



# Blue-Green Algae

Their significance  
and management  
within water  
supplies



Occasional Paper

4

# **Blue-green algae: their significance and management within water supplies**

Research of the  
Cooperative Research Centre for  
Water Quality and Treatment

In February 2002, the CRC for Water Quality and Treatment hosted a workshop on blue-green algae in drinking water systems. Over 65 members of the Australian water supply community attended this workshop, which featured speakers from across the CRC's cyanobacterial research program. The articles in this Occasional Paper summarise the research and issues discussed during the workshop.

All articles marked \* were originally presented at a workshop hosted by the American Water Works Association Research Foundation (AwwaRF) in 2001, and are copyright AwwaRF. The article authored by Ian Falconer was first printed in the journal *Phycologia* (Vol 40 no 3, pp228-233), and copyright is held by the International Phycological Society.

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Blue-green algae: their significance and management within water supplies.  
CRC for Water Quality and Treatment Occasional Paper 4

ISBN 187661613X  
Published by the CRC for Water Quality and Treatment 2002

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# BLUE-GREEN ALGAE: THEIR SIGNIFICANCE AND MANAGEMENT WITHIN WATER SUPPLIES

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## Introduction

Blue-green algae are a natural and widespread component of most aquatic systems, including streams, lakes, estuaries and the sea. They are cyanobacteria but as they have photosynthetic pigments and they look and behave like algae, they are commonly called blue-green algae.

Cyanobacteria are widely recognised as sources of taste and odours in water supplies. Many species impart disagreeable earthy or musty taste and odours that render the water unpalatable. With the identification of a number of toxic cyanobacterial metabolites there is increasing concern for health effects to consumers. It is the health risks that are the focus of this workshop.

The forms of cyanobacteria that are of most concern are those that form dense blooms. These are favoured by hot calm conditions and high nutrient loads. The combination of climate, the construction of large dams and the regulation of many of the rivers have resulted in Australia experiencing more difficulties with cyanobacteria than most other regions in the world.

The importance of cyanobacteria to the Australian water industry is reflected in the prominence given to this issue within the research programs of the CRC for Water Quality & Treatment. The CRC began in 1995 and a bid for a second term was successful in 2001. The research priorities are based on the major concerns of the water industry namely:

- Determination of the health risks.
- Development of monitoring methods for both the organisms and their toxins,
- Understanding of the factors which promote cyanobacterial growth leading to more effective control in source waters,
- Development of effective water treatment processes to destroy or remove toxins.

In February 2002, the CRC for Water Quality and Treatment hosted a workshop on blue-green algae in drinking water systems. Over 65 members of the Australian water supply community attended this workshop, which featured speakers from across the CRC's cyanobacterial research program. The articles in this Occasional Paper summarise the research and issues discussed during the workshop.

The research topics and the key participants are summarised in Table 1 (below). This represents the most comprehensive research program targeted at the needs of the water industry anywhere in the world. As a reflection of that international recognition the CRC has recently entered into joint funding agreements with the American Water Works Association Research Foundation (AwwaRF) for research into analytical methods for cyanotoxins and for the development of genetic methods for the rapid detection of toxic species.

**Table 1.** Summary of research by the CRC for Water Quality & Treatment

| Topic  | Key Researchers  | Students  |
|--|--|---|
| <b>Health risks</b>  |  |   |
| <ul style="list-style-type: none"> <li>• <b>Toxicology</b><br/>Animals subjected to microcystin and cylindrospermopsin</li> <li>• <b>Epidemiology</b><br/>Effects from exposure by drinking water &amp; recreation</li> <li>• <b>Guidelines</b><br/>Formulation of safe levels for drinking water and recreation.</li> </ul> | <ul style="list-style-type: none"> <li>• Ian Falconer, Adelaide University</li> <li>• Andrew Humpage, Australian Water Quality Centre (AWQC)</li> <li>• Glen Shaw, National Research Centre for Environmental Toxicology (NRCET)</li> <li>• Louis Pilotto, National Centre for Environmental and Public Health (NCEPH)</li> <li>• Ian Falconer, Adelaide University</li> <li>• Mike Burch, AWQC</li> </ul> | <ul style="list-style-type: none"> <li>• Suzanne Froschio, Adelaide University</li> </ul>   |
| <b>Monitoring</b>  |  |   |
| <ul style="list-style-type: none"> <li>• <b>Conventional taxonomy</b></li> <li>• <b>Genetic/molecular</b> techniques for identifying toxic species.</li> </ul>   | <ul style="list-style-type: none"> <li>• Peter Baker, AWQC</li> <li>• Chris Saint, AWQC</li> <li>• Brett Neilan, University of NSW (UNSW)</li> </ul>   | <ul style="list-style-type: none"> <li>• Kim Fergusson, University of South Australia (UniSA)</li> <li>• Melanie Kaebernick, UNSW</li> <li>• Carolina Beltran, UNSW</li> <li>• Daniel Tillett, UNSW</li> <li>• Michell Moffitt, UNSW</li> </ul>                             |
| <b>Toxin Analysis</b>  |  |   |
| <ul style="list-style-type: none"> <li>• Analytical methods developed for all the common toxins. Techniques include: HPLC, Mass Spectrometry, Capillary Electrophoresis, ELISA, and enzyme inhibition.</li> </ul>  | <ul style="list-style-type: none"> <li>• Brenton Nicholson, AWQC</li> <li>• John Papageoriou, AWQC</li> <li>• Carolyn Haskard, AWQC</li> <li>• Peter Hawkins, Sydney Water</li> <li>• Nimal Chandrasena, Sydney Water</li> <li>• Jing Cao, Sydney Water</li> </ul>   |   |
| <b>Ecology/Source Water Management</b>   |  |   |
| <ul style="list-style-type: none"> <li>• Flow manipulation in rivers</li> <li>• Mixing/destratification of reservoirs</li> <li>• Algicides</li> </ul>  | <ul style="list-style-type: none"> <li>• Mike Burch, AWQC</li> <li>• Justin Brookes, AWQC</li> <li>• Peter Hobson, AWQC</li> <li>• Peter Hawkins, Sydney Water</li> <li>• Dennis Steffensen, AWQC</li> </ul>   | <ul style="list-style-type: none"> <li>• Karen Westwood, Adelaide University</li> <li>• Rudi Regel, Adelaide University</li> <li>• David Lewis, Adelaide University</li> <li>• Hugh Wilson, Adelaide University</li> <li>• David Moore, University of Queensland</li> </ul> |
| <b>Water Treatment</b>   |  |   |
| <ul style="list-style-type: none"> <li>• Removal of cells by convention treatment &amp; membranes.</li> <li>• Adsorption by activated carbon.</li> <li>• Oxidation of toxins by chlorine and ozone</li> </ul>  | <ul style="list-style-type: none"> <li>• Mary Drikas, AWQC</li> <li>• Gayle Newcombe, AWQC</li> <li>• Chris Chow, AWQC</li> <li>• Brenton Nicholson, AWQC</li> </ul>   | <ul style="list-style-type: none"> <li>• Sam Brooke, UniSA</li> <li>• David Cook, UniSA</li> <li>• Lionel Ho, UniSA</li> </ul>  |

# TOXIC CYANOBACTERIAL BLOOM PROBLEMS IN AUSTRALIAN WATERS: RISKS AND IMPACTS ON HUMAN HEALTH

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I.R. Falconer 2001 Toxic cyanobacterial bloom problems in Australia waters: risks and impacts on human health. *Phycologia* 40: 228233

## ABSTRACT

Toxic cyanobacterial blooms have occurred throughout recorded history in Australia, as causes of livestock deaths and water unpalatability. Human injury from cyanobacterial toxins is rarely recorded, but members of two local populations in Australia have suffered from an acute toxicity believed to be a consequence of copper sulphate treatment of water blooms. Minor health effects have been correlated with cyanobacterial contamination of drinking water drawn from rivers. The World Health Organization has drawn up guideline values for microcystin in drinking water and recommendations for recreational waters. The major unknown is the potential for cancer stimulation by cyanobacterial toxins, particularly gastrointestinal cancers in Australia and other affluent countries and liver cancer in poorer nations.

## INTRODUCTION

The people of Australia are very familiar with toxic cyanobacterial blooms, because they have been a long-standing problem for agricultural and human drinking water supplies, as well as for the recreational use of water. Livestock poisoning by cyanobacteria was first described in the 19th century near Adelaide (Francis 1878), and the names of water-courses across the country, such as 'Poison Waterhole Creek,' may reflect the hazard from cyanobacterial toxicity. More recently, 1000 km of the Darling River carried a massive bloom of neurotoxic *Anabaena circinalis* Rabenhorst ex Borner & Flauhault, which killed an estimated 10,000 livestock and required emergency water supplies for several towns (New South Wales Blue-green Algae Task Force 1992). In three successive summers (1998-2000) in the centre of City of Adelaide, the Torrens Lake (no longer used for water supply) had a heavy bloom of toxic *Microcystis aeruginosa* (Kützinger) Lemmermann. This bloom was believed to be the cause of waterfowl deaths. Although livestock poisoning has been relatively common throughout farming history in Australia, cases of human and wildlife poisoning are rare, more through avoidance of drinking foul-smelling water than through an absence of toxicity in cyanobacterial blooms.

Effective assessment of the risk of cyanobacterial toxins to human health requires data that relate the dose of toxin to the clinical effects in a population. In general, when an adverse health effect has been suspected to have been caused by a cyanobacterial bloom in a water supply, no measurements of toxins have been undertaken. Even in the recent case of the deaths of more than 50 dialysis patients in Brazil from cyanobacterial toxicity, the best

assessment of toxic dose that could be achieved was the retrospective analysis of post-mortem samples of human liver and blood and of the filters from the treatment plant itself (Jochimsen *et al.* 1998; Pouria *et al.* 1998). As a result of the lack of adequate human exposure and toxicity data, animal toxicity data are often used for risk assessment. Approaches from human epidemiological data, relating illness to cyanobacterial contamination of drinking water, would provide effective measures of risk, if the problem of measuring toxin exposure would be overcome. In Australia, the major problem with toxic cyanobacteria in drinking water is with blooms of *M. aeruginosa* and the microcystin toxins they produce in supply reservoirs located in temperate areas. Although cyanobacterial cell numbers are regularly monitored in reservoirs, toxin concentrations may only rarely be measured in water supplies, even on occasions of confirmed toxic blooms in the source water. The other major potential hazards in water supplies in Australia are cylindrospermopsin, from the tropical *Cylindrospermopsis raciborskii* (Wotoszy\_ska) Seenaya & Subba Raju, saxitoxins from *A. circinalis*. All three toxic cyanobacteria occur as intermittent blooms in water supply reservoirs, and they are often controlled by the supply authority with applications of copper sulphate. This lyses the bloom cyanobacteria, liberating toxins into the water, making their removal in conventional water filtration plants difficult (Falconer *et al.* 1989).

## Health risks associated with cyanobacterial exposure through drinking water

Human health is of concern to each of us, having a direct impact on our lives through our well-being and that of



others. Indirectly, it has a major economic effect through the major and increasing costs of medical services. To obtain a perspective on the adverse effects likely to result from exposure to toxic cyanobacteria, assessments of morbidity (ill health) and mortality (death) from this cause need to be compared with those from other causes.

**Table 1.** Percentage mortality from different health conditions for Canada in 1994 from Thomas & Hrudey (1997).

| Condition               | Percentage mortality |
|-------------------------|----------------------|
| Infections diseases     | 2.1                  |
| Cancer                  | 28.7                 |
| Cardiovascular diseases | 36.3                 |
| Respiratory diseases    | 9.2                  |
| External causes         | 8.2                  |
| Congenital anomalies    | 0.6                  |

The rates of incidence of disease and mortality from disease are not tightly linked, since some diseases, such as chronic allergic conditions, have a high frequency and low mortality, whereas coronary occlusion has a high frequency and a high mortality. Study of death rates from different causes provides an indicator of the relative health importance of major sources of mortality. Table 1. gives some representative data for a first-world country, Canada. Each of these categories can be further subdivided to show the particular cause of death. Cancer deaths from the same Canadian statistics show that lung cancer (highest), colon cancer, breast cancer and prostate cancer, in that order, are the highest causes of mortality. Liver cancer (primary hepatocellular carcinoma), by contrast, is a minor cause of mortality in Canada (Thomas & Hrudey 1997).

An additional informative mode of expression of mortality data is to calculate the loss of life expectancy from various causes (Table 2, from Cohen 1991). This table dramatically indicates the relative impacts of lifestyle on life expectancy, as against exposure to recognised environmental hazards. Calculated in the same manner, the total loss of life expectancy for all infections diseases in males is 116 days; because this is an average figure, individual lives would in some cases be far longer if they had not contracted an infectious disease, whereas the lifespan of others would not be affected at all (Thomas & Hrudey 1997).

Exposure to toxins from cyanobacteria can be expected to influence both morbidity (ill health) and mortality. For example, the long-term oral consumption of *Microcystis Kützinger* toxins by mice has been demonstrated to cause chronic active liver injury and, in the same experiment, it also caused increased mortality through respiratory disease (Falconer *et al.* 1988). Cyanobacterial toxins (microcystin and nodularin) have also been experimentally implicated in carcinogenesis (Ito *et al.* 1997) and in tumour promotion (Ohta *et al.* 1994)

in skin and liver of rodents. Epidemiological evidence of increased rates of primary hepatocellular carcinoma in specific populations in China has been associated with drinking cyanobacterially contaminated drinking water (Yu, 1995). In China, liver cancer is a major component of cancer mortality, owing to a combination of endemic hepatitis in the population, aflatoxins in the diet, and the use of surface water supplies (Yu 1989, 1995). In the hyperendemic areas, the rate of liver cancer death reaches 60 per 100,000 population per year, compared with South Australia, with < 6 per 100,000 population per year (South Australian Cancer Registry). In Australia and other first-world countries, gastrointestinal cancer is very much more important, comprising about 26% of cancer deaths, of which a third are cancers in the colon (see Thomas & Hrudey 1997).

**Table 2.** Loss of life expectancy from social and health causes in the United States (from Cohen 1991).

| Cause                                  | Life expectancy loss (days) |
|--|-----------------------------|
| Being an unmarried male                | 3500                        |
| Being a male smoker                    | 2250                        |
| Being an unmarried female              | 1600                        |
| Being 30% overweight                   | 1300                        |
| Driving a motor vehicle                | 207                         |
| Being murdered                         | 90                          |
| Exposure to radon concentrations       | 30                          |
| Exposure to lead pollution             | 20                          |
| Exposure to pesticides in food         | 12                          |
| Exposure to airborne carcinogens       | 4                           |
| Exposure to drinking water carcinogens | 1.3                         |

Thus, in terms of population mortality rates and loss of life expectancy, cyanobacterial toxins in drinking water are unlikely to have impact in Western countries unless they are a hidden part of the cause of gastrointestinal cancer mortality. Epidemiological study is required to explore whether a relationship exists between human gastrointestinal cancer and cyanobacterial contamination of drinking water.

Cyanobacterial toxins may enter the body through oral consumption, inhalation or skin absorption, although the proportions can be expected to be widely different between toxins. Although the relative proportions have not been experimentally evaluated, non-recreational exposure can be expected to be almost entirely via the oral route. Cyanobacterial toxins comprise a very diverse group of organic molecules, with microcystins the only examples for which uptake data are available. Oral administration studies have shown that uptake of microcystin into the blood occurs through the gastrointestinal lining (Dahlem *et al.* 1989; Falconer *et al.* 1992b), whereas excretion is almost entirely through the faeces (for discussion, see Kuiper-Goodman *et al.* 1999). Thus, oral consumption of microcystin will result in the whole gastrointestinal tract lining being exposed to the toxin, with the potential for cell injury (Falconer *et al.*

1992b). Experimental assessment of the possible carcinogenic or tumour-promoting effects of exposure to this toxin have been carried out in the laboratory over six years (Falconer & Humpage 1996). Recent data have shown that the growth of aberrant crypt foci in the mouse colon is stimulated by *Microcystis* extracts in the drinking water (Humpage *et al.* 2000).

The World Health Organisation (WHO) expert group have discussed whether microcystin can be considered as a carcinogen, on the basis of the current experimental evidence. The strongest demonstrated effects are in the promotion of tumour growth, the tumour having been initiated previously by a recognized carcinogen (Fujiki & Suganuma 1993). The mechanism of tumour promotion by microcystin is understood, as it is the consequence of inhibition of specific protein phosphatase enzymes that play an essential part in the control of the cell cycle (Humpage & Falconer 1999). At present there is no agreement over the use of tumour promotion data for the derivation of guideline values for safe exposure to environmental contaminants.

The effects of cyanobacterial toxins on the general health and well-being of the human population (independent of cancer risk) can be estimated from animal data, as well as from epidemiological studies of the population itself. Acute animal toxicity data exist for the hepatotoxic microcystins and nodularin; the neurotoxic anatoxin-a, anatoxin-a(s) and the saxitoxins (paralytic shellfish poisons); and the cytotoxic lyngbyatoxin, aplysiatoxins and cylindrospermopsin (Kuiper-Goodman *et al.* 1999). Records of accidental animal deaths from drinking or eating cyanobacteria exist from 1878 onwards for each of these toxins (Francis 1878), apart from the marine lyngbyatoxin and aplysiatoxins. The majority of experimental animal studies have been of acute cyanobacterial toxicity, as well as have the studies of the numerous cases of accidental poisoning of livestock and domestic pets (Carbis *et al.* 1995; Falconer 1993). In one case in which the accidental poisoning of domestic animals caused deaths, surviving animals showed evidence of injury such as photosensitization and liver damage, lasting for weeks or months (Carbis *et al.* 1994, 1995).

No human deaths from oral consumption of cyanobacterial toxins have been demonstrated unequivocally, although strong presumptive evidence exists from a study of water-borne gastroenteritis in Brazil, in which 88 patients died from 2000 cases reported. In this outbreak no infectious agents were identified, and no heavy metal or agricultural chemical contaminants were found in the water supply. The reservoir had recently filled for the first time, and there was a substantial bloom of cyanobacteria, which resulted in the reservoir being dosed with copper sulphate. The timing of the outbreak coincided with the cyanobacterial bloom and ended after the copper dosing, with the authors concluding that cyanobacteria provided an

appropriate explanation for the serious outbreak (Teixera *et al.* 1993). Cyanobacterial toxicity was proved to be the cause of over 50 deaths from liver failure in a dialysis clinic in which microcystins were present in the dialysis fluid. In this case, the toxins were detected in the water filters supplying the dialysis water and the patients themselves (Jochimsen *et al.* 1998; Pouria *et al.* 1998).

Within Australia there have been two reported instances of human injury following toxic cyanobacterial contamination of a drinking water supply reservoir, and there has been one study of the health effects of drinking water supply reservoir, and there has been one study of the health effects of drinking river water containing cyanobacteria. In the first two cases, a cyanobacterial bloom in a drinking water reservoir resulted in complaints from the water consumers of bad taste and odour from the drinking water (Bourke *et al.* 1983, 1986). The controlling water authorities then treated the reservoirs with copper sulphate to lyse and remove the cyanobacteria. The clinically most serious cases occurred among the Aboriginal population of Palm Island, off the Queensland coast of Australia in 1979 (Byth 1980). Shortly after copper dosing of the reservoir, children were brought into the hospital with an unusual hepatoenteritis, initially showing acute tender liver enlargement, constipation, vomiting and headache. This was followed by bloody diarrhoea and loss of protein, electrolytes, glucose and ketones through the urine, with varying severity of dehydration. Severe cases were flown to the regional hospital, where they received intensive care with intravenous therapy (Byth 1980). A total of 140 children and 10 adults received hospital treatment. A toxic cyanobacterium was subsequently isolated from this reservoir and identified; it showed similar toxicity in animal studies to that shown in the children (Hawkins *et al.* 1985). Further research characterized a new alkaloid toxin, named cylindrospermopsin after the toxic species *C. raciborskii* from which it was isolated (Ohtani *et al.* 1992). Subsequent experimental studies in rodents have shown liver and kidney damage resulting from this toxin (Falconer *et al.* 1999b; Seawright 1999).

The second Australian example of population injury from cyanobacterial toxin in drinking water from reservoirs was less severe. A dense bloom of the hepatotoxic *M. aeruginosa*, widely responsible for livestock deaths in Australia during the last hundred years, was being monitored in the drinking reservoir supplying Armidale, NSW, in the summer of 1981. The bloom was terminated by copper sulphate addition to the reservoir retrap compared with that in residents of comparable areas with different water supplies, showed evidence of toxic liver damage coincident with the bloom and its termination (Falconer *et al.* 1983).

These events show that local populations can be affected by acute cyanobacterial toxicity, if high cyanobacterial cell densities are presented in drinking water reservoirs. This risk is exacerbated by the use of

algicides, which cause liberation of toxins from the cyanobacterial cells into the water. Without cell lysis, toxins can be reduced by removal of cyanobacteria by filtration in water treatment (Hrudey *et al.* 1999).

Rivers are also used directly from water supply in Australia, with simple chlorination for the prevention of enteric disease transmission. Eight towns along the lower Murray River were monitored for gastroenteritis and allergic conditions among the population and the data correlated with cyanobacterial cell counts in the raw water supply from the river. Significantly more gastroenteritis occurred in the population that drank chlorinated river water than among those drinking their own supply of unchlorinated rain water; the frequency of gastroenteritis was correlated with the log cyanobacterial cell count in the river (el-Saadi *et al.* 1995).

#### **Adverse health consequences of recreational exposure to cyanobacteria and their toxins**

Australia has the majority of its population in coastal regions, where there is a strong emphasis on sea bathing. Red tides of *Trichodesmium* Ehrenberg ex Gomong spp. occur during summer along the coastline at low frequency. They are associated with skin rashes in bathers, and it is general practice to issue health warnings against swimming in red tides. Brackish-water coastal lagoon and estuaries are also used extensively for recreation and are frequently subject to summer blooms of the hepatotoxic *Nodularia spumigena* Mertens (Jones *et al.* 1994). No human poisoning has been recorded as a result of ingestion of *N. spumigena*, but the toxin accumulates in marine mussels (Falconer *et al.* 1992a). This results in the prohibition of shellfish collection, because of the risk from toxin in the shellfish collection, because of the risk from toxin in the shellfish, *N. spumigena* scum also makes a very unpleasant environment for recreational swimming.

A waterbloom of *N. spumigena* in Lake Alexandrina, which is a shallow lake at the termination of the River Murray, caused numerous livestock deaths in 1878 and was the first scientifically documented case of cyanobacterial intoxication (Francis 1878). Major estuarine recreational areas used by the populations of the cities of Melbourne and Perth have had cyanobacterial blooms resulting in millions of dollars of lost tourist income and, in Perth, the expenditure of tens millions of dollars for remediation (Hosja *et al.* 2000).

Inland lakes and rivers are also subject to cyanobacterial blooms and are used for swimming, water skiing, sailing etc. An Australian epidemiological study was carried out linking swimming time and cyanobacterial cell count in the water to subsequent minor medical problems in people bathing in contaminated water. A dose response in skin or eye irritation and contaminated water. A dose response in skin or eye irritation and adverse

gastrointestinal effects was observed with an elevated adverse gastrointestinal effects was observed with an elevated 'odds ratio' of 3.44 for people bathing in the water for more than one hour at cyanobacterial cell counts about 5000 cells m<sup>-1</sup>. This low cell count does not correspond to any dose response to known toxins, and so the effects may have been due to allergens or lipopolysaccharide endotoxins (Pilotto *et al.* 1997). A more definite poisoning was reported among army recruits in England who ingested and inhaled toxic *M. aeruginosa* while swimming (Turner *et al.* 1990).

#### **Risk avoidance and safe levels of toxic cyanobacteria**

WHO recently established an expert group to examine evidence of adverse health effects attributed to toxic cyanobacteria and to report on the establishment of 'Guideline Values' for safe cyanobacterial contamination levels for drinking water and recreational water exposure (Chorus & Bartram 1999). For the derivation of drinking water guideline values, a standardized approach was taken, in which selected animal toxicity data, together with uncertainty (for safety) factors, were used to determine a 'Tolerable Daily Intake' (TDI, or Reference Dose). The only cyanobacterial toxin for which adequate data for subchronic oral toxicity were available was microcystin-LR, which is the most available and hence best studied of the approximately 60 variants, not necessarily including microcystin-LR (Falconer *et al.* 1994); however, this variant is among the most toxic. Microcystins are the most important cyanobacterial toxins in drinking water in temperate countries (see Chorus & Bartam 1999). The uncertainty factors applied were 10 for human variability, 10 for animal to human differences and 10 for limitations in the data (e.g. subchronic dosing compared with lifetime exposure, acute toxic effects or possible tumour promotion effects), giving a total uncertainty factor of 1000. This factor was applied to an experimental No Observed Adverse Effect Level of 40 µg kg<sup>-1</sup> body weight d<sup>-1</sup> of microcystin-LR established in mice (Fawell *et al.* 1994), to give a TDI of 0.04 µg kg<sup>-1</sup> d<sup>-1</sup> for lifetime oral exposure in people. Independent research that used pigs as a model for microcystin toxicity gave a very similar result (Falconer *et al.* 1994).

To determine the Guideline Value for safe drinking water, the following equation applies:

$$\begin{aligned}\text{Guideline Value} &= (\text{TDI} \times \text{bodyweight} \times \text{proportion of} \\ &\text{toxin consumed in drinking water})/\text{water intake}, \\ &= (0.04 \times 60 \times 0.8)/2.0 \\ &= 0.96\end{aligned}$$

(or effectively 1.0 µg l<sup>-1</sup> of microcystin-LR) (Falconer *et al.* 1999a), where TDI is 0.04 µg kg<sup>-1</sup> d<sup>-1</sup>, as above; a standard bodyweight is taken as 60 kg; standard water intake is taken as 2.0 litres day<sup>-1</sup>, and 80% of the microcystin intake is assumed to come from drinking water.

These calculations are based on microcystin-LR and are supported by an oral toxicity study carried out in pigs, which used a natural microcystin extract containing a range of toxic variants, not including microcystin-LR (Falconer *et al.* 1994). For the purpose of Australian National Guideline Values for microcystins in drinking water, the concept of toxicity equivalence to microcystin-LR has been used, since measurement of microcystin-LR alone would provide a deceptive underestimate or risk. Thus total microcystins are measured and converted to toxic equivalents of microcystin-LR (Australian National Health and Medical Research Council 2000). Thus, to provide a negligible health risk to the population for lifetime consumption, the concentration of total microcystin-LR equivalents should be below  $1.0\mu\text{g l}^{-1}$  of drinking water at the domestic tap. For notification of a 'Health Alert' for drinking water, a value of  $10.0\mu\text{g l}^{-1}$  has been proposed during a brief period of microcystin contamination (Fitzgerald *et al.* 1999).

At the present time no subchronic or chronic oral toxicity data have been published for other cyanobacterial toxins of importance in drinking water. In tropical regions, the toxin from *C. raciborskii* is potentially a source of adverse health effects in drinking water and data for acute toxicity are available (Falconer *et al.* 1999b; Seawright *et al.* 1999). These data are not appropriate for development of guideline values, owing to the difficulty of extrapolating from acute toxicity to chronic toxicity. Similar problems have prevented development of guideline values for saxitoxins (Kuiper-Goodman *et al.* 1999).

For safe recreational water use, a more complex set of criteria has been applied. This is owing to the very hazardous nature of concentrated scums of floating cyanobacteria, which may occur over a wide range of cell concentrations in the main water body if climatic conditions are suitable. Scums readily form overnight in calm warm weather, and concentrate by drifting down with the breeze to the shore line. Monitoring of lakes and rivers in Australia provides cell counts or equivalent measures of cyanobacterial concentration and notification of scums. For health purposes, actual toxicity measurement is not required, because most cyanobacterial blooms are toxic: it may reasonably be assumed, therefore, that a potential risk is present if cyanobacteria are in high concentrations (Sivonen & Jones 1999). For recreational waters, WHO have provided recommendations for a framework of risk assessment at three levels of cyanobacterial concentration in lakes and rivers, viz. 20,000 cells  $\text{ml}^{-1}$ ; 100,000 cells  $\text{ml}^{-1}$ ; and cyanobacterial scums. Health and monitoring advice are given for each (Falconer *et al.* 1999a).

## CONCLUSIONS

Adverse health effects from the consumption of cyanobacteria and their toxins are now sufficiently well understood for the WHO to aim to provide guideline values for safe drinking water. Consumption of water supplies conforming to these guidelines will prevent human injury from toxicity. Although death by acute poisoning is a rare, accidental event, unlikely to affect mortality figures or life expectancy of populations, the role of cyanobacterial toxins in cancer needs further evaluation. Rates of gastrointestinal cancer in first-world populations and liver cancer in developing countries may be affected by cyanobacterial contamination of drinking water, exerting a measurable effect on mortality rates.

## ACKNOWLEDGEMENTS

I would like to thank the University of Adelaide and the Cooperative Research Centre for Water Quality and Treatment for facilities and financial support.

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Accepted 28 February 2001.

This paper originally appeared in *Phycologia* vol 40 (3) pp 228-233.  
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# **CYANOBACTERIAL TOXINS – THE AUSTRALIAN PERSPECTIVE ON GUIDELINES AND MANAGEMENT**

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## **ABSTRACT**

Blue-green algae, which are more correctly referred to as cyanobacteria, are now recognised as a serious water quality problem with regard to both drinking water supply and recreational water use in Australia. The deterioration of our water resources through both poor land and catchment management, and water allocation practices, is now becoming better understood and acknowledged. Algal blooms are often a symptom of the resulting changes in water quality from these activities. The main water supply problems associated with cyanobacteria include offensive tastes and odours and the production of toxins. The concern surrounding toxicity and possible effects on human health has been the subject of considerable discussion between water resources and health agencies across Australia and internationally for quite a number of years. The World Health Organization (WHO) has recently acknowledged this concern by producing a monograph on the significance and management of toxic cyanobacteria in water resources and water supplies (1). The purpose of this paper is to discuss recent developments in relation to guidelines for cyanobacterial toxins in drinking water supplies and some of the implications of this for management. The National Health and Medical Research Council of Australia (NHMRC) recently reviewed cyanobacteria and their toxins as part of the rolling revision of the Australian Drinking Water Guidelines. The working party commenced work in 1998 and reviewed the current toxicological information and public health significance of the five classes of toxins found in cyanobacteria in Australia, which were considered to be of potential concern for public health. These are the hepatotoxins microcystin, nodularin and cylindrospermopsin, the saxitoxin class of neurotoxins, and the lipopolysaccharide endotoxins. The NHMRC working group derived a guideline of 1.3 µg/L for total microcystins (as microcystin-LR toxicity equivalents). This was different from the recent derivation of a guideline by the WHO for microcystin-LR of 1 µg/L. There was insufficient information to recommend a guideline for any of the other toxins at this time. A concurrent review of analytical methods for toxins concluded that HPLC with photo-diode array detection is currently the most robust method for monitoring microcystins in water to allow for compliance with the guideline. The main implication of the introduction of guidelines is the need for water supply operators and health authorities to monitor their drinking water for the respective toxins, rather than for cyanobacterial cell numbers, which may have been used as a surrogate for potential contamination in the past.

## **INTRODUCTION**

Blue-green algae, which are more correctly referred to as cyanobacteria, are now recognised as a serious water quality problem with regard to both drinking water supply and recreational water use in Australia. The deterioration of our water resources through both poor land and catchment management, and water allocation practices, is now becoming better understood and acknowledged. Algal blooms are often a symptom of the resulting changes in water quality from these activities. The conditions which favour the growth of cyanobacteria and lead to blooms are nutrient enrichment (largely

phosphorus but also nitrogen), warm temperatures, and calm stable water conditions such as those occurring in slow-flowing rivers and thermally stratified lakes. These conditions are often caused by human actions and activities, but can often be equally associated with natural climatic cycles which prevail over the wide geographic area of Australia. It has been argued that the growth of cyanobacteria may be favoured by simply impounding water in artificial storages in a hot dry climate such as ours. Similarly, highly variable river flows have always been a regular cyclical feature of the hydrology in a continent with regular droughts, however the regulation of large rivers, such as for example the Murray River, has led to an



overall reduction in flow volume and duration characteristics (2,3). The role of river regulation in the development of algal blooms and the effect on other aspects of riverine ecology needs to be better understood to allow for more informed management (4).

Notwithstanding these “thorny” water resource management issues, which require complex political and social solutions, the main water supply problems associated with cyanobacterial growth and proliferation in water supplies include offensive tastes and odours and the production of toxins. The concern surrounding toxicity and possible effects on human health has been the subject of considerable discussion between water resources and health agencies across Australia and internationally for quite a number of years (1,5,6). The purpose of this paper is to discuss recent developments in Australia in relation to guidelines for cyanobacterial toxins in drinking water supplies and the implications of this for management in that country.

The paper gives the recent guideline derivation for the group of cyanotoxin microcystins, developed as part of the process of the rolling revision of the Australian Drinking Water Guidelines (ADWG) by NHMRC/ARMCANZ (National Health and Medical Research Council/Agriculture and Resource Management Council of Australia and New Zealand).

This review has resulted in the production of four “Fact Sheets” for individual classes of toxins: microcystins, nodularin, saxitoxins, and cylindrospermopsin (Fact Sheets 17a-17d) (20; <http://www.health.gov.au:80/nhmrc/publications/synopses/eh19syn.htm>). The outcome of the review was that a guideline value was recommended for total microcystins (Fact Sheet 17a), and that no guideline values could be set for concentrations of nodularin, saxitoxins or cylindrospermopsin at this time due to the lack of adequate data (20).

This paper does not discuss guidelines for recreational exposure or the water quality issues associated with the use of algal and/or toxin contaminated water for agricultural purposes. These water issues are however, also under review through the National Health and Medical research Council in a revision of bathing water guidelines. The issues relating to the use of toxin-contaminated water for irrigated agriculture, stock watering and aquaculture are often raised in relation to potential for residues in produce. These topics will be reviewed in a discussion paper to be produced as part of the Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ) National Algal Management (NAM) Program in 2001/2002.

## CYANOBACTERIAL TOXINS IN AUSTRALIA

### Significance for drinking water quality

Cyanobacteria produce a range of toxic compounds that have a deleterious effect upon drinking water quality. Table one presents a list of the compounds which have been found to occur in cyanobacteria in Australia, their public health and water quality significance, and provides comments on a range of guideline and management issues. Some of the main points from the table are:

#### *Microcystins:*

The microcystin toxins are a group of cyclic peptide hepato- or liver toxins that are widely regarded as the most significant potential source of human injury from cyanobacteria on a world wide scale (7). This is arguably not the case in many geographic regions of Australia where other toxins may be more prevalent. Nevertheless blooms of toxic *Microcystis aeruginosa*, which is the predominant cyanobacterium producing microcystin, are widespread throughout southeastern Australia. Microcystins can occasionally be produced by *Anabaena* spp, however this appears to be rare in Australia. Microcystins have been implicated in causing liver damage in a human population exposed via reticulated town water supply where the source water contained blooms of *Microcystis* (8). In addition microcystins promote the growth of tumours in experimental animals (9,10), and the significance of this for humans who may be subject to chronic exposure via drinking water is unclear.

#### *Cylindrospermopsin:*

Cylindrospermopsin is an alkaloid toxin that has been isolated from two species of cyanobacteria in Australia – *Cylindrospermopsis raciborskii* (11) and recently from *Aphanizomenon ovalisporum* (12). *C. raciborskii* is a widespread bloom-forming organism in tropical and sub-tropical areas of Australia and this is a significant water quality issue due to the associated production of cylindrospermopsin. *C. raciborskii* also occurs in the phytoplankton in temperate regions but rarely forms blooms in the cooler climates (13). Cylindrospermopsin was implicated in a severe human poisoning episode associated with a bloom of the cyanobacterium *Cylindrospermopsis raciborskii* in a water supply reservoir on Palm Island, Queensland in 1979 (14). The experimental dosing of mice with extracts of *C. raciborskii* leads to widespread tissue and organ damage, primarily in the liver (14). Kidney damage is also observed on occasions and it has been proposed that *C. raciborskii* may produce other unknown toxins in addition to the characterised hepatotoxin cylindrospermopsin (7).

**Table 1. Cyanobacterial Toxins and Drinking Water Quality:** Major classes of toxins produced by cyanobacteria in Australia, their significance to drinking water quality, and comments on their guideline status and the implications for management of water supply.

| TYPE         | COMPOUND   | ORGANISMS   | EFFECTS   | DRINKING WATER QUALITY AND PUBLIC HEALTH SIGNIFICANCE   | GUIDELINE STATUS  | IMPLICATION FOR WATER MANAGEMENT  |
|--------------|--|---|---|---|---|---|
| Hepatotoxins | Microcystin  | <b>Microcystis aeruginosa</b><br>Anabaena spp.<br>in animal studies                                   | Liver Damage<br>Tumour promotion  | Acute toxicity in large water supply systems unlikely<br>Chronic liver damage with chronic exposure likely<br>The relationship between the animal tumour growth promotion properties of these toxins and human carcinogenicity needs to be determined | WHO Provisional Guideline : 1 µg/L for microcystin-LR (only) released 1998<br>Australian Guideline: 1.3 µg/L expressed as microcystin-LR toxicity equivalents (TE), released August, 2001 | The introduction of guidelines for microcystins obliges operators to monitor for the chemical compound, and this requires access to appropriate monitoring techniques<br>These guidelines include a reference to cell numbers - guideline is equivalent to 6,500 cells/mL , for a highly toxic population of <i>M. aeruginosa</i> |
| Neurotoxins  | Cylindrospermopsin   | Cylindrospermopsis raciborskii<br>Aphanizomenon ovalisporum   | Cytotoxic<br>Liver, kidney and other organ damage   | Risk of acute toxicity via drinking water supplies dependent upon circumstances<br>No information on effects of chronic exposure in animals although studies underway   | No WHO or other national guidelines available<br>No Australian Guideline  | This toxin is a significant water quality issue for tropical Australia<br>Cannot be disregarded as an issue in southern Australia   |
|              | Saxitoxin class of compounds (Paralytic Shellfish Poison – PSPs) | Anabaena circinalis   | Sodium channel blocking agent – acute poisoning results in death by paralysis and respiratory failure       | Acute toxicity in large water supply systems unlikely<br>Effects from chronic exposure not known<br>Public health significance unclear<br>No evidence of human illness from drinking water supply   | No WHO or other national guidelines available<br>No Australian Guideline  | Not likely to be as significant a drinking water quality issue as microcystins  |
| Endotoxins   | Lipopolysaccharides (LPS)  | Most cyanobacteria (outer cell wall component similar to LPS in cell walls of gram negative bacteria) | Implicated in<br>Gastro-intestinal disorders<br>Skin, eye irritation<br>Skin rashes<br>Respiratory symptoms | Less toxic than hepato – or neurotoxins<br>Effects from chronic exposure not known<br>Possibly significant for water supply in relation to bathing and showering  | No WHO or other national guidelines available<br>No Australian Guideline  | May become an issue for health investigation for recreational and drinking water, but lack of information makes it difficult to assess their significance<br>Poor information base makes guideline development unlikely at present  |

**Neurotoxins:**

One of the most common bloom-forming cyanobacteria in Australia is *Anabaena circinalis* and this has been shown relatively recently to often produce the saxitoxin (PSP – Paralytic Shellfish Poisons) class of neurotoxins (15,16). These toxins disrupt the normal signalling between nerves and muscles and can cause death by respiratory paralysis when given in high enough doses to both humans and other animals. The saxitoxins have been responsible for significant human illness and mortality in situations where they have been ingested with contaminated shellfish that accumulate the toxins in the marine environment (17). These toxins may not accumulate and appear not to produce chronic health effects in humans as they are cleared from the body relatively quickly (17). There is no evidence of human health effects caused directly from water that contains saxitoxin-producing cyanobacteria (18). The neurotoxins produced by *A. circinalis* may reservedly be considered as less of a threat to public health via public water supply than hepatotoxins. The reservations are that these are highly toxic compounds, which have not yet been the subject of medium- or long-term chronic animal studies, and there is little information on their occurrence in drinking water.

**Endotoxins:**

Lipopolysaccharides (LPS) are components of the cell wall of cyanobacteria (and all gram-negative bacteria) and they have been implicated as a potential cause of skin irritations and allergic reactions in cases of human exposure to cyanobacteria, usually in recreational waters (5,19). It is important to recognise that LPS could be produced in varying amounts by all cyanobacteria irrespective of whether they produce the better known hepato- or neurotoxins. Very little information exists on the presence of these compounds in water or cyanobacterial blooms in Australia. Given the potential for these compounds to cause contact skin irritations it follows that there could be adverse effects via water supplies from bathing or showering with cyanobacterial contaminated water. The likelihood of this is unknown and depends on the extent of contamination, the effectiveness of water treatment in removing these compounds, and the dose-response and sensitivity of the exposed population.

## GUIDELINES – CURRENT STATUS IN AUSTRALIA

The Australian Drinking Water Guidelines (ADWG) have been undergoing a process of rolling revision by NHMRC/ARMCANZ since 1999. The NHMRC/ARMCANZ Drinking Water Review Coordinating Group decided that guidelines for cyanobacteria and their toxins would be developed as part of the review for 1999/2000.

A working group completed a review of information on cyanobacteria and their toxins in relation to drinking water and public health in April 2000. This has resulted in the production of four "Fact Sheets" for individual classes of toxins: microcystins, nodularin, saxitoxins, and cylindrospermopsin (Fact Sheets 17a-17d) (20). The outcome of the review and subsequent consultation process was that a guideline value was recommended for total microcystins (Fact Sheet 17a), and that no guideline values could be set for concentrations of nodularin, saxitoxins or cylindrospermopsin due to the lack of adequate data. In relation to lipopolysaccharides (LPS) produced by cyanobacteria, the working group concluded at an early stage of their work that there was insufficient information to carry out a critical assessment on occurrence and significance of LPS and did not produce a fact sheet.

The process for guideline derivation for microcystins taken directly from NHMRC/ARMCANZ (20) was as follows:

**Derivation of Guideline**

$$1.3 \mu\text{g/L} = \frac{40 \mu\text{g/kg bodyweight per day} \times 70 \text{ kg} \times 0.9}{2 \text{ L/day} \times 1000}$$

where:

- 40 µg/kg body weight per day is the No Observed Adverse Effect Level (NOAEL) from a 13-week ingestion study with microcystin-LR in mice based on liver histopathology and serum enzyme level changes (21)
- 70kg is the average weight of an adult
- 0.9 is the proportion of total daily intake attributed to the consumption of water
- 2 L/day is the average amount of water consumed by an adult
- 1000 is the safety factor derived from extrapolation of an animal study to humans (10 for interspecies variability, 10 for intraspecies variability and 10 for limitations in the database, related particularly to the lack of data on chronic toxicity and carcinogenicity)

The guideline is derived for total microcystins and expressed as microcystin-LR toxicity equivalents (TE). This is because the total microcystin concentration should be considered in relation to potential health impacts.

The NHMRC/ARMCANZ (20) provided a comparison to the WHO guideline as follows: "The World Health Organization has recently undertaken an evaluation of the health-related information for cyanobacterial toxins (22,23,24). It was concluded that there are insufficient data to allow a guideline value to be derived for any cyanobacterial toxins other than microcystin-LR. The guideline recommended by the WHO for drinking water is 1 µg/L (rounded figure) for total microcystin-LR (free plus cell-bound), based on the Fawell

*et al.* (21) sub-chronic study. This guideline value for microcystin-LR is provisional, as the database is regarded as limited (23). The approach being taken for guideline derivation here is essentially similar to that used by WHO (24). The same ingestion study in mice was used to calculate the NOAEL. The Australian guideline of 1.3 µg/L total microcystin (as microcystin-LR TE) differs from the WHO provisional guideline of 1 µg/L microcystin-LR due to the incorporation of a different average body weight for an adult (70 kg versus 60 kg), and to a difference with regard to the proportion of the daily intake of microcystin being attributed to the consumption of drinking water. The proportion for the Australian situation is regarded to be 0.9, which is higher than 0.8 selected by WHO. This is due to lower potential exposure in Australia from other environmental sources, such as contaminated bathing water, and via dietary supplements potentially containing microcystins."

The NHMRC/ARMCANZ (20) also provided some guidance to assist with assessment of potential toxin contamination in the initial absence of toxin monitoring data as follows:

"In situations where *M. aeruginosa* occurs in drinking water supplies, and toxin monitoring data are unavailable, cell numbers can be used to provide a preliminary orientation to the potential hazard to public health. As an indication, for a highly toxic population of *M. aeruginosa* (toxin cell quota of 0.2 pg total microcystins/cell), a cell density of approximately 6,500 cells/mL is equivalent to the guideline of 1.3 µg/L microcystin-LR (TE), if the toxin were fully released into the water. It is important to note that this number is preliminary and for indicative purposes only, and for health risk assessment toxin determination is required."

As indicated above these findings are essentially similar to the position taken by the World Health Organization in their recent review of cyanotoxins as part of the continuing process of updating the second edition of the WHO Guidelines for Drinking Water Quality (GDWQ) (23). The various WHO working groups have indicated that at the time of their deliberations (1997/98) there was insufficient information available to derive guidelines for either neurotoxins or cylindrospermopsin.

A significant difference between the provisional WHO guideline and the NHMRC/ARMCANZ guideline is that the Australian guideline gives advice in relation to the concentration of total microcystins whereas WHO restricts its advice to the single compound microcystin-LR. Microcystin-LR is the most widespread variant in geographic terms and best characterised toxicologically of this class of hepatotoxins, of which there are in excess of 75 structural types. The Fact Sheet for microcystins

(17a) in the ADWG states that the Australian guideline for Total Microcystins is 1.3 µg/L expressed as toxicity equivalents of microcystin-LR.

The rationale for the Australian guideline covering total microcystins is that blooms of *Microcystis aeruginosa*, which is the most common toxin producing cyanobacterium in Australia, generally contain a range of variants of microcystin in varying amounts. Experience indicates that the number of variants in an individual sample can range from a few to up to more than 20 in some cases. It is the cumulative toxicity of the microcystins in total that represents the potential hazard to human health from ingestion via drinking water. Therefore the unit recommended for the quantitative expression of this cumulative toxicity in the guideline is total microcystins expressed as toxicity equivalents of microcystin-LR.

The process of guideline setting in general by NHMRC considers the requirements and current limitations of the measurement technology. In the case of analysis for cyanobacterial toxins the basic principles of techniques available for measuring compounds of a particular class vary considerably.

## IMPLICATION OF GUIDELINES FOR MANAGEMENT

In the Australian context drinking water guidelines are not meant to be mandatory standards but are designed to provide "guidance on what constitutes good quality drinking water (as distinct from water which is acceptable)" (25). The NHMRC/ARMCANZ also indicate that "guidelines provide a reference for use within the Australian administrative and legislative framework to ensure the accountability both of water authorities, as managers, and of State health authorities, as auditors of the safety of water supplies. The guidelines should not however, be construed as legally enforceable standards." (25) The definition and intention for use of guidelines issued by WHO is essentially similar. Chorus and Bartram (1) indicate that the primary aim of guidelines is the protection of public health and "the guideline values recommended for individual constituents of water are not mandatory limits. Rather they are intended to be used in the development of risk management strategies which include national or regional standards developed in the context of local or national environment, social, economic and cultural conditions that, if properly implemented, will ensure the safety of drinking-water supplies through the elimination, or reduction to a minimum concentration of constituents of water that are known to be hazardous to health."

In many cases the Australian Drinking Water Guidelines are being adopted by water authorities, either

in full or part, as their agreed levels of service for quality of water provided to consumers. They are seen as useful targets and performance indicators for audits of process performance. In cases of outsourcing or private operation of particular components of the water supply system (eg. water treatment plants), the guidelines are often used as the starting point to set contractual performance indicators. The guideline values may then become contract conditions. In some cases tighter limits than the guidelines may be set for some parameters, depending upon local sensitivity and requirements. Depending upon the contract, there may be penalties for exceedance. In one particular case of an outsourced water treatment operation, there is already a performance requirement for monitoring for cyanobacterial toxicity due to the local history of occurrence of cyanobacterial blooms (K. Craig, personal communication).

The requirement for monitoring of water supply for the specific toxic compounds is likely to be the most immediate consequence of the introduction of guidelines for cyanobacterial toxins. A consequence of the recommendation of such a guideline for microcystins is the potential requirement for identifying and quantifying specific toxin variants where contamination is suspected in a water supply. The process of guideline setting by NHMRC takes into account the requirement and current limitations of the measurement technology. In the case of analysis for cyanobacterial toxins the degree of sophistication and reliability of techniques for measuring the classes of compounds varies considerably.

A number of techniques are available for determining microcystin toxins in water (1). These range from rapid screening techniques based on ELISA and enzyme activity (protein phosphatase inhibition) assays, to quantitative chromatographic techniques based on High Performance Liquid Chromatography (HPLC) and more sophisticated (and expensive) Liquid Chromatography-Mass Spectrometry (LCMS). Analytical techniques based on either HPLC or LCMS can also be used for measuring quantities of saxitoxins or cylindrospermopsin in water. There has been very little work done on analytical techniques for cyanobacterial endotoxins, although this reflects the dearth of information on their occurrence and toxicity both here and overseas. Animal bioassays (mouse tests) are also available for screening the entire range of toxins. These tests have in the past always provided a definitive indicator of toxicity, although they cannot be used for precise quantification of compounds in water.

The revision of the NHMRC guidelines for toxins carried out a review of the status of analytical methods and a recently published report gives guidance on the selection of analytical techniques (26). The key finding of this review were as follows:

- The technique that provides the most reliable measurement for compliance with the ADWG for microcystins in water is high performance liquid chromatography (HPLC) with photo-diode array (PDA) detection. Liquid chromatography with mass spectral confirmation of toxin identity and quantification is also suitable if standards for the toxins present are available.
- For compliance monitoring in relation to the guideline, the concentrations of individual microcystins are determined by comparison against standards. The relative toxicity of microcystins other than microcystin-LR are then converted to microcystin-LR toxicity equivalents based on the ratio of their published LD<sub>50</sub> (mouse, i.p.) relative to that of microcystin-LR
- In situations where standards are unavailable for particular toxins in a sample it is necessary to use HPLC with PDA detection for analysis and to estimate the concentration, and therefore toxicity, of these microcystins against microcystin-LR as the analytical standard. In this case a slight overestimate of total microcystins (as microcystin-LR toxicity equivalents) may result.
- The technique of ELISA is useful for routine screening of water for toxin contamination. ELISA can however be regarded as a reliable measure of total microcystins (in microcystin-LR concentration equivalents) in situations where the sample is well characterised in terms of toxin composition. In this case the results should also be cross-calibrated initially and at periodic intervals against other techniques (HPLC-PDA). For samples containing toxins of unknown identities, it cannot provide a reliable quantitative result.
- Phosphatase inhibition assays show promise for monitoring microcystins in relation to the ADWG but require further development.
- Mouse bioassays are not suitable for determining microcystins at low concentration in water. These assays do not have sufficient sensitivity for application to water samples without impractical levels of pre-concentration. Mouse bioassays are useful for initial screening of highly concentrated cyanobacterial samples (eg "scums") of unknown toxicity. The results of a quantitative assay will indicate acute toxic effects and may also indicate the class of toxin (eg neurotoxin, hepatotoxin) from reactions and pathology in test animals. This can then allow for further quantitative testing by an alternative analytical method.
- Appropriate and careful handling of samples both prior to and during analysis is extremely important

to ensure an accurate determination of toxin concentration. Microcystin toxins are readily degraded both photochemical (ie in light) and microbial reactions. Samples should be kept refrigerated and in the dark prior to analysis, and should not be exposed to strong light during the preparation and analytical procedures.

## CONCLUSIONS

Australia has recently developed drinking water guidelines for the microcystin class of toxins. The guideline is derived for total microcystins is 1.3 µg/L and is expressed as microcystin-LR toxicity equivalents (TE). During the review process to produce this guideline it was concluded that no guideline values could be set for concentrations of nodularin, saxitoxins or cylindrospermopsin due to the lack of adequate data. The implication of the introduction of guidelines is the need for water supply operators and health authorities to monitor their drinking water for the toxin, rather than for cyanobacterial cell numbers. Previously algal counts have often been relied upon as a surrogate for potential toxin hazard. A review of the status of analytical methods for toxins concluded that the technique that provides the most reliable measurement for compliance with the ADWG for microcystins in water is high performance liquid chromatography (HPLC) with photo-diode array (PDA) detection. Liquid chromatography with mass spectral confirmation of toxin identity and quantification is also suitable if standards for the toxins present are available.

## ACKNOWLEDGMENTS

I would like to thank Mr Keith Craig (Vivendi Water Australia) for informative discussions on outsourcing contracts and Ms Jenny House for help in collating information and preparing the manuscript.

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## ALGICIDES FOR CONTROL OF TOXIC CYANOBACTERIA

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### ABSTRACT

Algicides have a role in management strategies for toxic cyanobacteria (blue-green algae). When used correctly they have the attraction of terminating the problem at the source in the reservoir, and this is a "once-off", if treatment is successful. The algicide of choice is copper sulphate. Copper sulphate has been used widely to control algal blooms in water supply storages and lakes for nearly 100 years. It is generally regarded as effective, economical and safe for operators to use, although copper can have adverse environmental impacts on the aquatic ecosystem. Other popular algicides include a range of copper chelates. The effectiveness of copper-algicide treatment is determined by a combination of chemical, physical and biological factors. Chemical factors include the characteristics of the receiving water. Factors such as the pH, alkalinity and dissolved organic carbon all determine copper speciation and complexation, which reduces the toxicity of copper in solution. Physical factors, particularly thermal stratification in the reservoir, affect the distribution of copper after application, which in turn may determine contact with the target organism. The important biological factor is the sensitivity of the target organism to copper. Cyanobacteria are generally regarded as being relatively sensitive to copper toxicity. The important point to remember about copper is that it is a broad-spectrum aquatic biocide, which will affect non-target species such as zooplankton and fish, and this can have significant adverse environmental effects. Local environmental regulations may determine the conditions under which algicides can be used.

There are several important issues to be aware of when treating toxic cyanobacteria with algicides. Firstly, they should be applied at the early stages of bloom development when cell numbers are low. This will reduce the potential for the release of high concentrations of intracellular toxin associated with dense blooms. Algicides will disrupt healthy cyanobacterial cells leading to the release of toxins and taste and odour compounds into solution. These dissolved toxins will then disperse and be diluted throughout the water body. Secondly, the lysis of cells by algicides compromises the effectiveness of toxin removal by conventional filtration. Intracellular toxins contained in intact cells are relatively well removed by flocculation and filtration steps in the treatment process. This can lead to reliance on adsorption by activated carbon or advanced oxidation to achieve effective removal of dissolved toxins from treated water. Thirdly, a withholding period may be required after algicide treatment to allow toxins in the reservoir to dilute, disperse and degrade.

### INTRODUCTION

Algae and cyanobacteria are a normal and essential component of the phytoplankton in aquatic ecosystems. Under certain conditions, usually a combination of high nutrient loadings and warm stable conditions they can grow excessively and form blooms. In drinking water reservoirs these blooms are of concern as they compromise the use of the water by producing off-flavours and in some cases toxins. Cyanobacteria have long been recognised as a cause of offensive tastes and odours in drinking water due to their production of

odour compounds such as geosmin and 2-methylisoborneol (1, 2, 3). More recent concern has been expressed regarding the implications for water supply of cyanobacterial toxins and human health (4).

Tastes, odours and toxins may require treatment with activated carbon in addition to the conventional water treatment processes. High cell biomass in the raw water can also reduce filter run times in treatment plants and add to the dissolved organic carbon (DOC) loading, which has implications for the formation of disinfection by products. Blooms are often unpredictable in terms of



incidence, intensity and duration, and the combination of these circumstances can lead to either impaired water quality or increased cost.

In some circumstances algicides are used in reservoirs to control algal or cyanobacterial growth and to prevent the associated water quality problems. In management terms this has the attraction and advantage of terminating the problem at source, and is a "once-off", if treatment is successful. The control of off-flavour problems at the source (ie. in - reservoir) with algicides may also be cheaper than treatment plant methods (5). This is because it can minimise the need for on-going treatment with PAC or GAC for an extended time if the blooms persist.

Copper sulphate has been used widely as an algicide to control algal blooms in water supply storages and lakes for nearly 100 years (6). It is generally regarded as effective, economical and safe to use for operators, although copper can have adverse environmental impacts on the aquatic ecosystem.

In some cases, however, treatment may be unsuccessful or partially successful. The effectiveness of copper algicide treatment is determined by three major factors (7):

- Chemical factors - the chemistry receiving water determines the speciation and thereby the toxicity of copper;
- Biological factors - the sensitivity of the target organism to copper;
- Physical factors - the distribution of copper in the reservoir after application may affect contact with the target organism.

This paper describes the commonly used algicides, the factors affecting the toxicity and mode of action of copper as an algicide, and gives advice on the use and limitation of applying algicides for the control of toxic cyanobacteria.

## ALGICIDES - CHEMICAL CONTROL OF ALGAE

Chemical control of algae in water supply storages has been a widespread water quality management practice for over 100 years. Copper sulphate has generally been the algicide of choice and records of its use date from 1890 in Europe (8), 1904 in the U.S. (9), and since at least the mid-1940's in Australia.

An extensive survey of water utilities in the U.S. and Canada in the 1980's indicated that copper sulphate is by far the most widely used algicide, although other alternatives are used under some circumstances (19). A summary of the range of compounds that have been used as algicides is given in Table I (below).

The properties, history of use and effectiveness of this range of algicides are discussed as follows:

**Copper sulphate:** Copper sulphate has been regarded as the algicide of choice because it is economical, effective, relatively safe and easy to apply, has no significant human health implications, and has been considered not to cause extensive environmental damage (6, 10). The last point has been a controversial issue for some time (11) because copper tends to accumulate in lake sediments (12, 13). In some cases it appears not to be remobilised and is bound permanently to the bottom sediments (12, 10). In contrast a study of 10 drinking water dugouts (small reservoirs) in Canada found that copper in the sediments, previously accumulated from copper sulphate treatments, was released back into the open water under low dissolved oxygen conditions in the hypolimnion in summer (14). It has also been suggested that sediment-bound copper could impact on the benthic macroinvertebrate community (13). It is important to remember that copper and other heavy metals differ from some other toxic contaminants in that they are not biodegradable, and once they have entered the environment their potential toxicity is controlled largely by their speciation or physico-chemical form (15, 16). Copper sulphate treatment has been shown to cause

**Table 1.** Common algicides, their formulations and key references (after Burch *et al.*, 91)

| COMPOUND                         | FORMULATION  | REFERENCES    |
|----------------------------------|--|---------------|
| Copper sulphate                  | $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  | 6, 20, 21, 19 |
| Copper II alkanolamine complex   | $\text{Cu Alkanolamine} \cdot 3\text{H}_2\text{O}^{++}$  | 22            |
| Copper - ethylenediamine complex | $[\text{Cu}(\text{H}_2\text{NCH}_2\text{CH}_2\text{NH}_2)_2(\text{H}_2\text{O})_2]^{++} \text{SO}_4$ | 22            |
| Copper - triethanolamine complex | $\text{Cu N}(\text{CH}_2\text{CH}_2\text{OH})_3 \cdot \text{H}_2\text{O}$                            | 22            |
| Copper citrate                   | $\text{Cu}_3[(\text{COOCH}_2)_2\text{C}(\text{OH})\text{COO}]_2$                                     | 19, 23, 6, 24 |
| Potassium permanganate           | $\text{KMnO}_4$  | 25, 20        |
| Chlorine                         | $\text{Cl}_2$  | 20            |
| Lime                             | $\text{Ca}(\text{OH})_2$   | 26            |
| Barley straw                     |  | 27, 28        |

short-term changes in phytoplankton abundance and species succession (17, 18). Fish kills may also occur following copper sulphate treatment, although it is not clear whether this is as a result of copper toxicity or oxygen depletion (13).

The widespread use of copper sulphate has given rise to a large amount of empirical information on dosing procedures and appropriate dose rates for the control of various algal species. A detailed summary of the relative toxicity of copper to a range of algae, including cyanobacteria, based on practical experience was given by Palmer (21) (Table II). Holden (20) provided a similar extensive table which gave concentrations required to eradicate different algae and indicated that the effective dose rate for most commonly occurring planktonic algae and cyanobacteria is in the range 0.5 - 2.0 mg L<sup>-1</sup> as copper sulphate (presumably CuSO<sub>4</sub>·5H<sub>2</sub>O). These doses are equivalent to 0.125 - 0.5 mg Cu L<sup>-1</sup>. Both of these authors recognised that the effective dose for a particular species in a particular water was affected by factors such as the abundance and physiological state of the algae, pH, temperature, alkalinity, and dissolved organic matter concentration of the water.

**TABLE II:** Relative toxicity of copper sulphate to cyanobacteria. Modified after Palmer (21).

| Group                            | Very Susceptible   | Susceptible   | Resistant                            |
|----------------------------------|--|---|--------------------------------------|
| Cyanobacteria (Blue-green algae) | <i>Anabaena</i> ,<br><i>Microcystis</i> ( <i>Anacystis</i> ),<br><i>Aphanizomenon</i> ,<br><i>Gomphosphaeria</i> ,<br><i>Rivularia</i> | <i>Cylindrospermum</i> ,<br><i>Planktothrix</i> ( <i>Oscillatoria</i> ),<br><i>Plectonema</i> | <i>Nostoc</i> ,<br><i>Phormidium</i> |

McKnight *et al.* (6) gave a more recent and thorough scientific assessment of the use of copper sulphate for the control of nuisance algae. They also indicate that there are wide differences in copper sensitivity among algal species. The relative growth inhibiting concentrations are given in terms of cupric ion activity (ie. [Cu<sup>2+</sup>]) for a range of phytoplankton derived from laboratory toxicity studies. The toxic cupric ion activities range from greater than 10<sup>-6</sup> to 10<sup>-11</sup> M (0.063 to 6.3 x 10<sup>-7</sup> mg Cu<sup>2+</sup> L<sup>-1</sup>) for species of diatoms, dinoflagellates, green algae and blue-green algae - a concentration range of over four orders of magnitude (6). It is worth noting that these toxic Cu<sup>2+</sup> concentrations are very much less than the usual doses applied as total copper in copper sulphate treatments. The relative toxicity is given in terms of ionic copper because it is believed that phytoplankton react principally to the concentration of Cu<sup>2+</sup> or loosely complexed copper rather than the total dissolved metal in water. The concentration of free cupric ion (Cu<sup>2+</sup>) can be reduced by complexation with both inorganic ligands under alkaline conditions, and organic ligands present in natural waters (6).

**Chelated Copper Algicides:** The problem of the reduced effectiveness of copper sulphate treatment in hard alkaline water has long been recognised (21, 19). Chelated copper algicides were developed to overcome the problems of the complexation and loss by precipitation of toxic copper under these circumstances. Examples of copper chelate algicides include copper ethanolamine, copper ethylene-diamine and copper-citrate (Table I). The chemical properties and application rates for these algicides are given by Humberg *et al.* (22). These chelated algicides are available as liquid formulations, and in some cases a granular form is also manufactured. This granular form along with large crystals of copper sulphate have been used for the control of odour producing benthic cyanobacteria growing at depth (9m) in a reservoir in California (29). This application is dependent upon the material sinking rapidly to the bottom and dissolving at depth.

Copper citrate has been used as an algicide in the U.S. (23, 19). It is available either as a commercial preparation (30) or by simultaneously dosing copper sulphate and citric acid (23). It is claimed that the use of citric acid as a chelating agent enhances the solubility of copper allowing it to remain in solution longer under alkaline conditions (23, 31).

McKnight *et al.* (6) suggested that the advantage of using synthetic copper chelating agents in hard, alkaline waters probably results from decreasing the supersaturation of malachite (Cu(OH)<sub>2</sub>CO<sub>3</sub>) and tenorite (CuO) and thereby the rate at which equilibrium with these insoluble forms (precipitates) is approached. It is possible that a longer time taken to reach equilibrium would result in the maintenance of toxic ionic Cu<sup>2+</sup> activities and the inhibition of algal growth for longer periods after dosing (6).

It has been suggested that the use of organic chelating agents is seemingly more expensive than the use of copper sulphate alone. However employing complexing agents may result in lower costs for the actual solubilised copper concentration, due to the potential for high complexation and precipitation loss of copper when using copper sulphate in certain waters (19). It is acknowledged that, despite their relatively widespread use in the U.S., the efficacy of chelated copper algicides in relation to water chemistry is poorly understood (19).

**Potassium Permanganate:** Potassium permanganate has been used as an algicide as early as 1935 (20). The survey of North American utilities in the 1980's, previously referred to, indicated that a small number use potassium permanganate relative to those who use copper sulphate (19). Fitzgerald (25) found that the dose range required to control algae and cyanobacteria was in the range 1 - 8 mg L<sup>-1</sup>.

**Chlorine:** Chlorine is used mainly for control of algae in water treatment works but has also been employed in reservoir situations (20). The effective dose rates would obviously be dependent on the chlorine demand of the water, but most algae are reportedly controlled by doses of free chlorine between 0.25 and 2.0 mg L<sup>-1</sup> (20).

**Lime:** Lime (both Ca(OH)<sub>2</sub> and CaCO<sub>3</sub>) has been used as an algicide to coagulate and precipitate phytoplankton cells out of the water column (32, 26). Unlike treatment with copper sulphate, the precipitation of cyanobacterial cells with Ca(OH)<sub>2</sub> does not appear to cause cell lysis and toxin release into the water (33, 34). Lime also functions to some extent as a longer - term algistat, reducing eutrophication by precipitating phosphorus from the water (35, 36). It appears that Ca(OH)<sub>2</sub> is more effective than CaCO<sub>3</sub> in precipitating phosphorus (35).

Many of the studies of both the mechanism and effects of liming for algal control have been carried out in eutrophic hardwater lakes or farm dugouts (dams) in Alberta, Canada (35, 32, 26). It is possible that the technique may be more effective in these conditions than in soft water. The dose rates used are also quite high, eg. 50 - 250 mg Ca(OH)<sub>2</sub> L<sup>-1</sup> (32), which would make the technique prohibitive for large lakes. Procedures for the application of lime, which involve pumping or spraying of a slurry, are given by Prepas *et al.* (37).

**Barley Straw:** The use of decomposing barley straw for the control of algae and cyanobacteria has been the subject of considerable recent interest and investigation (27, 28, 38; 39, 40). The effect of rotting barley straw in reducing the growth of filamentous green algae in a canal was first demonstrated by Welch *et al.* (27). Algistatic effects have been shown in laboratory cultures with the cyanobacterium *Microcystis aeruginosa* (28). The reasons for the inhibitory effects were suggested as being due to either, the production of antibiotics by the fungal flora, or the release of phenolic compounds such as ferulic acid and *p* - coumaric acid from the decomposition of straw cell walls (28). Inhibitory effects to cyanobacteria have also been demonstrated in reservoir trials with barley straw (40, 39).

There is some conflicting evidence on the efficacy of barley straw from Australia. Jelbart (38) failed to find any inhibitory effects from extracts derived from rotting straw on isolates of *M. aeruginosa*. Cheng *et al.* (41) also found no algicidal or algistatic effects from barley straw over a 6-month period in a comprehensive field trial using six experimental ponds.

These contradictory findings and the unknown identity of the phytotoxic compound/s in rotting barley straw would indicate that this technique is still too poorly understood to recommend for widespread use as an algal control measure, particularly in potable water supply situations.

## COPPER CHEMISTRY, SPECIATION AND TOXICITY IN NATURAL WATER

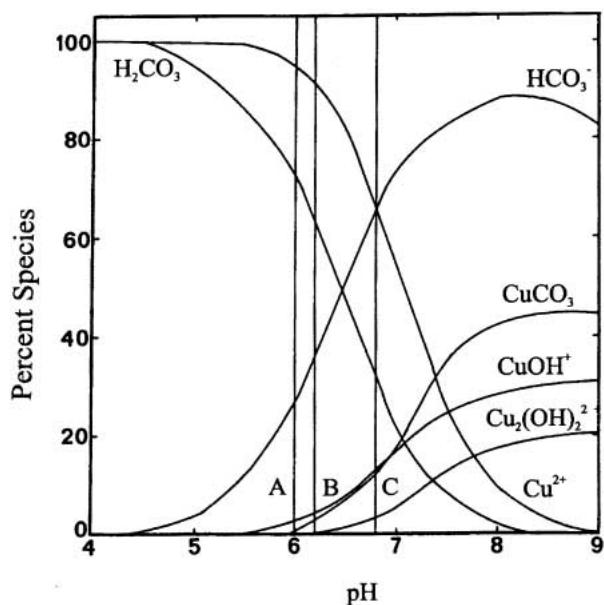
There have been many detailed reviews of the aquatic chemistry, speciation and aquatic toxicity of copper and other heavy metals in natural water (42, 43, 15, 16, 44, 45, 46). It is now generally accepted that the toxicity, bioavailability and transport of heavy metals in aquatic systems is determined by their speciation or physico - chemical form. This speciation and its influence on the toxicity of copper to algae has a determining influence on the success of copper algicide application in waters with different chemistry. The following is a brief review of aquatic chemistry of copper in natural water in relation to how this influences its use as an algicide.

Copper as copper sulphate (CuSO<sub>4</sub>·5H<sub>2</sub>O) generally dissolves readily in water to initially give the free cupric ion (Cu<sup>2+</sup>) in the hydrated form (aquo ion), [Cu(H<sub>2</sub>O)<sub>6</sub>]<sup>2+</sup> (42). The copper (II) in solution is a strong complexing agent and in the free cationic form has a strong tendency towards hydrolysis.

The resultant speciation of copper added to water will be dependent upon a number of competing reactions which are influenced by copper concentration, pH, redox potential and the types and concentrations of organic and inorganic ligands and colloidal surfaces present (45). The level of total copper in solution will also change with time and is dependent upon the kinetics and extent of the reactions which control the three processes of complexation, precipitation and adsorption. All of these processes ultimately determine bioavailability and toxicity. This is because most studies of the toxicity of heavy metals to aquatic organisms have indicated that the free ionic form (eg. Cu<sup>2+</sup>) or readily dissociable forms of copper are the most toxic species, and that toxicity is related to the activity of the free metal ion rather than the total metal concentration (47, 15, 16, 44, 48, 6, 49). This has been demonstrated with various algae (50, 51, 52, 53, 54, 55, 56, 57, 58).

The proportion of copper which is in the toxic form (ie. Cu<sup>2+</sup> or readily dissociable copper complexes) will depend upon the concentration and type of inorganic ligands (eg. hydroxide and carbonate) and organic ligands present in the water. The effect on metal toxicity of changes in pH, hardness, alkalinity and dissolved organic carbon in a particular water will be to alter the equilibrium between the ionic copper and the complexed forms and thereby change the concentration of the toxic form (59).

In relation to inorganic copper complexation in natural water it has been shown that pH and hardness (Ca, Mg, CO<sub>3</sub> / HCO<sub>3</sub> concentration) interact to control the ionic copper (Cu<sup>2+</sup>) concentration and thereby influence toxicity. Considering the inorganic system alone, the distribution of major copper species in relation



**FIGURE 1:** The speciation of copper (II) and carbonate as a function of pH. Diagram after Sylva (60).

to pH can be computed by chemical equilibrium models (60, 6, 19). A typical example of this is shown in Figure 1.

The figure indicates that below pH 7 free  $\text{Cu}^{2+}$  is the major species whereas above neutral the hydroxy - aquo complexes ( $\text{CuOH}^+$ ,  $\text{Cu}_2(\text{OH})_2^{2+}$ ), and various copper carbonate complexes ( $\text{CuCO}_3(\text{aq})$ , malachite(s) and tenorite(s)) are predominant. It can be seen that copper may start to precipitate at neutral to alkaline pH and be lost from solution at the copper concentrations and in the pH ranges commonly encountered during algicide application. The time course of this precipitation however may take several days or longer (6). The formation of copper carbonate complexes in hard alkaline water has been shown to reduce the toxicity of copper under these water chemistry conditions by reduction of free ionic  $\text{Cu}^{2+}$  (61, 62, 63).

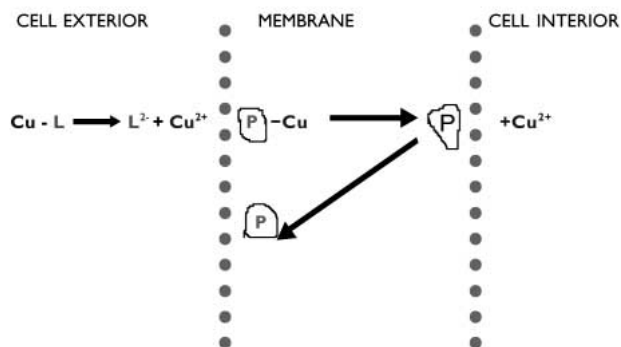
It is clear that copper complexation by inorganic ligands can affect its toxicity, but there is also evidence that in many freshwaters metals are mainly complexed with organic matter (15, 16, 64, 65, 66). It has been suggested that in freshwaters with high dissolved organic carbon (DOC) that the majority (98 - 100%) of copper is complexed by humic and fulvic acids and other natural adsorbents, and the bound copper is essentially non-toxic to aquatic organisms (67). Numerous studies with both laboratory cultures of algae and field populations have indicated that copper complexation by organic ligands reduces the toxicity of the added metal. (52, 24, 68, 17, 69, 70).

## MECHANISM OF COPPER TOXICITY

Copper is an essential micronutrient for growth of algae and cyanobacteria, and is required for various metabolic and enzyme processes (71). However, although it is a

required element, at concentrations higher than this essential "concentration window" copper is highly toxic to cells (15, 16). The mechanism of copper toxicity is likely to be concentration dependent. At the extremely high concentrations of both total and ionic copper encountered at the water surface during copper sulphate application (72), it is likely that the cell membranes are actually lysed by the oxidative activity of copper ions on membrane proteins. Early experimental studies with green algae (*Chlorella vulgaris*) showed that copper caused significant loss of cell potassium, indicating damage to membrane permeability (73). Evidence of cell lysis was also found in one study where membrane damage was seen within 24 hours in cultured cells of the cyanobacterium *Microcystis aeruginosa* following treatment with copper ( $0.64 \text{ mg Cu L}^{-1}$  as  $\text{CuSO}_4$ ) (34). The damage to cell integrity was confirmed by release of intracellular toxin from cells of a natural bloom of hepatotoxic *M. aeruginosa* similarly treated with copper sulphate (34). Jones & Orr (74) also found that the copper algicide COPTROL<sup>®</sup> caused cell lysis and release of microcystin into the water within 3 hours following the treatment of a dense bloom of toxic *M. aeruginosa*.

At lower concentrations metals need to be transported into the cell to exert physiological and toxic effects. Copper ions are believed to be transported into cells by a process of facilitated diffusion across the membrane (Figure 2) (48). This involves "shuttle" transport via a carrier protein which removes copper from its hydrophilic complex ( $\text{Cu} - \text{L}$ ) in solution and carries it, bound to a more hydrophobic complex, through the membrane to then be released into the cell where it is then bound possibly by thiol groups (48, 67). Copper availability for transport is therefore dependent upon strength of binding and rates of dissociation from the solution ligands at the membrane surface. This has implications for the use and toxicity of copper chelates as algicides.



**FIGURE 2:** Diagrammatic representation of the transport of copper complexes through a membrane and into the cell by facilitated diffusion. Diagram after Florence (48).

Once copper has entered the cell it can exert toxicity in a number of ways. One proposed mechanism is that copper may react with -SH groups in enzymes and free thiols (eg. glutathione) leading to inactivation of enzymes and processes of cell division (75, 76). The combined inhibitory effects of copper on both photosynthesis and growth have been established in numerous other studies with a range of algal and cyanobacterial species (77, 78, 79, 80, 71)

## CHEMISTRY AND TOXICITY OF CHELATED COPPER ALGICIDES

It is clear from the previous section that free ionic ( $\text{Cu}^{2+}$ ) or loosely complexed copper is the bioavailable fraction. For copper to exert intracellular toxicity to algae it must be dissociated from soluble copper complexes and be transported by the facilitated diffusion process into the cell (Figure 2). Most of the copper applied during copper sulphate dosing in hard alkaline waters with high DOC is likely to become rapidly and strongly complexed to inorganic ligands ( $\text{HCO}_3^-$ ,  $\text{CO}_3$ ) and organic ligands (DOC) and be rendered effectively non-toxic to algae. The rationale for adding copper as an organic chelate is to overcome this rapid complexation and loss of toxicity.

For copper chelated algicides to work effectively copper must stay in solution with the chelator and be progressively dissociated from the ligand at the membrane transport site or in solution. In this way the chelated algicide could provide a "copper - buffer capacity" by progressively dissociating to release  $\text{Cu}^{2+}$  either directly to cells or possibly to form toxic hydroxy-aquo complexes in solution. This model has been proposed to explain the toxicity of copper citrate as an algicide in alkaline water (19).

## COPPER SULPHATE APPLICATION METHODS

During the actual practice of copper sulphate dosing, the processes of copper solution, dispersal and complexation chemistry in relation to toxicity is likely to be highly dynamic. A study of copper sulphate treatments in South Australia showed that algae at the surface are exposed to exceedingly high concentrations of both ionic and total copper immediately after application (72). As the copper is subsequently diluted and dispersed the concentration of the most toxic ionic form ( $\text{Cu}^{2+}$ ) decreased to negligible levels, mainly due to complexation, within 60 - 120 minutes. By contrast the loss of total copper from the water column was relatively slow, with only 10 - 20 % reduction in added copper from solution within 24 hours. The method of application and kinetics of the various complexation reactions both have implications for successful treatment.

Physical factors such as water depth to be treated and the presence of thermal stratification influence the dispersal of surface applied copper and its contact with the target organism (72, 17, 81). The study by Burch *et al.* (72) demonstrated that when thermal stratification occurs and is combined with significant copper complexation this leads to rapid loss of the most toxic  $\text{Cu}^{2+}$  fraction shortly after application within the surface mixed zone. In this case only the target organisms in the surface mixed zone are likely to be exposed to toxic effects from the treatment.

The method of application of copper sulphate may have an important influence on copper dispersal and ultimately the toxicity and success of treatment. The traditional copper sulphate application method used in North America has been the dragging of burlap bags filled with  $\text{CuSO}_4$  crystals alongside or behind motorboats (82, 83, 17). In some cases, metal cages were used to replace sacks as long as 70 years ago (82).

Alternative application methods include pre-solution and spraying (84, 85); dry feeding via a belt-conveyor to the propeller stream and wake of the boat (83); the use of modified agricultural spreaders on barges (29); and dry scattering by a specially designed blower system (82). The application of dry copper sulphate from crop duster aircraft has also been reported (86).

## ALGICIDES AND TOXIC CYANOBACTERIA

Algicides have a useful role in management of the growth and proliferation of toxic cyanobacteria in reservoirs. They can be used to prevent or reduce to some extent the problems of toxins in the associated drinking water supply. Algicides can provide effective "once-off" short-term control of growth of cyanobacteria, at the source in the reservoir. This is useful in circumstances where alternative drinking-water sources are not available and other preventive measures are either not available or effective. Algicide treatment may be more cost-effective than toxin removal in drinking-water treatment plants, as has been suggested for the control of off-flavour problems (5), because an extended period of cyanobacterial contamination means there can be a requirement for costly enhanced treatment (eg PAC or GAC) for toxin removal.

Before applying algicides against toxic cyanobacteria it is important to be fully aware of both the environmental and practical problems with their use.

The environmental concerns are important in that the most commonly used algicide-copper sulphate, has wide ecological impact. Copper is a broad-acting aquatic biocide, which will also be toxic to non-target organisms such as zooplankton, other invertebrates and fish. It

should be used only in dedicated water supply reservoirs, and even then it is an unsatisfactory long-term solution. In many countries there are national or local environmental regulations which prohibit or limit the use of algicides due to their adverse environmental impact. This needs to be established prior to considering the use of algicides.

There are also important practical issues and algicides must be applied correctly to work effectively. It is important that if algicides are used they must be applied at the early stages of bloom development when cell numbers are low. This will reduce the potential for the release of high concentrations of intracellular toxin associated with dense blooms. Early application will also enhance the effectiveness of treatment as cyanobacterial cells can form a major part of the "copper demand" along with other organic matter in natural water (87).

Another consideration with using algicides is that they disrupt cyanobacterial cells and this leads to the release of toxins and taste and odour compounds from the cells. Studies have indicated that cyanotoxins are predominantly intracellular in healthy cells, and are only released into the water at an advanced stage of bloom decline, or following treatment with chemicals such as algicides (88). This release can be quite rapid and has been shown to occur within 3-24 hours in different studies (74, 34). These dissolved toxins will then disperse and be diluted throughout the water body, and will not be readily removed by conventional treatment processes of flocculation and filtration. Dissolved toxin then needs to be removed by adsorption with activated carbon (PAC or GAC) or by advanced oxidation (eg ozone). There is a well-known example of the danger of treating dense blooms with algicides from Australia (89). In this incident, which occurred on tropical Palm Island in 1979, many members of the community became ill with hepatitis following treatment of the water supply reservoir with copper sulphate for a cyanobacterial bloom problem (89). It has been retrospectively presumed that this illness may have been caused by the release of toxin into the water, which subsequently passed into the drinking water supply in significant concentrations (90).

It is good practice that if algicides are used to control toxic cyanobacteria then the reservoir should be isolated for a period to allow the toxins and odours to degrade. Unfortunately, there is very little information on the minimum withholding period in relation to toxin loss as it will depend upon local circumstances (ie temperature, microbial activity), but it could be in excess of 14 days (74).

In summary there are some practical guidelines given by Hrudehy *et al* (87) for the use of algicides for the control of toxic cyanobacteria:

1. Because cyanobacterial toxins are primarily intracellular, algicides must be used with great caution to avoid release of intracellular toxins. Algicides should be used at low cell numbers to avoid excessive toxins or off-flavours following rupture of the cells. This should be checked by monitoring.
2. Algicides may be used at higher cell numbers only if the reservoir can be taken out of service until the toxins and off-flavours degrade, or if treatment for removal of these toxins and other contaminants is available. In the latter cases the utility of algicide use should be assessed against the capability for whole cell removal offered by treatment processes, because cell removal may be the safer approach.
3. Algicides should only be used in waters where the environmental impacts are acceptable. Check with the local environmental agency.
4. It is important to know how effective the chosen algicide is in your waters. For example, copper may be less effective in waters with high dissolved organic carbon or at alkaline pH.

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# ECOLOGY AND MANAGEMENT OF CYANOBACTERIA IN RIVERS AND RESERVOIRS

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## ABSTRACT

There are three requirements for phytoplankton growth; a nutrient source, an adequate light supply and an inoculum from which the population can propagate. The cyanobacteria are well adapted to scavenge for these resources in stratified waterbodies. In the lower River Murray, Australia, low flow conditions can lead to stratification and a suitable habitat for cyanobacteria. During these periods *Anabaena* spp. tend to grow, whereas during high flow there is no stratification, and the diatom *Aulacoseira* dominates. Consequently, in regulated rivers the magnitude and timing of discharge can be manipulated to disrupt stratification every few days thereby controlling cyanobacterial growth. Similarly, in reservoirs the cyanobacteria tend to dominate when the water-column is stratified. At Myponga Reservoir *Anabaena* populations grew when the surface mixed layer became shallow and the cells remained entrained near the surface. Artificial destratification is one of few available management options available to control cyanobacteria. However, techniques are still required to effectively disrupt the surface mixed layer which escapes entrainment by conventional bubble plume aerators. The internal nutrient load can also be controlled with artificial destratification, which along with catchment sources of nutrients, contribute to the magnitude of the phytoplankton biomass.

## INTRODUCTION

The cyanobacteria are an extremely well adapted group of photo-autotrophic organisms which dominate the freshwater phytoplankton community during periods of calm, stratified conditions. Whilst the cyanobacteria have existed on earth for more than 2.5 billion years (1), there is a general opinion that "cyanobacterial blooms" are increasing in frequency due to anthropogenic eutrophication (2) and the regulation of waterways with weirs and dams.

The success of the cyanobacteria is, in part, attributable to the gas vesicles which provide buoyancy (3,4,5). The ability to float into the illuminated surface water in stratified water-bodies provides a distinct advantage over other phytoplankton which rely on turbulence to remain entrained. By floating upwards the cyanobacteria can significantly increase light capture and consequently increase productivity (6,7,8), nitrogen fixation (9) and growth (10).

There are three requirements for phytoplankton growth; a nutrient source, an adequate light supply and an inoculum from which the population can propagate. The growth rate of phytoplankton is determined by the rate of resource delivery and the maximum attainable biomass

is determined by magnitude of the nutrient resources (11,12).

Many features of lakes and reservoirs are dominated by the hydrodynamics, particularly the extent and duration of thermal stratification. The buoyancy of cyanobacteria enables them to float during periods of low turbulence when other species tend to sediment below the diurnal thermocline. The depth of the diurnal thermocline changes in response to wind mixing, air temperature and consequently on warm, calm nights a shallow diurnal thermocline persists. As the diurnal thermocline deepens, phytoplankton are mixed deeper, spend more time out of the euphotic zone, and may become light limited. Species such as *Microcystis aeruginosa* and *Anabaena circinalis* have maximal growth rates when they experience a daily light dose of photosynthetically active radiation (PAR: 400-700nm) of approximately 7 mol photons m<sup>-2</sup> d<sup>-1</sup> (13). This occurs when the diurnal thermocline is approximately the same depth as the euphotic depth (assuming maximum short wave radiation is 1000 Wm<sup>-2</sup>).

With a suitable physical environment established, nutrients and an inoculum are required for a cyanobacterial hazard to eventuate. Although cyanobacteria are often perceived as symptoms of eutrophication, the paradox is that they do not require

high nutrient concentrations to reach relatively high biomass. Concentrations of phosphorus less than  $0.01 \text{ mg L}^{-1}$  filterable reactive phosphorus (FRP) are considered to be growth limiting (14) and  $0.1 \text{ mg L}^{-1}$  soluble inorganic nitrogen is considered the minimum concentration to maintain growth during the growing season (12). Higher concentrations support rapid growth and higher biomass.

The risk to water quality from cyanobacteria arises firstly from taste and odour compounds, which are problematic at low cell concentrations (15) and secondly from toxins which become problematic at higher biomass (16).

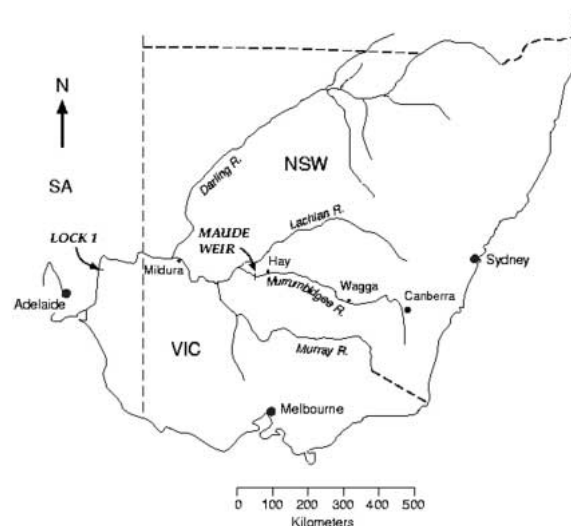
The aim of this paper is to describe the physical and chemical conditions which supported cyanobacterial growth in a lowland river and in a reservoir in South Australia. The principles of when cyanobacteria are likely to occur can be transferred to other water-bodies where the conceptual models can be tested. Management options for control of cyanobacteria by destratification and control of nutrient sources are discussed.

## METHODS

### Study Site - Lower River Murray

South Australia is heavily dependent on the lower River Murray for potable water and irrigation. In an average year the river supplies 50 per cent of South Australia's urban water consumption and in dry years this can increase to 90 per cent (17). South Australia receives a guaranteed water allocation which is specified in the Murray Darling Basin Agreement and is termed the 'entitlement flow'. This agreement between four states (Queensland, New South Wales, Victoria and South Australia), and the Commonwealth, guarantees South Australia 1850 GL per annum distributed in monthly allocations (17). This entitlement represents approximately 30% of the mean annual flow to South Australia (6570 GL).

The Murray-Darling river system drains the western slopes of the Great Dividing Range in south-east Australia. The River Murray originates in south-east New South Wales (NSW) and joins the Darling River which originates in north-east NSW. The Murray and Darling Rivers meet at Wentworth, which is 827 river km from the Murray Mouth where the river exits to the Southern Ocean. The combined length of the Darling and Murray rivers is 5500 km and the average annual discharge is 10,035 GL of which the Darling contributes about 10% (18). The lower River Murray, in South Australia, is regulated by six weirs which were built as aids to navigation and water diversion for irrigation (19). This river section is meandering and the flow is slow with a mean bed slope of  $1:360,000$  (19).



**Figure 1.** Map of South-eastern Australia showing major rivers of the Murray-Darling Basin and the lower River Murray in South Australia.

### Study Site – Myponga Reservoir

Myponga Reservoir is a flooded river valley impounded by a concrete arch-dam which was completed in 1962. Myponga Reservoir is located 70 km south of Adelaide, South Australia ( $S 35^{\circ} 24' 13''$   $E 138^{\circ} 25' 29''$ ). The reservoir has a capacity of 26800 ML at a full supply level of 211.7 m A.H.D. (Australian Height Datum), an average depth of 15 m with a maximum depth of 36 m. The mean retention time based on abstraction is approximately 3 years. The area of water-spread is  $2.8 \text{ km}^2$  and the catchment is  $124 \text{ km}^2$ . Water is removed from the reservoir via an off-take valve, located on the dam wall, at 195.2 m A.H.D. The multi-diffuser aerator was installed in 1994 and is located adjacent to the dam wall at a depth of 30 m. The aerator diffuser has 160 outlets over a length of 200 m and delivers air at  $120 \text{ L s}^{-1}$ . In addition to the aerator, there are two surface mounted mechanical mixers which were deployed in 1999 and pump water downward through a 15 m draft-tube at a rate of  $3.5 \text{ m}^3 \text{ s}^{-1}$ . The aerator and mixers are operated between October and March each year. Copper sulphate application occurs in response to *Anabaena circinalis* presence, and generally consists of one application in early January at a dose rate of  $2 \text{ mg L}^{-1}$ . Normal operation is to dose at low cell numbers ( $500\text{-}1000 \text{ cells mL}^{-1}$ ) to prevent taste and odour problems.

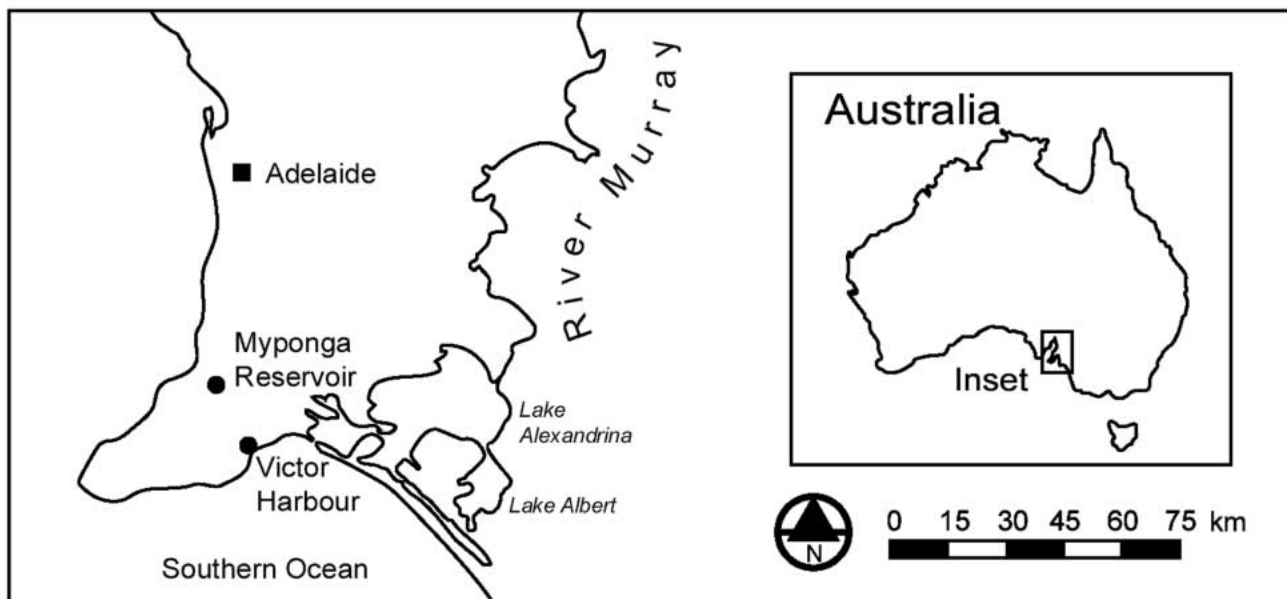


Figure 2. Myponga Reservoir in South Australia.

### Sampling and Enumeration of Phytoplankton

Water for phytoplankton enumeration and identification was sampled using a five meter hose-pipe to integrate the sample in vertical profile. Samples were preserved in Lugol's iodine and counted after 10x concentration in a settling column. Lower River Murray samples were collected at Morgan, 50km North of Lock 1 (Figure 1), and Myponga reservoir samples were collected at five sites in the reservoir. Murray River flow records were obtained from the Murray Darling Basin Commission.

Meteorological stations were deployed on both the lower River Murray and Myponga Reservoirs. Meteorological data was logged every 10 minutes for all instruments. The instrumentation installed on the stations monitor the following: temperature ( $^{\circ}\text{C}$ ) at 20 depths through the water column, wind speed ( $\text{ms}^{-1}$ ) and direction ( $^{\circ}$ ), relative humidity (%), air temperature ( $^{\circ}\text{C}$ ), incoming short-wave radiation ( $\text{Wm}^{-2}$ ), down-welling and up-welling long-wave radiation ( $\text{Wm}^{-2}$ ).

## RESULTS AND DISCUSSION

### Cyanobacteria in the lower River Murray - *Anabaena* and flow

Flow is a major factor determining the phytoplankton abundance and species composition in Australian lowland rivers (17,20,21). In the River Murray at Morgan there was a transition between the dominant species *Aulacoseira granulata* and *Anabaena* spp. which was highly dependent on flow (Figure 3). During periods of high flow ( $>10,000 \text{ ML day}^{-1}$ ) the diatom *Aulacoseira granulata* dominates. At low flows *Aulacoseira* tends to sediment out

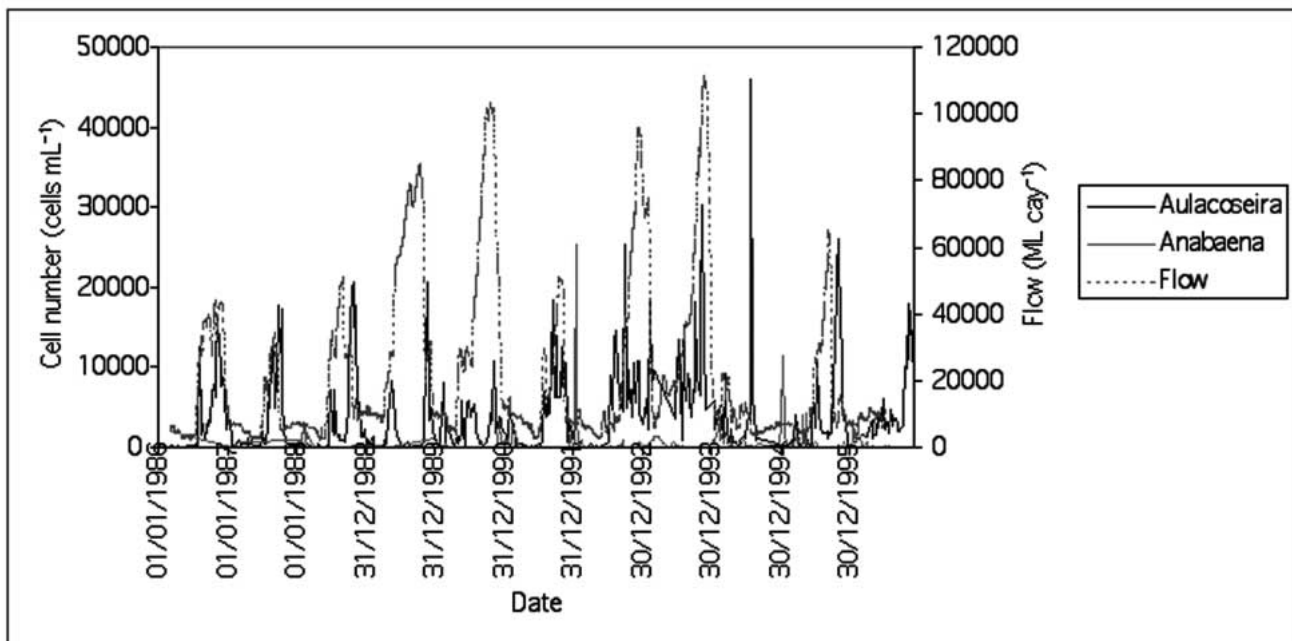
of the photic zone and growth cannot be sustained (22). The incidence of *Anabaena* spp. was usually restricted to daily flows less than  $6000 \text{ ML day}^{-1}$  (17) which coincides with periods of stratification (23,24).

On some occasions (1990-1991) relatively high concentrations of *Anabaena* were observed during high but receding flow. It is believed that these populations originated in lagoons adjacent to the main river channel but with an hydraulic connection (17,19,25). The wetland lagoons are shallow, provide a good habitat for cyanobacteria and often record higher cyanobacterial numbers than the main river channel. As the river hydrograph recedes the wetlands drain back into the main river channel and may contaminate the river with cyanobacteria.

### Observations from the lower River Murray

Baker *et al.* (25) tracked time-aligned parcels of water in a reach of the lower River Murray during summer 1994/1995 and calculated growth of the dominant species. Mean growth rates of *Anabaena circinalis* and *A. flos-aquae* were  $0.176$  and  $0.132 \text{ day}^{-1}$  respectively. However, maximum cell densities were only 2670 and 4560 cells  $\text{ml}^{-1}$  for each species which is low abundance and suggests that nutrients or grazing were limiting phytoplankton yield.

During the summer of 1995/1996 nutrient growth bioassays and FDA-conversion bioassays (26) revealed nitrogen limitation on several occasions in the lower River Murray and the nitrogen present was essentially unavailable for growth. In contrast phosphorus limitation was detected on only one occasion and between 8 and 70% of the total phosphorus was bioavailable (25).



**Figure 3.** River flow and the dominance of *Aulacoseira* or *Anabaena* at Morgan on the lower River Murray.

It is likely that although phosphorus may ultimately limit total phytoplankton biomass, low nitrogen concentrations in the River Murray favour heterocystic nitrogen-fixing species such as *Anabaena circinalis* and *Anabaena flos-aquae* while limiting the growth capacity of non-nitrogen fixing species such as *Microcystis aeruginosa*. Physical conditions in the lower River Murray during summer are similar to those that favour growth of *Microcystis* (27), but populations of *Microcystis* did not become established during the study period nor in any other year for which there are records. The particular combination of physical and chemical conditions presumably favoured the growth of *Anabaena* in preference to non-nitrogen fixing cyanobacteria, albeit at sub-optimum conditions.

In the summer of 1995/96 most of the water in the lower River Murray was sourced from the upper Murray and its tributaries, which historically have lower nutrient loads than the other major tributary, the Darling River (28). In the summer of 1996/97, a higher contribution of Darling River water resulted in elevated levels of both N and P in the lower River Murray and consequently the non-nitrogen fixing cyanobacterium *Planktothrix perornata* was found in relatively high abundance (29).

### Conceptual Model

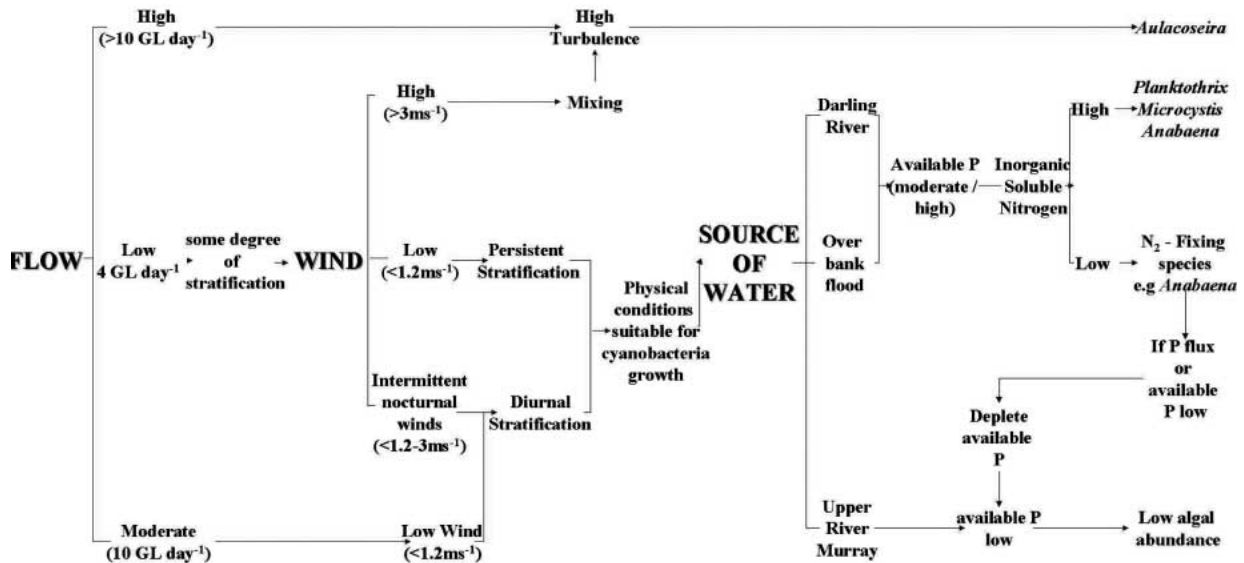
A conceptual model has been developed to summarise the physical and chemical factors governing the abundance of cyanobacteria in the lower River Murray (Figure 4). Flow is ranked as the primary factor affecting the development of cyanobacterial populations. High flow (>10,000 ML d<sup>-1</sup>) results in high turbulence and species such as the diatom, *Aulacoseira granulata* are favoured.

When flow is moderate (ca. 10,000 ML d<sup>-1</sup>), diurnal stratification occurs if wind strength is low (<1.2 ms<sup>-1</sup>). During periods of low flow, equivalent to summer entitlement flows (4,000 ML d<sup>-1</sup>), turbulence is sufficiently low to allow some degree of thermal stratification, provided that wind strength is low to moderate (<3 ms<sup>-1</sup>). Persistent stratification may result when wind speed is <1.2 ms<sup>-1</sup> (and flow is low), while diurnal stratification is more likely at wind speeds between 1.2 – 3 ms<sup>-1</sup>. Irrespective of flow, high wind speed (>3 ms<sup>-1</sup>) will disrupt thermal stratification and result in a mixed water column.

In the model, the source of water is the other environmental factor affecting algal dynamics. If water is sourced from the Darling River, or if over-bank flooding has occurred during the high spring flows originating from the upper River Murray, phosphorus concentrations may increase. When P concentration is high, nitrogen availability will then determine which species dominate. High soluble inorganic nitrogen availability favours non N<sub>2</sub>-fixing cyanobacterial genera such as *Microcystis* and *Planktothrix* and other phytoplankton. However, if nitrogen availability is low, nitrogen fixing genera, such as *Anabaena*, *Aphanizomenon*, *Anabaenopsis* and *Cylindrospermopsis* obtain a comparative advantage. If river flow originates from the upper River Murray and flow remains within the main channel, available phosphorus will be low and consequently phytoplankton abundance will remain low.

### RIVER MANAGEMENT

The correlation between the occurrence of *Anabaena* with low flow suggests that the manipulation of flow may be used to control cyanobacteria. Bormans and Webster



**Figure 4.** Conceptual model of the physical and environmental factors governing cyanobacterial abundance in the lower River Murray.

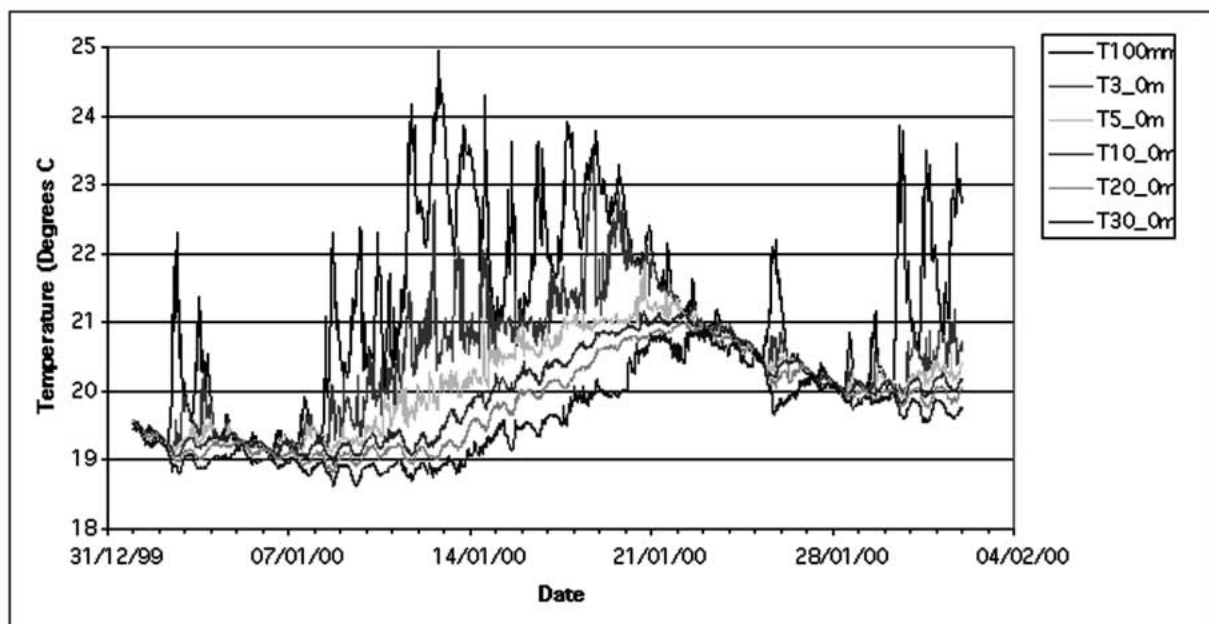
(24) developed a mixing criterion for turbid rivers which is applicable to many lowland rivers. In summary, the degree of stratification in a water column is determined by the relative supply rates of stratifying thermal energy and destratifying turbulent kinetic energy (TKE). In regulated rivers the magnitude and timing of discharge can be manipulated to disrupt stratification every few days thereby controlling cyanobacterial growth. Maier *et al.* (30) have modelled various flow scenarios for the control of stratification. They found that under current entitlement flow there is little risk of sustained blooms in the lower River Murray at Morgan. Consequently the best mechanism for cyanobacterial control is not to increase base flow but to disperse existing blooms with flow derived from reducing weir pool levels upstream.

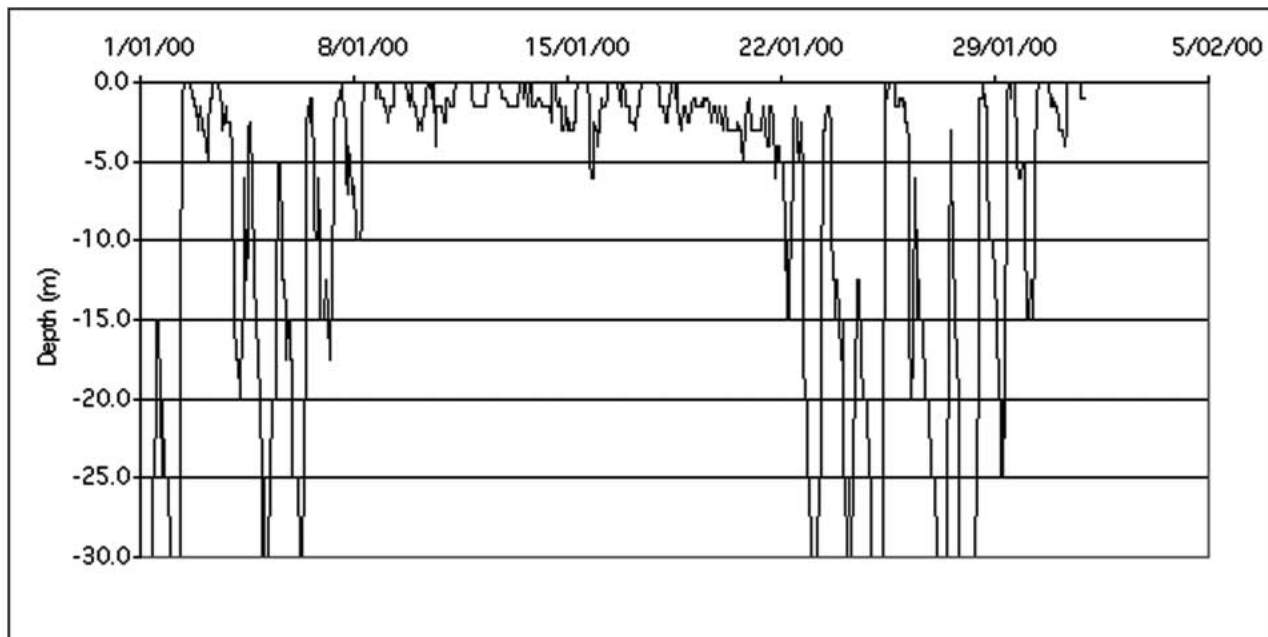
The proviso to this management strategy is that managers must consider the implications of this flow regime on aquatic macrophytes and benthic algae, which are adapted to particular water regimes, and provide a habitat for many organisms in the ecosystem.

### Cyanobacteria in Myponga Reservoir – Population Development

Myponga Reservoir is generally a well mixed site and cyanobacterial concentrations are low. However, during summer the physical conditions can become suitable for *Anabaena circinalis* to grow rapidly. Myponga Reservoir was well mixed in early January 2000, however, there was significant energising of the surface water between January 7 and January 21 (Figure 5). The result of this

**Figure 5.** Temperature profile at Myponga Reservoir





**Figure 6.** Depth of surface mixed layer, defined as the shallowest depth at which the temperature difference between two adjacent thermistors is 0.05 °C or greater.

persistent stratification was that the diurnal surface layer remained shallow (Figure 6) and the cyanobacteria were not entrained deep into the water column.

The euphotic depth in January was 3.6m (light attenuation coefficient:  $k_d=1.22$ ) and nutrient concentrations were sufficiently high to support rapid growth rate and high yield: Ammonia – 0.027 mg L<sup>-1</sup>, Filterable reactive phosphorus – 0.038 mg L<sup>-1</sup>, Total phosphorus – 0.051 mg L<sup>-1</sup>, Total Kjeldahl Nitrogen – 0.98 mg L<sup>-1</sup>, Nitrate and nitrite – 0.131 mgL<sup>-1</sup>. *Anabaena circinalis* was not detected on 14 December 1999, but was detected later in December, albeit at low numbers (Table 1). As the water column stratified, *A. circinalis* growth accelerated and by 10 January 2000 the highest recorded concentration was 3891. The mean growth rate between 4 January and 10 January was 0.36 day<sup>-1</sup> and concentrations were high enough to present a geosmin (taste and odour) threat to the treatment plant.

In this case the *A.circinalis* population was controlled with a chemical algicide on 11 January 2000, however, in reservoirs where algicides are not used, early warning of cyanobacterial risks can enable treatment, such as activated carbon dosing, to be scaled accordingly.

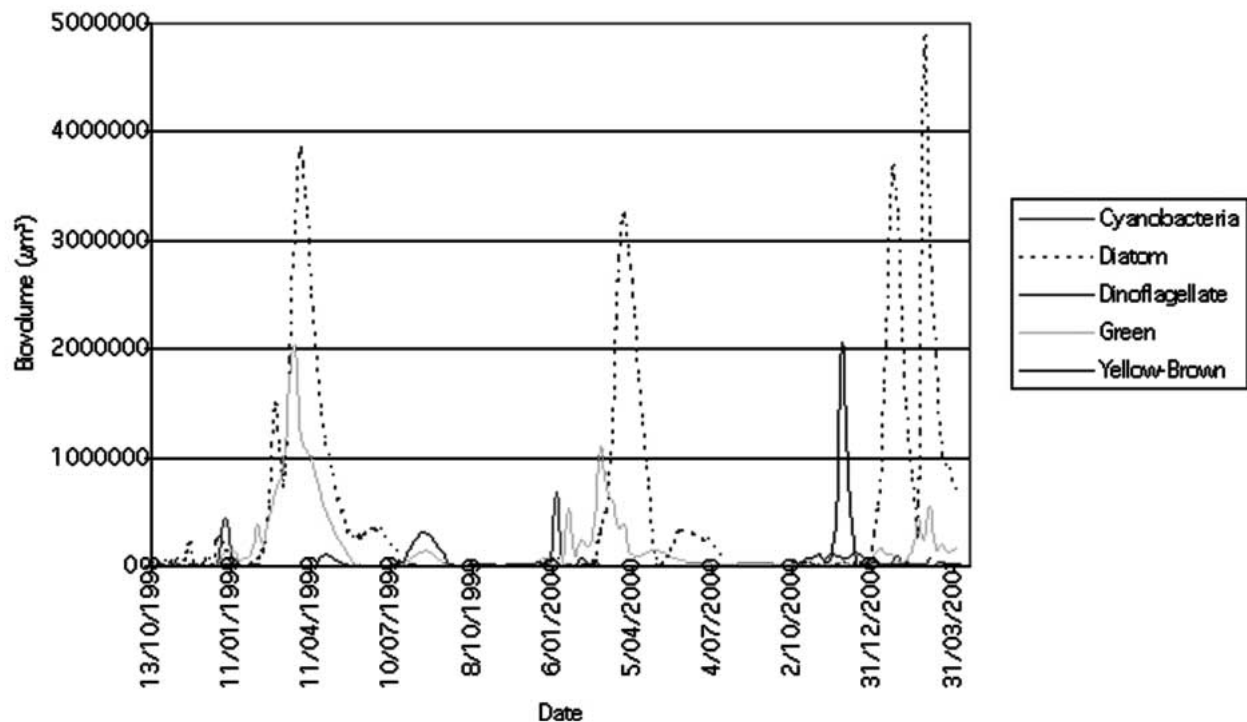
**Table 1.** *Anabaena circinalis* concentrations (cells mL<sup>-1</sup>) at five locations at Myponga reservoir. – signifies *A. circinalis* not detected in a 1mL, 10x concentrated sample.

| Location | 21/12/99 | 29/12/99 | 4/01/00 | 10/1/00 | 18/1/00 | 25/1/00 |
|----------|----------|----------|---------|---------|---------|---------|
| 1        | 4        | 9        | 43      | 3891    | 45      | 2       |
| 4        | –        | –        | –       | 2186    | 2       | –       |
| 5        | 29       | –        | –       | 146     | 12      | –       |
| 6        | –        | –        | 163     | 1470    | 30      | –       |
| 7        | –        | –        | 459     | 448     | 8       | –       |

Although there are two different destratifying systems in Myponga Reservoir, there is still strong persistent stratification in the surface layer as high nocturnal temperatures and low wind speed inhibit cooling. However, modelling studies have shown that the destratifiers have significantly reduced the period when *Anabaena* can grow (David Lewis unpublished data). The phytoplankton biomass at Myponga Reservoir is dominated by green algae and diatoms, which rely on turbulence to remain entrained, and the conditions when cyanobacteria grow is narrowed to a brief period in summer each year (Figure 7).

## MANAGEMENT OF CYANOBACTERIA IN RESERVOIRS

There are two main types of strategies which can be implemented to control cyanobacteria. These are loosely termed 'top-down' and 'bottom-up control'. Top down control is also termed bio-manipulation and is the management of higher trophic organisms to maximise grazing of algae and thus reduce cyanobacterial abundance. The bottom-up approach is to restrict the supply of essential nutrients and thus restrict growth. In this argument bottom-up control is extended to include the restriction to supply of all resources. Therefore the manipulation of the light climate, by artificial mixing, is also included in bottom-up control. There is some dispute



**Figure 7.** The relative abundance of the different phytoplankton groups in Myponga Reservoir.

about how effective bio-manipulation would be in Australian systems (31) and this paper will be limited to a discussion on bottom-up control.

### Artificial destratification

During stratification the hypolimnion is effectively separated from the atmosphere and becomes depleted of oxygen. Under these reducing conditions contaminants, such as ammonia, phosphorus, iron and manganese are resolubilised from the sediment. The role of bubble plume aerators is to weaken stratification and work synergistically with wind to mix the reservoir and to oxygenate the hypolimnion. To control contaminant resolubilisation the hypolimnion must receive sufficient oxygen to satisfy the sediment oxygen demand. Artificial destratification has been relatively successful at controlling the release of contaminants from sediments (32) but has been less successful in controlling cyanobacteria (33). The inability of bubble plume aerators to mix the stratified surface layers, outside the immediate influence of the plume, has meant there is still a habitat for buoyant cyanobacteria to exploit (34).

Advances in destratification technology for cyanobacterial control need to address methods to attack the stratified surface layer. Surface mounted mechanical mixers have been trialed in Myponga Reservoir with the intention of drawing warm water containing cyanobacteria and depositing it at depth via a draft tube (35). Improvements in mixer design will

increase mixing efficiency and the ability of the mixers to pull more surface water (36).

An alternative to surface mounted mechanical mixers is bubble plume aerators with multiple diffusers at a range of depths. The theory is to have a large diffuser line to generate large scale circulation cells and shallower lines to destratify the surface layer. To date there is limited data on how successful these systems are.

### Controlling the nutrient load

The major nutrient sources are the catchment, the internal load derived from sediment and in some cases from atmospheric deposition. The sources contributing to elevated nitrogen oxide species in the atmosphere include soil emission, fossil fuel combustion, lightning and biomass burning in forests and savanna (37). These sources, together with nutrients derived from catchment, are difficult for water managers to control. However, strategies to retain phosphorus in the catchment, such as gypsum application (38) and optimised fertiliser application, will reduce the sustainable algal biomass in lakes and reservoirs. In some instances catchments are naturally high in phosphorus and consequently attempts to reduce phosphorus to limiting levels would be unsuccessful. In these cases alternative strategies to control algae should be sought.

The nutrient source which reservoir managers often do have the ability to control is the internal nutrient load or sediment-derived nutrient load. The internal nutrient



load is most often controlled by oxygenation of the hypolimnion either by artificial destratification or by direct oxygen injection. In systems where the internal nutrient load contributes significantly to the total nutrient load, a reduction in nutrient release from sediment can significantly decrease the sustainable algal biomass (39).

In a typical phosphorus cycle, phosphorus is remobilised from sediment or decaying organic matter and entrained into the water column, where it is taken up by algae. From there the phosphorus is either passed on to higher levels of the food web or lost to the bottom as the algae sediment. In deep lakes the resolubilisation of P at the sediment is vertically separated from the algae and so each P molecule can only be accessed with entrainment from the hypolimnion to the epilimnion. In strongly stratified deep lakes this may happen only once or twice a year during significant 'over-turn' events. In contrast shallow lakes have the zone of P resolubilisation much closer to the zone of greatest productivity and a single molecule may be recycled a number of times during the growing season (13) and thereby sustain a high algal biomass for longer. This is why shallow lakes prove so much more difficult to restore than deep ones (14,13)

A lake remediation strategy which has been used extensively to decrease nutrient concentrations in waste treatment ponds and some natural lakes is sediment capping with phosphorus binding agents. However, some of the chemicals used to flocculate phosphorus in waste treatment facilities, such as alum, iron salts and lime, are not suitable for application in natural systems. Recently there have been advances in the use of modified clays to bind phosphorus and reduce the filterable reactive phosphorus in water-bodies. However, this technology is not readily available nor in wide use.

There is evidence emerging that desiccation/oxidation of sediments can significantly reduce the release of phosphorus from lake sediments upon re-wetting (40,41). This has been attributed to a number of interrelated factors including a shift in the bacterial community structure (loss of viable sulfate-reducing bacteria), increased carbon limitation in the dried sediments and aging of minerals with which P can be associated (42). Whilst it is not known how long the effects of desiccation will last, drying the lake sediments may be a suitable strategy to reduce P release in some shallow lakes. Many natural shallow lakes may have undergone seasonal wetting and drying before modification of the hydrology to maintain unnaturally high water levels.

The phosphorus binding capacity of sediments depends upon the sediment type and the number of sites available to absorb phosphorus. If the sediment has received phosphorus rich waters for a considerable period then the binding sites may be saturated and removal of this sediment by dredging may be an effective

strategy to reduce phosphorus concentrations in the receiving water.

## CONCLUSION

To successfully manage cyanobacteria in rivers, lakes and reservoirs there needs to be integrated management of the catchment, the water-body and the water treatment facility. The successful management scenario will differ between systems and vary depending on which species present the greatest risk. However, limiting light by artificial destratification and controlling nutrient resources remain the most sustainable strategies to control cyanobacteria.

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# THE APPLICATION OF GENETICS AND MOLECULAR BIOLOGY FOR DETECTING TOXIC CYANOBACTERIA AND THE BASIS FOR THEIR TOXICITY

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## INTRODUCTION

Australia has, reportedly, experienced toxic algal blooms for more than 100 years, however indigenous peoples would consider this an underestimation. Most attention to this water quality problem is due to the massive surface blooms that covered more than a thousand kilometres of the Murray-Darling-Barwon river system of inland eastern Australia during 1990-91. Toxic algal (cyanobacterial) blooms in drinking water supplies of Australia are comprised of the genera *Anabaena*, *Cylindrospermopsis*, and *Microcystis*. Occurrences of domestic and native animal poisonings have been reported for several years and bloom analyses have revealed the presence of both neuro- and hepatotoxic species of cyanobacteria. Species of *Lyngbya* and *Nodularia* have also been implicated in either cases of poisoning by ingestion or as allergens by external contact.

Eutrophication plays a variable role in the formation of algal blooms in Australia. As with the types of climatic regions, Australia also possesses a number of water body types of varied morphology and ecology. Typical of other countries, Australia experiences most algal blooms during the summer months when increased temperatures, high light, and water column stratification (resulting in increased phosphorous availability) occur. However, certain parts of Australia experience almost year-round summers that can often be accompanied by persistent populations of cyanobacteria. Particularly affected are those regions that have been deforested and receive nutrients via phosphate fertilisers, such as farming land. In addition human populations that release relatively untreated sewerage into water bodies present an ever-increasing stimulus for bloom formation.

Many of the species of cyanobacteria that affect water quality in Australia are similar to those commonly found internationally. Exceptions to this include the presence of the saxitoxin-producing species *Anabaena circinalis* and the toxic invasive species *Cylindrospermopsis raciborskii*. Since the mid twentieth century the Australian government has invested in recording the occurrence of algal blooms and also in the affects of cyanobacterial toxins on selected animal models. Countermeasures have

included monitoring the effects of nutrient inputs, zooplankton grazing, and monitoring drinking water for the presence of toxin. More drastic and expensive countermeasures that have been used include copper sulphate dosing to kill the algae and flushing of the water system to increase flow. These reactive responses to toxic cyanobacterial blooms are, however, insufficient for dealing with mass occurrences of cyanobacteria in the future and may increase the exposure of humans over time to potentially carcinogenic algal substances as well as being detrimental to the environment as a whole. Soon after the massive blooms of 1990 in Australia the Sydney Water Corporation and the University of New South Wales embarked on a program of research for the proactive management of drinking water supplies, with regards to algal blooms.

## CYANOBACTERIAL DETECTION AND IDENTIFICATION METHODS

Traditional microscopic assessment of cyanobacteria does not afford assessment of potential toxicity and similarly, detection of cyanotoxins in water does not stop the poisoning (acute or chronic) of consumers. Research into the genetics and evolution of cyanobacteria has allowed us to accurately identify toxic species of cyanobacteria in water and sediment prior to these species proliferating into a bloom. In particular, we have developed DNA-based tests that allow the identification of the toxic species *Anabaena circinalis*, *Cylindrospermopsis raciborskii*, *Lyngbya majuscula*, *Microcystis aeruginosa*, and *Nodularia spumigena*. DNA-directed protocols enable specific analysis of cyanobacteria in a sample and the species listed can be readily differentiated by studying genes such as those encoding phycocyanin, 16S rRNA, and various repetitive elements (1, 2, 3, 4). However, not all species of cyanobacteria are toxic and not even all strains of a so-called species have the potential to produce toxin. Therefore it was critical to more precisely define toxic cyanobacteria in terms of their threat to water quality. One way of doing this would be to ignore species diversity and directly target the potential for toxin production by detecting genes involved in toxin biosynthesis.

Cyanotoxins are similar to secondary metabolites produced by other bacteria and fungi, and have chemical structures including cyclic peptide, polyketides, and alkaloids. We have determined candidate genes involved in the biosynthesis of microcystin, cylindrospermopsin and nodularin. These genes are found exclusively in toxic strains of the species *Microcystis*, *Cylindrospermopsis* and *Nodularia*, respectively. Not only has the elucidation of the genetic basis for cyanotoxin production allowed for very accurate identification of toxic cyanobacteria, it has also provided clues to how toxin production might be regulated. For instance, under condition of high light *Microcystis* not only produces more toxin, it also exports this toxin into the surrounding water. Thus the level of cyanotoxin in water may not only be related to lysis of cells within a bloom but also because of the prevailing ecological conditions.

## SPECIFIC TYPES OF TOXIC CYANOBACTERIA AND THEIR CYANOTOXINS

### 1. *Anabaena circinalis* and saxitoxin production

Although there is a worldwide distribution of *A. circinalis*, there is a segregation of PSP toxin production among strains. Toxic American and European isolates of *A. circinalis* produce either anatoxin-a or anatoxin-a(s), while toxic Australian isolates produce PSPs exclusively and at high levels. Whether this PSP-producing geographical localisation to Australian fresh waters is due to the genetic diversity of strains or environmental factors has not been determined. In this study the genetic diversity between Australian and overseas *A. circinalis* isolates was determined by analysing the 16S rRNA gene sequences. Cyanobacterial cultures were filtered and washed-free of any contaminating heterotrophic bacteria prior to DNA extraction and the amplification of the 16S rDNA gene by universal primers. 16S rRNA gene phylogeny was also analysed to determine the population structure within Australian toxic and non-toxic *A. circinalis* strains.

At all taxonomic levels the sequence analysis of the 16S rRNA gene has proven to be the best approach for the phylogenetic classification of cyanobacteria. Problems with this technique occur due to the presence of contaminating heterotrophic bacteria in cyanobacterial cultures. In such cultures if conventional PCR primers are used to target highly conserved regions of 16S rRNA genes, a heterogeneous mixture of amplification products which cannot be directly sequence result. As a consequence, there has been a development of methods for the purification of cyanobacterial cultures through the removal of accompanying heterotrophic bacteria.

Phylogenetic analysis of 16S rDNA from PSP toxic and non-toxic sequences from 25 worldwide *A. circinalis* strains found no significant delineation between strains. The analysis failed to identify a reliable topology for the

strains, with very few branches having significant bootstrap values. We interpret the low bootstrap values and the high degree of sequence similarity (99%) among the strains to indicate that the 16S rDNA sequence is too highly conserved to pinpoint reliable differences among these closely related strains. This non-specific phylogenetic association among toxic and non-toxic strains could be explained in several ways. First, the misclassification of strains, resulting from insufficient taxonomic classification of experimental strains. Many strains used commonly in experimental papers are referred to by the current classification name which does not correspond to the often designated name, for example with *A. flos-aquae* also known as *Nostoc commune*. Within some taxa, more than one morphotype has been described, where the type description encompasses both morphological variants. For more than 50% of the strains in culture collections, the taxonomic names do not correspond to the morphological description of the taxon. Normal morphological characters have been altered by changing the culturing conditions. Second, The toxicity of cyanobacterial strains varies depending on environmental and hence culturing conditions, including nutrient variation, temperature and light exposure. The amount of toxin in cultured *A. circinalis* has been found to increase over the growth cycle due to the transformation of less toxic PSPs to the more toxic forms. Although not reported thus far, cyanobacterial strains grown in culture may undergo mutations which affect toxin production. An originally toxic environmental sample may be recorded as a non-toxic isolate due to toxicity determination occurring after a strain has been cultured for some time during which mutations could have occurred. Strain *A. circinalis* AWQC148C was tested by HPLC directly after isolation however, HPLC toxicity testing after culturing for three years found this strain to be non-toxic. Third, there are limitations with the techniques used to detect toxicity. The Na<sup>+</sup> channel  $\alpha$ -H-saxitoxin binding and saxiphillin  $\alpha$ -H-saxitoxin receptor assays have been found to bind a representative of the C-toxins, C1, with much lower affinity with the former showing cross-reactivity with unrelated Na<sup>+</sup> channel-binding toxins. HPLC analysis, although being the most reliable at detecting the individual PSPs, is a slow process requiring multiple chromatographic analyses. The mouse bioassay, although easy to perform and requires no special equipment is not applicable for the detection of individual PSPs and the results are affected by the susceptibility of mice strains, extract dilution, extract, matrices and sample preparation. Fourth, in the environment there may be horizontal gene transfer of toxic genes between *A. circinalis* strains.

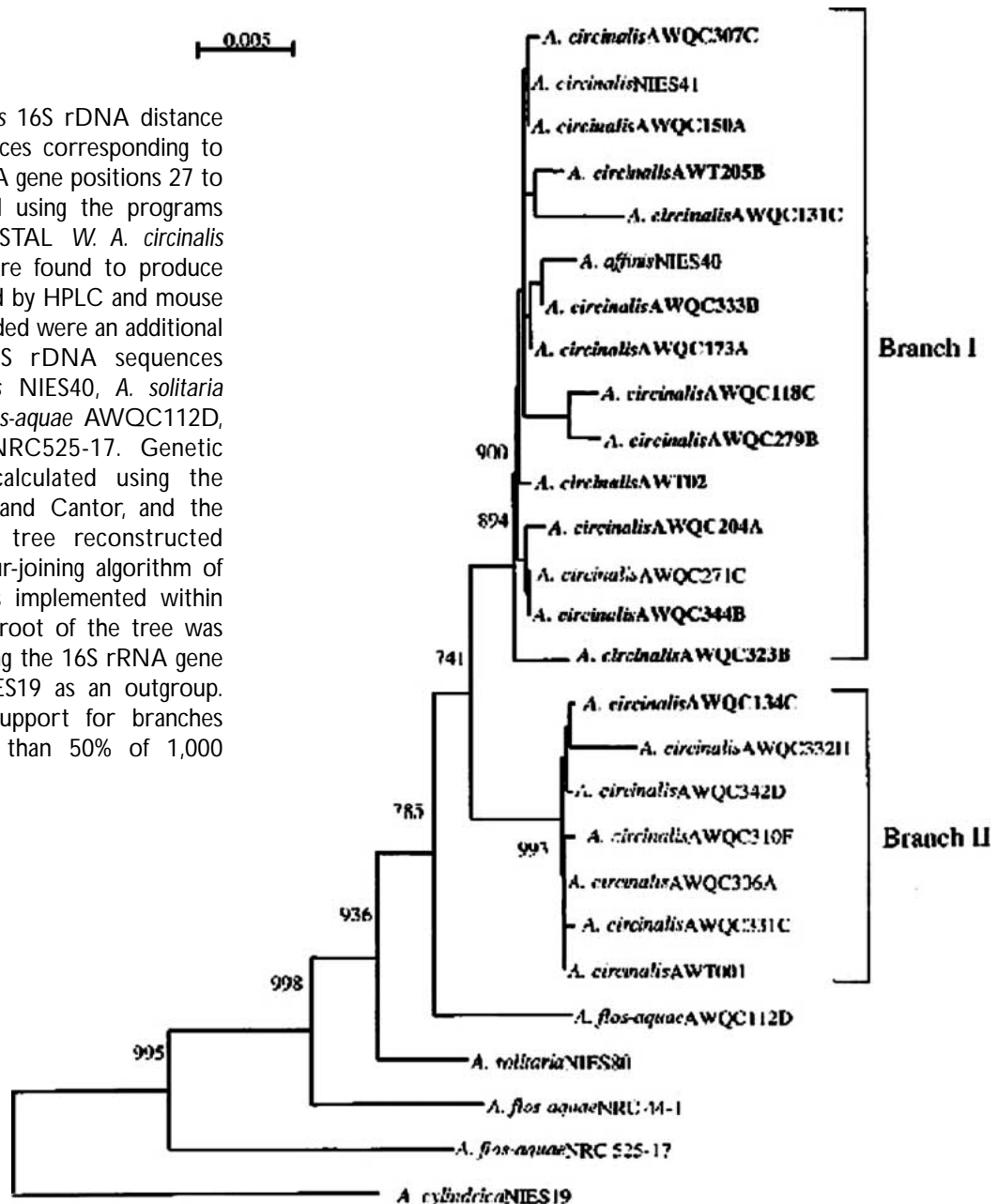
Although the 16S rDNA sequence data analysis found no consistent division between toxic- and non-toxic *A. circinalis* isolates from around the world, *A. circinalis* did form a distinct main phylogenetic species, with subspecies occurring within, cluster 1A, 1B, 2A and 2B. Two of these clusters, 2A and 2B contain *A. circinalis* strains interspersed with *A. flos-aquae* strains, casting doubt on

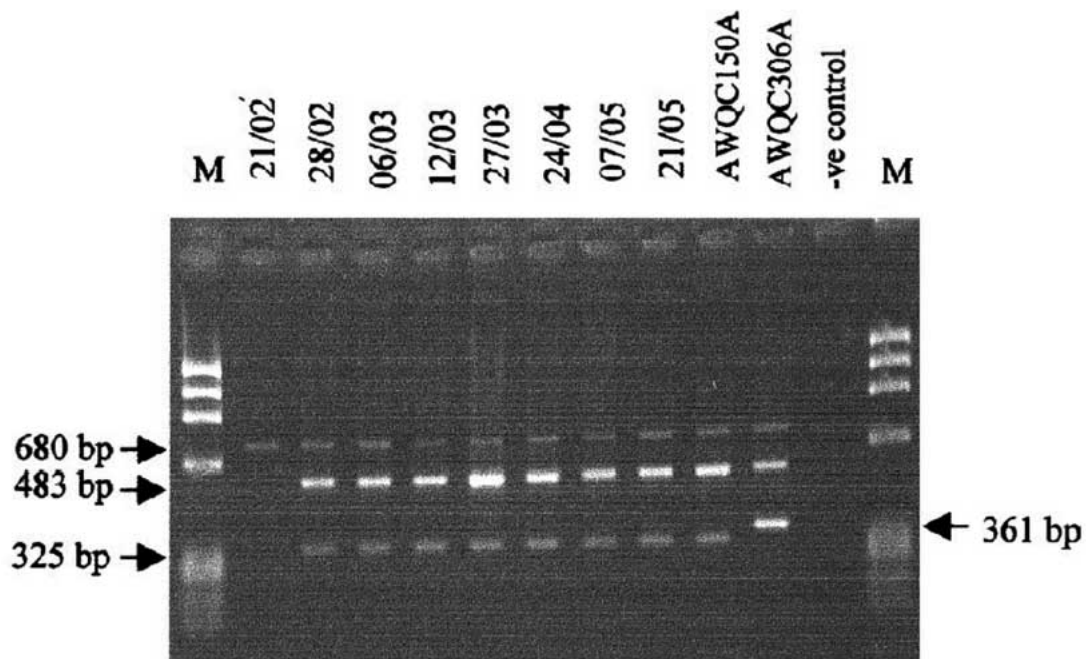
either the phylogenetic coherence of the taxon or the validity of the strain identification. The phenotypic characteristic used to differentiate *A. circinalis* from *A. flos-aquae* isolates is trichome spiral breath. Isolates with spiral breath greater than 50  $\mu\text{m}$  are classified as *A. circinalis* and spiral breath less than 50  $\mu\text{m}$  classified as *A. flos-aquae*. Studies on axenic cyanobacteria revealed that many characters employed to discriminate between species and genera are either not expressed in culture or vary with culture conditions. Trichome spiral breath is a morphological character that could be open to misclassification due to personal interpretation and could vary depending on culturing conditions, a character that is phenotypically plastic.

Australian or overseas isolates of *A. circinalis* did not demonstrate clustering based on geographical origin after

16S rDNA sequence analyses. The *A. circinalis* NIES-41 strain from Japan was found in cluster 1A and *A. circinalis* CCAP1403/25 from England in cluster 2A. This lack of segregation implicates that although there is a worldwide distribution of *A. circinalis*, and a segregation of PSP toxin production among strains the PSP-producing geographical localisation to Australian fresh waters is not due to the genetic diversity of strains but more likely to environmental factors. Since all *A. circinalis* strains have been found to belong to a single cosmopolitan species, perhaps what is needed is to investigate for anatoxin-a producing strains of *A. circinalis* in Australia and PSP producing *A. circinalis* strains overseas. Since *A. flos-aquae* was found not to form a distinct species from *A. circinalis* by genetic analysis then perhaps anatoxin-a producing *A. circinalis* has already been found here in Australia.

**Fig. 1a.** *A. circinalis* 16S rDNA distance tree. DNA sequences corresponding to the *E. coli* 16S rRNA gene positions 27 to 1494 were aligned using the programs PILEUP and CLUSTAL W. *A. circinalis* strains in bold were found to produce PSPs as determined by HPLC and mouse bioassay. Also included were an additional five *Anabaena* 16S rDNA sequences including *A. affinis* NIES40, *A. solitaria* NIES80, and *A. flos-aquae* AWQC112D, NRC44-1, and NRC525-17. Genetic distances were calculated using the method of Jukes and Cantor, and the phylogenetic tree reconstructed using the neighbour-joining algorithm of Saitou and Nei as implemented within CLUSTAL W. The root of the tree was determined by using the 16S rRNA gene of *A. cylindrica* NIES19 as an outgroup. Local bootstrap support for branches present in more than 50% of 1,000





**Fig. 1b.** Analysis of 16S rRNA gene using the *A. circinalis* branch I- and branch II-specific oligonucleotides. Lanes 21/02 to 21/05 correspond to PCR fragments from the environmental bloom samples collected on the dates indicated (day/month). Lanes AWQC105A and AWQC306A correspond to positive controls belonging to branch I and branch II, respectively. Lane -ve control, the negative control. Amplification with the phycocyanin primer pair PCaF plus PCbF yields a 680-bp product. Amplification with the *A. circinalis*-specific primer AC510R plus the cyanobacterial universal primer 27F1 yields a 483-bp product. Amplification with the branch I or branch II primer pairs ACB1F plus AC510R and ACB2F plus AC510R yields a 325- or 361-bp PCR product, respectively. The three PCR products from each sample were pooled, and a total of 6 ml was run on a 3% agarose gel in 13 TAE with 100 ng of fX174 HaeIII as DNA marker (lanes M). Reproduced from (5).

## II. *Cylindrospermopsis* and a potential mechanism for cylindrospermopsin biosynthesis

*Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju is a freshwater, planktonic cyanobacterium (Order Nostocales) which is of interest from a water quality perspective due to its ability to produce toxic compounds that can potentially affect the health of humans and animals. The alkaloid toxin cylindrospermopsin has been implicated in Australia's worst case of human poisoning attributed to a cyanobacterial toxin that resulted in severe hepat-enteritis among the children of an Aboriginal community in 1979. In more recent years this toxin has been implicated in cattle mortality and has been found to accumulate in the tissues of commercially cultured freshwater crayfish.

Two strains of *C. raciborskii* isolated from a domestic water supply in Brazil were recently shown to produce PSP toxins similar to those produced by *Anabaena circinalis* and some marine dinoflagellates raising further concerns over the presence of this cyanobacterium in potable and other water supplies worldwide. Padišák reported what is currently known of the geographical distribution of *C. raciborskii* and interestingly, this species, which was originally described as a taxon of only tropical interest has been increasingly reported from temperate regions of Australia, Europe, North and South America.

This new distribution of *C. raciborskii* has been noted only in the last ten years where it has been detected as a component of the phytoplankton of many previously unaffected water bodies.

With our growing knowledge of this cyanobacterium, there is a clear need to understand the genetic basis for some of the morphological and physiological differences observed between strains of this invasive species. In this paper we report on genetic variation between strains of *C. raciborskii* isolated from a range of countries (Australia, Brazil, Germany, Hungary, Portugal and the USA) by HIP1 repeated sequence PCR. This technique, which is based on structural genomic polymorphisms between defined cyanobacterial elements, has previously been shown to be effective in distinguishing between strains of *C. raciborskii* isolated from a range of water bodies in northern Australia that showed similar (> 99.8 %) 16s rRNA gene nucleotide sequences (6). This technique has also provided support for our earlier observations that there is no justification for separation of *Cylindrospermopsis* species based on gross differences in trichome morphology (ie. straight and coiled) (7) or on minor (although statistically significant) differences in vegetative cell or heterocyst dimensions. The strains used in this study have also been characterised by 16s rRNA gene sequencing to confirm the phyletic relationships between the globally diverse range of *Cylindrospermopsis* and also to some other cyanobacterial species known to produce

the toxic compounds cylindrospermopsin and PSP toxins, namely, *Umezakia natans* and *Aphanizomenon ovalisporum*.

*Cylindrospermopsis raciborskii* has been described in the literature as a highly adaptive cyanobacterium capable of considerable physiological and morphological plasticity. Our findings support that view. The strains investigated here showed a range of morphological characteristics, and an ability to produce a range of toxic compounds including cylindrospermopsin, PSPs and as yet unidentified toxic compounds. It is also clear that this species can no longer be considered a species of only tropical interest, nor can its distribution be considered as "pan tropical" given that its northern occurrences overlap with many circum-polar phytoplankton species. Blooms of this species have become a worldwide problem due to their production of toxic compounds (7).

The 16s gene nucleotide sequences of the 26 strains investigated here showed 99.1% similarity indicating that *C. raciborskii* is a genetically well-defined group. The genetic divergence determined for these strains of *C. raciborskii* is less than was found for a global study of 19 *A. circinalis* strains, which reported 98 % similarity in 16S rRNA gene nucleotide sequences (5).

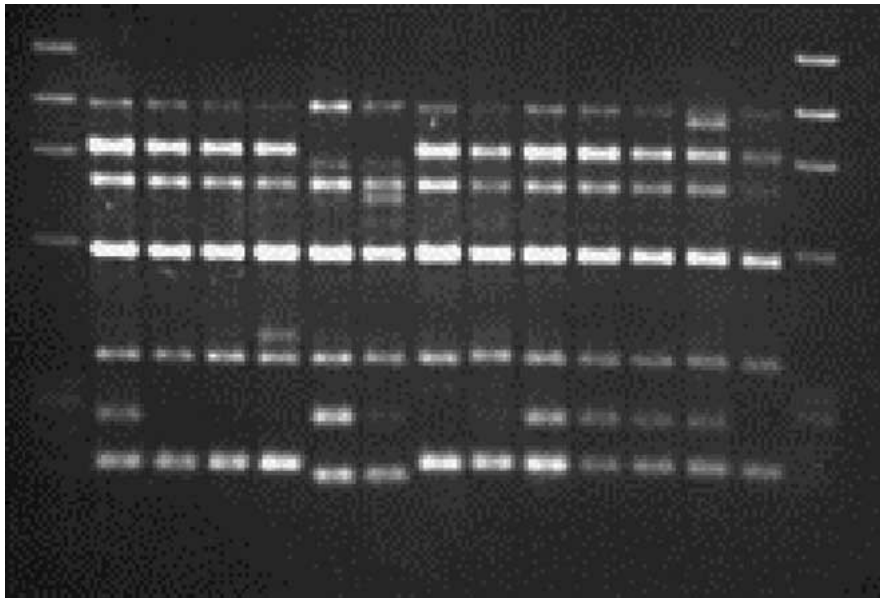
Despite the strong similarity between *C. raciborskii* strains, the phenogram constructed by alignment of the 16S rRNA gene nucleotide sequences indicated clustering of strains into geographically distinct groups, with strains from North and South America forming one group, European strains forming another group, and Australian strains constituting the third group. This observed relationship could not be validated statistically. The observed clustering of strains was nevertheless supported by the phylogenetic tree constructed from analysis of the HIP 1 PCR reactions (Figure 2) which indicated clear statistical differences in the DNA banding pattern of strains from different global locations. The strains assembled into two statistically validated groups. The first group contained strains from USA and Brazil; the second, European and Australian strains. Within the second group, the Australian and European strains showed statistically significant divergence. Within the European strains, the German and Portuguese strains were more closely related to each other than to the Hungarian strains, and within strains from Australia, the two strains isolated from water bodies in the north at latitudes < 20°S were more similar than those isolated from temperate regions of that continent.

While many of the strains investigated here have not been investigated thoroughly for the presence of toxic compounds, it is interesting to note that as yet, only Australian strains of *C. raciborskii* have been reported to produce cylindrospermopsin, and only Brazilian strains have been reported to produce PSP's. These two groups showed the greatest divergence with respect to both their 16S rRNA gene sequences (Figure 3) and their HIP1 genomic profiles (Figure 2). Furthermore, both Portuguese and Hungarian strains from the European sub-group have been shown to have a toxic effect in the mouse bioassay despite a confirmed absence of cylindrospermopsin and PSP toxins. It is possible that strains from these two sources could share a similar toxic compound/s.

The findings here do not permit any resolution concerning the origin source of *C. raciborskii*, however given the greater genetic similarity between European and Australian strains (as indicated by both 16s rRNA and HIP1 genomic profiles), it is possible that transfer of scientific samples from the Australasian region to Europe could have provided the source of this cyanobacterium which is now invading many previously unaffected European water bodies. Whilst this argument is purely speculative, it has been suggested that some physiological characteristics of *C. raciborskii*, including its high temperature demand and its inability to adapt to temperature fluctuations, indicate that this species possibly evolved in tropical lakes, with Australia or Africa as possible radiation points. Alternatively, it was further suggested that the naturally expanding distribution of *C. raciborskii* might be a useful indicator of global warming. Dispersal of *C. raciborskii* akinetes (resting spores) with migratory birds may be the most probable dispersal mechanism.

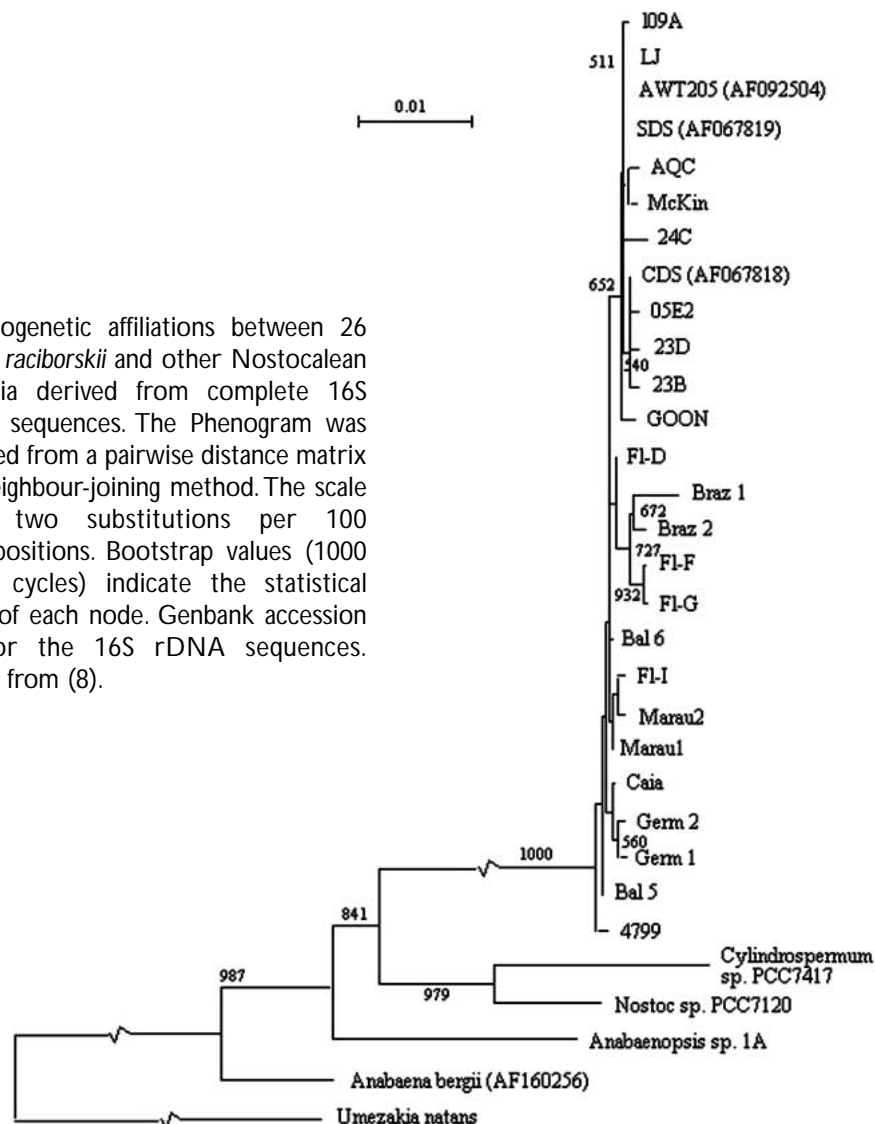
The HIP1 PCR technique is clearly a useful tool for distinguishing between strains of *C. raciborskii*. This technique confirmed observations evident in the 16s phylogenetic tree indicating that HIP 1 PCR may also be a useful taxonomic tool for some species of cyanobacteria with highly conserved 16s gene sequences. For data gathered on the genes related to the production of the toxin cylindrospermopsin please refer to the paper by Fergusson in this publication (also see Schembri MA, Neilan BA, Saint CP 2001). Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. Environmental Toxicology. 16: 413-421).





**Fig 2** Electrophoretic comparison of the PCR products formed in reactions primed with HipCA and HipTG primers for 26 strains of *C. raciborskii*, *A. cylindrica*, *U. natans* and a no DNA control. Reproduced from (8). Lane 1: Cylindrospermopsis (coiled); Lane 2: Cylindrospermopsis (straight); Lane 3: Townsville (coiled); Lane 4: Townsville (straight); Lane 5: Brazil 2; Lane 6: Brazil 1; Lane 7: A205; Lane 8: McKinlay (straight); Lane 9: Lake Julius; Lane 10: 24C; Lane 11: 23B; Lane 12: 23D; Lane 13: 5E. M:  $\phi$ X174 molecular weight markers. Samples were electrophoresed on a 3% agarose gel.

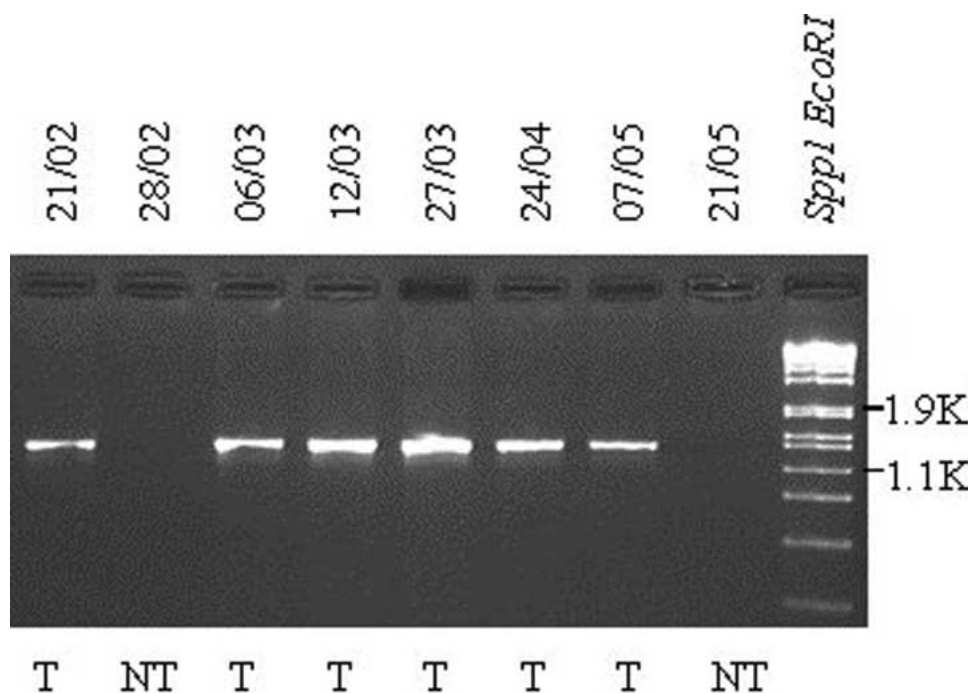
**Fig. 3** Phylogenetic affiliations between 26 strains of *C. raciborskii* and other Nostoclean cyanobacteria derived from complete 16S rRNA gene sequences. The Phenogram was reconstructed from a pairwise distance matrix using the neighbour-joining method. The scale represents two substitutions per 100 nucleotide positions. Bootstrap values (1000 re-sampling cycles) indicate the statistical significance of each node. Genbank accession numbers for the 16S rDNA sequences. Reproduced from (8).



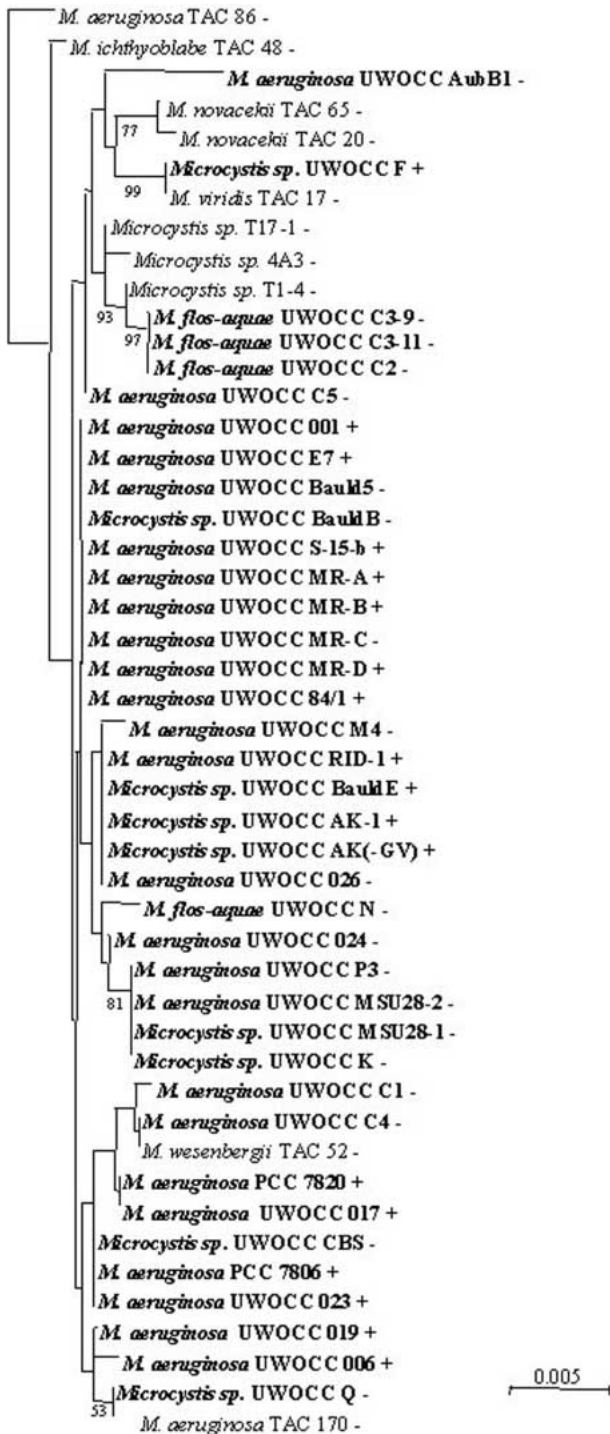
### *Microcystis* and microcystin synthetase

*Microcystis* spp., cyanobacteria that frequently occur as noxious blooms in eutrophic freshwaters, are of major concern because many strains produce cyclic heptapeptide toxins called microcystins. The microcystins are members of a family of more than 65 heptapeptides and share the common structure cyclo(D-Ala-L-X-DMeAsp-L-Z-Adda-D-Glu-Mdha), where X and Z are variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methyl-aspartic acid, and Mdha is N-methyl-dehydroalanine. Toxicity is mediated through the active transport of microcystin into hepatocytes by the bile acid organic anion transport system, followed by inhibition of eukaryotic serine/threonine protein phosphatases 1 and 2A. Acute poisoning, leading to death from massive hepatic hemorrhage, has been reported in both animals and humans. Chronic ingestion of sub-lethal doses has been demonstrated to induce primary hepatocellular carcinoma in rodents, and has been epidemiologically linked to primary liver cancer in humans. Water resource management has been complicated by the inability to differentiate between toxic and non-toxic *Microcystis* blooms without isolation and testing for toxin production. Several morphological and molecular studies have attempted to resolve the ambiguous relationship of *Microcystis* toxigenicity to its population structure. The relationship between toxigenicity and phylogeny within the cyanobacterial genus *Microcystis* is unclear. To

investigate this issue, we have designed PCR primers for the N-methyl transferase (NMT) domain of the microcystin synthetase gene *mcyA* and probed 37 *Microcystis* spp. cultures as well as several field samples. The NMT region was present in all 18 laboratory strains that gave positive reactions in the protein phosphatase inhibition assay for microcystin, but was absent in 17 non-toxic strains. Two other non-toxic strains, one of which had previously been reported to produce microcystin, possessed the NMT region. Detection of NMT-specific DNA in field samples corresponded to periods of toxicity as assessed by protein phosphatase inhibition. The *Microcystis* strains formed a monophyletic cluster based on 16S rRNA gene sequences, but comprised two groups with respect to phycocyanin intergenic spacer (PC-IGS) sequences. Toxic and non-toxic strains appeared to be erratically distributed within the PC-IGS and 16S rRNA trees. Sequence analysis of the NMT domain revealed two coherent groups. The genomic region immediately downstream of the *mcyABC* cluster in all 20 NMT-positive strains contained an ORF of unknown function (*uma1*) at a conserved distance from *mcyC*. All non-toxic strains also contained *uma1*, which is not co-transcribed with *mcyABC*. The consistent linkage of *mcyC* to *uma1* suggests that *mcyC* has not been frequently transferred into non-toxic strain via any mechanism involving insertion at random chromosomal locations. These results are discussed with respect to various mechanisms that could explain the patchy distribution of toxigenicity among the various *Microcystis* clades.

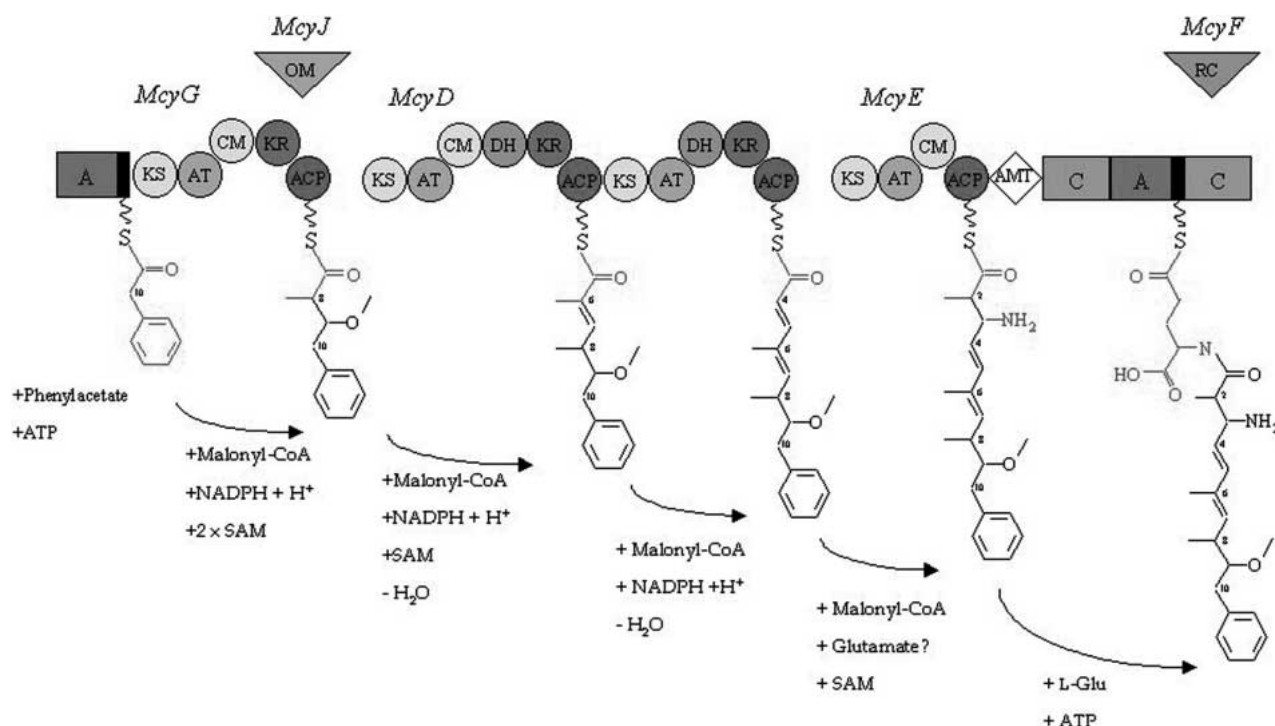


**Fig. 4.** PCR amplification of the N-methyl transferase region of microcystin synthetase from an environmental bloom. The NMT-specific PCR was performed using DNA isolated from the Botany Ponds cyanobacterial bloom samples collected in 1993 on the dates (day/month) indicated. Samples that proved toxic (T) or non-toxic (NT) by the phosphatase inhibition assay are indicated. Five microlitres aliquots of each PCR reaction were run on a 2% agarose gel in 1x TAE together with 100 ng of Spp-1 DNA digested with EcoRI. The gel was stained with ethidium bromide and photographed under UV transillumination. Reproduced from (9).

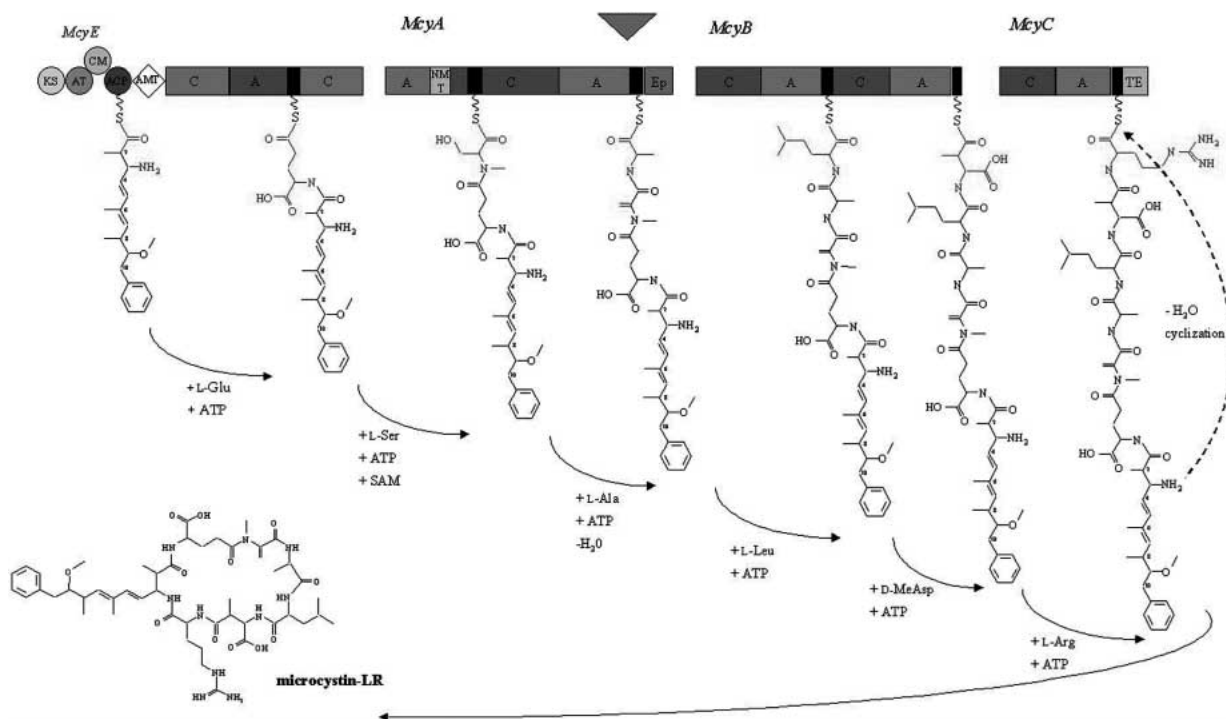


**Fig. 5.** *Microcystis* 16S rDNA distance tree. Strains in bold were sequenced during this study, with toxicity status (+/-) indicated after the name. Also included were an additional 10 other *Microcystis* 16S rDNA sequences. Genetic distances were calculated using the method of Jukes and Cantor and the phylogenetic tree reconstructed using the neighbour-joining algorithm as implemented within CLUSTAL W. The tree was rooted using the 16S rRNA gene of *Synechocystis* sp. PCC 6803 as an outgroup (branch not shown). Local bootstrap support for branches present in more than 50% of 1000 resampling are indicated at the relevant nodes. Reproduced from (9).

Of the known toxins produced by cyanobacteria, the microcystins are the most significant threat to human and animal health. These cyclic peptides are potent inhibitors of eukaryotic protein phosphatases (PP) type 1 and 2A. Synthesized nonribosomally, the microcystins contain a number of unusual amino acid residues including the  $\alpha$ -amino polyketide moiety Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid) (10). We have characterized the microcystin biosynthetic gene cluster from *Microcystis aeruginosa* PCC7806 (11). A cluster spanning 55 kb, composed of 10 bidirectionally transcribed open reading frames arranged in two operons (*mcyA-C* and *mcyD-J*), has been correlated with microcystin formation by gene disruption and mutant analysis. Of the 48 sequential catalytic reactions involved in microcystin synthesis, 45 have been assigned to catalytic domains within six large multienzyme synthases/synthetases (*McyA-E, G*), which incorporate the precursors phenylacetate, malonyl-CoA, S-adenosyl-L-methionine, glutamate, serine, alanine, leucine, D-methyl-isoaspartate, and arginine. The additional four monofunctional proteins are putatively involved in O-methylation (*McyI*), epimerization (*McyF*), dehydration (*McyJ*), and localization (*McyH*). The unusual polyketide amino acid Adda is formed by transamination of a polyketide precursor as enzyme-bound intermediate, and not released during the process. Here we describe the complete biosynthesis pathway of a complex cyanobacterial metabolite. The enzymatic organization of the microcystin assembly represents an integrated polyketide-peptide biosynthetic pathway with a number of unusual structural and enzymatic features. These include the integrated synthesis of a  $\alpha$ -amino-pentaketide precursor and the formation of b- and g-carboxyl-peptide bonds, respectively. Other features of this complex system also observed in diverse related biosynthetic clusters are integrated C- and N-methyltransferases, an integrated aminotransferase, and an associated O-methyltransferase and a racemase acting on acidic amino acids.



**Fig. 6.** Model for the formation of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) and predicted domain structure of McyG, McyD and McyE. Each circle and rectangle represents respectively a PKS or NRPS enzymatic domain. The putative amino transferase domain is represented by a diamond. The activity of the tailoring ORFs, McyJ & F, are shown as inverted triangles. KS, a-ketoacyl synthase; AT, acyl transferase; ACP, acyl carrier protein; ketoacyl reductase (KR); DH, dehydratase; CM, C-methyltransferase; OM, O-methyltransferase; A, aminoacyl adenylation; C, condensation; AMT, aminotransferase; RC, racemase. The NRPS thiolation motif is shown in black. Reaction order shows transfer and condensation of Adda to D-glutamate. Reproduced from (11).



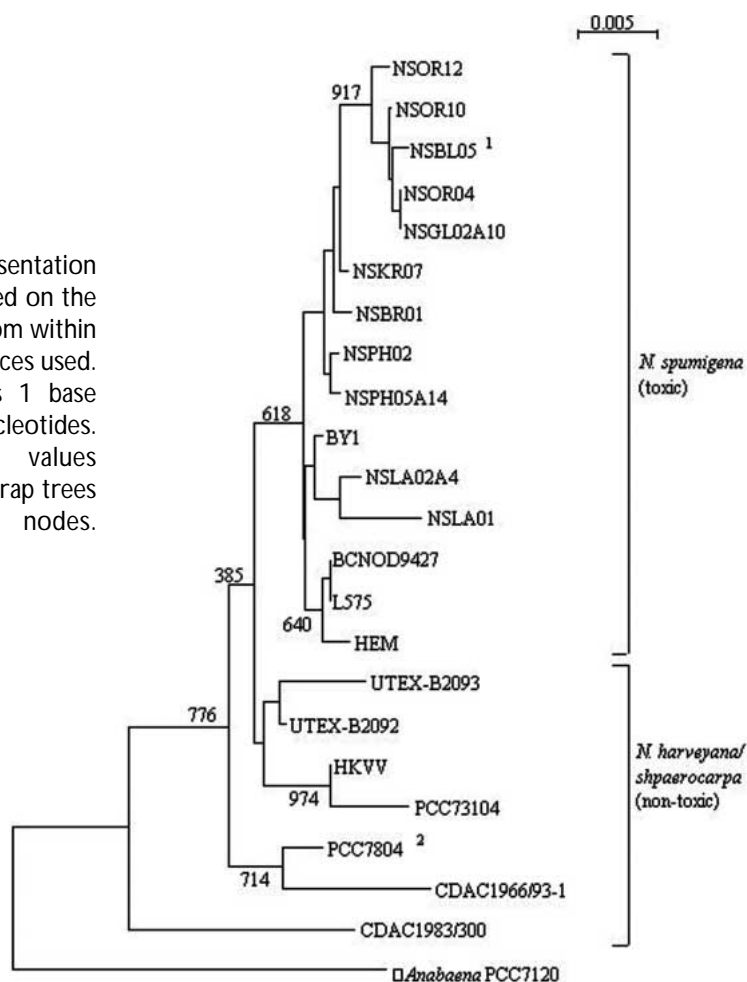
**Fig. 7.** Biosynthetic model for microcystin-LR and predicted domain structure of McyE, McyA, McyB, and McyC. Each circle and rectangle represents, respectively, a PKS or NRPS enzymatic domain. The amino transferase domain is represented by a diamond. The activity of the putative tailoring ORF, McyI, is shown as an inverted triangle. KS, a-ketoacyl synthase; AT, acyl transferase; CM, C-methyltransferase; ACP, acyl carrier protein; A, aminoacyl adenylation; C, condensation; NM, N-methyltransferase; Ep, epimerisation; TE, thioesterase; AMT, aminotransferase. The NRPS thiolation motif is shown in black. Aminoacyl activation and condensation order is predicted: L-Z-Adda, L-glutamate, L-methylserine, D-alanine, L-leucine, D-methyl-aspartate, L-arginine, cyclization. Reproduced from (11).

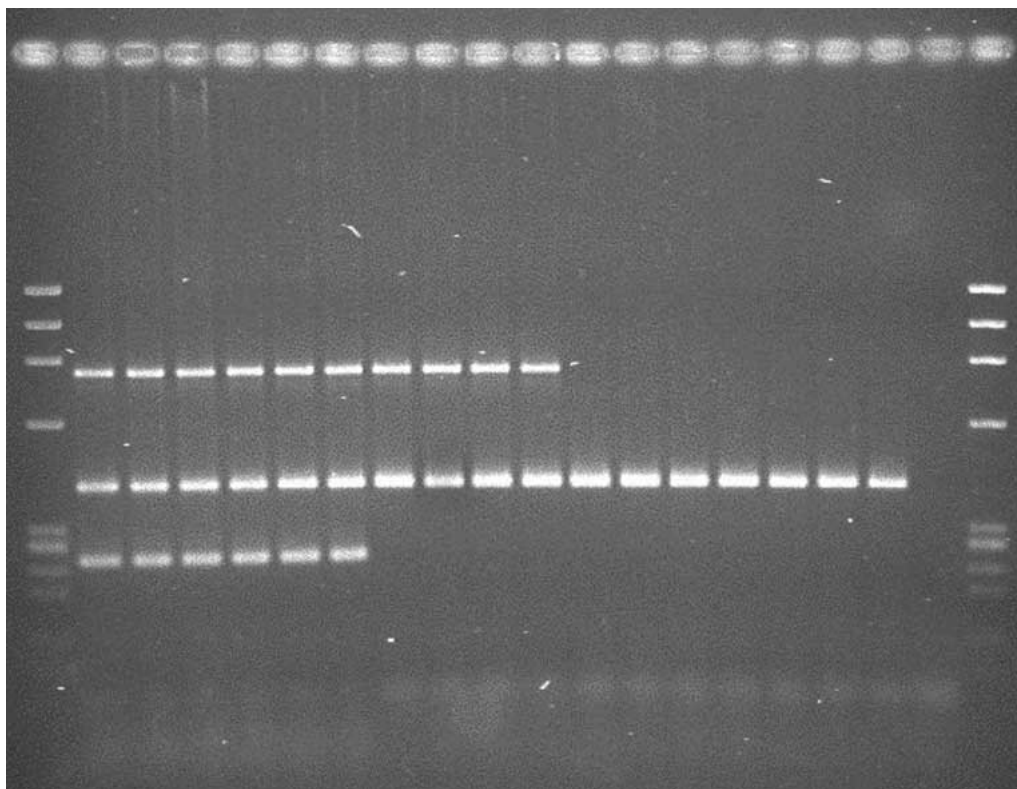
### ***Nodularia* and the putative nodularin synthetase**

The filamentous cyanobacterium *Nodularia* of the order Nostocales is bloom-forming, with the ability to produce toxic blooms. The toxin produced by *Nodularia*, nodularin, is a hepatotoxin, similar in structure to the heptapeptide toxin microcystin. Twenty-two strains of *Nodularia* representing the species *N. spumigena*, *N. harveyana* and *N. sphaerocarpa* were analysed for the production of toxin by protein phosphatase inhibition assay and sequenced over the 16S rDNA region. Phylogenetic analysis of *Nodularia* 16S rDNA sequences found *Nodularia* clustered into two main groups. A *N. spumigena* cluster was distinct from the benthic species *N. harveyana* and *N. sphaerocarpa*, which also formed a single cluster. There was no distinction between strains isolated from globally diverse locations. Nodularin-producing species were restricted to the single, evolutionally distinct cluster of *N. spumigena*. This is the first cyanobacterial toxin reported to be restricted to a single species. This has enabled the design of a specific 16S rRNA PCR for the rapid detection of nodularin-producing strains. Alignment of 16S rDNA sequences from toxic and non-toxic *Nodularia* with other members of the cyanobacteria allowed the design of both *Nodularia* generic and toxic *N. spumigena* specific primers (12).

The 16S rDNA sequence phylogeny in this study has attempted to analyse the taxonomy of *Nodularia* in relation to the production of nodularin as well as to analyse *Nodularia* in accordance to its geographical point of isolation. Results here show that nodularin production is restricted to a single phylogenetic cluster. Amongst this toxic cluster however, there appears to be no delineation between strains isolated from geographically distinct regions and there appears to be a continuous cline of globality amongst toxic bloom-forming *N. spumigena*, as was previously reported resulting from the analysis of *cpc*-IGS sequences of *Nodularia*. These results contradict the findings of Bolch *et al.*, 1999, that *Nodularia* display geographically distinct genotypes when analysed by *cpc*-IGS sequencing and RAPD-PCR. The benthic species analysed in this study were distinct from the *N. spumigena* cluster and there was no obvious phylogenetic difference between the two benthic species, *N. harveyana* and *N. sphaerocarpa*. Future analysis of these groups should be performed and we propose that these species be merged on the basis of lack of morphological and ecological distinction, as well as the high percentage sequence similarities and genetic distances determined in this study. Benthic strains of *N. spumigena* may also be reclassified within this newly merged group of benthic *Nodularia*. Analysis of the 16S rDNA phylogeny provides a rapid method for the detection of toxic bloom-forming *Nodularia* in environmental samples (12).

**Fig. 8.** Phylogenetic representation of the genus *Nodularia* based on the alignment of 1410 bases from within the 16S rRNA gene sequences used. The scale bar represents 1 base substitution per 100 nucleotides. Significant bootstrap values calculated from 100 bootstrap trees are indicated at the nodes. Reproduced from (12).





**Fig. 9.** Analysis of amplification of regions of the 16S rRNA gene using cyanobacterial and *Nodularia* specific oligonucleotide primers. Lanes 1-18 correspond to PCR fragments from the culture strains *N. spumigena* L575, HEM, BY1, NSOR10, NSBL05, NSPH05A14, *N. spumigena/sphaerocarpa* HKVV, *N. sphaerocarpa* 1966/93-1, *N. harveyana* PCC7804, *N. harveyana* 1983/300, *A. cylindrica* NIES19, *C. raciborskii* AWT205, *Lyngbya* AWT211, *Aphanizomenon* NIES81, *Synechocystis* PCC6803, *M. aeruginosa* PCC7806, *Nostoc* PCC7210 and no DNA control respectively. The marker lanes (M) contain PhiX174/HaeIII. Cyanobacterial specific primer produced a 400 bp fragment with universal primer 27f in all strains analysed (CYAN). *Nodularia* genus specific primer amplified a 780 bp fragment with 1490r universal primer in all *Nodularia* strains analysed in this study (NOD). Toxic specific primer amplified a 200 bp fragment with 1490r universal primer in all toxic planktonic bloom-forming *Nodularia* (TOX). Reproduced from (12).

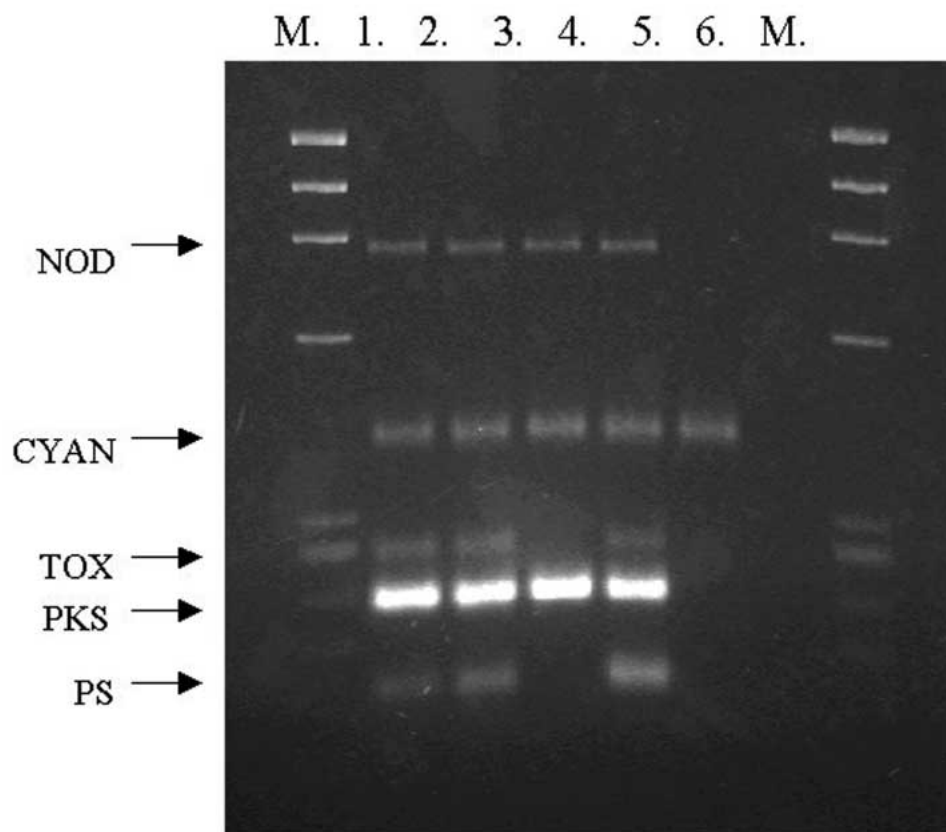
Nodularin is a hepatotoxin produced by the bloom-forming cyanobacterial species *Nodularia spumigena*. Putative peptide synthetase and polyketide synthase genes were detected in toxic strains of *Nodularia* by degenerate PCR. Using specific primer sets, peptide synthetase and polyketide synthase gene homologues were detected in nodularin-producing strains indicating a possible role of peptide synthetase and polyketide synthase enzyme complexes in the biosynthesis of nodularin. Strains of *Nodularia* isolated from around the world were also analyzed for the production of nodularin by the protein phosphatase 2A inhibition assay.

*Nodularia* blooms are highly toxic due to the associated production of the cyclic pentapeptide hepatotoxin nodularin. Nodularin production has been shown to be restricted to the planktic bloom-forming species *Nodularia spumigena* by phylogenetic analysis. Nodularin is similar in structure to the cyclic heptapeptide microcystin. Microcystin is produced non-ribosomally by a number of cyanobacterial genera including *Microcystis*, *Anabaena*, and *Oscillatoria*. Nodularin

contains a dehydroamino acid, N-methyldehydrobutyrine (Mdhb), two D-amino acids, D-glutamic (D-Glu) and D-erythro-B-methylaspartic acids (D-MeAsp) and the more common L-arginine (L-Arg) (Figure 1). Nodularin also contains the fatty acid C20 amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda). Both nodularin and microcystin share in common the amino acids Adda, D-Glu, D-MeAsp and L-Arg. The Mdhb residue found in nodularin is usually replaced by an N-methyldehydroalanine (Mdha) in microcystin. Due to structural similarity, we propose that a similar mechanism of toxin biosynthesis would be present in both microcystin and nodularin-producing cyanobacteria. Cyclic structure and conserved D- amino acids indicated the involvement of a peptide synthetase while the presence of the novel amino acid Adda supports similar polyketide synthesis. Using degenerate oligonucleotide primers previously described for the detection peptide synthetase genes in microcystin-producing species, a putative peptide synthetase region was sequenced in toxic *Nodularia*. Degenerate polyketide synthase oligonucleotide primers were also designed during this

study to sequence a putative polyketide synthase region specifically from *Nodularia*. We report, for the first time, that polyketide synthase-like genes have been detected in *Nodularia*. Specific primers were designed to determine the distribution of these putative peptide synthetase and polyketide synthase genes in toxic and non-toxic *Nodularia* strains. In this study, a protein phosphatase inhibition assay was used to determine the toxicity of *Nodularia* strains from around the world. Results were then utilized for the comparative study of possible toxin biosynthesis genes. We have identified peptide synthetase-like and polyketide synthase-like gene regions in *Nodularia*, the presence of which reflects the toxicity of the strain. The PP-2A inhibition assay was used to quantify the nodularin production by *Nodularia* strains using microcystins-LR as a standard. The increasing problem of *Nodularia* blooms in Australia and the Baltic sea has led to the need for rapid detection of toxic *Nodularia* blooms in

conjunction with current tests such as HPLC and organism bioassays. We have reported the design of specific oligonucleotide primers for the PCR detection of peptide synthetase and polyketide synthase genes in toxic *Nodularia* laboratory strains as well as in toxic environmental bloom samples (13). This specific PCR, when used in conjunction with previously described *Nodularia* specific 16S rRNA PCR, is able to distinguish toxic *Nodularia* from other cyanobacteria. In addition, the results show that the presence of both the peptide synthetase and polyketide synthase regions analysed correlate directly with the taxonomic group, *N. spumigena*, and associated nodularin-based toxicity of the sample. The presence of a polyketide synthase like gene in *Nodularia* has not been previously shown. In addition, the distribution of a *Nodularia*-specific peptide synthetase has not been examined in toxic and non-toxic strains of this genus.



**Fig. 10.** Analysis of the presence of peptide synthetase and polyketide synthase regions from an environmental sample containing a toxic *Nodularia* bloom and mixed cyanobacterial communities simulated to consist of *Nodularia*, toxic *Microcystis* and toxic *Anabaena*. Lanes 1-6 correspond to Lake Alexandrina bloom sample, mixed cyanobacterial community containing toxic *Nodularia*, mixed cyanobacterial community containing non-toxic *Nodularia*, toxic *N. spumigena* NSOR10, *Cylindrospermopsis raciborskii* A205 and a negative control respectively. The marker lanes (M) contain PhiX174/HaeIII. Cyanobacterial specific 16S rRNA PCR [16] amplified a 400bp fragment in all samples analysed (CYAN). *Nodularia* genus specific 16S rDNA PCR [5] amplified a 780 bp fragment from the bloom, mixed communities containing *Nodularia* and from NSOR10 (NOD). Toxic *N. spumigena* specific 16S rDNA PCR [11] amplified a 200 bp fragment from the bloom sample, the mixed community containing toxic *Nodularia* and from NSOR10 (TOX). Polyketide synthase specific primer set amplified a 300bp fragment (PKS) from the bloom sample, the mixed community containing toxic *Nodularia*, the mixed community containing toxic *Microcystis* and non-toxic *Nodularia* and from *N. spumigena* NSOR10. Peptide synthetase specific primer set amplified a 150bp fragment (PS) from the bloom sample, the mixed community containing toxic *Nodularia* and from NSOR10. Reproduced from (13).

## ACKNOWLEDGEMENTS

This paper is based on the research of R. Amanda Bass, Daniel Tillett, Leigh Hardman, Carolina Beltran, Melanie Kaebernick, Michelle Moffitt, Martin Saker, and several collaborators from Berlin, including Elke Dittmann, Hans von Dohren, and Tom Boerner. This work was financially supported by the CRC for Water Quality and Treatment, the Australian Research Council, the Alexander von Humboldt Stiftung, and the Deutsche Forschungsgemeinschaft.

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# IDENTIFICATION OF TOXIC CYANOBACTERIA USING DNA BASED TECHNOLOGY

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## ABSTRACT

A variety of gene based techniques are described that have been used to characterise the toxic cyanobacteria *Cylindrospermopsis raciborskii* and *Anabaena circinalis*. The techniques have not only been used to interpret the likely phylogeny of these species but have also been applied to their rapid detection in environmental samples. The nucleotide sequence of the DNA dependent RNA polymerase gene, *rpoC1*, has been used for phylogenetic analysis and the design of specific polymerase chain reaction (PCR) tests for detection. Short tandemly repeated repetitive (STRR) sequences were also used to create genetic profiles of individual isolates. A PCR test is also described which specifically identifies *Cylindrospermopsis* producing *C. raciborskii* and is capable of identifying other genera of cyanobacteria that produce this toxin.

## DISCUSSION

Algal blooms are being increasingly reported around the world in both marine and freshwater environments. Blue-green algae, more correctly termed cyanobacteria, are responsible for toxic blooms in freshwater systems and toxins may have hepatotoxic, cytotoxic, neurotoxic or allergenic effects on animals and humans. We have been applying molecular techniques to the analysis of two species of cyanobacteria of concern in Australia, *Cylindrospermopsis raciborskii* and *Anabaena circinalis*. *Cylindrospermopsis raciborskii* has been a problem in sub-tropical regions of Australia for some time but in recent years reports of toxic blooms of this organism have been made in South America, the USA, Europe and New Zealand. *C. raciborskii* is largely known for its production of the hepatotoxic alkaloid toxin cylindrospermopsin. Whilst several species of the genus *Anabaena* can produce toxins, in Australia thus far only *A. circinalis* has been demonstrated to produce toxins. *A. circinalis* can produce a range of neurotoxins including saxitoxin, neosaxitoxin and gonyautoxin, known collectively as paralytic shellfish poisons (PSPs).

The use of morphological criteria alone to characterise cyanobacteria is fraught with difficulty as it appears that many have the ability to change appearance depending on conditions in the field and the culturing methods used in the laboratory. In the case of trichomes of the heterocystous cyanobacterium *C. raciborskii* coiled and straight morphotypes are described. Trichomes can be looped or sigmoidal and these forms have been found to co-exist with straight trichomes in blooms. In the case of *Anabaena circinalis* a skilled microscopist is needed to

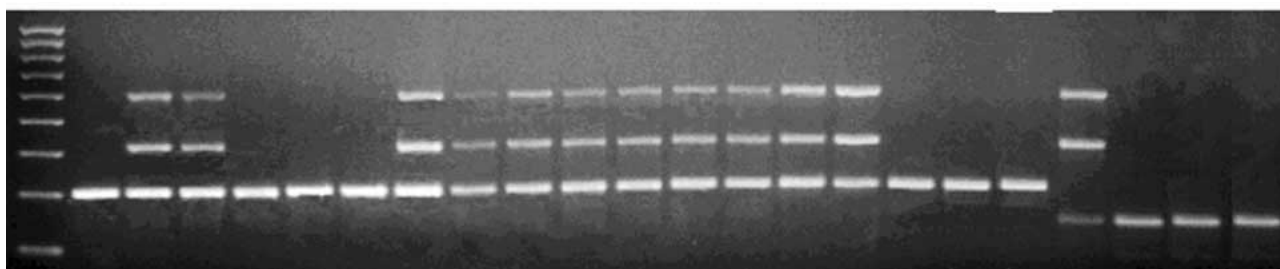
distinguish this species from, for example, *A. spiroides*, which is not known to be toxic in Australia.

We have used nucleotide sequence from a DNA dependent RNA polymerase gene (*rpoC1*) to characterise a range of isolates of *C. raciborskii* and members of the genera *Aphanizomenon* and *Anabaena*, including many *A. circinalis* isolates (Wilson *et al.*, 2000; Fergusson & Saint, 2000). The gene was used in preference to the 16S rRNA gene as it has been previously described as being more discriminatory (Palenik and Haselkorn, 1992). In the case of *C. raciborskii* all 13 isolates from geographically diverse areas of Australia and 2 Brazilian isolates showed 99.8 – 100% sequence homology over a 609 bp region analysed. These isolates consisted of both coiled and straight forms. This high level of similarity allowed a consensus sequence to be derived and this sequence is compared phylogenetically to other *rpoC1* sequences from cyanobacteria in Fig.1. In the case of *Aphanizomenon* and *Anabaena* a 612 bp *rpoC1* fragment was amplified from 26 isolates, 12 of which had been identified morphologically as *A. circinalis*. The data indicated that 2 genotypic groups of *A. circinalis* exist (termed I and II in Fig.1). Of interest is the fact that sequence analysis indicated that an isolate previously identified as *A. spiroides* was in fact *A. circinalis*. The significance of this is that in Australia *A. circinalis* is the only known toxic species of *Anabaena*, therefore misidentification on morphological criteria could result in a potentially toxic isolate being discounted.



The derived sequence from *rpoC1* analysis has permitted the design and implementation of PCR based diagnostic tests for both *C. raciborskii* and *A. circinalis*. These tests have been successfully applied to bloom material, for example a bloom thought to be *A. spiroides* which occurred in Bahia Blanca, Argentina, was confirmed as *A. circinalis*. By incorporating an internal control of known concentration the assays can also be made quantitative. The limit of detection is currently 2,000 cells but manipulation of the internal control can allow a diagnostic product to be produced of equal intensity to the control that indicates a specific level of cells/ml of water are present. This can be set at a level equivalent to that where bloom control strategies might be implemented.

The genes likely involved in cylindrospermopsin production by *C. raciborskii* have recently been characterised (Schembri *et al.*, 2001). By analogy to the situation with microcystin production it seems that polyketide synthase (*pks*) and peptide synthetase (*ps*) determinants are involved, as these genes are always present in cylindrospermopsin producing strains of *C. raciborskii* as well as *Aphanizomenon ovalisporum* and *Anabaena bergii*. They are always absent in non-toxic isolates. We have now developed a multiplex PCR test that will reveal the presence of these genes and distinguish *C. raciborskii* from other cylindrospermopsin producing genera (Fig. 3). This test can be applied to environmental samples.



**Fig. 3.** *C. raciborskii* multiplex PCR. Four bands are possible, termed 1–4 in descending order. The presence of 1, 2, & 3 indicates cylindrospermopsin producing *C. raciborskii* (Lanes 3 and 4 and 8-16); 3 indicates non-toxic *C. raciborskii* (Lanes 2, 5-7 and 17-19); 1, 2 & 4 indicates cylindrospermopsin producing *A. bergii* (Lane 20) and 4 alone indicates no cylindrospermopsin producing cyanobacteria present (Lanes 21-23).

## CONCLUSIONS

- Sequencing of the *rpoC1* gene from *C. raciborskii*, *A. circinalis* and a range of other cyanobacteria has permitted their phylogenetic relatedness to be established.
- Whilst coiled/straight, toxic/non-toxic isolates of *C. raciborskii* could not be distinguished, *A. circinalis* split into 2 groups, one of which contains predominantly toxin producing isolates.
- The derived sequence was used to design and implement *C. raciborskii* and *A. circinalis* specific PCR tests. These tests worked on bloom samples also.
- Genetic profiling using STRR sequence based PCR did distinguish coiled from straight *C. raciborskii* but not toxin producing from non-toxin producing.
- The genes associated with cylindrospermopsin production have been identified in *C. raciborskii*, *Aph. ovalisporum* and *A. bergii*. These determinants have also been targeted in a PCR test that distinguishes toxin producers from non-toxin producers.

## ACKNOWLEDGEMENTS

The authors would like to acknowledge the Cooperative Research Centre for Water Quality and Treatment and the SA Water Corporation for their support of this research.

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# INSTRUMENTAL METHODS FOR THE DETERMINATION OF CYANOBACTERIAL TOXINS

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## ABSTRACT

Cyanobacteria and their toxins are an emerging water quality issue for providers of drinking water and managers of waters used for recreational purposes. Robust and sensitive analytical methods are required for monitoring toxins and assessing their behaviour during water treatment. This paper reviews the toxins produced by cyanobacteria (cyanotoxins) and the methods currently available for their analysis.

## INTRODUCTION

Blooms of blue-green algae, more correctly known as cyanobacteria, are becoming increasingly common in water bodies as nutrient levels rise. These organisms are cosmopolitan; they have been recorded from every continent including Antarctica (Sivonen and Jones, 1999; Hitzfeld *et al.*, 2000). Cyanobacteria are now well known for their ability to produce potent toxins which have been responsible for numerous animal deaths (Kuiper-Goodman *et al.*, 1999). Cyanobacteria have also been implicated in episodes of human illnesses in various countries (Codd *et al.*, 1999). Recently deaths of dialysis patients in Brazil from water contaminated with cyanotoxins was reported (Jochimsen *et al.*, 1998). There is also epidemiological evidence from China of a link, in part, between cyanobacteria and cancer (Yu, 1994). The potential for adverse effects in the human population has resulted in cyanobacterial toxins (cyanotoxins) becoming an important water quality issue. Cyanobacteria produce several toxins, including neurotoxins, (anatoxins and saxitoxins), hepatotoxins, cylindrospermopsin and lipopolysaccharide (LPS) endotoxins (Sivonen and Jones, 1999).

## NEUROTOXINS

### Anatoxins

Toxins in this class identified to date are the neurotoxic alkaloids anatoxin-a, homoanatoxin-a and anatoxin-a(s). Anatoxins have been identified in cyanobacteria from the Northern Hemisphere, primarily *Anabaena flos-aquae* (eg Devlin *et al.*, 1977), a benthic *Oscillatoria* sp. (eg, James *et al.*, 1997) and at low levels in Japanese *Microcystis aeruginosa* (Harada *et al.*, 1993) but to date have not been found in southern Hemisphere countries such as Australia (Velzeboer *et al.*, 2000). A similar toxin, homoanatoxin-a, which differs in structure by an additional methylene group, has been identified from *Oscillatoria formosa* (Skulberg *et al.*, 1992). Anatoxin-a(s), a cholinesterase inhibiting toxin, is produced by *A. flos-aquae* (Matsunaga *et al.*, 1989) and probably *A. lemmermannii* (Henriksen *et al.*, 1997).

### Saxitoxins (Paralytic Shellfish Poisons (PSPs))

The neurotoxic saxitoxins or paralytic shellfish poisons (PSPs) are one of a number of groups of toxins more commonly produced by dinoflagellates in the marine

environment. These toxins have now also been found to be responsible for neurotoxicity in a number of cyanobacterial species; *Aphanizomenon flos-aquae* (eg, Ikawa *et al.*, 1985), *Lyngbya wollei* (Onodera *et al.*, 1997) and *Cylindrospermopsis raciborskii* (Lagos *et al.*, 1999). Saxitoxins are also produced by *Anabaena circinalis*, the only Australian cyanobacterium found to be neurotoxic to date (eg, Velzeboer *et al.*, 2000).

## PEPTIDE HEPATOTOXINS (MICROCYSTINS AND NODULARIN)

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 species of this cyanobacterium, as well as species of other genera (Sivonen and Jones, 1999). Similar highly toxic cyclic pentapeptides such as nodularin (Rinehart *et al.*, 1988) which is produced by *Nodularia spumigena*, normally a brackish water cyanobacterium, have also been recorded.

Variants in all amino acids in microcystins have now been reported resulting in over 60 compounds having been characterised to date (Sivonen and Jones, 1999). *M. aeruginosa* would appear to be the cyanobacterium most commonly found worldwide with microcystin-LR being the most common variant. However, it is common for more than one microcystin to be found in a particular strain of cyanobacterium (eg, Lawton *et al.*, 1995). The microcystin variants may also differ markedly in toxicity (Carmichael, 1992). The cyclic pentapeptide nodularins contains amino acids similar or identical to those found in microcystins.

## CYLINDROSPERMOPSIN

Cylindrospermopsin is an hepatotoxic alkaloid toxin isolated from various cyanobacteria; *C. raciborskii* (eg, Ohtani *et al.*, 1992), *Umezakia natans* in Japan (Harada *et al.*, 1994) and *Aphanizomenon ovalisporum* in both Australia (Shaw *et al.*, 1999) and Israel (Banker *et al.*, 1997). It has been implicated in cattle deaths in Queensland (Saker *et al.*, 1999) and in a severe outbreak of hepatoenteritis in the population of Palm Island, Queensland, Australia in 1979 following copper sulphate treatment to kill a heavy bloom of algae in a dam used as a drinking water supply (Byth, 1980). *C. raciborskii* is predominantly a tropical species but its range appears to be spreading rapidly.

## LIPOPOLYSACCHARIDE (LPS) ENDOTOXINS

The LPS endotoxins are perhaps the least understood of the toxins produced by cyanobacteria. These toxins are

constituents of the outer wall of both cyanobacteria and gram-negative bacteria (Keleti and Sykora, 1982). LPS endotoxins produced by cyanobacteria are less toxic than those produced by bacteria; however they may be responsible for illnesses such as gastroenteritis in human populations exposed to cyanobacteria.

## ANALYSES FOR CYANOBACTERIAL TOXINS

### General Considerations

Toxins in water bodies at the time of a bloom will be present in both the water (free, dissolved or extracellular toxins) and the cyanobacterial cells (intracellular toxins). Current analytical procedures only determine toxins in the dissolved or free state. Therefore the basis of any analytical procedure where toxins are contained in cellular material must include a step to extract or release intracellular toxin. The proportion of toxin which is intracellular appears to vary depending on the particular toxin class. Extraction of intracellular toxins requires lysis to rupture the cell wall to provide access of the extracting solvent to the toxin in the cell followed by dissolution of the toxin in the solvent. Cell lysis can be achieved by freeze-drying, often followed by sonication in solution, or by repeated freeze-thawing and/or sonication of whole cell material. Extraction is effected with water, organic solvents such as methanol or dilute acid, depending on the toxins. Procedures for optimum extraction of toxins can be compound specific.

At low concentrations, direct determination of toxins may not be feasible due to the inadequate sensitivity of the detection procedure. This necessitates a sample preconcentration step to increase the concentration of toxin in solution. This may also serve as a cleanup step by allowing removal of co-extracted material which may interfere in the analysis. These procedures are compound specific, and may also only have relevance to one detection protocol.

The stability of toxins in water samples has received little attention, but is critical to the accurate determination of toxin concentrations. Degradation of dissolved toxins during storage can lead to an underestimation of total toxin in the sample. Another issue with saxitoxins is their possible interconversion, a phenomenon which can significantly affect the concentration of individual components.

## NEUROTOXINS

### Anatoxins

*Extraction procedures.* Anatoxins can be successfully extracted from freeze-dried material using a range of

solvents such as alcohols or water, or a mixture of both, generally under acidic conditions (eg, Haugen *et al.* 1994). For anatoxin-a(s), the best solvent is aqueous acetic acid containing an alcohol (eg, Matsunaga *et al.*, 1989).

#### *Concentration/cleanup procedures*

No procedures have been published for the extraction of anatoxin-a(s) from water samples. For the extraction of anatoxin-a and homoanatoxin-a from water samples or aqueous extracts of cyanobacterial material, extractions have generally been carried out at alkaline pH where the anatoxins are present in their non-protonated free base form. Both solvent (eg, Himberg, 1989) and solid-phase (eg, Haugen *et al.*, 1994) extraction procedures have been employed. Extraction at neutral pH has employed weak cation exchange cartridges (eg, Harada *et al.*, 1989) or styrene-divinylbenzene cartridges in the presence of an ion-pairing reagent, followed by ultracentrifugation (Hormazabal *et al.*, 2000).

#### *Sample preservation*

Stevens and Krieger (1991) found that anatoxin-a was very labile in sunlight with a half life of 1-2 hours at normal water pH values (8-9). Non-photochemical degradation also occurred under alkaline conditions with half lives of a few days. At pH 6 in the dark, anatoxin-a was stable for several months. Thus samples should be kept in the dark and pH adjustment to around 6 if analyses are not performed immediately.

#### *Determination of anatoxins*

Anatoxin-a(s) has been determined in cyanobacterial material using chromatography by matching its retention time with that of a standard (Mahmood *et al.*, 1988; Henriksen *et al.*, 1997). In these cases confirmation was carried out by comparison of its toxicology with that of pure anatoxin-a(s).

Anatoxin-a has been determined directly in extracts using HPLC with detection at 227nm, the absorption maximum of anatoxin-a, (eg, Harada *et al.*, 1989) but the sensitivity is poor and the method is applicable only to cyanobacterial extracts. Sensitivity has been improved by using MS detection with selected ion monitoring (eg, Hormazabal *et al.*, 2000). Thin layer chromatography has been used for qualitative or semi-quantitative analysis (Kangatharalingam and Priscu, 1993). GC/MS analysis has been employed following conversion of anatoxin-a to more volatile derivatives, eg, following conversion to the acetyl derivative using acetic anhydride, whereby a detection limit of 1 µg/L can be achieved with water samples (eg, Himberg, 1989). Conversion of anatoxin-a to derivatives with electron capturing properties for analysis by GC/ECD has been used to increase sensitivity. Stevens and Krieger (1988) utilised derivatisation with trichloroacetic anhydride while Haugen *et al.* (1994) employed heptafluorobutyric anhydride to achieve a detection limit of 3 ng/L. Several improvements in sensitivity have been reported using HPLC procedures with both MS and fluorescence detection following

derivatisation (Takino *et al.*, 1999; James *et al.*, 1998). James *et al.* (1998) employed derivatisation with 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NDB-F) and with HPLC with fluorescence detection were able to obtain detection limits of better than 0.01 µg/L for anatoxin-a, homoanatoxin-a and degradation products. All methods which determine anatoxin-a should be applicable to homoanatoxin-a.

### **Saxitoxins**

#### *Extraction procedures*

Extraction of saxitoxins following lysis of cyanobacterial cells has generally employed aqueous acetic acid (eg, Rositano *et al.*, 1998). Rositano *et al.* (1998) found recoveries were highest where freeze-thawing was used in conjunction with 0.05M acetic acid. Ultrasonication was not necessary provided the sample was freeze-thawed three times. Based on results obtained with dinoflagellates (Ravn *et al.*, 1995), interconversion of some toxins would be expected to be minimal in this medium.

#### *Concentration/cleanup procedures*

Due to their hydrophilic nature, saxitoxins cannot be extracted and/or concentrated from water using organic solvents or reversed phase solid phase cartridges. However, reasonable sensitivity can be achieved by direct analysis using high performance liquid chromatography (HPLC) with post-column derivatisation and fluorescence detection (Rositano *et al.*, 1998).

Solid phase extraction procedures for the saxitoxins using graphitised carbon black cartridges were extensively evaluated by Rositano *et al.* (1998). The toxins were extracted in the presence of the ion-pairing reagent, heptanesulfonic acid, and eluted with aqueous methanol followed by aqueous acetic acid. Good reproducibility in the recoveries of saxitoxins found in Australian *A. circinalis* were obtained, apart from C2 which generally gave recoveries of less than 50%. This method is suitable for use with water samples, but still has room for improvement.

#### *Sample preservation*

The stability of saxitoxins in water samples has been investigated by Jones and Negri (1997) who demonstrated the conversion of the relatively non-toxic C-toxins to the much more toxic dc-GTXs. While the conversion occurred at a significant rate at 25°C, no data were presented to indicate that refrigeration might not be suitable as a preservation method in the shorter term. Preservation techniques for water samples containing saxitoxins therefore requires investigation.

The extractant used in the analysis of saxitoxins also has relevance to the stability of the toxins in the sample extract. Saxitoxins extracted from neurotoxic Australian *A. circinalis* were found to be stable in 0.05M acetic acid at room temperature and at 4°C for at least 2 weeks (Rositano *et al.*, 1998). Therefore aqueous acetic may be considered to be the best choice for extracting saxitoxins



from cyanobacterial material. Concentrations would not appear to be a critical variable if within the range 0.01 - 1.0M.

#### *Determination of saxitoxin*

##### *Instrumental Analyses (High Performance Liquid Chromatography, Capillary Electrophoresis)*

Extensive development of high performance liquid chromatographic (HPLC)-based procedures for saxitoxins has been carried out in relation to the determination of these compounds in contaminated shellfish. Of the published methods, those based on HPLC with fluorescence detection following post-column oxidation are the most versatile and useful, being both highly specific and sensitive.

HPLC analytical procedures for saxitoxins (PSPs) in dinoflagellates or contaminated shellfish using post-column derivatisation fall into two groups. Both involve ion-pair chromatography with fluorescence detection following post-column derivatisation. Sullivan (1990) utilised two different chromatographic systems, ie, two analytical methods; but with this system it is not possible to resolve some decarbamoyl (dc) toxins from carbamate toxins. Oshima *et al.* (1989, 1995) overcame this limitation by using three analytical systems which can unequivocally resolve and quantify the range of saxitoxins. These three systems are utilised on the basis of the charge on the parent molecule; one separates STX and neoSTX, including the dc derivatives (charge of +2); one separates the GTXs, including B toxins and dc derivatives (charge of +1) and one separates the C toxins (charge of 0).

The method of Oshima *et al.* (1989) has been found to be readily transferable to the saxitoxins produced by cyanobacteria and has served as the basis of published methods (eg, Negri *et al.*, 1997; Rositano *et al.*, 1998). However, there is still the possibility that peaks with retention times matching those of saxitoxins may be observed when there is no toxin present. Onodera *et al.* (1996) have reported an approach whereby if there is some doubt as to whether an HPLC peak is a saxitoxin, the analysis is repeated without post-column oxidant. If responses of peaks of interest change in same way relative to authentic standards, then such behaviour can be used as confirmation of identity.

Analytical procedures based on the method of Oshima *et al.* (1989) are lengthy but are now well documented and can be automated. Instrumentation is relatively expensive but readily available for this analysis. Mass spectrometry (MS) also has potential application for the detection of saxitoxins separated by liquid chromatography. However the mobile phases necessary for efficient separation of the various toxins are not compatible with MS systems, thereby limiting this approach at present (Quilliam, 1996).

Lawrence and coworkers (eg, Lawrence *et al.*, 1995) have developed an HPLC procedure with pre-column oxidation to produce fluorescent derivatives of saxitoxins in contaminated shellfish. While this procedure is much simpler than the post-column oxidation method, certain saxitoxins produce the same derivative. Thus only a total toxin concentration is determined for some compounds which limits its usefulness.

A number of procedures based on separation of saxitoxins using capillary electrophoresis (CE) have been developed for use in monitoring contaminated shellfish. Detection has been with UV or mass spectrometry (eg, Thibault *et al.*, 1991) as interfacing CE with MS is much simpler than interfacing LC with MS. These procedures, while for the most part giving good resolution of the various saxitoxins, suffer in their lack of sensitivity and robustness, making them unsuitable for trace determinations.

##### *Enzyme Linked Immunosorbent Assays (ELISAs)*

A number of immunoassay procedures for saxitoxins have also been published. Again these have been developed for application to contaminated shellfish. These assays are highly sensitive to the individual toxins against which antibodies have been generated, however they all show poor cross-reactivity to other saxitoxins. In particular if antibodies have been generated against STX, there is virtually no response to the C toxins (Cembella and Lamoureux, 1993). While such procedures should be applicable to saxitoxins from cyanobacteria, this response to toxin structure rather than toxicity will limit their usefulness to water quality monitoring. If the toxin profile in samples being tested is relatively constant, it may be possible to calibrate the assay against such a profile and therefore obtain a result in toxin equivalents. However most commercial assays have been developed for saxitoxin itself which means they will have poor cross reactivity to the C-toxins, which are the predominant toxins in some cyanobacteria such as neurotoxic Australian *A. circinalis*, and thus will be very poor in determining these compounds.

##### *Other Assays*

Other assays for saxitoxins, again developed for determining these compounds in contaminated shellfish, include neuroreceptor and cytotoxicity assays (Cembella *et al.*, 1995). The use of cultured mouse neuroblastoma cells is a particularly important development in this field (eg, Jellett *et al.*, 1995). The use of such systems directly measures overall toxicity of samples. These assays should be applicable to determining saxitoxins produced by cyanobacteria but require evaluation. These assays are unlikely to be sufficiently sensitive for direct determination of toxin concentrations in water, and therefore a concentration step may be necessary. However they have considerable potential.

## PEPTIDE HEPATOTOXINS (MICROCYSTINS AND NODULARIN)

### *Extraction Procedures*

The effective extraction of intracellular toxins is necessary as in healthy blooms containing peptide hepatotoxins, the major proportion of the toxin present is contained within cells (eg, Jones and Orr, 1994). It is only when a bloom collapses, or is treated with an algicide such as copper sulphate, and cells die that toxins are released to the surrounding water in significant concentrations (eg, Jones and Orr, 1994). Some lysis of cyanobacterial cells occurs during the freeze-drying process employed when isolating cyanobacterial material for investigation, and this assists in subsequent extraction into solution. Freeze-thawing also achieves this end, and has been utilised in a number of analytical procedures for the peptide hepatotoxins (eg, Lawton *et al.*, 1994).

Extraction of microcystins from lysed cells has been carried out using a number of solvent systems including acetic acid/water mixtures (Harada *et al.*, 1988b) or alcohol/water mixtures (eg, Brooks and Codd, 1986). Water, in conjunction with sonication has also been shown to be applicable to live cells (Rositano and Nicholson, 1994). Methanol/water mixtures have now been investigated in some detail (eg, Fastner *et al.*, 1998). Overall, the use of 75% methanol as recommended by Fastner *et al.* (1998), rather than pure methanol, would appear to be optimal for a range of microcystins. Three extractions are required for complete extraction of toxins (Lawton *et al.*, 1994; Fastner *et al.*, 1998; Ward *et al.*, 1997). Simple lysis/extraction procedures such as heating in a boiling water bath and using a microwave oven employing water as solvent were also found effective for a range of microcystins including hydrophobic variants such as microcystins -LW and -LF (Metcalf and Codd, 2000). These procedures, including sonication, are preferable where solvents such as methanol interfere in the subsequent analytical determination.

In terms of determining the intracellular component, the procedure of Lawton *et al.* (1994), ie, filtration and freeze-thawing, prior to solvent extraction, would appear to be the most appropriate. As indicated, extraction with 75% methanol will give the best recovery of a range of microcystins.

### *Concentration/Cleanup Procedures*

At low concentrations, the direct determination of hepatotoxins is generally not feasible due to the poor sensitivity of the analytical method, and a preconcentration step is required. Concentration can be carried out by solid-phase extraction, usually using an octadecyl (C18) coated silica adsorbent cartridge. The sample is passed through a cartridge which retains the toxins by adsorption. Toxins are eluted from these cartridges using solvents such as methanol. These methanol extracts may be reduced in volume by

evaporation, thereby resulting in toxins being concentrated relative to the original water sample.

The properties of the microcystins and nodularin can influence their extraction from water using these C18 cartridges. While the extraction efficiency of a number of microcystins and nodularin is generally reported to be high, most studies have generally focused on the more common microcystins such as microcystin-LR (eg, Gathercole and Thiel, 1987; Harada *et al.* 1988a; Lawton *et al.*, 1994). The recovery of more hydrophobic microcystins such as microcystin-LW can, however, be very poor under some circumstances (Lawton *et al.*, 1994). Not all commercially available C18 extraction columns are equivalent and microcystin-RR could only be efficiently recovered using C18 cartridges with endcapped silanol groups (Ojanpera *et al.*, 1995). Thus the recovery of toxins of interest with the particular C18 extraction cartridges to be used should be carefully checked to determine the effectiveness of the analytical procedure.

These cartridges also extract the naturally occurring organic matter (NOM) present in water, and following elution and concentration of toxins from the cartridge, the NOM is also isolated and concentrated. This co-extracted NOM can interfere in the chromatographic separation by producing peaks which may obscure the toxin peaks, thus making quantification imprecise. A procedure involving selective elution of toxins versus co-extracted interferences such as NOM has been developed to minimise this problem (Tsuji *et al.*, 1994b). Thus solid phase extraction can be used not only to concentrate toxins present in a sample but also to effect a cleanup by selectively eliminating interferences. Immunoaffinity columns have also recently been developed for concentration and cleanup of toxins, (eg Kondo *et al.*, 2000) and show great promise due to their high specificity for microcystins. Other options for extraction include solid-phase extraction disks (Tsuchiya and Watanabe, 1997) or cartridges with other solid-phase packings, eg, cyano (Pyo and Lee, 1994) but have not been extensively evaluated.

### *Sample Preservation*

Pure microcystins and nodularin are relatively stable in aqueous solutions of high purity or sterile water (eg, Watanabe *et al.*, 1992). However, in the presence of pigments, photochemical degradation can occur relatively quickly (Tsuji *et al.*, 1994a). Therefore water samples which contain cellular material and dissolved microcystins may be relatively unstable to sunlight with respect to toxin concentrations. These samples should be kept in the dark. Microcystins in solution appear stable to chemical breakdown by hydrolysis (Harada *et al.*, 1996b) so the pH at which water samples are kept is not important. These toxins may, however, be susceptible to microbiological degradation.

The microbial degradation of microcystins and nodularin in water bodies has been demonstrated (eg, Cousins *et al.*, 1996; Jones *et al.*, 1994), and the same behaviour will apply to water samples, unless some means of preservation is employed. In all cases degradation will depend primarily on the level of degrading organisms and temperature. Microbial activity and hence degradation can be slowed by refrigeration of samples (Heresztyn and Nicholson, 1997). Adding a preservative which kills microorganisms, eg, copper, may be useful procedure to minimise degradation. However such preservative will kill cyanobacteria, thereby releasing toxins, and will only be useful in the determination of total toxin content.

The stability of toxins in sample extracts must also be considered. While refrigeration or freezing of sample extracts is generally accepted as preventing degradation, Gjølme and Utkilen (1996) showed that microcystin-RR had varying stability at 4°C in various solvents. While the toxin was stable in methanol and 75% methanol over 32 days, in water and 5% acetic acid little toxin was left after this time. These results indicate that methanol or aqueous methanol are preferred extraction solvents if there is likely to be delay between sample extraction and analysis.

Another factor to consider is the possible adsorption of microcystins on materials which contact samples or extracts during analysis. Hyenstrand *et al.* (2001) investigated the adsorption of microcystin-LR on plastic (polypropylene) microcentrifuge tubes and pipette tips, and on glass. Significant losses by adsorption to plastics and glass occurred with aqueous solutions or solutions containing less than 25% methanol. 75% methanol which appears to be the best compromise for extracting microcystins of different hydrophobicities from cellular material may be the solvent mixture of choice for handling microcystins to prevent these losses by adsorption.

#### *Determination of Microcystins and Nodularin*

*High performance liquid chromatography (HPLC) Separation.* The most common instrumental analytical procedures for the determination of microcystins and nodularin employ high performance liquid chromatography (HPLC). The toxins are separated from each other and other co-extracted compounds using a reversed phase C18 packed column (eg, Harada *et al.*, 1988a; Lawton *et al.*, 1994), internal surface reversed phase column (Meriluoto and Eriksson, 1988) or ion exchange column (Gathercole and Thiel, 1987) and an aqueous mobile phase containing methanol or acetonitrile. This approach has received extensive application in the determination of toxins in cyanobacterial material. The mobile phase can determine whether certain toxins are resolved from each other, and from co-extracted compounds, since for accurate quantification, good resolution is required. For example, microcystins -LR and -YR co-elute with an acetonitrile/ammonium acetate mobile phase but can be resolved with methanol-based mobile phases (Harada *et al.*, 1988a). For similar microcystins, a combination of

mobile phases may be necessary to separate these compounds for unequivocal identification.

#### *Detection*

Having separated the toxins, the next stage is detection. The detection step is critical in that it must unambiguously respond to a toxin with high sensitivity. Co-extracted material such as the naturally occurring organic matter in water may interfere by masking the responses produced by the toxins. In this case cleanup procedures as recommended by, eg, Tsuji *et al.* (1994b) are required. Detection limits will depend on concentration factors attained and also on the sample volume. Tsuji *et al.* (1994b) achieved a detection limit of around 0.02 µg/L for individual microcystins with a 5 L water sample and MS detection.

The most common means of detecting these toxins following chromatographic separation is by UV absorbance. Most microcystins and nodularin have a UV absorption maximum at 238 nm (Lawton *et al.*, 1994, 1995). However those with aromatic amino acid constituents such as microcystin-LW which contains tryptophan have absorbance maxima at lower wavelengths, 222 nm (Lawton *et al.*, 1994, 1995). The wavelength of the UV detector of the HPLC equipment can be set at these values to record the responses of microcystins in sample extracts separated on the HPLC column. However other components in the sample extract can also have some absorbance at this wavelength and such an approach can only be regarded as producing a tentative result as to the presence of a toxin. In addition Ikawa *et al.* (1999) recently showed that common additives in plastics could contaminate water samples, co-elute with microcystins and have sufficient UV absorbance at 238nm to produce erroneous results. Thus plastic materials must be avoided, or their suitability checked, in the analysis of these microcystins using HPLC.

For accurate quantification, microcystins must be unequivocally identified, and analytical standards must be available. A standard can be used to indicate, to some extent, the identity of the toxin present, ie, matching retention time of a peak in a sample chromatogram with that of an authentic standard can be used as a tentative indication of identity of a toxin. Retention times cannot be used as unambiguous indicators of the presence of a particular toxin as toxins of similar structure can co-elute. It is, however, possible to estimate toxin concentrations, even when the toxin is not identified and standards are not available, as long as there is confidence that a microcystin is being detected. The literature indicates that microcystins have similar absorption coefficients (eg, Watanabe *et al.*, 1988). Thus they have similar sensitivities when analysed by HPLC with detection at 238 nm. Hence total concentrations can be estimated by reference to a known microcystin standard with a relatively high degree of accuracy.

A photo-diode array (PDA) detector which not only records the UV response but also the spectrum of a separated analyte provides much better evidence of the presence of a microcystin than using single wavelength detection. The typical spectrum of a microcystin with an absorbance maximum at 238 nm (or occasionally 222 nm in the case of microcystins containing tryptophan) provides a greater degree of confidence that a microcystin is present (Lawton *et al.*, 1994, 1995). Even so, identifying peaks in chromatograms as microcystins when concentrations are low and spectra are not well defined, is still difficult and depends on the experience of the analyst. Detection limits considerably less than 1 µg/L for individual microcystins should be attainable with appropriate concentration and cleanup procedures using this detection method.

Mass spectrometry as a detection method following HPLC separation provides a much better solution to the problem of unequivocally identifying microcystins, as microcystins produce characteristic ions in their mass spectra (eg, Lawton *et al.*, 1995). LC/MS with various interface and ionisation configurations has been reported for the determination of microcystins (eg, Lawton *et al.*, 1995; Tsuji *et al.*, 1994b; Zweigenbaum *et al.*, 2000). With a 5 L water sample and MS detection, Tsuji *et al.* (1994b) achieved a detection limit of around 0.02 µg/L for individual microcystins. MALDI-TOF mass spectrometry has also been successfully applied as a detection method following LC separation (Robillot *et al.*, 2000). Derivatisation of microcystins prior to LC/MS analysis has also been reported as a technique to assist in identifying microcystins (Sherlock *et al.*, 1997).

From the mass spectrum which acts as a fingerprint, a microcystin can be identified as long as a mass spectrum of an authentic standard is available. A useful extension to this technique is MS/MS detection where the fragmentation pattern can be used to greatly assist in determining the identities of unknown microcystins (eg, Lawton *et al.*, 1995). However, as with any analytical procedure which can unequivocally identify a toxin, standards are necessary for accurate quantification. Although work to date has shown different microcystins to have similar responses when determined with PDA detection, the same is not true of MS detection, ie, the total ion current depends markedly on the particular microcystin. Thus concentrations of unknown microcystins, or microcystins for which standards are not available, cannot be reliably estimated with this method.

Another approach to improving sensitivity and selectivity in the HPLC determination of microcystins and nodularin is the use of derivatising reagents. For example, James and James (1991) reported a post-column system whereby the arginine residue of microcystin-LR was derivatised with a fluorescent reagent. Such an approach obviously limits the application to toxins containing arginine, eg, microcystin-LR, and toxins such as microcystin-LA would not be determined.

#### *Capillary Electrophoresis (CE)*

Other separation techniques such as capillary electrophoresis (CE) and related techniques must also be considered for the separation and quantification of the peptide hepatotoxins (eg, Boland *et al.*, 1993). These lack sensitivity compared with HPLC procedures and are not suitable for routine monitoring of water without further development. Sensitivity has been increased by CE separation of fluorescently derivatised microcystins and detection using a laser-induced fluorescent detector (Li *et al.*, 1999). However, this also requires further evaluation.

#### *Thin Layer Chromatography (TLC)*

TLC procedures for the separation of microcystins have been reported (eg, Ojanpera *et al.*, 1995) and with appropriate detection systems, UV spectra of the separated components can be recorded. From their characteristic UV spectra, microcystins can be identified in a manner analogous to PDA detection in HPLC. Quantitative results are possible, but the method should be considered a screening procedure until more development is carried out.

#### *MMPB method*

A gas chromatographic (GC) method based on oxidation of microcystins which splits the Adda side chain to produce 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB) which is then determined, either by GC or GC/MS (as its methyl ester) (Sano *et al.*, 1992; Kaya and Sano, 1999), or by HPLC/fluorescence detection (after conversion to a fluorescent derivative) (Sano *et al.*, 1992), has been reported. A detection limit of 0.43 ng microcystin was reported (Kaya and Sano, 1999); for water samples the detection limit will depend on the concentration factor. The GC/MS approach was used to monitor microcystins in Japanese lakes (Tanaka *et al.*, 1993) and in sediments (Tsuji *et al.*, 2001). Harada *et al.* (1996a) reported a similar method whereby the MMPB was determined directly without derivatisation using GC/MS or LC/MS. This approach results in a total toxin concentration which can be expressed in terms of microcystin-LR.

#### *Enzyme linked immunosorbent assays (ELISAs)*

Various researchers have demonstrated that antibodies could be generated against microcystins, eg, Kfir *et al.* (1986) produced monoclonal antibodies against microcystin-LA which bound a range of other microcystins with equal efficiency. On this basis it would be expected that an ELISA based on these antibodies would show good cross reactivity and thus detect a wide range of microcystins. However, Chu *et al.* (1989) generated antibodies against microcystin-LR and found cross reactivities to vary between good (microcystin-RR) to poor (microcystin-LA). Nevertheless this procedure which was very sensitive was employed for determining microcystin content in environmental samples with reasonable success although the limitations of poor cross reactivity and therefore poor detection of some compounds were admitted (Chu *et al.*, 1990). Like all

ELISAs, cross reactivity depends on structural similarity which may not necessarily reflect similar toxicity. A more recent study by Nagata *et al.* (1995) produced similar results. Although the response to microcystin-LA was low (26% of that of microcystin-LR) with the antibody most sensitive to microcystin-LR, the results indicate that such an assay might be most useful as a semi-quantitative screen. This ELISA has been used to determine microcystin concentrations in waters from various countries such as China where there are high levels of primary liver cancer (Ueno *et al.*, 1996). The detection limit of the assay in this study was 0.05 µg/L.

An and Carmichael (1994) used an ELISA for determining microcystin content of cyanobacterial material. They evaluated the cross reactivity of a number of microcystins and found a poor correlation between reactivity and acute toxicity. In particular some toxic microcystins were poorly or not detected. This again highlights the point that, since ELISA techniques can greatly underestimate the concentration with some variants, they cannot be relied on as quantitative assays. They are, however, useful screening tools. Other ELISAs have recently been reported (eg, Metcalf *et al.*, 2000a; Zeck *et al.*, 2001) and may find application following further evaluation.

Rivasseau *et al.* (1999b) evaluated a commercial kit, the EnviroGard kit which is now distributed by EnSys, for determining microcystins in water samples. They found interferences leading to false positive results with waters spiked with low levels of microcystin-LR. The limit of quantification was therefore estimated as 0.2 µg/L. The matrix effect could be eliminated by using solid phase extraction for both concentrating the microcystins and cleanup. With samples containing cellular material the agreement was not so good, with the ELISA producing higher results than HPLC. However, this appeared to be due partly to cross-reacting microcystin variants which could not be quantified by HPLC. Cleanup using C18 cartridges improved the agreement between the two procedures, indicating some matrix interferences as well. Metcalf *et al.* (2000b) also evaluated commercial kits and found them susceptible to interferences which made them useful screening tools but limited their use for quantitative analyses. Commercial ELISA test kits are also now available from Mitsubishi Corporation, Japan and are based on the method of Nagata *et al.* (1995).

#### *Phosphatase inhibition assays*

The peptide hepatotoxins, the microcystins and nodularin, inhibit enzymes (protein (serine/threonine) phosphatases) responsible for the dephosphorylation of intracellular phosphoproteins (MacKintosh *et al.*, 1990). The types which are most inhibited by cyclic peptide hepatotoxins are Type 1 and a subset of Type 2 called protein phosphatase-2A. The extent of the inhibition of this reaction by these toxins can therefore be used as a measure of toxin concentrations. The basis of this type of procedure then is the measurement of phosphate release

from a phosphorylated protein in the presence of a phosphatase enzyme preparation and an inhibitor such as microcystin. Phosphatase inhibition by cyanobacterial hepatotoxins such as microcystins and nodularin can be determined using <sup>32</sup>P radiolabelled substrates (eg, MacKintosh *et al.*, 1990) and measuring the radiolabelled phosphate produced. This technique has been used for the measurement of microcystins in environmental samples (eg, Sim and Mudge, 1993; Jones and Orr, 1994; Lambert *et al.*, 1994). It has been applied to monitoring water samples with a detection limit of around 0.1 µg/L or less (eg, Lambert *et al.*, 1994). The method is sensitive but suffers from the major drawback that the <sup>32</sup>P isotope has a short half-life (approximately 14 days), which means that the labelled proteins for the assay have to be prepared on a regular basis. Such proteins are not commercially available and require reasonably sophisticated procedures for their preparation. Furthermore they use radioactive ATP and commercial enzymes in their preparation, both of which are quite expensive. Finally there is the fact that many routine laboratories are not set up to carry out radioactive determinations.

The problems of radiolabelled assays led to investigations of colorimetric procedures for the determination of microcystins and nodularin. The main approach has been to use a substrate such as *p*-nitrophenyl phosphate and determine the coloured *p*-nitrophenol released from the substrate in the presence of protein phosphatase and microcystin (or nodularin) (eg, An and Carmichael, 1994; Ward *et al.*, 1997; Heresztyn and Nicholson, 2001). Metcalf *et al.* (2001) combined the colorimetric assay with an immunoassay to produce a novel assay with high sensitivity and specificity for microcystins which appears to have potential. Other approaches reported, but with only limited assessment, have utilised substrates such that highly fluorescent or luminescent products are produced (eg, Isobe *et al.*, 1995). These assays then potentially have considerable sensitivities.

The colorimetric and fluorescent based phosphatase inhibition assays are extremely attractive in that they have high sensitivity, and, although used primarily to investigate toxins in cyanobacterial material, have the potential to be applied to water samples without a preconcentration step. These are toxicity-based measurements in that the inhibitory effect should be related to toxicity. However, acute toxicity in mammals will also depend on the ease of transport of the toxin across the cell membrane which will, in turn, be related to the hydrophobicity of the toxin. This might be illustrated by microcystin-RR which inhibits PP1 as strongly as microcystin-LR (Rivasseau *et al.*, 1999a). However, the acute toxicity of this hydrophilic microcystin is around 10-fold less than microcystin-LR (Carmichael, 1997). Thus the relationship between toxicity and phosphatase inhibition cannot be considered to be exact.

Phosphatase inhibition assays have received some evaluation in relation to their applicability in directly determining microcystins in water. Rivasseau *et al.* (1999a) observed a matrix effect leading to false positive results when employing *p*-nitrophenyl phosphate as substrate and PP2A. The effect was considered to be due to the mineral content of the water complexing with the EDTA in the assay medium and affecting the free nickel concentration. However, Heresztyn and Nicholson (2001) found no evidence of a matrix effect when employing manganese ion in the assay medium. This may indicate a more robust protocol, ie, the free manganese ion concentration which affects sensitivity is not influenced by the mineral content. The detection limit of the method was around 0.2 µg/L.

## CYLINDROSPERMOPSIN

### Extraction Procedures

Eaglesham *et al.* (1999) utilised freeze thawing of water samples for the extraction of cylindrospermopsin; however the effectiveness of this procedure was not validated. Other reported extraction procedures utilised methanol (Harada *et al.*, 1994), aqueous methanol (Banker *et al.*, 1997) and 5% acetic acid (Hawkins *et al.*, 1997) for freeze-dried material. While water probably is an adequate extraction solvent for this hydrophilic toxin, extraction for quantitative determination of intracellular toxin as a component of the total water concentration requires further attention.

### Sample Preservation

Studies of cylindrospermopsin adsorption on materials and degradation have been reported which are relevant to the storage of samples collected for analysis (Chiswell *et al.*, 1999). Cylindrospermopsin is strongly adsorbed by polyethylene which precludes its use as a sample container. No adsorption by Pyrex glass was found. Cylindrospermopsin is stable in the dark in water and little loss by chemical decomposition occurs over several weeks between pH 4 and 10, and at temperatures up to 50°C. However, sunlight rapidly degrades cylindrospermopsin in the presence of cellular material. Therefore samples should be stored in the dark. In contrast to blooms of *Microcystis*, a large proportion of cylindrospermopsin in environmental samples appears to be present as extracellular toxin.

### Determination of Cylindrospermopsin

Cylindrospermopsin can be determined by HPLC with detection at 262nm (Harada *et al.*, 1994). It is hydrophilic which precludes its extraction and concentration from water samples with solid phase extraction cartridges such as C18. Without extraction/concentration this HPLC procedure would appear to have insufficient sensitivity and specificity for monitoring water samples.

A method for monitoring cylindrospermopsin using LC/MS/MS has recently been reported (Eaglesham *et al.*, 1999). This method was sufficiently sensitive to be applicable directly to water samples without an extraction/preconcentration step. Concentrations down to around 1 µg/L can be readily quantified. These results indicate the power of MS/MS as a detection method in chromatography for providing a high degree of specificity without the need for sample cleanup.

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# WATER TREATMENT OPTIONS FOR CYANOBACTERIA AND THEIR TOXINS

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## ABSTRACT

When treating water subject to a cyanobacterial bloom the first priority should be removal of intact cells using separation techniques such as coagulation or membrane filtration. Chlorination and ozonation are effective for the destruction of residual dissolved microcystins and cylindrospermopsin at moderate water temperatures. Possible temperature effects on these oxidation reactions are currently unknown. Anatoxin-a can be effectively removed using ozone, although chlorine is relatively ineffective. Oxidation techniques do not appear to be the best method for the treatment of saxitoxins under normal treatment plant operating conditions. Powdered activated carbon can be effective for the removal of all toxins, except, perhaps, microcystin LA, provided the appropriate carbon, and the correct dose is applied. Granular activated carbon filters show a limited lifetime for the adsorption of most microcontaminants, including cyanotoxins. The biodegradation of cyanotoxins across GAC filters shows great potential as a treatment process.

## INTRODUCTION

Toxic cyanobacteria (blue-green algae) have now been reported in 27 countries and are found on all continents, including Antarctica. Drinking water authorities worldwide are faced with the challenge of treating contaminated water, or the possibility of a toxic bloom occurring sometime in the future. Knowledge of reliable treatment options, applicable in a range of conditions, for the cells themselves, and the toxins they produce, is of crucial importance to the international water industry.

The chemical structures of the most common cyanotoxins are shown in Figures 1-4.

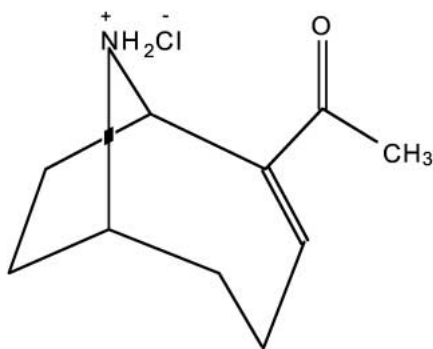


Figure 1. Anatoxin-a

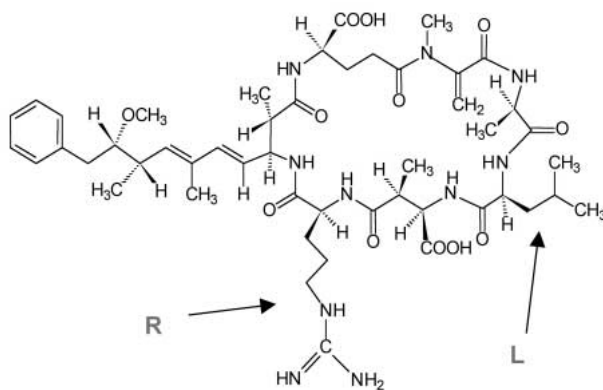
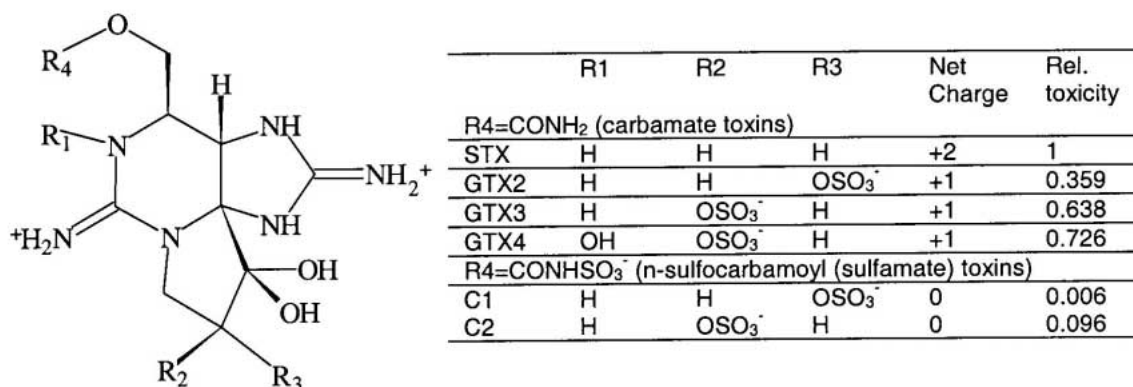
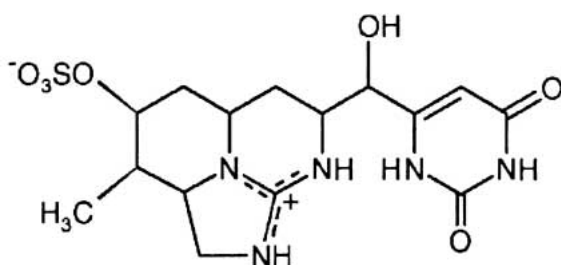


Figure 2. Microcystin LR, the most common of the 70 microcystin variants identified so far. The two amino acids indicated are leucine (L) and arginine (R). The different microcystins contain various amino groups in these two positions.



**Figure 3.** Saxitoxin class of cyanotoxins. Rel. toxicity indicates the toxicity relative to the most toxic analogue saxitoxin (STX).



**Figure 4.** Cyindrospermopsin

In this paper the authors present an overview of water treatment options for a range of cyanobacteria and cyanotoxins, based on literature information and the comprehensive experimental program on cyanobacteria undertaken at the Australian Water Quality Centre.

## MATERIALS AND METHODS

### Materials

#### Toxin Standards

Anatoxin-a and was obtained from Calbiochem Corporation (California, USA). Microcystin-LR was obtained from Sapphire Bioscience (Sydney, Australia).

#### Cyanobacterial Cell Material

For the jar test studies, a laboratory strain of *Microcystis aeruginosa* (MIC 338), known to produce microcystin-LR, was grown in 12 L ASM-1 medium (1) at 25°C under continuous illumination (~ 80 mM photons m<sup>-2</sup> s<sup>-1</sup>). For pilot plant experiments cultures were grown in BG-11 medium (2) with 1% CO<sub>2</sub> gas which gave a higher final cell yield (10<sup>7</sup> cells mL<sup>-1</sup>) at the time of harvesting. Cultures were harvested at the late exponential phase of growth, which corresponded to 10 days after inoculation.

#### Toxin Extracts

Microcystin LR and LA spiking material was extracted and purified from a local toxic bloom of *Microcystis aeruginosa*, Adelaide, Australia. For the chlorination work,

live cells were used, or the material was used after freeze-drying. Saxitoxin spiking material was purified from a toxic scum of *Anabaena circinalis* from Victoria, Australia. This material had a toxin profile characteristic of Australian strains of *A. circinalis* (3), ie, C1 and C2 toxins predominated with lesser quantities of GTX2, GTX3 and saxitoxin (STX). Details are given in (4).

#### Membranes

Two commercially available flat sheet membranes, micro filtration (MF) and ultra filtration (UF), usually used for spiral-wound elements were evaluated in this study. The MF membrane was manufactured by DESAL (Desalination Systems Inc., CA, U.S.A.) and made of polyvinylidene fluoride (PVDF) with 0.3µm pore size (approximate). The UF membrane, PS-100-H, was manufactured by Hoechst (Hoechst Aktiengesellschaft, Wiesbaden, Germany) and made of polysulphon with a molecular weight cut off (MWCO) of about 100000 (pore size approximately 0.01µm).

#### Membrane Filtration Unit

A test cell was designed by Rheinisch-Westfälisches Institut für Wasserchemie und Wassertechnologie (IWW) and made from acrylic plastic for use in this study (5).

### Chemicals

Copper sulphate stock solution was prepared by dissolving AR grade copper sulphate pentahydrate in Milli-Q<sup>®</sup> water. Aluminium sulphate stock solution (20,000 mgL<sup>-1</sup> as Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·18H<sub>2</sub>O) was prepared in Milli-Q<sup>®</sup> water from liquid aluminium sulphate (approximately 7.5% Al<sub>2</sub>O<sub>3</sub>) obtained from a water treatment plant.

## Methods

### Analysis of Toxins

Microcystins were analysed by HPLC with photo-diode array (PDA) detection (6) modified from Meriluoto and Eriksson (7). Anatoxin was determined by GC with electron capture detection following derivatisation according to the method of Stevens and Krieger (8) or by HPLC with fluorescence detection following derivatisation with NBD-F according to James and Sherlock (9). Saxitoxins were determined by HPLC with post-column derivatisation and fluorescence detection (10) as modified from Oshima (11). Mouse bioassays were carried out by intraperitoneal injection of 1.0 mL of extract into 20 g mice.

### Cell Density

*M. aeruginosa* cells were counted on a compound microscope in a Sedgewick-Rafter counting chamber after preservation in Lugol's iodine. Cell counts were carried out to a minimum precision of 20%.

### Cell Viability

Cyanobacterial cells were stained with fluorescein diacetate (FDA) and propidium iodide (PI). FDA stains cells with an intact cell membrane and active esterases. PI stains cells that have damaged cell membranes (12, 13). A minimum number of 100 cells were counted to achieve a precision greater than 10%.

### Jar Test

A FMS6V variable speed, six paddle gang stirrer with 76 mm [3.0 in.] diameter flat paddle impellers (SEM, Australia) and Gator jars were used. Water samples (2 L) were placed on the gang stirrer with six samples tested at a time, and the coagulant added while stirring at 230 rpm ( $G=480 \text{ s}^{-1}$ ). After 1 minute the speed was reduced to 25 rpm ( $G=18 \text{ s}^{-1}$ ) for 14 minutes for flocculation to occur. The samples were allowed to settle for 15 minutes. The settled water samples were then filtered under gravity through Whatman No.1 papers. The optimum dose was the minimum dose required to achieve the following treated water quality: turbidity < 0.5 NTU, color < 10 HU and aluminium <  $0.2 \text{ mgL}^{-1}$ .

### Effect of Coagulant on Cyanobacterial Cells

Batches of *M. aeruginosa* culture were concentrated by centrifugation at 8000 rpm for 10 min., mixed with natural water and left overnight at 25°C under constant illumination. The water was then divided into samples of equal volume to enable triplicate testing of the effect of copper sulphate or aluminium sulphate, compared with controls. Samples were taken for analysis initially, after 4 hours and after 24 hours. Cell density and cell viability were determined to establish the initial conditions of the experiment.

### Pilot Plant Experiment

An in-house designed pilot plant was used in this study to simulate the conventional water treatment process as described in Drikas et al. (15)

### Chlorophyll-a

Samples were filtered through Whatman GF/C filter paper and the chlorophyll was extracted using 10 mL ethanol (95%). The optical densities of the extracts at 665 and 750 nm were determined spectrophotometrically and chlorophyll-a concentrations were determined using the equations derived by Wintermans and de Mots (16).

### Membrane characterisation and operation

The pure water flow rate for each membrane was determined by using Milli-Q® water in Dead-End (DE) mode. The transmembrane pressure drop was adjusted to 200 kPa. In this study, both Dead-End (DE) and Cross-Flow (CF) modes were evaluated with the two selected membranes. In these experiments the membrane was backwashed after 500 mL of permeate was collected. Finally, a recirculation mode was tested using UF membrane in cross flow mode to simulate the conditions in an actual membrane filtration process. In this operation mode, the concentrate was pumped back to the pressurised vessel which increased the duration of the filtration process and also concentrated the cyanobacterial cells in the solution.

### Chlorination

Oxidation experiments were carried out on pure microcystin LR (m-LR), freeze dried material containing m-LR and microcystin LA (m-LA), live cells of a culture of *M. aeruginosa* containing mostly m-LR and live cells of a natural bloom of *N. spumigena*. Details are given in Nicholson et al. (17) and Rositano (6).

Chlorination of saxitoxins was carried out with semi-purified material. Reservoir water was dosed with the semi-purified material, and then with sufficient chlorine to produce a residual of 0.5 mg/L after 30 minutes. The pH of samples was adjusted before chlorine dosing and again after the 30 minutes contact time. Because of the relatively large chlorine doses required, there was substantial pH changes during the experiments. The pH at the end of the experiments was taken as the reaction pH. Initial toxin concentrations ranged from approximately 150 µg/L for C2 through to 10 µg/L for saxitoxin itself (18).

### Ozonation

Ozone stock solution was added to 250 mL of test solution and allowed to react for 5 minutes. Residual ozone was purged using nitrogen. Full details are given in (19).

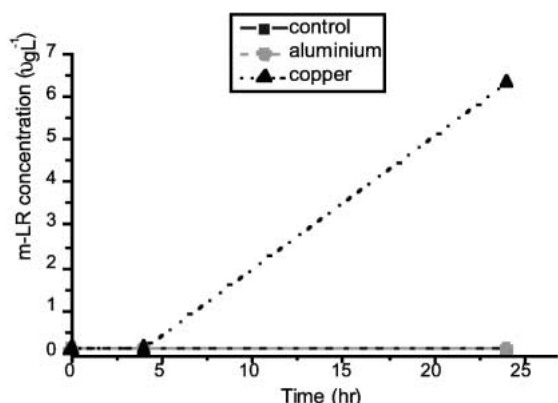
### Powdered and Granular Activated Carbon Application

Equilibrium and kinetic experiments using powdered activated carbon were undertaken as described elsewhere (20). Granular activated carbon pilot and laboratory studies are described in (4).

## RESULTS AND DISCUSSION

### Conventional Treatment

#### Effect of Water Treatment Chemicals on Cyanobacterial Cells



**Figure 5:** The effect of chemicals on microcystin release. Copper sulphate dosage,  $0.25 \text{ mgL}^{-1} \text{ Cu}$  and aluminium sulphate dosage,  $0.10 \text{ mgL}^{-1} \text{ Al}$ .

Copper sulphate has been shown to be toxic to *M. aeruginosa* at concentrations lower than  $1.0 \text{ mgL}^{-1}$  ( $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ ) and was used to demonstrate algicidal effects (21). The effects of stoichiometrically equivalent concentration of both copper ( $0.25 \text{ mgL}^{-1} \text{ Cu}$ ) and aluminium ( $0.10 \text{ mgL}^{-1} \text{ Al}$ ) on *M. aeruginosa* cells were compared. In order to account for the variability associated with using cultured organisms and to provide reliable results, the experiments were repeated three times on three separate occasions. Cell density and cell viability for *M. aeruginosa* cells in control and alum treated samples indicated that alum had no impact on the number or viability of the cells.

The copper treated samples at 24 hours were the only samples where a significant microcystin concentration was detected ( $6.3 \pm 0.1 \text{ µgL}^{-1}$ ) indicating significant cell damage and toxin release caused by copper (Figure 5).

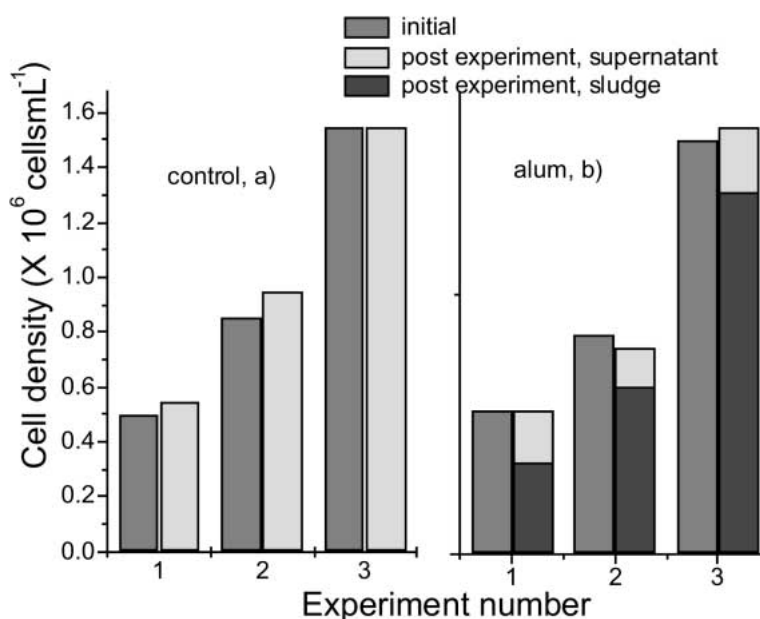
As considerably higher concentrations of alum are used in water treatment plants, the effect of higher concentrations of aluminium over 24 hours were evaluated. The optimum alum dose required for the natural water used in these experiments was  $60 \text{ mgL}^{-1}$  as alum. Given that underdosing of alum is known to result in more available aluminium, samples were also dosed at half this concentration. As predicted the soluble aluminium concentrations were higher in the sample dosed with half the optimum dose than for the optimum dose. Also the aluminium concentrations in the samples containing *M. aeruginosa* cells were lower than in the samples without cells, suggesting significant reduction in soluble aluminium by complexation to cell material.

The results indicated that dosing of alum at concentrations used in water treatment, and at considerably lower than optimum dose, where residual aluminium levels are higher, does not appear to be toxic to *M. aeruginosa* over a 24 hour period (14). Similar results were obtained in a previous study using *Anabaena circinalis* examining the release of geosmin, responsible for earthy/musty odour, using a similar experimental procedure (12). The use of ferric chloride as a coagulant also showed that cultured *M. aeruginosa* and *A. circinalis* cells were not damaged by the chemical treatment and hence there was no additional release of cell metabolites into the water (13).

#### Jar Tests

The effectiveness of flocculation at an optimum alum dose ( $65 \text{ mgL}^{-1}$  as alum) in removing cells of *M. aeruginosa* under mixing conditions which simulate water treatment was evaluated. Cell density and viability were determined

**Figure 6.** Cumulated cell density for *M. aeruginosa* from flocculation experiments, control a), and alum, b)



in the sludge and the supernatant, before and after the addition of alum, and compared with control samples. The pH of the water with cells present was reduced from 9.4 - 9.7 to 7.2 by the addition of alum. Results of 3 replicates are shown in Figure 6. The results of cell density before the addition of alum and after mixing and settling of the cells in both the sludge and supernatant show 70%, 72% and 83% of the cells were removed in experiment 1, 2 and 3 respectively (e.g., percentage removal =  $100 \times (\text{number of cells in raw water} - \text{number of cells in supernatant}) / \text{number of cells in the raw water}$ ). There was no significant effect of alum dosing ( $p > 0.05$ ) on cell viability with cells remaining intact both in the supernatant and in the sludge in all three experiments. The results in Table 1 show that flocculation did not cause additional release of m-LR into the water.

This experiment, and the pilot plant work described below, used cultured cells of toxic *M. aeruginosa* to provide a consistent and reliable source of material over the study. This cultured material differs physically from naturally occurring field populations of *M. aeruginosa* in being comprised of small regular colonies, and many single and pairs of cells with much less mucilage than natural material. The field populations can contain very large (macroscopic) colonies with greater amounts of mucilage (acid-mucopolysaccharides) surrounding them. The differences in the surface physico-chemical properties of these two forms of *M. aeruginosa* are not known. Although there may be some differences in binding with alum this has not been investigated, however it is anticipated that it will not be significantly different.

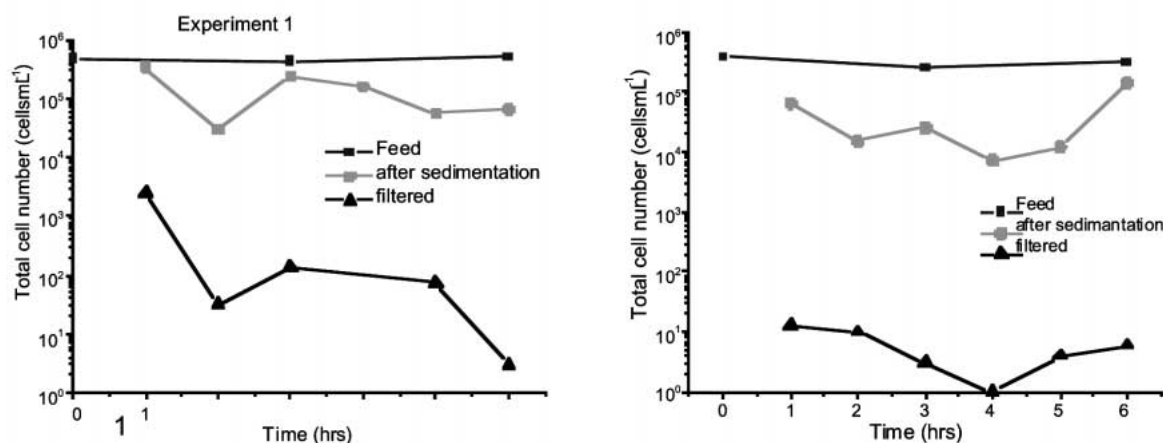
#### Pilot Plant Experiments

The pilot experiment provided a evaluation of the effect of conventional water treatment process on *M. aeruginosa* cells. The optimum alum dose was determined to be  $70 \text{ mgL}^{-1}$  at pH 6.2 using standard jar test procedures for South Para Reservoir water with the addition of cyanobacterial cells. Samples of raw (feed), before filtration (after sedimentation) and filtered (finished) water were monitored during the 6 hour run. The quality of the feed water (turbidity, colour and pH) did not change during the experiment. In general, the pilot plant performed well and the water quality parameters of the finished water during the experimental period were below the required limits (turbidity  $< 0.5 \text{ NTU}$ , colour  $< 10 \text{ HU}$  and residual aluminium  $< 0.2 \text{ mgL}^{-1}$ ) over the entire experimental period. The addition of the algae appeared to aid the flocculation process, resulting in better finished water quality in the experiment with algal cells present compared with the control. Algae provide particulate matter as a nucleus for flocculation and allow faster settling; this was particularly apparent over the initial 2 hours of the experiments.

The cell density results in the feed of the duplicate experiments are presented in Figure 7. The cell densities in the feed were between  $4 \times 10^5$  and  $5 \times 10^5$ , and  $3 \times 10^5$  and  $4 \times 10^5 \text{ cells mL}^{-1}$  for experiments 1 and 2 respectively. The average cell densities after sedimentation were  $1.5 \times 10^5$  and  $4.4 \times 10^4 \text{ cells mL}^{-1}$  for experiments 1 and 2 respectively. These results indicate 70 and 85 % of the cells were removed by sedimentation in experiments 1 and 2 respectively. The cell density in the finished water was generally low, except at the beginning of the experiment when the system has not achieved its

**Table 1:** The dissolved m-LR concentration for samples obtained from flocculation experiments (jar tests).

|            | Microcystin-LR concentration ( $\mu\text{gL}^{-1}$ ) |                           |                           |
|------------|--|---------------------------|---------------------------|
|            | Experiment 1   | Experiment 2              | Experiment 3              |
| Control    | $10.8 \pm 0.7$                                       | $< 0.1$ (detection limit) | $0.2 \pm 0.3$             |
| After Alum | $2.7 \pm 0.1$  | $< 0.1$ (detection limit) | $< 0.1$ (detection limit) |



**Figure 7:** The cell density at different sampling locations.

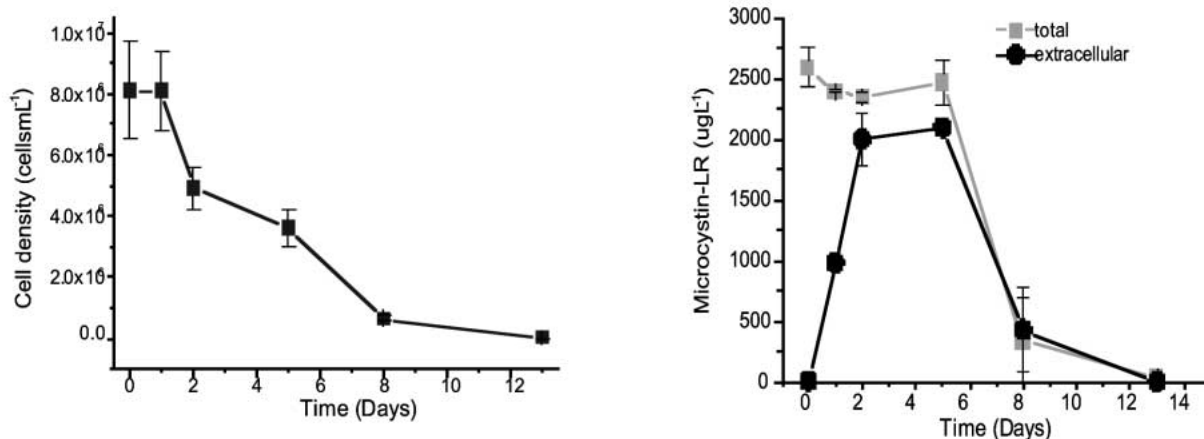


optimum performance. The average cell density in the finished water in experiment 1 was  $60 \text{ cells mL}^{-1}$  while in experiment 2,  $6 \text{ cells mL}^{-1}$  were found. These figures are 0.01 and 0.002% of the feed, respectively.

The cell viability results (as % FDA-active cells) indicated that close to 100% of cells were FDA-active in the feed in both experiments and that the cells in the sludge were between 93 and 99% FDA-active. Based on these results, the cells present in the sludge have minor damage compared with the feed. The cells in the backwash water also showed minor damage; cell viability results in both experiments were 99 and 95% FDA-active, respectively.

The initial dissolved (free extracellular) concentrations of m-LR in the feed for both experiments were approximately  $2 \mu\text{g L}^{-1}$ . This is probably due to the natural toxin release from the cells which occurred during culturing (22). In the feed, the m-LR concentration gradually increased to  $4 \mu\text{g L}^{-1}$  at the end of experiment 1 while in experiment 2 it was constant over the experimental period. In general, the dissolved m-LR concentrations after sedimentation and in the finished water were comparable to the feed for both experiments (slightly higher in experiment 1 and slightly lower in experiment 2). These results indicate that the pilot scale conventional water treatment does not release toxin above the background concentrations. However, conventional water treatment processes are not capable of removing extracellular m-LR.

During the experiments, the sludge chamber was emptied every two hours. In order to assess the degradation of the cells in the sludge, the sludge remaining at the completion of the experiment was sampled over a period of 14 days (15). The cell viability results presented by percentage FDA-active dropped below 10% after one day and therefore cell viability measurement was ceased. The cell density in the sludge dropped to half its initial value after 2 days and continuously decreased throughout the experimental period (Figure 8). After day 8, the cell number was close to zero. The total microcystin concentration stayed constant in the first 6 days and dropped sharply to zero in day 8. The extracellular microcystin (released solely from microcystis cells) initially started at zero on day 0 and increased until day 2 when the extracellular concentration was essentially equivalent to the total microcystin concentration. This increase in extracellular microcystin indicated the breakdown of cells and release of toxin. From this result, complete cell breakdown occurred after only 2 days of the experiment. After day 5, both total and extracellular microcystin concentrations decreased indicating degradation of microcystin. This finding agrees reasonably well with Jones and Orr (23) for the degradation of microcystin after chemically treating a *Microcystis aeruginosa* bloom with a copper algicide. In that study, the microcystin persisted at high levels for 9 days before degradation commenced.



**Figure 8:** Cell density of *M. aeruginosa* and extracellular and total microcystin-LR concentrations present in the sludge during the experiment.

### Recommendations

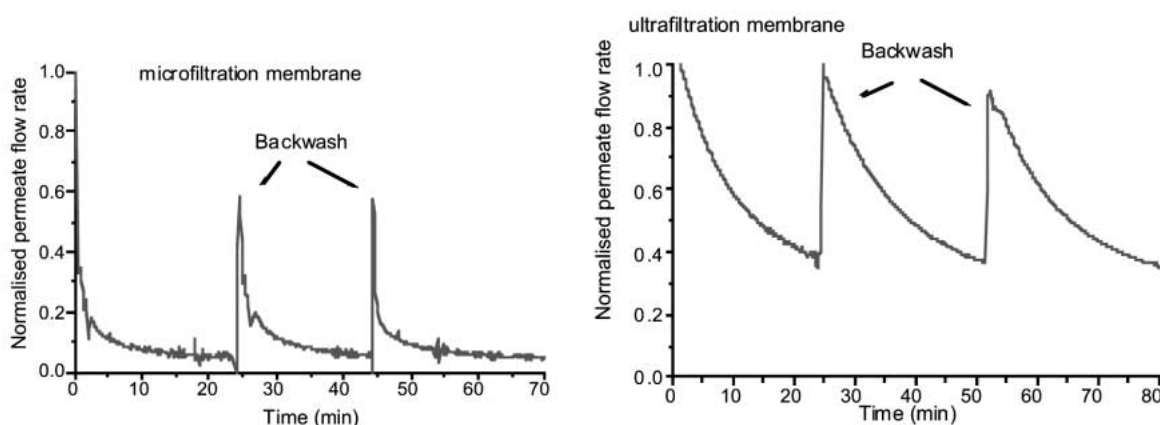
Chemicals such as copper sulphate, or oxidants such as chlorine, lyse algal cells and release algal toxins. To maximise the efficiency of removal of both algal cells and their metabolites pre-oxidation should not be used. Under these conditions, cells can be effectively physically removed prior to the application of any oxidation process. For cultured *M. aeruginosa* or *A. circinalis* this can be achieved using aluminium sulphate or ferric chloride under normal operating conditions, without damage to cells causing additional release of dissolved toxin. However, under normal bloom conditions it is highly likely that the algal cells will be in various stages of their growth cycle with some algae already in decline and releasing algal toxins. It will therefore be necessary to include a treatment step to remove the extracellular toxins. It is recommended that toxin concentration in the treated water is monitored during bloom situations as there is no suitable surrogate to ensure the safety of the water.

Again, under normal operating conditions, rapid sludge removal from sedimentation basins will ensure very little additional dissolved toxin is released from the settled cells. Supernatant from sludge drying beds should not be recycled back to the head of the plant or the raw water source until all toxin has degraded.

### Membrane Filtration

Under the test conditions with cyanobacterial culture, the MF membrane showed a high rate of initial flow rate decline in both Dead-End (DE) and Cross-Flow (CF) modes (Figure 9). A similar observation was reported by Gatenholm et al. (24) using MF and UF membranes for *E. coli* separation. The faster flux decline for MF may be accentuated by the initial high permeate flux of the MF membrane transporting a higher number of cells to the membrane surface with consequent rapid buildup of cell mass. However, the permeate flux of MF and UF in both operation modes shortly before backwashing is almost the same. This is a strong indication for cake filtration, as almost the same mass of cells were removed by the membranes just before backwashing. Under this situation, the permeate flux was controlled by the layer. This means that the hydraulic resistance of the attached layer was much higher than the resistance of the membrane alone.

The recovery of the permeate flux following backwashing (Figure 9) was higher for the UF membrane; suggesting that *M. aeruginosa* cells were harder to remove from the MF membrane than from the UF membrane. A possible explanation for this may be the difference in surface roughness for the two tested membranes. It is likely to be more difficult to remove cells from a rough membrane surface, such as the MF membrane, than from a smoother surface, such as the UF membrane. Higher rates of irreversible pore blockage on the membrane's surface, leading to a smaller active pore area could account for reduced relative flow rates for the MF membrane.



**Figure 9.** Normalised permeate flow rate and permeate flux of MF membrane and UF membrane in CF mode.

In this study direct microscopic examination (cell count) and total biomass as determined by chlorophyll-a measurement, were used to assess the removal efficiency of the cyanobacteria. Results for cell removal efficiency as indicated by cell numbers and biomass in the backwash, permeate, and the amount remaining on the membrane are given in Table 2. Apart from some minor leakages, the removal efficiency was high for both membranes and modes of operation. The slight leakage of whole cells through the membrane which were then detected in the permeate by microscopic examination and chlorophyll-a measurement (particularly for MF in CF mode) was likely to be due to the poor sealing in the test apparatus. The results indicated *M. aeruginosa* was harder to remove from the microfiltration membrane (30% of the feed left on the membrane) than the ultrafiltration membrane (10% of the feed left on the membrane). This is certainly an important issue because if membrane washing cannot completely remove the deposited algae, toxin may be released when the cell dies.

Cell viability measurements to detect the percentages of living, damaged and dead cells were undertaken. No increase in dead cells was found in the backwash water in most cases, except MF-DE. However, in general there were  $\approx$  2-5% more damaged cells in the backwash water compared with the feed. This increase was clearly shown in the recirculation mode, where the damaged cells increased from 4% to 10% during the experiment (Table 3). This is in contrast to the finding of Petrusevski *et al.* (25) who used UF as a means of concentrating algal cells and found no cell damage after the process. However, the experimental setup and the aim of the study were different. The algal cells that were not damaged were obtained from the concentrate stream rather than the backwash water as in our case.

In all cases there was no significant increase ( $p < 0.05$ ) in the level of dissolved microcystin-LR in the permeate compared with the feed. Although cell damage was noted, it apparently did not result in any further release of microcystin in the finished water during cell removal.

**Table 2:** The distribution / partitioning of cells of *M. aeruginosa* through the compartments of the membrane filtration system after operation for three cycles. Removal/ retention was measured by both chlorophyll-a and direct microscope cell counts. As cell counts were not undertaken on the membranes, the percent present on the membranes calculated from Chlorophyll-a measurement was used.

| Chlorophyll-a Measurement |         |                    |                    |                 | Cell Count         |                    |                    |                    |
|---------------------------|---------|--------------------|--------------------|-----------------|--------------------|--------------------|--------------------|--------------------|
|                           |         | Back Wash/Feed (%) | Permeate/ Feed (%) | On Membrane (%) | Total <sup>a</sup> | Back Wash/Feed (%) | Permeate/ Feed (%) | Total <sup>b</sup> |
| MF-DE                     | Cycle 1 | 80                 | 2*                 | 44              | –                  | 56                 | 1                  | –                  |
|                           | Cycle 2 | 81                 | 2*                 | 34              | –                  | 44                 | 1                  | –                  |
|                           | Cycle 3 | 89                 | 1*                 | 48              | –                  | 74                 | 1                  | –                  |
|                           | Average | 83                 | 2                  | 38*             | 124                | 58                 | 1                  | 97                 |
| MF-CF                     | Cycle 1 | 40                 | 1                  | –               | –                  | 53                 | 1                  | –                  |
|                           | Cycle 2 | 41                 | 1                  | –               | –                  | 74                 | 3                  | –                  |
|                           | Cycle 3 | 46                 | 5                  | 32              | –                  | 58                 | 9                  | –                  |
|                           | Average | 43                 | 2                  | 32              | 77                 | 62                 | 4                  | 98                 |
| UF-DE                     | Cycle 1 | 93                 | 0                  | –               | –                  | 118                | 0                  | –                  |
|                           | Cycle 2 | 85                 | 1                  | –               | –                  | 90                 | 3                  | –                  |
|                           | Cycle 3 | 87                 | 1                  | –               | –                  | 89                 | 1                  | –                  |
|                           | Average | 89                 | 1                  | 9 <sup>=</sup>  | 99                 | 99                 | 1                  | 109                |
| UF-CF                     | Cycle 1 | 45                 | 0                  | –               | –                  | 46                 | 0                  | –                  |
|                           | Cycle 2 | 28                 | 0                  | –               | –                  | 33                 | 0                  | –                  |
|                           | Cycle 3 | 30                 | 0                  | 9               | –                  | 32                 | 0                  | –                  |
|                           | Average | 34                 | 0                  | 9               | 43                 | 37                 | 0                  | 46                 |

\*: repeat on a separate occasion

<sup>=</sup>: from UF-CF

Total<sup>a</sup>: Back Wash / Feed (Chlorophyll) + Permeate / Feed (Chlorophyll) + On Membrane (Chlorophyll)

Total<sup>b</sup>: Back Wash / Feed (Cell Count) + Permeate / Feed (Cell Count) + On Membrane (Chlorophyll)

Feed Concentration: in the order of  $10^5$  cells mL<sup>-1</sup> or approximately 20  $\mu$ g L<sup>-1</sup> chlorophyll-a.

**Table 3.** The condition of the cells in back wash water after recirculation using ultrafiltration membrane.

| Time (hrs) | Back Wash Water Live (%) | Injured (%) | Dead (%) |
|------------|--------------------------|-------------|----------|
| 0 (feed)   | 96                       | 4           | 0        |
| 1          | 97                       | 3           | 0        |
| 2          | 96                       | 3           | 0        |
| 3          | 93                       | 7           | 0        |
| 4          | 90                       | 10          | 0        |

In the experiments with the UF membrane, particularly in the recirculation experiment, the amount of microcystin was significantly lower ( $p < 0.05$ ) in the permeate than in the feed. This suggested that the particular UF membrane employed may have rejection properties or adsorption ability for microcystin. A simple batch test utilising a small section of membrane in contact with a solution containing microcystin-LR compared with a control confirmed that the presence of the membrane caused some 20 - 30 % reduction in toxin concentration (13).

This removal of microcystin would not be expected for UF membranes but should occur for membranes with lower molecular weight cut off such as nanofiltration membranes. Hart and Stott (26) evaluated the effect of nanofiltration for the removal of microcystin spiked into natural water at concentrations between 5 - 30  $\mu\text{g L}^{-1}$  and detected below 1  $\mu\text{g L}^{-1}$  in the permeate. Australian studies with nanofiltration membranes also showed that microcystin LR and nodularin at 8  $\mu\text{g L}^{-1}$  were removed from River Murray water spiked with toxin (27).

### Recommendations

Micro and ultrafiltration membrane systems appear suitable processes for the removal of cyanobacterial cells. Whilst this study showed some evidence of cell damage, there was no significant increase in dissolved toxin concentration in the permeate. In contrast, the particular type of UF membrane employed was found to remove some dissolved microcystin. This is not expected for UF membranes but would be expected for nanofiltration or reverse osmosis systems.

It was not possible to completely remove all the *M. aeruginosa* cells from the MF membrane and this is an important issue because if membrane washing cannot completely remove the deposited algae, toxin may be released when the cell dies. The extent to which cyanobacteria adhere to the membrane surface would depend on the surface characteristics of both the algae and the membranes and would need to be considered when choosing membranes for removal of cyanobacteria.

### Chlorination

#### *Microcystins and nodularin*

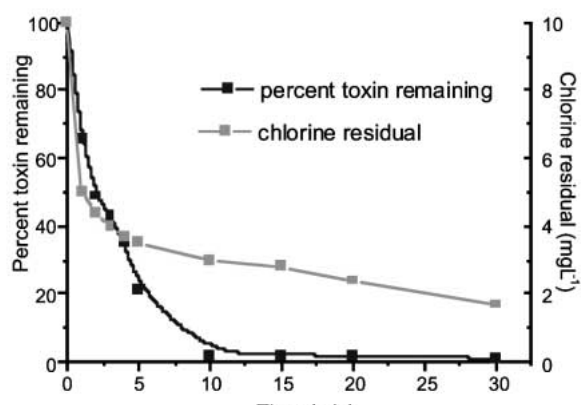
Microcystins in an extract of freeze-dried material were rapidly destroyed by chlorine, with the destruction of toxins correlating well with loss of toxicity as determined by mouse bioassay (Table 4). Based on the reported acute toxicities of microcystins LR and LA (50  $\mu\text{g kg}^{-1}$ ), (28), a lethal dose would be around 1  $\mu\text{g}$  per mouse for a 1 mL injection, which agrees well with the analytical results. Results for individual toxins were the same, total toxin concentration is given.

**Table 4.** Microcystin concentrations and mouse bioassay results after chlorination of an extract of toxic *M. aeruginosa* with aqueous chlorine. Contact time = 30 min

| Chlorine dose ( $\text{mg L}^{-1}$ ) | Toxin concentration determined by HPLC ( $\mu\text{g L}^{-1}$ ) | Microcystin injected ( $\mu\text{g}$ ) | Mouse bioassay result |
|--------------------------------------|---|--|-----------------------|
| 0                                    | 192   | 3.8                                    | +                     |
| 0.2                                  | 174   | 3.5                                    | +                     |
| 0.5                                  | 164   | 3.3                                    | +                     |
| 0.8                                  | 154   | 3.1                                    | +                     |
| 1.0                                  | 4   | 0.08                                   | -                     |
| 2.0                                  | 4   | 0.08                                   | -                     |

+ = acutely toxic; - = non-toxic

The removal of toxins by chlorine was found to be very dependent on pH. The destruction of microcystin decreased markedly at pH above 8 for sodium and calcium hypochlorite and above pH 9 for gaseous chlorine. This behaviour reflects the effect of pH on the oxidising potential of hypochlorous acid. Under alkaline conditions ( $\text{pH} > 8$ ) chlorine is present predominantly as the hypochlorite ion which is a weaker oxidant than hypochlorous acid, the main species present under neutral to acid conditions.



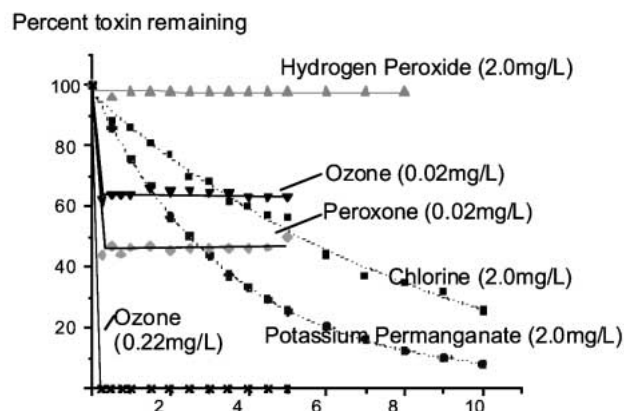
**Figure 10.** Percent toxin remaining, and chlorine residual vs. time for a culture of *M. aeruginosa*

Chlorine was also found to be effective in destroying toxins present in live cells of both *M. aeruginosa* and *N. spumigena* (6,17). Presumably chlorine is effective at rapidly lysing the cells, thereby releasing the toxins where they react rapidly with chlorine. Figure 10 shows the results with a culture of *M. aeruginosa* where a 10 mgL<sup>-1</sup> dose of chlorine reduced the concentration of microcystins from 46 to < 1 µgL<sup>-1</sup> (98% removal) in 30 minutes.

With live cells of *N. spumigena* collected from a bloom, nodularin was reduced from a concentration of 440 µgL<sup>-1</sup> to < 1 µgL<sup>-1</sup> in 30 minutes. At this time the chlorine residual was 0.5 mgL<sup>-1</sup>. On the basis of these and other results, it was concluded that chlorine treatment of cyanobacteria was effective in destroying hepatotoxins such as microcystins and nodularin as long as a free chlorine residual of at least 0.5 mgL<sup>-1</sup> was present after 30 minutes and the pH was less than 8. For toxin removal to be effective, it is important that the chlorine demand of the water be satisfied. In waters with a relatively high demand, removal is slower than with pure toxins in high purity water due to the competitive reactions with the natural organic material.

The effective destruction of hepatotoxins by chlorine is contrary to earlier results reported in the literature (29-31). Hoffmann (29) reported removal of toxicity, determined by mouse bioassay, as a function of chlorine dose. It can now be calculated from the acute toxicities of these toxins that insufficient chlorine was used in these experiments. In the work reported by Keijola et al. (30) and Himberg et al. (31), where toxin removal was determined by HPLC, the low chlorine doses utilised were probably consumed by the naturally occurring humic material present.

**Other Oxidants.** Other oxidants were also investigated as a means of destroying microcystins. Chloramine and hydrogen peroxide were ineffective but potassium permanganate was more effective than chlorine in oxidising pure m-LR (6).



**Figure 11.** Percent m-LR remaining vs time for a range of oxidants. Initial m-LR concentration, 1 mgL<sup>-1</sup>.

Figure 11 shows the effect of various oxidants on the concentration of m-LR (starting concentration, 1 mgL<sup>-1</sup>). With live cells, toxin removal was very much slower, and potassium permanganate was not as effective as chlorine. This suggests that chlorine is more efficient than potassium permanganate at lysing the cells even though it is a weaker oxidant (6). Again toxins in extracts were oxidised at a slower rate than toxins in pure water due to competitive reactions of the natural organic matter.

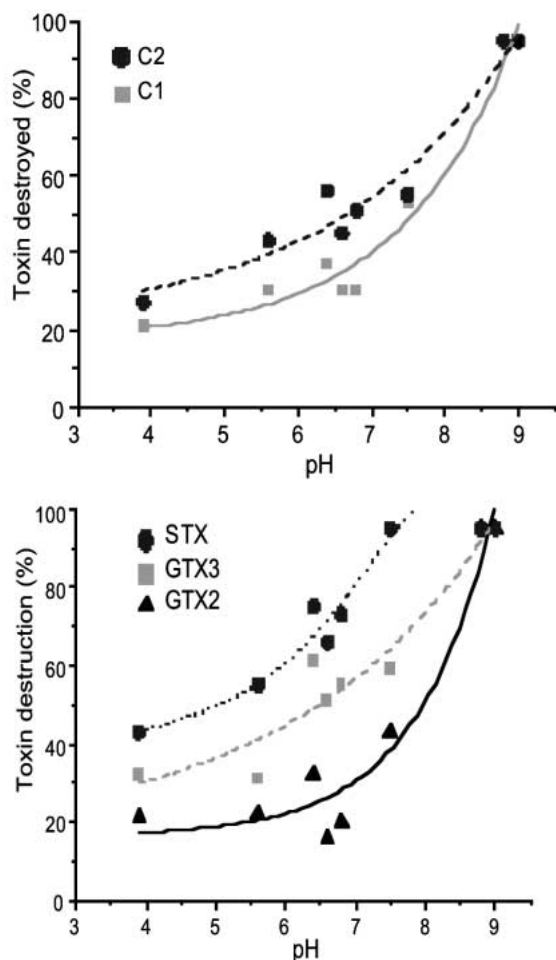
#### Anatoxin-a

Chlorine was found to be relatively ineffective in oxidising anatoxin-a. Anatoxin-a was dosed in to reservoir water at a concentration of 20 µgL<sup>-1</sup> and treated with varying doses of chlorine. In all experiments the pH was between 6 and 7. At the highest chlorine dose (15 mgL<sup>-1</sup>), after 30 minutes contact time the free chlorine residual was 4.5 mgL<sup>-1</sup> but toxin removal was only 16%. Similar results have been reported previously (30, 32, 33).

#### Saxitoxins

Destruction of saxitoxins by chlorine was dependent both on pH and the particular toxin (Figures 12). The form of the chlorinating agent was unimportant when the pH was taken into account. The order of ease of removal of the saxitoxins was STX > GTX3 ~ C2 > C1 > GTX2. A high removal was possible at pH 9 provided a residual of 0.5 mgL<sup>-1</sup> free chlorine was present after 30 minutes contact time (18). Removal as a function of pH was not linear with the degree of removal increasing rapidly at around pH 7.5. The more effective removal at higher pH was unexpected as chlorine is known to be a weaker oxidant under these conditions. However the more effective removal may be due to the toxin molecule being present in an unprotonated form at higher pH and therefore more susceptible to oxidation. This is supported by the fact that in the detection of saxitoxins using post-column oxidation, sensitivity, which depends on the oxidation of these toxins to fluorescent derivatives, increases as the pH increases from 6.5 (34). Thus oxidation, at least to form fluorescent derivatives, is more efficient as the pH increases.

The feasibility of using chlorine to remove saxitoxins will depend on the pH of the water, the chlorine dose, initial concentrations of toxins and the degree of removal required. Removal may be improved by pH adjustment. Although saxitoxins are not detected by chemical analysis after chlorination under optimum conditions, there is no indication as to the nature of the oxidation products.



**Figure 12.** Percent saxitoxin destroyed by chlorine as a function of pH. Chlorine dose was sufficient to provide a  $0.5 \text{ mgL}^{-1}$  residual after 30 minutes.

#### *Cylindrospermopsin*

Cylindrospermopsin, like the microcystins, is very susceptible to oxidation by chlorine. Oxidation is very rapid with  $> 99\%$  removal in the pH range 6 – 9 under conditions where a chlorine residual of  $0.5 \text{ mgL}^{-1}$  is achieved (35).

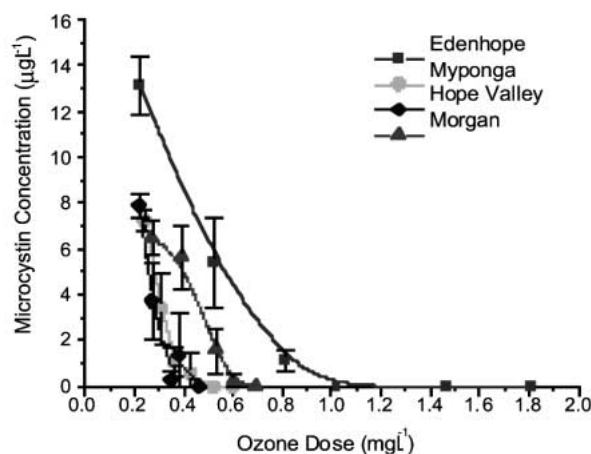
#### **Recommendations**

Chlorination is an effective, simple method for the destruction of microcystins and cylindrospermopsin. At the moderate water temperatures studied so far, the doses of chlorine required to maintain a residual in the distribution system would be expected to result in toxin-free water. However, it is well known that the reaction

with natural organic material in water to form disinfection by-products is strongly dependent on temperature, and it is possible that at lower temperatures the rate of reaction with cyanotoxins would be greatly reduced. This aspect of the application of chlorine requires further research. The oxidation of saxitoxins by chlorine is effective only at high pH ( $> 8-9$ ). Therefore pH control must be applied for the utilisation of this method for removal of the saxitoxin class of cyanotoxins. Chlorination would not be recommended as a method for the treatment of drinking water containing anatoxin-a.

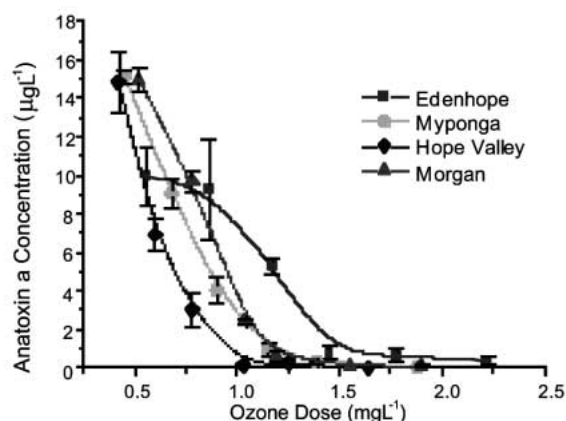
#### **Ozonation**

##### *Microcystins*



**Figure 13.** Microcystin concentration as a function of ozone dose in four treated waters.

Ozonation of microcystins LR and LA (m-LR and m-LA) was undertaken in a batch reactor in four treated waters. For details of water quality, see Rositano et al. (19). There was a strong effect of water quality which was related to the ozone demand of the water. In all waters the microcystins were both destroyed to below detection at an ozone dose sufficient to retain a residual of molecular ozone for five minutes (Figure 13).



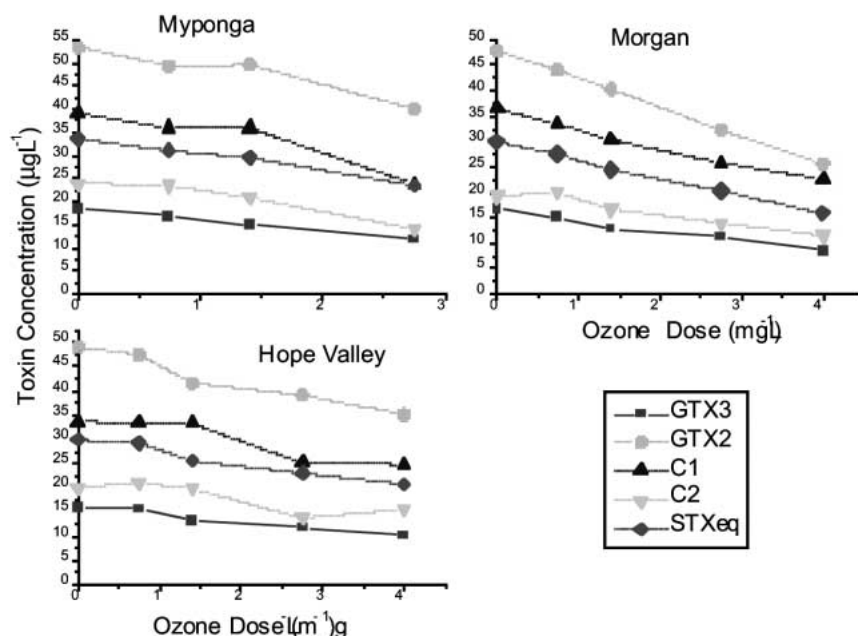
**Figure 14.** Anatoxin-a concentration as a function of ozone dose in four treated waters.

#### Anatoxin-a

Results for anatoxin-a were similar to those obtained for microcystins (Figure 13), with slightly higher ozone residuals required for the destruction to below detection (Figure 14).

#### Saxitoxins

A mixture of saxitoxins was exposed to ozone under the same conditions as described above. In this case, all of the toxins appear to be fairly recalcitrant to oxidation by ozone (Figure 15).



**Figure 15.** Saxitoxin concentration as a function of ozone dose.

#### Cylindrospermopsin

Aldridge et al (35) reported effective degradation of cylindrospermopsin using ozone in laboratory trials. The conditions of the experiment were unclear, as the toxin spiking solution was only semi-purified, and added significantly to the ozone demand of the water. However, the authors reported that the reaction required low contact times, and doses of ozone that were below the demand of the water.

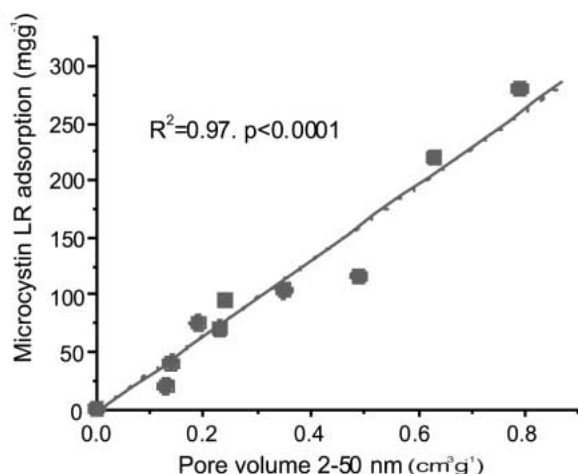
#### Recommendations

At moderate temperatures ( $> 16^{\circ}\text{C}$ ), ozone is effective for the destruction of microcystins and anatoxin-a under the conditions of dose and contact time usually employed for the deactivation of *Cryptosporidium* and *Giardia*. An ozone dose insufficient to retain a residual for longer than 1 minute should not be relied upon for the destruction of the toxins. There is anecdotal evidence that microcystin is not destroyed under such conditions at low temperatures. This aspect of the ozonation of toxins requires further study, as microcystins are found in a wide range of climates. Saxitoxins would not be destroyed completely under the conditions suggested for anatoxin-a and microcystins. It is possible that cylindrospermopsin is susceptible to ozonation, due to the double bond in its structure. However, further work is required to determine the effect of water quality conditions, and ozone CT values (concentration  $\times$  time) required in a range of waters.

#### Activated Carbon

##### Powdered Activated Carbon

**Microcystins.** The information available in the literature on the adsorption of m-LR onto activated carbon indicates that, as with the adsorption of most microcontaminants, the removal efficiency is dependent on the type of activated carbon and the water quality conditions (26, 36, 37).

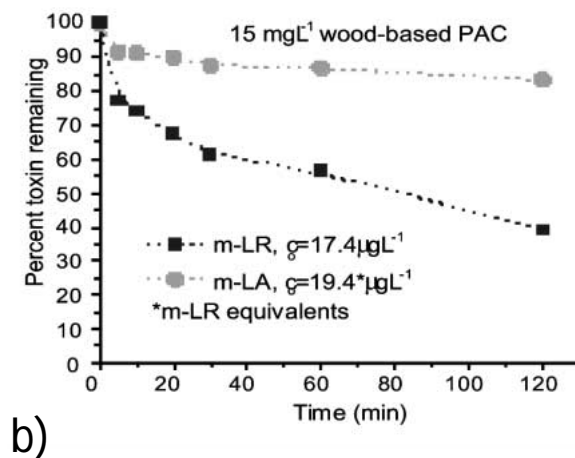
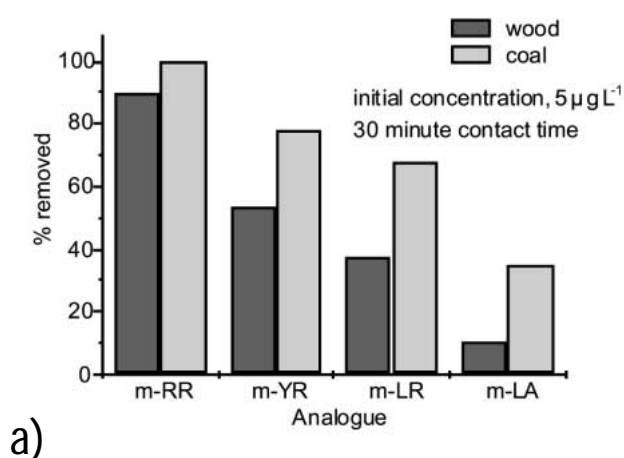


**Figure 16.** Microcystin LR adsorption vs. pore volume of activated carbons

Several studies have shown that chemically-activated wood-based powdered activated carbons (PACs) are superior for the adsorption of this toxin (26,36,38). Donati et al. (36) related this to the volume of large pores (2-50 nm) in the carbons, appropriate in size for the m-LR molecule (molecular weight 994). Figure 16 shows the relationship between m-LR adsorption at equilibrium, and the volume of pores in the range 2-50 nm. A clear linear relationship is seen, indicating a strong dependence on pore volume, and limited influence of the surface chemistry of the carbons.

Microcystin LR is seldom the only microcystin present in a toxic algal bloom, and in many regions m-LR is not the most commonly-occurring variant (39). Very little information is available in the literature on the effect of water treatment processes on other variants. The only published investigation of the adsorption of microcystin variants other than m-LR used relatively impure toxin extracts (40). The authors suggested differences seen in the adsorption of the microcystin variants could have been due to different contaminant levels in the spiking material. The UKWIR undertook a computer modelling study to compare the octanol/water partition coefficients of nine microcystin variants (41). With this information, and molecular size data, the authors concluded that the variants should respond similarly to water treatment processes, and, in particular, that the variants would adsorb onto activated carbon to the same, or greater, extent as the commonly-studied variant microcystin LR.

Figure 17 shows the large differences observed in the adsorption of four microcystin variants onto two activated carbons. The results are counter-intuitive as the adsorption increases as the hydrophobicity decreases, and molecular weight increases, when the opposite trend could be expected. Electrostatic effects are assumed to be the cause of this trend, and these are currently under investigation. Figure 17 b displays the large difference in the adsorption of m-LR and m-LA as a function of time. Computer modelling of kinetics of adsorption was used to predict the PAC doses required to reduce the two compounds to below the WHO guideline of  $1.0 \mu\text{g L}^{-1}$  in 60 minutes. The results are given in Table 5. Clearly, the differences in adsorption between microcystin variants will have a significant effect on treatment options available to water suppliers.



**Figure 17.** Differences in adsorption behaviour of microcystin variants



**Table 5.** Predicted PAC doses required to obtain a concentration of 1  $\mu\text{gL}^{-1}$  after 60 minutes contact

| Inlet concentration ( $\mu\text{gL}^{-1}$ ) | m-LA PAC dose ( $\text{mgL}^{-1}$ ) | m-LA PAC dose ( $\text{mgL}^{-1}$ ) |
|---|-------------------------------------|-------------------------------------|
| 10  | 38                                  | >>100                               |
| 5   | 29                                  | 95                                  |
| 2   | 15                                  | 50                                  |

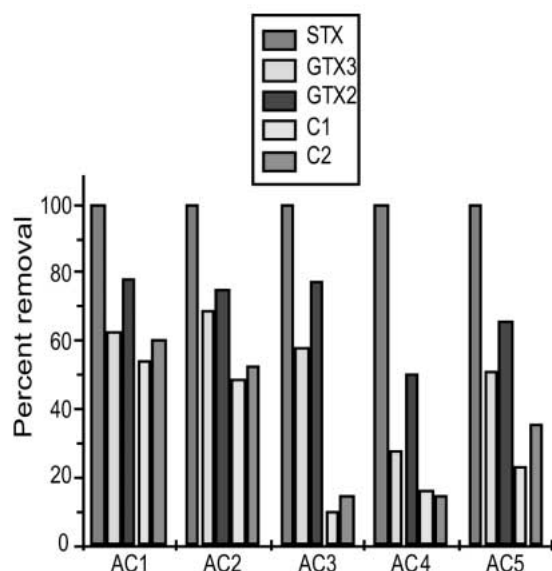
#### Anatoxin-a.

Very limited information exists on the adsorption of anatoxins by powdered activated carbon. The removal of anatoxin-a was studied on one activated carbon, a wood-based carbon found to be suitable for m-LR removal (26). As the structure, in particular the size, of the two compounds is very different, it is unlikely that the most suitable carbon would be the same for both compounds. Keijola *et al.* (30) also investigated the adsorption of anatoxin-a. They observed reasonable removals, however, no details are available on the activated carbon used, or the water quality after spiking with the algal material. There is potential for the method to be successful, however, a systematic study into the effect of activated carbon type, water quality and contact time is required before recommendations can be made.

#### Saxitoxins.

Figure 18 shows the percent removals from a mixture of saxitoxins by five powdered activated carbons.

**Figure 18.** Percent removal of saxitoxins for five activated carbons. Contact time = 1 hr, carbon dose = 30  $\text{mgL}^{-1}$ .



As a general trend, the adsorption of the compounds decreases as  $\text{STX} > \text{GTX} > \text{C}$ . As that is also the order of toxicity of the compounds, PAC is effective for the overall removal of toxicity. Although the charge of the compounds shows a similar trend ( $\text{STX}(+2) > \text{GTX}(+1) > \text{C}(0)$ ) this is unlikely to be the major effect on adsorption as the carbon with the most positive surface charge (AC2) also displays the highest adsorption. The size of the compounds in solution follows the trend  $\text{STX} < \text{GTX} < \text{C}$  and it is likely that the relationship between the size of the compound and the pore volume distribution of the activated carbon plays the major role.

#### Cylindrospermopsin.

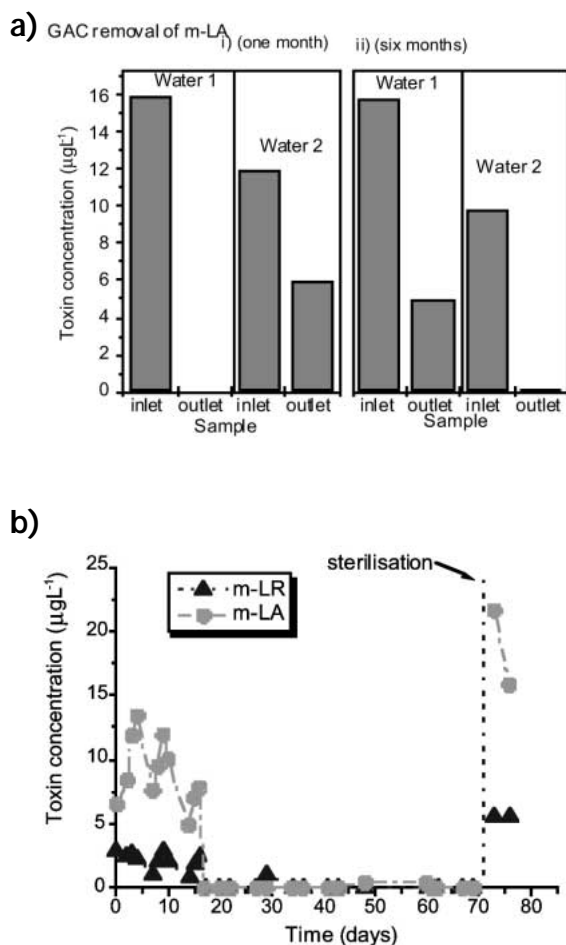
There is no information available in the international peer-reviewed literature on the removal of cylindrospermopsin by PAC. Bailey *et al.* (42) reported good removals with a wood-based PAC at doses below 30  $\text{mgL}^{-1}$ , and contact time of 30 minutes, and Cullen (43) obtained a maximum of 60% removal for a range of activated carbons at doses around 6  $\text{mgL}^{-1}$ . Aldridge *et al.* (35) reported 50% removal of the toxin with a dose of only 2.7  $\text{mgL}^{-1}$  when the initial concentration was approximately 2.5  $\mu\text{gL}^{-1}$ . No details were given regarding the contact time or type of activated carbon. As with anatoxin-a, a systematic study of the adsorption of this toxin is required.

#### Granular Activated Carbon

##### Microcystins.

Figure 19 a) shows the removal of m-LA during pilot plant trials at two water treatment plants. The pilot GAC filters were fed with treated water prior to chlorination. At intervals, the GAC filter feed water was spiked with a mixture of m-LR and m-LA. In Figure 19 ai) the inlet and outlet m-LA concentrations after 1 month of running the filters are shown. In water 1 the GAC filters remove both m-LR and m-LA to below detection after one month. In water 2 there is already breakthrough of m-LA above acceptable limits. Microcystin LR also broke through, but at a lower level. This can be attributed to the higher level of DOC in water 2, providing more competition for adsorption sites, and reducing the adsorption of the toxins. Figure 19 aii) shows the results of a similar spiking trial, after 6 months of running the plants. In this case the opposite trend is seen, water 1 shows breakthrough, due to the adsorption of NOM on the carbon, occupying adsorption sites, and reducing the capacity of the carbon for microcystins. In contrast, after 6 months both toxins were removed to below detection across the GAC filter in water 2. This was attributed to biodegradation of the toxins across the GAC filter in water 2, whereas the microorganisms colonising the GAC in water 1 did not appear to have the ability to biodegrade the microcystins.

The biodegradation of m-LR is well established. Jones and Orr (23) reported the rapid biodegradation of m-LR after the treatment of a toxic bloom of *Microcystis aeruginosa* with an algicide. However, the biodegradation only occurred after a lag phase of several days. The authors suggested the initial period was required for the microorganisms to acclimatise to the toxin as a food source. This lag phase in microcystin biodegradation has been reported by a number of authors (44,45). The UKWIR have reported significant biodegradation of m-LR on GAC filters (32, 46). Figure 19 b).shows the removal of both microcystins across a lab-scale GAC filter over a 68 day period. The inlet concentration was maintained at approximately 20  $\mu\text{g L}^{-1}$  of each toxin. Initially breakthrough of both toxins was seen, as the GAC was taken from one of the pilot plants, and had been in use for 3 months. After 16 days the removal mechanism clearly shifted from adsorption to biodegradation, with both toxins removed to below detection. After 68 days the carbon was removed, and sterilised by drying using rotary evaporation at 40°C. The inlet concentration was increased to around 30  $\mu\text{g L}^{-1}$  of each toxin. The removal prior to sterilisation was clearly biodegradation, as the sterilisation procedure resulted in significant toxin breakthrough.



**Figure 19.** Microcystin removal across GAC filters, adsorption and biodegradation.

#### Anatoxin-a.

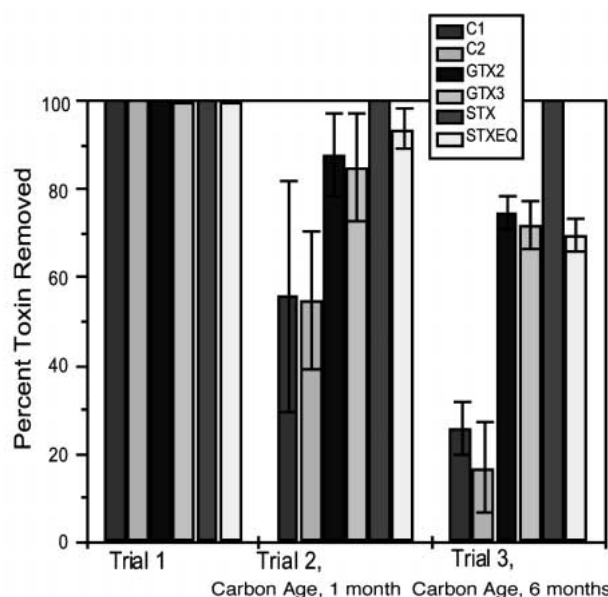
GAC appears to be effective for the removal of anatoxin-a for a period of time (32, 42, 46). Carlile (32) used small scale column tests to model full scale GAC bed life, and predicted breakthrough after around 15 weeks. This could be expected to be prolonged by biodegradation within the GAC filter, as was reported with slow sand filtration of anatoxin-a (42). The information currently available does not allow the confident recommendation of GAC filtration for this toxin.

#### Cylindrospermopsin.

No data available.

#### Saxitoxins

Figure 20 shows the percent removal of saxitoxins by the granular form of AC1 (Figure 18) over a 6 month laboratory investigation. A mixture of saxitoxins was spiked into the influent water to the GAC column at the beginning of the 6 month investigation (trial 1), after 1 month (trial 2) and after 6 months (trial 3). The trends in removal are the same as illustrated in Fig. 18. After 6 months the removal of toxicity, measured in saxitoxin equivalents, was still satisfactory- approximately 70%.



**Figure 20.** Saxitoxin percent removals during laboratory-scale GAC trial using Hope Valley water

#### Recommendations

In general, mesoporous carbons are the best for the removal of microcystins, that is, carbons with a large number of large pores. Chemically-activated wood-based carbons are often highly mesoporous, although recently a coal-based carbon has shown superior adsorption properties (Figure 17). If several variants of microcystin are present, which is most frequently the case, assessment of activated carbon should not be based on the adsorption of m-LR alone. It appears that m-RR is

very readily removed, whereas activated carbon adsorption should not be relied upon for the removal of m-LA. PAC dose requirements for microcystin removal at a particular water treatment plant can be predicted using adsorption models. From this information, water authorities can make an informed choice regarding water treatment options. GAC lifetime for the removal of microcystins would be limited under most conditions in the absence of biodegradation. More research is required regarding the potential for utilising biodegradation of microcystins under a range of water quality conditions. Although the limited information available suggests anatoxin-a and cylindrospermopsin could be removed using activated carbon, a systematic study of a range of carbons and a range of waters is required prior to confident application of the adsorbent. There also appears to be potential for the use of biodegradation for anatoxin-a and cylindrospermopsin; the requirements for further research are the same as for microcystins.

Both PAC and GAC are effective for the removal of saxitoxins, primarily due to the effective removal of the most toxic of the analogues, STX, and GTX toxins. Microporous carbons (with pores < 2 nm) such as good quality coal and coconut carbons, are recommended.

### Other Treatment Methods

UV photolysis has been found to be effective for the destruction of some toxins; however, the conditions required are often outside the range of practical water treatment application. For example, Carlile (32) studied the UV irradiation of anatoxin-a at 254 nm and found reasonable removal of toxin at doses two orders of magnitude higher than those usually utilised for disinfection. In the same study the author found microcystin LR (m-LR) was more readily destroyed than anatoxin-a. Cylindrospermopsin was found to degrade rapidly in sunlight in the presence of algal extract, while in its pure form, in the absence of other organic material, the toxin did not degrade (47). This effect was also seen with m-LR in the presence and absence of organic material (6). Titanium dioxide has been used successfully as a catalyst for the destruction of m-LR and cylindrospermopsin by UV photolysis (48-50). Although not currently accepted as a drinking water treatment process, this technique may be viable at some future stage.

### CONCLUSIONS

When treating water subject to a cyanobacterial bloom the first priority should be removal of intact cells using separation techniques such as coagulation or membrane filtration. The sludge/backwash from these processes should be kept separated from the treatment process until the toxins within the cells have been released and

degraded. This will avoid the release of toxins back to the head of the plant, or the raw water source. Residual dissolved toxins can be treated using oxidation techniques or powdered/granular activated carbon. Chlorination and ozonation are effective for the destruction of microcystins and cylindrospermopsin at moderate water temperatures. Presently, little information is available regarding the effect of temperature on these oxidation processes, and the efficacy of these treatments should be investigated under the range of temperatures and oxidant doses used at the water treatment plant affected. Anatoxin-a can be effectively removed using ozone, although chlorine is relatively ineffective. Oxidation techniques do not appear to be the best method for the treatment of saxitoxins under normal treatment plant operating conditions. Powdered activated carbon can be effective for the removal of all toxins, except, perhaps, m-LA, provided the appropriate carbon, and the correct dose is applied. However, under conditions of high toxin concentration over a prolonged period this option may become prohibitively expensive. Microcystin variants show a significant range of adsorbabilities, and that should be taken into account when activated carbon application is considered. Granular activated carbon filters show a limited lifetime for the adsorption of most microcontaminants, including cyanotoxins. However, the biodegradation of cyanotoxins across GAC filters shows great potential as a treatment process, particularly if the optimum conditions for biodegradation can be identified, and perhaps imposed on the filter.

### ACKNOWLEDGMENTS

The financial assistance of the Urban Water Research Association of Australia, Water Services Association of Australia, Department of Industry, Science and Tourism, American Water Works Association Research Foundation and United Water International is gratefully acknowledged. We also wish to thank our fellow researchers Michael Burch, Christopher Chow, Cos Donati, Jenny House, Rolf Gimbel, Stefan Panglisch, Dennis Steffensen, Renate Velzeboer, David Cook, Janina Morrison, Joanna Rositano, Najwa Slyman, Kurt Lehmayr, Lionel Ho, Chris Saint, Claudia Sauerland, Jenny Morrall, Tom Woods for their contributions to this study.

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