Urban Water Research Association of Australia

By-Products Formed in the Destruction of Algal Toxins by Oxidants such as Chlorine

Research Report No. 126

URBAN WATER RESEARCH ASSOCIATION OF AUSTRALIA (UWRAA)

The Association is a Division of the Water Services Association of Australia. UWRAA has the charter to develop and manage a portfolio of research to support the business plan for the urban water industry developed by the Water Services Association of Australia.

The UWRAA Research Report series presents information resulting from research projects supported by the Association and is published as a record of the work undertaken and as a means of disseminating the research findings. The Association also encourages the presentation of findings by the researchers in professional journals and at conferences. The Association's reports are indexed on STREAMLINE, the national water data base.

For further details contact:
Dr John Langford
Executive Director
Water Services Association of Australia
Level 7
469 Latrobe Street Telephone: (03) 9606 0678
Melbourne Vic 3000 Fax: (03) 9606 0376

Urban Water Research Association of Australia

By-Products Formed in the Destruction of Algal Toxins by Oxidants such as Chlorine

J Rositano, P M Bond, B C Nicholson

Australian Water Quality Centre

Research Report No 129
October 1997

ISBN 1 876088 31 1

FOREWORD

This report is based on UWRAA Research Project No WS-62 'By-Products from the Destruction of Algal Toxins by Oxidants such as Chlorine'. Organisational responsibilities for the project were as follows:

Sponsoring Authority : South Australian Water Corporation

Research Agency : Australian Water Quality Centre

Project Officer : Dr Brenton Nicholson, Australian Water Quality Centre

Principal Researchers : Joanna Rositano, Peter Bond, Australian Water Quality Centre

This project was funded by the Urban Water Research Association of Australia and the South Australian Water Corporation.

SYNOPSIS

Previous studies carried out at the Australian Water Quality Centre showed that chlorination was effective in oxidising and thus removing acute toxicity of cyanobacterial peptide hepatotoxins as determined by HPLC and mouse bioassay. Although acute toxicity was removed, it was not known whether the by-products from chlorination of the hepatotoxins were capable of producing subacute toxic effects. The aim of this project therefore was to determine the by-products produced from the chlorination of microcystins and to assess their potential toxicity.

A range of toxin producing and non-toxin producing cultures of *Microcystis aeruginosa* were chlorinated to determine the effect on measures of toxicity. The more common disinfection by-products as well as total halogenated organic components were also measured. Toxicity of the cultures was monitored via specific and non-specific toxicity assays such as the mouse bioassay, HPLC analysis, protein phosphatase inhibition assay and Ames mutagenicity assay in order to determine any trend between acute or subacute toxicity and chlorination of the toxin producing *Microcystis* cultures.

Protein phosphatase inhibition decreased after chlorination in strains of *Microcystis* which produced toxin. This was consistent with the elimination of acute toxicity in mice and HPLC analysis results. No correlation could be made between the results of Ames mutagenicity testing and the presence of microcystins. There was also no recognisable trend between any of the toxicity assay results and the presence of chlorinated disinfection by-products. It was found that chlorination of the various *Microcystis* cultures yielded similar chlorinated by-products. However different ratios of these compounds were found which implies the presence of different organohalogen precursors in some strains.

CONTENTS

FOREWORD ii

SYNOPSIS iii

LIST OF FIGURES vi

LIST OF TABLES vii

1 INTRODUCTION

1.1 Toxic Cyanobacteria 1

1.2 Cyanobacterial Toxins 2

1.3 Toxicology of Peptide Hepatotoxins 5

1.4 Control of Peptide Hepatotoxins

1.4.1 Activated Carbon 6

1.4.2 Oxidation

1.4.2.1 Chlorine 6

1.4.2.2 Chloramines 7

1.4.2.3 Potassium Permanganate 7

1.4.2.4 Ozone 8

1.4.2.5 Other Oxidants 8

1.5 Project Aim 9

2 MATERIALS AND METHODS

2.1 Cyanobacterial Materials 11

2.2 Growth and Preparation of Cyanobacterial Cultures for Chlorination Experiments. 13

2.2.1 Concentration of *M. aeruginosa* Cultures 15

2.2.2 Cell Counting Procedure 16

2.3 Chlorination of *M. aeruginosa* Cultures

2.3.1 Experiment 1 Strain PCC7820 16

2.3.2 Experiment 2 Strain 338 17

2.3.3 Experiment 3 Strain 023 17

2.3.4 Experiment 4 Strain 031 17

2.4 Analysis of Chlorinated Cultures

2.4.1 Toxicity Testing

2.4.1.1 Microcystin Analysis by HPLC 18

2.4.1.2 Mouse Bioassay 21

2.4.1.3 Phosphatase Assay 21

2.4.1.4 Ames Mutagenicity Assay 21

2.4.2 By-Products Analysis

2.4.2.1 Trihalomethane Analysis (THM) 22

2.4.2.2 Adsorbable Organic Halogen (AOX) 22

2.4.2.3 Haloacetic Acids (HAAs) 22

2.4.2.4 Preparation of Diazomethane Solution. 26

2.4.2.5 Haloacetonitriles (HANs) and Chloral Hydrate 27

2.4.2.6 Chlorophenols 28

3 RESULTS AND DISCUSSION

3.1 Toxicity of *Microcystis aeruginosa* Cultures After Chlorination 31

3.2 By-Products from the Chlorination of *Microcystis* Cultures 38

4 SUMMARY AND CONCLUSIONS 45

5 RECOMMENDATIONS 46

6 ACKNOWLEDGMENTS 47

7 REFERENCES 48

8 APPENDIX 57

LIST OF FIGURES

1	Peptide hepatotoxins (1) Microcystin-XY and (2) Microcystin-LR	3
2	Alkaloid neurotoxins (1) Anatoxin-a and (2) Anatoxin-a(s)	4
3	Basic structure for the Paralytic Shellfish Poisons	4
4	Cylindrospermopsin	5
5	200x and 400x magnification images of culture PCC7820 showing the *Microcystis* strain PCC7820 and the contaminant strain	12
6	HPLC chromatograms of strains PCC7820 (A), 338 (B), 031 (C) and 023 (D)	13
7	Cyanobacterial cultures being grown up on a large scale	15
8	Determination of chlorine demands for experiments 1-4	18
9	Flow diagram of analyses carried out after 30 minutes chlorination	19
9 cont	Flow diagram of analyses carried out after 24 hours chlorination	20
10	Standard curves for monochloroacetic acid, dichloroacetic acid and trichloroacetic acid	26
11	Apparatus for the generation of diazomethane	28
12	Calibration curves for chloral hydrate and dichloroacetonitrile	30
13	The phosphorylation/dephosphorylation cycle	32
14	Microcystin-LR mutagenicity dose response curve with *Salmonella typhimurium* strain TA98 without S9	37
15	Distribution of chlorination by-products for strains PCC7820, 338, 023, and 031	40

LIST OF TABLES

I	Cell count ratio and toxicity of cultured PCC7820	12
II	Retention time data for haloacetic acids	24
III	Recovery data for the haloacetic acids from Milli-Q water	24
IV	Retention time data for chloral hydrate and haloacetonitriles	28
V	Recoveries of haloacetonitriles and chloral hydrate from spiked solutions of a non-toxic *M. aeruginosa* culture and Milli-Q water at various concentrations	29
VI	Summary of toxicity results for strains PCC7820 and 338	33
VII	Summary of toxicity results for strains 023 and 031	34
VIII	Total trihalomethane analysis results	43
IX	Haloacetic acids analysis results	43
X	Chloral hydrate and haloacetonitriles analysis results	44
XI	Adsorbable organic halogen analysis results	44

1 INTRODUCTION

1.1 Toxic Cyanobacteria

The increasing prevalence of algal blooms arising through increasing eutrophication of water bodies is placing greater pressures on the uses of water, particularly for drinking. In particular the blue-green algae, more correctly known as cyanobacteria, are well known for their ability to produce tastes and odours, thereby reducing the suitability of affected water for this purpose (Izaguirre *et al.*, 1982; Slater and Blok, 1983) . Some cyanobacteria can also produce potent toxins which have been responsible for numerous animal deaths (Schwimmer and Schwimmer, 1968; Carmichael *et al.*, 1985; Beasley *et al.*, 1989; Carmichael and Falconer, 1993).

Toxic cyanobacteria have been recorded from 31 countries which includes every continent except Antarctica (Carmichael and Falconer, 1993). Areas which have well documented occurrences of toxic cyanobacteria include North America, Southern Africa, Australia, UK and Scandinavia and may reflect the distribution of researchers rather than the distribution of the organisms. Toxicity is widespread through the cyanobacteria with 41 species from 19 genera reported as toxic (Scott, 1991).

In Australia there have been several documented incidents involving deaths of sheep, cattle, horses, turkeys and honey bees associated with blooms of *Microcystis, Anabaena* and *Nodularia* (Mulhearn, 1959; McBarron and May, 1966; May and McBarron, 1973; McBarron *et al.*, 1975; Main *et al.,* 1977; Bowling, 1992; Negri *et al.*, 1995).

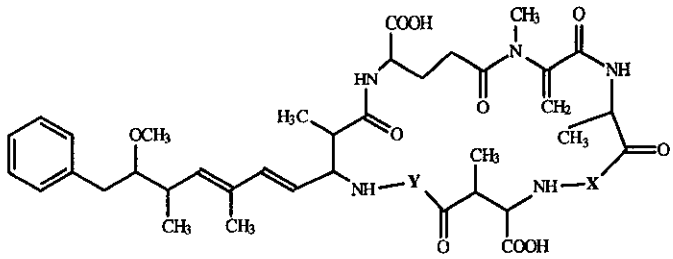
While the unpalatable appearance of water affected by heavy algal blooms has probably prevented significant human consumption with consequent fatalities, there is increasing evidence that low-level exposure may have sub-lethal health effects in man with reports of such incidents from North America, (Tisdale, 1931a,b; Veldee, 1931; Dillenberg and Dehnel, 1960; Lippy and Erb, 1976; Billings, 1981) the United Kingdom (Turner *et al.*, 1990) and Africa (Zilberg, 1966).

In Australia the most dramatic incident was at Palm Island 1979 when 139 children and 10 adults became seriously ill after drinking water affected by a heavy cyanobacterial bloom (Bourke *et al.*, 1983). Subsequent research suggested that the causative organism was the cyanobacterium *Cylindrospermopsis raciborskii* (Hawkins *et al.*, 1985). Falconer *et al.* (1983a) reported evidence of liver damage attributable to the presence of *Microcystis aeruginosa* in the water supply of Armidale, New South Wales. More recently gastrointestinal and skin complaints have been associated with recreational exposure to *Nodularia spumigena* (Soong *et al.*, 1992) and *Anabaena circinalis* (El Saadi *et al.*, 1995; El Saadi and Steffensen, 1996). There is also epidemiological evidence from China of a link between cyanobacteria and cancer (Yu, 1994; Ueno *et al.*, 1996).

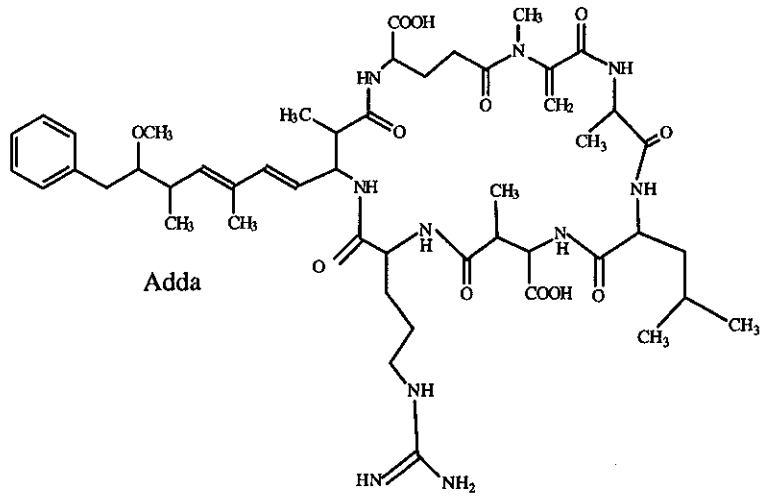
1.2 Cyanobacterial Toxins

Cyanobacteria are known to produce hepatotoxins, neurotoxins and lipopolysaccharide (LPS) endotoxins (Codd *et al.*, 1989; Carmichael, 1992, 1994). The hepatotoxins are cyclic peptides with the most frequently encountered compounds being the microcystins, cyclic heptapeptides produced most commonly by *Microcystis aeruginosa* but also by other genera such as *Oscillatoria, Anabaena and Nostoc* (Skulberg *et al.*, 1993). *Nodularia spumigena*, normally a brackish water cyanobacterium, can produce a similar cyclic pentapeptide, nodularin, which is equally as toxic as the most toxic microcystins (Rinehart *et al.*, 1988). A similar pentapeptide, motuporin, has recently been isolated from a marine sponge (de Silva *et al.*, 1992). The structures of the peptide hepatotoxins are shown in Figure 1.

Microcystins initially appeared to contain 5 invariant and 2 variant amino acids. One of the invariant amino acids is a unique β-amino acid called Adda. A 2 letter suffix (XY) is ascribed to each individual toxin to denote the 2 variant amino acids. X is commonly leucine, arginine or tyrosine, and Y, arginine, alanine or methionine. Recently some variants of the "invariant" amino acids have been reported, eg, desmethyl amino acids and/or replacement of the 9-methoxy group of Adda by an acetyl moiety. Currently there are over 40 variants of microcystin which have been characterised (Carmichael, 1994). Of these over 40 compounds, microcystin-LR would appear to be the microcystin most commonly found in cyanobacteria. It is also common for more than one microcystin to be found in a particular strain of cyanobacterium (Namikoshi *et al.*, 1992; Lawton *et al.*, 1995). The microcystin variants may also differ in toxicity (Carmichael, 1992).



(1)

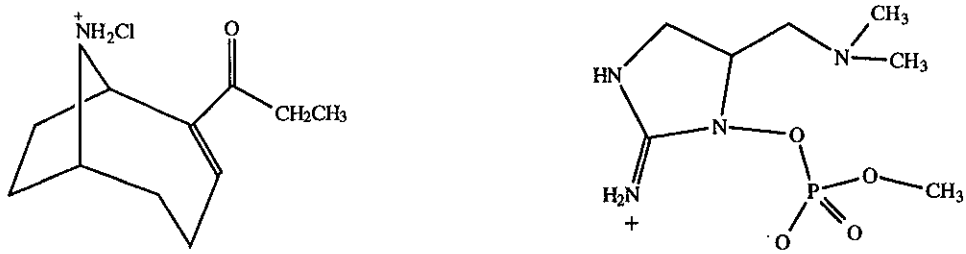


(2)

Figure 1. Peptide hepatotoxins (1) Microcystin-XY and (2) Microcystin-LR

Nodularin is known only from *Nodularia spumigena*. This cyclic pentapeptide contains amino acids similar or identical to those found in microcystins, namely arginine, glutamic acid, β-methylaspartic acid, N-methyl-dehydrobutyrine and also Adda (Rinehart *et al.*, 1988).

Neurotoxins include anatoxin-a and anatoxin-a(s) which are produced by *Anabaena flos-aquae* in the northern hemisphere (Figure 2). Neurotoxicity in *Anabaena circinalis* in Australia has recently been found to be due to a group of compounds called the paralytic shellfish poisons (PSPs) (Figure 3) (Humpage *et al.*, 1994). This group of toxins is usually encountered in the marine environment where they are produced by dinoflagellates, the organisms responsible for "red tides", so-called as their blooms cause a red discolouration of the water (Anderson, 1994).



(1) (2)

Figure 2. Alkaloid neurotoxins (1) Anatoxin-a and (2) Anatoxin-a(s)

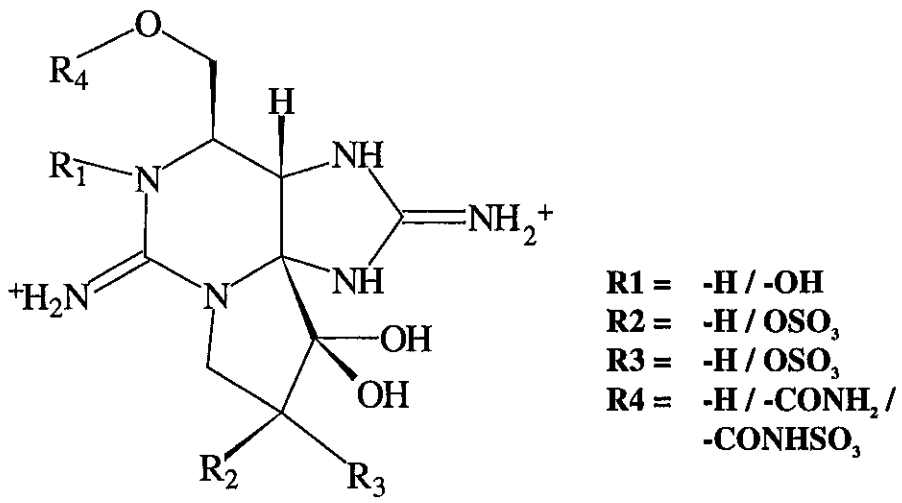


Figure 3. Basic structure for the Paralytic Shellfish Poisons

Toxic dinoflagellates are consumed by shellfish, thereby rendering them toxic. Human illnesses and deaths have occurred on a number of occasions through the consumption of shellfish contaminated in this way (Kao, 1993). The LPS endotoxins, also commonly produced by certain bacteria, may be involved in episodes of allergic reactions and minor illnesses such as gastroenteritis in humans who have come in contact with cyanobacterial blooms (Codd *et al.*, 1989).

A more unusual cyanobacterial toxin is cylindrospermopsin (Figure 4) which has been isolated from *C. raciborskii* (Ohtani *et al.*, 1992) and *Umezakia natans* (Harada *et al.*, 1994). It is an hepatotoxic alkaloid which principally affects the liver but other organs such as the kidney are also affected (Hawkins *et al.*, 1985, 1997; Terao *et al.*, 1994).

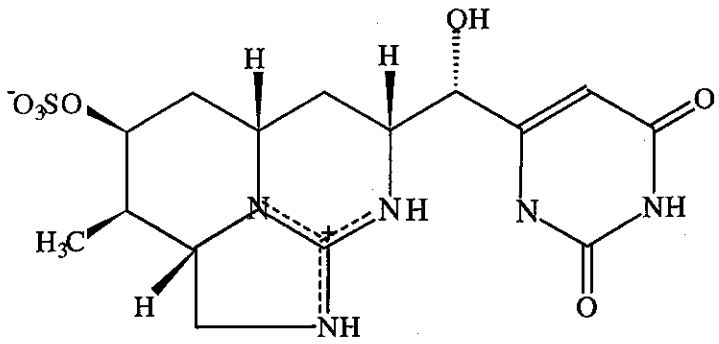


Figure 4. Cylindrospermopsin

1.3 Toxicology of Peptide Hepatotoxins

Of these chemically diverse groups of toxins, the peptide hepatotoxins (microcystins and nodularin) are probably the most important because of their apparent chronic toxicity. A link between exposure to microcystins and tumour promotion has been shown in experiments with laboratory animals (Falconer and Buckley, 1989; Falconer, 1991; Nishiwaki-Matsushima *et al.*, 1992; Fawell *et al.*, 1994; Fujiki *et al.*, 1996). Nodularin has very similar characteristics but also has now been reported to be a liver carcinogen (Ohta *et al.*, 1994; Fujiki *et al.*, 1996).

Microcystins inhibit enzymes responsible for the dephosphorylation of intracellular phosphoproteins (MacKintosh *et al.*, 1990). These enzymes are called protein (serine/threonine) phosphatases and are classified into two groups called Type 1 and Type 2 (Shenolikar, 1994). The types which are most inhibited by cyclic peptide hepatotoxins are Type 1 and a subset of Type 2, protein phosphatase 2A. The inhibition of these enzymes appears to be related to the hepatotoxicity of these toxins (Runnegar *et al.*, 1993; Toivola *et al.*, 1994) and is probably also related to their tumour promotion properties. Recent studies on the high incidence of liver cancer in certain parts of China suggest that the causative agents are microcystins (Yu, 1994; Ueno *et al.*, 1996).

While there is still considerable uncertainty as to the public health risks associated with chronic exposure to peptide hepatotoxins in drinking water, preliminary investigations indicate that drinking water quality guidelines for microcystins could be around 0.5 - 1.0 μg/L (Jones *et al.*, 1993a; Falconer *et al.*, 1994; Kuiper-Goodman *et al.*, 1994). There is thus an urgent need to control toxin concentrations to below these levels in drinking water.

1.4 Control of Peptide Hepatotoxins

1.4.1 Activated Carbon

Water treatment options for the control of peptide hepatotoxins in drinking water have been summarised by Drikas (1994). Activated carbon was found to be effective as early as 1976 (Hoffmann, 1976) for removal of toxins produced by *M. aeruginosa* but more detailed water treatment studies were not reported until later. These studies showed both powdered and granular activated carbons to be effective for the removal of either pure toxins or toxins from cyanobacterial material (Falconer *et al.*, 1983b, 1989; Keijola *et al.*, 1988; Himberg *et al.*, 1989; Lahti and Hiisvirta, 1989; James and Fawell, 1991; Pieronne, 1993; Jones *et al.*, 1993b; Bernazeau, 1994; Craig and Bailey, 1995; Lambert *et al.*, 1996). Factors affecting the adsorption of microcystin-LR by activated carbons has also been studied in detail (Donati *et al.*, 1993, 1994). While activated carbon can be highly effective, there are a number of problems associated with the use of this technique. These include cost, difficulties in introducing activated carbon into water treatment plants not designed for this purpose, differences in effectiveness between different carbons and determining when adsorption capacities are exhausted.

1.4.2 Oxidation

1.4.2.1 Chlorine

An alternative approach which has been investigated in some detail is the use of oxidants commonly employed in water treatment for disinfection. Early research with chlorine, the most common oxidant used for disinfection of drinking water, showed it to be ineffective in destroying peptide hepatotoxins (Hoffmann, 1976; Keijola *et al.*, 1988; Himberg *et al.*, 1989). However

recent work (Nicholson *et al.*, 1993, 1994; Rositano and Nicholson, 1994; Rositano, 1996) has shown that, provided a chlorine residual of at least 0.5 mg/L was present after 30 minutes contact time, chlorination was effective in the destruction of the hepatotoxins, microcystin and nodularin. Initial toxin concentrations were in the range of 130-300 µg/L which were considered representative of highly contaminated water. The destruction of toxins was pH dependent; chlorinating agents such as calcium and sodium hypochlorite were not as effective at high dose rates due to elevation of pH.

The work described by Hoffmann used a very low chlorine/toxin ratio and although the final pH was not mentioned the initial pH was 8.5 which would significantly reduce the chlorine efficiency and may explain why chlorine was found to be ineffective (Nicholson *et al.*, 1994). The work described by Keijola *et al.* (1988) and Himberg *et al.* (1989) used low chlorine doses (0.5 mg/L) which would be expected to be rapidly consumed by reaction with the high level of oxidisable material present in the water (potassium permanganate values of 35 - 48 mg/L). The chlorine would therefore not be available to react with the toxins.

1.4.2.2 Chloramines

Chloramination of dilute algal extract solutions at a concentration of 20 mg/L monochloramine did not have any significant effect on the toxins after a contact time of up to 5 days (Nicholson *et al.*, 1994; Rositano and Nicholson, 1994; Rositano, 1996). This is not unexpected given that monochloramine has a weaker oxidising capacity compared with chlorine. There does not appear to be any benefit in pursuing the use of chloramination for the removal of algal toxins.

1.4.2.3 Potassium Permanganate

Potassium permanganate is a relatively strong oxidant and is used in water treatment for the oxidation of iron and manganese. A dose of 1 mg/L potassium permanganate removed 95% of microcystin-LR at an initial concentration of 200 µg/L after 30 minutes (Rositano and Nicholson, 1994; Rositano, 1996). However oxidation of toxins in cyanobacterial material was relatively poor, suggesting that potassium permanganate was unable to access intracellular toxin by either

penetrating the cell wall or lysing the wall of cyanobacterial cells, thereby releasing toxin to the surrounding medium where oxidation could occur (Rositano, 1996).

1.4.2.4 Ozone

Ozone is one of the most powerful oxidants known and has been used effectively for disinfection and the oxidation of a wide range of compounds in water treatment. Keijola *et al.* (1988) showed that preozonation at 1 mg/L was sufficient to completely remove toxicity caused by both hepatotoxins and anatoxin-a. Himberg *et al.* (1989) further determined that the removal efficiency was dependent on ozone dose. This work has been summarised by Lahti and Hiisvirta (1989). James and Fawell (1991) also confirmed that ozonation removed microcystin-LR and further showed that ozonation did not result in the formation of 'new' toxic products sufficient to cause acute poisoning or signs of illness in mice within 24 hours. The effectiveness of ozone has also been reported by Bernazeau (1994).

Rositano and Nicholson (1994) found that the rate of oxidation of microcystin-LR with ozone was extremely fast with 99% of the toxin removed in 15 seconds. It was also found that with water containing cyanobacterial material, higher ozone dose rates were required but toxin removal was complete with concentrations of ozone just above the ozone demand of the water; ie, ozonating to produce a residual of only 0.05 mg/L was sufficient to completely remove microcystins. Again toxin destruction was pH dependent (Rositano and Nicholson, 1994; Rositano, 1996; Rositano *et al.*, 1997). Pieronne (1993) showed that nodularin was also very rapidly and effectively oxidised by ozone while the removal of anatoxin-a was slower than the hepatotoxins; 92% removal was reached after 60 seconds with a residual of 0.11 mg/L.

1.4.2.5 Other Oxidants

A number of other oxidants are available for disinfection; in particular, hydrogen peroxide, peroxone (a mixture of ozone and hydrogen peroxide) and irradiation with ultraviolet light.

Hydrogen peroxide at a dose of 20 mg/L removed only 17% of microcystin from a freeze dried algal extract with a peroxide residual of 11.2 mg/L after one hour (Rositano and Nicholson, 1994;

Rositano, 1996). Considering the high oxidation potential of hydrogen peroxide this may be considered surprising; however hydrogen peroxide is not used alone as an oxidant in water treatment because of its poor chemical kinetics (Glaze, 1990) and this may also be the reason for the poor reaction with the microcystins.

Ultraviolet light is a very efficient means of disinfecting water at the point of use and this could prove a simple solution for a single household using water containing toxins. The stability of microcystin-LR to UV irradiation was investigated (Rositano and Nicholson, 1994). Although more than 90% removal occurred within 30 minutes, the radiation dose required to achieve this was far in excess of the average radiation delivered by commercially available disinfection units. Static units with high irradiation intensities would be required.

Hydrogen peroxide has been used in combination with both ozone and UV to improve the oxidation efficiency of each process. Preliminary evaluation of UV/hydrogen peroxide did not result in any increased removal of microcystin-LR above that obtained with UV alone (Rositano and Nicholson, 1994). Peroxone appeared to be slightly more effective than ozone alone in oxidising microcystin (Rositano, 1996).

1.5 Project Aim

The effectiveness of oxidation for the removal of hepatotoxins is a significant discovery and offers, in the case of chlorine, a relatively cheap, simple solution for removal of these toxins. However of concern is whether oxidation of these toxins, while removing the toxicity, introduces other noxious by-products. In the case of chlorine, destruction of microcystins is accompanied by the elimination of acute toxicity as determined by mouse bioassay (Nicholson *et al.*, 1993, 1994; Rositano and Nicholson, 1994; Rositano, 1996). Nevertheless the formation of by-products with lower but potentially chronic toxicity has not been addressed.

The aim of this project was to evaluate the formation of by-products during chlorine oxidation. Chlorine was selected as it is the most common of the oxidants employed in water treatment and, compared with ozone, is a much cheaper and convenient option for incorporation into water treatment processes for toxin control. The initial plan was to identify specific by-products of

microcystin-LR and nodularin over a range of chlorine:toxin ratios. However it rapidly became apparent that the quantities of pure toxins required, and the complexity of the procedures required for the isolation/identification of by-products made this approach impractical given the relatively short duration of the project. Consequently an approach based on identifying and quantifying more general indicators of toxicity following chlorination of toxic and non-toxic cultures of cyanobacteria was adopted.

Analyses for specific and non-specific toxicity included:

- HPLC analysis for microcystins
- mouse bioassay
- phosphatase inhibition (^{32}P protein phosphatase assay)
- Ames mutagenicity

It was recognised that the chlorination of algal cultures would produce disinfection by-products (DBPs) at levels comparable to those obtained from the chlorination of humic material (Briley *et al.*, 1980). This has significant implications on the chlorination of drinking water supplies contaminated with cyanobacteria. In August 1996, Australian drinking water quality guidelines for DBPs were released (NHMRC/ARMCANZ, 1996) and covered the most commonly identified chlorination by-products such as the trihalomethanes (THMs), haloacetic acids (HAAs), haloacetonitriles (HANs), chloral hydrate, chloropicrin, cyanogen chloride and the chlorophenols. On this basis the opportunity was taken to analyse for a number of these compounds as they directly related to the health implications of chlorinating drinking waters affected by blooms of toxic and non-toxic *M. aeruginosa*.

The disinfection by-products determined were:

- trihalomethanes (THMs)
- haloacetic acids (HAAs)
- haloacetonitriles (HANs)
- AOX (a more general measure of halogenated by-products)
- chlorophenols

2 MATERIALS AND METHODS

2.1 Cyanobacterial Materials

Four strains of *Microcystis aeruginosa* which produced varying concentrations of total microcystin per freeze dried weight (as determined by high performance liquid chromatographic (HPLC) analysis and mouse bioassay) were used in this project.

Strain PCC7820 was obtained from Professor G.A. Codd of the University of Dundee, Scotland and contained four microcystins identified as microcystins-LW, -LR, -LF and -LY and has an LD_{50} of 30 mg/kg from previously published data. The HPLC toxin profile however was very different from that published by Codd and coworkers (Lawton *et al.*, 1994).

This strain unfortunately had become contaminated with another strain of *M. aeruginosa*. Both were easily differentiated by the difference in cell diameter as strain PCC7820 was twice the diameter of the foreign strain (Figure 5). Five 12 L cultures and 2 1 L cultures were grown under identical conditions having been inoculated with the same stock inoculum at 1% v/v. The ratio of PCC7820 to foreign *Microcystis* cells varied considerably from culture to culture (Table I). Subsamples of cultures 4, A, and B (100 mL) were extracted and analysed by HPLC to determine the toxin content. The results suggest that the foreign strain is either non-toxic or produces very little toxin since there is quite a significant difference in cell concentrations of the foreign strain between these samples but not a significant difference in toxin content. Strain PCC7820 (which included the foreign strain) was used for one experiment. It was then decided to use a *M. aeruginosa* strain which produces a higher concentration of microcystins.

A second toxic *M. aeruginosa* strain, MIC 338 was donated by Dr Gary Jones of CSIRO Division of Water Resources. This strain was collected from Shepparton, Victoria in 1991. The culture produced between 1500-1800 mg/g dry weight of microcystin. This strain produced 4 toxic components, microcystin-LR and 2-desmethyl LR which coeluted by HPLC and another two components which have not yet been characterised (Figure 6).

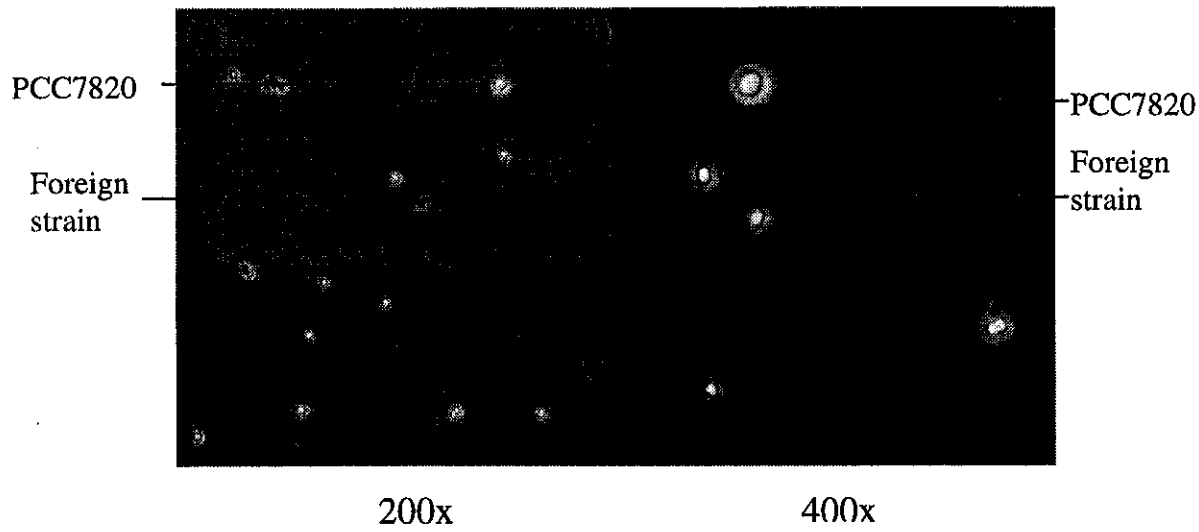


Figure 5. 200x and 400x magnification images of culture PCC7820 showing the *Microcystis* strain PCC7820 and the contaminant strain

TABLE I

CELL COUNT RATIO AND TOXICITY OF CULTURED PCC7820

Culture	Cell Count (cells/mL)		Ratio	Microcystin
	PCC7820	Foreign Strain	PCC7820/ Foreign	Concentration (μg/100mL)
1 (12 L)	420 000	90 000	4.7:1	*
2 (12 L)	417 000	190 000	2.2:1	*
3 (12 L)	400 000	128 000	3.1:1	*
4 (12 L)	538 000	161 000	3.3:1	0.75
5 (12 L)	421 000	95 000	4.4:1	*
A (1 L)	300 000	450 000	0.7:1	0.39
B (1 L)	400 000	3 000 000	0.1:1	0.92

* Toxin concentration was not determined

Strain 023 was collected from the River Murray near Mildura, Victoria in 1991 and contained one minor toxic component as determined by HPLC analysis with PDA detection (Figure 6). Mouse bioassay at 500 mg/kg resulted in a non-toxic response. A second strain of *M. aeruginosa*, strain 031, which produced a non-toxic response by mouse bioassay at 500 mg/kg, was obtained from Carcoar Dam, New South Wales in 1992. It contained no toxic components by HPLC analysis.

2.2 Growth and Preparation of Cyanobacterial Cultures for Chlorination Experiments

All cultures were grown in BG11 media (Rippka *et al.*, 1979) which consisted of the following:

$NaNO_3$	1500 mg/L	Na_2C0_3	20 mg/L
K_2HPO_4	30.5 mg/L	Trace Metals	
$MgSO_4.7H_2O$	75 mg/L	H_3BO_3	2.86 g/L
$CaCl_2.2H_2O$	36 mg/L	$MnCl_2.4H_2O$	1.81 g/L
Citric Acid	6 mg/L	$ZnSO_4.7H_2O$	0.222 g/L
Ferric Ammonium		$Na_2MoO_4.2H_2O$	0.39 g/L
Citrate	6 mg/L	$CuSO_4.5H_2O$	0.0792 g/L
Na_2EDTA	1 mg/L	$Co(NO_3)_2.6H_2O$	0.0496 g/L

Media was autoclaved at 121 kPa for 40 minutes and cooled slowly to prevent the precipitation of salts. Ferric ammonium citrate was added aseptically and the media was then pH adjusted to 7.4. Media was inoculated under sterile conditions with the appropriate culture of *Microcystis* in its late log growth phase at 1 % v/v. The *Microcystis* cultures were grown in 12 L of media in 15 L Nalgene containers at 25°C under continuous light at 60 µmoles/m^2/sec (Figure 7). The cultures were left to grow for 7-8 days with gentle shaking twice daily.

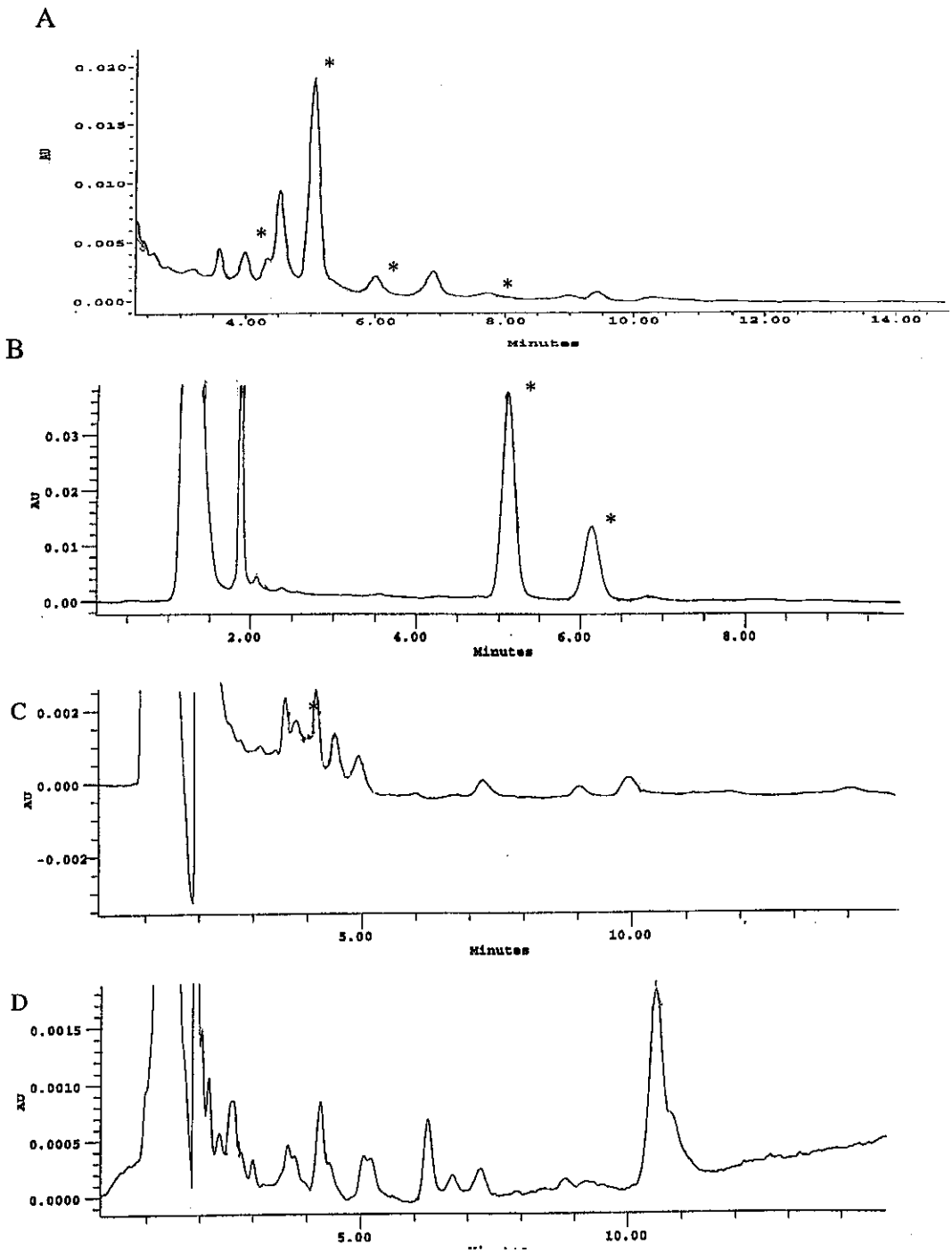


Figure 6. HPLC chromatograms of strains PCC7820 (A), 338 (B), 031 (C), and 023 (D). Toxic components are represented by an asterisk. Strain 338 shows only 2 peaks, both containing two co-eluting components, all of which are toxic. Despite the large number of peaks in D none of these show UV spectra typical of microcystins.

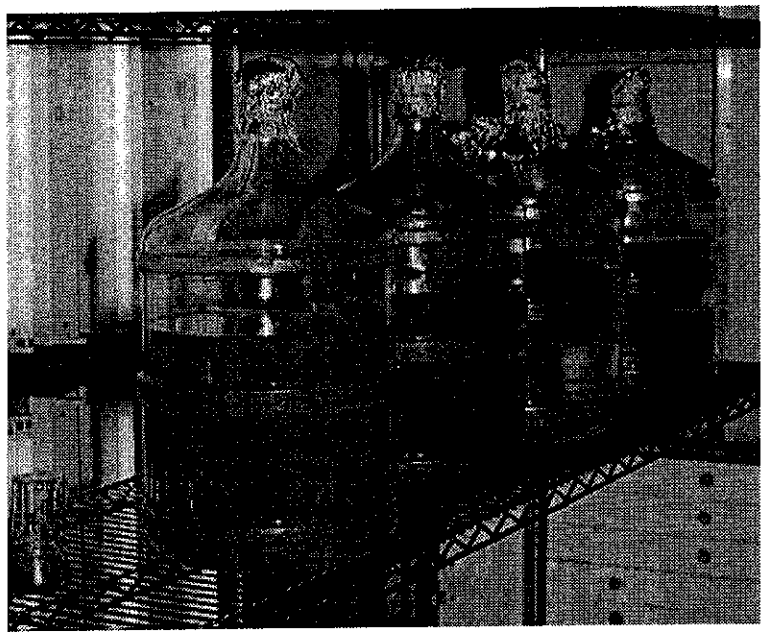


Figure 7. Cyanobacterial cultures being grown up on a large scale

2.2.1 Concentration of *M. aeruginosa* Cultures

Various researchers have found that *M. aeruginosa* produces the highest level of toxicity at the mid-log or late-log phase of growth (Van der Westhuizen and Eloff, 1983; Watanabe and Oishi, 1985) It was decided therefore to use the cultures at this stage in their growth. Cell counts were carried out daily from seven days growth to determine when the cultures had reached late log phase. Cultures which produced microcystins were concentrated to ensure acute toxicity by mouse bioassay. This was achieved by centrifuging batches of culture at 8000 rpm for 10 minutes with no brake, decanting the supernatant and combining the cells. The decanted media was filtered through a 0.45 μm cellulose acetate filter to remove any remaining cyanobacterial cells and this solution was used as a media control in the chlorination experiments for strains PCC7820 and 338. Strains 023 and 031 did not require a cell concentration step as these strains did not produce toxins.

2.2.2 Cell Counting Procedure

A 1.5 mL sample was stained with a 1 % v/v Lugols iodine solution (50 µL). The sample was left to stand for 20 minutes, vortex mixed and transferred to a 1 mL Sedgwick-Rafter counting chamber. The chamber was placed in a humid environment and allowed to stand for a further 30 minutes to allow the cells to settle to the bottom of the chamber without evaporation of the sample. Five fields of view were counted under phase contrast using an Olympus BH-2 microscope at either 200x or 400x magnification. The cell number per millilitre was calculated by multiplication of the total cell count by a known factor for each objective. Errors due to volume variation of the counting chamber were also taken into account.

2.3 Chlorination of *M. aeruginosa* Cultures

2.3.1 Experiment 1 Strain PCC7820

Concentration of the cultures resulted in a cell concentration of 1.5 x 10^6 cells/mL PCC7820 and 1.8 x 10^6 cells/mL of the foreign strain of *M. aeruginosa*. The total organic carbon content was 5.5 mg/L, chlorophyll *a* 217 µg/L, and the pH was adjusted to 7.5 by the slow addition of 0.1M hydrochloric acid. The microcystin content of a 100mL sample of culture was determined by HPLC analysis to ensure acute toxicity by mouse bioassay and was determined to be 1.2 µg/100 mL. The chlorine demand of the culture was determined by chlorinating four 100 mL subsamples at various chlorine doses and determining the residual by DPD/FAS titration (Standard Methods, 1976) after 30 minutes and was found to be 5.2 mg/L (Figure 8). Four 1.8 L volumes of the culture were poured into 2 L vessels, three of these were dosed with a concentrated chlorine solution to give a final dose of 8.0 mg/L chlorine, the other solution was used as a control. Two 1.8 L volumes of BG11 media used to grow the cyanobacteria were filtered through a 0.45 µm cellulose acetate membrane, one volume was chlorinated at the same dose whilst the other was left non-chlorinated. All solutions were left standing for 30 minutes with occasional stirring. After 30 minutes a chlorine residual was determined for each vessel and then quenched with approximately 100 mg sodium sulphite. Appropriate volumes from each vessel were apportioned for the various analyses to be carried out as outlined in Figure 9.

2.3.2 Experiment 2 Strain 338

A culture of strain 338 was concentrated as in Experiment 1, however the pH was adjusted by the addition of 10% v/v 0.1M phosphate buffer (pH 7) to ensure that the pH was maintained at pH 7 on the addition of chlorine solution. The final cell concentration was 340 000 cells/mL, TOC 5.1 mg/L, chlorophyll *a* 179 µg/L and initial microcystin content of 6.9 µg/100 mL. It should be noted that the initial microcystin content was different from the microcystin content at the time of the experiment as the experiment was carried out a day after the initial screening and the culture was still growing. The chlorine demand was determined to be 18.6 mg/L (Figure 8) and subsequently the cultures were chlorinated at 20 mg/L following the same protocol as in Experiment 1. This procedure was repeated with a further 4 x 1 L of culture and 2 x 1 L of media, however these solutions were quenched after 24 hours and the volumes apportioned for the appropriate analyses (summarised in Figure 9).

2.3.3 Experiment 3 Strain 023

As in Experiment 2 the pH was adjusted by the addition of 10 % v/v 1M phosphate buffer. The final cell concentration was 460 000 cells/mL, TOC 2.5 mg/L, chlorophyll *a* 124 µg/L and initial microcystin content of 0.27 µg/100 mL. The chlorine demand was 8.1 mg/L (Figure 8) and the chlorine dose applied was 12 mg/L. BG11 media was used as a media control in this experiment such that one vessel was chlorinated and the other was unchlorinated. The experiment was repeated with 1 L vessels and left for 24 hours before quenching.

2.3.4 Experiment 4 Strain 031

As in the previous experiment the pH was adjusted by the addition of 10% v/v 1M phosphate buffer (pH 7). The final cell concentration was 514 000 cells/mL, TOC 2.2 mg/L, chlorophyll *a* 57 µg/L and chlorine demand 6.0 mg/L (Figure 8). Microcystins could not be detected by HPLC. The solutions were dosed at 8 mg/L chlorine. The following flow diagram (Figure 9) illustrates the analyses carried out for each experiment. Chlorination of each strain was carried out in triplicate and in each experiment there was a control which consisted of the culture without chlorination but with quenching after 30 minutes or 24 hours. Two media controls (blanks)

included one chlorinated and another non-chlorinated for both the 30 minute and 24 hour chlorination. Each analysis was carried out in duplicate except for those which were carried out by subcontractors such as phosphatase inhibition assay and AOX . These analyses are quite expensive and so analyses were minimised.

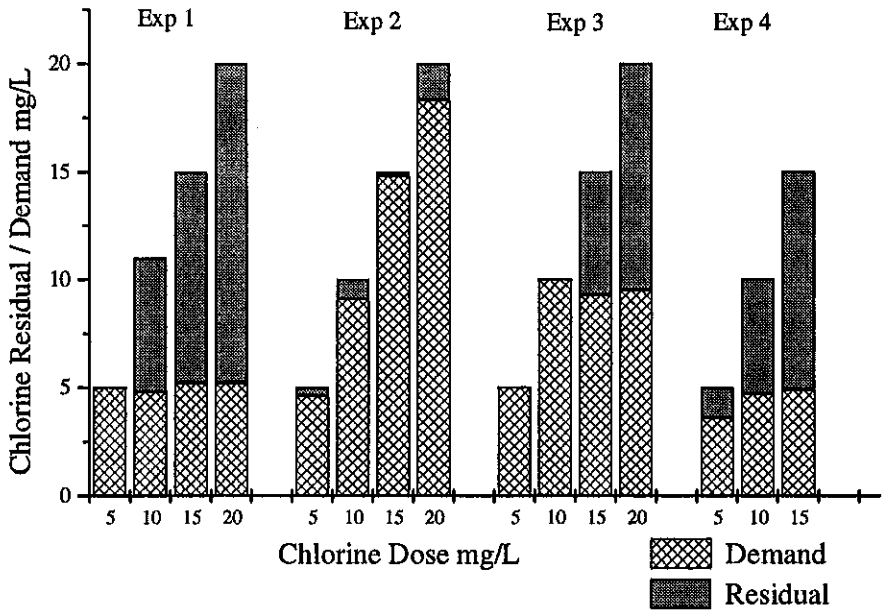


Figure 8. Determination of chlorine demands for experiments 1-4 : (Exp 1) 5.2mg/L, (Exp2) 18.3mg /L, (Exp 3) 9.4mg/L and (Exp4) 4.7mg/L

2.4 Analysis of Chlorinated Cultures

2.4.1 Toxicity Testing

2.4.1.1 Microcystin Analysis By HPLC

Duplicate 100 mL culture samples were freeze-thawed then filtered through Whatman GF/C filters. The filter paper was extracted twice with 5 mL 100% methanol whilst the filtrate was passed through a Waters 500 mg C18 solid phase extraction cartridge which had been preconditioned with methanol (15 mL) followed by water (15 mL). The cartridge was washed

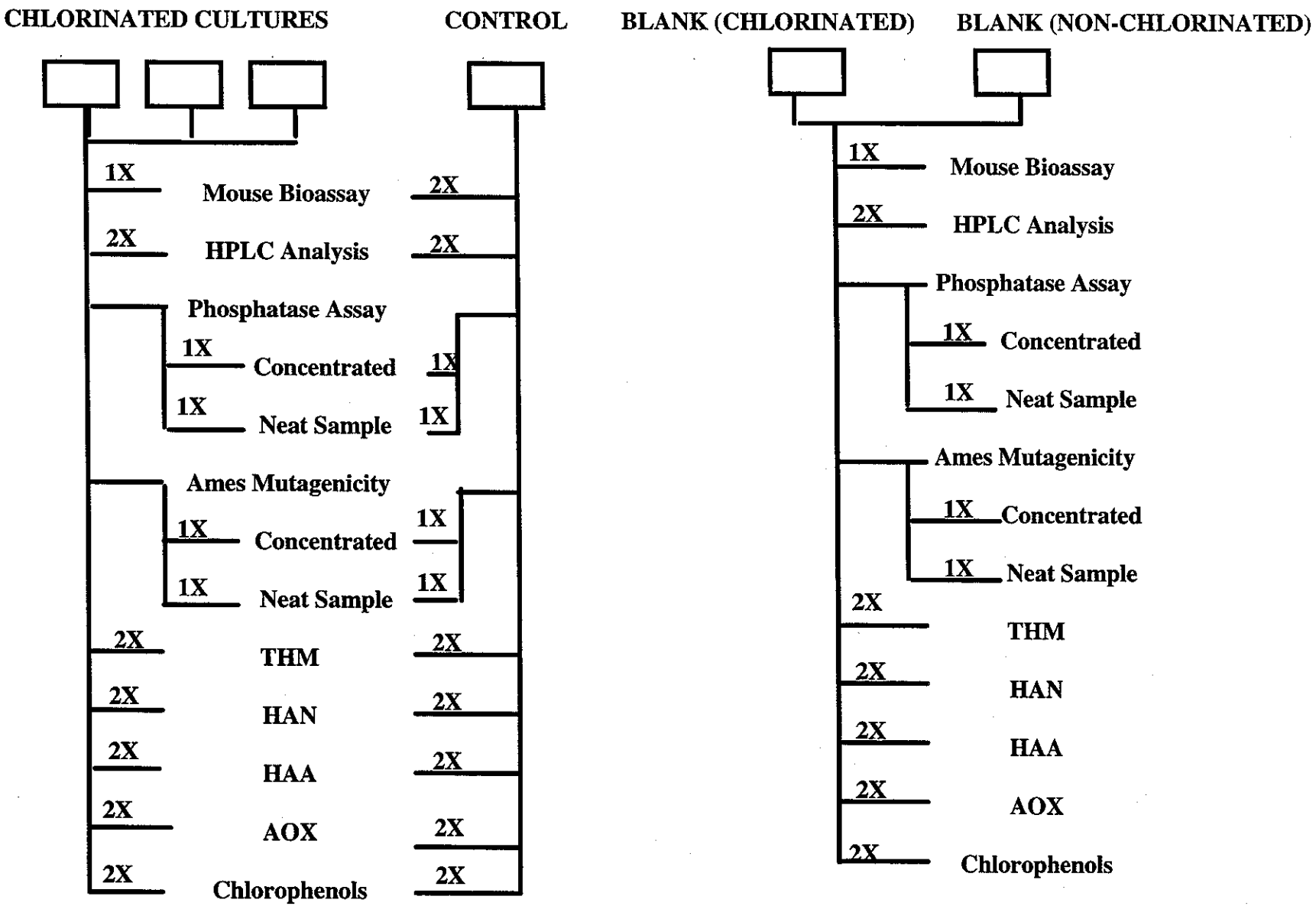


Figure 9. Flow diagram of analyses carried out after 30 minutes chlorination

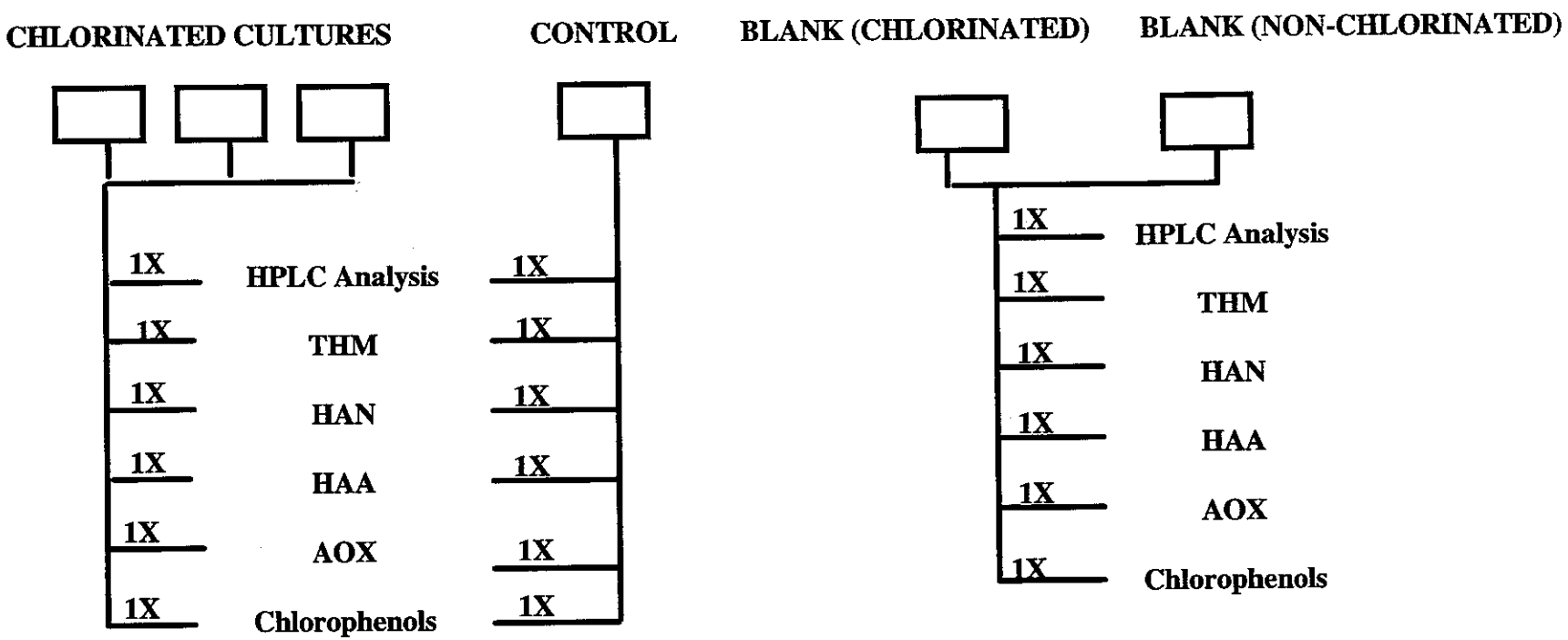


Figure 9 (cont.). Flow diagram of analyses carried out after 24 hours chlorination

with water followed by 10% and 20% methanol before eluting the column with 100% methanol. The methanol fractions from the GF/C filter and the solid phase extraction cartridge were combined and evaporated to dryness under a stream of nitrogen at 40°C. The residue was taken up in 500 µL methanol followed by 500 µL water, and the combined methanol/water fraction filtered through a PVDF 0.45 µm Gelman Acrodisc. A 50 µL injection was analysed by HPLC using a 27% acetonitrile/0.1M pH 7 phosphate buffer mobile phase at 1 mL/minute. The system consisted of all Waters equipment including a 996 photodiode array detector monitoring at 240 nm, 600E quaternary pump, 717plus autosampler and a Waters Symmetry C18, 5 µm 3.9 x150 mm analytical column.

2.4.1.2 Mouse Bioassay

500 mL volumes were extracted according to the method described above except that the combined methanol extracts were dried and taken up in 2.5 mL of Milli-Q water (Millipore Corp., USA). The bioassays were carried out by the Institute of Medical and Veterinary Science, Adelaide, South Australia. Two 1 mL samples were injected intraperitoneally into 20 g white female Balb/c mice. Death within 1-6 hr with the characteristic enlarged liver was used as a measure of toxicity.

2.4.1.3 Phosphatase Assay

Assays were carried out by Dr A.T. Sim and Ms L.-M. Mudge at the University of Newcastle according to the procedure of Sim and Mudge (1993). Experimental samples were analysed as the neat sample and as concentrated samples by extraction of 100 mL by the extraction technique outlined in section 2.4.1.1 except that the combined dried extracts were made up to 1 mL in Milli-Q water.

2.4.1.4 Ames Mutagenicity Assay

100 mL samples were extracted according to the method outlined (2.4.1.1). They were made up to 2 mL in Milli-Q water and aliquots of 100 µL were tested according to the revised method of Maron and Ames (1983) using *Salmonella typhimurium* strains TA98, TA100 and TA102. S9 mix was used as a metabolic activator for strains TA98 and TA100. Azide and mytomycin C

were used as positive controls for strains TA100 and TA102 respectively while aflatoxin, used in conjunction with S9, was used as a positive control for TA98 and TA100. This procedure was also repeated with the corresponding non-concentrated samples. Microcystin-LR standard solution at 5 μg/mL (Calbiochem Corp., USA) was also tested.

2.4.2 By-Products Analysis

2.4.2.1 Trihalomethane (THM) Analysis

Chloroform, bromodichloromethane, dibromochloromethane, and bromoform were analysed by capillary gas chromatography (GC) with electron capture detection (ECD) using a Perkin-Elmer HS 40 headspace sampler at 45°C and Varian 3400 gas chromatograph. The column was a DB-624, 30 m x 0.32 mm ID and 1.8 μm film thickness (J&W Scientific, USA). The column was set at 60°C for 1 minute, then ramped to 180° C at 30° C/minute. Standards were obtained from Aldrich Chemicals (Wisconsin, USA).

2.4.2.2 Adsorbable Organic Halogen (AOX)

AOX analyses were carried out by Levay and Co. Environmental Services (Ian Wark Institute, University of South Australia). 10 mL Samples were diluted to 100 mL with Milli-Q water followed by the addition of 5 mL of 17 mg/L sodium nitrate solution and 50 mg activated carbon. The samples were then shaken for 30 minutes and analysed on a Euroglas ECS 1000 AOX Analyser (Euroglas, The Netherlands).

2.4.2.3 Haloacetic Acids (HAAs)

Two methods for the analysis of the chlorinated acetic acids; monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), and trichloroacetic acid (TCAA) were attempted. They were US EPA methods 552 and 552.1 (USEPA, 1990, 1992) which entail methyl esterification with diazomethane and acidified methanol respectively. The acidified methanol method (552.1) resulted in low yields in comparison to the diazomethane esterification; the latter was the method of choice and is described below. The methyl-*tert*-butyl ether (MtBE) used in this method was distilled and dried over calcium sulfate prior to use.

A 30mL water sample was acidified (pH < 0.5) using concentrated sulphuric acid. MtBE (3 mL) was added followed by copper sulphate pentahydrate (3 g) and sodium sulphate (12 g, acidified to pH < 4 by the addition of 0.1 mL concentrated sulfuric acid to 100 g sodium sulfate in ethyl ether which was then evaporated). The vials were shaken by hand for 3 minutes to break up any lumps and then shaken for a further 30 minutes using a mechanical shaker. The ether layer was transferred to a 2.0 mL volumetric flask and approximately 0.3 mL was evaporated by blowing down under a gentle stream of nitrogen. 1,2,3-Trichloropropane at a concentration of 5.2 g/L (80 µL) was added as an internal standard. Diazomethane solution (see 2.4.2.4 for method) was added to bring the final volume to 2.0 mL. After 30 minutes, 0.2 g silica gel (60 - 100 mesh, activated), was added and allowed to be in contact with the diazomethane for 15 minutes. GC vials were filled and analysed within 48 hours (2 µL injections).

Samples were analysed using a Varian 3500 capillary gas chromatograph with electron capture detector (GC-ECD), Varian autosampler and a DAPA software system. The primary analytical column was a DB-1701, 30 m x 0.25 mm ID, 0.25 µm film thickness (J&W Scientific, USA) using hydrogen as the carrier gas. The injector temperature was 200°C with a splitless delay of 30 seconds. The column oven was programmed at 50°C for 10 minutes, reaching 210°C ramping at 10°C /minute, and a final hold time of 10 minutes. The detector temperature was 290°C. Confirmation of analytes was done using a DB-5 capillary column, 30 m x 0.32 mm ID, 0.25 µm film thickness (J&W Scientific, USA). Retention times are given in Table II.

A calibration curve was produced for each of the 3 chloroacetic acid esters spiked in Milli-Q water. Peak areas were compared with the area of an internal standard to produce a response ratio. Triplicate analysis of 5 concentrations was done for the range 0 - 50 µg/L; recovery data are given in Table III. Linear responses were found with good correlation for each of the acids, although monochloroacetic acid was difficult to detect. The linear range for the acids was found to extend to at least 50 µg/L (Figure 10).

TABLE II

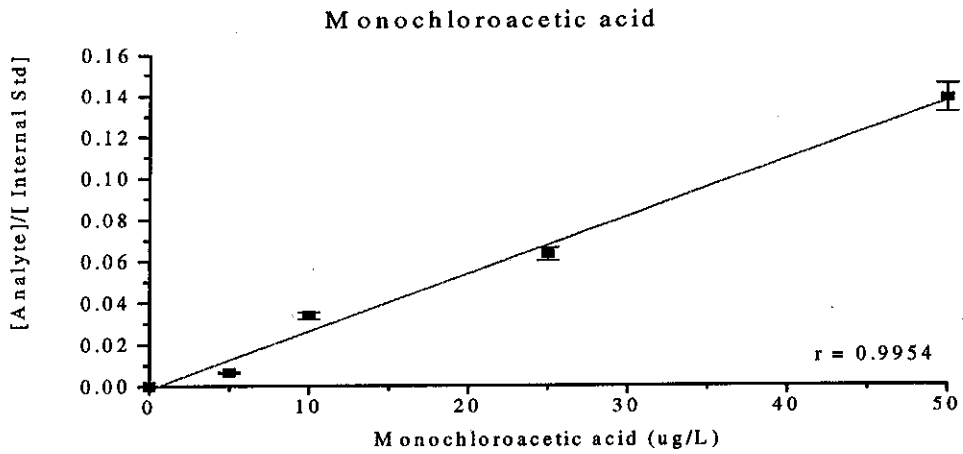
RETENTION TIME DATA FOR HALOACETIC ACIDS

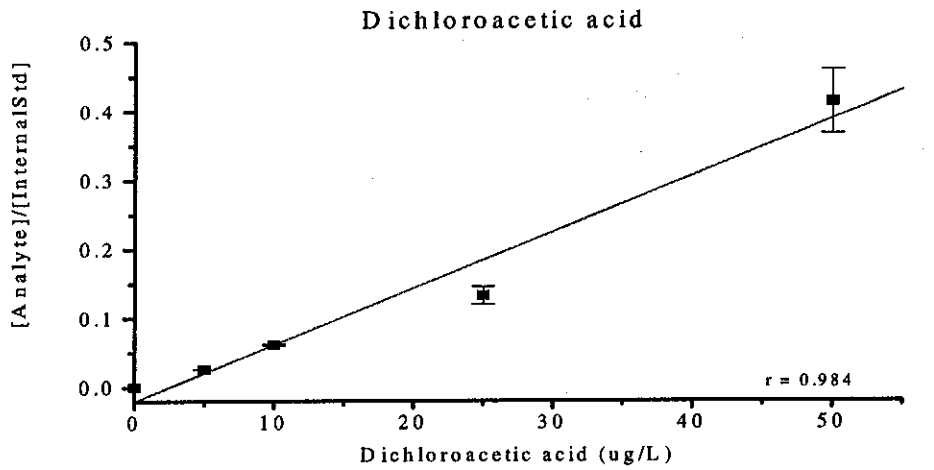
	Retention Time (Minutes)	
Analyte	**DB-1701**	**DB-5**
Monochloroacetic Acid	3.18	2.2
Dichloroacetic Acid	5.02	3.5
Trichloroacetic Acid	7.21	6.5
Internal Standard (1,2,3-Trichloropropane)	8.60	5.7

TABLE III

RECOVERY DATA FOR THE HALOACETIC ACIDS FROM MILLI-Q WATER

Concentration	**Recovery (%)**		
(µg/L)	**Monochloroacetic Acid**	**Dichloroacetic Acid**	**Trichloroacetic Acid**
0	0	0	0
5	65	86	77
10	60	90	91
25	68	112	70
50	73	99	81





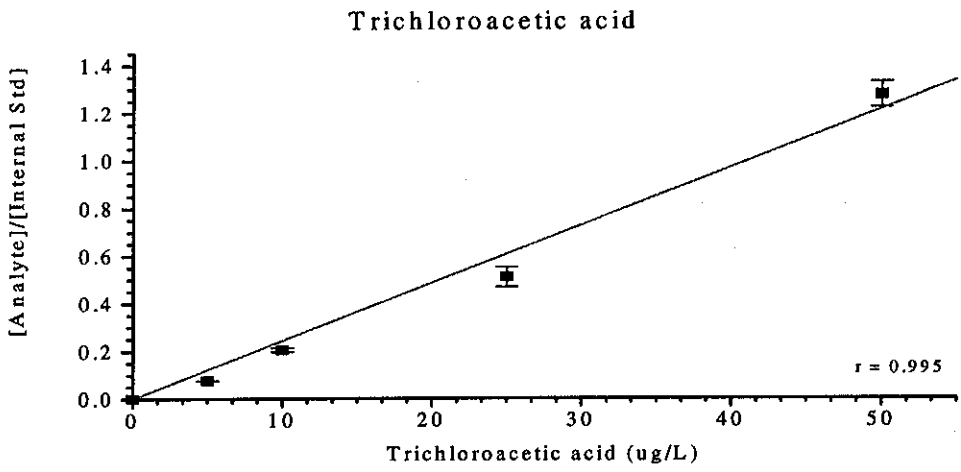


Figure 10. Standard curves for monochloroacetic acid, dichloroacetic acid and trichloroacetic acid

2.4.2.4 Preparation of Diazomethane Solution.

Diazomethane has the potential to explode, therefore generation of diazomethane was carried out behind a protective shield. Glassware had polished glass joints and no sharp edges.

A solution of diazomethane was prepared by the base catalysed decomposition of N-nitroso-N-methyl 4-toluenesulfonamide (pTSN). A solution of pTSN (2 g) in methoxyethanol (10 mL) was added dropwise to a stirred solution of methanolic KOH (10% w/w, 25 mL). A flow of nitrogen was maintained through the solution at a rate of approximately 2 bubbles per second. As diazomethane is generated the solution turns opaque yellow and the diazomethane is transferred and collected in an adjoining flask containing MtBE (50 mL) by the stream of nitrogen (Figure 11). The generation of diazomethane has ceased when the solution turns clear. The yellow solution of dissolved diazomethane in MtBE is then effectively stoppered and stored in the freezer until required.

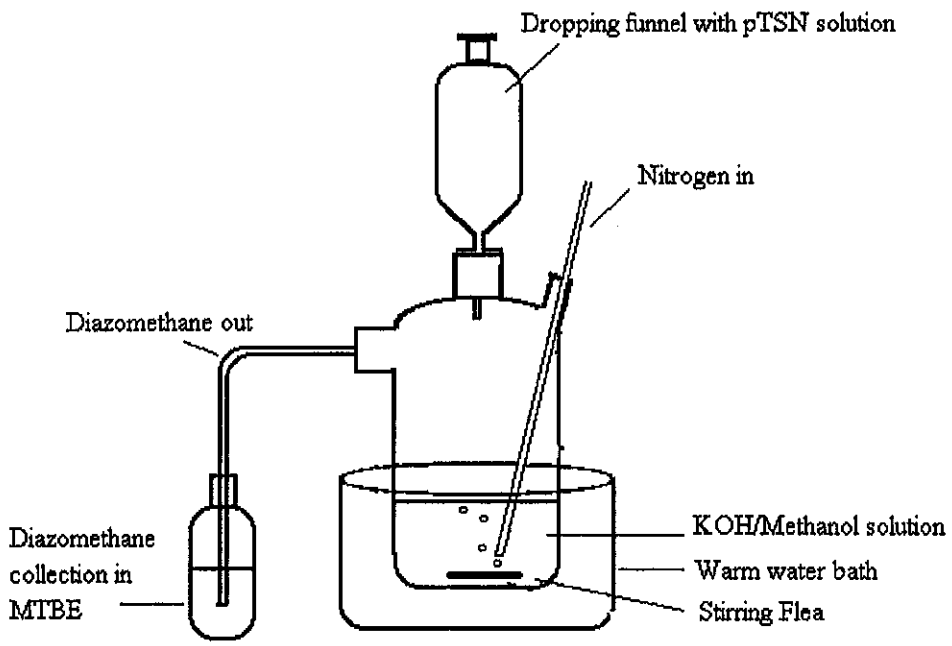


Figure 11. Apparatus for the generation of diazomethane

2.4.2.5 Haloacetonitriles (HANs) and Chloral Hydrate

Trichloroacetonitrile, dichloroacetonitrile, bromochloroacetonitrile and chloral hydrate were analysed according to a modified version of US EPA method 551 (USEPA, 1990).

Glass screw cap vessels (40 mL) were cleaned scrupulously using detergent, rinsed with tap water followed by demineralised water and finally with acetone. They were then oven dried. To a 35 mL sample was added sodium chloride (8 g) which was dissolved by manually shaking followed by the addition of MtBE (2 mL). The samples were shaken by hand for 2 minutes and the ether layer transferred to a GC autosampler vial. An internal standard was not added to the samples, instead an external calibration curve for each of the analytes was established by extracting quadruplicate samples of Milli-Q water spiked with the analytes at 5.0 µg/L, 2.0 µg/L, and 0.05 µg/L. The calibration curve was linear over this range and concentrations were determined by direct extrapolation from these curves (Figure 12). Dilution of the samples was sometimes necessary for quantification within this range.

The short retention time of trichloroacetonitrile on the DB 1701 column (Table IV) resulted in coelution of this analyte with other unknown extracted materials. It was not found necessary, however, to prepare a calibration curve from the DB-1 column data as only traces of this analyte were found to be present in samples. Recoveries for various extractions from spiked cultures of *M. aeruginosa* and Milli-Q water are given in Table V. Initial recoveries for chloral hydrate from both a spiked culture of *M. aeruginosa* and Milli-Q water were consistently low and this compound could not initially be detected at the lower levels. A number of measures where taken to improve the recoveries such as careful purification and drying of reagents, the capillary column washed frequently with various solvents and the electron capture detector replaced, which improved the sensitivity and allowed detection at 0.05 µg/L. These data are also shown in Table V.

The samples were analysed on a Varian 3500 capillary GC with electron capture detector and DAPA workstation. A DB-1701 column (30 m x 0.25 mm ID, 0.25 µm film thickness) was used as the primary analytical column with hydrogen as the carrier gas. The oven was set at 40°C for 9.67 minutes, ramped to 120°C at 6°C/min, held for 10 minutes then ramped to 150°C at 6°C/min and held for a further 5 minutes. The injector temperature was 200°C and

detector temperature 290°C. The presence of analytes of interest was confirmed by analysis on a BP-1 column (25 m x 0.25 mm ID, 1 µm film thickness). The initial oven temperature was set at 35°C for 9 minutes, increased to 120°C at 1°C/min, then to 210°C at 10°C/min.

TABLE IV

RETENTION TIME DATA FOR CHLORAL HYDRATE AND HALOACETONITRILES

	Retention Time (Minutes)	
Analyte	**DB-1701**	**DB-1**
Chloral Hydrate	2.57	10.75
Dichloroacetonitrile	4.57	9.75
Trichloroacetonitrile	2.05	8.31
Bromochloroacetonitrile	10.28	-

2.4.2.6 Chlorophenols

The analysis of 2,4,6 trichlorophenol, 2,3,4,6 tetrachlorophenol and pentachlorophenol was carried out according to the AWQC laboratory method OC07 and is briefly described below.

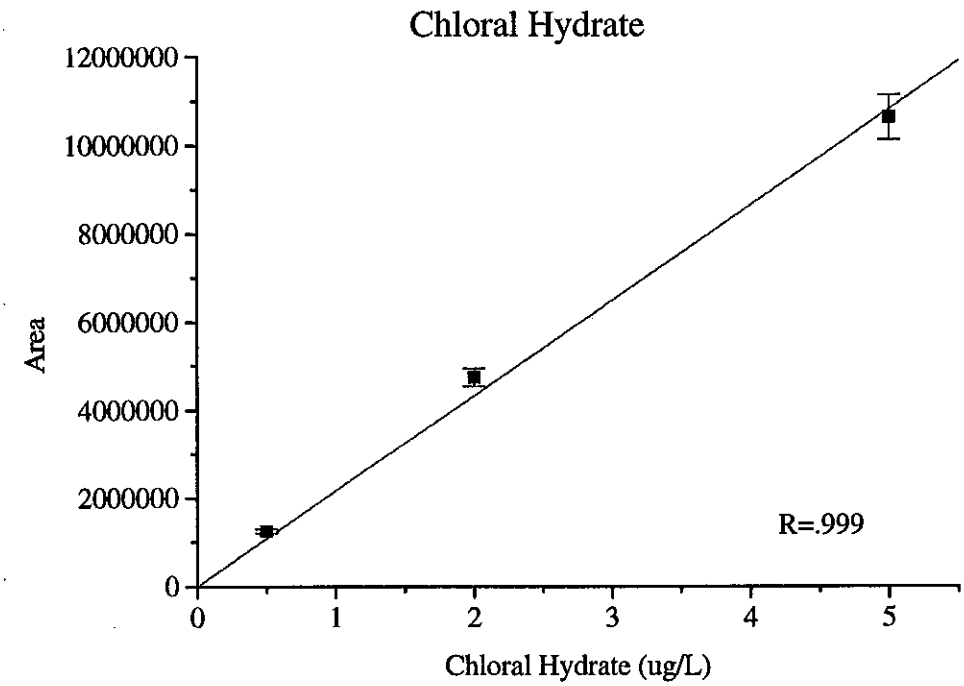
The samples (200 mL) were filtered through a 0.45 µm filter membrane and acidified to pH 2 with sulfuric acid (2M). Internal standard was added (2,4,6 tribromophenol, 0.5 mL of a 250 µg/L solution), and the sample passed through a C18 solid phase extraction cartridge (500 mg). The analytes were eluted with 5 mL isopropanol which was transferred to a volumetric flask (250 mL) containing 45 mL Milli-Q water. The pH was adjusted to 9 with sodium hydroxide (0.1M) and potassium carbonate added. The analytes were derivatised by adding acetic anhydride and swirling for approximately 3 minutes, then extracted in hexane (5 mL) after making to the mark with Milli-Q water. The extracts were analysed by GC/ECD.

Analyte concentrations are determined by comparing the response factors with those of standards made up in Milli-Q water, derivatised and analysed in the same manner. GC columns used were a DB-5 (15 m x 0.25 mm ID, 0.25 µm film thickness) and a DB-1701 (15 m x 0.25mm ID, 0.25 µm film thickness)(J&W Scientific, USA).

TABLE V
RECOVERIES OF HALOACETONITRILES AND CHLORAL HYDRATE FROM SPIKED SOLUTIONS OF A NON-TOXIC *MICROCYSTIS AERUGINOSA* CULTURE AND MILLI-Q WATER AT VARIOUS CONCENTRATIONS[a]

	Chloral Hydrate			Dichloroacetonitrile			Bromochloroacetonitrile		
	Mean	Stdev	%Recovery	Mean	Stdev	%Recovery	Mean	Stdev	%Recovery
Concentration µg/L									
Culture[b]									
0	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.05	nd	nd	nd	80685	446438	63	nd	nd	nd
2	473665	53258	15	3150804	240401	91	2104787	202499	102
5	1857603	303055	21	4861593	192940	61	4185100	419404	88
Milli-Q[c]									
0	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.05	nd	na	nd	53688	na	68	nd	na	nd
0.1	nd	na	nd	52433	na	33	nd	na	nd
0.5	nd	na	nd	420420	na	64	172087	na	87
2	484318	na	15	2021633	na	121	814572	na	127
5	2847975	na	38	4651419	na	65	2431809	na	71
0	nd	nd	nd	nd	nd	nd			
.05	1250374	48855	62.8	1857549	50921	119			
2	4739084	201445	72.3	6048837	713970	128			
5	10620046	500024	66	13484085	575421	114			

(a) No recoveries were obtained for trichloroacetonitrile due to its short retention time and co-elution with other materials
(b) Four replicates
(c) Single analysis



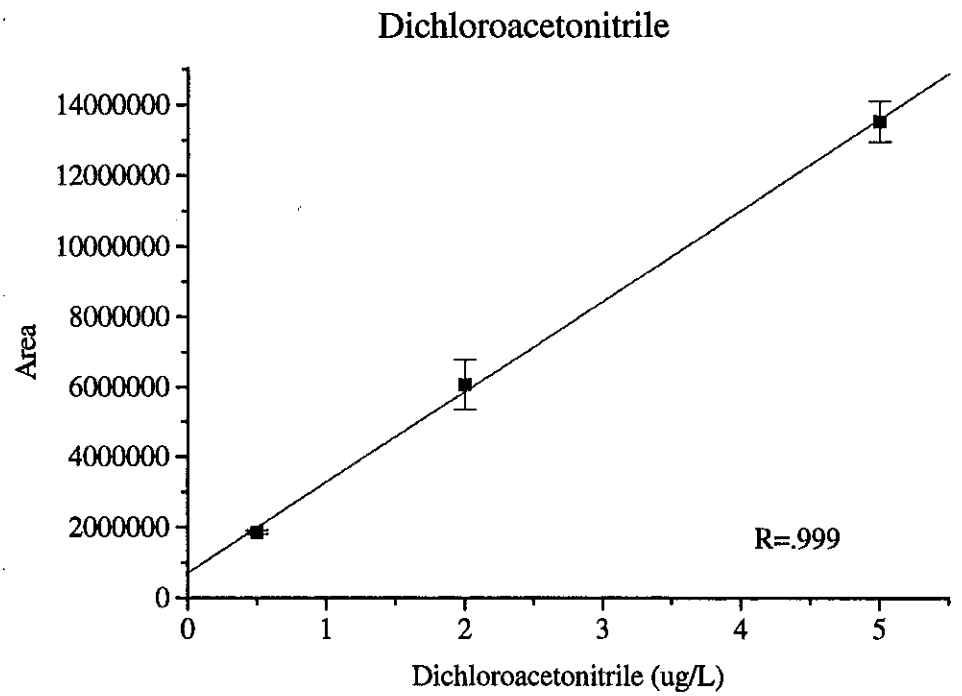


Figure 12. Calibration curves for chloral hydrate and dichloroacetonitrile

3 RESULTS AND DISCUSSION

3.1 Toxicity of *Microcystis aeruginosa* Cultures After Chlorination

The chlorinated cultures were assessed for potential toxicity by

- **HPLC Analysis.** To determine the concentration of microcystins present in the culture before and after chlorination.

- **Mouse Bioassay**. The mouse bioassay, although a crude and relatively insensitive technique, allows confirmation of the acute toxic effects of microcystin in cultures of *M. aeruginosa* and/or any acute toxic effects of other highly toxic compounds apart from microcystins.

- **Phosphatase Inhibition Assay**. Microcystins are known to inhibit protein phosphatase activity at extremely low levels (MacKintosh *et al.*, 1990; Nishiwaki-Matsushima *et al.*, 1991). It was expected that there would be correlation between the phosphatase inhibition, HPLC analysis and mouse bioassay data which would indicate the effectiveness of chlorination in eliminating phosphatase inhibition due to the microcystins. The samples were tested as the neat solution and as an extract solution which was a 100 fold concentration. Figure 13 illustrates the basis of the phosphatase inhibition assay used in this study. ^{32}P labelled glycogen-phosphorylase a is generated by the action of phosphorylase kinase and ATP^{32}. The ^{32}P is then cleaved by the action of protein phosphatases 1 and 2A and is measured in solution after precipitation of the proteins. Phosphatase inhibition of PP1 and PP2A by microcystins results in decreased concentrations of ^{32}P. An underestimation of toxin concentration is possible due to the presence of endogenous protein phosphatases in the sample. This increases the total phosphatase activity thereby producing higher concentrations of ^{32}P. Underestimation of toxin concentration may also be due to the presence of small peptides with bound phosphate arising from proteolysis of the glycogen-phosphorylase which remain in solution after precipitation of the larger proteins.

- **Ames Mutagenicity.** The Ames mutagenicity test was used as a screening technique to determine potential mutagenicity of samples before and after chlorination. Mutagenicity could be derived from the algal metabolites and/or their chlorinated products which would include of course any microcystins and their by-products.

A summary of results for the toxicity analyses is given in Tables VI and VII.

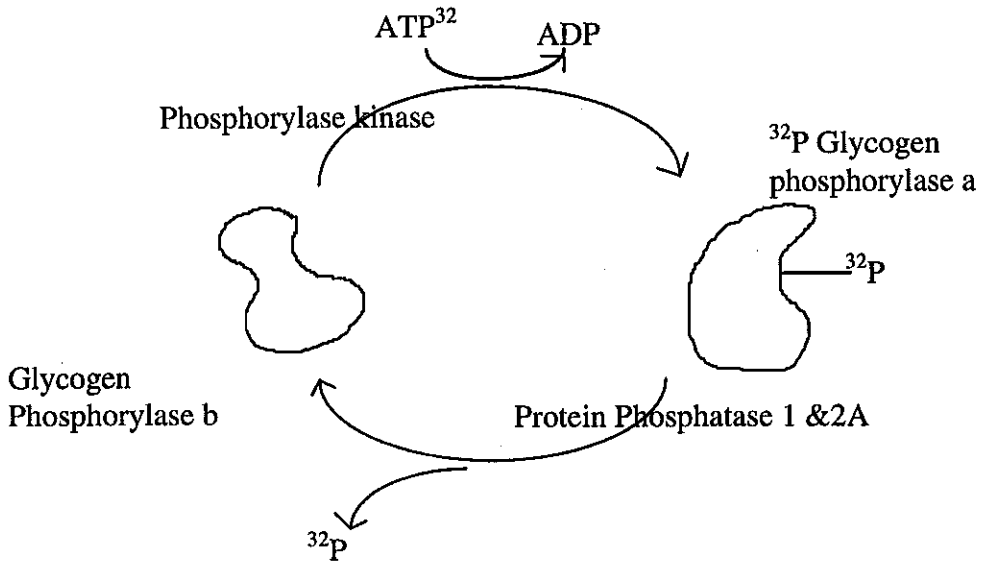


Figure 13. The phosphorylation / dephosphorylation cycle. ^{32}P labelled glycogen-phosphorylase a is generated by the action of phosphorylase kinase and ATP^{32} . The ^{32}P is then cleaved by the action of protein phosphatases 1 and 2A and measured in solution after precipitation of the proteins. Phosphatase inhibition of PP1 and PP2A by microcystins results in decreased concentrations of ^{32}P.

To obtain an acute toxic response by mouse bioassay due to hepatotoxicity from microcystins, the concentration of toxins in a solution or culture (taking into account the concentration factor of 100x) would need to be between 10 and 20 µg/L assuming that the LD_{50} of the microcystins present were between 50-100 µg/kg. As expected from the HPLC results, a positive mouse bioassay response was obtained from strains PCC7820 control and 338 control and non-chlorinated media, whilst a negative response was obtained for the other strains and samples. (Tables VI and VII) The results obtained from the phosphatase inhibition analyses generally supported this assumption with the exception of the PCC7820 'Control neat' sample. In this case the reported concentration of 2.6 µg/L would be insufficient to cause an acutely toxic response by mouse bioassay.

TABLE VI

SUMMARY OF TOXICITY RESULTS FOR STRAINS PCC7820 AND 338

Microcystis Strain/Assay		Control	Chlorinated Blank	Non-Chlorinated Blank	Chlorinated Culture 30 min	Chlorinated Culture 24 hr
PCC7820						
Mouse Bioassay		AT	NT	NT	NT	NT
HPLC (μg/L)		18.9	ND	ND	ND	NA
Phosphatase Assay (μg/L)	Neat	2.6	2.4	4.0	1.6(0.6)	NA
	Conc	50	NA	NA	0.32 (0.2)	NA
Ames Mutagenicity	Neat	M* TA98±S9	NM	NM	NM	NA
	Conc	M TA98±S9	NM	M TA98±S9	M TA98±S9	NA
338						
Mouse Bioassay		AT	NT	AT	NT	NT
HPLC (μg/L)		60.7	ND	31.5	ND	NA
Phosphatase Assay (μg/L)	Neat	15.0	0.3	8.1	1.6(0.7)	NA
	Conc	151	NA	NA	0.22 (0.3)	NA
Ames Mutagenicity	Neat	NM	NM	NM	NM	NA
	Conc	NM	NM	NM	NM	NA

AT = Acutely toxic to mice, NT = not acutely toxic to mice, ND = not detected by HPLC analysis, M = mutagenic by Ames mutagenicity assay, NM = non-mutagenic by Ames mutagenicity assay, NA = data not available, * = weak mutagenic response. TA98±S9 = *S. typhimurium* strain TA98 with and without the metabolic activator S9 mix.

TABLE VII

SUMMARY OF TOXICITY RESULTS FOR STRAINS 023 AND 031

Microcystis Strain/Assay		Control	Chlorinated Blank	Non-Chlorinated Blank	Chlorinated Culture 30 min	Chlorinated Culture 24 hr
023						
Mouse Bioassay		NT	NT	NT	NT	NT
HPLC (μg/L)		2.8	ND	ND	ND	ND
Phosphatase Assay (μg/L)	Neat	4.8	3.9	2.4	3.1(2.0)	NA
	Conc	0.05	0.25	0.11	3.8 (6.2)	NA
Ames Mutagenicity	Neat	NM	M TA98±S9	M TA98±S9	M TA98±S9	NA
	Conc	NM	NM	NM	NM	NA
031						
Mouse Bioassay		NT	NT	NT	NT	NT
HPLC (μg/L)		ND	ND	ND	ND	NA
Phosphatase Assay (μg/L)	Neat	1.9	0.1	1.8	1.5(0.5)	NA
	Conc	.01	.01	.02	2.0 (0.9)	NA
Ames Mutagenicity	Neat	NM	M TA98±S9	M TA98±S9	M TA98±S9	NA
	Conc	M TA98±S9	M TA98±S9	M TA98±S9	M* TA98±S9	NA

AT = Acutely toxic to mice, NT = not acutely toxic to mice, ND = not detected by HPLC analysis, M = mutagenic by Ames mutagenicity assay, NM = non-mutagenic by Ames mutagenicity assay, NA = data not available. TA98±S9 = *S. typhimurium* strain TA98 with and without the metabolic activator S9 mix.

There was at times significant variation between the 'neat sample' and the expected 'concentrated sample' results obtained from the phosphatase inhibition assay. These results could not be explained as they were too inconsistent and the sample set too small to draw any conclusions. The assay produced a positive response for nearly all the samples analysed, however microcystins were not always detected by HPLC and mouse bioassay was not sensitive enough to achieve a positive response at the lower concentrations. These findings may be due to the following-

- The assay is not specific for the microcystins alone. Any substance which inhibits protein phosphatases 1 and 2A will interfere and be determined as microcystin. These substances may be produced by the cells or could be a product of the chlorination/oxidation reaction.

- All the strains tested in our study may be producing low levels of toxin which may not be detected by mouse bioassay or HPLC analysis.

- Incomplete oxidation of the microcystins may result in a chlorinated or smaller molecule which still displays the ability to inhibit protein phosphates 1 and 2A.

Despite the presence of phosphatase inhibition in all the samples analysed, the general trend was of a decrease in inhibition after chlorination for strains PCC7820 and 338, both of which contained acutely toxic levels of microcystins, and low level inhibition both before and after chlorination for strains 023 and 031. Similarly, in a study by Lambert *et al.* (1994) in which raw and treated drinking waters were monitored for microcystins by protein phosphatase inhibition assay over a period of 33 days, low levels of toxin in the range of 0.89 to 0.09 μg/L were detected in every sample of both the raw and treated water.

Phosphatase inhibition assay has also been used to determine levels of microcystins in various monitoring studies. Jones and Orr (1994) monitored by both HPLC and phosphatase inhibition assay the biological decay of microcystin-LR in a small enclosed lake after algicide treatment. Whilst a similar trend existed for microcystin release into water and degradation by both HPLC and phosphatase monitoring, protein phosphatase inhibition increased dramatically before degradation commenced. Their results indicate that a biotransformation of either the microcystin or another substance present in the lake had occurred which caused increased protein phosphatase

inhibition, and was not detected by HPLC analysis. A similar *in vitro* biodegradation study of microcystin-LR by Lam *et al.* (1995) did not show an increase in protein phosphatase inhibition prior to decay.

The Ames mutagenicity tests did not show any trend of increased mutagenicity after chlorination. Each strain of *Microcystis* displayed completely different mutagenicities for the various samples analysed. Mutagenicity was only observed with strain TA98, both with and without the S9 mix. It was expected that the non-chlorinated media would be non mutagenic in all experiments, however, it proved to be mutagenic for strains 023 and 031. The media control for the last two experiments was fresh BG11 media plus the quenching agent, sodium sulfite. The media control for strains PCC7820 and 338 was BG11 media which had been used to grow the cultures. This had had the cells removed by filtration and also contained the quenching agent. It can be deduced from this that one or more of the initial components of the BG11 media was mutagenic to the TA98 strain.

The microcystin producing strain PCC7820 displayed a mutagenic response for the 'neat' and concentrated 'control' samples with TA98 with and without S9 mix. This was expected as it was found that standard microcystin-LR at a concentration of 5 μg/mL produced a mutagenic and dose dependent response with strain TA98 without S9 mix (Figure 14). In contrast, the more toxic strain 338 did not produce any mutagenic response from any sample which was surprising since this strain of *Microcystis* also produces microcystin-LR. These analyses were repeated to yield the same negative response. It can only be concluded from these results that one or more components of the samples from Experiment 2 (strain 338) was toxic to the *Salmonella* strains used, thereby killing most of the bacteria resulting in very low colony counts.

Tsuchiya *et al.* (1992) and Juttner (1983) identified organic sulfur compounds from strains of *Microcystis aeruginosa* which have been found to produce thionyl chloride on chlorination. Ames mutagenicity testing of thionyl chloride resulted in a positive mutagenic response (Tsuchiya *et al.*, 1992). It was noted by Tsuchiya *et al.* (1992) that mutagenic activity also occurred with strains TA98 with and without S9 and TA100 without S9 after chlorination of these *Microcystis* cultures. Whilst mutagenicity in the chlorinated cultures cannot be directly attributed to thionyl chloride, it is likely that it is a contributing factor in total mutagenicity of these cultures.

Sample Volume (μL)	Number of Revertants			Mean	Standard Deviation
25	330	335	340	336.7	6.2
50	790	770	800	787	12.5
100	1340	1400	1560	1433	93

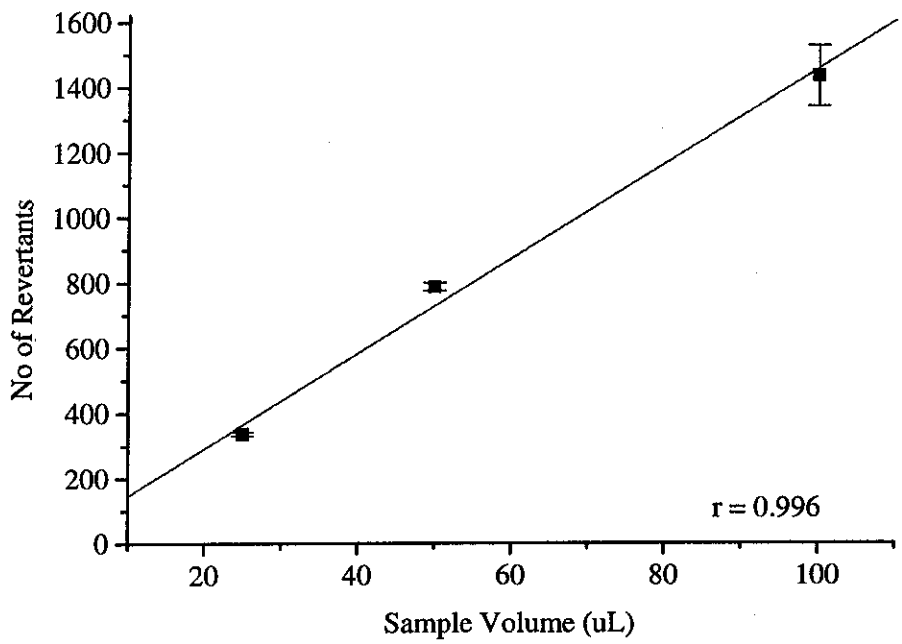


Figure 14. Microcystin-LR mutagenicity dose response curve with *Salmonella typhimurium* strain TA98 without S9

Closed loop stripping followed by GC/MS analysis of *Microcystis* culture 031 positively identified two disulfide compounds along with other organic compounds commonly found in algal cultures (Appendix). This may be a contributing factor to the mutagenicity of the chlorinated neat samples from strain 031 and possibly strain 023. Mutagenicity due to volatile compounds would not be expected in the concentrated sample as they would be purged from the sample during the extraction procedure. These results are however inconclusive due to the potential mutagenicity of the media to the *Salmonella* strain TA98 as shown by the positive response of the unchlorinated media control. Clearly, further detailed studies would need to be carried out to assess toxicity of the chlorinated cultures by Ames mutagenicity assay.

3.2 By-Products from the Chlorination of *Microcystis* Cultures

Strains PCC7820 and 338 were chlorinated at a cell biomass which resulted in acute toxicity to mice prior to chlorination when 100 mL of culture was extracted, concentrated and subsequently injected intraperitoneally in mice. The concentration of cells at the time of chlorination for cultures PCC7820 and 338 was different due to the different growth characteristics of the two strains. Strain PCC7820 produced less toxin than strain 338 and therefore cell numbers needed to be higher to produce an acutely toxic effect to mice. Surprisingly the TOC of each culture was similar despite the large difference in cell numbers (cell numbers 1.5×10^6 and 3.4×10^5 cells/mL, TOC 5.5 and 5.1 mg/L for strains PCC7820 and 338 respectively). This is probably due to the difference in the size of the cells, *Microcystis* 338 being slightly larger than PCC7820 and contained noticeably more mucilage material than PCC7820 by examination under the microscope. This may also account for the increased chlorine demand for strain 338.

Non-toxic strains 023 and 031 which were chlorinated at approximately 5×10^5 cells/mL had similar TOC values and chlorine demands.

Chlorination of the two microcystin producing cultures resulted in relatively minor or non detectable concentrations of the chlorinated disinfection by-products (DBPs) such as trihalomethanes (THMs), haloacetic acids (HAAs), haloacetonitriles (HANs), chlorophenols and chloral hydrate, especially after 30 minutes contact time (Tables VIII-X). They did however produce large amounts of other unknown chlorinated organic substances after both 30 minutes

and 24 hours chlorination. This group was measured by the total adsorbable organic halogen (AOX) minus the known detectable chlorinated by-products. The relative distribution of the detected chlorinated by-products for experiments 1 to 4 are represented by the pie graphs in Figure 15. In all cases after 24 hours chlorination the relative proportion of unknown AOX has decreased whilst the other DBPs have increased. The AOX value for the chlorinated media control is significantly lower in all cases than for the chlorinated culture. This implies that chlorination of the cyanobacterial cells results in a high concentration of organohalogen precursors which over time produce the smaller detected DBPs. This is consistent with studies by Wachter (1982) and Oliver and Shindler (1980) who demonstrated that the production of THMs from the chlorination of cyanobacteria or cyanobacterial extracellular products increases with time. Studies by Krasner *et al.* (1996) on the chlorination of raw waters containing TOC values of 2 mg/L have shown that levels of disinfection by-products such as THMs, HAAs and HANs also increases over time. Nicholson *et al.* (1990) also reported the increase in THM levels with time while AOX levels remained relatively constant following chlorination at a water filtration plant. It is not surprising therefore that the levels of the other chlorination by-products from experiments 1 to 4 such as chloral hydrate, and those mentioned previously have increased after 24 hours.

As expected, the same trend appeared for strains 023 and 031; however strain 023 produced a significantly higher concentration of THMs and HAAs at both 30 minutes and 24 hours reaction time compared with the other strains despite similar or lower cell biomass. It has been identified that the ability of algal biomass and extracellular products to generate THMs varies between species and growth phases of a culture (Thompson, 1978; Oliver and Shindler, 1980; Van Steenderen *et al.*, 1988). This then is most likely the case for the generation of HAAs also.

Cultures of strains 023, and 031 had similar AOX values after 30 minutes chlorination (Table XI), however, after 24 hours chlorination the proportion of chlorinated DBPs and AOX was quite different. This difference in proportions of DBPs produced by each culture suggests a difference in the nature of the precursors. This is similar to the behaviour of chlorine with natural organic matter (NOM). It has been shown that the consumption of chlorine by NOM is greatest with the phenolic humic substances and that there exists a correlation between the formation of DBPs such as chloroform, dichloroacetic acid, and trichloroacetic acid with the phenolic component of aquatic humic substances (Harrington *et al.*, 1995; Reckhow *et al.*, 1990).

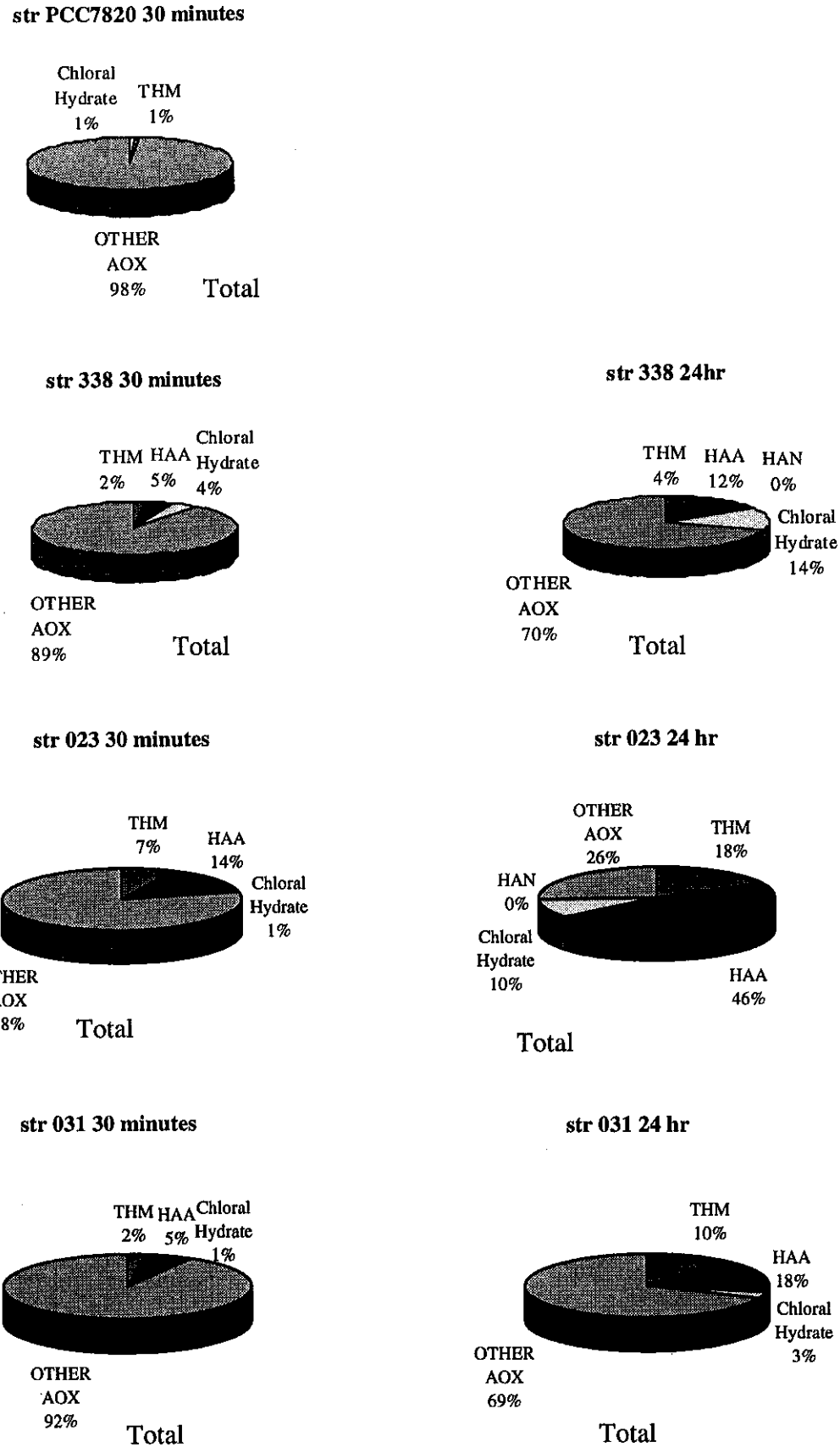


Figure 15. Distribution of chlorination by-products for strains PCC7820, 338, 023 and 031

A summary of results of chlorination DBPs is given in Tables VIII to XI. No chlorophenols were detected in any of the samples. The main chlorinated by-products detected included chloroform, chloral hydrate, trichloroacetic acid, dichloroacetic acid and dichloroacetonitrile. Traces of trichloroacetonitrile were also detected. Similar results were obtained in a study by Tsuchiya and Watanabe (1994) on the chlorination of *M. aeruginosa* strain TAC51 where the predominant disinfection by-products were formaldehyde, chloroform, chloral hydrate, dichloroacetonitrile and bromodichloromethane. Formaldehyde was detected in that study at relatively high levels (>1000 μg/L). Formaldehyde was not determined in this study.

Detected levels of chloral hydrate for experiments 2 and 3 after 24 hours chlorination exceeded the guideline limit of 50 μg/L. Whilst it is not expected that cyanobacterial cell concentrations would often reach levels of 5 x 10^5 cells/mL in water entering a water treatment plant, the additive effect of by-products produced from naturally occurring organic matter should be considered and it is possible that guideline values could be exceeded under some conditions.

It was not possible to identify by-products specific to the chlorination of toxic strains of *Microcystis* in this study as it would be necessary first to characterise the products from the chlorination of pure microcystin. Time and resources were not available for a more detailed study, however some indication of the sites of attack can be found in the literature. Given the structure of the peptide hepatotoxins, the primary site of oxidation would be expected to be the conjugated diene of the Adda side chain. This Adda site is extremely important for toxicity and geometrical isomerism around these double bonds is sufficient to eliminate acute toxicity (Harada *et al.*, 1990a,b) and also to significantly reduce inhibition of protein phosphatases (Nishiwaki-Matsushima *et al.*, 1991; Fujiki *et al.*, 1996).

Hydrogenation or ozonolysis of this diene system has also been reported to produce an inactive compound (Namikoshi *et al.*, 1989). Oxidation of this diene system of Adda has been employed in analytical methods for the indirect determination of microcystins by determining the concentration of one of the oxidation products. Sano *et al.* (1992) utilised oxidation with potassium permanganate and sodium periodate to produce 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) which was determined by gas GC or HPLC. This method has been employed for the determination of microcystins in Japanese lakes (Tanaka *et al.*, 1993). Harada *et al.* (1996)

employed ozone as the oxidant to produce MMPB which was determined by gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). Based on these results, oxidation would thus be expected firstly to cleave the Adda side chain to produce MMPB as one product and to eliminate acute toxicity. It is difficult to speculate as to what might be the sub-lethal toxicity of the oxidation products. These would need to be tested to determine their likely effects if present as contaminants in drinking water. However the considerable reduction in acute toxicity following oxidation may suggest that the by-products are relatively benign. The trends observed in the toxicity studies described in section 3.1 support this conclusion.

TABLE VIII
TOTAL TRIHALOMETHANE ANALYSIS RESULTS*

M. aeruginosa strain	Control (n=2)	Chlorinated Media (30min) (n=2)	Non-Chlorinated Media (30min) (n=2)	Chlorinated Culture 30 minutes (n=6)	Chlorinated Culture 24 hours (n=3)
PCC7820	3	nd	nd	2.8 (±0.8)	na
338	nd	2	nd	3.7 (±0.5)	17.0 (±1.0)
023	nd	7.5	nd	28.5 (±6.5)	90.0 (±8.0)
031	nd	26.5	nd	9.2 (±1.2)	39 (±10.0)

* Trihalomethanes determined include $CHCl_3$, $CHCl_2Br$, CH_2Br_2, $CHBr_3$, $CHClBr_2$. However the only analyte detected was chloroform. All results in µg/L; nd = not detected at a detection limit of 1.0µg/L; na = data not available; () = standard deviation

TABLE IX
HALOACETIC ACIDS ANALYSIS RESULTS*

M. aeruginosa strain	Control (n=2)	Chlorinated Media (30min) (n=2)	Non-Chlorinated (30min) Media (n=2)	Chlorinated Culture 30 minutes (n=6)	Chlorinated Culture 24 hours (n=3)
PCC7820					
Monochloroacetic acid	nd	nd	nd	nd	na
Dichloroacetic acid	nd	nd	nd	<1.0	na
Trichloroacetic acid	nd	nd	nd	nd	na
0338					
Monochloroacetic acid	nd	01.0	6.0	3.5 (4.2)	nd
Dichloroacetic acid	nd	1.5	nd	3.4 (0.7)	19
Trichloroacetic acid	nd	3.1	nd	4.8 (1.2)	28.3
023					
Monochloroacetic acid	nd	nd	nd	nd	nd
Dichloroacetic acid	nd	14.7	nd	52.2 (11.1)	208.5 (31.5)
Trichloroacetic acid	nd	2.3	nd	3.7 (0.9)	26.6 (6.2)
031					
Monochloroacetic acid	nd	nd	nd	nd	nd
Dichloroacetic acid	nd	14.4	nd	18.4 (1.7)	57.5 (30.8)
Trichloroacetic acid	nd	<1.0	nd	3.7 (0.4)	10.3 (6.1)

* All results in µg/L; ()= standard deviation, nd = not detected with detection limit of 1.0 µg/L

TABLE X
CHLORAL HYDRATE AND HALOACETONITRILES ANALYSIS RESULTS*

M. aeruginosa strain	Control (n=2)	Chlorinated Media (30min)(n=2)	Non-Chlorinated Media (30min) (n=2)	Chlorinated Culture 30 minutes (n=6)	Chlorinated Culture 24 hours (n=3)
PCC7820					
Chloral Hydrate	nd	0.5	nd	1.8(±0.2)	na
Dichloroacetonitrile	nd	d	nd	d	na
Trichloroacetonitrile	nd	nd	nd	nd	na
0338					
Chloral Hydrate	nd	3.0	nd	10.1 (±0.5)	58.3 (±4.9)
Dichloroacetonitrile	nd	d	nd	d	0.8 (±0.1)
Trichloroacetonitrile	nd	nd	nd	d	d
023					
Chloral Hydrate	nd	nd	nd	3.0 (±1.8)	52.0 (±3.2)
Dichloroacetonitrile	nd	nd	nd	d	0.5 (±0.3)
Trichloroacetonitrile	nd	nd	nd	d	d
031					
Chloral Hydrate	nd	nd	nd	3.5 (±0.1)	11.8 (±1.5)
Dichloroacetonitrile	nd	nd	nd	nd	nd
Trichloroacetonitrile	nd	nd	nd	nd	nd

* All results in µg/L; () = standard deviation; nd = not detected; na = data not available

TABLE XI
ADSORBABLE ORGANIC HALOGEN (AOX) ANALYSIS RESULTS*

M. aeruginosa strain	Control (n=2)	Chlorinated Media (30min)(n=2)	Non-Chlorinated Media (30min) (n=2)	Chlorinated Culture 30 minutes (n=6)	Chlorinated Culture 24 hours (n=3)
PCC7820	38.5	26	4	368.8 (±21.1)	na
338	17	26	4	247.2 (±24.8)	406 0(±18.0)
023	20	25 (190)**	nd	404.8 (±68.7)	513 (±45.0)
031	21	24 (144)**	5	410 (±53.5)	376(±13.0)

* All results in µg/L; () = standard deviation; nd = not detected; na = data not available; ()** = 24hr chlorination

4 SUMMARY AND CONCLUSIONS

The results presented indicate that chlorination is capable of removing acute toxicity from hepatotoxic cyanobacterial material as determined by HPLC and mouse bioassay. Although acute toxicity was eliminated, it appeared that subacute toxicity was still apparent as detected by the phosphatase inhibition assay. However toxicity by phosphatase inhibition was also apparent in the strains of *Microcystis* which did not show acute toxicity in mice at 500 mg/kg and at concentrations of 2.8 μg/L and less than 0.5 μg/L as determined by HPLC analysis. It cannot be assumed therefore that phosphatase inhibition at these low levels is due to the presence of microcystins or toxic by-products until an analytical technique specific for these compounds is developed that will enable comparative analysis at levels less than 0.5 μg/L. Despite a positive response for phosphatase inhibition from nearly all the samples analysed, the strains of *Microcystis* which contained toxins by HPLC analysis all showed a trend towards decreased phosphatase inhibition after chlorination.

A correlation between the production of by-products from the chlorination of *M. aeruginosa* cultures and the toxicity of the cultures as determined by Ames mutagenicity cannot be made from these results. Toxicity of the cultures after chlorination as determined by Ames mutagenicity assay could not be attributed to microcystin by-products as non-toxic strains were also mutagenic.

The detected by-products from the chlorination of toxic and non-toxic strains of *M. aeruginosa* include chloroform, chloral hydrate, trichloroacetic acid, dichloroacetic acid, dichloroacetonitrile and traces of trichloroacetonitrile. Large concentrations of unknown chlorinated by-products were also produced as determined by adsorbable organic halogen (AOX) analysis.

From the results of this study, it is clear that the possibility exists for high levels of DBPs to be produced in treated drinking waters if water supporting a bloom of cyanobacteria entering a water treatment plant were prechlorinated. To prevent this from occurring, either the intact cyanobacterial cells would have to be removed before chlorination or sophisticated water treatment techniques would have to be employed for the removal of by-products.

5 RECOMMENDATIONS

The original aim of this project was not sufficiently achieved. Further work is needed to determine the specific by-products from the chlorination of the microcystin toxins and subsequent assessment of the toxicity of these by-products to ensure that safe drinking water is being delivered to communities where drinking water supplies are affected by hepatotoxic cyanobacterial blooms.

6 ACKNOWLEDGMENTS

The researchers would like to thank the following people and organisations for their assistance during the course of this project:

- Staff of the Australian Water Quality Centre especially members of the Organic Chemistry, Biology, and Water Treatment units for allowing the use of tightly stretched instruments and facilities

- The Mass Spectrometry Unit for carrying out the closed loop stripping analyses and assessing the mass spectral data

- Marilena Marchesan of the Microbiology Unit for carrying out Ames mutagenicity assays

- Dr Alistair Sim and Lisa Mudge of the University of Newcastle for carrying out protein phosphatase inhibition assays

The Urban Water Research Association of Australia and SA Water Corporation without whose funding this project would not have been possible

7 REFERENCES

Anderson, D.M. (1994) Red tides. *Sci. Am.* **271(2)**, 52-58.

Beasley, V.R., Cook, W.O., Dahlem, A.M., Hooser, S.B., Lovell, R.A. and Valentine, W.M. (1989) Algae Intoxication in Livestock and Waterfowl. *Vet. Clin. North Am. : Food Animal Prac.* **5**, 345-361.

Bernazeau, F. (1994) Can Microcystins Enter Drinking Water Distribution Systems? In: Steffensen, D.A. and Nicholson, B.C., (Eds.) *Toxic Cyanobacteria: Current Status of Research and Management*, pp. 115-118. Adelaide, South Australia: Australian Centre for Water Quality Research.

Billings, W.H. (1981) Water-Associated Human Illness in Northeast Pennsylvania and its Suspected Association with Blue-Green Algae Blooms. In: Carmichael, W.W., (Ed.) *The Water Environment: Algal Toxins and Health*, pp. 243-255. New York and London: Plenum Press.

Bourke, A.T.C., Hawes, R.B., Neilson, A. and Stallman, N.D. (1983) An Outbreak of Hepato-Enteritis (The Palm Island Mystery Disease) Possibly Caused by Algal Intoxication. *Toxicon* **Suppl. 3**, 45-48.

Bowling, L. (1992) *The Cyanobacterial (Blue-Green Algae) Bloom in the Darling/Barwon River System, November-December 1991*, Department of Water Resources, NSW, Technical Services Report, 49 pp.

Briley, K.F., Williams, R.F., Longley, K.E. and Sorber, C.A. (1980) Trihalomethane Production from Algal Precursors. In: Jolley, R.L., Brungs, W.A. and Cumming, R.B. (Eds.) *Water Chlorination: Environmental Impact and Health Effects*, **Vol. 3**, pp. 117-129. Michigan: Ann Arbor Science Publishers.

Carmichael, W.W. (1992) Cyanobacteria Secondary Metabolites - The Cyanotoxins. *J. Appl. Bacteriol.* **72**, 445-459.

Carmichael, W.W. (1994) Toxins of Cyanobacteria. *Sci. Am.* **270(1)**, 64-72.

Carmichael, W.W. and Falconer, I.R. (1993) Diseases related to Freshwater Blue-Green Algal Toxins, and Control Measures. In: Falconer, I.R., (Ed.) *Algal Toxins in Seafood and Drinking Water*, pp. 187-209. London: Academic Press.

Carmichael, W.W., Jones, C.L.A., Mahmood, N.A. and Theiss, W.C. (1985) Algal Toxins and Water-Based Diseases. *CRC Crit. Rev. Environ. Control* **15**, 275-313.

Codd, G.A., Bell, S.G. and Brooks, W.P. (1989) Cyanobacterial Toxins in Water. *Water Sci. Technol.* **21(3)**, 1-13.

Craig, K. and Bailey, D. (1995) Cyanobacterial Toxin Microcystin 'LR' Removal Using Activated Carbon - Hunter Water Corporation Experience. *Proc. 16th AWWA Fed. Convent. (Aust.)* **2**, 579-586.

De Silva, E.D., Williams, D.E., Andersen, R.J., Klix, H., Holmes, C.F.B. and Allen, T.M. (1992) Motuporin, a Potent Protein Phosphatase Inhibitor Isolated from the Papua New Guinea Sponge *Theonella swinhoei* Gray. *Tetrahedron Letts.* **33**, 1561-1564.

Dillenberg, H.O. and Dehnel, M.K. (1960) Toxic Waterbloom in Saskatchewan, 1959. *Can. Med. Assoc. J.* **83**, 1151-1154.

Donati, C., Drikas, M., Hayes, R. and Newcombe, G. (1993) Adsorption of Microcystin-LR by Powdered Activated Carbon. *Proc. AWWA 15th Federal Convent. (Aust.)* **2**, 332-338; *Water (Aust.)* **20(3)**, 25-28.

Donati, C., Drikas, M., Hayes, R. and Newcombe, G. (1994) Microcystin-LR Adsorption by Powdered Activated Carbon. *Water Res.* **28**, 1735-1742.

Drikas, M. (1994) Control and/or Removal of Algal Toxins. In: Steffensen, D.A. and Nicholson, B.C., (Eds.) *Toxic Cyanobacteria: Current Status of Research and Management*, pp. 93-101. Adelaide, South Australia: Australian Centre for Water Quality Research.

El Saadi, O. and Steffensen, D.A. (1996) *Epidemiological Evidence of Algal Toxins in Drinking Water and Recreational Waters. Research Report No.104.* Melbourne: Urban Water Research Association of Australia

El Saadi, O., Esterman, A.J., Cameron, S. and Roder, D.M. (1995) Murray River Water, Raised Cyanobacterial Cell Counts, and Gastrointestinal and Dermatological Symptoms. *Med. J. Aust.* **162**, 122-125.

Falconer, I.R. (1991) Tumor Promotion and Liver Injury Caused by Oral Consumption of Cyanobacteria. *Environ. Toxicol. Water Qual.* **6**, 177-184.

Falconer, I.R. and Buckley, T.H. (1989) Tumour Promotion by *Microcystis* sp., a Blue-Green Alga Occurring in Water Supplies. *Med. J. Aust.* **150**, 351

Falconer, I.R., Beresford, A.M. and Runnegar, M.T.C. (1983a) Evidence of Liver Damage by Toxin from a Bloom of the Blue-Green Alga, *Microcystis aeruginosa. Med. J. Aust.* **1**, 511-514.

Falconer, I.R., Burch, M.D., Steffensen, D.A., Choice, M., and Coverdale, O.R. (1994) Toxicity of the Blue-Green Alga (Cyanobacterium) *Microcystis aeruginosa* in Drinking Water to Growing Pigs, as an Animal Model for Human Injury and Risk Assessment. *Environ. Toxicol. Water Qual.* **9**, 131-139.

Falconer, I.R., Runnegar, M.T.C., Buckley, T., Huyn, V.L. and Bradshaw, P. (1989) Using Activated Carbon to Remove Toxicity from Drinking Water Containing Cyanobacterial Blooms. *J. Am. Water Works Assoc.* **81(2)**, 102-105.

Falconer, I.R., Runnegar, M.T.C. and Huynh, V.L. (1983b) Effectiveness of Activated Carbon in the Removal of Algal Toxin from Potable Water Supplies: A Pilot Plant Investigation. *Proc. 10th AWWA Federal Convent. (Aust.)* 26-1-26-8.

Fawell, J.K., Guppy, M. and Chipman, K. (1994) *Toxins from Blue-Green Algae: Tumour Promotion by Microcystin-LR - Preliminary* in vitro *Studies*, Marlow, Buckinghamshire: Foundation for Water Research.

Fujiki, H., Sueoka, E. and Suganuma, M. (1996) Carcinogenesis of Microcystins. In: Watanabe, M.F., Harada, K., Carmichael, W.W. and Fujiki, H., (Eds.) *Toxic Microcystis*, pp. 203-232. Boca Raton: CRC Press Inc.

Glaze, W.H. (1990) Chemical Oxidation. In: Pontius, F.W., (Ed.) *Water Quality and Treatment: A Handbook of Community Water Supplies*, 4th edn. pp. 747-779. New York: McGraw-Hill, Inc.

Harada, K.-I., Matsura, K., Suzuki, M., Watanabe, M.F., Oishi, S., Dahlem, A.M., Beasley, V.R. and Carmichael, W.W. (1990a) Isolation and Characterization of the Minor Components Associated with Microcystins LR and RR in the Cyanobacterium (Blue-Green Algae). *Toxicon* **28**, 55-64.

Harada, K.-I., Murata, H., Qiang, Z., Suzuki, M. and Kondo, F. (1996) Mass Spectrometric Screening Method for Microcystins in Cyanobacteria. *Toxicon* **34**, 701-710.

Harada, K.-I., Ogawa, K., Matsuura, K., Murata, H., Suzuki, M., Watanabe, M.F., Itezono, Y. and Nakayama, N. (1990b) Structural Determination of Geometrical Isomers of Microcystins LR and RR from Cyanobacteria by Two-Dimensional NMR Spectroscopic Techniques. *Chem. Res. Toxicol.* **3**, 473-481.

Harada, K.-I., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M.F., Watanabe, M. and Terao, K. (1994) Isolation of Cylindrospermopsin from a Cyanobacterium *Umezakia natans* and its Screening Method. *Toxicon* **32**, 73-84.

Harrington, G.W., Bruchet, A., Rybacki, D and Singer, P.C. (1996) Characterization of Natural Organic Matter and its Reactivity with Chlorine. In: Minear, R.A. and Amy, G.L., (Eds.) *Water Disinfection and Natural Organic Matter: Characterization and Control*, ACS Symposium Series 649, pp. 138-158. Washington: American Chemical Society.

Hawkins, P.R., Chandrasena, N.R., Jones, G.J., Humpage, A.R. and Falconer, I.R. (1997) Isolation and Toxicity of *Cylindrospermopsis raciborskii* from an Ornamental Lake. *Toxicon* in press.

Hawkins, P.R., Runnegar, M.T.C., Jackson, A.R.B. and Falconer, I.R. (1985) Severe Hepatotoxicity Caused by The Tropical Cyanobacterium (Blue-Green Alga) *Cylindrospermopsin raciborskii* (Woloszynska) Seenaya and Subba Raju Isolated from a Domestic Water Supply Reservoir. *Appl. Environ. Microbiol.* **50**, 1292-1295.

Himberg, K., Keijola, A.-M., Hiisvirta, L., Pyysalo, H. and Sivonen, K. (1989) The Effect of water Treatment Processes on the Removal of Hepatotoxins from *Microcystis* and *Oscillatoria* Cyanobacteria: A Laboratory Study. *Water Res.* **23**, 979-984.

Hoffmann, J.R.H. (1976) Removal of *Microcystis* Toxins in Water Purification Processes. *Water (SA)* **2(2)**, 58-60.

Humpage, A.R., Rositano, J., Bretag, A.H., Brown, R., Baker, P.D., Nicholson, B.C. and Steffensen, D.A. (1994) Paralytic Shellfish Poisons from Australian Cyanobacterial Blooms. *Aust. J. Mar. Freshwater Res.* **45**, 761-771.

Izaguirre, G., Hwang, C.J., Krasner, S.W. and McGuire, M.J. (1982) Geosmin and 2-Methylisoborneol from Cyanobacteria in Three Water Supply Systems. *Appl. Environ. Microbiol.* **43**, 708-714.

James, H. and Fawell, J.K. (1991) *Detection and Removal of Cyanobacterial Toxins from Freshwaters, Report FR0211*, Marlow, Buckinghamshire: Foundation for Water Research.

Jones, G. and Orr, P.T. (1994) *In situ* Release and Degradation of Microcystin Following Algicide Treatment of a *Microcystis aeruginosa* Bloom in a Recreational Lake. *Water Res.* **28**, 871-876.

Jones, G., Burch, M., Falconer, I. And Craig, K. (1993a) Cyanobacterial Toxicity. In *Algal Management Strategy. Technical Advisory Group Report.* Canberra: Murray-Darling Basin Commission.

Jones, G., Minatol, W., Craig, K. and Naylor, R. (1993b) Removal of Low Level Cyanobacterial Peptide Toxins from Drinking Water Using Powdered and Granular Activated Carbon and Chlorination - Results of Laboratory and Pilot Plant Studies. *Proc. AWWA 15th Federal Convent. (Aust.)* **2**, 339-346.

Juttner, F. (1983) Volatile Odorous Excretion Products of Algae and their Occurrence in the Natural Aquatic Environment. *Water Sci. Technol.* **15**, 247-257.

Kao, C.Y. (1993) Paralytic Shellfish Poisoning. In: Falconer, I.R., (Ed.) *Algal Toxins in Seafood and Drinking Water*, pp. 75-86. London: Academic Press.

Keijola, A.M., Himberg, K., Esala, A.L., Sivonen, K. and Hiisvirta, L. (1988) Removal of Cyanobacterial Toxins in Water Treatment Processes: Laboratory and Pilot-Scale Experiments. *Toxicity Assessment* **3**, 643-656.

Krasner, S.W., Sclimenti, M.J., Chinn, R, Chowdhury, Z.W. and Owen, D.M. (1996) The Impact of TOC and Bromide on Chlorination By-Product Formation. In: Minear, R.A. and Amy, G.L. (Eds.) *Disinfection By-Products and Water Treatment: The Chemistry of Their Formation and Control*, pp. 59-90. Boca Raton, CRC Lewis Publishers.

Kuiper-Goodman, T., Gupta, S., Combley, H. And Thomas, B.H. (1994) Microcystins in Drinking Water: Risk Assessment and Derivation of a Possible Guidance Value for Drinking Water. In: Steffensen, D.A. and Nicholson, B.C., (Eds.) *Toxic Cyanobacteria: Current Status of Research and Management*, pp. 67-73. Adelaide: Australian Centre for Water Quality Research.

Lahti, K. and Hiisvirta, L. (1989) Removal of Cyanobacterial Toxins in Water Treatment Processes; Review of Studies Conducted in Finland. *Water Supply* **7, Barcelona**, 149-154.

Lam, A.K-Y., Fedorak, P.M., and Prepas, E.E. (1995) Biotransformation of the Cyanobacterial Hepatotoxin Microcystin-LR, as Determined by HPLC and Protein Phosphatase Inhibition Bioassay. *Environ. Sci. Technol.* **29**, 242-246.

Lambert, T.W., Boland, M.P., Holmes, C.F.B. and Hrudey, S.E. (1994) Quantitation of the Microcystin Hepatotoxins in Water at Environmentally Relevant Concentrations with the Protein Phosphatase Bioassay. *Environ. Sci. Technol.* **28**, 753-755.

Lambert, T.W., Holmes, C.F.B. and Hrudey, S.E. (1996) Adsorption of Microcystin-LR by Activated Carbon and Removal in Full Scale Water Treatment. *Water Res.* **30**, 1411-1422.

Lawton, L.A., Edwards, C., Codd, G.A. (1994) Extraction and High Performance Liquid Chromatographic Method for the Determination of Microcystins in Raw and Treated Waters. *Analyst* **119**: 1525-1530.

Lippy, E.C. and Erb, J. (1976) Gastrointestinal Illness at Sewickley, Pa. *J. Am. Water Works Assoc.* **88**, 606-610.

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P. and Codd, G.A. (1990) Cyanobacterial Microcystin-LR is a Potent Inhibitor of Protein Phosphatases 1 and 2A from both Mammals and Higher Plants. *FEBS Letts.* **264**, 187-192.

Main, D.C., Berry, P.H., Peet, R.L. and Robertson, J.P. (1977) Sheep Mortalities Associated with the Blue Green Alga *Nodularia spumigena*. *Aust. Vet. J.* **53**, 578-581.

Maron, D.M., and Ames, D.N. (1983) Revised Method for the *Salmonella* Mutagenicity Test. *Mutat. Res.* **113**:173-215

May, V. and McBarron, E.J. (1973) Occurrence of the Blue-Green Alga, *Anabaena circinalis* Rabenh., in New South Wales and Toxicity to Mice and Honey Bees. *J. Aust. Inst. Agric. Sci.* **39**, 264-266.

McBarron, E.J. and May, V. (1966) Poisoning of Sheep in New South Wales by the Blue-Green Alga *Anacystis cyanea* (Kuetz.) Dr. and Dail.. *Aust. Vet. J.* **42**, 449-453

McBarron, E.J., Walker, R.I., Gardner, I. and Walker, K.H. (1975) Toxicity to Livestock of the Blue-Green Alga *Anabaena circinalis*. *Aust. Vet. J.* **51**, 587-588.

Mulhearn, C.J. (1959) Beware Algae! They Can Poison Livestock. *J. Dept. Agric. South Aust.* **62**, 406-408.

Namikoshi, M., Rinehart, K.L., Dahlem, A.M., Beasley, V.R. and Carmichael, W.W. (1989) Total Synthesis of Adda, the Unique C_{20} Amino Acid of Cyanobacterial Hepatotoxins. *Tetrahedron Letts.* **30**, 4349-4352.

Namikoshi, M., Rinehart, K.L., Sakai, R., Stotts, R.R., Dahlem, A.M., Beasley, V.R., Carmichael, W.W. and Evans, W.R. (1992) Identification of 12 Hepatotoxins from a Homer Lake Bloom of the Cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: Nine New Microcystins. *J. Org. Chem.* **57**, 866-872.

Negri, A.P., Jones, G.L. and Hindmarsh, M. (1995) Sheep Mortality Associated with Paralytic Shellfish Poisons from the Cyanobacterium *Anabaena circinalis*. *Toxicon* **33**, 1321-1329.

NH&MRC/ARMCANZ (1996) *Australian Drinking Water Guidelines 1996*. Canberra: National Health and Medical Research Council/Agriculture and Resource Management Council of Australia and New Zealand.

Nicholson, B.C., Hayes, K.P. and Thomas, P.M. (1990) Chlorinated Byproducts of Drinking Water Disinfection. *Proc. Conf. 'Halogenated Organics and the Environment'*, Adelaide: Australian Centre for Water Treatment and Water Quality Research

Nicholson, B.C., Rositano, J. and Burch, M.D. (1994) Destruction of Cyanobacterial Peptide Hepatotoxins by Chlorine and Chloramine. *Water Res.* **28**, 1297-1303.

Nicholson, B.C., Rositano, J., Humpage, A.R. and Burch, M.D. (1993) Removal of Algal Toxins in Water Treatment Processes. *Proc.15th AWWA Federal Convent. (Aust.)* **2**, 327-331.

Nishiwaki-Matsushima, R., Nishiwaki, S., Ohta, T., Yoshizawa, S., Suganuma, M., Harada, K.-I., Watanabe, M.F. and Fujiki, H. (1991) Structure-Function Relationships of Microcystins, Liver Tumor Promoters, in Interaction with Protein Phosphatase. *Jpn. J. Cancer Res.* **82**, 993-996.

Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W. and Fujiki, H. (1992) Liver Tumor Promotion by the Cyanobacterial Peptide Toxin Microcystin-LR. *J. Cancer Res. Clin. Oncol.* **118**, 420-424.

Ohta, T., Sueoka, E., Iida, N., Komori, A., Suganuma, M., Nishiwaki, R., Tatematsu, M., Kim, S.J., Carmichael, W.W. and Fujiki, H. (1994) Nodularin, a Potent Inhibitor of Protein Phosphatases 1 and 2A, is a New Environmental Carcinogen in Male F344 Rat Liver. *Cancer Res.* **54**, 6402-6406.

Ohtani, I., Moore, R.E. and Runnegar, M.T.C. (1992) Cylindrospermopsin: A Potent Hepatotoxin from the Blue-Green Alga *Cylindrospermopsis raciborskii*. *J. Am. Chem. Soc.* **114**, 7941-7942.

Oliver, B.G. and Shindler, B.D. (1980) Trihalomethanes from the Chlorination of Aquatic Algae. *Environ. Sci. Technol.* **14**, 1502-1505.

Pieronne, P. (1993) *Report on French-Australian Industrial Research Program.* Adelaide: Australian Centre For Water Quality Research.

Reckhow, D.A., Singer, P.C. and Malcolm, R.L. (1990) Chlorination of Humic Materials: By-product Formation and Chemical Interpretation. *Environ. Sci. Technol.* **24**, 1655-1664

Rinehart, K.L., Harada, K.-I., Namikoshi, M., Chen, C., Harvis, C.A., Munro, M.H.G., Blunt, J.W., Mulligan, P.E., Beasley, V.R., Dahlem, A.M. and Carmichael, W.W. (1988) Nodularin, Microcystin, and the Configuration of Adda. *J. Am. Chem. Soc.* **110**, 8557-8558.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stainer, R.G. (1979) Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *J. Gen. Microbiol.* **111,** 1-61.

Rositano, J. (1996) *The Destruction of Cyanobacterial Peptide Toxins by Oxidants Used in Water Treatment. Urban Water Research Association of Australia, Research Report No. 110,* Melbourne, Victoria: Urban Water Research Association of Australia.

Rositano, J. and Nicholson, B.C. (1994) *Water Treatment Techniques for the Removal of Cyanobacterial Toxins from Water*, Adelaide: Australian Centre for Water Quality Research, Report 2/94.

Rositano, J., Nicholson, B.C. and Pieronne, P. (1996) Destruction of Cyanobacterial Toxins by Ozone. *International Ozone Association Conference, Sydney*; *Ozone Sci. Eng.*, in press.

Runnegar, M.T., Kong, S.M. and Berndt, N. (1993) Protein Phosphatase Inhibition and *In vivo* Hepatotoxicity of Microcystins. *Am. J. Physiol.* **265**, G224-G230.

Sano, T., Nohara, K., Shiraishi, F. and Kaya, K. (1992) A Method for Micro-Determination of Total Microcystin Content in Waterblooms of Cyanobacteria (Blue-Green Algae). *Int. J. Environ. Anal. Chem.* **49**, 163-170.

Schwimmer, M. and Schwimmer, D. (1968) Medical Aspects of Phycology. In: Jackson, D.F., (Ed.) *Algae, Man, and the Environment*, pp. 279-358. New York: Syracuse University Press.

Scott, W.E. (1991) Occurrence and Significance of Toxic Cyanobacteria in Southern Africa. *Water Sci. Technol.* **23, Kyoto**, 175-180.

Shenolikar, S. (1994) Protein Serine/Threonine Phosphatases - New Avenues for Cell Regulation. *Annu. Rev. Cell Biol.* **10**, 55-86.

Sim, AT., and Mudge, L-M. (1993) Protein Phosphatase Activity in Cyanobacteria-Consequences for Microcystin Toxin Analysis. *Toxicon*, **31**:1179-1186

Skulberg, O.M., Carmichael, W.W., Codd, G.A. and Skulberg, R. (1993) Taxonomy of Toxic Cyanophycaea (Cyanobacteria). In: Falconer, I.R., (Ed.) *Algal Toxins in Seafood and Drinking Water*, pp. 145-164. London: Academic Press.

Slater, G.P. and Blok, V.C. (1983) Isolation and Identification of Odorous Compounds from a Lake Subject to Cyanobacterial Blooms. *Water Sci. Technol.* **15**, 229-240.

Soong, F.S., Maynard, E., Kirke, K. and Luke, C. (1992) Illness Associated with Blue-Green Algae. *Med. J. Aust.* **156**, 67

Standard Methods (1976) *Standard Methods for The Examination of Water and Wastewater*, 14th Edition, p. 329. Washington: American Public Health Association.

Tanaka, Y., Takenaka, S., Matsuo, H., Kitamori, S. and Tokiwa, H. (1993) Levels of Microcystins in Japanese Lakes. *Toxicol. Environ. Chem.* **39**, 21-27.

Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M.F., Harada, K.I., Ito, E. and Watanabe, M. (1994) Electron Microscopic Studies on Experimental Poisoning in Mice Induced by Cylindrospermopsin Isolated from Blue- Green Alga *Umezakia natans*. *Toxicon* **32**, 833-843.

Thompson, BC. (1978) *Trihalomethane Formation Potential of Algal Extracellular Products and Biomass.* Masters Thesis. Blacksburg, Virginia: Virginia Polytechnic Institute and State University.

Tisdale, E.S. (1931a) Epidemic of Intestinal Disorders in Charleston, W. Va., Occurring Simultaneously with Unprecedented Water Supply Conditions. *Am. J. Public Health* **21**, 198-200.

Tisdale, E.S. (1931b) The 1930-1931 Drought and its Effect Upon Public Water Supply. *Am. J. Public Health* **21**, 1203-1218.

Toivola, D.M., Eriksson, J.E. and Brautigan, D.L. (1994) Identification of Protein Phosphatase 2A as the Primary Target for Microcystin-LR in Rat Liver Homogenates. *FEBS Letts.* **344**, 175-180.

Tsuchiya, Y. and Watanabe, M.F. (1994) Identification of Chlorination By-Products from the Culture of *Microcystis aeruginosa. Proc. Fourth International Symposium on Off-Flavours in the Aquatic Environment*, pp. 88-91. Adelaide: International Association on Water Quality.

Tsuchiya, Y., Watanabe, M.F. and Watanabe, M. (1992) Volatile Organic Sulfur Compounds Associated with Blue-Green Algae from Inland Waters of Japan. *Water. Sci. Technol.* **25(2)**, 123-130.

Turner, P.C., Gammie, A.J., Hollinrake, K. and Codd, G.A. (1990) Pneumonia Associated with Cyanobacteria. *Brit. Med. J.* **300**, 1440-1441.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F., Park, H.-D., Chen, G.-C., Chen, G. and Yu, S.-Z. (1996) Detection of Microcystins, a Blue-Green Algal Hepatotoxin, in Drinking Water Sampled in Haimen and Fusui, Endemic Areas of Primary Liver Cancer in China, by Highly Sensitive Immunoassay. *Carcinogenesis* **17**, 1317-1321.

US EPA (1990) *Methods for the Determination of Organic Compounds in Drinking Water, Supplement 1.* Cincinnati: Environmental Systems Laboratory Office of Research and Development, US Environmental Protection Agency.

US EPA (1992) *Methods for the Determination of Organic Compounds in Drinking Water, Supplement 2.* Cincinnati: Environmental Systems Laboratory Office of Research and Development, US Environmental Protection Agency.

Van der Westhuizen, A.J. and Eloff, J.N. (1983) Effects of Culture Age and pH of Culture Medium on the Growth and Toxicity of the Blue-Green Alga *Microcystis aeruginosa.* *Z. Pflanzenphysiol. Bd.* **110,** 157-163.

Van Steenderen, RA. Scott, W.E. and Welch, D.I. (1988) *Microcystis aeruginosa* as an Organohalogen Precursor. *Water (SA)* **14,** 59-62.

Veldee, M.V. (1931) An Epidemiological Study of Suspected Water-Borne Gastroenteritis. *Am. J. Public Health* **21,** 1227-1235.

Wachter, J.K. (1982) *Characterisation of Organohalide Formation Upon Chlorination of Algal Extracellular Matter.* Sc. Dissertation. Pittsburgh: University of Pittsburgh.

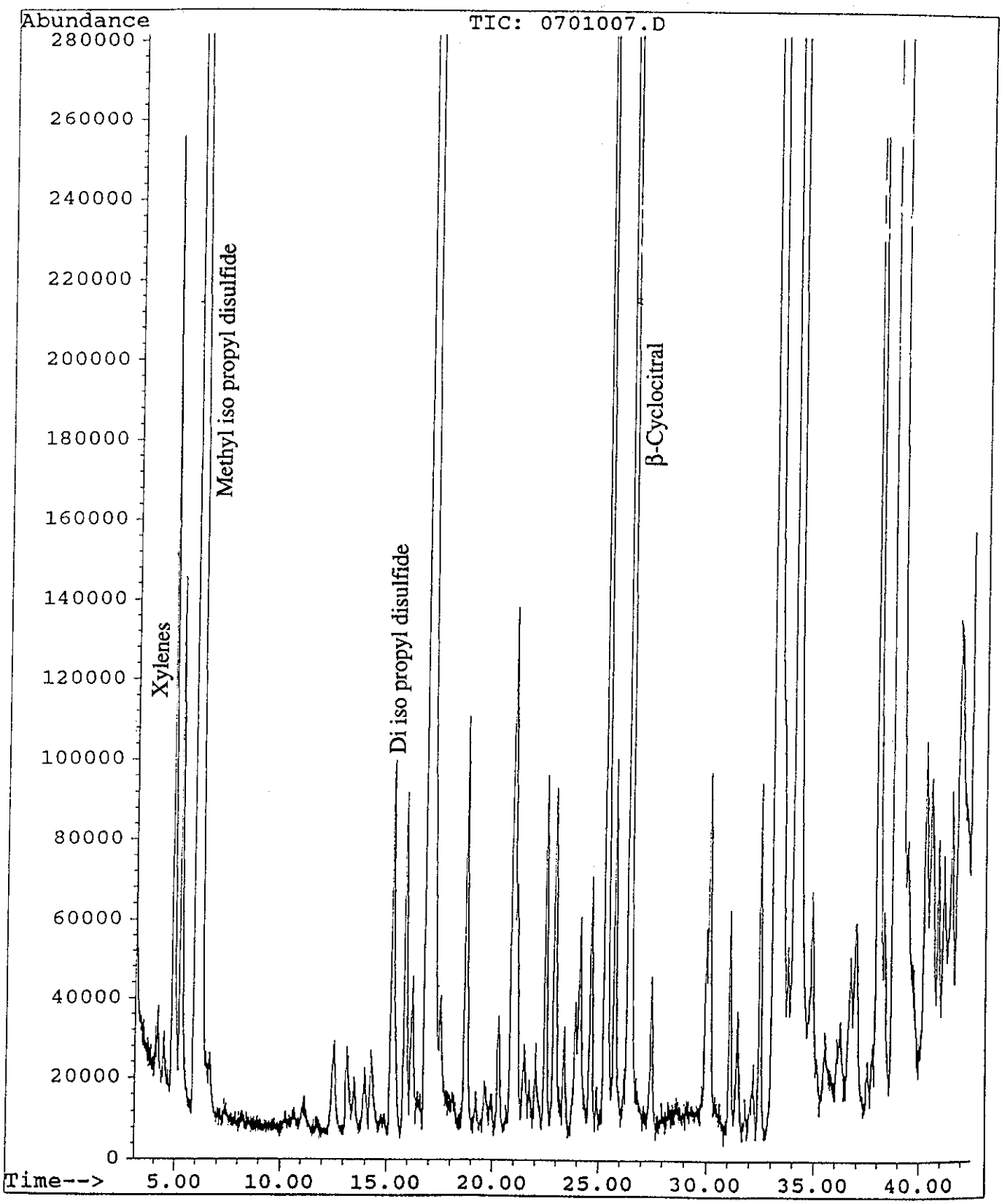
Watanabe, M.F. and Oishi, S. (1985) Effects of Environmental Factors on Toxicity of a Cyanobacterium (*Microcystis aeruginosa*) Under Culture Conditions. *Appl. Environ. Microbiol.* **49,** 1342-1344.

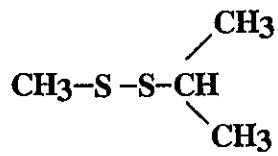
Yu, S.-Z. (1994) Blue-Green Algae and Liver Cancer. In: Steffensen, D.A. and Nicholson, B.C., (Eds.) *Toxic Cyanobacteria: Current Status of Research and Management,* pp. 75-85. Adelaide: Australian Centre for Water Quality Research.

Zilberg, B. (1966) Gastroenteritis in Salisbury European Children - A Five-Year Case Study. *Centr. Afr. J. Med.* **12,** 164-168.

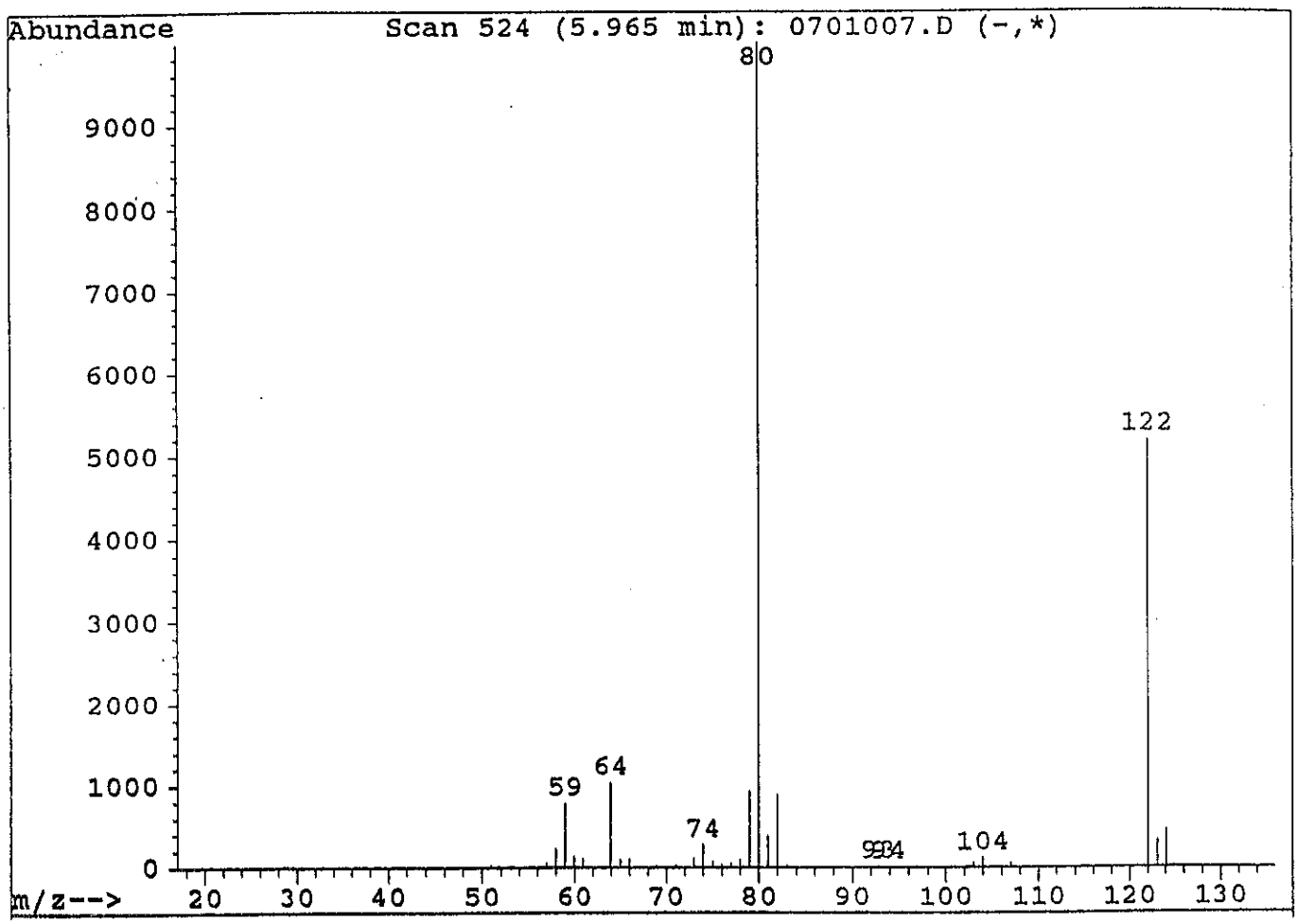
8 APPENDIX

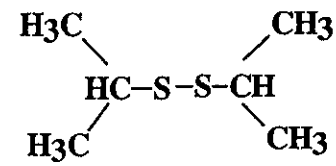
Closed loop striping analysis followed by GC/MS analysis resulted in the following GC scan with identification of the labelled compounds by mass spectral library match.



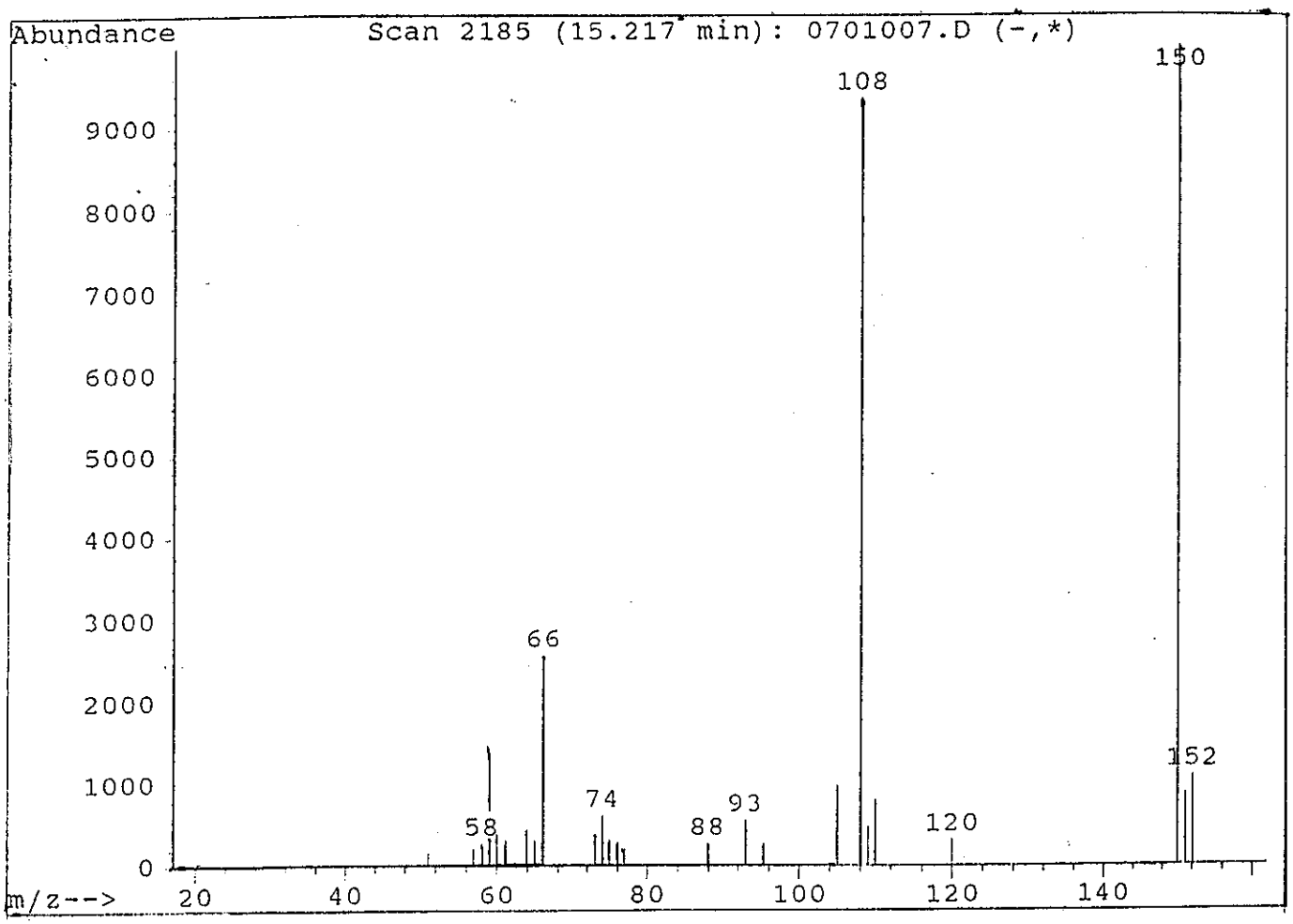


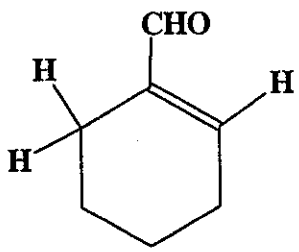
Methyl isopropyl disulphide



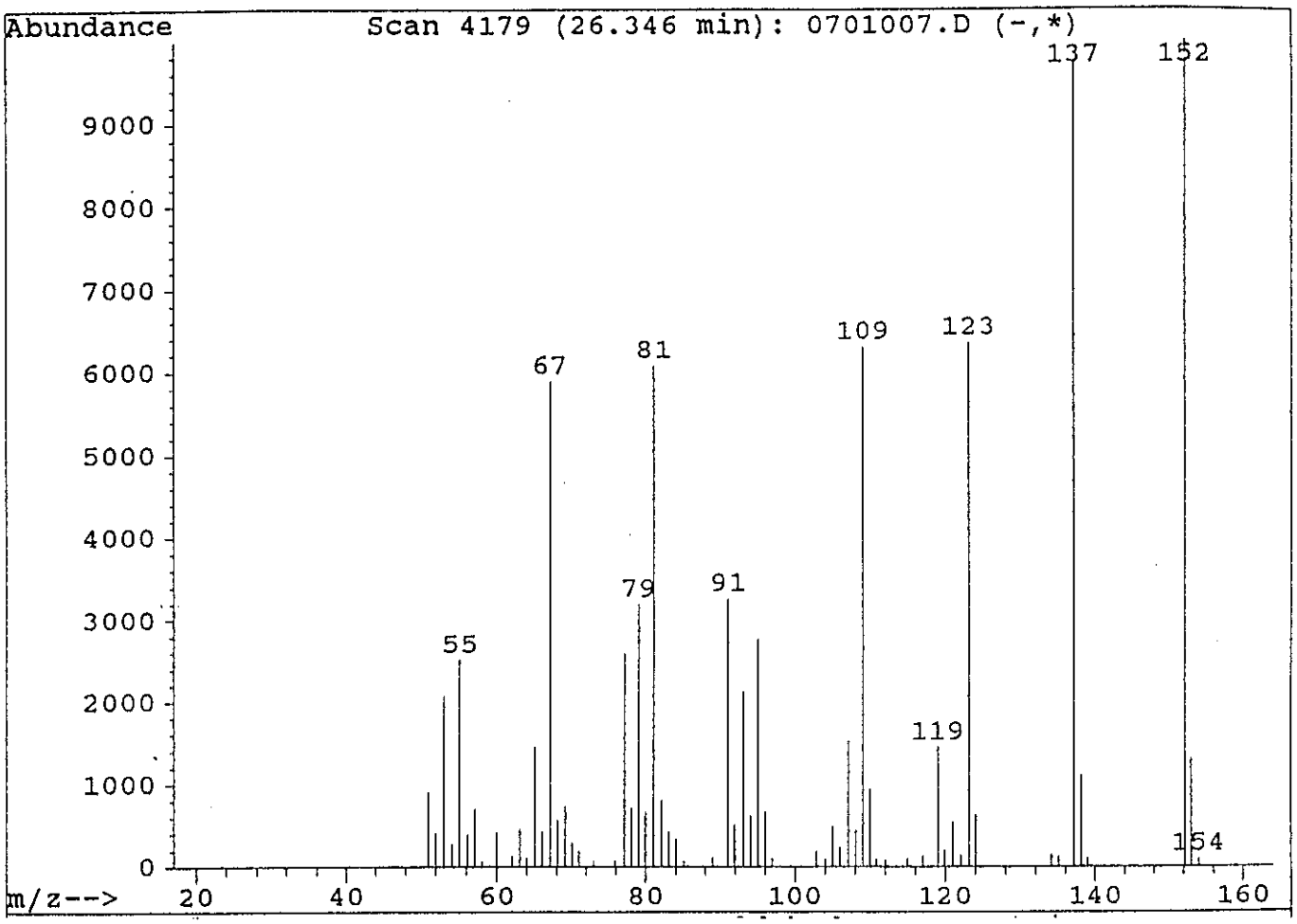


Di isopropyl disulfide





β-Cyclocitral



UWRAA RESEARCH REPORTS

Report Number	Title	Author
1	Trickling filter – solids contact process: Pilot plant studies.	M. Laginestra
2	A model of water pricing for Melbourne, Sydney and Perth	P. B. Dixon P. M. Norman
3	Taste generation associated with chloramination	M. Kerslake
4	Bacterial regrowth in water supplies	K. Power L. A. Nagy
5	Leakage management: Assessing the effect of pressure reduction on losses from water distribution systems	B. Horvath
6	Improving communication with the public on water industry policy issues	B. E. Nancarrow G. J. Syme
7	Water use efficiency of domestic appliances	I. J. Beith D. J. Horton
8	Sewage fermentation units to increase degradable COD fraction	P. J. Bliss D. Barnes P. R. Evans I. Law
9	Review of artificial destratification of water storages in Australia	T. F. McAuliffe R. S. Rosich
10	Taste thresholds of mono-chloramine and chlorine in water	R. O'Halloran C. Veres
11	Chromatographic analysis of chloramines using electrochemical detection	R. O'Halloran Hai Lin Ge P. Spizziri
12	Glass reinforced plastic bore casing for large diameter and deep bores	R. Bowyer
13	A guide to improving communication with the public on water industry policy issues	B. E. Nancarrow G. J. Syme
14	Fouling and cleaning of fine bubble ceramic dome diffusers	K. J. Hartley
15	Chloramination of Water Supplies	P. M. Thomas (ed)
16	The 1988 Australian Winter Storms Experiment: Report on aircraft observations	J. B. Jensen
17	Pipeline assets: Life cycle management and economic life	R. Vass M. Anderson R. Lewis D. Samson
18	Development of empirical model for tradewaste discharges to small treatment plants	Camp Scott Furphy
19	PRELIM users guide (Amended): Australian Version	Camp Scott Furphy
20	Chemical regeneration of activated carbon: Preliminary studies	G. Newcombe
21	Management and display of dam surveillance data	D. M. Stirling G. L. Benwell A. B. Mumane
22	Evaluation and demonstration facilities for primary sensors	J. A. Lanaway M. Cavey
23	Modelling and design of reservoir aeration destratification systems	D. P. Lewis J. C. Patterson J. Imberger R. P. Wright S. G. Schadlow
24	Modelling optimum conditions for reservoir destratification using mechanical mixers	R. M. A. Velzeboer J. A. Cugley J. C. Patterson
25	Methods for detection of *Giardia* and *Cryptosporidium* in water: A preliminary assessment	C. A. Bee P. E. Christy B. E. Robinson
26	Toxic cyanobacteria in water supplies: Analytical techniques	D. J. Flett B. C. Nicholson
27	Tracing toxic discharges to sewers by analysis of biofilms	D. Oliver T. Watson
28	Electronic meter reading: Link between water meter and house	P. J. Reid J. S. Renwick M. F. Prior
29	Identification of common noxious cyanobacteria: Part 1 – Nostocales	P. Baker
30	Forecasting water demand using weather data	M. N. Viswanathan
31	Effects of controls on water consumption	M. N. Viswanathan
32	Biological removal of iron from groundwater: Preliminary studies	M. N. Viswanathan
33	Statistical modelling of water main failures	E. Tsui G. Judd
34	Stratification, mixing and water quality in Darwin water supply reservoirs	R. Lukatelich D. Robertson K. Boland J. Imberger J. Patterson
35	Performance auditing in the Australian urban water industry	S. O'Kane I. Parry D. Blunden D. Herring
36	Microbiological studies on enhanced removal of phosphates from sewage	R. C. Bayly J. W. May G. Vasiliadis G. N. Rees
37	Magnetite and microwaves in sewage effluent treatment	D. R. Dixon A. J. Ware
38	Polymer based electrode for the selective detection of dichloramine	Y. Lin G. G. Wallace
39	Current cost asset valuation: Methodology	J. Dyke

UWRAA RESEARCH REPORTS

Report Number	Title	Author
40	Community analysis of household water pressure satisfaction	G. J. Syme B. E. Nancarrow B. J. Bishop P. VanderWal
41	Assessment of coagulants for water treatment	C. Donati
42	Coagulants for water treatment: A generic guide	ACWQR
43	Optimal prices for urban water: A general equilibrium model applied to Melbourne	P.B. Dixon D.J. Baker
44	Applications of the Streaming Current Detector in water treatment	W. Barron D. R. Dixon M. Pascoe
45	Sydney coastal stormwater study	W. G. Rowlands et.al.
46	Identification of common noxious cyanobacteria: Part 2 – Chroococcales and Oscillatoriales	P. Baker
47	Levelling using the Global Positioning System	A. P. Armstrong P. A. Collier F. J. Leahy
48	Allocation of sewerage costs to customer segments	R. Hood P. Geary
49	Impact of urban lawns on nutrient contamination of an unconfined aquifer	M. L. Sharma D. E. Herne P. G. Kin J. D. M. Byme
50	Early warning system for hazardous substances in sewage	R. O'Halloran B. A. Sexton N. H. Pilkington
51	Management model for trade waste discharges to small treatment plants (including PRELIM VERSION 4.0 Users Guide)	Camp Scott Furphy
52	Automatic meter reading: Link between meters and billing centre (Combined utilities trial)	B. Phey A. Leong Z. Balazic
53	Prediction of perceived odour strength and type from composition of sewage odour mixtures	D. G. Laing A. Eddy D. J. Best
54	Tracer studies using bacteriophage to predict the fate of viruses in the marine community: Preliminary assessments	B. J. Richardson A. L. Charlton S. Currie P. Ashton I. Lowther
55	Development of a water quality analyser suitable for unattended use in rivers and streams	G. W. Skyring I. A. Johns J. A. Cugley
56	Enhancement of nitrification in wastewater lagoons	P. M. Gross
57	Identification of critical water supply assets	PPK Consultants
58	Water in Our Environment: Education Project	H. Breidahl D. Cliffe H. Henderson
59	Electronic household water meter: Investigation into a cost effective design	Z. Balazic A. Leong
60	Domestic greywater reuse: Preliminary evaluation	B. Jeppesen
61	Chemical regeneration of activated carbon: A feasibility study	G. Newcombe
62	Tracing toxic discharges to sewers by analysis of biofilms (Stage 2)	W. H. Lock
63	Production of Jerusalem artichoke hybrids under irrigation using urban wastewater	M. Parameswaran
64	Control of pitting corrosion of copper tubes in potable waters	R. J. Taylor P. H. Cannington
65	Measurement of Total Factor Productivity in major water utilities: Melbourne case study	I. Manning E. Molyneux
66	Assimilable organic carbon as a measure of bacterial growth potential in water supplies	K. C. Tapang M. Drikas L. E. Bennett
67	Simultaneous peak water demands in residential areas	J. Henstridge G. J. Syme B. E. Nancarrow
68	Installation damage: Effect on lifetimes of uPVC pipes subjected to cyclic pressure	L. S. Burn
69	Safety aspects of the design of roadways as floodways	R. J. Keller B. Mitsch
70	Regional development implications of wastewater reuse: Werribee case study	D. Hunter W. Smith L. Nagy P. Jacob
71	Treatment of electroplating wastes using new-generation membrane technology	A. G. Fane Y. R. Shen
72	Stochastic economic approach to headworks augmentation timing	G. Kuczera W. S. Ng
73	Domestic greywater reuse: Overseas practice and its applicability to Australia	B. Jeppesen D. Solley
74	Decision support systems for the water industry: An Object-Oriented approach	J. M. Edwards
75	Chemical characterisation and olfactometric measurement of odours from sewage treatment process units	R. Kaye N. Mulhem D. Stone
76	Utilisation of sewage sludge for minesite rehabilitation: Rix's Creek Mine Trial	C. P. Phillips
77	A benchmarking methodology for the Australian water industry	I.R.C. Eggleton
78	Water quality effects of aeration/ destratification at Harding Reservoir, W.A.	R.S. Rosich T.A. McAuliffe

UWRAA RESEARCH REPORTS

Report Number	Title	Author
79	Heavy metals and organics in domestic wastewater	W. H. Lock
80	Effluent reuse: Land and wet weather storage requirement	J. M. Anderson T.J. Ruge
81	Graphical interactive pipe network analysis program	B.L. Berghout
82	Survey of pipeline rehabilitation techniques	Gutteridge Haskins & Davey
83	Bioavailability of aluminium from drinking water: Co-exposure with foods and beverages	J. Walton G. Hams D. Wilcox
84	Dezincification of brass in potable waters	D. Nicholas
85	Behaviour of aluminium during water treatment	P. Zhang M. McCormick J. Hughes M. Brymner
87	Benchmarking and best practice for urban waterway management	C. Aitken
88	Quantification of factors controlling the development of *Anabaena circinalis* blooms	J.A. Winder D.M.H. Cheng
89	Urban water, markets and the Hilmer reform process	R. Maddock N. Gonzalez
90	Remote sensing electronic device for hydrogen sulphide in the atmosphere	D.G. Laing D. Barnett G.G. Wallace
91	Simultaneous peak flows for medium density residential areas	J. Henstridge G.J. Syme B.E. Nancarrow S. Martens S. Gilbert
92	Die-off of human pathogens in stored wastewater sludge and sludge applied to land	R. Gibbs C. Hu G. Ho I. Unkovich P. Phillips
93	Benchmarking the economic performance of Australian urban water authorities	London Economics
94	Biological Nutrient Removal Plants: Review of Full-Scale Operation	K. Hartley
95	Stormwater Management in Australia: Community Perceptions, Attitudes and Knowledge	B E Nancarrow B S Jorgensen G J Syme
96	Development of a high resolution water quality model	Ch Zoppou S Roberts
97	Landfarming hydrocarbon wastes	W E Razzell P Griffin F Boevink
98	Water treatment plants for small communities	A B Roberts J A DeLaine
99	The role of biofilm and sediment accumulation and of chlorine tolerance in bacterial regrowth	K N Power R P Schneider K C Marshall
100	Phosphorus in Detergents: Its Contribution to Eutrophication in Australian Inland Waters	P Cullen A Heretakis A Herington
101	Disinfection of Wastewater Effluent: A Review of Current Techniques	C Hamilton
102	The effect of irrigation on Blue Gum (*Eucalyptus globulus*) water uptake	R H Froend G W Chester J K Marshall
103	Principles for Setting Developer's Contributions for Urban Water Infrastructure	G A Draper J F Thomas P B Mcleod
104	Epidemiological Evidence of Algal Toxins in Drinking Water and Recreational Waters	O El Saadi D A Steffensen
105	Detection of Cyanobacterial Peptide Toxins by a Non-Radioactive Protein Phosphatase Inhibition Assay	J F Wheldrake A Bilney L Rosenberg
106	Water Treatment Sludge: Potential for Use as a Soil Ameliorant	M Ahmed C Grant J Oades
107	Model Guidelines for Domestic Greywater Reuse for Australia	B Jeppesen
108	Advance Warning of Cyanobacterial Toxicity	A T R Sim J A P Rostas
109	Guidelines on the Quality of Stormwater and Treated Wastewater for Injection into Aquifers for Storage and Reuse	P Dillon P Pavelic
110	The Destruction of Cyanobacterial Peptide Toxins by Oxidants Used in Water Treatment	J Rositano
111	Application of Duckweed in Treating Municipal Wastewater	R A Leng
112	Alternative Overseas Water Treatment and Supply Practices	P Nadebaum N Johnston T Priestley R Vass
113	Ammonia Removal from Sirofloc® STP treated Sewage using Australian Natural Zeolite	N A Booker E L Cooney

UWRAA RESEARCH REPORTS

Report Number	Title	Author
	Environmental Management Guidelines for the Australian Water Industry	S Mills
114	Predicting the Failure Performance of Individual Water Mains	K Mavin
115	Drinking Water Disinfection By-Products Relevant to the 1996 NHMRC/ARMCANZ Guidelines	K L Simpson K P Hayes
116	Sustainable Urban Water Systems: Issues and Opportunities	M Mouritz P Newman
117	Water Sensitive Urban Design: A Tool for Urban Integrated Catchment Management - a Case Study of Bayswater	M Mouritz P Newman
118	An Evaluation of an Integrated Urban Water Management System: Palmyra Case Study	M Mouritz P Newman
119	Critical Evaluation of Domestic Irrigation Equipment	The Australian Irrigation Technology Centre
120	New Concepts in Sludge Dewatering	P Scales S Johnson D Labbett D Dixon
121	Impact on Water Quality of Gross Pollutants	M Abood S J Riley
123	Determination of the Hepatotoxin Cylindrospermopsin Produced by the Cyanobacterium *Cylindrospermopsis Raciborskii*	P M Bond B C Nicholson
124	The Microbiological Oxidation and Removal of Manganese from Drinking Water by a Continuous Recycle Fluidized Bioreactor	L I Sly L Bryant E Larsen D R Dixon

Report Number	Title	Author
	Other Reports	
200	Review of Drinking Water Treatment Coagulants	Gutteridge Haskins & Davey
201	Biological Aspects of Aluminium in Food and Water Supply	F Cumming
	Occasional Papers	
No 1	Water Pricing and the Marginal Cost of Water	R Warner
No 2	Systematic Environmental Management in the Water Industry: Toward Best Practice	S Mills
	A Guide to Wetland Invertebrates of Southwestern Australia	J Davis