"	"	"	Kwa Makutha	6.2	5	4.4	29
	"	"	"	"	6.2	"	4.0	36
	"	"	"	"	6.2	10	4.0	36
	"	"	"	"	6.2	"	2.8	55
	"	"	"	"	6.2	20	0.4	94
	"	"	"	"	6.2	"	1.2	81
4	Camp's Drift	23.7	**Sutcliffe 207CP**	Mixed Std	3.3 (LR)	5	3.0	7
	"	"	"	"	3.3 (LR)	"	2.5	22
	"	"	"	"	3.2 (nod)	"	2.9	10
	"	"	"	"	3.2 (nod)	"	3.1	3
	"	"	"	"	2.9 (YR)	"	2.6	10
	"	"	"	"	2.9 (YR)	"	2.3	21
	"	"	"	"	3.3 (LR)	10	2.1	34
	"	"	"	"	3.3 (LR)	"	2.2	30
	"	"	"	"	3.2 (nod)	"	3.0	6
	"	"	"	"	3.2 (nod)	"	2.0	38
	"	"	"	"	2.9 (YR)	"	1.9	35
	"	"	"	"	2.9 (YR)	"	1.9	35
	"	"	"	"	3.3 (LR)	20	1.5	55
	"	"	"	"	3.3 (LR)	"	1.6	51
	"	"	"	"	3.2 (nod)	"	1.6	49
	"	"	"	"	3.2 (nod)	"	2.0	37
	"	"	"	"	2.9 (YR)	"	0.6	80
	"	"	"	"	2.9 (YR)	"	0.8	77

Table 12.1 cont. Microcystin toxin removal by PAC

Test	*Test Water*	*Temp °C*	*PAC*	*Toxin Spike*	*Initial Toxin Conc. µg.ℓ⁻¹*	*PAC Dose mg.ℓ⁻¹*	*Final Toxin Conc. µg.ℓ⁻¹*	*Percent Removal*
4	Camp's Drift	23.7	**Sutcliffe 207CP**	Kwa Makutha	6.2	5	5.2	17
	"	"	"	"	6.2	"	5.1	18
	"	"	"	"	6.2	10	5.0	19
	"	"	"	"	6.2	"	4.1	34
	"	"	"	"	6.2	25	3.8	38
	"	"	"	"	6.2	"	3.7	41
5	DV Harris	23.7	**Aktacarb PWT**	Mixed Std	3.1 (LR)	5	2.9	6
	"	"	"	"	3.1 (LR)	"	2.6	14
	"	"	"	"	2.9 (nod)	"	3.5	0
	"	"	"	"	2.9 (nod)	"	2.1	29
	"	"	"	"	2.6 (YR)	"	2.2	15
	"	"	"	"	2.6 (YR)	"	1.9	28
	"	"	"	"	3.4 (RR)	"	1.6	52
	"	"	"	"	3.4 (RR)	"	1.8	46
	"	"	"	"	3.1 (LR)	10	2.4	21
	"	"	"	"	3.1 (LR)	"	1.8	40
	"	"	"	"	2.9 (nod)	"	2.7	10
	"	"	"	"	2.9 (nod)	"	2.2	24
	"	"	"	"	2.6 (YR)	"	1.6	38
	"	"	"	"	2.6 (YR)	"	1.3	50
	"	"	"	"	3.4 (RR)	"	1.1	68
	"	"	"	"	3.4 (RR)	"	0.8	77
	"	"	"	"	3.1 (LR)	25	0.7	78
	"	"	"	"	3.1 (LR)	"	0.7	77
	"	"	"	"	2.9 (nod)	"	0.9	71
	"	"	"	"	2.9 (nod)	"	0.8	72
	"	"	"	"	2.6 (YR)	"	0.1	96
	"	"	"	"	2.6 (YR)	"	0.3	90
	"	"	"	"	3.4 (RR)	"	0.1	96
	"	"	"	"	3.4 (RR)	"	0	100
	"	"	"	Kwa Makutha	6.2	5	4.5	9
	"	"	"	"	6.2	"	4.2	15
	"	"	"	"	6.2	10	3.7	25
	"	"	"	"	6.2	"	3.7	26
	"	"	"	"	6.2	25	1.3	68
	"	"	"	"	6.2	"	1.6	73

Test	*Test Water*	*Temp* °C	*PAC*	*Toxin Spike*	*Initial Toxin Conc.* $\mu g.\ell^{-1}$	*PAC Dose* $mg.\ell^{-1}$	*Final Toxin Conc.* $\mu g.\ell^{-1}$	*Percent Removal*
6	DV Harris	23.7	**Sutcliffe WF**	Mixed Std	2.7 (LR)	5	1.1	59
	"	"	"	"	2.7 (LR)	"	0.8	71
	"	"	"	"	3.0 (nod)	"	1.0	67
	"	"	"	"	3.0 (nod)	"	0.6	81
	"	"	"	"	2.5 (YR)	"	0.5	80
	"	"	"	"	2.5 (YR)	"	0.3	87
	"	"	"	"	3.2 (RR)	"	0.2	94
	"	"	"	"	3.2 (RR)	"	0.2	95
	"	"	"	"	2.7 (LR)	10	0	100
	"	"	"	"	2.7 (LR)	"	0	100
	"	"	"	"	3.0 (nod)	"	0	96
	"	"	"	"	3.0 (nod)	"	0	100
	"	"	"	"	2.5 (YR)	"	0	100
	"	"	"	"	2.5 (YR)	"	0	100
	"	"	"	"	3.2 (RR)	"	0	100
	"	"	"	"	3.2 (RR)	"	0	100
	"	"	"	"	2.7 (LR)	25	0	100
	"	"	"	"	2.7 (LR)	"	0	100
	"	"	"	"	3.0 (nod)	"	0	100
	"	"	"	"	3.0 (nod)	"	0	100
	"	"	"	"	2.5 (YR)	"	0	100
	"	"	"	"	2.5 (YR)	"	0	100
	"	"	"	"	3.2 (RR)	"	0	100
	"	"	"	"	3.2 (RR)	"	0	100
	"	"	"	Kwa Makutha	6.2	5	2.2	56
	"	"	"	"	6.2	"	1.8	64
	"	"	"	"	6.2	10	0	100
	"	"	"	"	6.2	"	0	100
	"	"	"	"	6.2	25	0	100
	"	"	"	"	6.2	"	0	100
7	DV Harris	23.8	**Zetachem**	Mixed Std	3.0 (LR)	5	1.9	37
	"	"	"	"	3.0 (LR)	"	1.3	57
	"	"	"	"	3.2 (nod)	"	2.6	18
	"	"	"	"	3.2 (nod)	"	1.5	54
	"	"	"	"	2.8 (YR)	"	1.4	52
	"	"	"	"	2.8 (YR)	"	1.2	57
	"	"	"	"	4.0 (RR)	"	0.8	86
	"	"	"	"	4.0 (RR)	"	0.6	81
	"	"	"	"	3.0 (LR)	10	0.4	86
	"	"	"	"	3.0 (LR)	"	0.5	84
	"	"	"	"	3.2 (nod)	"	0.4	86
	"	"	"	"	3.2 (nod)	"	0.4	87
	"	"	"	"	2.8 (YR)	"	0.2	94
	"	"	"	"	2.8 (YR)	"	0.2	92
	"	"	"	"	4.0 (RR)	"	0.1	98

	"	"	"	"	4.0 (RR)	"	0	100

Test	*Test Water*	*Temp* ℃	*PAC*	*Toxin Spike*	*Initial Toxin Conc.* $\mu g.\ell^{-1}$	*PAC Dose* $mg.\ell^{-1}$	*Final Toxin Conc.* $\mu g.\ell^{-1}$	*Percent Removal*
7	DV Harris	23.8	**Zetachem**	Mixed Std	3.0 (LR)	25	0	100
	"	"	"	"	3.0 (LR)	"	0	100
	"	"	"	"	3.2 (nod)	"	0	100
	"	"	"	"	3.2 (nod)	"	0	100
	"	"	"	"	2.8 (YR)	"	0	100
	"	"	"	"	2.8 (YR)	"	0	100
	"	"	"	"	4.0 (RR)	"	0	100
	"	"	"	"	4.0 (RR)	"	0	100
	"	"	"	Kwa Makutha	6.2	5	5.4	0
	"	"	"	"	6.2	"	4.6	9
	"	"	"	"	6.2	10	3.2	49
	"	"	"	"	6.2	"	2.0	60
	"	"	"	"	6.2	20	0	100
	"	"	"	"	6.2	"	0	100
8	DV Harris	23.9	**Sutcliffe WF**	Mixed Std	3.0 (LR)	5	1.1	63
	"	"	"	"	3.0 (LR)	"	1.0	68
	"	"	"	"	3.2 (nod)	"	0.8	76
	"	"	"	"	3.2 (nod)	"	0.7	80
	"	"	"	"	2.7 (YR)	"	0.4	85
	"	"	"	"	2.7 (YR)	"	0.4	87
	"	"	"	"	3.4 (RR)	"	0.2	94
	"	"	"	"	3.4 (RR)	"	0.2	94
	"	"	"	"	3.0 (LR)	10	0.2	95
	"	"	"	"	3.0 (LR)	"	0.2	95
	"	"	"	"	3.2 (nod)	"	0.2	93
	"	"	"	"	3.2 (nod)	"	0.2	93
	"	"	"	"	2.7 (YR)	"	0	100
	"	"	"	"	2.7 (YR)	"	0	100
	"	"	"	"	3.4 (RR)	"	0	100
	"	"	"	"	3.4 (RR)	"	0.1	98
	"	"	"	"	3.0 (LR)	25	0	100
	"	"	"	"	3.0 (LR)	"	0	100
	"	"	"	"	2.9 (nod)	"	0	100
	"	"	"	"	2.9 (nod)	"	0	100
	"	"	"	"	2.8 (YR)	"	0	100
	"	"	"	"	2.8 (YR)	"	0	100
	"	"	"	"	4.0 (RR)	"	0	100
	"	"	"	"	4.0 (RR)	"	0	100
	"	"	"	Kwa Makutha	6.2	5	3.3	35
	"	"	"	"	6.2	"	3.1	38
	"	"	"	"	6.2	10	0.8	84
	"	"	"	"	6.2	"	0.90	81
	"	"	"	"	6.2	25	0	100

	"	"	"	"	6.2	"	0	100

Test	*Test Water*	*Temp* ℃	*PAC*	*Toxin Spike*	*Initial Toxin Conc.* $\mu g.l^{-1}$	*PAC Dose* $mg.l^{-1}$	*Final Toxin Conc.* $\mu g.l^{-1}$	*Percent Removal*
9	DV Harris	23.0	Sutcliffe 207CP	Mixed Std	2.7 (LR)	5	1.8	33
	"	"	"	"	2.7 (LR)	"	2.1	23
	"	"	"	"	3.2 (nod)	"	2.6	0
	"	"	"	"	3.2 (nod)	"	3.3	18
	"	"	"	"	2.7 (YR)	"	1.8	36
	"	"	"	"	2.7 (YR)	"	2.1	24
	"	"	"	"	3.3 (RR)	"	2.8	13
	"	"	"	"	3.3 (RR)	"	1.9	42
	"	"	"	"	2.7 (LR)	10	1.5	45
	"	"	"	"	2.7 (LR)	"	1.7	39
	"	"	"	"	3.2 (nod)	"	1.9	42
	"	"	"	"	3.2 (nod)	"	2.2	32
	"	"	"	"	2.7 (YR)	"	1.3	53
	"	"	"	"	2.7 (YR)	"	1.3	52
	"	"	"	"	3.3 (RR)	"	1.0	69
	"	"	"	"	3.3 (RR)	"	1.0	69
	"	"	"	"	2.7 (LR)	25	1.5	44
	"	"	"	"	2.7 (LR)	"	1.8	33
	"	"	"	"	3.2 (nod)	"	2.4	26
	"	"	"	"	3.2 (nod)	"	2.8	12
	"	"	"	"	2.8 (YR)	"	0.8	74
	"	"	"	"	2.8 (YR)	"	0.8	70
	"	"	"	"	3.3 (RR)	"	0.5	86
	"	"	"	"	3.3 (RR)	"	0.3	90
	"	"	"	Kwa Makutha	6.2	5	4.0	11
	"	"	"	"	6.2	"	3.7	16
	"	"	"	"	6.2	10	2.8	38
	"	"	"	"	6.2	"	3.0	34
	"	"	"	"	6.2	25	0.3	92
	"	"	"	"	6.2	"	0.3	92

Comparison of the removal of microcystin-LR by various PACs from the samples spiked with the kwaMakutha extract is shown in Figs. 12.5 and 12.6.

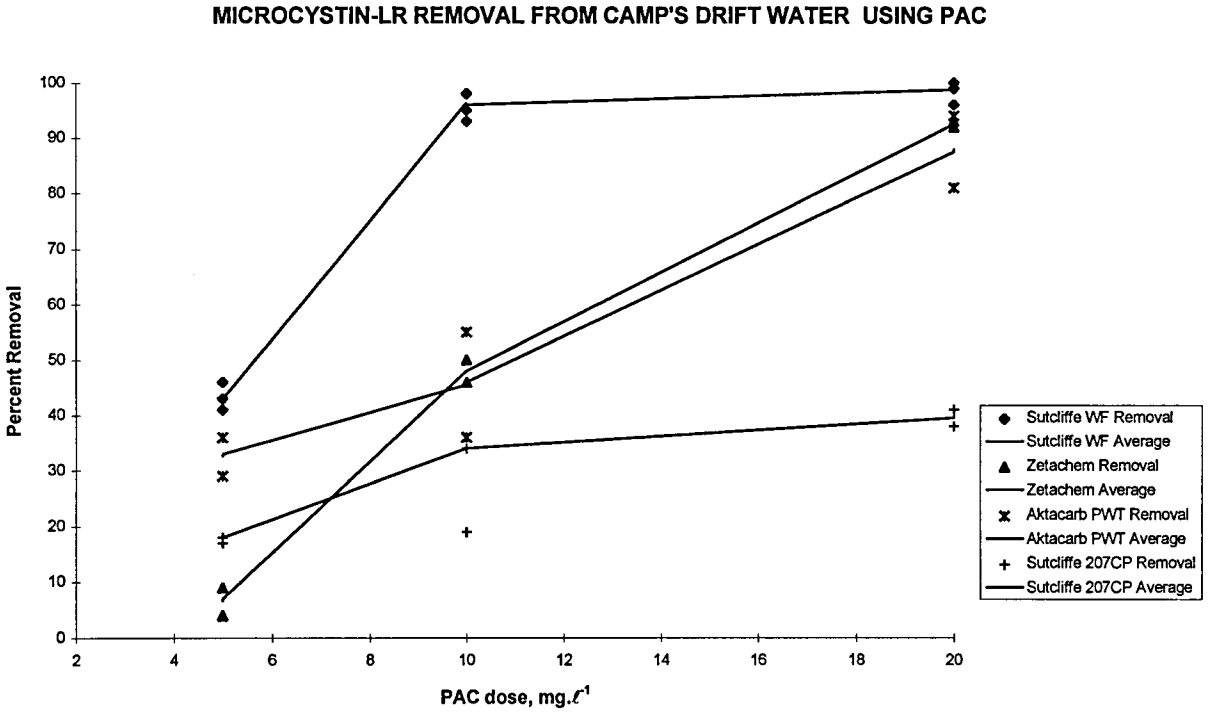


Fig. 12.5 Comparison of the Microcystin-LR removal from Camp's Drift raw water using different PACs

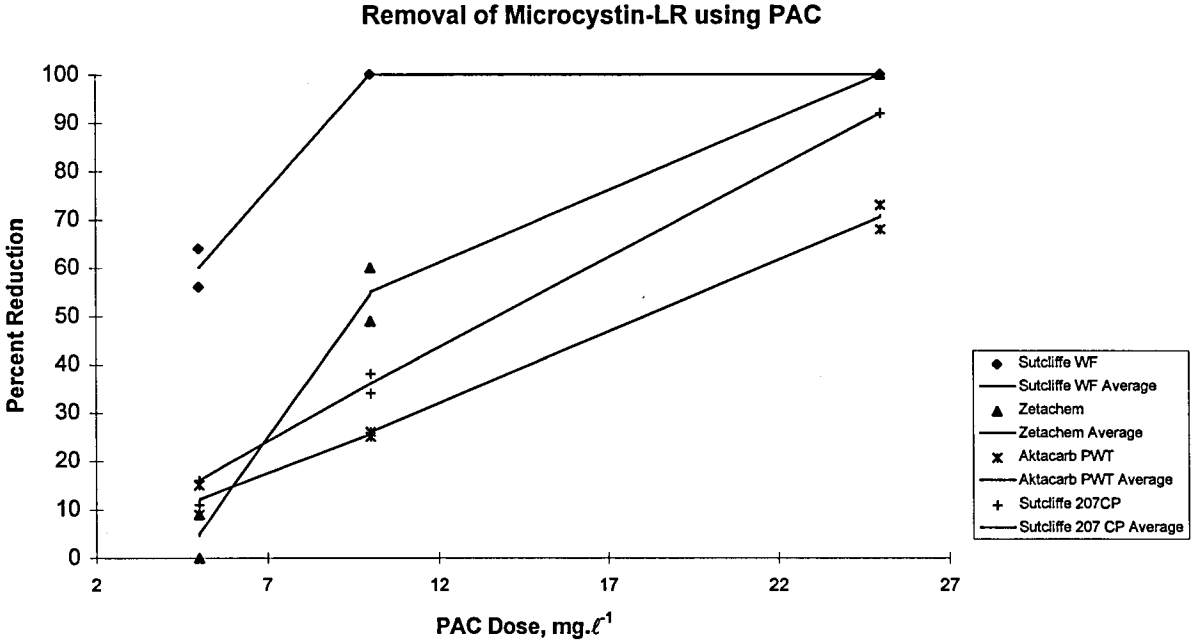


Fig. 12.6 Comparison of the Microcystin-LR removal from DV Harris raw water using different PACs

12.4 Discussion

Removal of the microcystin toxins varied depending on the type and dose of PAC used as well as the type of toxin standard spiked into the test water. In Test 1, where Camp's Drift raw water was spiked with the kwaMakutha extract, the microcystin-LR concentration (6.9 $\mu g.\ell^{-1}$) was reduced by 93-98%, with a Sutcliffe WF PAC dose of 10 $mg.\ell^{-1}$. Using a PAC dose of 20 $mg.\ell^{-1}$, the toxin was 96-100% removed. Using the mixed toxin standard in DV Harris water (Test 6) the toxins having individual concentrations of 2.5 to 3.2 $\mu g.\ell^{-1}$, were completely removed at a PAC dose of 10 $mg.\ell^{-1}$.

For most of the tests the removal of the pure mixed toxins was greater than the microcystin-LR removal from the extract-containing samples. This is probably due to the competitive demand from the other organics in the algal extract and is more noticeable at the 5 and 10 $mg.\ell^{-1}$ PAC doses.

The Zetachem PAC produced 81-89% removal of the mixed toxins at a dose of 10 $mg.\ell^{-1}$ and 46-50% removal of the microcystin-LR in the extract-containing sample. Although the removal of the individual toxins varied, there was no clear evidence to suggest selective adsorption of particular toxins to the PACs tested. Comparison of the toxins from the different types of water showed similar removal for samples spiked with the toxin extract, but the removal of the mixed toxins were slightly better from the DV Harris water at the 10 $mg.\ell^{-1}$ dose. A Zetachem dose of 25 $mg.\ell^{-1}$ completely removed both the mixed toxins as well as the microcystin-LR from the toxin containing extract.

Compared to the other wood sourced carbons (Sutcliffe WF and Zetachem), the Aktacarb PWT carbon produced the poorest removal, as shown in Figs. 97 and 98. At the 10 $mg.\ell^{-1}$ PAC dose, the mixed toxins (2.6-3.4 $\mu g.\ell^{-1}$) were between 10 and 77 % removed whereas the microcystin-LR from the extract containing sample was reduced by 19-55%.

The Sutcliffe 207CP PAC was the only carbon tested that was obtained from a coal source. At the 10 $mg.\ell^{-1}$ dose, the mixed toxins were 6-38 % removed from the Camp's Drift water and 32-69% removed form the DV Harris water. At the same PAC dose, the removal of microcystin-LR from the extract-containing samples was 19-34% and 34-38% from the Camp's Drift water DV Harris water, respectively. At the highest PAC dose of 20 $mg.\ell^{-1}$ the toxin removal from the extract samples was 38-41% from the Camp's Drift water and at 25 $mg.\ell^{-1}$, the removal was 92% from DV Harris water.

12.5 Conclusion

The Sutcliffe WF PAC was the most effective carbon tested for the removal of microcystin toxins. For a mixture of pure toxins spiked into raw water, at concentrations ranging from 2.7 to 4.0 $\mu g.\ell^{-1}$, a PAC dose of 10 $mg.\ell^{-1}$ removed the toxins by 93 to100%. For test samples spiked with a toxic algal extract having a microcystin-LR concentration of 6.2 - 6.9 $\mu g.\ell^{-1}$, a PAC dose of 10 $mg.\ell^{-1}$ removed the toxin by 81-100%, whilst a PAC dose of 20 $mg.\ell^{-1}$ produced 96-100% removal. The Zetachem PAC produced 73-100% removal of the mixed toxins at a PAC dose of 10 $mg.\ell^{-1}$ and complete removal of the microcystin-LR (6.2 $\mu g.\ell^{-1}$) from the extract-containing samples at a PAC dose of 25 $mg.\ell^{-1}$.

At similar toxin and PAC concentrations, the Sutcliffe 207CP and the Aktacarb PACs failed to produce complete removal of the toxins at the highest dose of 25 $mg.\ell^{-1}$.

PUBLICATIONS AND PAPERS

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2. Paper presented to IAWQ 19th Biennial Conference, Vancouver, 1998:
R.W. Moollan , B. Rae, A. Verbeek, Removal of microcystin toxins by chlorination, ozonation and powdered activated charcoal, *Water Quality International,* Book 10, 134-141.

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APPENDIX A

Principle Groups of Blue-greens

Order (Family)	*Representative Genera*
Chroococcales	Gloeothece, Synechoccus, (Anacystis, Agmenellum) Gloeocapsa, Chroococcus, Synechocystis, Microcystis, Merismopedia
Chamaesiphonales	Chamaesiphon, Dermocarpa Dermocarpella, Chroococcidiopsis
Pleurocapsales	Xenococcus, Myxosarcina, Pleurocapsa, Hyella
Nostocales	Oscillatoria, Microcoleus, Spirulina, Pseudanabaena, Plectonema, Lyngbyna, Phormidium, Schizothrix
Nostocaceae	Anabaena, Aphanizomenon, Nostoc, Nodularia, Anabaenopsis, Cylindrospermum
Rivulariaceae	Calothrix, Dichothrix, Gloeotrichia, Rivularia
Scytonemataceae	Scytonema, Tolypothrix,
Stigonematales	Mastigocoleus, Nostochopsis, Mastigocladus, Westiella, Fischerella, Hapalosiphon, Stigonema, Chlorogloeopsis

Confirmed Toxin-producing Strains

Microcystis aeruginosa Kutz.
*Microcystis viridis (a. Br.)*Lemm.
Microcystis wesenbergii Kom.
Nodularia spumigena Mertens
Nostoc rivulare Kutz.
Oscillatoria actissima Kuff
Osillatoria agardhii/rubescens group
Oscillatoria nigro-viridis Thwaites
Anabaena circinalis Rabenh.
Anabaena flos-aquae (Lyngb.) Breb.
Anabaena spiroides var. *contracta* Kleb.
Anabaena variabilis Kutz.
Aphanizomenon flos-aquae (L.) Ralfs
Oscillatoria agardhii Gom.
Cylindrospermopsis raciborskii (Wolos)
Lyngbya wollei

Taken from Carmichael (1994)

APPENDIX B

MICROCYSTIN TOXINS ISOLATED AND CHARACTERISED

	Toxin	*Formula*	*M.Wt*
1.	microcystin-LA	$C_{46}H_{67}N_7O_{12}$	909
2.	microcystin-LAba	$C_{47}H_{69}N_7O_{12}$	923
3.	microcystin-AR	$C_{49}H_{68}N_{10}O_{12}$	952
4.	microcystin-YA	$C_{49}H_{65}N_7O_{13}$	959
5.	[D-Asp^3, Dha^7]microcystin-LR	$C_{47}H_{70}N_{10}O_{12}$	966
6.	D-Asp^3]microcystin-LR	$C_{48}H_{72}N_{10}O_{12}$	980
7.	[D-ha^7]microcystin-LR	$C_{48}H_{72}N_{10}O_{12}$	980
8.	[D-MAdda^5]microcystin-LR	$C_{48}H_{72}N_{10}O_{12}$	980
9.	microcystin-LF	$C_{52}H_{71}N_{10}O_{12}$	985
10.	microcystin-LR	$C_{49}H_{74}N_{10}O_{12}$	994
11.	[D-Asp^3,D-Glu(OCH$_3$)^6]microcystin-LR	$C_{49}H_{74}N_{10}O_{12}$	994
12.	[(6Z)-Adda^5]microcystin-LR	$C_{49}H_{74}N_{10}O_{12}$	994
13.	[L-Ser7]microcystin-LR	$C_{48}H_{74}N_{10}O_{13}$	998
14.	microcystin-LY	$C_{52}H_{71}N_7O_{13}$	1001
15.	microcystin-HilR	$C_{50}H_{76}N_{10}O_{12}$	1008
16.	[D-Asp^3, ADMAdda^5]microcystin-LR	$C_{49}H_{72}N_{10}O_{12}$	1008
17.	[D-Glu-OCH$_3$^{6}]microcystin-LR	$C_{50}H_{76}N_{10}O_{12}$	1008
18.	[D-Asp^3,Dha^7]microcystin-RR	$C_{47}H_{71}N_{13}O_{12}$	1009
19.	[L-MeSer^7]microcystin-LR	$C_{49}H_{76}N_{10}O_{13}$	1012
20.	[ADMAdda5]microcystin-LR	$C_{50}H_{74}N_{10}O_{13}$	1022
21.	[D-Asp^3,ADMAdda^5]microcystin-LHar	$C_{50}H_{74}N_{10}O_{13}$	1022
22.	[D-Asp^3]microcystin-RR	$C_{48}H_{73}N_{13}O_{12}$	1023
23.	[Dha^7]microcystin-RR	$C_{48}H_{73}N_{13}O_{12}$	1023
24.	microcystin-FR	$C_{52}H_{72}N_{10}O_{12}$	1028
25.	microcystin-M(O)R	$C_{48}H_{72}N_{10}O_{12}S$	1028
26.	[Dha^7]microcystin-HphR	$C_{52}H_{72}N_{10}O_{12}$	1028
27.	[D-Asp^3,Dha^7]microcystin-HtyR	$C_{51}H_{70}N_{10}O_{13}$	1030
28.	[Dha^7]microcystin-YR	$C_{51}H_{70}N_{10}O_{13}$	1030
29.	[D-Asp^3]microcystin-YR	$C_{51}H_{70}N_{10}O_{13}$	1030
30.	microcystin-YM(O)	$C_{51}H_{69}N_7O_{14}S$	1035
31.	[ADMAdda^5]microcystin-LHar	$C_{51}H_{76}N_{10}O_{13}$	1036
32.	microcystin-RR	$C_{49}H_{75}N_{13}O_{12}$	1037
33.	[(6Z)-Adda^5]microcystin-RR	$C_{49}H_{75}N_{13}O_{12}$	1037
34.	[D-Ser^1,ADMAdda^5]microcystin-LR	$C_{50}H_{74}N_{10}O_{14}$	1038
35.	[ADNAdda^5,MeSer^7]microcystin-LR	$C_{50}H_{76}N_{10}O_{14}$	1040
36.	[L-Ser^7]microcystin-RR	$C_{48}H_{75}N_{13}O_{13}$	1041
37.	[D-Asp^3,MeSer^7]microcystin-RR	$C_{48}H_{75}N_{13}O_{13}$	1041
38.	microcystin-YR	$C_{52}H_{72}N_{10}O_{13}$	1044
39.	[D-Asp^3]microcystin-HtyR	$C_{52}H_{72}N_{10}O_{13}$	1044
40.	[Dha^7}microcystin-HtyR	$C_{52}H_{72}N_{10}O_{13}$	1044
41.	microcystin-(H$_4$)YR	$C_{52}H_{76}N_{10}O_{13}$	1048
42.	[D-Glu-OC$_2$H$_3$(CH$_3$)OH^6]microcystin-LR	$C_{52}H_{80}N_{10}O_{13}$	1052
43.	microcysin-HtyR	$C_{53}H_{74}N_{10}O_{13}$	1058
44.	[L-Ser^7]microcystin-HtyR	$C_{52}H_{74}N_{10}O_{14}$	1062
45.	microcystin-WR	$C_{54}H_{73}N_{11}O_{12}$	1067
46.	[L-MeLan^7]microcystin-LR	$C_{52}H_{81}N_{11}O_{14}S$	1115

Taken from Carmichael

APPENDIX C

MICROCYSTIN TOXICITY
(Intraperitoneal Lethal Dosage Values)

LD_{50} values based on mouse or rat	$LD_{50}(\mu g.kg^{-1})$
nodularin	50
[D-Asp^1]nodularin	75
[DMAdda^3]nodularin	150
[6(Z)-Adda^3]nodularin	>2000
[D-Glu-OCH_3^{4}]nodularin	>1200
dihydronodularin	
[D-MeAbu^5]nodularin	150
[L-MeAbu^5]nodularin	150
microcystin-LR	50
microcystin-LA	50
microcystin-YR	70
microcystin-RR	600
microcystin-AR	250
microcystin-LY	90
microcystin-FR	250
microcystin-WR	150-250
microcystin(O)	56
microcystin-M(O)R	700-800
microcystin-HtyR	80-100
microcystin- HilR	100
[D-Asp^3]microcystin-LR	50
[D-Asp^1]microcystin-RR	250
[D-ha^7]microcystin-LR	250
[L-MeSer^7]microcystin-LR	150
[L-MeLan^7]microcystin -LR	1000
[D-Glu-OCH_3^{6}]microcystin-LR	>1000
[D-Glu-$OCH_2H_3C(CH_3)OH$^{6}]microcystin-LR	>1000
[DMAdda^5]microcystin-LR	90-100
[ADMAdda^5]microcystin-LR	60
[D-ASp^3,ADMAdda^5]microcystin-LR	160
[ADMAdda5]microcystin-LHar	60
[6(Z)-Adda^5]microcystin-LR	>1200
[6(Z)-Adda^5]microcystin-RR	>1200
[L-MeAla^7]microcystin-LR	85
[D-MeAla^7]microcystin-LR	100

Toxicity of aqueous solutions of lyophillised *Microcystis* cultures (Tustin, et al., 1973) to Vervet monkeys was as follows:
Monkeys receiving 200 and 500 $\mu g.kg^{-1}$ died after the fourth and third intragastric exposure respectively, whereas two receiving 200 mg kg^{-1} died after the twelfth exposure.
An intraperitoneal dose of 200 $\mu g.kg^{-1}$ caused death after 2 hrs
Oral toxicity had no apparent effect after doses of 200 $\mu g.kg^{-1}$ followed by 1000 $\mu g.kg^{-1}$ a day later.

APPENDIX D

Summary of the Recommended Procedures for the Determination of Microcystin Toxins.

1.....

Sample Preparation

Two 1 ℓ samples were filtered through Whatman GF/C filter paper. (The algal cells retained on the filter paper would be used for the determination of the intracellular toxin content)

Each 1 ℓ filtrate was treated with sodium sulphite solution (100 μℓ;1 g per 100 ml) to eliminate free chlorine, then shaken vigorously and allowed to stand for a few minutes.

One of the samples was spiked with microcystin-LR (150 μℓ of a solution containing 5μg toxin per 150 μℓ).

The samples were then sub-divided into 500 mℓ portions, to which trifluoroacetic acid (TFA) (5 mℓ ; 10%)solution was added.

The samples were filtered once more and methanol (5 mℓ) was added prior to C_{18} solid-phase extraction.

C_{18} Cartridge Conditioning Procedure

Cartridges (Isolute C18 trifunctional, 1 g per 3 mℓ) were conditioned by passing through 10 mℓ of methanol followed by 10 mℓ of water.

Extraction & Recovery of the Toxins from Water

Each water sample (500 mℓ) was drawn through Teflon tubing onto the cartridge (attached to a vacuum manifold) at a flow rate not exceeding 10 $m\ell.min^{-1}$.

After extraction, each cartridge was washed consecutively with 10 mℓ of 10%, 20% and 30% (v/v) aqueous methanol and air was sucked through the cartridge for approximately 30 minutes to minimise the amount of water eluted with the sample.

The toxins are then eluted with 3 mℓ of 0,1% (v/v) trifluoroacetic acid (TFA) in methanol.

The eluate was collected, placed on a hot block (at 45°C) and blown to dryness using high purity nitrogen gas.

The residue was then re-suspended in 100 μℓ of methanol and transferred to a micro-centrifuge tube (1,5 mℓ) and re-dried.

After re-suspension in 75 μℓ of 75% aqueous methanol, 25 μℓ of this solution was injected into the HPLC.

Detection limits : 0.1 $\mu g.\ell^{-1}$ (for real samples)
: 0.03 $\mu g.\ell^{-1}$ (standards)

Intracellular Toxin Analysis

The filter paper and cyanobacterial cells were placed into a suitable container and freeze-thawed prior to extraction in 20 mℓ of methanol for 1 hr. The liquor was decanted into a pear shaped rotary evaporation flask and evaporated under reduced pressure at 40°C. The procedure was repeated twice in the same rotary vessel. The residue was re-suspended in 250 μℓ of methanol and 25 μℓ injected onto the HPLC system.

Identification of Microcystin Toxins and Nodularin

Microcystin toxins and nodularin are reported to have either one of the two characteristic UV spectra shown below. One has a local absorption maximum at 238 nm and the other which is

typical of microcystin toxins containing tryptophan has two local maxima, one at 222 nm and the other at 238 nm.

While the use of reversed phase HPLC with photodiode array detection has commonly been used for the analysis of microcystin toxins, the novel feature of the paper was the inclusion of the UV spectral identification of this group of toxins.
Peaks in the HPLC chromatograms having the UV spectrum of Fig (a) would be identified as a microcystin toxin or nodularin, and those having the UV spectrum of Fig. (b) would be identified as microcystin toxins containing a tryptophan residue.

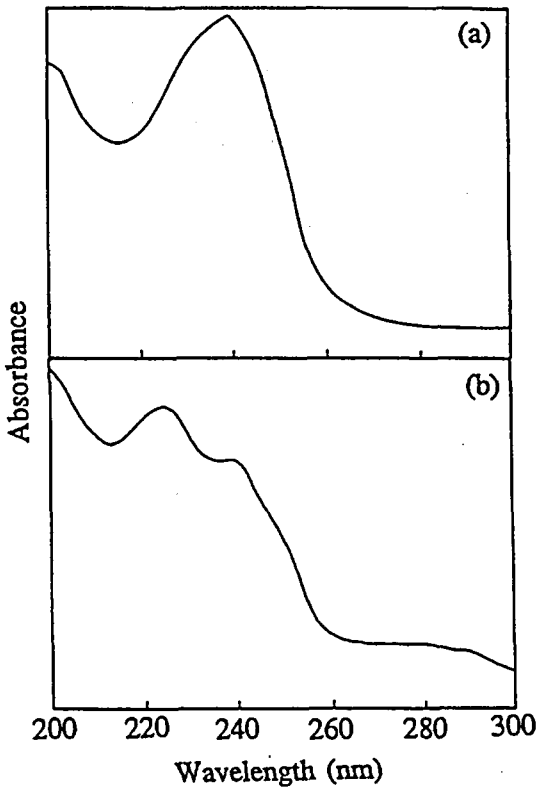


Fig (a) Characteristic UV spectrum of microcystin toxins
(b) Characteristic UV spectrum of microcystin toxins containing tryptophan

The following inconsistencies may be observed in the paper by Lawton *et al.*[36]:

1. The detection limits and calibration graphs were obtained using the experimental procedure, i.e., the toxins were added to raw and treated water samples and recovered. The toxin peak areas of the recovered solutions were plotted against the mass of toxin on the column (in 25 μl) to produce the calibration graphs. Since the calibration graphs are linear for all the toxins tested, it implies that the percentage recoveries from the water were uniform, but there would be no means of determining whether all toxins were fully recovered.

2 There was no methodology supplied of how the percentage recoveries of the microcystins and nodularin in spiked water samples were calculated. It is uncertain how any recovery losses could be obtained (as indicated in Table 5) if the same procedure as the one used for the determination of the calibration graphs was applied.

In addition to the inconsistencies in the determination of the percentage toxin recoveries, it is also noted that the toxin recoveries of microcystin-RR and -LR were generally lower for the treated waters compared to the untreated waters (which are expected to contain more organic componds than the treated waters). The recoveries of microcystin-LW were consistently low and again its recovery from raw waters was considerably higher than from the treated waters.
The high recoveries ranging up to 147% also required justification. HPLC peak areas of duplicate injections are expected to vary but the method used for the calculation of the percentage recoveries would show how the over-recoveries could have been achieved. Either the peak areas of the toxins spiked into the water sample were co-eluting with that of other peaks in the sample chromatogram or the concentrations of the toxin standard solutions spiked into the test samples were underestimated.

3. The precision data for the recoveries of the microcystins: -RR, -LR,-LY,-LW,-LF and nodularin from Loch Rescobie raw water and Dundee tap waters had the actual recoveries adjusted, based on the recovery of microcystin-LR alone. It is not clear why this specific toxin was chosen to adjust the recoveries of the other toxins, (except that it may be more readily available). The figures in Table 8 are indicative of this adjustment but those in Table 7 do not correspond. Either there has been a recording error or some other method of adjusting the actual recoveries has been used.

The recovery adjustment forms part of the recommended procedure in that a spike of only microcystin-LR was added to the raw samples and its recovery measured. It is not clear how the recoveries of the other toxins can be calculated or identified if they are not spiked into the sample water since the identification of the toxins are also based on their retention times. It would appear that the percentage recovery of microcystin-LR is calculated and applied for the other toxins identified as being microcystins or nodularins by their characteristic UV spectrum.
If this was the case, the precision tests, where the toxins were spiked into the water and the actual recoveries determined for each of the toxins, should contain percentage recoveries which are very similar for all the toxins. From the actual recoveries shown, this was not achieved. The actual recoveries from the Loch Rescobie raw water varied from 20% to 130% and the adjusted recovery values ranged from 24% to 152%. These percentage values indicate that the toxins are not recovered to the same extent from the same sample matrix and that the method used to adjust the recoveries cannot be a reliable means to quantify the amount of toxins in the water sample.

4. In the recommended procedure, the sample filtrates were treated with sodium sulphite to remove the residual free chlorine. The samples were then spiked with an aliquot of microcystin-LR and an aliquot of trifluoroacetic before extraction. The reason for adding the trifluoroacetic acid was not mentioned and it is not stipulated whether the sodium sulphite should always be added to the samples or only when chlorine is present.

1. S.L. Kenefick, S.E. Hrudey, E.E. Prepas, N. Motkosky & H.G. Peterson, *Wat. Sci Tech.*, 1992, 28 (No 2), pp 147-154.

Extraction and analysis of microcystins and nodularin
The method of Harada et al (1988) was modified as follows: 5% (v/v) acetic acid (5 ml) was added to about 100 mg of freeze-dried homogenised cells, left in the dark for 1 hr and sonicated for 3 × 30 seconds. The solution was then left in the dark for another hour and centrifuged once more. The combined supernatants were added together and applied to a C_{18} cartridge and washed with 10 ml of 5% (v/v) acetic acid, 10 ml of water and 10 ml of 10% (v/v) aqueous methanol. The microcystins were eluted with 5 ml of methanol and evaporated. The residue was re-dissolved in 750 µl of solvent (10 mM ammonium acetate:acetonitrile, 74:26, v/v) and centrifuged. The supernatant was filtered (0.22µm) and 20 µl of the filtrate injected onto an HPLC system - Waters 6000A pump, HP 1040A diode array detector, HP85 data processor. C_{18} µBondapak 30 cm × 3.9 mm column (Waters), using a solvent system of 10 mM ammonium acetate : acetonitrile (74:26 v/v) at a flow rate of 1 ml min^{-1} with UV detection at 238 nm.

Extraction and analysis of Anatoxin -a
The method of Himberg (1989) was modified : Methanol (25 ml) plus conc. HCl (0.25 ml) was added to approximately 50 mg of freeze-dried homogenised cells. The solution was sonicated for 15 min. and centrifuged. The supernatants were filtered through Gelman Acro 50 filters and the filtrate evaporated nearly to dryness and the residue re-dissolved in 1 ml of chloroform. Acetic anhydride (1 ml) was added and allowed to react for 16 hours at 50°C. The reacted solution was

evaporated nearly to dryness until no smell of the acetic acid. The residue was re-dissolved in chloroform and applied to a disposable silica cartridge and washed with 7.5 ml of acetone:chloroform (1:19) v/v. The acetylated anatoxin-a was eluted with 2 ml of methanol, concentrated to 100 μl and made up in the chloroform to give a final volume of 1 ml.
The chloroform solution was then used for GC-MS - 10 μl of 1-acetylpiperidine (50:50 in chloroform) was added as the internal standard. GC-MS system in SIM (selective ion mode) consisted of an HP 5890 gas chromatograph with 5970 mass selective detector. HP 59940 ChemStation for data acquisition and DB-1 column, 30 m × 0.25 mm I.D, 0.25 μm thick stationary phase.
Detector temperature was set at 250°C and the oven temperature programmed from 80°C to 275°C at 15°C /min. with a final hold temperature of 240°C. 1 μl injections were made using an injector pressure of 70 kPa and injection temperature of 240°C. Ions m/z = 207, 165, 164, 136 were monitored for acetylanatoxin-a; and m/z = 84 and 124 for the internal standard.

3. I.M. Birk, U. Matern, I. Kaiser, C. Marten & J. Weckesser, *Journal of Chromatography*, 1988, 449, 423-431.

Extraction and analysis of microcystins and nodularin
Buffers :
(A) 0,1M sodium phosphate, pH 8,0; (B) 27 mM Tris, 142 mM glycine pH 8,2-8,5; (C) 150 mM Tris-HCl pH 8,8

Toxin Isolation :
Lyophilised microcystis cells (10-30g) was extracted for 20 min. at room temp with water-saturated n-butanol (300 ml) containing 1% (v/v) acetic acid. The solution was centrifuged (1850g, 15 min.) and the pellet re-extracted. The supernatants were pooled and dried at reduced pressure (35°C). The residue was re-dissolved in water and the pH adjusted to 8. The lipids were extracted with diethyl ether by gentle mixing and subsequent centrifugation (1830g, 4°C, 5 min.). The aqueous phase separated from non-toxic interlayer and ether phase. The aqueous portion was dried under reduced pressure (35°C) and residue applied to silica gel reversed phase 18 TLC plates in methanol, and fluorescent-quenching band at R_f 0,2 was extracted into propan-2-ol. The extract was dried and the residue re-dissolved in chloroform:methanol (7:3, v/v). The solution was filtered through HV 0,45 filter prior to HPLC separation on Partisil.

Native polyacrylamide gel electrophoresis (PAGE) :
Gradient PAGE was performed on slabs (1 mm × 100 mm × 100 mm, 11,5 -18% acrylamide) at 15 mA in buffers B (electrode) and C (separation). Developed gels were cut into 2 mm sections and extracted with buffer A . Protein extracts were determined spectrophotometrically (mg protein per ml = 1,55 E280 - 0,76 E260) at 240 nm.

HPLC :
A linear gradient from 30 to 80% methanol-acetic acid (99:1, v/v) in chloroform-acetic acid (99:1, v/v) at a flow rate of 2 ml min^{-1} (analytical) or 10 ml min^{-1} (preparative). Elution was monitored at 254 nm.

Spectrometry :
UV spectra were recorded using a UVIKON 810 spectrophotometer. ^{1}H (300 MHz) and ^{13}C NMR (75 Mhz) was used with tetramethylsilane as an internal reference. Fast atom mass bombardment (FAB-MS) was performed on Kratos MS 50 MS RF spectrometer with glycerol as a matrix.

Amino acid analysis :

The toxin was hydrolysed in 6M hydrochloric acid (0,5 ml) for 24 hours at 105°C in sealed tubes. The samples were subjected to amino acid analysis in a Biotronik LC 6001 amino acid analyser. Elution of amino acid derivatives monitored at 570 and 440 nm.

4. C. Martin, K. Sivonen, U. Matern, R. Dierstein & Jurgen Weckesser, *FEMS Microbiology Letters*, 1990, 68, 1-6.

Extraction and analysis of microcystins and nodularin
Lyophilized cells (5g) were extracted in in 400 ml 5% (v/v) aqueous acetic acid, ultrasonicated, stirred for 30 min. at room temperature and centrifuged (20000 × g). The supernatants were collected.

C_{18} cartridges were conditioned with methanol (15 ml) and twice distilled water. The supernatant was passed through the cartridge and eluted with methanol (15 ml). The eluate was evaporated to 5 ml *in vacuo* and diluted to 30% (v/v) methanol with water and filtered using 0,45μm Millex filter.

Ion exchange chromatography: The eluate (2 mg) was dissolved in 30% methanol (30 ml)and applied with a syringe to a preconditioned 1 ml ACCELL QMA ion exchange cartridge (Waters). After washing with water (5 ml), the toxins were eluted with 0,02M ammonium bicarbonate in 30% methanol (5 ml).
A column (1,6 × 5 cm) was slurry packed with ACCELL QMA anion exchange resin (Waters) in 30% methanol and the diluted C_{18} eluates (15 ml) were applied to these column at a flow rate of 1 ml min^{-1}). The column was washed with water (15 ml) prior to elution with 30 ml 0,02M ammonium bicarbonate in 30 % methanol.

Reversed phase HPLC : The toxin fraction from the ion exchange column was purified further on a semi-preparative ODS column (250 × 10 mm , 10μm Bio Rad, F.R.G.) or an analytical ODS column (250 × 4,6 mm , 5μ Beckman) using a linear gradient of acetonitrile/water from 45% B in A to 55% B in A within 30 min using a flow rate of 2 ml min^{-1} and 1 ml min^{-1} respectively.
solvent A = (10% acetonitrile with 0,1% trifluoroacetic acid in water, v/v)
solvent B = (90% acetonitrile with 0,1% trifluoroacetic acid in water, v/v).
Absorption was monitored at 214 and 238 nm.

UV spectroscopy : Absorption spectra from 200 nm to 280 nm were taken in 50% acetonitrile in water containing 0,1% trifluoroacetic acid.

5. K. Harada, K. Ogawa, K. Matsuura, H. Nagai, H. Murata, M. Suzuki, Y. Itezono, N. Nakayama, M. Shirai & M. Nakano, *Toxicon*, 1991, 29 (No. 4/5), pp 479-489.

Extraction, purification and characterisation of microcystins and nodularin
Toxin Purification : Dried cells were extracted with 5% aqueous acetic acid 3 times for 30 min. while stirring. Combined extracts centrifuged at 9300g for 1 hr and the supernatants applied to a reversed phase silica gel column (12 × 4,3 cm I.D., Chromatorex ODS). The column was washed with 150 ml water; water:methanol (8:2) 500 ml and eluted with 400 ml of methanol to give the toxin fraction. The fraction was chromatographed on reversed phase silica gel (91 × 1,1 cm, Chromatorex ODS) with methanol:0,05M sodium sulphate (6:4) as mobile phase to give semi-pure toxin. The fractions were desalted with ODS cartridges.
Toxin A was purified by TOYOPEARL HW-40 column chromatography (91 × 1,1 cm I.D.) and Toxin B purified by preparative HPLC with methanol: 0,05 M sodium sulphate (6:4) as mobile phase.

HPLC : Reversed phase isocratic conditions using a Nucleosil $5C_{18}$ column (150 × 4,6 mm, Chemco Scientific Co) and a Cossmosil $5C_{18}$-P column (250 × 10 mm, Naclai Tesque) for

preparative separations. Mobile phases - methanol:0,05 M phosphate buffer (pH 3) (58:42) and methanol:0,05 M sodium sulphate (6:4). Flow rate of 1 ml min^{-1} and 2 ml min^{-1} were used for analyses and preparative separations respectively. UV absorbance was at 238 nm.

TLC : Silica gel plates (Kieselgel 60 F254, Merck)were used. Solvent systems were as follows- chloroform:methanol:water = 65:35:10 (lower phase) and ethyl acetate:isopropanol:water = 4:3:7 (upper case). Samples were detected with iodine and short wavelength UV.

Amino Acid Analysis: Purified peptides, hydrolysed in 6N HCl for 24 hrs. Amino acids were derivatised with phenylisothiocyanate before application on the column and the phenylthiocarbamyl amino acids were analysed using a Waters Pico Tag HPLC. Derivatives were loaded on a C_{18} (150 × 4,6 mm) column and eluted over 8 min. with 0 to 60% gradient of acetonitrile in 0,138 M aqueous sodium acetate. Flow rate = 1 ml min^{-1}. Absorption wavelength was at 254 nm.

Chemical Analysis : Fast atom bombardment (FAB) mass spectrometry using a double focusing JEOL (Tokyo) JMS-HX110 was used. The fast atom beam was operated at 6 kV using a Xenon gas and operated at 10 kV accelerating potential. Glycerol and 1N HCl was used as the matrix. Samples adjusted to 10μg/ml.
Nuclear magnetic resonance (NMR) recorded on 400 mHz JEOL JNM-GSX 400 and GX-400 spectrometers equipped with $^{1}H/^{13}C$ dual probe. Samples were dissolved in 0,7 ml of CD_3OD with trimethylsilane as internal standard. UV spectra and specific rotations were measured with a Shimadzu UV-VIS and JASCO DIP-181 polarimeter.

Non-destructive methods using 2-D NMR include:
DQF-COSY = double quantum filter correlation spectroscopy (Piantini, 1982)
HMQC = ^{1}H-detected multiple quantum coherence (Bax & Subramanian, 1986)
HMBC = heteronuclear multiple bond correlation (Bax & Summers, 1986)
These methods assign all carbons and protons so that constituent amino acids in microcystins are definitely confirmed.

6. J. Meriluoto, J. Eriksson, K. Harada, A. Dahlem, K. Sivonen & W. Carmichael, *Journal of Chromatography*, 1990, 509, 390-395.

Extraction and analysis of microcystins and nodularin
Preparation of Field Samples : Lyophilised M. aeruginosa cells were used to test extraction efficiency. 10g of algal material was placed in 1,5 ml polypropylene Eppendorf tubes, extracted for 5 min. in a bath sonicator using 100μl/mg of acetonitrile:0,1 M potassium dihydrogen phosphate 15:85 pH 6,8 adjusted with 10M potassium hydroxide after addition of acetonitrile. Sample centrifuged 10000g for 10 min.
Supernatant collected and pellet re-extracted (1×, or 2×) spun once more and injected into HPLC

Internal Surface Reverse Phase (ISRP)-HPLC consisted of a Shimadzu LC -7a pump, SPD 6A detector at 238 nm, CR-5A integrator, 250 × 4,6 mm ID GFF-S5-80 ISRP column (by Regis Chemical Morton, USA) and a Rheodyne 0,45μm filter (Cotati, CA, USA)
Mobile phase contained acetonitrile:0,1M potassium dihydrogenphosphate (pH 6,8 adjusted with 10 M potassium hydroxide after addition of acetonitrile) (15:85)
Flow rate = 1 ml min^{-1} and 20μl injection volumes were used.

7. J. An & W.W. Carmichael, *Toxicon*, 1994, 32 (No. 12), pp. 1495-1507.

Purification and detection of microcystins and nodularin

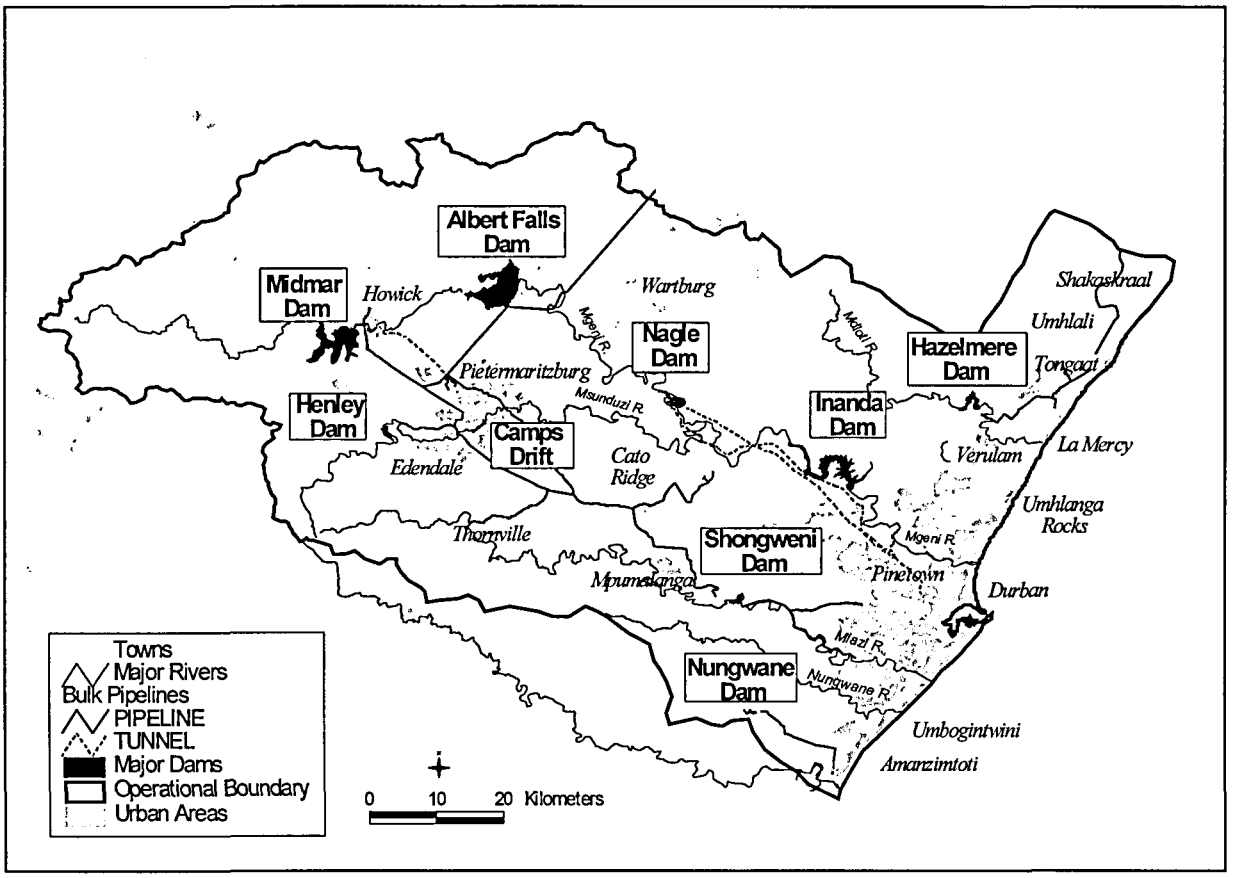
The toxin purification method of Krishnamurthy, *et al.*, (1986) was modified. Freeze-died *Microcystis aeruginosa* cells were extracted in water:methanol:butanol (75:20:5) overnight at room temperature (22°C), with stirring. The extract was centrifuged (20000 × g) for 30 to 60 min. or filtered over glass wool and then centrifuged. The supernatant was air dried at 25-30°C overnight to remove methanol and butanol. The concentrate was applied to a Bond Elut C_{18} reverse -phase column. The column washed with water, 20% methanol and the microcystin-LR eluted with 80% methanol. The eluate was dried overnight at 20-35°C. Preparative HPLC using a Waters Prep Pak C_{18} cartridge (47 × 300 mm, 55 -105μm particle size, 125 Å pore size) was used to purify the toxin. Mobile-phase solvents used were 28% acetonitrile and 72% 20 mM NH_4OAc), pH 5.0, with a flow rate of 30 ml min^{-1} and monitored at 238 nm. Toxins were air dried overnight to remove the acetonitrile. Microcystin-LR was then desalted on another Waters Prep Pak C_{18} column, followed by gel chromatography on Toyo Pearl HW-40 F using 100% methanol for the separation of LR from the remaining pigments. The estimated purity of microcystin-LR was 95-98% by analytical HPLC (methanol(55%)/55 mM Na_2SO_4 (45%), pH 6.8) and Pico Tag amino acid analysis.
MCYST-LR (0.5 mg) was dissolved in 1 ml methanol and diluted to working concentrations of 0.5, 1.5, 10 and 50 ng ml^{-1} with 0.01 M phosphate buffer saline (PBS), pH 7.4. The final methanol concentration was less than 1%.

Protein Phosphatase Inhibition Assay :
Source of protein phosphatase 1 : Catalytic sub-unit of rabbit skeletal muscle protein phosphatase 1 as expressed in E. coli.
Buffers : PBS = phosphate buffered saline, sodium phosphate buffer, 0.01 M containing 0.15 M NaCl, pH 7.5.
BSA = bovine serum albumin in 100 ml PBS.
Procedure : PP1 activity was determined by measuring the rate of colour production from the liberation of p-nitrophenol phosaphate (Sigma) using the microtiter plate reader at 37°C. PP1 was diluted in 50 mM TRIS-HCl, pH 7.4, 1 mg ml^{-1} BSA, 1 mM $MnCl_2$ and 2 mM dithiothreitol (DTT). The reaction of enzyme and substrate was done in reaction buffer containing 50 mM Tris-HCl, pH 8.1, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5 mg/ml BSA. MCYST-LR standards ranging from 0.5 to 100 ng ml^{-1} were incubated with PP1 for 4 min. at room temperature. The reaction was started by addition of substrate (pNPP=5 mM) reaction buffer. The plate was read at 405 nm using a microplate reader.

8. ELISA method developed by Waters
Waters have recently (1995) developed an ELIZA test kit (Enviroguard QuantiTube Test Kit) which can be used to rapidly determine the total concentration of microcystin toxins in a water sample.

APPENDIX E

The Umgeni Water Catchment



APPENDIX F

Allen's BG-11 Medium for the Culture of Cyanobacteria

Microelement Stock Solution (1000 mℓ) :

Add :	H_3BO_3	2.680 g
	$MnCl_2.4H_2O$	1.810
	$Na_2MoO_4.2H_2O$	0.391
	$ZnSO_4.7H_2O$	0.222
	$CuSO_4.5H_2O$	0.079
	$Co(NO_3)2.6H_2O$	0.049

Dissolve in deionised water (1000 mℓ)

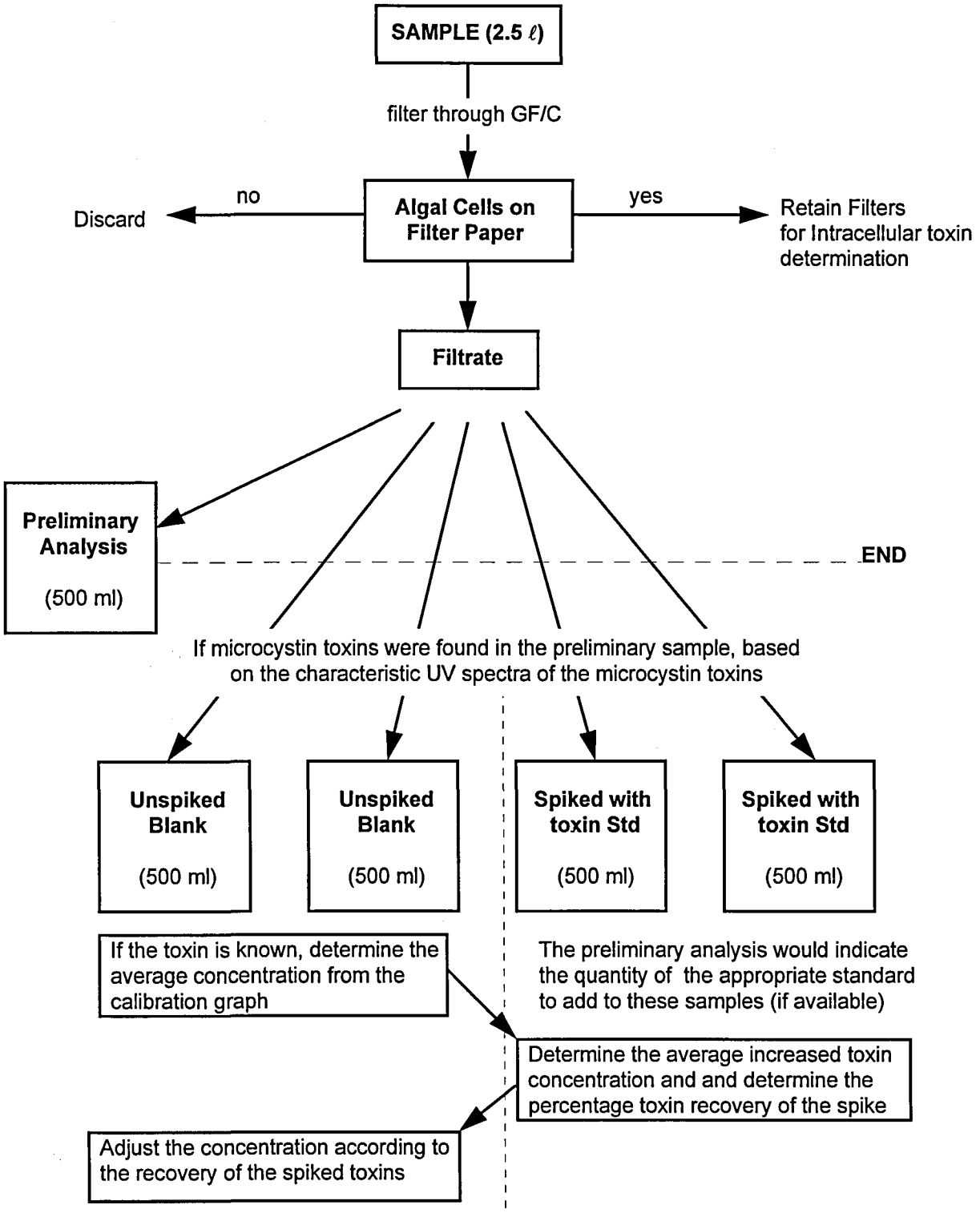
BG-11 Stock Solution :

Add:	EDTA	0.003g
	$FeCl_3.6H_2O$	0.003
	$NaNO_3$	1.590
	K_2HPO_4	0.039
	$MgSO_4.7H_2O$	0075
	$Na2CO_3$	0.020
	$Ca(NO_3)_2.4H_2O$	0.020
	$Na_2SiO_3.5H_2O$	0.0.43
	Citric Acid	0.006
	Microelement Stock solution	1 mℓ

Adjust the pH of the final medium to 7.8 with 1M HCl and 1M NaOH

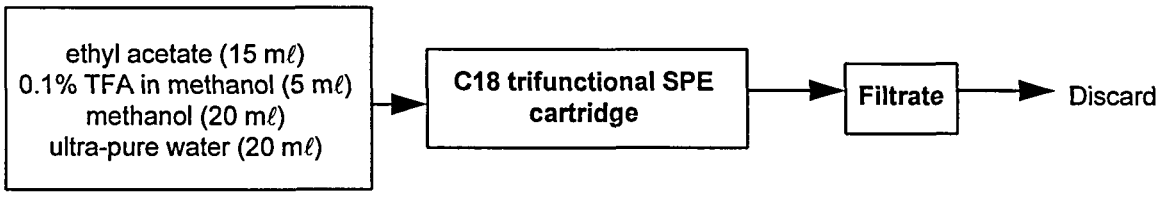
APPENDIX G

Summary of Extracellular Microcystin Toxin Analysis

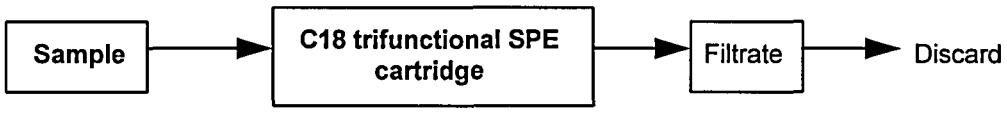


Solid-phase Extraction Procedure

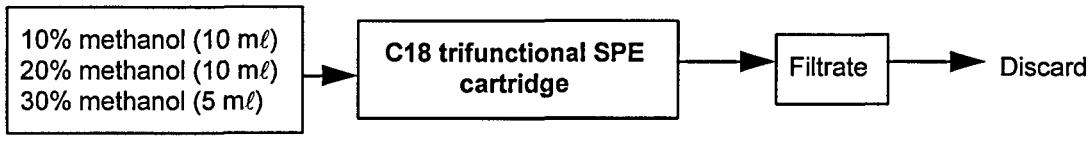
1. *Cartridge Conditioning*



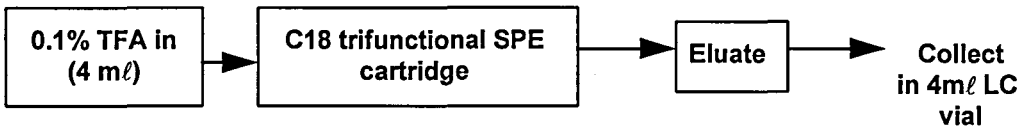
2. *Solid-phase extraction*



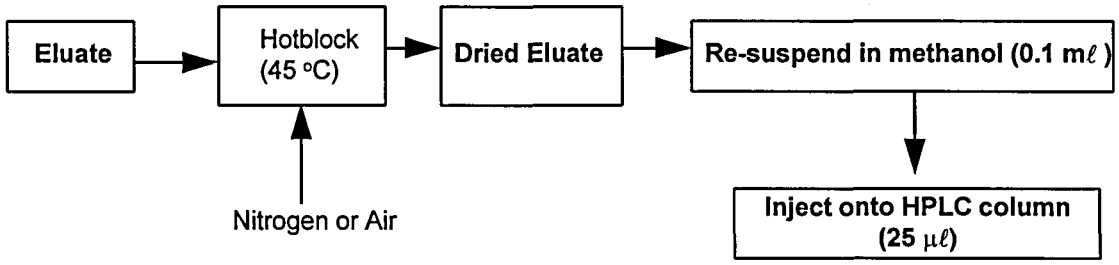
3. *SPE clean up*



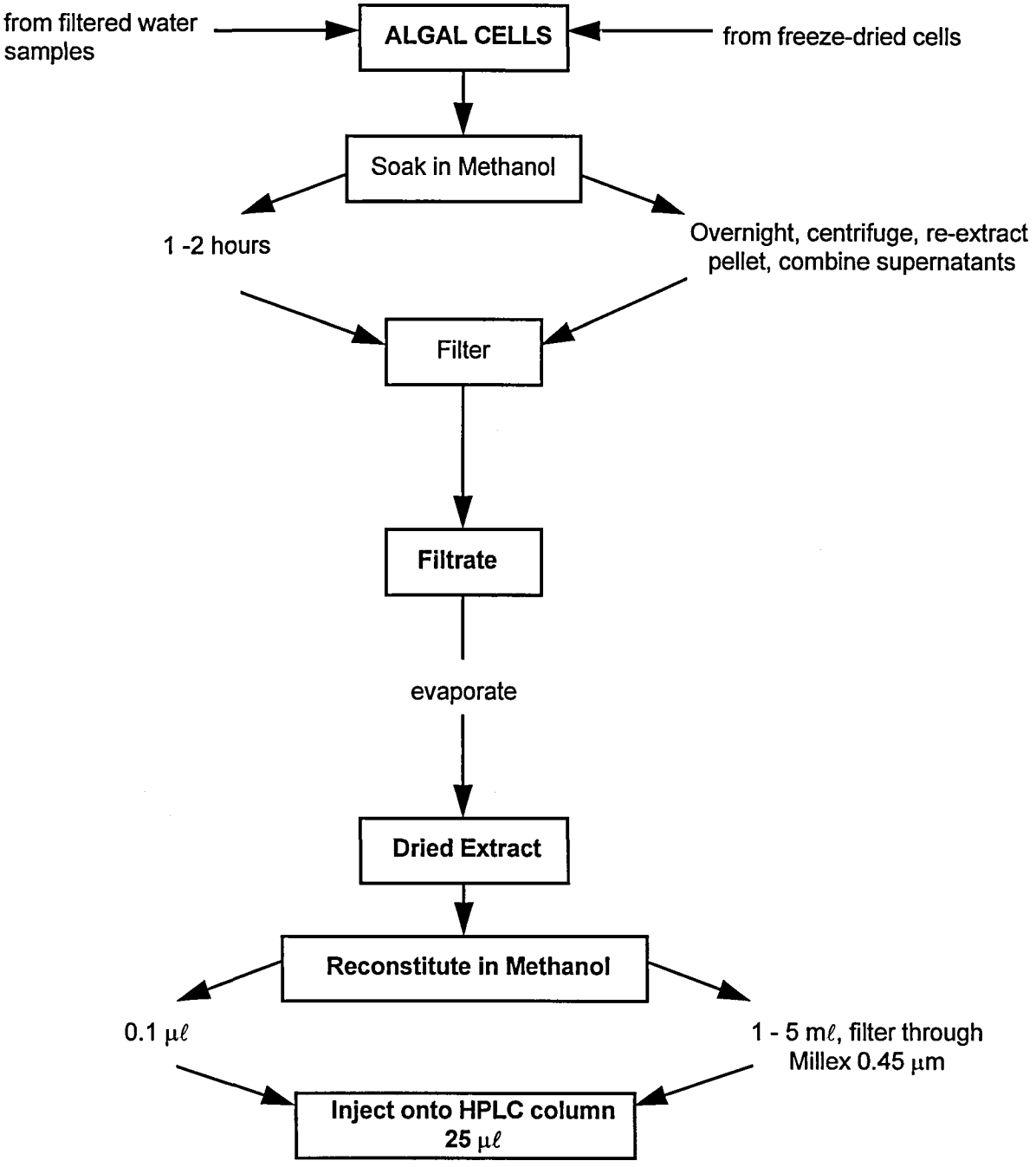
4. *Elution*



5. Concentration



Summary of Intracellular Microcystin Toxin Analysis



APPENDIX H

PAC Specifications

AKTACARB PWT PREMIUM

Supplier	:	Aktacarb (PTY) Ltd (Germiston, SA)
Source	:	wood
Particle size	:	15 - 100 μm (95%)
Ash	:	2 - 6%
Iodine #	:	800 - 1000 $mg.g^{-1}$
Methylene Blue #	:	180 - 210 $mg.g^{-1}$
Phenol Adsorption	:	4 - 5%
pH	:	alkaline
Moisture	:	3 - 6%

SUTCLIFFE WF

Supplier	:	Active Chemicals (Pty) Ltd (Boksburg, SA)
Source	:	wood (China)
Iodine #	:	1000 - 1100 mg.g-1
Ash	:	8%

SUTCLIFFE 207CP

Supplier	:	Sutcliffe Speakman Carbons (Lancashire, UK)
Particle size	:	< 200 μm (95%)
pH	:	10
Ash	:	5%
Moisture	:	10% (w/w)

ZETACHEM

Supplier	:	Zetachem Speciality Chrmicals (Pty) Ltd
Particle size	:	200 μm (95%)
Ash	:	< 6%
Moisture	:	< 7%
Iodine #	:	> 950 $mg.g^{-1}$ (95%)
Methylene Blue #	:	> 150 $mg.g^{-1}$

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SECTION II

USE OF PROTEIN PHOSPHATASE ENZYME INHIBITION AS A DETECTION AND QUANTIFICATION METHOD FOR THE PRESENCE OF *MICROCYSTIS* TOXINS IN DRINKING WATER

Report to the

WATER RESEARCH COMMISSION

by

R C Clark

Environmentek : CSIR
P O Box 395
PRETORIA
0001

Project Worker : Dr Richard Clark

EXECUTIVE SUMMARY

Section II deals with the use of enzyme inhibition methods for the detection and quantification of microcystins in drinking water and is divided into two parts.

Part I deals with a novel attempt to use protein electrophoresis as a means of monitoring enzyme-mediated substrate-product concentration changes in assay mixtures, thereby avoiding the use of radioactive phosphorus isotopes in the assay. The results did not indicate success, but such a method may nevertheless still be possible, if sufficient time and effort can be devoted to the determination of the correct conditions for conducting electrophoresis.

Part II deals with the use of the radiolabelled method developed by Codd and co-workers. The following conclusions were drawn from this study:

> With care, it should be possible to detect as little as 5 pg of microcystin-LR in a 20 µl assay aliquot, which could be increased to 40 µl in volume. This equates to a sensitivity of 5 ng/20 ml, or 0.25 µg/ 1000 ml, or half of these concentrations in the case of the larger aliquot. This is well below the nominally recommended human safety limit of 1µg per litre of water (Codd 1994, see Page 8). No toxin concentration steps are therefore needed in the procedure.
>
> The dam samples taken were not shown to contain measurable amounts of microcystins in concentrations over 1µg per litre.
>
> Additions of a microcystin-LR standard to these dam water samples gave analytical results that show that matrix effects from all other substances in these water samples does not interfere with the assay.

The overall conclusion is that for routine screening of water samples, the radiolabelled substrate and enzyme inhibition assay is the best available means for such tests. Positive or suspect samples could then be studied further by other methods, including HPLC, for confirmatory evidence, as well as for the resolution and identification of the specific toxins present.

The field of algatoxin analysis is a very active and rapidly evolving one, and it is likely that improved and more economical methods will avail themselves in due course. Presentation in the form of a commercially available kit would meet the ideals of the water quality analyst.

TABLE OF CONTENTS

SECTION 2

LIST OF FIGURES

LIST OF TABLES

Tabulated information is all closely related to the associated text content, and tables have therefore not been either labelled or numbered.

PLATES **PAGE**

1. INTRODUCTION

This project arose originally from a proposal, submitted to the Water Research Commission, in September 1992.

Motivation was on the grounds that there was a paucity of information, both in terms of quantity as well as reliability, on the contamination of South African water supplies by *Microcystis* toxins (termed as microcystins; a group of chemically variable algal secondary metabolites, with a cycloheptapeptide ring as a common structural feature). Much of the problem was ascribed to shortcomings in the available methods of algatoxin analysis, and it was recommended that investment be made in further development of existing analytical protocols to increase sensitivity, as well as to improve resolution and identification of the numerous structural variants of these toxins, all of which have dissimilar toxicological potencies.

Umgeni Water undertook development work on high performance liquid chromatography (HPLC), as an analytical system, and concentrate on novel pre-chromatographic sample chemical derivitisation methods, to raise analytical sensitivity and specificity with respect to microcystins.

The CSIR undertook to investigate the reported phenomenon of phosphoprotein phosphatase inhibition by microcystins, and pursue the following programme, quoted verbatim from the September 1992 proposal document:

> Development of extraction procedures for protein phosphatase enzyme systems from difference sources (plant and animal) for toxin assay.
>
> Evaluation of the most suitable protein phosphatase preparation to detect toxins. Evaluations will include actual water samples fro algal bloom-containing dams such as Hartebeespoort and Inanda Dams.
>
> Detailed monitoring in critical water supplies (for at least a year period). Vaal dam and Inanda dam are the main target impoundments.
>
> Development of the most suitable remedial actions.

Further investigation into the literature, and after interaction with Prof Geoff Codd (University of Dundee, Scotland) by telephone and by correspondence, it appeared that since the relevant protein phosphatases have conserved structures, and are ubiquitous in occurrence, there would be little worthwhile information gained from a survey of these enzymes from different biological sources.

A serious disadvantage of the enzyme inhibition assay available at the time was that it required the used of radioactive material. Many technicians refuse to do handle such material, and anyone doing so, is required by law to be registered and medically examined at regular intervals, and the work area has to be fitted and finished according to the requirements deemed necessary by the authorities for nuclear safety for the isotope selected for use. There is also need for a liquid scintillation counter, and such equipment is a high capital cost. The radiolabelled ATP is expensive and has to be air-freighted frozen from a foreign supplier, synthesised just before use, as the usable life of the isotope (^{32}P) is about six weeks only.

Since the emphasis of the proposal had been on method development, the CSIR decided to seek an alternative to use of radiolabelled isotopes, but still exploiting the phenomenon of protein phosphatase inhibition by microcystins. Part I of the report deals with this development work, and the conclusions drawn therefrom.

At the Steering Committee Meeting, held in June 1995, criticism was levelled at the work, in that it was seen to concern itself too much with method development, and did too little to gather information about microcystin concentration measurement in water of South African dams and reservoirs. The decision was then taken to use prevailing published methods, used in overseas leading laboratories with reasonable success, to collect this information from "real samples" of South African dam waters.

The decision then was to use the standard inhibition assay method, largely attributed to Codd and co-workers at Aberdeen University, and assay a toxin standard at range of dilutions, in distilled water, and in waters drawn from local dams, to establish whether there was any matrix effect on the accuracy of this assay. Abnormally high rainfall during the summer of 1996 resulted in massive through-flows of water through all dams, diminishing the prospect of finding any natural microcystin contaminations in samples therefrom.

There is a so-called colourimetric assay (non-radioactive), based on the breakdown of paranitrophenyl phosphate as a substrate, and gaining analytical specificity through the use of the purified protein phosphatase itself. While this may in time prove to be a more attractive option, the enzyme is currently of limited availability, and is extremely costly.

Part II of this report deals with the methods, results, discussion and the conclusions drawn from the use of the protein phosphatase inhibition assay method for the detection and quantification of microcystin.

Key reference sources of information are the following documents, detailed in the reference section of this report. There is the conference publication, edited by Codd, Jeffries, Keevil, and Potter (1994), and two key papers; Honkanen, Zwiller, Moore, Daily, Khatra, Dukelow, and Boynton (1990), and MacKintosh, Beattie, Klumpp, Cohen, and Codd (1990). A further paper, describing a

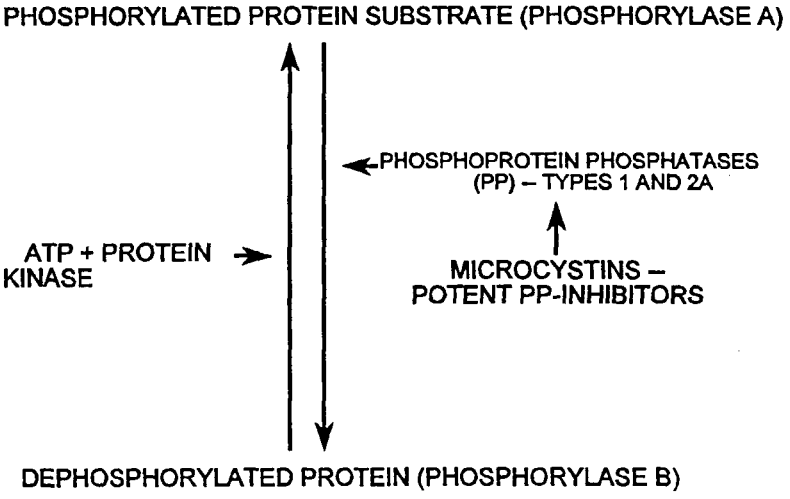
colourimetric assay, by An and Carmichael (1996), was not pursued, as preliminary enquiries revealed that the recombinant PP-1 required was only of limited availability at extreme cost. No doubt, with time, this could change, especially if commercial exploitation as a commercial kit takes place.

2. PART I: AN ATTEMPT TO DEVELOP AN ENZYME INHIBITION ASSAY FOR THE DETECTION AND QUANTIFICATION OF MICROCYSTINS, WITHOUT THE USE OF RADIO-ISOTOPES, BY THE USE OF ELECTROPHORETIC METHODS FOR MONITORING THE PROGRESS OF THE ENZYME REACTION

2.1 Principle of the methods used

As was pointed out in the introductory section of this report, the objective of this section of the work was to look for a novel method of enzyme inhibition assay for microcystins, without the use of radioactive isotopes.

The following reaction schematic gives the principle of the assay method:



A kit is supplied, containing phosphorylase b, ATP, and protein kinase, from which one first has to prepare phosphorylated protein substrate (phosphorylase a). For radiolabelled assay methods, ^{32}P-ATP or ^{33}P-ATP is also mixed with the "cold" (unlabelled) ATP provided in the kit, so obtaining a radiolabelled substrate for the assay. This procedure is described in detail in Part II of this report. Preparation of unlabelled phosphorylase a was carried out as for the work in Part II, except that no supplementary radio-labelled ATP was added to the reaction mixture.

Dephosphorylation of the substrate (phosphorylase a) to product (phosphorylase b) is promoted largely by protein phosphatase (PP) types 1 and 2a, both of which are readily inactivated by the presence of microcystins. The protein phosphatases are prepared as a crude extract from germinating peas, the detail being provided in Part II. Although such an extract will contain many different phosphatase types, presentation of a pure substrate for enzymic attack compels the dephosphorylation process to be specific for these target enzymes being monitored in the assay. Picogram quantities of microcystin in an assay tube can radically impair enzyme efficiency, slowing dephosphorylation rate, and so diminishing the rate of increase of concentration of phosphorylase b, and

conversely the rate of decrease of concentration of phosphorylase a. Thus, the PP serves as a "molecular amplifier", allowing the detection of a target substance (microcystin) at picogram level, through observation of a secondary reaction (dephosphorylation) at concentrations that are many orders of magnitude greater than the target substance. It is through this, that such extreme sensitivity of the method is achieved. It is also a toxicological parameter, which will indicate the combined toxicological effects of microcystins in a mixture (which could be present in natural water samples), together with synergistic/antagonistic mutual effects that may prevail. Not to be overlooked is the possibility that PP-inhibitory effects could come from other substances which are not necessarily microcystins, or from material that is not of algal origin.

Attachment of a phosphate radical to a protein will change the protein's charge composition, and the isoionic point will consequently be altered. This, in turn, should alter the migratory behaviour of the protein in aqueous solution, when exposed to an electric field. This phenomenon can be studied by the technique termed electrophoresis. Micro-methods have been developed, in which very small samples can successfully be studied, achieving fine resolution of complex protein mixtures. Two such methods are PAGE (polyacrylamide gel electrophoresis) and CE (capillary electrophoresis).

PAGE was carried out on a system provided by Pharmacia, termed their "PHAST" system, 50 by 50 mm "Phastgel" sheet gels. This is a two-dimensional system, on which several samples may be run alongside one another, including standard substances, as well as marker dye, movement of which may be used as an internal reference. CE was carried out on a system provided by Beckman, termed their P/ACE™ System 2000. This is a small-bore capillary system, and being one-dimensional, there can only be one sample per run. Resolution can be exceedingly high, if the correct operating conditions are used.

Each assay sample will consist of a complex mixture of many proteins, many of which will arise from the crude pea extract that contains the PP, kinase, as well as bovine serum albumin, which is included in the ingredients of the kit, to stabilise the material. The only protein expected to change charge during the course of the assay would be the phosphorylase a, changing to phosphorylase b. In inhibited assays, no such change would take place. Since accurate quantification of components in electrophoresis is difficult, one would want to aim for observable "end-points", such as equal quantities of both phosphorylases a and b, which would be indicative of a 50% extent of dephosphorylation reaction in an assay tube. These would be equivalently sized spots on PAGE, or peaks of equal area on P/ACE.

2.2 Methods and results

Section II describes preparation of microcystin-LR stock solution, (0.5 mg) in 10% methanol-water (5 ml). Microcystin "assay" solution was made from stock (5 µl) in 10% methanol-in-water (1000 ml).

The trichloroacetic acid provided in the kit was not used.

Protein phosphatase assay buffer (10 ml) was made up in accordance with the kit's instructions, and kept at 2 - 4°C (refrigerator) or on ice, when being used on the bench.

One snap-frozen tube of pea extract was thawed, and kept on ice.

2.2.1 Microcystin-inhibited protein phosphatase reactions

Inhibition assays were carried out in tapered Eppendorf "3810" 1.5 ml snap-top plastic microtest-tubes, in batches of eight. The set was made up as follows:

20 µl protein phosphatase solution (pea extract)
20 µl protein phosphatase buffer solution
X µl microcystin assay solution, vortex-mix
Y µl 10% methanol in water solution, vortex-mix
20 µl phosphorylase a substrate solution, vortex-mix

Quantities of X and Y used, and the identification mark of each tube, are as follows:

	O	I	II	III	IV	V	VI*
X µl microcystin assay solution	0	0.6	3	6	12	30	30
Y µl 10% methanol in water	30	29.4	27	24	18	0	0

* In the case of VI incubation at 30°C was omitted from the procedure. Blocking with microcystin stock solution followed make-up of assay tube, which was then put in ice-water.

Tubes 0 to V were immediately placed on a 30°C thermostatted water bath for 10 minutes, and then withdrawn.

5 µl microcystin stock solution was then added to each tube, to completely block any further dephosphorylation, and the tubes kept in ice-water in the refrigerator for electrophoretic study the following day.

2.2.2 PAGE electrophoretic method

This was carried out on a Pharmacia "PHAST" system, on 50 x 50 mm PhastGel sheet gels. Details of the method are given in Pharmacia's Laboratory Separation Division's PhastSystem™ Separation Technique File No 120; Native-PAGE.

The native-PAGE system has the advantage of retaining structural and conformational integrity of proteins under examination, and is thus less likely to generate artefactual

data.

A 1 µl sample applicator was used.

Running pH was 8.8, in PhastGel gradient gels, 8 to 25% (Code 17-0542-01) . PAGE Buffer strips (Code 17-0517-01) were used. Run time was 60 minutes, after which the standard Coomassie blue staining-destaining procedure, supplied with the equipment, was followed. Gels were photographed after the background had cleared completely (2 to 3 days).

2.2.3 P/ACE™ high-performance capillary electrophoresis (HPCE)

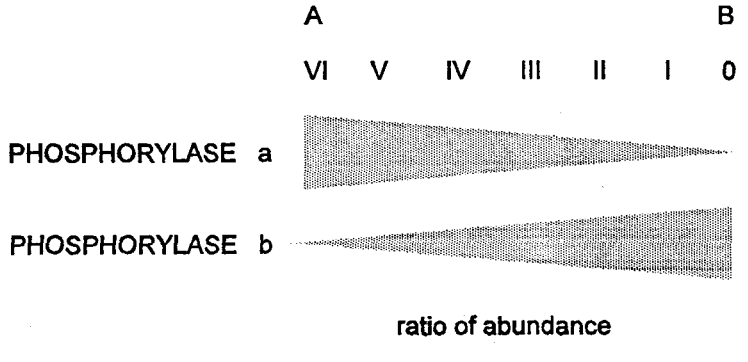
This was performed on the Beckman P/ACE System 2000 according to the standard method that they provide in the literature with the equipment.

Absorbance was measured at 214 nm.
Capillary was 270 mm by 50 µ.
Buffer system was 100 mM borate + KOH to pH 8.3
Samples were pressure-loaded at the anode end.
Operating voltage was 30 kV.

3. RESULTS AND DISCUSSION

An increase in the concentration of the "assay" microcystin in each sample should lead to a decrease in dephosphorylation rate of phosphorylase a to b.

The expected relative compositions of phophorylase a and b in the sample are depicted diagrammatically as follows, the width of the wedge representing abundance:



Considering the factor of molecular charge alone, it would be expected that the phophorylase a, with an extra phosphate, which is an additional anionic substituent, would have a diminished mobility relative to phosphorylase b, when migrating towards the cathode (negative electrode) on electrophoresis. It is possible, however, that other factors, such as a molecular conformational change on phosphorylation, could also influence electrophoretic mobility.

3.1 PAGE electrophoresis results

Photographic record of the gel has been given in Figure 1. In order to accommodate samples in a single run, samples II, III and IV were omitted, since the initial purpose was to determine whether it was possible to see differences with respect to electrophoretic pattern in the "extreme cases".

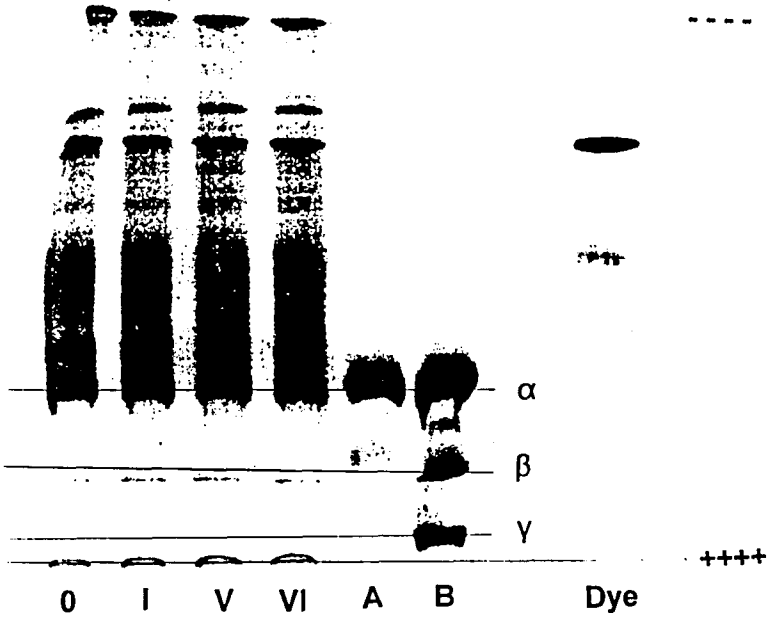
An electrophoretic run, with electrode polarity reversed, was also carried out, confirming that no bands had been inadvertently lost through back-migration of components into the electrode region behind the sample loading area.

It has first to be pointed out that a heterogeneous protein background will arise from the following sources:

the crude protein extract from the germinating garden peas

bovine serum albumin, that has been included as a stabilising agent in the protein phosphatase assay buffer of the Gibco-BRL kit.

FIGURE 1: PHOTOGRAPHIC RECORD OF PAGE ELECTROPHORETOGRAM



This material would give rise to the general background in the tracks O to VI and to the indicator dye track, which also contains a small amount of bovine serum albumin.

A very clear change in electrophoretic pattern can be seen on conversion of phosphorylase b to phosphorylase a. The two slower bands for b (ß and gamma), after phosphorylation, run with the single major band for a (alpha) This is a mobility *increase* on phosphorylation, contrary to expectation. Whether b, as three bands, represents three iso-enzymes, perhaps one of which may have more than a single phosphorylation site per enzyme molecule, would not be known, without detailed information regarding the origins of the material. The presence of phosphorylatable protein that is not phosphorylase b (and does not interfere with the radiolabel assay procedure) can also be a possibility. Phosphorylase a moves as a single major band (alpha) with only a faint band (beta) of slower mobility behind it, which could be a trace of phosphorylase b, arising either from an incomplete phosphorylation reaction, or subsequent breakdown of phosphorylase a during recovery procedures. The use of a membrane concentrator can lead to the loss of small molecular weight proteins below the cut-off limit of the membrane, and influence the electrophoretic pattern.

Electrophoretic origins for samples O, I, V and VI are all stained, unlike that for the other samples, and this is ascribed to denatured and/or insoluble proteinaceous material in the seed extract that was unable to penetrate the gel matrix. In retrospect, it would have been useful to have run a channel with the seed extract alone, for the purposes for comparison. The strong band (alpha), found in both b and a, is present in samples 0 to VI, the second slowest band (beta) being only very faint, and not showing any clear gradation in intensity from 0 through to VI. What would have been expected was a strongest beta band in 0, and a weakest beta band in VI.

3.2 Conclusions from the PAGE results

Phosphorylases b and a (tracks B and A) show a difference in PAGE electrophoretic patterns, presumably arising from the difference in protein phosphorylation. The diminished intensities of bands beta and gamma in track A could be because either or both of these bands belong to phosphorylase b. Difficult to explain is the higher migration speed of alpha towards the cathode, which would not be expected of a species of increased phosphorylation; but protein molecular conformational change may also be involved.

Samples in tracks 0, I, V, and VII show no notable differences, not even track 0, incubated with no microcystin. Bands beta and gamma do not appear in any significant abundance in these assay samples. Since the biochemistry of the assay has been shown to "work" in Part II of this report, it can only be assumed that the compositional differences being looked for on this electrophoretogram cannot be seen against the general protein background.

Use of a membrane concentrator in the recovery step of phosphorylase a could lead to loss of some small molecular weight components, which might make some unexpected contribution to the difference between the

electrophoretograms of Sample A and B.

Background contributed by extraneous proteinaceous components, such as bovine serum albumin and pea seed cell sap proteins, has a higher electrophoretic mobility range than that attributable to the phosphorylases, and should therefore not be expected to interfere with phosphorylase a and b band identification.

3.3 P/ACE electrophoretic results

Runs were at 30 kV; monitor wavelength was 214 nm.
Depiction of the capillary configuration can be given as follows:

The higher-mobility alpha band (as seen on PAGE) should emerge at the detector first (shortest emergence time) on P/ACE. Electro-osmotic flow through the capillary is from + to -, namely, co-directional with the protein sample migration.

Print-outs for P/ACE runs run are in Figures 2 to 7. X-axis units are minutes. Not all samples were loaded, for the same reason of economy of effort given for the PACE runs, sufficient information being available from the "extreme-case" conditions. Since with P/ACE, it is not possible to load precise and repeatable sample volumes per run, overall peak sizes from run to run may differ, but relative sizes of peaks within a run should accurately reflect relative concentrations of components in that sample. Where required, reliable quantification with P/ACE requires the inclusion of internal sample standards, to which unknown peaks may be related.

P/ACE, like PAGE, shows distinctly different patterns for Samples A and B, these being phosphorylase a and b. Ultraviolet absorbance, used in P/ACE may be a more trustworthy reflection of quantity than protein-dye affinity, used in PAGE.

P/ACE results of the assay test samples, like the PAGE electrophoretograms, show insufficient differences that could be interpreted as meaningful compositional changes arising from different rates of dephosphorylation taking place in each of the test samples.

3.4 Conclusions from the P/ACE results

Like PAGE, P/ACE shows a difference in electrophoretic pattern between phosphorylases a and b. On the basis of this result, one would expect to be able to make an approximate estimate of the relative composition of these components in an assay mixture.

Like PAGE, P/ACE shows no evidence of any significant protein compositional differences between the various dephosphorylation assay samples, with, or without microcystin addition.

The presence of extraneous proteinaceous components from sources such as the pea seed cell sap, or bovine serum albumin, may mask or interfere in some way with detection and identification of phosphorylase a and b.

4. CONCLUSIONS FROM THE ELECTROPHORESIS TECHNIQUES USED

Work carried out in Part II of this project shows that the biochemistry of the method is unlikely to be suspect.

The only inference that can be drawn is that the protein compositional changes, occurring during the course of dephosphorylation of phosphorylase a to b, are not detectable by the two methods electrophoretic methods investigated.

Enzyme inhibition assay of microcystins by electrophoretic analysis of the reaction products is not possible under the experimental conditions that were used for this work.

Figure 2: P/ACE electrophoretogram, Sample B

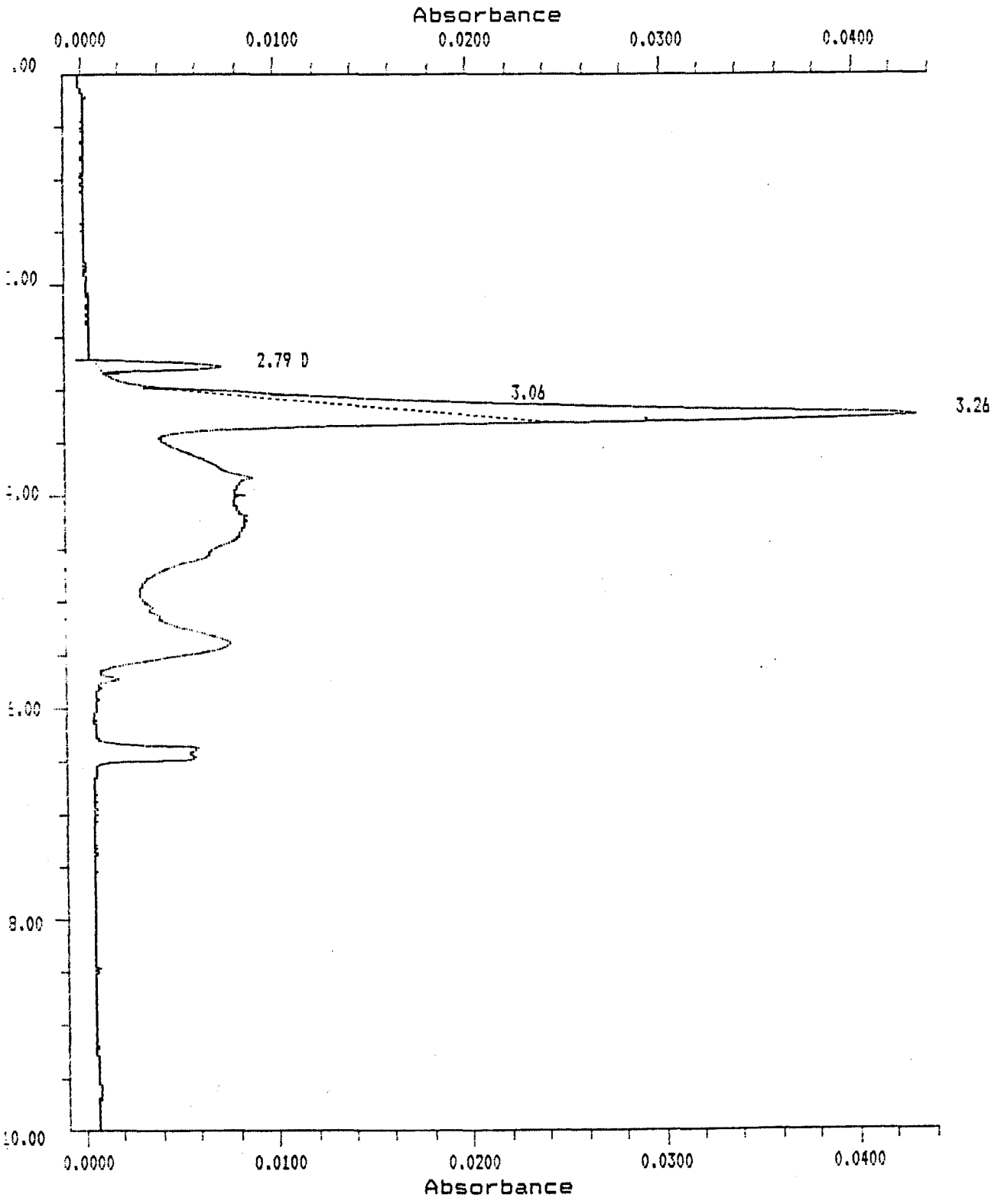


Figure 3: P/ACE electrophoretogram, Sample A

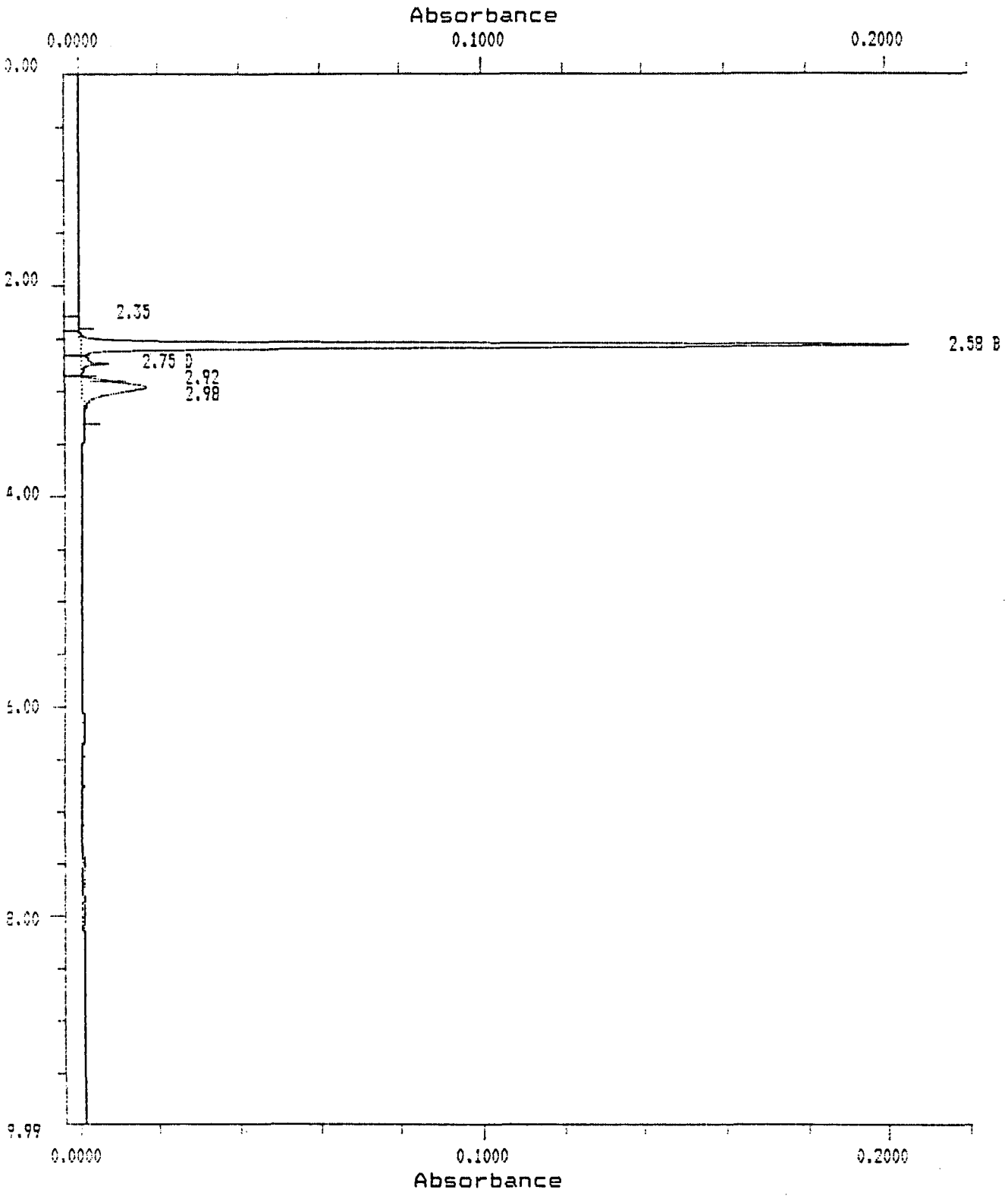


Figure 4: P/ACE electrophoretogram, Sample O

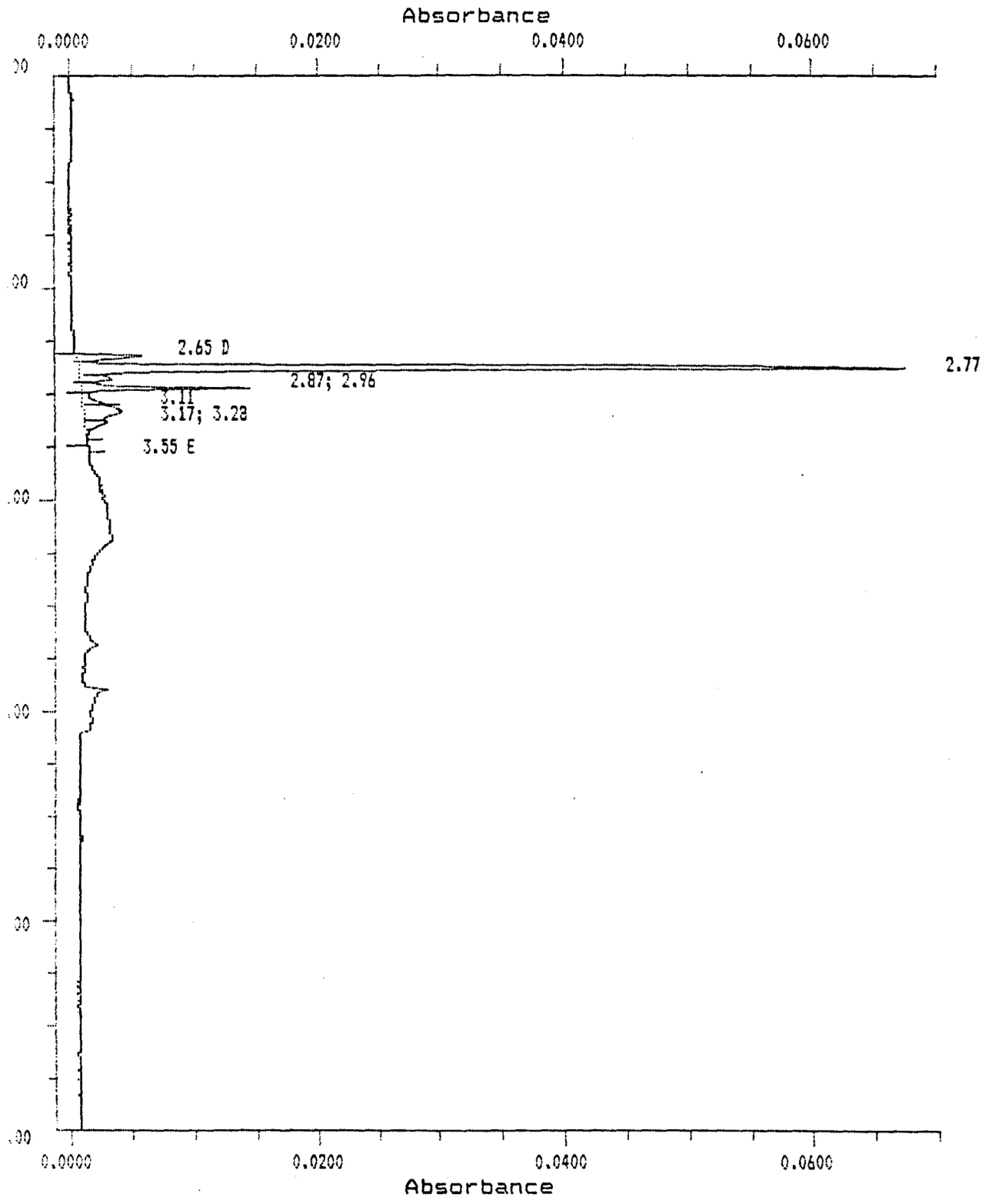


Figure 5: P/ACE electrophoretogram, Sample I

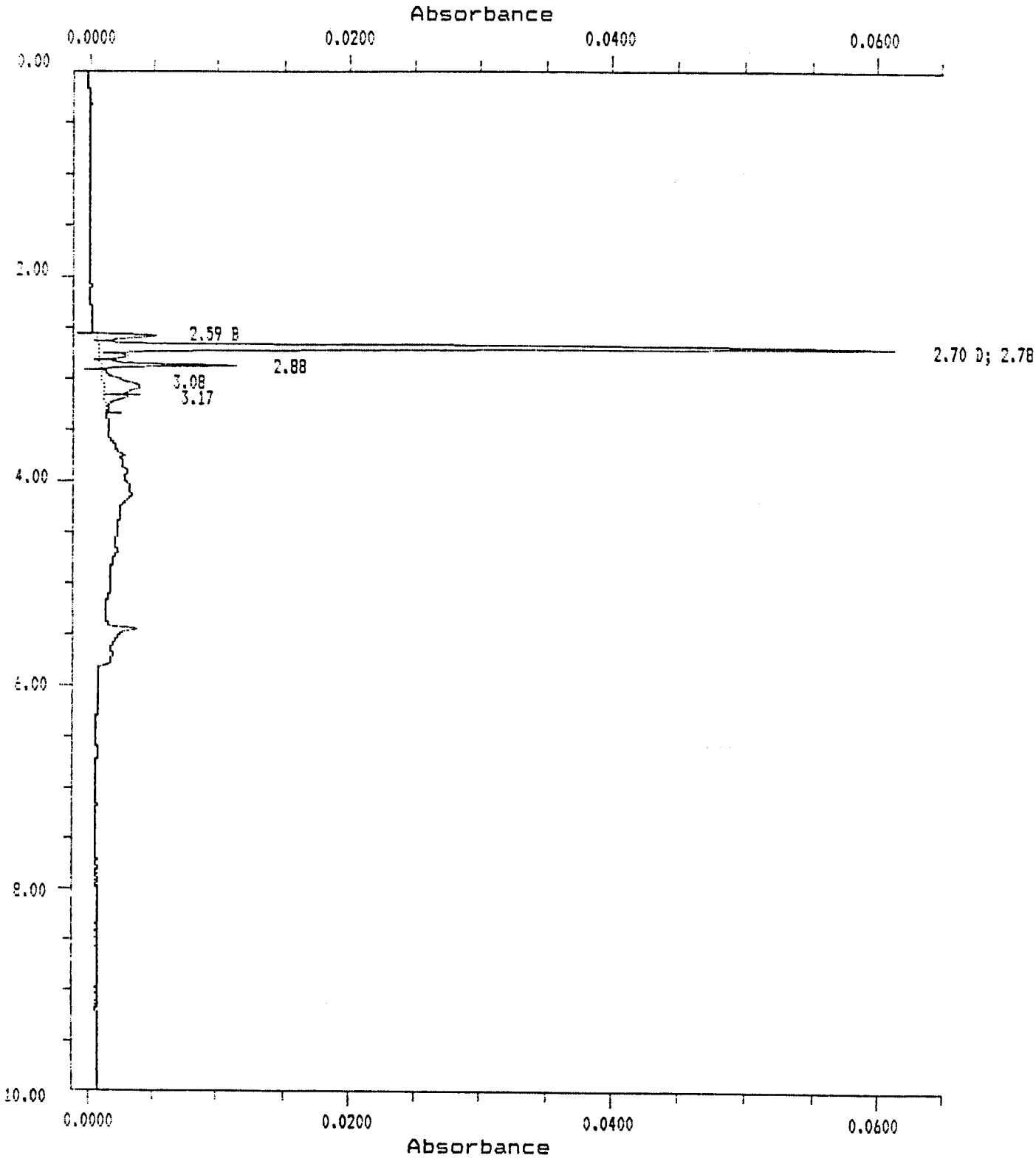


Figure 6: P/ACE electrophoretogram, Sample V

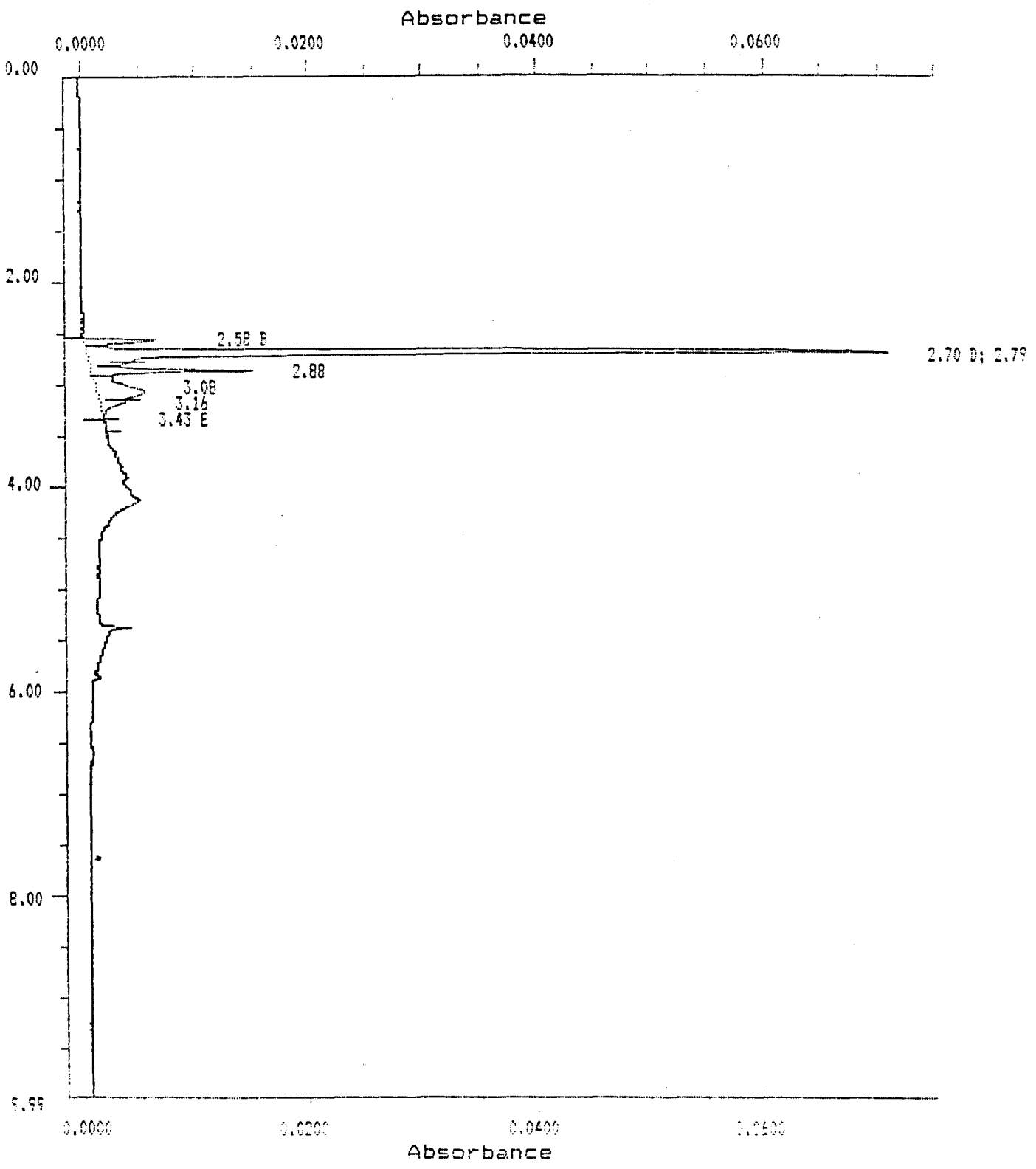
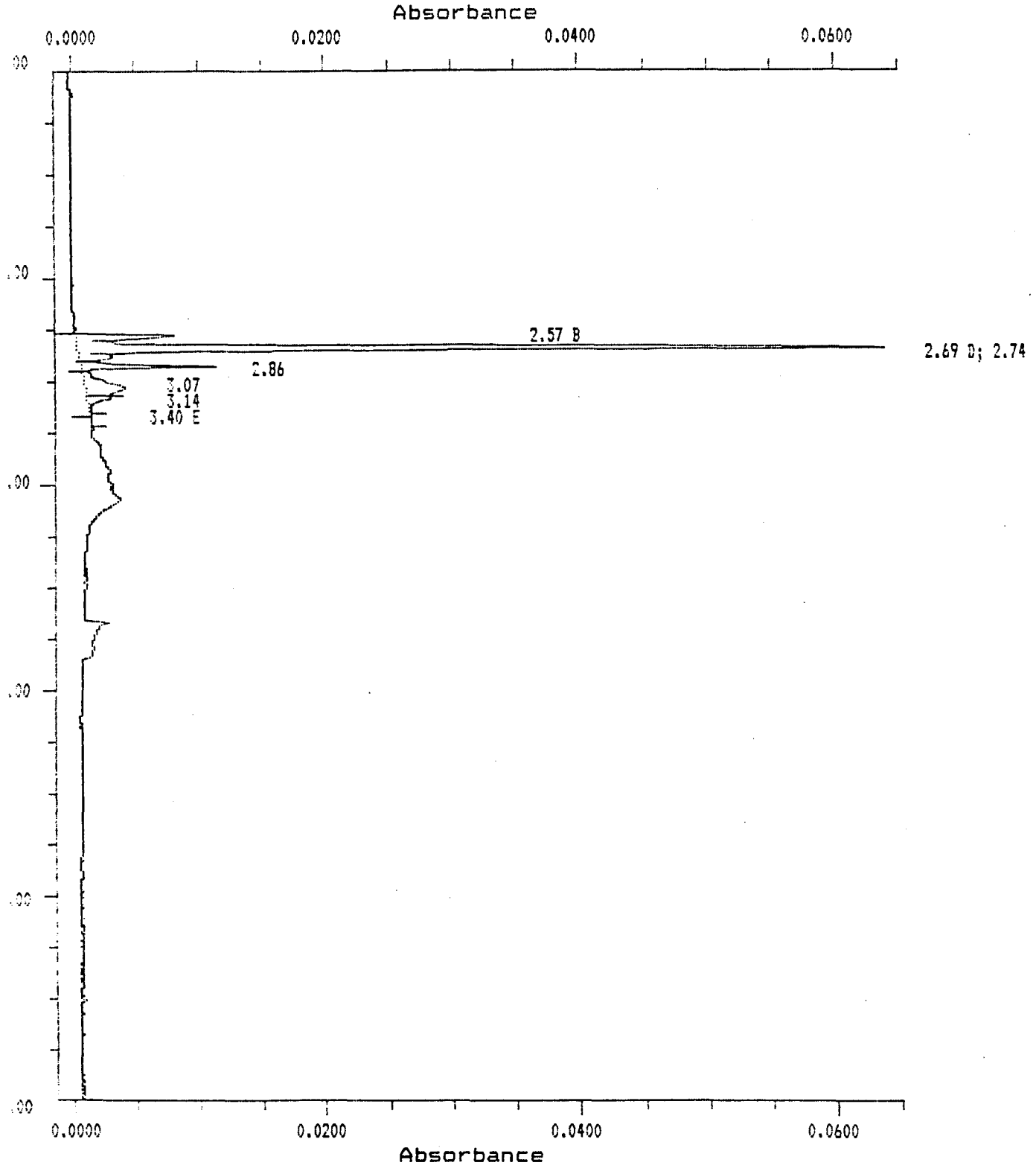


Figure 7: P/ACE electrophoretogram, Sample VI



5. PART II: USE OF ^{33}P-LABELLED PHOSPHORYLASE A IN AN ENZYME INHIBITION ASSAY FOR THE PRESENCE OF MICROCYSTINS.

The principle on which the assay system is based has been described in Part I of this report. The difference in the following work is that release of radioactive inorganic phosphate from the protein is monitored with liquid scintillation counter, which gives a measurable parameter of high selectivity and sensitivity, without significant interference by any other materials present in the system.

^{33}P has been selected for this work, in preference to the ^{32}P, which is the isotope in more common use. The reason for this choice is because of a less hazardous radioactive emission (reduced Bremsstralung risk), and a longer half-life, which extends the usable time of the isotope after receipt. The tabulated data below from (Amersham Life Sciences) shows the difference between the two isotopes:

	Half-life, days	emission	range in air and water, mm
^{32}P	14.3	high beta energy; 1.7MeV	7900 and 0.8
^{33}P	25.4	low beta energy; 0.25MeV	490 and 0.6

The assay method was kindly provided by Professor Codd (private communication), and is based closely on the protocol that has been supplied with the GibcoBRL Life Technologies kit, catalogue number 13188-016, "Protein phosphatase assay system".

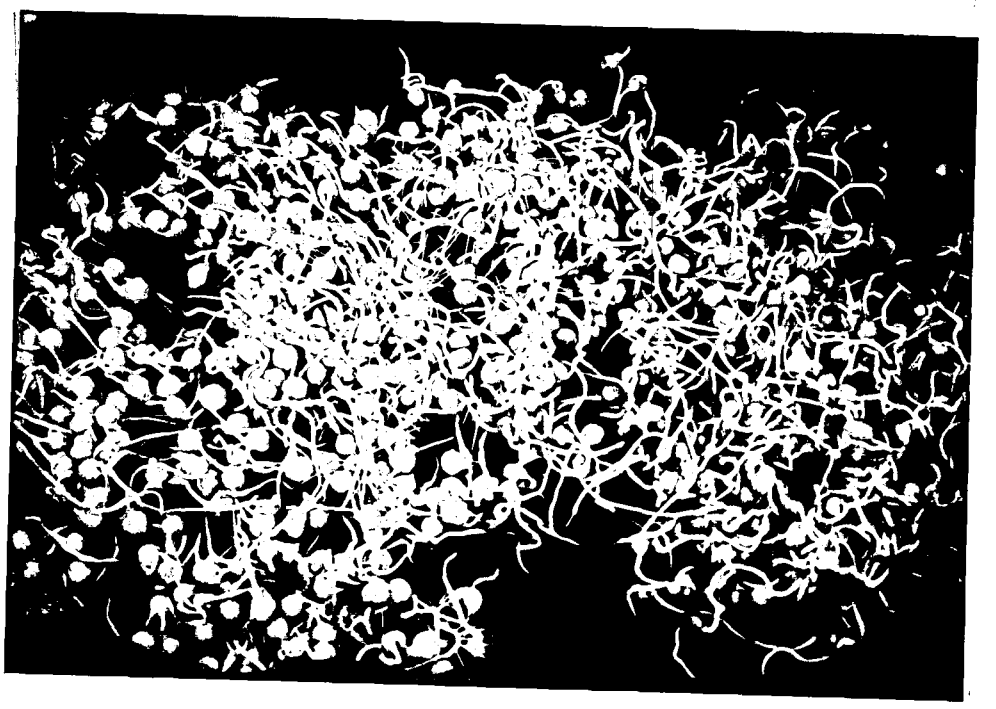
5.1 Preparation of the crude protein phosphatase extract (referred to as the enzyme)

One seed packet of garden peas (about 50) were germinated on a wet layer of geofabric, covered by a second geofabric layer, for about five days. The photograph (Plate 1) overleaf, shows the state of growth at which they were harvested. Seedlings with heathy and vigorous growth were hand-picked out to a total mass of 48 g. Buffer (96 ml), being 50 mM Tris-HCl plus 0.1 M EGTA, 0.1% mercaptoethanol, adjusted to pH 8.0 with NaOH, was added to the seeds in a Waring Blendor, and the material homogenised for 40 seconds at full speed. The pulp was transferred to Sorval stainless steel SS-43 centrifuge tubes and spun at 11 500 rpm (RCF = 16 000 x g) for 20 minutes at 5°C. The material was then carefully poured through about five layers of muslin, which retained solid and fatty scum, giving an opalescent filtrate, which was pipetted in 1 ml aliquots into 2 ml Eppendorf tubes. These were immediately closed and snap frozen in liquid nitrogen, and then stored at -70°C in a low-temperature deep freeze until required for use, labelled as enzyme stock.

5.2 Preparation of the radiolabelled substrate

This was carried out with the use of the Gibco-BRL LIfe Technologies "Protein

Plate 1: Photograph of germinated pea seeds. Only the batch on the right were harvested. Those on the left had a coloured substance applied to them, suspected to be a fungicide, and were not used, due to risk that this could influence experimental results.



phosphatase Assay System", Catalogue No 3188SA, essentially following the instructions provided in the literature enclosed with the kit. The gamma-^{33}P-ATP was supplied by Amersham Life Science. Quantity was 25 µl, 260 µCi, labelled at >1000 Ci/ mmol. All manipulations were carried out in a registered radioisotope laboratory, wearing disposable gloves, monitoring equipment and work area for contamination by means of a hand-held Geiger-Muller counter (Eberline model HP-160). All radio-isotope-containing solid and liquid waste was placed in appropriate containers, for collection and authorised disposal. At the end of laboratory work, personal urine (1 ml) was mixed with scintillant (10 ml) and counted, to establish whether any personal contamination had taken place during the course of this project.

Both the ^{33}P-labelled ATP, one tube of kinase/substrate mix, and one tube of the phosphorylation reaction buffer were thawed in ice. Water (55 µl), was added to the label (25 µl), and the solution transferred with a zipette (disposable tips) to the thawed phosphorylation reaction buffer. Mixing was effected by gentle aspiration with the
mixture was then transferred to the kinase/substrate tube, mixed again
tion, and put into the themostatted water bath at 30°C for 60 mins. At
eriod, ammonium phosphate solution (90% saturated) (1 ml) was ad
ette aspiration carried out, and the material then left on ice for 30 min.
n spun for 20 mins at 10 000 rpm of a Sigma 101S microcentrifuge
2 - 4°C. The supernatant was drawn off with a zipette and discarded in
aste container. Wash solution (45% saturated ammonium sulphate)(1
was added to the labelled protein precipitate, also on ice, which was t
d by careful pipette aspiration/discharge action. The material
again in the cold as before, and the supernatant again discarde
uid waste. This washing/centrifugation cycle was repeated a further th
four times), before dissolving the precipitate in solubilisation buffer (1
perature with pipette aspiration, and transferring the liquid to the h
he membrane concentrator provided in the kit. This was followed wi
f solubilisation buffer at room temperature, to complete material tran
to the concentrator. The concentrator collection vial was first cut to ab
to accommodate the unit in the rubber insert for glass tubes in a Sor
of a table-top centrifuge. A suitably weighted counterbalance (unu
concentrator) was put in the opposite well of the rotor. The centrifuge was run at room temperature, while using a Strobotac stroboscopic lamp to check rotor speed (6000 rpm) as well as to freeze the image of the revolving concentrator, making it possible to observe the descending meniscus of the fluid in the concentrator reservoir, and to stop centrifugation when about 400 µl remained. The centrifuge was stopped, contents of the collection vial discarded to radioactive waste, and the concentrator reservoir replenished with solubilisation buffer to a reservoir contents volume of 2 ml. Centrifugation was resumed until the reservoir contents volume was again reduced to 400 µl. Permeate in the collection vial, and the vial itself, were appropriately discarded to radioactive waste, and the retentate in the concentrator reservoir transferred with a zipette to a 5 ml tapered glass screw-capped Pierce Reactivial. The concentrator reservoir was washed with solubilisation buffer three times (3 by 0.5 ml aliquots), and these washings all added to the Reactivial, total volume finally being brought to about 3.1 ml with solubilisation buffer. After mixing, this solution was distributed in 0.5 ml

phosphatase Assay System", Catalogue No 3188SA, essentially following the instructions provided in the literature enclosed with the kit. The gamma-^{33}P-ATP was supplied by Amersham Life Science. Quantity was 25 µl, 260 µCi, labelled at >1000 Ci/ mmol. All manipulations were carried out in a registered radioisotope laboratory, wearing disposable gloves, monitoring equipment and work area for contamination by means of a hand-held Geiger-Muller counter (Eberline model HP-160). All radio-isotope-containing solid and liquid waste was placed in appropriate containers, for collection and authorised disposal. At the end of laboratory work, personal urine (1 ml) was mixed with scintillant (10 ml) and counted, to establish whether any personal contamination had taken place during the course of this project.

Both the ^{33}P-labelled ATP, one tube of kinase/substrate mix, and one tube of the phosphorylation reaction buffer were thawed in ice. Water (55 µl), was added to the label (25 µl), and the solution transferred with a zipette (disposable tips) to the thawed phosphorylation reaction buffer. Mixing was effected by gentle aspiration with the zipette. This mixture was then transferred to the kinase/substrate tube, mixed again by gentle aspiration, and put into the themostatted water bath at 30°C for 60 mins. At the end of this period, ammonium phosphate solution (90% saturated) (1 ml) was added, mixing by zipette aspiration carried out, and the material then left on ice for 30 min. The tube was then spun for 20 mins at 10 000 rpm of a Sigma 101S microcentrifuge in a cold room at 2 - 4°C. The supernatant was drawn off with a zipette and discarded in the radioactive waste container. Wash solution (45% saturated ammonium sulphate)(1 ml), cooled in ice, was added to the labelled protein precipitate, also on ice, which was then re-suspended by careful pipette aspiration/discharge action. The material was centrifuged again in the cold as before, and the supernatant again discarded to radioactive liquid waste. This washing/centrifugation cycle was repeated a further three times (total of four times), before dissolving the precipitate in solubilisation buffer (1 ml) at room temperature with pipette aspiration, and transferring the liquid to the head chamber of the membrane concentrator provided in the kit. This was followed with a rinse (1 ml) of solubilisation buffer at room temperature, to complete material transfer from the tube to the concentrator. The concentrator collection vial was first cut to about half its height to accommodate the unit in the rubber insert for glass tubes in a Sorvall SS-34 rotor of a table-top centrifuge. A suitably weighted counterbalance (unused concentrator) was put in the opposite well of the rotor. The centrifuge was run at room temperature, while using a Strobotac stroboscopic lamp to check rotor speed (6000 rpm) as well as to freeze the image of the revolving concentrator, making it possible to observe the descending meniscus of the fluid in the concentrator reservoir, and to stop centrifugation when about 400 µl remained. The centrifuge was stopped, contents of the collection vial discarded to radioactive waste, and the concentrator reservoir replenished with solubilisation buffer to a reservoir contents volume of 2 ml. Centrifugation was resumed until the reservoir contents volume was again reduced to 400 µl. Permeate in the collection vial, and the vial itself, were appropriately discarded to radioactive waste, and the retentate in the concentrator reservoir transferred with a zipette to a 5 ml tapered glass screw-capped Pierce Reactivial. The concentrator reservoir was washed with solubilisation buffer three times (3 by 0.5 ml aliquots), and these washings all added to the Reactivial, total volume finally being brought to about 3.1 ml with solubilisation buffer. After mixing, this solution was distributed in 0.5 ml

aliquots into 2 ml tapered Eppendorf tubes, stored closed at 2-4°C before use, labelled as substrate solution. Concentrators were consigned to radioactive waste.

5.3 Standard toxin stock solution of microcystin-LR

Microcystin-LR (0.5 mg), was supplied by Gibco-BRL. The specified weight was assumed to be correct, and the material was dissolved in a small amount of methanol by injection of the solvent with a Hamilton syringe through the rubber septum of the ampoule. The same syringe was used to transfer this solution to a 5 ml volumetric flask. A succession of small methanol-water washings from the ampoule to the 5 ml volumetric flask were made, until the microcystin-LR was in 10% water in methanol solution to the 5 ml mark on the volumetric flask. This has been termed the microcystin stock solution, with a concentration of 100 µg/ ml (100 ng/µl, or 10 000 pg/µl), and was stored at 2-4°C.

5.4 Activity assay of the protein phosphatase enzyme in the germinated pea extract (no added microcystin)

Protein phosphatase assay buffer was made up from stock solutions "A" and "B" in the kit, as instructed, and kept at 2-4°C. 20% TCA solution was also prepared according to directions and stored at 2-4°C.

Tapered Eppendorf "3810" 1.5 ml snap-top plastic micro-test tubes (vials) were used for each sample assay, a batch of 8 being processed at a time, for manageable control over incubation times and other manipulative procedures. Use of multiple gang pipettes and other aids could make it possible in routine practice to increase the samples batch sizes.

A snap-frozen vial of pea extract (crude protein phosphatase enzyme) was taken from the -70°C freezer, and thawed in water at room temperature, before storing at 2-4°C.

A set of eight Eppendorf assay tubes were put in ice, and made up, as indicated below:

tube number	1	2	3	4	5	6	7	8
added pea extract, µl	0	5	20	50	75	100	0	0
added assay buffer, µl	100	95	80	50	25	0	100	100
enzyme solution conc., %	0	5	20	50	75	100	0	0

A further 20 µl assay buffer was added to each tube, followed by 20 µl of the labelled substrate solution. Each tube was vortexed and placed in the 30°C thermostatted bath for 10 min, after which they were returned to the ice, and 20% TCA (180 µl) added. The tubes were again vortexed, allowed to stand for a further 10 min, and then centrifuged at 10 000 rpm on the microcentrifuge for 10 min. The tubes were returned to ice, and aliquots of supernatant (200 µl) were carefully drawn off, each being placed in a screw-

capped 20 ml glass scintillation vial containing scintillation fluid (Packard Ultima Gold)(10 ml). Each vial was then well shaken. A ninth vial, identified as "total", contained scintillation fluid (10 ml) and an aliquot of the labelled substrate (20 µl). All samples were counted on a Packard Minaxi-ß Tri-Carb-4000 Series liquid scintillation counter, set to count over the time required for collection of a statistically valid number (5 min or less). Since ^{33}P was the only isotope present, the energy acceptance window could be wide, and the machine was set up (keV units) as follows:
Region A: LL - UL = 0 - 249
Region B: LL - UL = 8 - 249
Region C: LL - UL = 0 - 0
Radioactivity, as disintegrations per minute (dpm) was recorded as given by the scintillation counter print-out , DPM1/K. After each run, a count on a ^{14}C standard was executed to verify that the instrument was functioning correctly.

^{32}P radioactivity data, for this experiment, were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	25.6	146	290	275	313	344	12.4	15.2	588

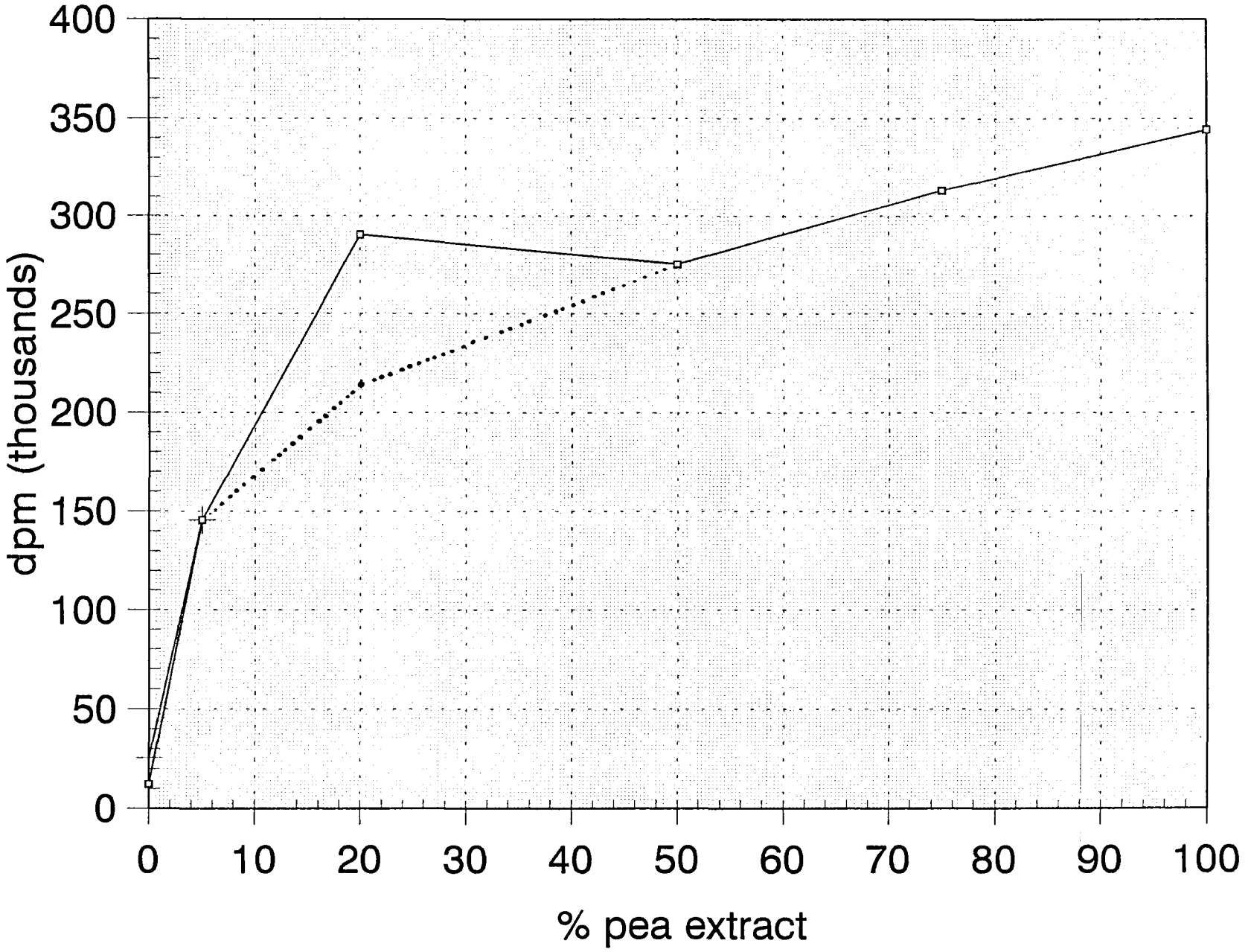
These data have been depicted graphically in Figure 8, overleaf.

Value for 20% pea extract may be suspect, possibly due to accidental aspiration of labelled protein precipitate while drawing off TCA supernatant, and the dotted line gives a speculated value, which would provide the expected shape of dephosphorylation curve. Since 200 µl is drawn for counting from the total sample volume of 340 µl, a proportionate reduction of the recorded "tot" would be 345 x 10^3 dpm, which would be the expected maximum from any assay vial in this batch. 100% Pea extract results in total dephosphorylation. Taking note of the advice (Codd - private communication) that for assay inhibition studies, the amount that the enzyme break-down of the substrate over the measured period should not exceed 30%, it is clear that the pea extract enzyme activity is far too great to use without dilution. Labelling is thorough, as can be seen from the large dpm per assay, and for 30% phosphate release, a supernatant dpm of about 110 x 10^3 dpm per vial (marked on the graph) would require a pea enzyme stock dilution to a concentration below 4% v/v. Cognisance has to be taken of the fact that diluted enzyme solutions are less storage stable, and have to be used as soon as possible after preparation.

5.5 Comments on manipulative methods of the experimental technique

The whole procedure is "fiddly", making exacting demands of anyone's eye-hand co-ordination. The small Eppendorf vial, made of opalescent plastic, makes vision of the pipette tip and protein pellet unclear, increasing the risk of contaminating supernatant with precipitated protein, resulting in erroneous dpm data, especially for small degrees of dephosphorylation. The inconvenience of wearing gloves, risk of spillage (especially any liquid close to the edge of the "snap-top", as will always be after vortex mixing),

Figure 8: Pea extract activity concentration plot



cross-contamination, demands the skill of keeping all material down in the bottom of the vial and not as droplets on the upper sides, and the care needed to keep times and temperature all the same, add to the scope for error. "Blank" counts (tubes 1, 7 and 8, for this experiment) are a useful indicator of operator practice skill - the lower, the better. It can be seen in this project how practice has lead to a decrease in these blank values as the work proceeded.

5.6 Inhibition assay Experiment No1

The same batch of pea extract was diluted the following day, 30 µl plus 970 µl of assay buffer, the material being stored at 2-4°C before use.

Microcystin stock solution (25 µl, containing 2.5 µg microcystin) was diluted with distilled water to 1000 ml. This diluted toxin was thus 2.5 µg/ 1000 ml, or 2.5 ng/ ml, or 2.5 pg/µl.

A set of eight Eppendorf tubes were put in ice, and made up, as indicated below:

tube number	1	2	3	4	5	6	7	8
dil. pea extract, µl	20	20	20	20	20	20	0	0
assay buffer, µl	20	20	15	10	5	0	40	40
dil. microcystin, µl	0	0	5	10	15	20	0	0
nett microcystin, pg	0	0	12.5	25	37.5	50	0	0

Labelled substrate (20 µl) was added to all the tubes, as well at to a "total" vial of scintillant as described before. All Eppendorf tubes were vortexed, incubated, and treated with TCA as has been described in the previous experiment.

Scintillation counting data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	28.1	7.15	11.1	11.3	7.01	6.11	12.9	11.2	632

Tube 1 depicts phosphorus release in the absence of inhibitor ("uninhibited"). Volumetric corrections for "total", as described for the enzyme dilution assay, would give a theoretical maximum count per sample of 544 x 10^3 dpm.

Tube 1 indicates that the uninhibited dephosphorylation of the substrate is well below the recommended maximum of 30% (180 x 10^3 dpm), and is less than expected on the basis of the dilution curve of the previous experiment, but may be due to loss of

enzyme activity on standing in the diluted state, even on storage at 2-4°C. It needs to be prepared as a fresh dilution from snap-frozen pea enzyme stock each day.

Tube 2, effectively a duplicate of 1, has an unexpectedly low count, and may be due to the enzyme reaction having failed to proceed through poor sample handling, or some other unaccountable cause. Tubes 3,4,5 and 6 show progressive decreases in released phosphate, attributable to microcystin inhibition of the reaction. Tube 3 counts are less than half that of tube 1 (over 50% inhibition, discounting any contribution by background). Tube 3 contained 12.5 pg of microcystin-LR. Codd claims the sensitivity of the method to be in the range of 10 - 100 pg, with which this would agree. Figure 9 overleaf indicates that within the range, there is a lack of linearity, as well as scatter, indicating caution in any attempt to quantify the microcystin content from the degree of inhibition in a particular tube, unless conditions are first established using a set of standard concentration of microcystins, where linearity and repeatability can be demonstrated. Tubes 7 and 8 should have had low dpm (which was achievable in succeeding experiments). It can only be assumed that contamination of supernatant with labelled protein precipitate took place; 200 µl had to be drawn from a total of 240 µl, allowing very little margin for manipulative error. In succeeding experiments, disposable tips were changed on the zipette for all aliquots drawn and discharged, to ensure that this was no source of cross-contamination. The drawn aliquot of TCA supernatant was later reduced from 200 µl to 180 µl, as is explained later in the report.

5.7 Inhibition assay Experiment No 2

This was a repeat for Experiment 1, but with the same stock pea extract diluted, 100 µl with 900 µl assay buffer.

Radioactivity counts were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	20.8	19.5	5.35	2.99	3.86	3.20	1.78	2.74	583

Tubes 1 and 2, with no inhibition, agree well as duplicates, but it is evident in spite of higher enzyme concentration, released phosphate is evidently lower than with Experiment 1 and may be due to loss of enzyme activity of the stored pea extract at 2 - 4°C, over the period between the two experiments (ca 5 hours). Interestingly, the effect of microcystin inhibition at this lower overall enzyme titre per tube is even more dramatic than for the first experiment, showing how this low enzyme activity favours high inhibition sensitivity in the assay. "Blank" tubes 7 and 8 show an appropriately low background radioactivity release, being about half of the released radioactivity of the microcystin inhibited tubes.

Figure 10 overleaf gives a graphical presentation of the data, with only about a quarter of the uninhibited radioactivity being released in the presence of 12.5 pg microcystin. Sample loads of an excess over 10 pg are not quantifiable, as the enzyme activity appears to be overwhelmed by toxin inhibition, giving a plateau.

Figure 9: Experiment 1

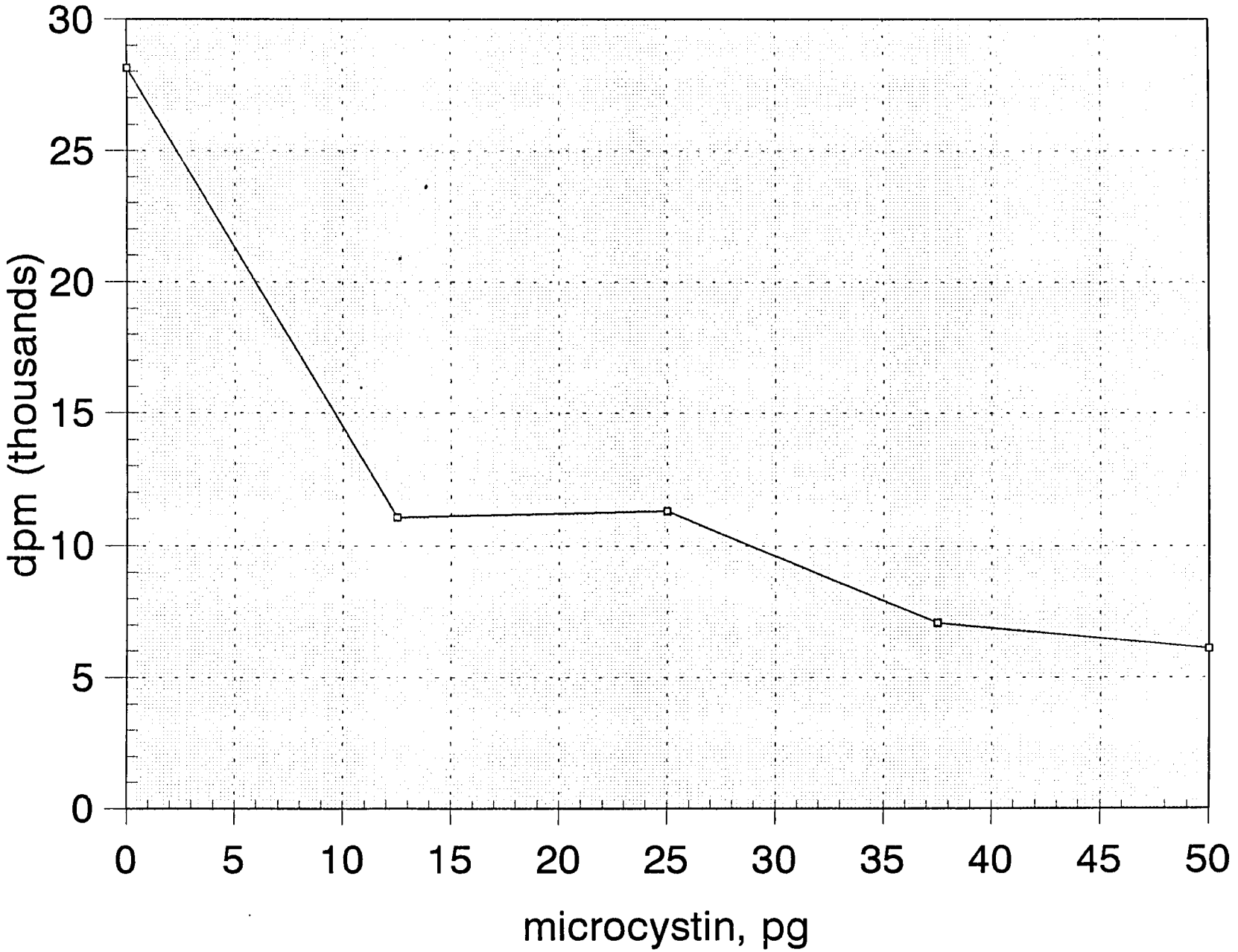
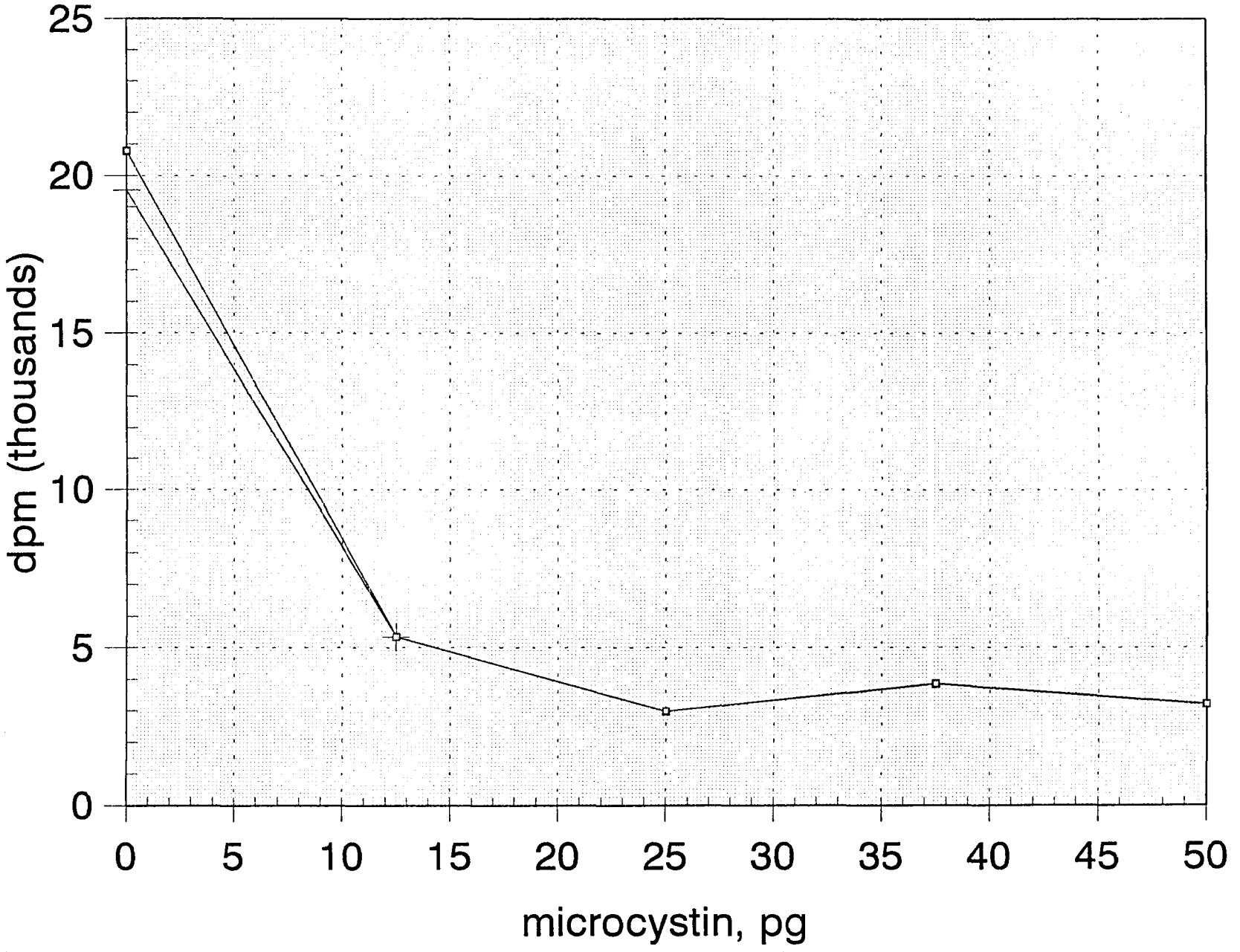


Figure 10: Experiment 2



5.8 Inhibition assay Experiment No 3

This was the first of a set of experiments involving samples of water from dams in the Pretoria locality. All sites were near each dam wall, away from water influent points to the dams. Clean 5 l plastic cans with caps were used, and the water collected as follows, on 23 February 1996, which was after a period of heavy regional and general rain:

Hartebeestpoort Dam (identified as H): This was collected at the end of the boarding jetty at the Transvaal Yacht Club, beyond a band of bright green algae at the water's edge. A small amount of this algal material was suspended in the water sample taken.

Bon Accord Dam (identified as B): This was collected at the end of the boarding jetty in front of the hotel. There was no sign of green from any algae, but the water had a light brown turbidity.

Roodeplaat Dam (identified as R): This was collected at the end of the boarding jetty alongside the motor-boat launch ramp at the public boating area. Like Hartebeestpoort dam, there was a band of wind-drifted algae along the water's edge. The water sample contained a small amount of this algal material.

Control water (identified as W): This was distilled water.

All samples were put into a dark cold room (2 - 4°C) within an hour of collection. Assay work was completed within ten days of the date of water collection from the dams.

Water samples were all filtered through Whatman No 1 paper prior to further study, to ensure that no spurious results could arise from toxin adsorption to solid surfaces, or its partition into non-aqueous material such as fats, oils or greases. The microcystins have a detergent-like molecular structure, with the ADDA side chain being hydrophobic (a surface adsorbing site) and a heptapeptide ring, which is hydrophilic (a water-associating site). At very low concentrations of added toxin, it could easily "disappear" from the body of the water by coating to the container wall, such as glass or plastic, or to dirt, algal cells or any other foreign material in the water, possibly resulting in erroneously low assay titres being measured.

A fresh batch of snap-frozen pea extract enzyme was thawed and diluted 1:1 with assay buffer, for the next experiment. As before, this diluted enzyme was stored at 2 - 4°C (refrigerator) before use.

A set of assay tubes, containing samples of filtered unadulterated dam water (no added microcystin), were made up in ice as follows:

tube number	1	2	3	4	5	6	7	8
dil. pea extract, µl	20	20	20	20	20	20	0	0
assay buffer, µl	20	20	15	15	15	15	40	40
dam water, µl	0	0	5	5	5	5	0	0
dam sample identity	-	-	H	B	R	W	-	-

Labelled substrate (20 µl) was added to each tube, and the remainder of the experiment carried out in the way already described. An exception was that the final aliquot of TCA supernatant drawn for scintillation counting, was reduced from 200 µl to 180 µl, to diminish the risk of accidental contamination by labelled protein precipitate. Since radioactive incorporation in the substrate was high, reduction of this volume was unlikely to impact upon analytical sensitivity. Scintillation data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	274	245	277	241	271	278	9.03	6.70	553

Data are plotted in Figure 11 overleaf.

"Blanks" 7 and 8 are adequately low, though tube 7 was accidently bumped after centrifugation, and shows the importance of not disturbing precipitated material after it has settled at the base of the tube. "Uninhibiteds" 1 and 2 are over half that of the "total", which after dilution correction, would be 415 x 10^3 dpm, indicating that the enzyme activity of the diluted pea extract was higher than that required for the recommended 30% activity release in an assay. Values for the four waters, tubes 3, 4, 5 and 6, are similar, taking the data scatter into account, and not significantly lower than tubes 1 and 2, showing that these water samples did not exert measurable inhibition on the dephosphorylation reaction under the conditions of this experiment.

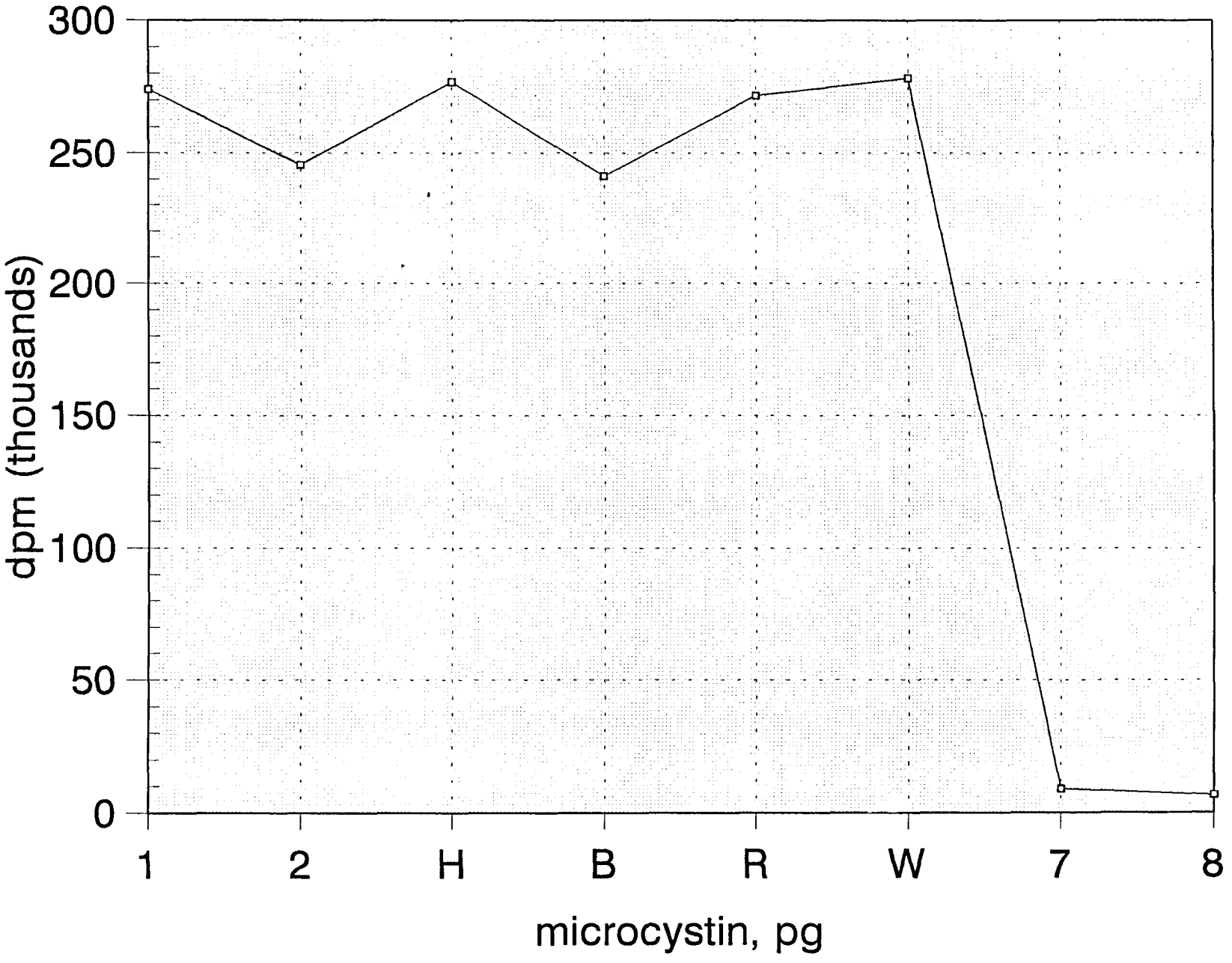
5.9 Inhibition assay Experiment No 4

This was a repeat of experiment No 3, with the exception that the four filtered water samples were "spiked" with microcystin-LR stock at 10 µl / 1000 ml. This would give a content of 1 µg toxin per 1000 ml water for assay (1 ng/ml, or 1 pg/µl). The 5 µl sample taken for each tube would then contain 5 pg of added toxin per tube.

The same 1:1 diluted enzyme used for the previous experiment was also used for this experiment.

A set of assay tubes were made up exactly the same way as for the previous run, except in this case, the "spiked" dam water was used. The tubes were then processed

Figure 11: Experiment 3



as before. Scintillation data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	334	324	297	293	278	193	3.54	2.67	496

Data are plotted overleaf in Figure 12.

Degree of dephosphorylation for the uninhibited controls (tubes 1 and 2) was again higher than recommended, being about 80% instead of the 30% of the maximum possible 372 x 10^3 dpm, the dilution-corrected value from the "total" dpm. Blanks 7 and 8 were both low, showing that bench technique was now improving. The three dam waters showed an observable degree of phosphorylation inhibition. Distilled water showed significantly more inhibition. However, taken in the context of the results of subsequent experiments, it is more likely that interference with the progress of the enzyme reaction in this vial may have taken place from incomplete mixing, or some other manipulative error, and highlights the necessity for multiple sample analyses, for routine analytical work. It could be concluded that 5 pg was just detectable under the conditions of this experimental run, in spite of the excessively high enzyme activity used.

5.10 Inhibition assay Experiment No 5

This experiment was a repeat of Experiment No 4, except that aliquots of "spiked" waters per assay tube were increased, as follows:

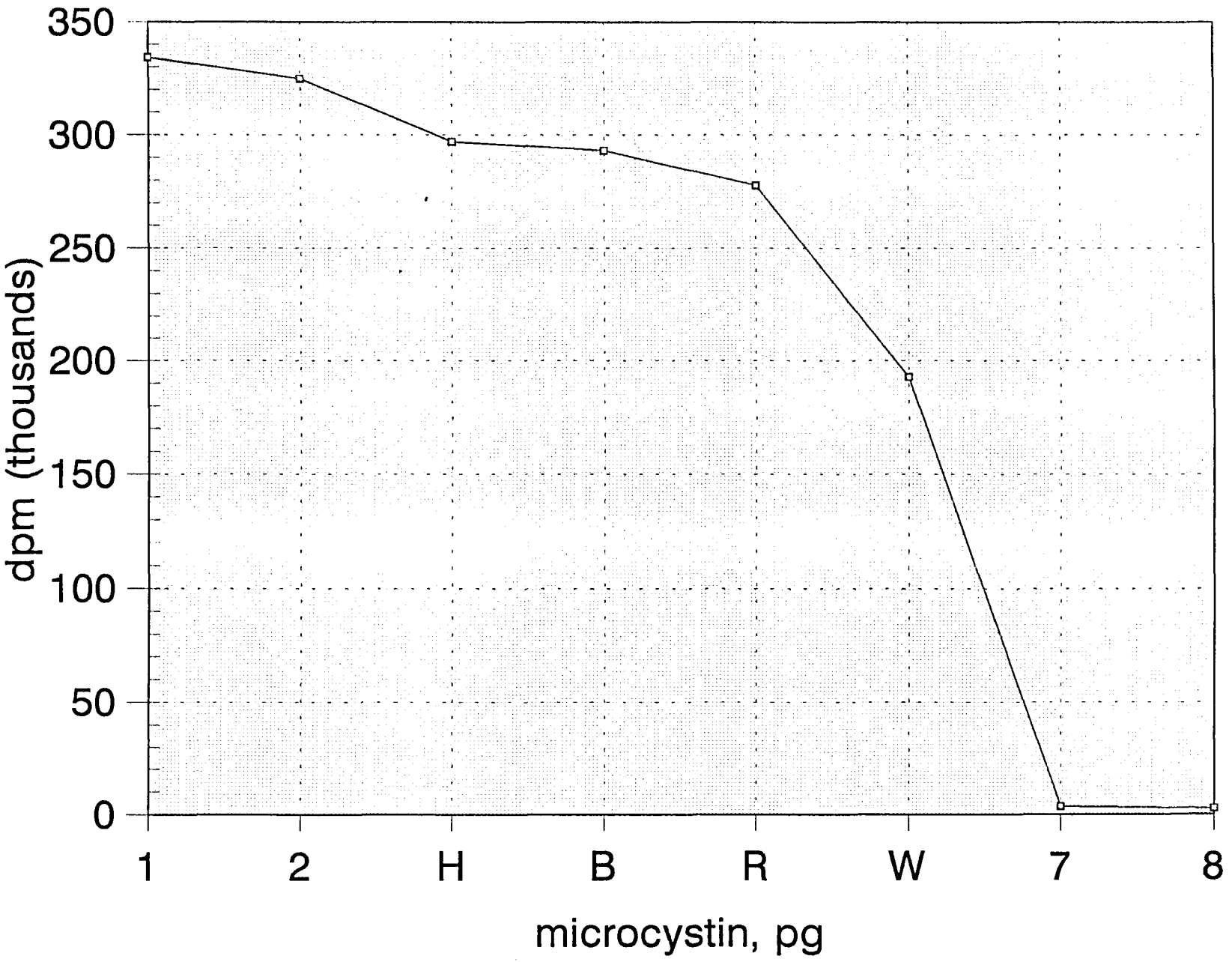
tube number	1	2	3	4	5	6	7	8
dil. pea extract, µl	20	20	20	20	20	20	0	0
assay buffer, µl	20	20	10	10	10	10	40	40
dam water, µl	0	0	10	10	10	10	0	0
dam sample identity	-	-	H	B	R	W	-	-

Amount of added microcystin-LR per assay tube was thus increased to 10 pg. The remainder of the protocol was the same as for the previous experiments, including the continued use of the pea extract dilution of 1:1 in assay buffer.

Scintillation data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	291	303	236	285	248	281	4.93	2.30	544

Figure 12: Experiment 4



Data are plotted in Figure 13 overleaf. "Blank" tubes 7 and 8 are low, showing minimal background contamination by either unprecipitated phosphoprotein or free phosphate. "Uninhibited" tubes 1 and 2 agree well as duplicates, but with high dpm, again show that the added enzyme was excessively active, and should be used at greater dilution. Tubes H, B, R, and W show evidence of some inhibitory activity, but data scatter does not allow one to conclude that inhibition in these tubes has been clearly demonstrated.

5.11 Inhibition assay Experiment No 6

A further 20 µl of microcystin stock was added to each of the water samples, making up the total stock solution content per each litre of test water to 30 µl. 10 µl sample aliquots drawn for each assay tube will thus have contained 30 pg of microcystin-LR per assay tube. The procedure was then carried out in the same way as for Experiment No 5, again using the same batch of 1:1 diluted enzyme.

Scintillation data from the TCA supernatants are as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	283	272	230	199	224	204	-	3.35	541

Data are plotted overleaf in Figure 14.

Counts for tube 7 were lost through malfunction of the scintillation counter. The reading is assumed to have been the same as blank tube 8, and is plotted thus. Uninhibited reactions in tubes 1 and 2 again have an excessive phosphate release, due to an excessive pea enzyme activity, as a consequence of insufficient dilution of the stock frozen material. Nevertheless, an observable level of inhibition can be seen from the graph, points for H, B, R, and W being grouped lower than the "uninhibited" plots of tubes 1 and 2. All counts were lower than for the previous runs on this batch of enzyme, and may be due to loss of the enzyme activity during storage.

5.12 Inhibition assay Experiment No 7

In this experiment, a fresh batch of snap-frozen pea enzyme was thawed, and made up as 250 µl thawed solution with 750 µl of assay buffer. Aliquots of microcystin added to each tube thus remained at 30 pg, but this toxin was now being "challenged" with lower enzyme activity. Procedure was otherwise unchanged.

Scintillation data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	265	242	194	175	178	212	4.21	4.09	450

Figure 13: Experiment 5

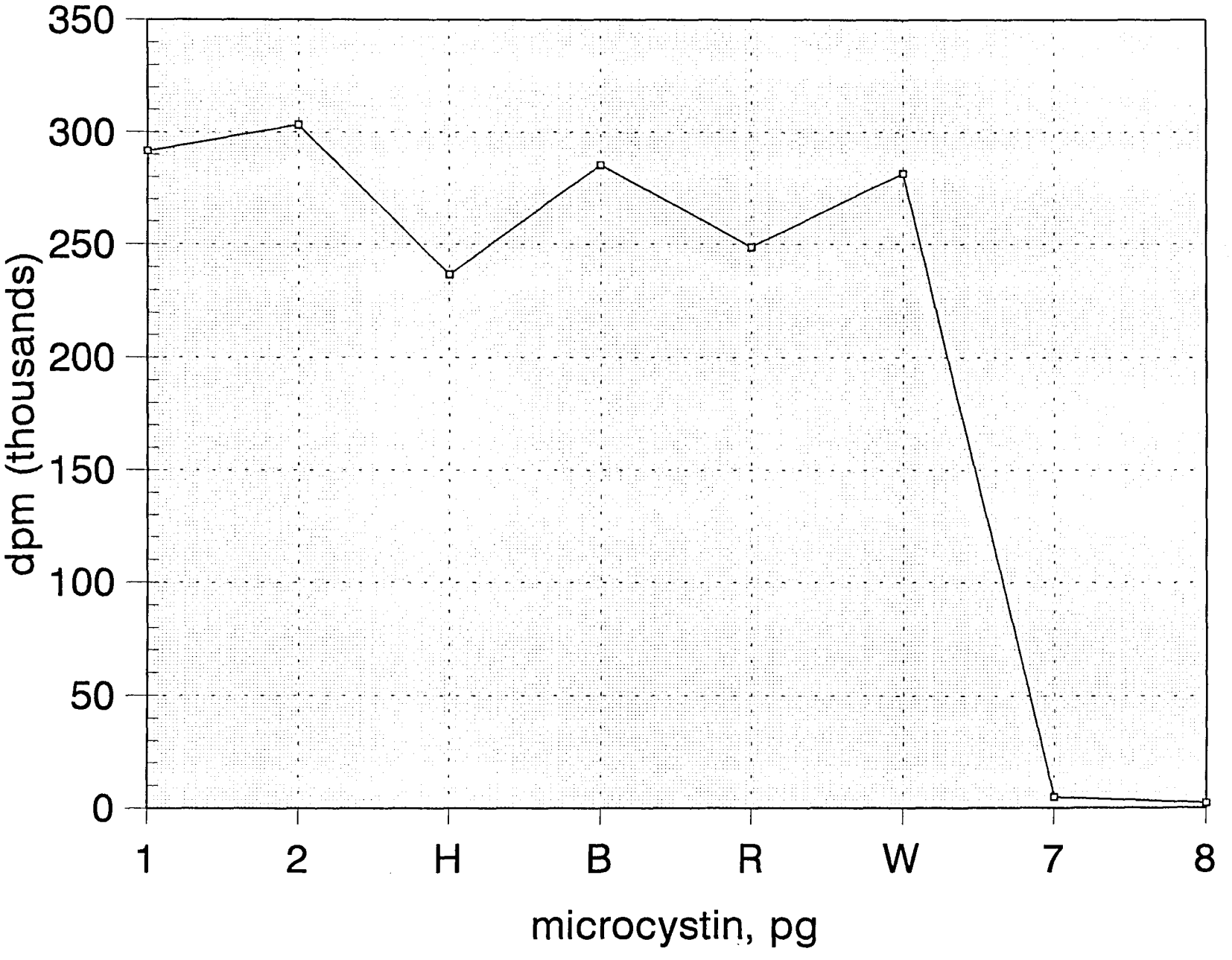
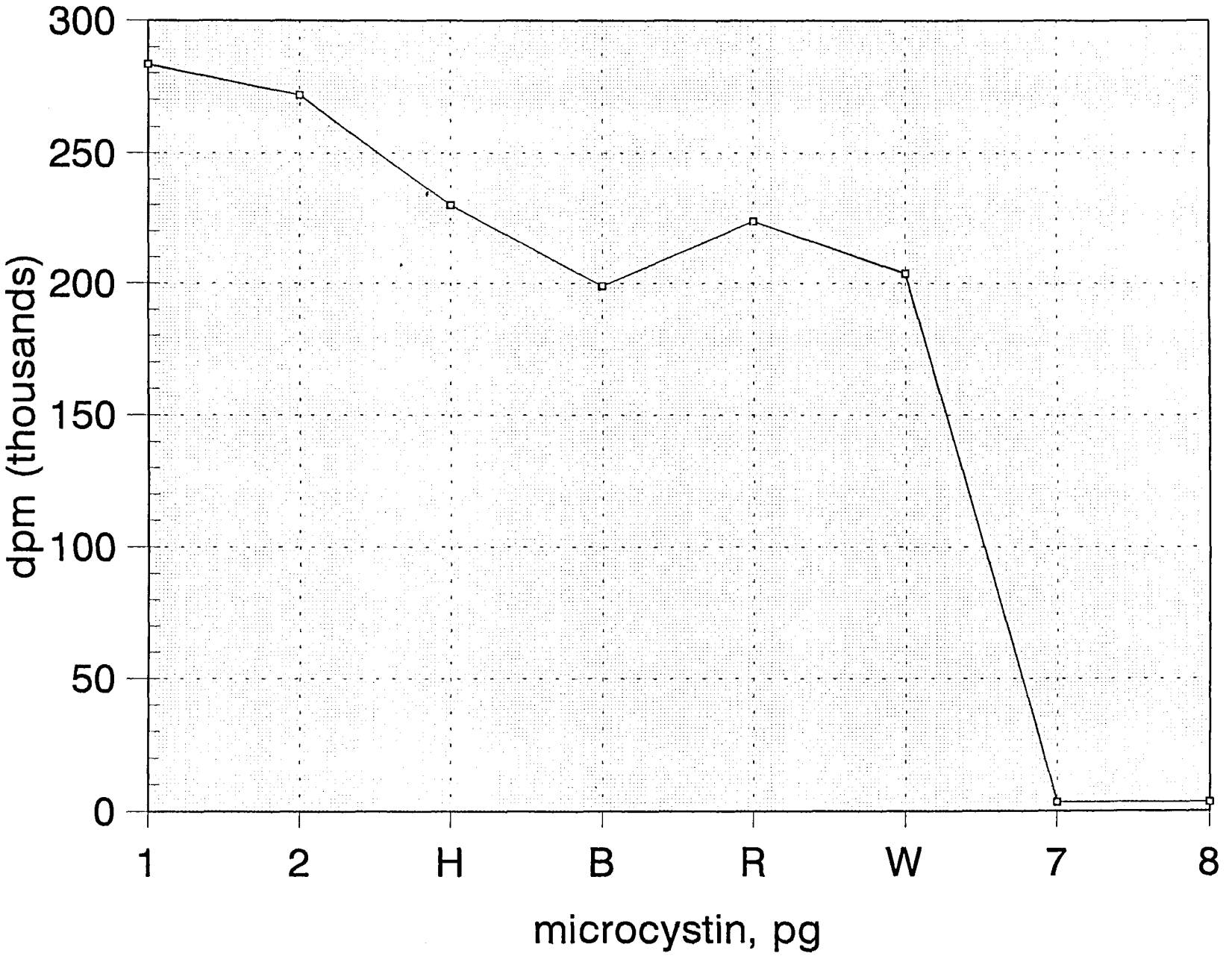


Figure 14: Experiment 6



Data are plotted overleaf in Figure 15. Reduced enzyme activity from a greater dilution of the stock pea enzyme extract has resulted in a relatively lower count for "uninhibited" tubes 1 and 2, though the values are still insufficiently low to fulfill the requirement of only 30% dephosphorylation. Nevertheless, the inhibitory action of the "spiked" waters is clearly observable, with the exception of the distilled water value, counts for which are higher than for the dam waters, and might be due to manipulative error (accidental contamination by labelled protein precipitate). Blanks 7 and 8 are low, showing that general manipulative practice has improved.

5.13 Inhibition assay Experiment No 8

This was a repeat of Experiment 7, except that the balance of the 25% concentrated pea extract was diluted by addition of a further 1 ml of assay buffer, giving it a nett concentration of about 12%, with respect to the snap-frozen enzyme stock solution.

Scintillation data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	211	217	60.0	40.3	29.6	127	2.61	1.39	492

Data are plotted overleaf in Figure 16.

"Blank" tubes 7 and 8 gave low dpm. Improvement in assay sensitivity through greater dilution of the enzyme is immediately evident from the graph. "Uninhibited" counts in tubes 1 and 2 are down to about 200 x 10^3 dpm (which is still not below the recommended 30% "uninhibited" dephosphorylation). Nevertheless, waters H, B, and R are diminished values of about 50 x 10^3 dpm, a quarter of the dpm in tube 1 or 2. Again, as for Experiment 7 (Figure 8), the distilled water shows less inhibition. While it is possible to speculate some form of microcystin toxic synergism with material in the dam waters, absent from the distilled water, it may also be possible that an error of addition of toxin stock to the distilled water might have taken place. A more concerning, but unlikely possibility would be that the dam waters already contain natural toxin in them.

5.14 Inhibition assay Experiment No 9

This was a re-execution of Experiment 8, to assess repeatability of the method.

Scintillation data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	147	143	35.5	63.0	36.3	92.8	2.29	1.74	467

Figure 15: Experiment 7

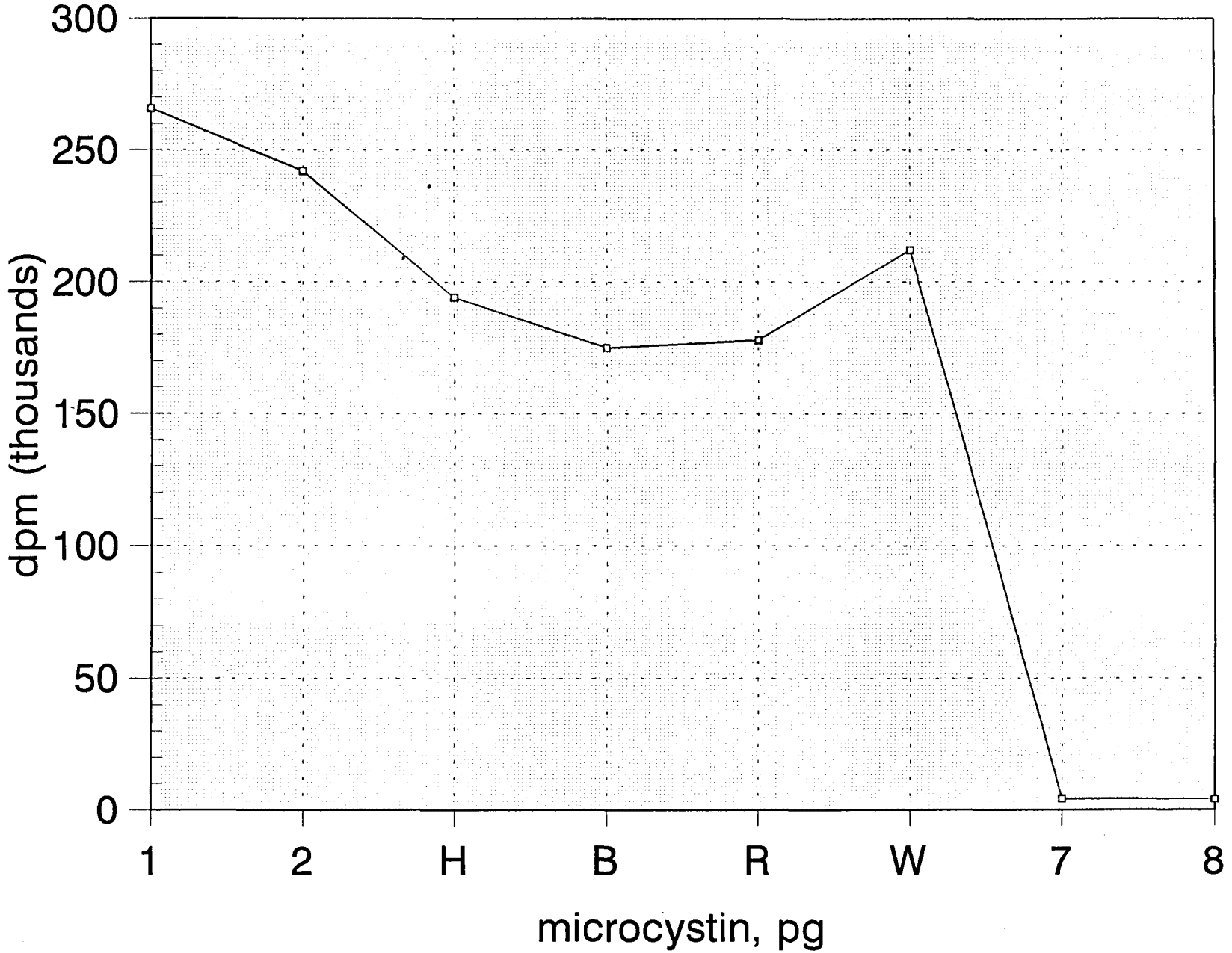
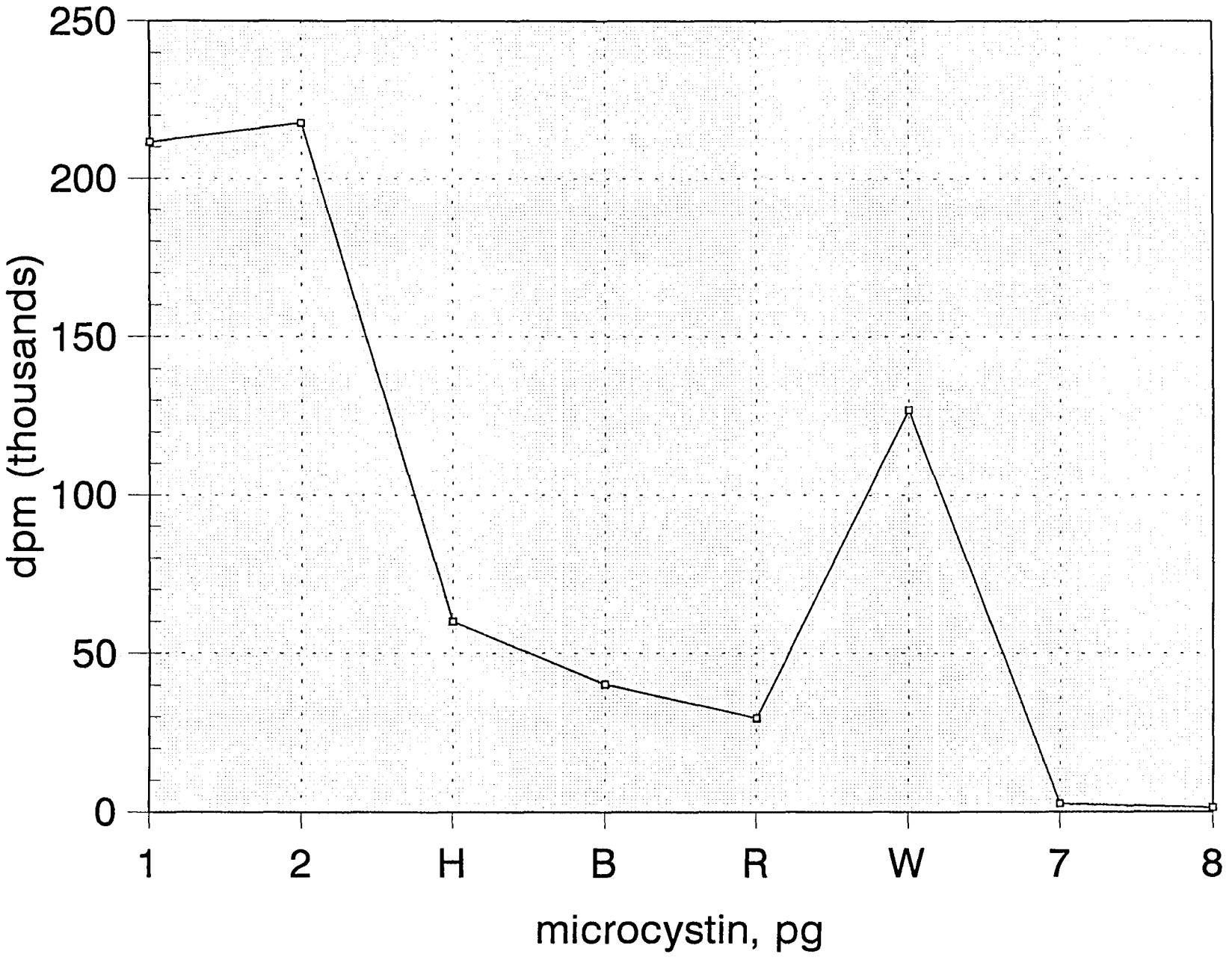


Figure 16: Experiment 8



Data are plotted overleaf in Figure 17. "Uninhibited" dpm for tubes 1 and 2 fell from about 200 x 10^3 in Experiment 8 to about 150 x 10^3 in this Experiment 9, which cannot be attributed to loss of enzyme activity through storage, as this activity is back to about 220 x 10^3 dpm for "uninhibiteds" in Experiment 10. The difference could be attributable to a factor such as a shortened incubation time for this batch, although every effort was made to adhere rigorously to a uniform procedure. Although overall dpm for all samples was lower, Inhibitory ratios (sample dpm divided by uninhibited dpm) for H, R and W were similar for Experiments 8 and 9, showing these results to be reproducible. Distilled water again showed the inexplicably higher value relative to the dam waters. Tube B, however, gave a higher result in Experiment 9 than in Experiment 8, and again demonstrates the limits of obtaining absolutely consistent data by this method.

5.15 Inhibition assay Experiment No 10

This final experiment was with the use of filtered water samples, to which no added microcystin was present. The diluted enzyme solution used for Experiments 9 and 8 was used, and the procedure was otherwise unchanged.

Scintillation data from the TCA supernatants were as follows:

Tube No	1	2	3	4	5	6	7	8	tot
dpm x 10^3	224	231	204	221	144	235	1.77	1.98	500

Data are plotted in Figure 18 overleaf.

Values for all waters were similar to that for "uninhibited" tubes 1 and 2, with the exception of tube R for Roodeplaat dam, which showed a lower value, and was consistently so in plots for the previous Experiments 9, 8 and 7, but not for 6 , or 3. No reliable conclusion would be possible, without collection of more data. Considering that in Experiment 8, 30 pg of microcystin-LR per assay tube reduced "uninhibited" counts of about 220 x 10^3 dpm to about 50 x 10^3 dpm, it can be reasoned that the dam water contains significantly less than this quantity per aliquot taken (10 µl). 30 pg/10 µl is equivalent to 3 ng/ ml, or 3 µg/ litre. Safe limits for human health are generally quoted as 1 µg/l.

6. DISCUSSION AND CONCLUSIONS FROM THE WORK DONE

The work carried out in this project has firstly shown that the use of ^{33}P in place of ^{32}P can be made, without apparent detriment to the performance of the assay, and with the advantages of reduced radiation risk to the user, together with a useful life that is almost double that of ^{32}P.

The work also shows that an above-usual demand of skill and practice, and care is made on the analyst, and that the best results can only be expected if it is a routine

Figure 17: Experiment 9

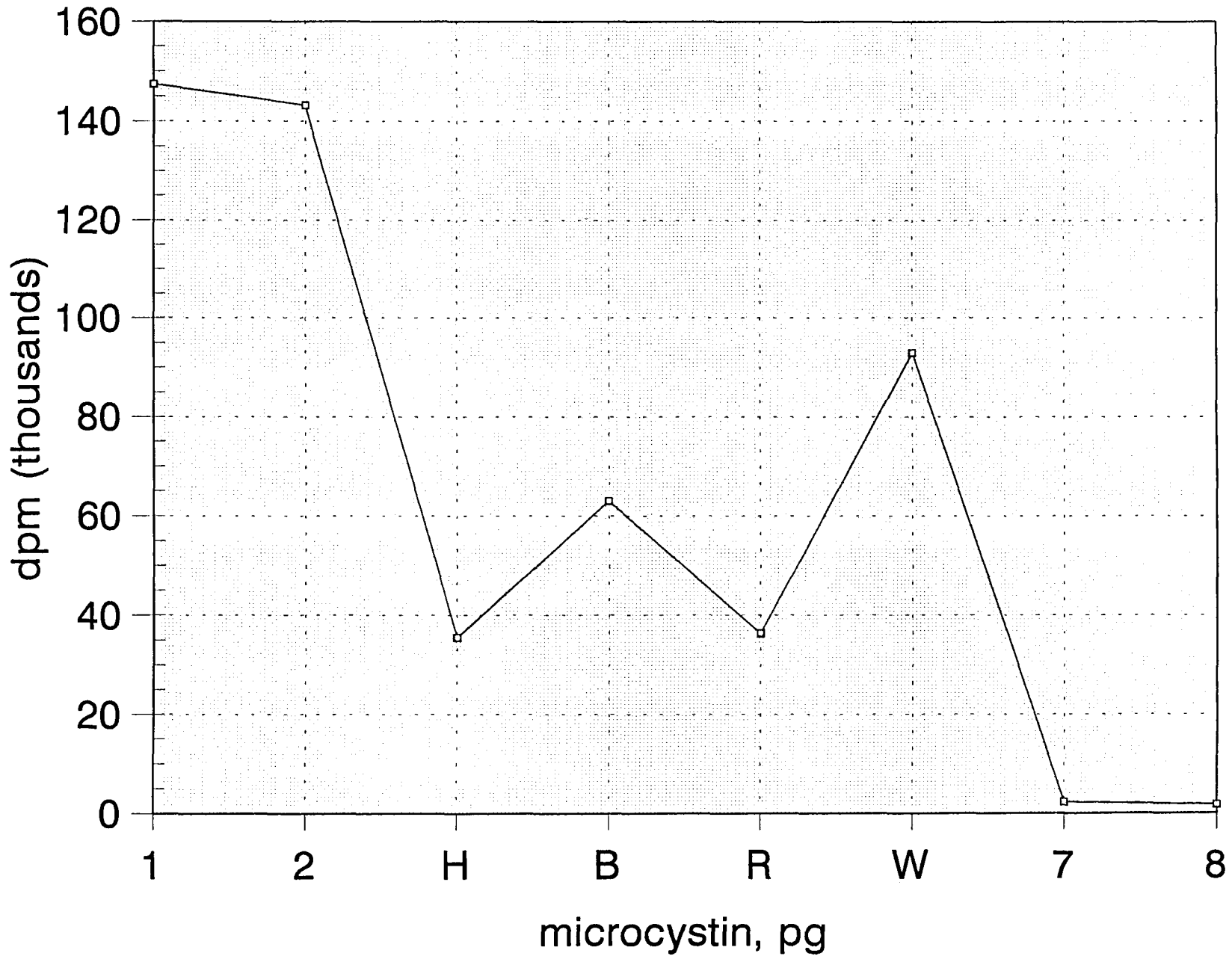
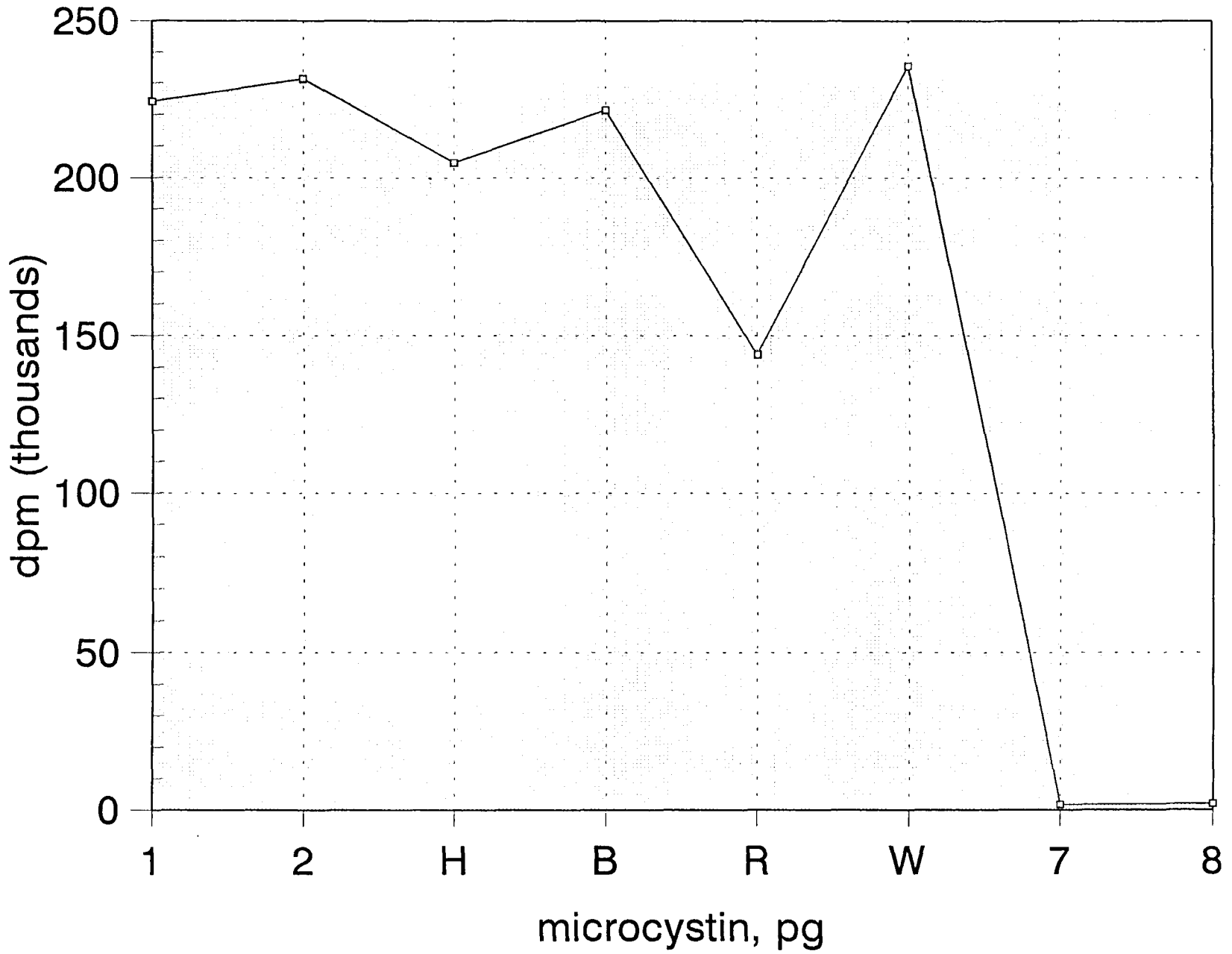


Figure 18: Experiment 10



operation in an environment that is dedicated to this work. An example of an adverse problem with this project was having to take material from the radioactivity room on the ground floor to the microfuge in a cold room on the second floor at the opposite end of the building. This was also illegal in terms of permission under which radioactive isotopes may be used. Without the financial restraints imposed upon this project, it would have been possible to continue work, until the ideal reaction conditions were found, where high sensitivity and reproducibility could be achieved. The method, as it has been carried out with this work, will certainly detect 1 µg toxin per litre of water, and there is scope for improvement, through refinement and practice of bench procedures. It could be possible to make up the assay buffer, using test sample water as a diluent for the stock, so increasing the volume of test water in each assay tube to 40 µl, raising the sensitivity four-fold (<0.25 µg toxin/litre of water).

6.1 Costs

The assay kit, with material for 300 assays, was air-freighted from UK, frozen, at a cost of about R2000-00. Labelled ATP cost about R1000-00, and scintillation fluid was R500-00 for 5 l. These items alone will contribute about R11-00 to the cost of each test. Microcystin-LR standard is currently about R1000-00 for 0.5 mg, and may require regular repurchase for availability of fresh authentic standards for method calibration. Other microcystin variants cost much more, depending on there rarity/availability. Not accounted for is the cost of consumables such as disposable pipette tips and protective gloves, Eppendorf tubes and scintillation vials. All these materials are imported items, and are thus subject to price variations linked to the foreign exchange rate. Capital items needed are the scintillation counter, contamination monitor, standard centrifuge and microcentrifuge, crushed ice machine and refrigerator. The largest expense of all would be the radioactivity laboratory, fitted out in accordance with nuclear safety standards. Costs are also incurred in the containment and disposal of radioactively contaminated solid and liquid waste. Manpower costs would depend largely on scale of operation, being greatest for intermittent work on sample analysis. The preparation of the enzyme is a tedious operation as is the labelling of the substrate, and can become waste if not used within the period of its working life.

6.2 Disadvantages

Cost, mentioned above, is a primary problem.

Operator health risk from accidental contact with ^{33}P is another problem.

The need to "keep your hand in" to get good results is essential.

The method measures phosphoprotein phosphatase enzyme inhibition only, (a hepatotoxicological parameter) and offers a warning of the possible presence of microcystins. Inhibition may also arise from other toxic materials that are not microcystins. Conversely, other algatoxins that are not microcystins may not be detected, as they do not inhibit phosphoprotein phosphatases, but are nevertheless a significant health risk.

7. REFERENCES

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