

Cylindrospermopsin in
whole cell extracts and live cultures
of *Cylindrospermopsis raciborskii*:
ecotoxicity, bioaccumulation and management

Susan Heather White Kinnear

School of Biological and Environmental Sciences
Faculty of Science, Engineering and Health
Central Queensland University

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Abstract

Cylindrospermopsin (CYN) is an alkaloid toxin produced by at least six blue-green algal genera. In Australia, the most common producer organism is *Cylindrospermopsis raciborskii*. This research determined the toxic effects and potential for toxin bioaccumulation associated with toxin-producing *C. raciborskii*, using environmentally relevant exposure scenarios. Short-term semi-static renewal ecotoxicity tests were conducted on four freshwater test organisms, using freeze-thawed *C. raciborskii* whole cell extracts or live cultures of *C. raciborskii* containing CYN.

Duckweed (*Spirodela oligorrhiza*) demonstrated variable responses to whole cell extract exposures containing 0 – 500 $\mu\text{g L}^{-1}$ of CYN. Growth stimulation and growth inhibition were recorded from the duckweed, depending on toxin exposure concentrations and length of the incubation period. Chlorophyll content of *Spirodela* was also affected by toxin exposure, with small peaks in chlorophyll *a* recorded in conjunction with exposure to 8 $\mu\text{g L}^{-1}$ CYN. Changes to frond morphology (chlorosis, necrosis) and reduced root lengths were also noted, but these effects were not consistent throughout all trials. Bioconcentration of free CYN was not detected in the tissues of *Spirodela*; small quantities of toxin recovered from the tissues probably represented toxin sorption to the cell walls.

Growth of water thyme (*Hydrilla verticillata*) was stimulated by exposure to whole cell extracts of *C. raciborskii* containing a maximum of 400 $\mu\text{g L}^{-1}$ CYN. Exposure to the test solutions appeared to promote the redistribution of plant

resources in *H. verticillata*, and the possible benefits of this, particularly with respect to increased root production, were considered. Effects on the chlorophyll content of *Hydrilla* were variable and included decreases in total chlorophyll content and changes to the chlorophyll *a:b* ratio. Bioconcentration of free toxin was not detected in the tissues of *Hydrilla*.

In trials with *Melanoides tuberculata*, exposure to whole cell extracts or live algal cultures did not significantly affect the behaviour or relative growth rates of adult snails. However, changes in the number of hatchlings released from parent snails were recorded: exposure to whole cell extracts corresponded with increased numbers of hatchlings compared with controls, whereas decreases in hatchling numbers compared with controls were recorded from treatments containing live *C. raciborskii*. Both bioconcentration and bioaccumulation occurred in the soft tissues of snails, although exposure to whole cell extracts resulted in only minor tissue contamination compared with that from live *C. raciborskii* exposures. Bioaccumulation of the analog deoxy-CYN was recorded in the soft tissues. *M. tuberculata* did not bioconcentrate CYN in the shell.

Tadpoles of the cane toad (*Bufo marinus*) featured the most dramatic responses to the test solutions. Exposure to live *C. raciborskii* cultures resulted in up to 66% mortality of *B. marinus*, whereas all tadpoles survived exposure to whole cell extracts of comparable CYN concentrations. Decreases in the time spent swimming and relative growth rates were recorded from surviving tadpoles during both types of exposure regimes. Histological examination of *Bufo* tissues revealed tissue injuries to multiple organs, with particular severity noted in the

liver, intestine, nephric ducts and gill epithelia. The extent of cellular damage was similar in whole cell extract (containing a maximum of $400 \mu\text{g L}^{-1}$ CYN) and the live culture trials (containing a maximum of $232 \mu\text{g L}^{-1}$), despite the unequal toxin concentrations. Bioconcentration of CYN was not evident during the whole cell extract trial, whereas exposure to live cultures resulted in maximum average tissue concentrations of $895 \mu\text{g toxin kg}^{-1}$ fresh weight.

A secondary aim of the thesis was to identify and address gaps in management approaches for toxin bioaccumulation and possible environmental effects associated with toxin-producing *C. raciborskii* blooms. A predictive management strategy was developed to determine the likelihood of tissue contamination in aquatic organisms inhabiting water bodies affected by blooms. The ten-step framework for predicting bioaccumulation risk was based upon characteristics of cyanotoxin bioavailability, exposure and uptake routes during the progression of a toxic bloom. Key concepts included monitoring changes in toxin availability throughout the progression of a toxic bloom, and the prediction of bioaccumulation risks based on threshold toxin values for selected aquatic organisms. These threshold values were re-examined following the completion of the laboratory trials.

Current approaches for the management of blooms with respect to environmental risks were discussed, and procedures for the proper evaluation of environmental risks associated with *Cylindrospermopsis* blooms were assessed. These included adequate detection and monitoring systems, setting of environmental guideline values, and options for the control and remediation of toxic blooms.

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Cylindrospermopsis raciborskii:
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Susan Heather White Kinnear
(B. Environ. Sci.; B. Sci. (Biol) (Hons))

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School of Biological and Environmental Sciences
Faculty of Science, Engineering and Health
Central Queensland University

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Note to Reader

To avoid repetition, this thesis has been arranged into four sections. Section one includes an evaluation of relevant literature (chapter one), the development of a predictive model from which bioaccumulation risk could be assessed (chapter two), and common materials and methods for all trials (chapter three). These chapters set the context for the experimental trials. Experimental results are divided into those for aquatic plants (section two) and aquatic animals (section three); key concepts raised from the laboratory trials are discussed at the end of each section. Section four evaluates the research data for its contribution to current knowledge on CYN toxicity and the environmental implications of toxin-producing *C. raciborskii* blooms (chapter ten). Toxic algal bloom management protocols are evaluated in view of safeguarding ecological systems, and the predictive management model is re-examined (chapter eleven). Final conclusions and areas for further study are identified in chapter twelve.

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Abbreviations and units of measure

Abbreviations and units of measure used in this thesis, in alphabetical order:

ANOVA	(univariate) analysis of variance
ARNAT	Australian Research Network for Algal Toxins
ASM1	blue-green algal culturing media
ASTM	American Society for Testing and Materials
BAF	bioaccumulation factor (tissue concentration divided by total toxin concentration)
BCF	bioconcentration factor (tissue concentration divided by extracellular toxin concentration)
Chl <i>a</i>	chlorophyll <i>a</i>
Chl <i>b</i>	chlorophyll <i>b</i>
Chl <i>a:b</i>	chlorophyll <i>a:b</i> ratio
CQU	Central Queensland University
CYN	cylindrospermopsin (fraction not specified)
CYN _{EXC}	extracellular cylindrospermopsin (aqueous, non-cell bound); toxin that is outside the cell wall due to natural cell leakage or artificial cell lysis (e.g. freezing)
CYN _{INC}	intracellular cylindrospermopsin (cell-bound)
CYN _{TOT}	total cylindrospermopsin (sum of intracellular and extracellular fractions)
Deoxy-CYN	deoxy-cylindrospermopsin
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Environmental Protection Agency (United States EPA)
EXC	extracellular (toxin); lysed toxins, non-cell bound toxins
Free-CYN	cylindrospermopsin that is not bound and/or has not been metabolically modified or conjugated; able to be detected via HPLC/MS-MS
Free-deoxy-CYN	deoxy-cylindrospermopsin that is not bound and/or has not been metabolically modified or conjugated; able to be detected via HPLC/MS-MS
GI ₅₀	concentration required to exert growth inhibition in 50% of the population in a given time
GSH	glutathione
GST	glutathione-S-transferase
h	hour(s)
HPLC	high performance liquid chromatography
IC ₅₀	concentration required to exert inhibition in 50% of the population in a given time
IP	intraperitoneal
INC	intracellular; toxin contained within cyanoprokaryote cells
ICTC	International Conference on Toxic Cyanobacteria
K _{OW}	octanol-water partition coefficient
LC ₅₀	concentration required to exert 50% lethality in a given time
LPS	lipopolysaccharide(s)
MANOVA	multivariate analysis of variance
MC	microcystin

[Abbreviations continued]

MS or MS/MS	mass spectrometry or tandem mass spectrometry
NOAEL	no observed adverse effect level
ng L ⁻¹	nanograms per litre
OECD	Organisation for Economic Cooperation and Development
PCR	polymerase chain reaction
POD	peroxidase activity
PP	protein phosphatases
PSI	protein synthesis inhibition
PSPs	paralytic shellfish poisons
Q _{CYN}	the quantity of cylindrospermopsin per cell
QHSS	Queensland Health Scientific Services (analytical laboratory)
RGR	relative growth rate(s)
ROS	reactive oxygen species
RM ANOVA	repeated measures analysis of variance
RT-PCR	reverse transcriptase ‘real-time’ polymerase chain reaction
TDI	tolerable daily intake(s)
µg L ⁻¹ or µg mL ⁻¹	micrograms per litre or micrograms per millilitre
WHO	World Health Organisation

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Declaration and statement of access

The research and discussion presented in this thesis are the original work of the author and has not been submitted at any tertiary institute or university for any other award.

Any material which has been presented by any person or institute is duly referenced, and a complete list of all references is presented in the bibliography.

.....

Susan H.W. Kinnear

I, the undersigned author of this thesis, understand that the Central Queensland University will make this work available with the university library, and that it will be accessible to library users and other approved libraries. This thesis should not be copied, or closely paraphrased without the consent of the author, and written acknowledgement of the assistance gained from this work. Beyond this, I do not wish to place any restrictions on access to this thesis.

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Susan H. W. Kinnear

List of publications arising and conference attendances

Statement of authorship

The following manuscripts were published or accepted for publication during the candidate's period of enrolment and relate to the thesis content. S. H. White conducted all experimental work, including trial preparations, all monitoring, data collection and analyses, and developed each of the manuscripts with input from L. Duivenvoorden and L. Fabbro. G. K. Eaglesham conducted all toxin analyses on water and tissue samples and provided intellectual input.

..... (S.H.W. Kinnear (nee White))

Published manuscripts

White, S.H., Duivenvoorden, L. J. & Fabbro, L. D., 2005. A Decision-making Framework for Ecological Impacts Associated with the Accumulation of Cyanotoxins (Cylindrospermopsin and Microcystin). *Lakes and Reservoirs: Research and Management* 10: 25-37. (Corresponds with chapter two).

White, S.H., Duivenvoorden, L. J. & Fabbro, L. D., 2005. Absence of free-cylindrospermopsin bioconcentration in water thyme (*Hydrilla verticillata*). *Bulletin of Environmental Contamination and Toxicology* 75 (3): 574-583. (Corresponds with parts of chapter five).

White, S.H., Duivenvoorden, L. J., Fabbro, L. D. & Eaglesham, G. K., 2006. Influence of intracellular toxin concentrations on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata*). *Toxicon* 47(5): 497-509. (Corresponds with parts of chapter seven).

Copies of published papers have been included at the end of this thesis (Appendix F).

In submission

White, S.H., Duivenvoorden, L. J. & Fabbro, L. D. Sublethal responses in *Melanoides tuberculata* following exposure to *Cylindrospermopsis raciborskii* containing cylindrospermopsin. (Submitted in December 2005; corresponds with parts of chapter seven).

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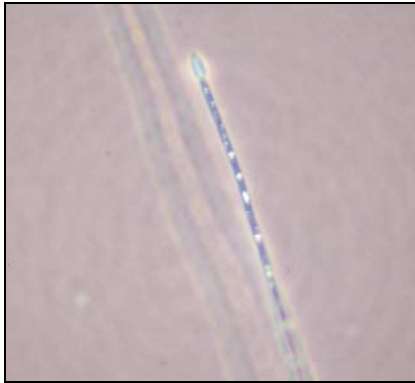
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SECTION I

Cylindrospermopsis raciborskii
and cylindrospermopsin:
background information and methods



Cylindrospermopsis raciborskii, x400

Chapter one: review of literature

1.1 Cyanoprokaryotes

Cyanoprokaryotes (cyanobacteria, blue-green algae) are non-nucleated organisms characterized by an ability to synthesize chlorophyll *a* (Whitton & Potts 2000) but which lack membrane-bound organelles and sexual reproduction (Komárek & Anagnostidis 1986). The cyanoprokaryotes are a diverse and adaptive group, occupying a broad range of habitats including Antarctic ice shelves and volcanoes. However, they are most commonly of interest as a component of the phytoplankton populations of freshwater, estuarine and marine environments. Of particular importance are species known to form blooms, especially where these are associated with the production of potent toxins.

1.2 Occurrence and effects of toxic blooms

Some cyanoprokaryote genera have superior growth abilities due to the presence of buoyancy-regulating gas vacuoles, and the capability to capture a variety of light wavelengths and undertake nitrogen fixation (Bowling 1994). Cyanoprokaryote dominance has been recorded under conditions of low light penetration, water column stability, warm water temperatures and high nutrient availability but low nitrogen to phosphorus ratios (Fabbro & Duivenvoorden 1996; Jacoby *et al.* 2000; Hesse & Kohl 2001; Saker & Griffiths 2001). Rapid cell division often leads to cell concentrations reaching 'bloom' proportions, currently defined in Australia as $> 15,000 \text{ cells mL}^{-1}$ (Jones *et al.* 2002).

Sometimes, cyanoprokaryote blooms are associated with the production of poisonous compounds known as cyanotoxins. Toxin production is highly variable within and

between blooms according to speciation, genetic composition, and the prevalence of conditions that allow maximal toxin production by individual cells (Carmichael 2001b).

Toxic blooms are known to exert acute and chronic lethal and sublethal effects on a range of terrestrial and aquatic organisms. The reviews of Schwimmer & Schwimmer (1968), Beasley *et al.* (1989), Carmichael & Falconer (1993) and Duy *et al.* (2000) provide substantial evidence of animal deaths associated with intoxication by cyanoprokaryotes. Mortalities have been recorded from livestock, domestic fowl, pets and wildlife; extensive fish kills are also common. Effects on humans have also been comprehensively reviewed (see Falconer 2001; Carmichael *et al.* 2001). Several accounts have linked adverse effects in terrestrial species with intoxication by algal blooms (for example, Matsunaga *et al.* 1999). However, the accuracy of these might be questioned, particularly prior to the 1980s when understanding of cyanotoxins was limited due to non-rigorous reporting of bloom events.

1.3 *Cylindrospermopsis raciborskii*

Cylindrospermopsis raciborskii Woloszynska Seenayya et Subba Raju 1972 is a filamentous and heterocystous cyanoprokaryote (Komárek & Anagnostidis 1989) that is the namesake, and one of several producer species, of the toxin cylindrospermopsin (CYN) (Ohtani *et al.* 1992). The species may also produce several other compounds, including the analog deoxy-cylindrospermopsin (deoxy-CYN), and the paralytic shellfish saxitoxins (Lagos *et al.* 1999; Norris *et al.* 1999a; Molica *et al.* 2002).

C. raciborskii has been recognised by several alternate names including species of *Anabaenopsis*, *Anabaena*, *Cylindrospermum*, *Aphanizomenon* and *Raphidiopsis* (Chiswell *et al.* 1999); since *Cylindrospermopsis* has only recently been included in identification keys, it has been misidentified in the past (Chapman & Schelske 1997). The ecology of *C. raciborskii* has been reviewed previously (Chiswell *et al.* 1997; Padisák 1997). Recently, global climate change has been examined as a possible trigger for the more widespread distribution, frequency and duration of toxic *C. raciborskii* blooms, particularly in temperate areas (Garnett *et al.* 2003; Neilan *et al.* 2003; Briand *et al.* 2004; Chonudomkul *et al.* 2004).

C. raciborskii blooms are globally common occurrences, with *Cylindrospermopsis* often a dominant species and frequently found to be toxic (for example, Branco & Senna 1994; Bouvy *et al.* 2000; Fastner *et al.* 2003; Saker *et al.* 2003; Bouaïcha & Nasri 2004). In Australia, *C. raciborskii* blooms have been documented from rivers, impoundments, lakes, ponds and dams in Queensland (Saker *et al.* 1999a; Saker & Eaglesham 1999; Shaw *et al.* 1999; McGregor & Fabbro 2000) and in other states (Baker 1996). In the local central Queensland area, *C. raciborskii* has been detected in the Fitzroy River (Fabbro 1999) and is widely distributed throughout the Fitzroy River Catchment (Dr Larelle Fabbro, Senior Research Scientist, Centre for Environmental Management, Central Queensland University, pers. comm.).

Limited information is available regarding the percentage of natural *C. raciborskii* blooms that produce toxin (McGregor & Fabbro 2000). Several studies have examined parameters that affect the toxin content of natural and cultured populations of *C. raciborskii*. Recently reviewed by Griffiths and Saker (2003), these have

included pH, light, temperature, nutrient and mixing regimes (Branco & Senna 1994; Fabbro & Duivenvoorden 1996; Chiswell *et al.* 1997; Chiswell *et al.* 1999; Saker & Griffiths 2000; Bormans *et al.* 2004)). Growth rates also appear to be coupled with toxin production (Hawkins *et al.* 2001), and it is suggested that the size of vegetative cells provides some indication of likely toxin content (Hawkins *et al.* 2001; Saker & Neilan 2001).

The toxicity of CYN may also be highly variable between isolates (Saker & Griffiths 2000), and toxin production does not appear to be based on the Australian geographical origin of the species (Schembri *et al.* 2001). Mesocosm experiments have demonstrated that *C. raciborskii* toxin quotas appear to be higher in tropical rather than sub-tropical environments (Garnett *et al.* 2003). The molecular mechanisms of toxin production are generally poorly understood (Schembri *et al.* 2001).

1.4 Cylindrospermopsin

1.4.1 Structure and properties

CYN is a tricyclic alkaloid cytotoxin first isolated and identified in 1992 (Ohtani *et al.* 1992). Structurally, the molecule is a sulfated guanidinium zwitterion (Figure 1.1). CYN is highly water-soluble, and has a relatively low molecular weight of 415 Daltons (Sivonen & Jones 1999; Shaw *et al.* 2000). CYN is most commonly associated with blooms of *C. raciborskii* (in Australia, Thailand, Hungary, New Zealand and the United States), but is also associated with blooms of *Umezakia natans*,

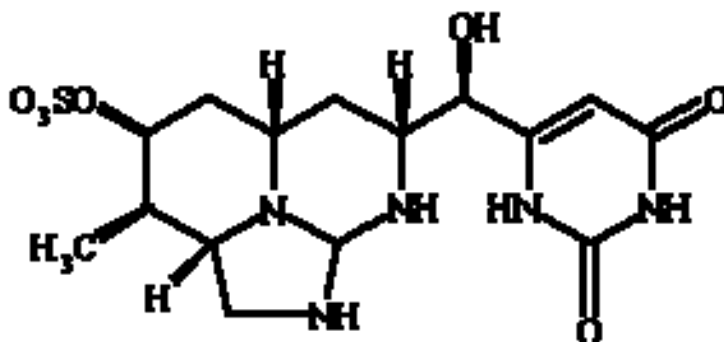


Figure 1.1 Chemical structure of cylindrospermopsin. (Reproduced from Cyanosite (2005), with permission from the webmaster, Dr Mark Schneegurt).

Aphanizomenon ovalisporum, *Aph. flos-aque*, *Anabaena bergii* var *limnetica* and *Raphidiopsis curvata* (Shaw *et al.* 1999; Schembri *et al.* 2001; Stirling & Quilliam 2001; Li *et al.* 2001a; Li *et al.* 2001b; Fergusson & Saint 2003; Preußel *et al.* 2006). Recently, CYN has also been detected from blooms of benthic *Lyngbya wollei* in south-east Queensland, Australia (Marc Seifert, National Research Centre for Environmental Toxicology, pers. comm.).

CYN is relatively stable to heat, light and pH (Chiswell *et al.* 1999), although pure CYN breaks down rapidly in sunlight when cell pigments are present (90% complete in two to three days). Photocatalytic degradation of CYN appears to be pH dependent, being most efficient at pH 9.0 (Chiswell *et al.* 1999). During naturally toxic blooms it is not unusual for between 70 to 98% of CYN to be dissolved in the water column (Carson 2000). Extracellular CYN also accounts for a considerable amount of total CYN in laboratory cultures, especially those in the postexponential growth phase (Hawkins *et al.* 2001; Griffiths & Saker 2003).

1.4.2 Typical environmental concentrations

Toxin concentrations associated with Australian *C. raciborskii* blooms have ranged from below detection limits to up to 589 µg CYN L⁻¹ (Saker & Eaglesham 1999; Saker 2000; Saker & Griffiths 2001). Baker (1998, p. 45) also detected 1.5 mg L⁻¹ in a sample from a 1996 North Pine Dam bloom. A study by McGregor and Fabbro (2000) during October 1997 to June 1999 found that the maximum average toxin concentration of water storages within Queensland peaked at 18.9 µg L⁻¹ in Cania Dam.

1.4.3 Detection

Until recently, a lack of chemical, biochemical and cell-based detection methods meant detection of CYN relied entirely on mouse bioassay. However, comparisons between human, mouse and cattle deaths indicate this might not be the best approach, since mouse bioassays could underestimate toxicity (Shaw *et al.* 2002). Animal ethics considerations have also meant that bioassays are increasingly being avoided.

Harada *et al.* (1994) developed the first CYN screening method using reverse phase high performance liquid chromatography (HPLC) coupled with photo diode array detection. CYN has an easily identifiable peak and maximum UV absorbance at 262nm (Ohtani *et al.* 1992; Hawkins *et al.* 1997). Use of HPLC/mass spectrometry (MS) with electrospray results in a detection limit of 200 µg L⁻¹ (Carson 2000). In contrast, HPLC combined with tandem mass spectrometry (MS/MS) allows detection at much lower levels (Eaglesham *et al.* 1999). Detection via HPLC followed by photo diode array represents a less expensive alternative to HPLC-MS/MS, however this may not adequately detect trace quantities of CYN (Welker *et al.* 2002). Toxin

detection by HPLC-MS/MS is therefore currently the method of choice, achieving a $1 \mu\text{g L}^{-1}$ detection limit (Nicholson & Burch 2001).

The use of cell-free rabbit reticulocyte systems and polymerase chain reactions for CYN detection has also been demonstrated (Frosio *et al.* 2001; Fergusson & Saint 2003). Purity of CYN samples can be determined via nuclear magnetic resonance (NMR) and mass spectrometry (Baker 1998; Eaglesham *et al.* 1999). Most recently, Kubo *et al.* (2005) demonstrated that double-cartridge fractionation of CYN might be more effective in removing both hydrophobic and hydrophilic (anionic) compounds. An important limitation of current detection techniques is the inability to detect CYN that becomes bound or changed (for example, metabolised) within living cells. It is expected that CYN may bind to living tissues because of its structural features (Duy *et al.* 2000), and because similar problems have been experienced with detection of other cyanotoxins. This problem is considerable since CYN concentrations in the tissues of aquatic organisms might be underestimated.

1.4.4 Toxicity

The mechanism(s) of action for CYN remain(s) unclear (Seawright *et al.* 1999). In the past, toxicological studies have been limited by a lack of pure standard material with which to experiment (Norris *et al.* 2001a). CYN is known to be capable of inhibiting protein synthesis, however this does not appear to occur via protein phosphatase inhibition (Chong *et al.* 2002). It also appears that additional modes of toxicity exist in some isolates (Hawkins *et al.* 1997; Carmichael 2001a). Possibly, synergisms between CYN and other cell components (Norris *et al.* 1999) could lead to these increases in toxicity.

1.4.4.1 Uptake

Uptake of CYN is poorly understood. The sulfate group on the CYN molecule is not required for cell entry or toxicity (Runnegar *et al.* 2002). Since CYN affects the liver, the toxin could require similar transport carriers as those for the hepatotoxin, microcystin. Bile acid transporters appear to be utilized initially (Chong *et al.* 2002), however a secondary system may be present since bile acid inhibition only provides protection against CYN toxicity for periods up to 72 h. In addition, several whole-animal studies have reported tissue damage in organs that do not use bile acid transporters (Falconer 2005), including cytotoxic effects in the eye, spleen, kidney, lungs, thymus and heart (Terao *et al.* 1994; Falconer 1999; Shaw *et al.* 2000). Chong *et al.* (2002) theorized that uptake via passive diffusion might be responsible, given the relatively small molecular weight of CYN. Alternately, Runnegar *et al.* (2002) considered that CYN was unlikely to permeate cell walls since it is so hydrophilic.

1.4.4.2 Binding

Binding of CYN following uptake has been minimally studied. Norris *et al.* (2001b) reported that protein adducts were formed in the liver of CYN-dosed mice; these compounds could be formed by CYN binding. Recent work by Froscio *et al.* (2003) could also be interpreted as evidence for toxin binding. Here, an hepatocyte cell line was exposed to CYN for one hour, then removed from the toxin media, washed and incubated for a further two hours. Rates of protein synthesis inhibition were measured; this occurred during and after toxin exposure and rinsing. This indicated not only that CYN was taken up into the cell, but also, possibly, that the toxin became trapped inside the cell, indicating bound toxin (Froscio *et al.* 2003).

1.4.4.3 Mechanism(s) of action

The CYN molecule appears to require a uracil moiety (pyrimidine ring) for toxicity, since cleavage of this group by chlorination results in the loss of toxicity (Banker *et al.* 2001). Loss of the hydroxyl group also results in reduced *in vivo* activity (Runnegar *et al.* 2002). Exertion of CYN toxicity may require several mechanisms of action, but toxic effects appear to relate principally to protein synthesis inhibition (PSI).

The ability of CYN to cause PSI was originally demonstrated by Terao *et al.* (1994) using intraperitoneally (IP) injected mice. The presence of distinct morphological changes, such as proliferation of membrane systems like the smooth endoplasmic reticulum and Golgi apparatus, led to suspicions of PSI. This was subsequently confirmed, with globin synthesis completely inhibited in rabbit reticulocytes following exposure to 48 µg L⁻¹ purified CYN (Terao *et al.* 1994). The full onset of CYN toxicity is delayed, however, the component that is caused by PSI occurs rapidly and is non-reversible (Froscio *et al.* 2003). Furthermore, unlike other mechanisms of CYN toxicity (see below), PSI does not appear to require metabolic activation to exert toxicity, since the enzymes required for bioactivation were not present in the cell-free system used in the study (Froscio *et al.* 2003).

Cylindrospermopsin also inhibits glutathione (GSH) synthesis in mammalian hepatocytes (Runnegar *et al.* 1994; 1995), evidenced by dissociation of ribosomes from the endoplasmic reticulum and a sequence leading to cell death (Duy *et al.* 2000; Humpage & Falconer 2003). Depletion of GSH appears to result from synthesis inhibition, rather than conjugation with CYN or its metabolite(s) (Runnegar *et al.*

1995; Norris *et al.* 2002). Since GSH is critical to cellular defence by reducing oxidative damage and aiding in the metabolism of xenobiotics (Carson 2000; Pastore *et al.* 2003), cells with reduced GSH are predisposed to CYN toxicity (Runnegar *et al.* 1995). Whilst CYN toxicity relates to the loss of basic detoxication cell functions (Runnegar *et al.* 1995), this is not considered the primary cause for hepatotoxicity: depletion generally precedes toxicity and synthetic chemicals can reduce GSH at far greater levels without producing death or liver damage (Norris *et al.* 2002).

Production of metabolites during CYN breakdown in the liver appears to result in the presence of compounds more toxic than the parent molecule (Runnegar *et al.* 1994). It has been suggested that it is these metabolites (activated CYN), which are responsible for eventual cell death, whereas the native CYN molecule causes only PSI (Froscio *et al.* 2003). Toxic metabolites are considered likely to form as by-products from cytochrome P-450 processes (Terao *et al.* 1994; Shaw *et al.* 2000), given that P-450 inhibition offers some protection from CYN toxicity (Runnegar *et al.* 1995; Froscio *et al.* 2003). Humpage *et al.* (2005) noted that CYN toxicity was lower in day-old primary hepatocytes compared with fresh hepatocytes, seemingly because the former had lost metabolic capacity. Additionally, vacuolation and necrosis of hepatocytes is best noted from the periacinar region of the liver, the principal site for xenobiotic metabolism by P-450s (Shaw *et al.* 2000).

Exposure to CYN may be associated with strand breakage or mutation during replication, with the formation of DNA adducts resulting from intercalation of CYN's uracil group with DNA nucleotides, and/or hydrogen bonding with nucleotide base pairs (Reisner *et al.* 2004; Falconer 2005). Genotoxic action of CYN is evidenced by

DNA strand breakage in mice (Shen *et al.* 2002), DNA fragmentation in mouse hepatocytes (Humpage *et al.* 2005), and loss of chromosomes in human cell lines (Humpage *et al.* 2000). Although no direct genotoxic effect of CYN was found in studies of Chinese hamster ovaries, this result might have been influenced by the absence of CYN biotransformation (Fessard & Bernard 2003).

Loss of DNA from the nucleus may also occur due to strand fragments being lost to the cell cytoplasm (and the resultant formation of micronuclei) (Falconer 2005). CYN may also affect the functioning of the mitotic spindle during cell division, causing whole chromosomes to be lost during replication (Falconer 2005). It has also been suggested that PSI could be initiated by binding of CYN, or its metabolite, to DNA or RNA (particularly uridine) (Reisner *et al.* 2004), resulting in transcription or translation interruptions (Shaw *et al.* 2000).

The nucleotide structure of CYN and the presence of the guanidine and sulphate groups have aroused suspicion that CYN may be a carcinogen (Ohtani *et al.* 1992; Humpage *et al.* 2000; Shen *et al.* 2002). However, there are conflicting reports regarding tumour promotion by CYN: Falconer and Humpage (2001) suggested that it was possible, whereas Chong *et al.* (2002) found CYN did not inhibit protein phosphatase 2A (which controls proliferation) and thus was unlikely to be a tumour promotant.

Several other toxic mechanisms for CYN have been reported. Kiss *et al.* (2002) suggested that CYN might be neuroactive towards gastropod cell membranes. The *in vivo* toxicity remains largely unknown (Humpage *et al.* 2000). Bernard *et al.* (2003)

suspected highly concentrated doses of CYN were cardiotoxic, based on their own experimental results and those of Seawright *et al.* (1999), Harada *et al.* (1994) and Saker *et al.* (1999a). However, the difference between Bernard *et al.*'s test concentrations (10 – 20 mg mL⁻¹) and naturally occurring CYN concentrations (generally, below 5 mg L⁻¹) should be noted: there was no relevance to toxin concentrations recorded from water.

1.4.4.4 Effects associated with exposure

In 1979, CYN toxicity was implicated in the 'Palm Island Mystery Disease', where toxin exposure was responsible for the hospitalisation of over one hundred children suffering acute gastroenteritis (Byth 1980; Hawkins *et al.* 1985). Since then, extensive studies have helped describe the symptoms of CYN intoxication. Hepatotoxicity and general cytotoxicity are two of the primary consequences of CYN exposure. The kidney also appears to be a chief target for toxicity (Falconer *et al.* 1999). Liver damage in mice results from gavaged (force-fed) exposure to both *C. raciborskii* cell lysates and purified CYN (Seawright *et al.* 1999). Falconer *et al.* (1999) noted that oral toxicity was in general 25-fold lower than IP toxicity, although extent of histological damage does not appear to be influenced by route of administration.

In mammals CYN hepatotoxicity proceeds by four distinct phases – protein synthesis inhibition, membrane proliferation, fat droplet accumulation and cell death. Toxicity is characterised initially by accumulation of ribosomes from the rough endoplasmic reticulum into the cytoplasm of hepatocyte cells, and ends with a terminal phase of severe hepatic necrosis (Terao *et al.* 1994). In the final stages of toxicity (100h in

mice), ribosomes detach from the rough surface endoplasmic reticulum; nuclei appear dense, rounded and smaller; membrane proliferation occurs; vacuoles appear in the cytoplasm of central vein hepatocytes accompanied by fat droplet production, necrotic debris is evident and, eventually, 100% of hepatocytes are destroyed (Terao *et al.* 1994; Shaw *et al.* 2000).

CYN is also injurious to the gut lining and causes kidney malfunction and blood vessel injury (Duy *et al.* 2000). Dilation of kidney cortical tubules and changes in glomerular red cell content have been reported, with IP injection resulting in the loss of overall kidney structure within 24 h (Falconer *et al.* 1999). The toxin is also cytotoxic to the eye, spleen, kidney, lungs, thymus and heart of dosed mice (Terao *et al.* 1994; Falconer 1999; Shaw *et al.* 2000).

Acute toxicity values for CYN vary according to length of exposure, ingestion method, toxin type (purified standard compared with cell extracts) and producer strain. The LD₅₀ of CYN for IP injected laboratory mice was reported at 2,100 µg kg⁻¹ for 24h exposure, but only 200 µg kg⁻¹ for exposure over five to six days (Ohtani *et al.* 1992). In contrast, the oral LD₅₀ for mice falls within the range 4,400 – 6,900 µg kg⁻¹, although variability in individual susceptibility was noted (Seawright *et al.* 1999). Similarly, Shaw *et al.* (2000) reported an oral LD₅₀ of 6,000 µg kg⁻¹ using a freeze-dried cellular suspension of *C. raciborskii*. Repeated daily doses via IP injection for fourteen days produced a no observed effect level of 1 µg kg day⁻¹ in mice (Shaw *et al.* 2000). One peculiarity of CYN toxicity is that when administered intraperitoneally, higher doses of toxin appear to actually prolong the survival time of mice (Hawkins *et al.* 1985).

Few studies have calculated toxicity values for organisms other than mice. Metcalf *et al.* (2002b) found LC₅₀ values for *Artemia salina* decreased with increasing exposure time, and that tests using this species were more sensitive than mouse bioassay, with the 24h LC₅₀ for purified CYN being 8.1 mg L⁻¹. The 48h LD₅₀ for locusts is reportedly 131.4 mg kg⁻¹, although values were five to six times lower if the extracts were done with methanol (Hiripi *et al.* 1998).

Only two studies have examined CYN toxicity towards plant species. Vasas *et al.* (2002) demonstrated inhibitory effects of CYN on *Sinapis* mustard seedling metabolism, with 50% growth reduction apparent at 18.2 mg L⁻¹. A dose-dependent relationship with growth inhibition was apparent for both the crude extract and purified CYN (Vasas *et al.* 2002). Most recently, Metcalf *et al.* (2004) demonstrated that pollen germination of tobacco plants was reduced following exposure to between 5 – 1,000 mg L⁻¹.

The use of aquatic organisms in CYN toxicity studies appears limited. Nogueira *et al.* (2004a) reported that *C. raciborskii* strains caused high mortality, reduced growth, and changes to fecundity in *Daphnia magna*, with 100% of subjects dead following 72 h exposure to CYN-containing *C. raciborskii*. *C. raciborskii* blooms may be linked with changes in zooplankton and phytoplankton community structure, via preferential food selection (Leonard & Paerl 2005). Krzyżanek *et al.* (1993) reported dying out of the benthic fauna to correspond with blooms of *Aphanizomenon flos-aquae*: toxin concentrations of the bloom were not examined but *A. flos-aquae* is now known to produce CYN (Preußel *et al.* 2006), amongst other toxins. Saker and Eaglesham (1999) examined histological effects of CYN on redclaw crayfish, but no

abnormalities were noted. Richey *et al.* (2001) examined possible effects of *C. raciborskii* on alligators from Lake Griffin (Florida), however the toxin dose was not clearly stated, and it was difficult to ascribe any adverse effects solely to algal toxicity.

Large animal deaths have been attributed to CYN after three cows and ten calves died in northwest Queensland during 1997 (Saker *et al.* 1999b); histological examinations were consistent with hepatotoxin poisoning. The calf livers presented as pale and swollen, with the gall bladder, small intestine and heart also affected.

1.5 Deoxy-cylindrospermopsin

C. raciborskii also produces the CYN analog, deoxy-CYN. Deoxy-CYN is thought to arise from the loss of the oxygen molecule from the uracil group, and the hydroxyl group from the uracil bridge, although the sulfate group remains (Norris *et al.* 1999). The potential toxicity of deoxy-CYN is currently under study. Early studies using IP-injected mice showed no effects of deoxy-CYN after five days (Norris *et al.* 1999). Contrarily, synthetic deoxy-CYN has been shown to inhibit both protein synthesis and glutathione production *in vitro* in a rabbit reticulocyte lysate system (Looper *et al.* 2005).

Recent work presented at the 2005 workshop of the Australian Research Network for Algal Toxins (ARNAT) compared the IC₅₀ values of deoxy-CYN and CYN for 24 h and 48 h using four different cell lines (Neumann *et al.* 2005). The values were strikingly similar, leading the authors to conclude that deoxy-CYN toxicity probably proceeds by the same mechanism as that of CYN. However, the test concentrations

(0.9 – 4.6 mg L⁻¹) had little environmental relevance, given that deoxy-CYN usually represents just a fraction of CYN values. It was also speculated that deoxy-CYN could be produced as a precursor to CYN, and is later hydroxylated to CYN.

1.6 Bioaccumulation: critical definitions

Bioaccumulation, simply defined, is a process whereby organisms take up toxicants such that their internal concentration exceeds that available in the environment (Walker *et al.* 2001; Burkhard *et al.* 2003). Based on the nature of uptake mechanisms, bioaccumulation can be further divided into bioconcentration, where toxicants are sourced from water only (non-dietary routes), and bioaccumulation, where toxicants are contained in both food and water supplies (Mackay & Fraser 2000; Voutsas *et al.* 2002; van der Oost *et al.* 2003).

Uptake sources for cyanotoxins may include dissolved (aqueous) toxins, toxin-laden algal cells or toxin-laden tissues. Uptake can therefore potentially occur via dermal exposure, oral consumption of cells or tissues, and/or (accidental) drinking of suspended particles and aqueous concentrations. Strictly, uptake of extracellular toxins should thus be termed ‘bioconcentration’ whilst uptake of either intracellular or total toxins (intracellular and extracellular) is ‘bioaccumulation’. Bioconcentration factors (BCFs) and bioaccumulation factors (BAFs) are used to describe the relationship between tissue toxin concentrations in comparison to toxins available in the surrounding environment: specifically, BCF is tissue toxin/extracellular toxin; whilst BAF is tissue toxin/total toxin or tissue toxin/intracellular toxin.

An additional issue in defining bioaccumulation is the location of the toxicant within the target tissues. For example, Streit (1998) defines accumulation as ‘the uptake, storage and accumulation of contaminants by organisms from the environment’. This implies that toxicants must be wholly intracellular. For algal toxins, however, adsorption may also occur, allowing toxin to become associated with the tissues of aquatic biota, whilst not being truly intracellular. Both intracellular and adsorbed toxin components have ecological importance in terms of implications for toxin transfer to successive trophic levels. Hence, for the purposes of this thesis, accumulated toxin will include ‘cyanotoxin concentrations present in or on the cells of an aquatic organism’. These concentrations may also include bound and unbound intracellular fractions, though the former cannot be quantified by current detection techniques.

1.7 Bioaccumulation of algal toxins

The uptake potential of cyanotoxins may be considerable. Firstly, all aquatic organisms experience direct contact with lysed toxins in the water column; many also are vulnerable to ingestion of toxin-laden cells via algal grazing. Furthermore, due to the evolution of aquatic organisms alongside cyanoprokaryotes, mechanisms to resist toxicity might have developed (Christoffersen 1996). This could enable some organisms to accumulate substantial toxin concentrations without side effects, and so provide excellent vectors for toxin transfer in the food chain (Sivonen & Jones 1999). To date, studies relating to bioaccumulation of freshwater algal toxins have been mostly devoted to the hepatotoxin, microcystin (MC). The following section examines trends in bioaccumulation of MC and other cyanotoxins, before describing work that has been done on CYN (section 1.6).

1.7.1 Existing studies

Bioaccumulation of MC has been demonstrated in organisms ranging from aquatic plants, zooplankton, invertebrates to vertebrates (Table 1.1). This toxin was first isolated from the genus *Microcystis* (Rinehart *et al.* 1994), but may also be produced by other cyanoprokaryotes including *Anabaena*, *Oscillatoria* and *Nostoc* (Sivonen & Jones 1999). Considerable work has also been conducted on marine toxins, including nodularin (Sipiä 2001; Sipiä *et al.* 2001b; Sipiä *et al.* 2001a; Kankaanpää *et al.* 2002; Karjalainen *et al.* 2003; Lehtonen *et al.* 2003), the paralytic shellfish poisons saxitoxin, yessotoxin and okadaic acid (MacKenzie *et al.* 2004; Nogueira *et al.* 2004b; Pereira *et al.* 2004).

1.7.2 Influences on toxin uptake risk

1.7.2.1 Nature of exposure regime

The influence of toxin exposure concentrations on bioaccumulation potential of algal toxins is not yet clear. Higher exposure concentrations are linked with increased bioaccumulation values; however, the significance of such concentrations is likely to vary with exposure route and exposure time. For example, Bury *et al.* (1995) showed that even at relatively low levels of MC exposure ($16 - 17 \mu\text{g L}^{-1}$), gill ventilation rates of the brown trout (*Salmo trutta*) would allow exposures of nearly 2 mg of toxin per day.

Increasing the period of toxin exposure appears to have minimal effects on toxin yields from tissues due to the rapid onset of steady-state conditions. Babcock-Jackson (2000) reported that MC accumulation in zebra mussels (*Dreissena polymorpha*) was not increased by extended exposure periods to over one day, since equilibrium was reached within 24 h. Steady-state conditions in mussels have been reached in as

Table 1.1 Examples of microcystin bioaccumulation studies with aquatic organisms. NA = not applicable; DW = dry weight; WW = wet (fresh) weight. HPLC = high performance liquid chromatography; ELISA = enzyme linked immunosorbent assay; LC-MS = liquid chromatography-mass spectrometry

Organism	Exposure	Accumulated concentration ^a	Target organ/s	Extraction and detection method	Study
Aquatic plants					
<i>Ceratophyllum demersum</i> <i>Elodea canadensis</i> <i>Vesicularia dubyana</i>	2.5 mg L ⁻¹ MC-LR over 7 days	71 µg g ⁻¹ WW 40 µg g ⁻¹ WW 110 µg g ⁻¹ WW	<i>Elodea</i> : leaves, then shoots & stems. Trace amounts in roots.	HPLC	Wiegand & Pflugmacher (2001)
Zooplankton					
Tropical zooplankton communities (<i>Brachionus</i> , <i>Moina</i> , <i>Ceriodaphnia</i> and <i>Metacyclops</i>)	<i>M. aeruginosa</i> bloom	0.3 – 16.4 µg g ⁻¹ DW	NA	ELISA	Ferrão-Filho <i>et al.</i> (2002b)
Lake zooplankton populations	MC-LR	> 70 µg g ⁻¹ WW	NA	Methanol extraction; HPLC	Kotak <i>et al.</i> (1996)
Snails					
<i>Physa gyrina</i>	MC-LR	121 µg g ⁻¹ WW	NA	Methanol extraction	Kotak <i>et al.</i> (1996)
<i>Lymnaea stagnalis</i>	MC-LR	96 µg g ⁻¹ WW	NA		
<i>Helisoma trivolvis</i>	MC-LR	11 µg g ⁻¹ WW	NA		
<i>Lymnaea stagnalis</i>	72 µg MC-LR g ⁻¹ dry weight for 24 hours	136 ng g ⁻¹ WW	Alimentary tract (82.8 %); digestive gland (16.9%). Intestine		Zurawell (2001)
<i>Sinotaia histrica</i>	0.473 µg L ⁻¹ total MC 0.476 µg L ⁻¹ total MC 20.1 ± 6.1 µg L ⁻¹ (lab trial)	2.7 µg g ⁻¹ DW 3.2 µg g ⁻¹ DW; 0.42 – 2.65 µg per individual per day, (average 1.48 µg)	Hepatopancreas Intestine Hepatopancreas		Ozawa <i>et al.</i> (2003)

Cont'd over...

Organism	Exposure	Accumulated concentration ^a	Target organ/s	Extraction/detection method	Study
Mussels/Clams					
<i>Unio douglasiae</i>	Live <i>Microcystis</i> cells over 15 days at 15°C and 25°C	130 µg g ⁻¹ (15°C) DW; 250 µg g ⁻¹ (25°C) DW	Hepatopancreas measured only	Butanol/methanol extraction; HPLC	Yokoyama & Park (2003)
<i>Unio douglasiae</i> <i>Anodonta woodiana</i> <i>Cristaria plicata</i>	<i>Microcystis</i> containing MC-LR and -RR	420 µg g ⁻¹ DW 12.6 µg g ⁻¹ DW 297 µg g ⁻¹ DW	Hepatopancreas measured only	Butanol/methanol extraction; HPLC	Yokoyama & Park (2002)
<i>Mytilus edulis</i>	Natural <i>M. aeruginosa</i> bloom	336.9 µg g ⁻¹ WW (GC/MS) 0.204 µg g ⁻¹ WW (PP assay)	NA	Lemieux oxidation with GC/MS; Methanol extraction & PP assay	Williams <i>et al.</i> (1997)
<i>Mytilus edulis</i>	10 ⁵ cells mL ⁻¹ toxic <i>M. aeruginosa</i> over 4d	10.7 µg g ⁻¹ DW	NA	Butanol/methanol extraction; ELISA	Amorim & Vasconcelos (1999)
<i>Anodonta grandis simpsonia</i>	3 days @ 51-55 µg L ⁻¹ dissolved MC-LR (laboratory trial)	No accumulation	Measured whole	Methanol extraction; PP assay	Prepas <i>et al.</i> (1997)
	12 – 28 days @ 0 - 8.3 µg L ⁻¹ MC-LR (field exposure)	24 – 527 ng g ⁻¹ DW	Measured whole		

Cont'd over...

Organism	Exposure	Accumulated concentration ^a	Target organ/s	Extraction/detection method	Study
Crayfish / shrimp					
<i>Procambarus clarkii</i>	Toxic <i>Microcystis</i> strain	2.9 µg g ⁻¹ DW	Intestine, hepatopancreas	Butanol/methanol extraction; ELISA	Vasconcelos <i>et al.</i> (2001)
<i>Pacifastacus leniusculus</i>	Toxic <i>Planktothrix agardhii</i>	Trace amounts only	Only studied hepatopancreas	Methanol, HPLC	Lirås <i>et al.</i> (1998)
<i>P. clarkii</i> (and others)	Natural exposure in contaminated lake	0.05 – 9,97 µg total MC g ⁻¹ DW	Stomach, intestine, gonad, gills, muscle ^{bc}	Butanol/methanol extraction; LC-MS	Chen & Xie (2005)
Fish					
<i>Tilapia rendalli</i>	Toxic <i>Microcystis</i> in the Jacarepaguá Lagoon	29.9 ng g ⁻¹ (muscle, mean) ^a WW 6.5 µg g ⁻¹ (liver, mean) WW 67.8 µg g ⁻¹ (viscera, mean) ^b WW	Measured only muscle, liver and viscera	Methanol extraction; ELISA	Magalhães <i>et al.</i> (2001)
<i>Oreochromis niloticus</i>	<i>Microcystis</i> bloom in fish farm pond	> 800 ng g ⁻¹ (gut) WW; > 500 ng g ⁻¹ (liver) WW; > 400 ng g ⁻¹ (kidney) WW; > 100 ng g ⁻¹ (muscles) WW	Gut, liver, kidney, muscles	Methanol extraction; ELISA	Mohamed <i>et al.</i> (2003)

^amaximum recorded during the study; ^bin order from highest accumulation to lowest; ^chepatopancreas also studied, but toxin concentrations not included since they might have been underestimated.

little as two to five days (Williams *et al.* 1997; Wiegand & Pflugmacher 2001; Nogueira *et al.* 2004b). However, in the natural environment, steady state conditions may be difficult to maintain due to the fluctuating availability of cyanotoxin fractions.

It is also conceivable that a threshold tissue concentration could be reached, whereby aquatic organisms are triggered to reduce feeding to avoid death from toxicity. In studies of saxitoxin bioaccumulation in *Daphnia magna*, Nogueira *et al.* (2004b) considered the lack of any relationship evident between tissue concentrations and time might have related to the possible gradual intoxication of animals, leading to decreased feeding rates (and hence toxin uptake).

1.7.2.2 Size of organism

Size may be critical to bioaccumulation risk because of the relationship between surface area to volume ratios and transdermal uptake rates. For example, Wiegand & Pflugmacher (2001) found higher uptake rates of MC-LR in *Vesicularia* moss compared with *Elodea* and *Ceratophyllum*, probably since the moss had a larger surface area to volume ratio and lacked the protective cuticle found on the macrophytes. Christoffersen (1996) examined aquatic microorganisms and found larger organisms to be generally more tolerant to higher toxin concentrations. Smaller organisms, including juveniles, may also have increased susceptibility to bioaccumulation because of limited mobility, especially those living in inshore littoral areas where scums aggregate (Kotak *et al.* 1996).

1.7.2.3 Trophic level

Trophic interactions between toxic algae and consumer organisms are likely to significantly influence toxin accumulation. On a gram-for-gram basis, accumulated levels of MC appear to be higher in snails and mussels (reported maximum 121 $\mu\text{g g}^{-1}$ and 420 $\mu\text{g g}^{-1}$, respectively) than in fish (reported maximum 6.5 $\mu\text{g g}^{-1}$; Table 1.1). This probably reflects the fact that grazing and filter-feeding organisms are likely to ingest more toxin-laden cells than are omnivorous species. Oral consumption rates correspond well with MC uptake: both Kotak *et al.* (1996) and Yokoyama & Park (2002) concluded that accumulation was a direct result of grazing activity in gastropods. In addition, daily variations in toxin accumulation by *Procambarus* crayfish were suggested to correlate with feeding and resting phases (Vasconcelos *et al.* 2001). Toxin accumulation may also be affected by metabolic rates, since these affect consumption rates (Ferrão-Filho *et al.* 2002b). This is particularly important for large animals with high energy requirements (Christoffersen 1996), or when grazing rates change due to reproductive status or season (Vanderploeg *et al.* 2001), thus promoting increased bioaccumulation susceptibility.

Trophic interactions may also affect food web transfers: Ferrão-Filho *et al.* (2002a) concluded that zooplankton could more efficiently transfer MC to higher trophic organisms than could other organisms such as molluscs. Little is known regarding cyanotoxin transfer in food webs (Kotak *et al.* 1996). Pflugmacher & Wiegand (2001) examined MC-LR bioaccumulation in aquatic organisms of different trophic feeding levels (detritivores, herbivores and predacious water

beetles) but found only grazers to have detectable levels of toxins, though a strong possibility of MC-LR transfer to terrestrial food webs was noted.

1.7.3 Target tissues

Once internalised, microcystin appears to have a tendency for storage in the liver or analogous structures, although toxin has also been recovered from several other organs (Table 1.1). Mohamed *et al.* (2003) and others have reported MC from the gut, but if toxins are simply lying in the alimentary canal, rather than being taken up, these values do not represent true bioaccumulation. Target tissues for bioaccumulation in plants have been poorly studied: comparisons between toxin levels in leaf, shoot or root sections have been reported on only two occasions (Pflugmacher *et al.* 2001; Yin *et al.* 2005).

1.7.4 Metabolism and depuration

Elimination of microcystins from the plasma is possible via both urinary and faecal excretion (Falconer 1993). Elimination of pseudofaeces is thought to be one way in which mussels rid themselves of MC (Vasconcelos 1995), whilst bile may play an important role in MC elimination in fish (Tencalla & Dietrich 1997). Increases in glutathione (GST) activity and the presence of MC metabolites suggested a detoxication pathway was present in aquatic plants: if so, reduction of MC may occur through detoxication processes in the cytosol and chloroplasts (Pflugmacher *et al.* 1999a; Pflugmacher & Wiegand 2001).

Depuration rates and patterns of MC removal also vary between species. Duy *et al.* (2000) suggested MC depuration is generally bi-phasic, with a rapid, initial

rate followed by a prolonged period of gradual clearance. This has since been demonstrated to occur in some snails and mussels (Zurawell 2001; Yokoyama & Park 2003). Clearance times – the periods over which tissues become free of toxins – also vary considerably within and between organisms and for different organs. Zurawell *et al.* (1999) reported clearance times in *Lymnaea* snails to vary from 4h in the gut to 100h in the digestive gland. Marine mussels studied by Williams *et al.* (1997) cleared > 96% of MC over 4 days. Seasonal temperature fluctuations may also affect depuration rates: *Lymnaea* snails were shown to decrease depuration rates in cooler waters (Zurawell 2001).

1.8 Bioaccumulation of CYN

Several of the features of CYN indicate bioaccumulation potential of the toxin. Both the molecular size and shape of compounds are known to influence accumulation potential (Shaw *et al.* 2000), and CYN is a relatively small and stable molecule (415 Da) (Ohtani *et al.* 1992). Unlike other toxins, CYN is also present as primarily extracellular toxin (Hawkins *et al.* 2001; Griffiths & Saker 2003), and is persistent (Chiswell *et al.* 1999) thus increasing the likelihood of being ingested in drinking water. Lastly, given the toxic effects of CYN, it is conceivable that normal cell depuration and detoxification processes may be disrupted during or following exposure, so allowing toxin residues or metabolites to accumulate.

1.8.1 Existing studies

Despite the likelihood for CYN bioaccumulation, limited studies have been conducted using this toxin: only four works have been published to date

(excepting those arising from this thesis). Norris *et al.* (2001b) showed CYN accumulated into the liver and kidney of mice within six hours of dosing via IP injection. The bioaccumulation rate progressively decreased over five to seven days, after a single dosing event. Norris *et al.* (2001b) also demonstrated that up to 73% of toxin was excreted (in urine and/or faeces) within twelve hours.

Saker and Eaglesham (1999) studied bioaccumulation in the crayfish *Cherax quadricarinatus* and the rainbow fish *Melanotaenia eachamensis*. In the crayfish, toxin was detected from both the muscle ($900 \mu\text{g kg}^{-1}$ freeze-dried tissue) and hepatopancreatic tissues ($4,300 \mu\text{g kg}^{-1}$ freeze-dried tissue) after specimens were collected from an aquaculture pond containing $589 \mu\text{g L}^{-1}$ CYN. Toxin was also recovered from the visceral tissue of rainbow fish *Melanotaenia eachamensis* ($1,200 \mu\text{g kg}^{-1}$ freeze-dried tissue). Laboratory trials with the crayfish demonstrated bioaccumulation could occur within fourteen days of toxin exposure, with hepatopancreatic and abdominal tissues containing over 1,000 and $200 \mu\text{g toxin kg}^{-1}$ freeze dried tissue, respectively (Saker & Eaglesham 1999).

Saker *et al.* (2004) exposed the swan mussel, *Anodonta cygnea*, to total CYN values ranging from $14 - 90 \mu\text{g L}^{-1}$ over sixteen days. This resulted in maximum dry weight tissue accumulations of $61,500 \mu\text{g kg}^{-1}$ in the haemolymph, $5,900 \mu\text{g kg}^{-1}$ in the viscera and $2,900 \mu\text{g g}^{-1}$ for whole-body tissues. Toxin was also detected in tissues of the mantle, foot and gonad. The relative distribution of CYN in the tissues changed over the trial period, although bioaccumulation generally occurred within two day's exposure (Saker *et al.* 2004). The authors

also studied the mussels during a fourteen-day depuration period, after which time almost 50% of the toxin remained in the tissues. Depuration was found to be bi-phasic; small increases in tissue toxin concentrations were thought to indicate mobilisation of tissue-bound CYN (Saker *et al.* 2004).

Nogueira *et al.* (2004a) have also reported CYN from the tissues of the cladoceran *Daphnia magna* following exposure to *C. raciborskii*. However, bioaccumulation was not evident, with bioaccumulation factors of 0.71 and 0.46, for 24 h and 48 h exposures, respectively.

1.8.2 Possible uptake route(s)

The water solubility and relatively low molecular weight of CYN may indicate increased bioavailability and the possibility of uptake via passive transport. Chong *et al.* (2002) has already theorized that passive diffusion might be responsible for CYN uptake in rat hepatocytes. On the other hand, active transport mechanisms of CYN have not been well studied. In mammalian hepatocytes, bile acid transporters are used in the early stages of uptake, but an unknown, secondary system is also thought possible (Chong *et al.* 2002).

In aquatic plants, toxin uptake may also be regulated by external factors unrelated to algal blooms. For example, heavy metal uptake is dependent on available metal concentrations, the growth form of plant, cuticle thickness and type of absorption mechanism (Guilizzoni 1991). Uptake rates are generally lowered in senescent compared with actively growing plants (Guilizzoni 1991).

Thus, a growth inhibition effect, if present, may actually protect the plant from bioaccumulation.

1.8.3 Possible ecological effects

Bioaccumulation of CYN is ecologically significant: increased toxin loads in the tissues may increase the lethal and sublethal toxicities associated with exposure. The extensive range of effects associated with direct cyanotoxin exposure in aquatic animals, and evidence of their dose-dependency (Sivonen & Jones 1999) provides a compelling argument for the need to study the bioaccumulation of cyanotoxins. In aquatic environments, all community members can be considered at risk of toxicity, since toxins influence most habitats (Christoffersen 1996). Furthermore, bioaccumulation may allow toxins to pass via the food web to organisms not otherwise exposed to them, including terrestrial fauna that prey on aquatic organisms.

Clements and Newman (2002, p.14) note that toxic responses include those at biochemical, individual, population, community and ecosystem levels, each having progressively greater ecological relevance, despite being less specific. At the organ level, the severity of effects from bioaccumulants depends on whether a toxin (or a metabolite) reaches a site of action, and if it is biologically active when it does so (Sijm & Hermens 2000). For CYN this is difficult to study as the site(s) of action remain(s) largely unknown. However, an awareness of organ-specific accumulation and effects is crucial, since concentration data calculated using other tissues might incorrectly link accumulation levels with

given effects, if preferential storage in one organ is not accounted for (Beek *et al.* 2000).

At the organismal level, bioaccumulation itself is not hazardous until bodily concentrations exert sublethal or lethal effects (Feijtel *et al.* 1997). The lethal body burden is the accumulated concentration at which death occurs and may vary between species because of different uptake and depuration rates, toxicodynamics, sensitivity, and lipid content (Franke 1996). Bioaccumulation may also impart benefit at the organismal level. Deterring prey by taste reception is known to occur in some aquatic organisms (Mebs 1998), however this is yet to be shown for cyanotoxins.

Species-level effects may include interruptions to reproductive cycles and development, causing eventual reductions in abundance. Complex phenomena such as fertility changes or other generational effects have been quoted in association with bioaccumulation (Franke *et al.* 1994) and occur with MC toxicity in plants (Casanova *et al.* 1999). Population changes may result from toxicity specifically towards developmental stages of organisms (Oberemm 2001). Landsberg (2002) noted that reduced species diversity was often recorded in combination with toxic cyanobacterial blooms. Other species-level toxic effects may also include changes to interactions such as hunting for prey, and behavioural changes (Oberemm 2001). Some toxic blooms can be prolonged or recurrent (for example, in Australia's Murray-Darling River System (Baker & Humpage 1994)). If these contain CYN, there is the potential to completely

cover entire and successive breeding seasons, preventing germination and the development of larval and young life stages.

Food web transfers may also be affected by toxin bioaccumulation and its accompanying toxicity. This is important with respect to top-down biomanipulation approaches to toxic bloom management: consumers may remove toxic phytoplankton from the water (Boon *et al.* 1994; Matveev *et al.* 1994), only to accumulate it into other food web compartments, such as zooplankton and fish. Food chain transfers may also represent human risk regarding organisms for consumption (crayfish, mussels and fish). To date, such transfers have never been studied for CYN, representing a significant information – and hence management – gap.

1.8.4 Management implications

In Australia, monitoring and management of algal blooms is currently controlled at national and state level (Jones *et al.* 2002; QDNR 2003). Such protocols, however, predominantly focus on human health consequences rather than environmental risk. Currently, the level of risk for a water body is assigned based on cell and toxin concentration data, but these data are not interpreted for likelihood of bioaccumulation nor environmental effects. Hence, current management approaches do not adequately address all aspects of cyanoprokaryote toxicity, especially ecological risk(s) for aquatic ecosystems.

Ecological risk assessment and management of other toxicants (heavy metals, pesticides) in Australian aquatic systems is addressed within the framework of

the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC 2000). This framework enables holistic management of natural aquatic environments by considering environmental, economic, social and conservation values. Generally, trigger values are calculated using available ecotoxicity testing data for a range of species, from which a value is calculated that is considered to offer a certain level of protection (for example, 95% protection). Consequently, exceeding trigger values indicates a need for responses such as further investigation, amelioration or remediation. However, the guidelines do not currently have provision for direct cyanotoxicity to aquatic flora and/or fauna, nor for the potential ecological implications resulting from cyanotoxin bioaccumulation.

The toxicity and bioaccumulation of CYN remain poorly understood. However, the possible ecological risks posed by cyanotoxin bioaccumulation suggest that their inclusion is vital to manage algal blooms effectively for ecosystem and human health. Moreover, neither current algal management strategies nor the ANZECC water quality guidelines make provision for the management of effects associated with cyanotoxin bioaccumulation and exposure. Clearly, there is a need to address this information gap, and to integrate resulting data to develop adequate management strategies aimed at minimising ecological and human health threats posed by cyanotoxin bioaccumulation. In particular, the increased incidence of toxic *Cylindrospermopsis* blooms signals a need for greater understanding of this species, and particularly its toxin, to ensure adequate and appropriate management strategies.

1.8.4.1 The use of predictive modelling

Management of toxic cyanoprokaryote blooms is particularly challenging. Due to the water solubility and stability of cyanotoxins in light, costly and complex chemical procedures are needed to accelerate decomposition and removal (Haider *et al.* 2003). Reactive management strategies are inadequate to manage cyanotoxin exposure problems. Rather, management approaches that are able to predict likely periods of bioaccumulation risk, and their associated toxicity, are better equipped to minimise the toxic effects of blooms.

Several studies have indicated that predictive modelling could be suitable for cyanotoxin bioaccumulation. For example, Yokoyama and Park (2002) found bioaccumulation in the bivalve *Unio douglasiae* could be predicted from the concentration of intracellular MC in suspended solids. However, bioaccumulation was later noted to closely track both ambient toxin and temperature levels (Yokoyama & Park 2003). Both Zurawell *et al.* (2001) and Kotak *et al.* (1996) found MC concentrations in snails to mirror changes in the toxicity and abundance of the phytoplankton population.

Predictive models for bioaccumulation and toxicity are often based on quantitative structure activity relationships, which use physical and chemical characteristics to arrive at the likely biological behaviours for particular substances. In the past, octanol-water partition coefficients (K_{OW}) have been favoured for bioaccumulation studies. Generally, values of $\log K_{OW} > 3$ are considered to have bioaccumulation potential (Franke *et al.* 1994). For example,

K_{OW} values estimated for MC-LR, -LY, -LW and -LF using HPLC techniques were 2.16, 2.92, 3.46 and 3.56, respectively (Ward & Codd 1999).

To date, a K_{OW} value has not been examined for CYN. However, the use of K_{OW}s is disadvantageous in many respects: there are no considerations of active transport mechanisms, diffusion behaviour, metabolism, accumulation into specific organs or tissues, and uptake and depuration kinetics (Franke *et al.* 1994; Franke 1996).

Predicting cyanotoxin bioaccumulation is particularly challenging since the exposure regimes associated with toxic blooms are not straightforward. Unlike synthetic toxicants (heavy metals, pesticides), cyanotoxins are produced within living organisms: toxin content of blooms may be highly variable depending on the number and physiological status of algal cells (Orr & Jones 1998), and on various environmental conditions (Utkilen & Gjølme 1995; Saker & Griffiths 2000). Furthermore, toxins may be available as intracellular (cell-bound), extracellular (non-cell bound or aqueous) and tissue-bound toxin fractions, which means that toxin bioavailability is largely determined by the status of the toxic algal population.

Bioaccumulation studies must also make consideration for potentially large variability in uptake rates, uptake routes and the detoxication/depuration capabilities of different animals and trophic levels. Effective modelling of cyanotoxin bioaccumulation is unique in having to address the multiple issues of exposure regimes, uptake rates, metabolism and depuration. Avenues for

suitable predictive modelling of bioaccumulation of cyanotoxins are discussed further in chapter two.

1.9 Summary and research questions

C. raciborskii is a toxin-producing alga increasingly being recorded from tropical Australia and elsewhere globally. CYN, a toxin produced by *C. raciborskii*, has been only recently characterised and is poorly studied compared with other cyanotoxins. The effects of CYN exposure in many aquatic plants, invertebrates and vertebrates have yet to be studied. Similarly, the potential for CYN bioaccumulation, where compounds in the tissues of animals or plants exceed concentrations available in the environment, has been only poorly studied.

Presently, Australia lacks adequate management strategies to address the ecological risks posed by toxic *C. raciborskii* blooms, and specifically CYN toxicity and bioaccumulation. Predictive modelling is a possible avenue by which adequate management strategies for toxic booms can be developed.

Clearly, there is an urgent need to address the information gaps regarding CYN toxicity and bioaccumulation, and to develop adequate management strategies aimed at reducing the ecological effects of toxic algal blooms. The core research questions arising from this review of literature therefore are:

1. Does cylindrospermopsin bioaccumulate in aquatic organisms, and if so, which ones?

(H₀: Bioaccumulation does not occur in any of the test species)

2. Do adverse effects (sublethal, lethal) occur in conjunction with bioaccumulation and/or exposure to cylindrospermopsin in each tested species?

(H₀: Lethal and sublethal effects do not occur in conjunction with either bioaccumulation or exposure to cyanotoxins in any of the test species).

3. What conditions influence the potential for bioaccumulation and exposure effects in each tested species?

(H₀: Changes in exposure regimes (concentration, time) have no effect on bioaccumulation or its effects in any test species).

4. How can bioaccumulation of cylindrospermopsin in tropical freshwater systems be best managed?

Chapter two: management of cyanotoxin bioaccumulation via predictive modelling

Note to reader: material in this chapter has been published in **White, S.H.**, Duivenvoorden, L. J. & Fabbro, L. D. (2005). A Decision-making Framework for Ecological Impacts Associated with the Accumulation of Cyanotoxins (Cylindrospermopsin and Microcystin). *Lakes and Reservoirs: Research and Management* 10: 25-37.

2.1 Challenges in modelling cyanotoxin bioaccumulation

Bioaccumulation processes are complex, with the nature and extent of bioaccumulation depending on two main elements: the bioavailability of the toxicant; and species-specific factors including feeding, metabolism, growth dilution and uptake efficiencies (McCarty & Mackay 1993). For cyanotoxins, however, bioavailability is itself governed by many factors, including the type and concentration of available toxins, their spatial and temporal variability, and their uptake routes. Primarily, these complexities result from the fact that living cells produce cyanotoxins, which is unlike most other toxicants (pesticides, heavy metals).

2.2 Influences on toxin bioavailability

2.2.1 Toxin production

Toxin production by cyanoprokaryotes is influenced by many factors, although the significance of these remains poorly understood. Whilst a single species (or strain) can be identified as being toxigenic, this does not necessarily mean toxin production occurs during blooms (Chorus *et al.* 2001). Moreover, increased cell densities of cyanoprokaryotes do not always correspond with increased toxicity (Bickel *et al.*

2000; Jacoby *et al.* 2000). Many studies have focussed on finding triggers for toxin production and the circumstances under which toxin production increases. Mostly, these have examined the relationships between energy status and cell division rates and the toxin content of cells (for example, Orr & Jones 1998; Bickel *et al.* 2000). Others have linked environmental parameters, particularly nutrient, temperature and light conditions, with toxin cell quotas (Saker & Griffiths 2000; Long *et al.* 2001; Saker & Neilan 2001). Toxin decomposition processes have also been examined with respect to time, pH, temperature, light, water quality and microbial activity (for example, Lahti *et al.* 1997b; Chiswell *et al.* 1999).

Toxin production is also variable because of the possibility of more than one toxin, or toxin variants (sometimes, congeners (Dietrich 2005)), occurring in a given bloom. For example, over sixty-five MC variants have been identified (Carmichael 2001a), although a natural bloom rarely contains more than ten (Geoff Eaglesham, Senior Chemist, QHSS, pers. comm.). *C. raciborskii* may produce more than one variant of CYN and/or toxin analogs (Hawkins *et al.* 1997; Falconer 1999; Norris *et al.* 1999; Norris *et al.* 2002). The implications of these are that predictions of toxicity levels based only on the presence and/or cell concentrations of a toxigenic species are not sufficient – rather, sampling of blooms is essential to confirm toxicity.

2.2.2 The presence of different toxin fractions

Cyanotoxins may be present as intracellular (INC), extracellular (EXC) or tissue-bound components. Here, intracellular toxins are defined as those held within algal cells (cell bound); extracellular are those toxins released from cells into the surrounding water (aqueous, non-cell bound). Tissue-bound toxins are those found

within animal tissues, and these may be associated (reversibly or non-reversibly bound) with protein phosphatases or other cellular enzymes. Strictly speaking, tissue toxins ought not to include those lying in the alimentary canal, since these have not yet been metabolised. As Codd *et al.* (1999a) notes, few studies have examined the compartmentalisation of different toxin fractions with respect to exposure routes. However, the three fractions represent a significant issue in bioaccumulation modelling, because of their different bioavailabilities. As noted by Dietrich (2005), the form and type of toxin exposure allows for different risk scenarios. In the case of bioaccumulation risk, for example, there will be a difference in the relative importance of dietary (intracellular) to aqueous (extracellular) exposures according to the organism in question, and its likely uptake routes.

Toxin fraction composition is different for each cyanotoxin. For example, MC is usually intracellular (Berg *et al.* 1987; Watanabe *et al.* 1992; Lahti *et al.* 1997b; Park *et al.* 1998; Long *et al.* 2001) although Wiedner *et al.* (2003) found extracellular concentrations of MC to be 20% higher in cultures with higher light irradiance. Aboal & Puig (2005) reported the INC/EXC ratio of MC to be variable between different reservoirs, although peaks in dissolved concentrations always followed peaks in intracellular concentrations. In contrast, CYN is largely extracellular throughout natural blooms (Chiswell *et al.* 1999; Carson 2000; Norris *et al.* 2001; Metcalf *et al.* 2002a). Moreover, in cultured strains, up to 50% extracellular toxin has been recorded in some isolates (Saker & Griffiths 2000; Hawkins *et al.* 2001; Norris *et al.* 2001). Exceptions to this situation may occur where *C. raciborskii* is in the most rapid growth phase, with large proportions of INC toxin evident (Hawkins *et al.*

2001). This predominantly extracellular nature of CYN is in direct contrast to almost all other cyanotoxins, excepting anatoxin-a production by *Anabaena flos-aquae* (Bumke-Vogt *et al.* 1996 in Hawkins *et al.* 2001).

The bioavailability of each of these toxin fractions is critical because of their influence on uptake route. Intracellular and tissue-bound toxins may pose significant risks for organisms that ingest toxin-laden cells, through either grazing activity or accidental ingestion. On the other hand, plants and other organisms vulnerable to transdermal uptake may be at greater risk from extracellular toxin. Tissue-bound toxins represent risk for consumers not exposed to toxins directly (for example, terrestrial birds). Thus, the availability of a particular toxin fraction may be more significant for bioaccumulation potential in a given set of organisms (for example, intracellular toxins for grazers).

2.2.3 Temporal variation

Temporal variation of cyanotoxin fractions occurs because of production, decomposition and uptake during the progression of a toxic bloom. In general, the dynamics of fraction availability can be mapped throughout a bloom (Figure 2.1). Here, the early stages of a toxic bloom consist mostly of intracellular toxin, consistent with production and storage within cyanoprokaryote cells. However, as the bloom ages, some cells die and lyse, releasing small amounts of extracellular toxins into the water. Meanwhile, newly made cells continue to add to intracellular toxin levels.

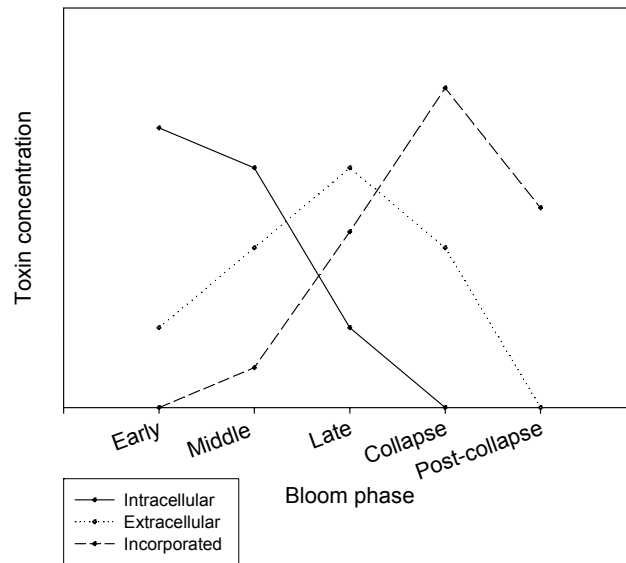


Figure 2.1 Speculated change in toxin fractions with bloom progression

In the latter stages of a bloom, this situation is reversed: more cells lyse, the production of intracellular toxin is slowed, and a transition to extracellular toxin occurs. Tissue-bound (incorporated) toxins may occur in some aquatic organisms, if they are capable of toxin accumulation. Finally, during bloom collapse, extracellular toxins may either persist or be rapidly degraded (Lahti *et al.* 1997a; Chiswell *et al.* 1999; Duy *et al.* 2000). Tissue-bound toxins continue to be present until depuration and/or clearance occurs by a variety of possible mechanisms (Falconer 1993; Zurawell 2001; Beattie *et al.* 2003; Yokoyama & Park 2003; Soares *et al.* 2004). The longevity of toxin fractions may also be different, contributing to their presence throughout a bloom: Lahti *et al.* (1997b) reported that dissolved MC was more persistent compared with MC in particulate material.

Christoffersen (1996) suggested the worst time for injurious bloom effects to occur was in the later stages, when cells lyse and release large amounts of toxins into the water. However, this may not necessarily be true for bioaccumulation effects, since

intracellular toxin could be of similar or greater importance than the extracellular. That is, certain exposure routes may become more or less important at different times during the bloom. Hence, organisms with different uptake routes will be susceptible to bioaccumulation at different times: plants are unlikely to be affected by intracellular toxins, and hence may not be predisposed to bioaccumulation early in bloom formation. Conversely, algal grazers could be considered at higher risk during this time. This is a key issue to identifying peak bioaccumulation hazard times for different aquatic organisms, but presents a difficulty in modelling, because of the need to know the concentrations of each toxin fraction.

The timing of toxic blooms is also critical to bioaccumulation potential, since seasonal temperature changes help determine species dominance with cyanoprokaryote assemblages as well as their accompanying toxicity (Saker & Griffiths 2000). For example, a negative correlation between temperature and toxin cell quotas has been documented for CYN (McGregor & Fabbro 2000), thus indicating greater risk of toxin production in cooler months. Furthermore, short-interval bloom periodicity—especially for autumn and winter bloom species—may represent decreased exposure times. Toxic blooms may also coincide with sensitive life stages of some animals (moulting, hatching), thus predisposing them to greater toxin exposure.

2.2.4 Spatial variation

The bioavailability of toxin fractions may also change spatially. Since intracellular toxins are held within living cells, their distribution is not homogeneous in a system. Concentrations of toxin may occur at certain depths, since gas-vacuolate cyanoprokaryote cells are able to regulate buoyancy according to light preferences.

For surface species, wind-induced concentrations may also occur. Similarly, senescing cells may sink and create an abundance of extracellular toxins near benthic habitats. The spatial variability of tissue-bound toxins is almost unlimited as these are essentially mobile according to the animal (or plant) in which they are contained. These spatial variations mean that habitats may predispose some aquatic organisms to certain toxin fractions—for example, benthic animals may frequently be in contact with extracellular toxins, whilst floating aquatic plants are situated alongside toxic algal scums (intracellular toxin).

2.2.5 Individual uptake rates

A final challenge in predictive modelling of bioaccumulation potential is the individual capabilities of aquatic animals to respond to cyanotoxin availability. Since most toxicity studies have not examined aquatic animals, predictions of bioaccumulation and its effects are very difficult. Some organisms may be able to minimise or avoid exposure to cyanotoxins, so decreasing their likelihood for bioaccumulation. For example, Zurawell (2001) surmised that sessile filter feeders (such as bivalves) might be liable to the greatest exposure to cyanotoxins as they are unable to move away, and have limited alternatives for food. However, this may also have given them the opportunity to develop evolutionary tolerance to the toxins.

The physiology of aquatic animals may also affect bioaccumulation potential: in some organisms, interactions between toxins and internal proteins may occur (Kankaanpää *et al.* 2002), whilst others may convert toxins to a different form or compound, particularly CYN (Runnegar *et al.* 1994). This introduces the possibility of toxin

conjugates being accumulated, or for incomplete residues to pass up the food chain, so contributing to biomagnification.

2.3 A model for cyanotoxin bioaccumulation and risk assessment

Clearly, the nature of cyanotoxins – especially their temporal and spatial variability, and the resulting implications for exposure – makes the task of developing a predictive model for bioaccumulation difficult. However, the potential ecological risks associated with cyanotoxin bioaccumulation makes a clear case for developing such a model. Ideally, this model should examine toxin availability for each aquatic organism by physical sampling of water bodies, combined with a consideration of likely exposure to, and uptake of, each of the toxin fractions. It should also lend itself towards meaningful results that can be used in ecological risk assessment of lakes and reservoirs affected by toxic cyanoprokaryote blooms.

2.3.1 Background

Three factors are critical in enabling bioaccumulation of cyanotoxins: one, that the toxin is in a form available for uptake by a given organism (fraction availability); two, that the toxin is available in high enough levels that bioaccumulation occurs (toxin threshold); and three, that the toxin remains in this form, and at these levels, for a period long enough to allow accumulation into tissues (toxin exposure).

Fraction availability has been discussed earlier in the context of influencing bioaccumulation by aquatic organisms, through associations with different uptake

routes. Logically, it could be concluded that organisms which have only one uptake route (for example, aquatic plants), are not at risk of bioaccumulation unless the toxin fraction associated with that route is present. However, aquatic organisms with multiple uptake routes may favour one fraction over another.

The second influence on bioaccumulation potential is the bioavailable concentrations of each toxin fraction. Presumably, a threshold exists below which toxin accumulation does not occur, if the metabolic capabilities of aquatic organisms regulate or prevent accumulation. It is difficult to theorise a threshold for MC from current literature, due to disparate reporting of exposure levels (for example, toxin per dry weight of cells compared with toxin content in water).

Toxin thresholds will be different for each species, according to their metabolic capabilities. An example of this is two animals exposed to the same bioavailable toxin levels (Figure 2.2). The first animal ('non regulator') has no metabolic capability to process (depurate, detoxify, metabolise) toxin, so, once present, the toxin accumulates into the tissues. The second animal ('regulator'), however, is capable of processing small levels of the toxin ($< 4 \mu\text{g L}^{-1}$), hence preventing toxin bioaccumulation. However, this capability fails at higher toxin levels, and eventually, a threshold is reached where toxins accumulate into the tissues ($12 \mu\text{g L}^{-1}$). The identification of minimum toxin thresholds required to produce cyanotoxin bioaccumulation is critical in being able to manage toxic blooms.

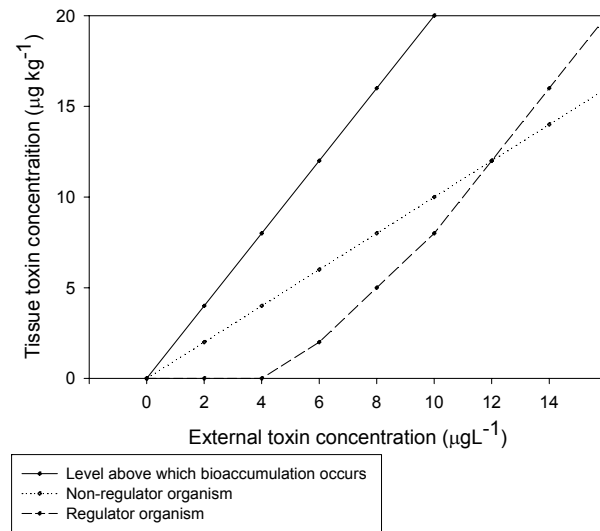


Figure 2.2 Bioaccumulation thresholds in animals of different metabolic capabilities.

Thirdly, there must be a minimum exposure time associated with the capability to accumulate toxins in aquatic organisms. This may vary depending on the relative importance of a given uptake route. For example, organisms that accumulate toxins originating from dermal exposure may need only short periods of toxin contact. In contrast, bioaccumulation rates of organisms that ingest toxin-laden cells are likely to depend on feeding activity. Exposure time and consequent bioaccumulation may also be affected by metabolism rates between species (Yokoyama & Park 2002).

Lastly, to predict the range and extent of ecological effects likely in a given species, the most critical factor is that internal tissue concentrations are at or above hazardous levels. Combined with this should be a strong knowledge base regarding the likely sublethal or lethal effects corresponding to these toxin levels. To date, almost no data are available regarding the links between internal toxin concentrations and death, loss in reproductive success or other sublethal effects. Basing ecological risk assessments on internal concentrations is more reliable than doing so using water concentrations, because the issues of uptake and metabolism have already been resolved (Sijm &

Hermens 2000). However, problems such as preferential storage of toxins into some organs must be first acknowledged, since these could lead to underestimation of true tissue toxin levels.

From the above discussion, five main elements have emerged which require consideration in a model for bioaccumulation and its ecological effects:

1. knowledge of actual toxin concentrations in the water, including the concentrations of intracellular and extracellular fractions;
2. understanding of the possible toxin uptake routes for each aquatic organism, such that the influence of each toxin fraction in contributing to bioaccumulation can be gauged;
3. knowledge of the minimum concentration thresholds above which bioaccumulation occurs in each organism (and, by default, their regulatory capability);
4. knowledge of the minimum exposure time necessary for bioaccumulation in each organism;
and, for prediction of ecological effects,
5. knowledge of the internal tissue toxin concentrations, and the sublethal and lethal effects associated with these, for each aquatic organism.

2.3.2 The model framework

The following model (Figure 2.3) is a decision-tree framework that combines a predictive modelling approach to toxic algal bloom management – specifically, bioaccumulation – together with ecological risk assessment based on internal concentration effects. A worked example is provided later (see 2.3.3). In general, the

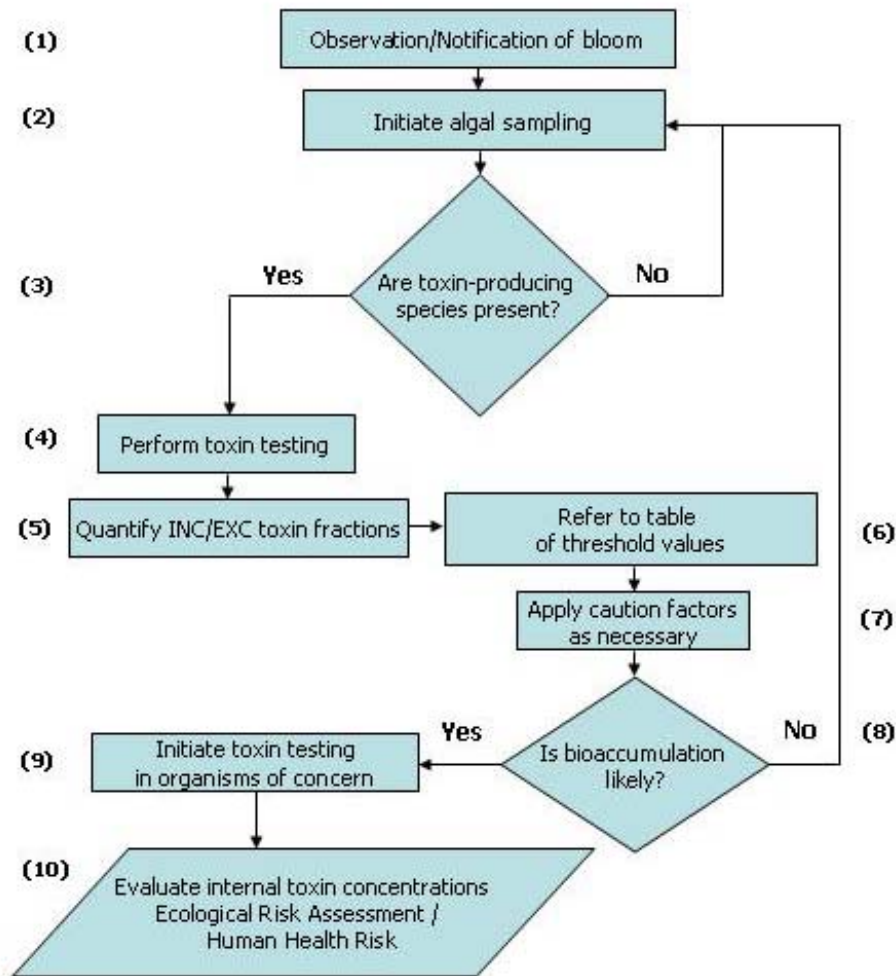


Figure 2.3 Proposed model for monitoring and management of cyanotoxin bioaccumulation and its ecological effects.

model relies on collection and examination of water samples for toxin analyses, followed by an evaluation of what these toxin levels mean in terms of bioaccumulation potential for a range of aquatic organisms. In the context of other risk characterisation and management strategies for toxic cyanobacterial blooms, this model sits entirely within the ‘situation assessment’ action plan outlined by Codd *et al.* (2005b).

The step-wise process (Figure 2.3) begins with monitoring of water bodies likely to be affected by toxic cyanoprokaryote blooms, based on prior history and/or current

conditions (1). Algal sampling is conducted (2), with samples examined for potentially toxigenic species (3). Where these are present at concerning levels, toxin testing is initiated (4). The levels at which testing is necessary may vary for different cyanoprokaryote species: for example, McGregor & Fabbro (2000) suggest that *C. raciborskii* cell densities $> 20,000 \text{ cells mL}^{-1}$ should be examined for toxicity.

At this stage of the framework (step 4), all procedures are already nationally accepted algal monitoring protocols. However, in the national protocols, toxin results are evaluated according to guideline limits for public health concerns, not for bioaccumulation. Furthermore, toxin results are reported as total toxin concentrations. In contrast, this model requires separate quantification of the intracellular and extracellular toxin fractions (5), which are critical in determining bioaccumulation potential.

Next, toxin results are evaluated by making a prediction as to whether or not the conditions have made bioaccumulation likely. This prediction is made using a table of threshold values for aquatic organisms (6), as exemplified for MC (Table 2.1). Notably, the values in this are completely arbitrary; current knowledge of cyanotoxin bioaccumulation processes in aquatic animals is still poor. In practice, the risk threshold values will be based on the results of laboratory-executed and field-validated ecotoxicity experiments. The table works by identifying a species of concern and its likely uptake route(s) for cyanotoxins, and then provides the specific threshold value(s) above which bioaccumulation is deemed likely. Using the table, evaluation of bioaccumulation risk is made easier, particularly because

Table 2.1 Bioaccumulation risk threshold values for microcystin (example only, values are arbitrary). * non-essential uptake routes; NA = not applicable; NR = not required (one uptake route is considered to be of greater significance).

Species	Primary exposure route(s)	Intracellular threshold	Extracellular threshold	Caution Factor(s)
<i>Lemna</i> sp. (duckweed)	Transdermal	NA	1 µg L ⁻¹	NR
<i>Tubifex</i> sp. (tube worm)				NR
	Transdermal; oral	2 µg L ⁻¹	1 µg L ^{-1*}	
<i>Thiara</i> sp. (herbivorous snail)	Oral	0.5 µg L ⁻¹	6 µg L ^{-1*}	NR
<i>Eusthenia</i> sp. (carnivorous stonefly)	Oral (tissue-bound)	NA	4 µg L ⁻¹	NR
		(do not graze on algae)		
<i>Cherax</i> sp. (omnivorous crayfish)	Oral; Transdermal	4 µg L ⁻¹	3 µg L ⁻¹	Reduce values by ½ in presence of two sub-threshold values.
<i>Bidyanus</i> sp. (silver perch; omnivorous)	Oral (tissue- bound); transdermal; drinking	10 µg L ⁻¹	4 µg L ⁻¹	Reduce values by 1/2 in presence of two sub-threshold values.

providing the singular threshold values for each toxin fraction allows the problem of multiple uptake routes to be addressed: it matters not how many routes are available, as long as each one of the thresholds is not exceeded.

In many animals, one uptake route in particular will represent the primary or most significant source of toxin. For example, it is reasonable to expect that algal grazers such as snails may receive most of their toxin ‘dose’ from intracellular toxins. Although transdermal uptake is also possible, it would be minor in comparison. The threshold values for minor routes are still supplied in Table 2.3, because they could be used to aid in decision-making. For example, where the concentrations of available toxin for the major uptake routes are close to, but not exceeding the given threshold, the minor route could be used as a supplement to determine if there is risk of bioaccumulation. This reflects the possibility of additive or synergistic effects of more than one toxin uptake route in a given animal.

In some animals, however, two or more uptake routes may have the same level of significance (that is, both are major uptake routes). For this reason, caution factors (step 7) have been introduced into the table (Table 2.1). Caution values have been used in other ecotoxicological applications, often to account for the differences between laboratory measured lethal effects and the expected sublethal effects occurring in the field, or short versus long-term experimental data (Markich & Camilleri 1996). Here, however, their role is to ensure that several sub-threshold fraction values are not mistakenly interpreted as being risk-free. This is achieved by allowing the caution values act to decrease the singular

thresholds, after which the risk from each fraction should be re-evaluated (steps 6- 8) using the new (reduced) threshold value. Using such factors provides ‘an extra margin of safety beyond the known or estimated sensitivities of aquatic organisms’ (Stephan *et al.* 1985).

Returning to the model, if any of the singular thresholds have been exceeded, (before or after application of caution factors), tissue testing is initiated for those organisms considered at risk of bioaccumulation (Figure 2.3, step 9). Finally, the results of these tests can be compared against known sublethal and lethal toxicities for similar internal concentrations in order to assess the likely ecological effects of bioaccumulation (10). This final step will also require a table showing internal tissue concentrations with their corresponding effects; however, as no such data yet exists, this table is not provided.

The decision making process followed in this model may need to be repeated several times in the event of a particularly long toxic bloom, especially where early toxin sampling indicates levels below threshold values. In this event, monitoring should be continued in order to ensure levels do not rise above risk concentrations later. Incorporating exposure times into this model is difficult, because of the need to introduce almost continuous toxin testing to document fluctuations in toxins between tests. It may therefore be useful to initiate toxin testing at critical stages of a bloom, for example, the ‘middle’, ‘late’ and ‘collapse’ stages (Figure 2.1).

2.3.3 A worked example

A decision pathway for managers of a reservoir stocked with *Cherax* crayfish may be as follows. Early in the bloom, algal sampling and enumeration identifies the predominance of toxic *Microcystis*, prompting the need for toxin analysis. The first toxin analysis is performed; results obtained are $3 \mu\text{g L}^{-1}$ intracellular toxin and $2 \mu\text{g L}^{-1}$ extracellular toxin. The risk evaluation values for *Cherax* are $4 \mu\text{g L}^{-1}$ for the intracellular fraction and $3 \mu\text{g L}^{-1}$ for the extracellular fraction (Table 2.2). Based on individual toxin fractions, no risk thresholds have been exceeded for *Cherax*. However, both routes are considered of major uptake significance, which means caution factors must be introduced. In addition, since more than two sub-threshold values are present, each individual threshold must be reduced by half. As a result of applying these caution factors, the thresholds are exceeded (see Table 2.2 below), and tissue toxin testing should be initiated, and the results used to determine possible ecological effects of bioaccumulation.

Table 2.2 Example of threshold values after application of caution factors.

Toxin fraction	Toxin level ^a	Previous threshold	Caution Factor	New threshold	Result
Intracellular	$3 \mu\text{g L}^{-1}$	$4 \mu\text{g L}^{-1}$	Half	$2 \mu\text{g L}^{-1}$	Exceeded
Extracellular	$2 \mu\text{g L}^{-1}$	$3 \mu\text{g L}^{-1}$	Half	$1.5 \mu\text{g L}^{-1}$	Exceeded

^amicrocystin-LR equivalents

2.3.4 Model evaluation

This model is unique in addressing most of the challenges posed by predictive modelling of cyanotoxin bioaccumulation values. Firstly, variability in toxin production by cyanoprokaryotes is addressed by regular toxin testing, based on the species composition of the bloom. Likewise, the temporal change in toxin fraction variability is accounted for because the toxin tests actually separate out

the intracellular and extracellular fractions. Lastly, the responses of aquatic organisms to cyanotoxin exposure (uptake, metabolism and adverse effects) are each considered in their own right, by dividing the risk evaluation table according to species. If this table can be formulated using laboratory and field derived values, the number of species is limited only by available data sets.

This model does not represent the processes of uptake and depuration rates, nor the possibility of metabolic transformation or conjugation of toxins, though these have been studied (Zurawell 2001; Pflugmacher 2002; Yokoyama & Park 2003). Similarly, the possibility of tissue-bound toxins as a further uptake route for some animals (via biomagnification) has not been considered. Incorporating all of these complexities may risk underestimating the bioaccumulation hazard, especially if all components are not properly accounted for (Franke *et al.* 1994). Rather, the model establishes when bioaccumulation risk is present for a given set of organisms, and allows this information to be used as a screening method for bioaccumulation. Following this, tissue testing is performed and assessments of ecological (and human) health risks are performed on known data. Direct quantification of toxin levels within tissues will always remain the most trustworthy and effective tool for determining if tissues are safe for consumption (MacKenzie *et al.* 2004).

The benefits of this model include ease of integration into current algal management approaches, since steps one through four are already standard operational procedures. In addition, the model is relatively simple for lay-people to understand, so minimising training requirements. There are no requirements

for extra testing or data collection methods other than those used at present—except for performing toxin analyses on intracellular and extracellular, rather than total, fractions. This model acts as a screening device to avoid the possibility of undertaking unnecessary and costly tissue samples.

Drawbacks to the model include higher costs for toxin analyses, since the cell-bound and non-cell bound fractions are determined separately. Repeated sampling may be necessary during long bloom periods. The model also has a certain degree of error: toxin results may not exactly reflect toxin fractions in the sampled water body, due to variability in sampling (collection, transport and storage) and toxin detection methods. MacKenzie *et al.* (2004) noted the difficulties in obtaining truly representative subsamples during phytoplankton monitoring. In particular, lysing of cells and/or degradation of dissolved fractions in storage may underestimate actual toxin concentrations (Nicholson & Burch 2001). Hence, evaluating these levels will give only gross indication of the bioaccumulation risk.

Although they have not been examined in this model, many other factors may contribute to uptake and bioaccumulation rates; most notably the effects of changing environmental parameters including temperature and pH. In addition to these problems, the model is limited by spatial variability of the toxin fractions. It would be time consuming and costly to design a toxin testing protocol aimed at reflecting the spatial variability in intracellular and extracellular toxin fractions, and probably impossible to do so for tissue-bound toxins.

Using the risk threshold values and caution factors has several advantages. Firstly, the issue of multiple toxin routes is addressed, as each has its own threshold value. Secondly, each organism can be considered separately, so avoiding interspecies error associated with different uptake and metabolic processes. However, it is vital that risk and caution values are based on the results of laboratory-executed and field validated ecotoxicity experiments: other arbitrary values have been criticised because of their lack of theoretical basis.

One downfall of using the risk threshold approach is that it presumes uptake will always occur in the presence of toxin fractions. This may be likely for intracellular toxin: oral consumption of MC appears to correspond well with toxin uptake in gastropods (Kotak *et al.* 1996; Yokoyama & Park 2002), but the situation is uncertain for tissue-bound and extracellular toxins. Possible errors in using the thresholds may also result from size differences, even within the same species. Smaller organisms will be more susceptible to transdermal toxin uptake due to their high surface area to volume ratios (Wiegand & Pflugmacher 2001). Younger life stages and juveniles may also have increased susceptibility because their mobility is limited, often to inshore littoral areas where scums aggregate (Oberemm 2001). Equally, larger animals with higher consumption rates may be subject to higher oral uptake rates (van der Oost *et al.* 2003).

2.4 Summary and research questions

The range of organisms potentially affected by cyanotoxin bioaccumulation during toxic blooms and the vital ecological roles these play in aquatic systems indicates a clear need to introduce a suitable programme to enable algal-bloom

managers to predict bioaccumulation and its consequences. Such an approach may then allow preventative or remediation steps to be taken with the aim of reducing ecological and human effects. The predictive model thus makes a valuable contribution to decision-making frameworks for algal blooms.

The management model proposed above provides clear settings in which bioaccumulation and ecotoxicity experiments can be designed. Specifically, the core research questions arising from this analysis are:

1. Do the relative concentrations of intracellular and extracellular toxin fractions significantly affect CYN bioconcentration or bioaccumulation in aquatic organisms?

(H_0 : That CYN uptake will be identical at identical toxin exposure concentrations, regardless of the ratio of INC/EXC fractions).

2. Does total toxin concentration (intracellular and/or extracellular) significantly impact CYN bioconcentration or bioaccumulation in aquatic organisms?

(H_0 : That CYN uptake is identical at identical toxin exposure concentrations).

Chapter three: general materials and methods

Many of the materials and methods used were common to all experiments: they are described here to avoid repetition in later chapters.

3.1 Culture of *Cylindrospermopsis raciborskii* for toxin

C. raciborskii cultures were grown in glass Schott bottles in either a controlled-temperature room at $25 \pm 2^{\circ}\text{C}$ under continuous light (approximately $4 - 8 \mu\text{mol photon m}^{-2}\text{s}^{-1}$; cool white fluorescent tubes); or on open laboratory shelves at room temperature ($21 - 24^{\circ}\text{C}$). Cultures comprised a straight *C. raciborskii* strain (CQU-FR001; isolated from Fitzroy River on 3rd December, 1993 by Dr. Larelle Fabbro), which is known to reliably produce CYN in culture. Saxitoxin was not found in the cultures when tested using sodium channel and saxiphilin radioreceptor assays (Llewellyn *et al.* 2001). Previous tests also indicated absence of saxitoxin (Baker 1998).

Cultures were grown in ASM-1 algal media (Appendix A). Solutions were adjusted to pH 7.8 – 7.9 using hydrochloric acid (1 molar) and/or sodium hydroxide (1 molar) and buffered with the addition of HEPES buffer (N-2'-hydroxyethylpiperazine-N-2'-ethanesulfonic acid) prior to autoclaving. After inoculation, lids were loosely placed on bottles and cultures were gently mixed periodically. Subculturing using aseptic techniques took place on an as-needs basis to maintain cultures free of contaminants and to maintain them in the exponential growth phase (optimal for toxin production). Cultures were checked for bacterial contamination by preparing streaks on agar media plates and slope tubes.

Handling, storage and transport of toxic cultures was done in accordance to a risk assessment conducted with reference to current research data and the guidelines of the National Health and Medical Research Council of Australia (NHMRC & NRMMC 2004). Where possible, glass equipment was used to minimise toxin adsorption to plastics (Nicholson & Burch 2001); high density polyethylene bottles were used for storage of frozen samples. Contaminated plastics and unwanted culture materials were disposed of after treating with chlorine bleach.

3.2 Trial preparation and procedures

3.2.1 Test procedures

Where possible, experimental procedures followed the guidelines provided by the Organisation for Economic Cooperation and Development (OECD 2002) and the American Society for Testing and Materials (ASTM 2003). Reference toxicants were not used, since these are not considered mandatory by either standard, and there are many published examples of algal toxin studies which have not used these (for example, Oberemm *et al.* 1999; Romanowska-Duda & Tarczyska 2002; Mitrovic *et al.* 2005).

All trials were conducted as semi-static renewal tests: frequent replacement of test solutions was necessary to ensure actual toxin concentrations remained close to nominal test concentrations. Trials were usually conducted over seven or fourteen day test periods, excepting some of the initial trials run with *Spirodela oligorrhiza*. These time periods were chosen to reflect environmental relevance, since *Cylindrospermopsis* blooms in the natural environment may last from several days to several months. Also, since earlier studies have shown that bioaccumulation of CYN

can occur in as little as two days (Saker *et al.* 2004), one and two-week incubation periods were considered suitable for investigations of bioaccumulation. CYN test concentrations were chosen to reflect naturally occurring concentrations from blooms in Queensland (Baker, 1998; McGregor & Fabbro, 2000).

3.2.2 Preparation and cleaning of test chambers and equipment

Test chambers for all trials were thoroughly washed prior to use: chambers were placed through one dishwasher cycle with Deconex laboratory detergent, followed by a further cycle containing no detergent, and then rinsed using the relevant control water for each trial. After use, test chambers were rinsed with bleach to remove any CYN prior to dishwashing for the next trial. Some chambers also required scrubbing by hand to remove algal material. Additional glassware used in the preparation of treatment solutions and renewal (for example, measuring cylinders and beakers) was immersed in 5% bleach solution overnight, before being thoroughly washed by hand (using dishwashing detergent) and rinsed in tap and diluent water prior to next use.

3.2.3 Measurement of physical and chemical parameters

Disturbance of test organisms was minimal during water quality analyses; analyses were performed on solutions after organisms were transferred into fresh treatments. In all trials, the following measurements were taken using calibrated equipment: photosynthetically active radiation (LiCor LI-250 meter fitted with Quantum Sensor); conductivity (TPS LC84) and pH (TPS LC80A or TPS 80A). In trials involving animals, dissolved oxygen (TPS WP-82Y) and total ammonia concentrations (Aquasonic or Aquarium Pharmaceuticals Inc., freshwater total ammonia salicylate test kits) were also measured. Ambient air (plant trials) or water (animal trials)

temperatures were monitored at hourly or half-hourly intervals using Hastings StowAway Tidbits; reported values are the average of two or three data loggers.

3.3 Analysis of free (non-bound) cylindrospermopsin

Presently, CYN that is bound or metabolically modified within plant or animal tissues cannot be detected. All reported CYN values relate solely to CYN as judged against pure standard material (non-bound, free toxin), hence, total toxin concentrations may have been underestimated.

3.3.1 Culture and test solution samples

Samples were frozen for transport. Analyses were conducted at Queensland Health Scientific Services (QHSS; Kessels Rd, Coopers Plains) using HPLC/tandem mass spectrometry (full method provided in Appendix B).

3.3.2 Plant and animal tissues

Plant and animal tissues were partially prepared at CQU prior to toxin analyses by QHSS. Freeze-drying was done using a Virtis Sentry freeze-drier with Alcatel vacuum pump; the time interval required to completely dry samples was checked by freezing dummy samples for each test organism until stable weights were reached (< 1% change). Typically, the time required was 24 – 48 h, depending on initial sample biomass. The sample containers (plastic tissue culture tubes or centrifuge tubes) used during freeze-drying were also checked to ensure these did not change weight due to being processed (< 0.3% and < 0.1% change, respectively, over 24 h). Tissue homogenisation was conducted by an Ultra Turrax running at 24,000 rpm for

one minute. Analysis of free CYN was conducted at QHSS using HPLC/tandem mass spectrometry (full method in Appendix C).

3.4 Analysis of deoxy-cylindrospermopsin

A set of standards for deoxy-CYN was developed in late 2004. Hence, deoxy-CYN values (both in culture and in tissues) are available only for trials run after this date (selected *Hydrilla verticillata*, *Melanoides tuberculata* and *Bufo marinus* trials). Again, it should be noted that only free (non-bound or not metabolically changed) deoxy-CYN concentrations were detected via the HPLC method. Methods for deoxy-CYN detection in culture, water and tissue samples are provided alongside the CYN techniques in Appendices B and C.

3.5 Statistical methods and graphing

All statistical analyses were carried out in SPSS v11.5.0 or v12.0.1. Results from ecotoxicity trials were examined using univariate, repeated measures (RM) or multivariate analyses of variances (ANOVAs) using $\alpha = 0.05$. These analyses tested for significant effects of ‘treatment’ (toxin exposure concentration), ‘time’ (exposure time) and ‘interaction’ (treatment and time combined).

Prior to conducting tests in the ANOVA family (ANOVA, RM ANOVA, MANOVA), normal distributions of samples were checked by observing placement of data on P-P plots (Quinn & Keough 2002). Rather than the use of Levene’s test, which may be too sensitive in the case of smaller sample sizes (Underwood 1997), homogeneity of variances was assumed where the ratio of the largest to smallest variance was not greater than 4:1, since ANOVAs are relatively robust to

heterogeneous datasets (Quinn & Keough 2002). If possible, datasets were transformed to achieve homogeneity.

In cases where variances were heterogeneous (ratios >4) and could not suitably be transformed, ANOVAs were still conducted, but the $\alpha = 0.01$ significance level was adopted. Since heterogeneous variances contribute to a large Type 1 error rate, lowering of the significance level helped to ensure that significant outcomes, where they occurred, would still be reliable (Underwood 1981, p. 535). Non-significant outcomes of ANOVAs are not affected by variance heterogeneity.

A posteriori tests (multiple pairwise comparisons) were used to identify significant differences between treatment groups (CYN exposure concentrations). These included Tukey tests, or, where equal variances could not be assumed, Dunnett's T3 test based on the Studentized maximum modulus using $\alpha = 0.01$. Dunnett's test is capable of adequately controlling Type 1 error rates even when run under heterogeneous variances and for smaller sample sizes (Hsiung & Olejnik 1994 in Kromrey & La Rocca 1995; Quinn & Keough 2002).

Dendograms and multidimensional scaling plots were generated in Primer v5.0. Regression equations were calculated using SigmaStat v3.0. All other graphing was done using SigmaPlot v.8.0.

3.6 A note regarding the use of live cultures and freeze-thawed whole cell extracts

The presence of both deoxy-CYN and other cellular substances (such as lipopolysaccharides) within *C. raciborskii* cultures represents an important problem

regarding toxicity tests. To maximise environmental relevance, all trial work conducted for this thesis used whole-cell extracts or live *C. raciborskii* cultures, rather than purified toxin standards. However, this means that any adverse effects of exposure cannot be solely ascribed entirely to CYN. In the interests of accuracy, therefore, most of the conclusions reached regarding the toxicity of CYN are done so in the knowledge that the contribution of other substances (such as LPSs or undescribed toxins) cannot be discounted, particularly if synergistic or additive effects are possible.

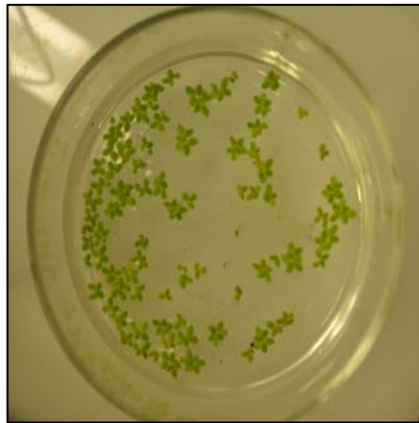
Whole cell extracts (crude extracts) of *C. raciborskii* were prepared by repeated freeze-thawing of live *C. raciborskii* cultures. Although other techniques such as sonication are used in studies of other cyanoprokaryotes, it has been found that freeze-thawing is effective in breaking open the cells of *C. raciborskii* (Geoff Eaglesham, pers. comm.). This may be due to the fact that *C. raciborskii* trichomes are not encapsulated by mucilage, which makes cell lysis difficult in some other species (such as colonies of *Microcystis*).

SECTION II

Bioconcentration risk and toxicity in aquatic macrophytes



Hydrilla verticillata



Spirodela oligorrhiza



Experimental setup for plant trials

General introduction to section II

Toxic effects of CYN exposure have not yet been described in aquatic macrophytes. However, some modes of CYN toxicity are applicable to plant cells, including inhibition of protein synthesis (Terao *et al.* 1994), inhibition of glutathione synthesis (Runnegar *et al.* 1995; Runnegar *et al.* 1994), and possibly genotoxicity (Shen *et al.* 2002; Humpage *et al.* 2000). Cylindrospermopsin toxicity has already been demonstrated in some terrestrial species (Metcalf *et al.* 2004; Vasas *et al.* 2002); several studies have also demonstrated cyanotoxic effects in aquatic plants, but these have been restricted to microcystin exposures. Thus, it is expected that CYN exposure may result in adverse effects in aquatic macrophytes.

Bioconcentration of CYN in aquatic plants also remains unstudied. Aquatic macrophytes experience continuous exposure risk during toxic algal blooms: they cannot move to avoid toxicity, and dissolved toxins can diffuse into almost all macrophyte habitats (those where floating, submerged or emergent plants are found). Furthermore, as many aquatic plants are structurally simple organisms, they may not have well-developed mechanisms to resist or minimise toxin accumulation.

Toxin deposition into plant cells ultimately relies on a viable uptake route, including passive or active transport into the cell. As plants do not ‘ingest’ live cyanoprokaryote cells, extracellular toxins are the only toxin fraction associated with uptake risk. This is why toxin accumulation into plant tissues represents ‘bioconcentration’ as opposed to ‘bioaccumulation’ (see section 1.4, p. 17).

However, as CYN from cultures and natural blooms is typically represented by high proportions of extracellular, rather than intracellular, toxin (Griffiths & Saker 2003; Hawkins *et al.* 2001; Carson 2000), CYN exposure may represent a significant bioconcentration risk for aquatic plants.

Chapters four and five examine CYN exposure and bioconcentration in two aquatic macrophytes: the floating duckweed, *Spirodela oligorrhiza*; and the submerged, rooted water thyme, *Hydrilla verticillata*. Both species could reasonably be expected to experience toxic blue-green algal blooms in their natural habitat. Chapter six evaluates the results of the two earlier chapters in the context of the environmental implications of *C. raciborskii* exposure in aquatic macrophytes.

Chapter four: ecotoxicity and bioconcentration risk in *Spirodela oligorrhiza*

4.1 Introduction

Spirodela oligorrhiza (Kurz) Hegelm is a native Australian floating aquatic plant commonly found in waterways throughout central Queensland. The species is characterised by elliptic leaves usually a few millimetres to one centimetre long, with two to five fronds per plant (Aston 1973; Sainty & Jacobs 1994). *Spirodela* is important to aquatic food webs, representing a significant food source for many animals. Along with the closely-related *Lemna* sp., *Spirodela* has been recognised as a useful bioassay and monitoring organism (Smith & Kwan 1989; Frankart *et al.* 2002), primarily because it is a structurally simple vascular plant that reproduces quickly (Wang 1990). The following trials investigated relative growth rates, chlorophyll content, plant abnormalities and bioconcentration risk in *S. oligorrhiza* during exposure to *C. raciborskii* whole cell extracts containing 0 – 500 µg L⁻¹ extracellular CYN (CYN_{EXC}). The development of suitable test methods for CYN and *Spirodela* was also addressed.

4.2 Methods

4.2.1 Culture of test organisms

S. oligorrhiza plants were obtained from an aquaculture pond at Central Queensland University (CQU) and cultured for several weeks prior to experimentation. Plants were grown in shallow plastic dishes under a continuous light source, using a gently aerated growth medium of 'Thrive' fertiliser and distilled water. Plants were rinsed well with tap water prior to use to minimise nuisance algal growth. Species

identification was conducted using Aston (1973). Plants selected for experimental use were always healthy, rapidly growing and visually free from contaminants.

Cylindrospermopsin was sourced from mass cultures of toxic *C. raciborskii*. For each trial, several 500 mL or 1 L cultures of *C. raciborskii* were pooled in a five litre conical flask, mixed well and then decanted into smaller storage containers. In trials one to four (see below), one randomly-chosen subsample was despatched to Queensland Health Scientific Services (QHSS) for CYN determination, and this value was used in all subsequent calculations for preparation of test concentrations. In trials five to seven, the source culture toxin concentration was obtained from the average of duplicate subsamples. Cultures were stored frozen prior to use to ensure toxin was in the extracellular (dissolved) form (CYN_{EXC}).

4.2.2 Trials to determine bioaccumulation and ecotoxicity

One range-finding (pilot) trial was conducted, followed by three full-scale trials. Trials were conducted under near-constant temperature conditions and continuous photoperiod (approximately 40 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$; Osram Lumilux cool white fluorescent tubes). Continuous photoperiod conditions do not reflect the natural light regime for *S. oligorrhiza*, but are recommended by both OECD Draft Guidelines for *Lemna* (OECD 2002) and the United States Environmental Protection Agency (EPA) test guidelines for aquatic plant toxicity tests (USEPA 1996). Conductivity and pH were monitored at plant harvest intervals; temperature was measured continuously throughout all trials. Elevated pH values are common in naturally occurring *C. raciborskii* blooms (McGregor & Fabbro 2000; Briand *et al.* 2002); thus, to maximise environmental relevance, no attempt was made to buffer pH during trials.

Test solutions (whole cell extracts of *C. raciborskii*) were prepared by thawing frozen *C. raciborskii* cultures of known toxin concentration and diluting to the desired CYN concentration using autoclaved ASM1 media (for recipe, see Appendix A). Controls ($0 \mu\text{g L}^{-1}$) were prepared with ASM1 media only. Dispersing agents or solvents were not required since CYN is highly water soluble (Sivonen & Jones 1999). Treatments were always assigned randomly to dishes; these were repositioned daily following inspection for frond counts. Test vessels were glass crystallisation dishes covered with watch glasses to minimise evaporation. Test solutions (150 mL) and controls were replaced by half (75 mL) at three-day intervals throughout all trials to ensure toxin concentrations remained at the nominal values.

Test procedure - trial one (pilot trial)

A range-finding test was conducted using nominal concentrations of 0, 5, 10, 50 and $100 \mu\text{g L}^{-1}$ CYN_{EXC} (administered as *C. raciborskii* whole cell extract) and a 21-day exposure period. Nutrient limitation in the duckweed was considered unlikely to occur over this period, since the ASM1 culturing media was nutrient enriched and replenished regularly. Treatments were prepared with nine replicates ($n = 45$). On day zero of the trial, *S. oligorrhiza* plants were added to each test vessel to obtain approximately one-third dish coverage.

Total frond area (percentage coverage of the test dishes) of *S. oligorrhiza* was calculated at two- to three-day intervals throughout the trial, by individually photographing dishes (Nikon Coolpix 995) and digitising the image, as recommended by the OECD guidelines (OECD 2002). Images were kept to a consistent size by shooting overhead photographs from a set tripod with preset zoom magnification.

The percent (%) coverage of plants was calculated in Jasc PaintShop Pro v7: this was achieved by selecting fronds with the ‘magic wand’ tool, cropping to the selection and decreasing the colour depth to a black and white image. Next, a histogram was generated to indicate the total number of black pixels (fronds). This value was compared with total pixel number to obtain percentage coverage. This method was validated prior to the experiment; variability in frond selection was shown to produce, at maximum, 0.5% variation in pixel output. Relative growth rate (RGR) of *S. oligorrhiza* based on increase in percentage cover was then calculated using the formula of Hunt (1990). The photographs also allowed a detailed study of any deformations in daughter fronds and chlorosis or necrosis.

Three replicates per treatment were harvested on days seven, fourteen and twenty-one. These tissues were pooled within treatments, thoroughly rinsed by sieving with 250 mL distilled water, blotted dry on paper towel and weighed. The tissues were then halved for use in analysis of chlorophyll content and CYN concentration. Two test solutions were randomly chosen and sent to QHSS for CYN determination using HPLC/MS-MS (limit of detection $0.2 \mu\text{g L}^{-1}$). Full details of this method are provided in Appendix B.

Test procedure - trials two to four

Three trials (trials two, three and four) investigated nominal concentrations of 0, 100, 250, 350 and $500 \mu\text{g L}^{-1}$ CYN_{EXC} (as *C. raciborskii* whole cell extracts) over a twelve-day exposure period. Each treatment concentration was prepared with six replicates ($n = 30$). In contrast to the pilot trial, 25 fronds (single or multiple-frond plants) were used to begin each of the trials. This is higher than the frond number recommended

for use in the OECD protocol for *Lemna* inhibition tests (OECD 2002), however this quantity was necessary to ensure that adequate biomass was available for chlorophyll and toxin analyses. Frond percentage coverage was recorded at two-day intervals and calculated as described above.

The number of fronds per dish was also recorded daily (distinct fronds as distinguished with the naked eye, using the same assessor throughout all trials). Separate RGR were calculated from both the percentage cover and frond count data, using the formula of Hunt (1990). Inclusion of frond count and total frond area (percentage cover analyses) data is considered the most effective combination for measuring sensitivity in duckweed, especially where only small effects are present (Eberuis *et al.* 2002). Three replicates per treatment were harvested on days six and twelve, and the tissues used for chlorophyll analyses (trial two) or CYN determinations (trials three, four). Two reserved test solutions were randomly chosen and sent to QHSS for CYN determination.

4.2.3 Trials for ecotoxicity using revised methods

The response of *Spirodela* in trials one to four was highly variable. Moreover, these trials appeared to be invalid since plants in control dishes recorded slow doubling times (> 2.5 days), and because CYN and pH values in the test solutions were not satisfactory (see below). Hence, three further semi-static ecotoxicity trials (hereafter, trials five, six and seven) were conducted using a revised test method. Test procedures were changed to achieve suitable growth of control plants, and to minimise problems of pH change and the loss of CYN from the test solutions. Wherever possible, these were done in accordance with the OECD guidelines for

testing of *Lemna* sp. (OECD 2002), and/or the USEPA guidelines for aquatic plant toxicity using *Lemna* (USEPA 1996).

Plants were obtained from a CQU mesocosm approximately six weeks prior to use, and cultured aseptically under continuous light at $110 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Osram Lumilux cool white fluorescent tubes). Plants were rinsed with a weak sodium hypochlorite solution periodically to prevent contamination. The nutrient media was modified Hoagland's Basal Salt Solution (Australtec Pty Ltd, Australia), prepared by dissolving 1.63 g powder in one litre of distilled water, and buffering to pH 6.8 using 1M NaOH. This pH is slightly higher than that usually used for growing *Lemna* in modified Hoagland's solution (normally, 4.8 – 5.2 (USEPA 1996)), but achieved best growth of the *Spirodela*. It also better represented the natural environment of duckweed (Riethmuller *et al.* 2003). Media was prepared two days in advance to allow the pH to stabilise and refrigerated for subsequent use. Cultures were subcultured approximately twice weekly, to minimise contaminants and maintain exponential growth. The culture was deemed healthy since there was a high incidence of multiple-frond plants and no signs of leaf discolouration. Growth rates of the inoculum culture were checked periodically; the doubling time was always < 2.5 days.

Trials lasted for seven days each. Test dishes were the same as those used in the earlier tests (trials one to four). Since growth stimulation had been recorded from plants exposed to treatments containing $100 \mu\text{g L}^{-1}$ CYN in earlier trials (see results, Table 4.4), these trials examined the lowest dose required to prompt a stimulatory response in the *Spirodela*. Nominal test concentrations were reduced to $100 \mu\text{g L}^{-1}$ or less, and spaced more closely than those of trials two to four, to investigate more closely the response(s) of *Spirodela*. Treatment solutions (150 mL) were prepared by

diluting a freeze-thawed culture of *C. raciborskii* to 0, 7.5, 15, 30, 60 and 120 $\mu\text{g CYN L}^{-1}$ using modified Hoagland's Basal Salt Solution. Each test concentration was prepared using five replicates (total $n = 30$). The growth media was buffered to pH 6.8 prior to the addition of the cell extracts; however, test solutions were not buffered after the addition of *C. raciborskii* cell extract, nor during the trial, since this would have reduced environmental relevance (see 4.2.2). Furthermore, according to the USEPA guidelines, changes in test solution pH after the addition of the test chemicals should be recorded, but not adjusted (USEPA 1996). The pH of each batch of fresh solutions, and the spent treatment solutions, were recorded at two-day intervals throughout the trials.

Test solutions (150 mL, including controls) were renewed at two-day intervals using freshly prepared media with *C. raciborskii* cell extract added. Here, test dishes were washed with tap water and rinsed in nutrient media to minimise nuisance green algal growth, prior to being refilled with the test solutions. Toxin analyses (CYN and deoxy-CYN) were conducted on selected treatment preparations on day zero and on all treatments (pooled samples of five replicates) on days four and seven. In one treatment per trial, five replicates were analysed for CYN separately to indicate the variability between replicates.

On day zero, ten healthy fronds of *S. oligorrhiza* were added to each dish, using two, three or four-frond plants. The dishes were randomly placed on a laboratory bench and were repositioned daily, to avoid differences in light intensity on the bench. Trials were run under continuous light at $110 \mu\text{mol photon m}^{-2}\text{s}^{-1}$; temperature was monitored in two surrogate test vessels containing tap water using Hastings Stowaway Tidbit Data Loggers.

Frond counts were conducted daily as for trials one to four. Plants were also examined for any signs of necrosis, chlorosis, gibbosity, loss of buoyancy, colony breakup or other changes to frond morphology. Plants were harvested at the end of each trial (day seven), dried on paper towel and weighed (fresh biomass). The samples were then stored frozen in the dark (< 3 days) before being analysed for chlorophyll content, as described in 4.2.4.

Relative growth rates and doubling times for the seven-day period were calculated from the frond count data. The tests were considered acceptable if the doubling times of control plants were < 2.5 days, regardless of incubation day (OECD 2002). The percentage change in frond numbers (growth inhibition or growth stimulation) was calculated by subtracting the average control frond number ($n = 5$) from the fronds recorded in each treatment dish. Fresh weights recorded on day seven were also compared in this way.

4.2.4 Chlorophyll analyses

Chlorophyll samples (trials one, two, five, six and seven) were pooled within treatments to obtain sufficient tissue mass. In trial one, a high amount of plant biomass was available due to higher initial percent coverage, so treatments were halved prior to analysis ($n = 2$ for each treatment). Determinations were carried out in the dark using spectrophotometric determination (Hitachi U-2000) of chlorophyll *a* and *b*, using the standard method of the U.S. Environmental Protection Agency (USEPA 1994), modified using 80% chilled acetone extraction and the chlorophyll calculation equations described in Inskeep and Bloom (1985). All measurements were taken in duplicate and the average value used.

4.2.5 Plant toxin analyses

Toxin analyses were carried out on tissue samples from trials three and four only. Fresh *S. oligorrhiza* tissues were weighed ($n = 3$ for each treatment), frozen, freeze-dried and despatched to QHSS. There, samples were frozen, thawed and homogenised with 5mL HPLC grade water for one minute. The samples were then filtered (0.45 micron, Millex-HV) and analysed for free (non-bound) CYN only, using HPLC/Electrospray/tandem Mass Spectrometry (limit of detection, $0.2 \mu\text{g L}^{-1}$; or 1.0 nanogram per 5mL tube) (Eaglesham *et al.* 1999; Norris *et al.* 1999). Spike recoveries for tubes of *Spirodela* averaged 84.4% for CYN ($n = 5$). Full details of the extraction and detection methods are provided in Appendix C.

4.2.6 Toxin adsorption to plant cell walls

A short trial was conducted to determine whether free CYN in tissues was represented by toxin binding (= adsorption) to the cell wall, rather than intracellular uptake (= bioconcentration). Plants were exposed to $250 \mu\text{g L}^{-1}$ CYN_{EXC} as *C. raciborskii* whole cell extract for seven days under the same test conditions as trials two to four. On day zero, approximately 40 fronds of *S. oligorrhiza* were added to each of five crystallisation dishes. After seven days, the plants in each dish were removed and subject to different rinsing treatments, as follows:

- (1) tissues sieved to remove treatment solution only;
- (2) tissues sieved to remove treatment solution, followed by washing of tissues on sieve using 250 mL of distilled water;
- (3) tissues sieved to remove treatment solution, followed by immersion of tissues in a glass beaker with 250 mL distilled water, agitating with a glass rod for approximately 30 seconds, then re-sieving to remove excess water;

- (4) as for (2) but using 250 mL of distilled water mixed with 1 mL of Tween 80 (a polyoxyethylene sorbitan monooleate wetting agent); or
- (5) as for (3) but using 250mL of distilled water mixed with 1mL of Tween 80.

Plant tissues were blotted dry with paper towel, weighed, freeze-dried, reweighed and transported to QHSS for analysis of free CYN content.

4.2.7 Sampling, mixing and analysis of CYN culture strength

Unusual results were obtained from the CYN decomposition trial (see 4.3.6), which prompted a further trial to check the suitability of *C. raciborskii* sampling and mixing procedures, and the reliability and accuracy of analytical procedures for CYN detection. *C. raciborskii* cultures were pooled in a five litre conical flask, mixed and decanted (as described in 4.2.1). A randomly chosen subsample was sent to QHSS for CYN analysis, and the remaining samples frozen. After approximately one week, these samples were thawed overnight in a fridge and remixed thoroughly, by pooling in a large glass tank and stirring with a metal spoon for at least fifteen minutes. Sample containers were filled by subsampling from the tank. Five of these samples were randomly chosen and analysed for CYN at QHSS.

4.2.8 Statistical analyses

Highly variable data were considered suitable for analysis only where the ratio of the largest to smallest variances was less than 4:1 (Quinn & Keough 2002). If this variance ratio was exceeded and datasets could not be adequately transformed, data were analysed using a lowered ($\alpha = 0.01$) significance level (Underwood 1981). For trials one to four, two-way repeated measures ANOVAs (SPSS for Windows v11.5.0)

were used to detect differences in *S. oligorrhiza* relative growth rates (frond coverage, frond number), with between-subjects effects referring to ‘nominal exposure concentration’ and within-subjects effects referring to ‘exposure time in days’. For trials five to seven, one-way repeated measures ANOVAs (SPSS for Windows v12.0.1) were used to analyse frond count and fresh biomass data.

Sphericity was checked using Mauchly’s test: where this value was significant, multivariate p values (Roy’s Largest Root) for time and interaction were reported instead of the Sphericity Assumed Statistic. Tukey tests (homogeneous data) or Dunnett’s T3 tests (heterogeneous data) were used to detect significant differences between nominal exposure concentrations. Within-subjects contrasts examined changes in exposure time. Values for chlorophyll a , b , total chlorophyll and the ratio of chlorophyll $a:b$ present in *Spirodela* were analysed using MANOVAs; Post-hoc Tukey tests ($\alpha = 0.05$) were used to identify significantly different treatments.

4.3 Results

4.3.1 Water quality and measured CYN concentrations

Water quality was similar across trials one to four (Table 4.1). Large differences in treatment pH were recorded throughout these trials, particularly in high CYN-containing treatments, and towards the end of the incubation periods. In contrast, water quality remained constant throughout trials five to seven, including within and between treatments (Table 4.1). The conductivity was far higher in trials five to seven compared with the earlier trials, ranging from 1,420 – 1,961 $\mu\text{S cm}^{-1}$.

Table 4.1 Test conditions and water quality characteristics measured during trials one to seven. Water quality values are as recorded on harvest days.

Trial number	Photoperiod	Temperature (°C)	pH	Conductivity (µS cm ⁻¹)
Trial 1 (pilot)	Continuous	29 ± 2°C		
Control			8.0 – 9.9	250 – 350
5 µg L ⁻¹			7.8 – 10.0	260 – 300
10 µg L ⁻¹			8.2 – 10.0	280 – 300
50 µg L ⁻¹			8.0 – 9.7	240 – 320
100 µg L ⁻¹			7.8 – 10.5	250 – 350
Trial 2	Continuous	29 ± 2°C		
Control			7.9 – 9.7	220 – 240
100 µg L ⁻¹			8.2 – 10.0	230 – 290
250 µg L ⁻¹			8.0 – 9.4	250 – 300
350 µg L ⁻¹			8.00 – 9.9	240 – 300
500 µg L ⁻¹			8.1 – 9.6	250 – 500
Trial 3	Continuous	27 ± 1°C		
Control			8.9 – 10.0	280 – 330
100 µg L ⁻¹			9.1 – 10.3	290 – 330
250 µg L ⁻¹			8.5 – 10.3	290 – 350
350 µg L ⁻¹			8.5 – 10.6	300 – 360
500 µg L ⁻¹			8.9 – 10.0	330 – 390
Trial 4	Continuous	27 ± 1°C		
Control			8.7 – 10.8	390 – 470
100 µg L ⁻¹			8.6 – 10.8	350 – 390
250 µg L ⁻¹			8.8 – 10.3	310 – 450
350 µg L ⁻¹			8.4 – 10.3	260 – 400
500 µg L ⁻¹			9.0 – 10.5	310 – 390
Trial 5 ^a	Continuous	26 ± 2°C		
Control			6.1 – 6.8	1694 – 1790
7.5 µg L ⁻¹			5.8 – 6.8	1700 – 1790
15 µg L ⁻¹			5.3 – 6.8	1705 – 1740
30 µg L ⁻¹			6.3 – 6.8	1700 – 1730
60 µg L ⁻¹			6.2 – 6.9	1656 – 1675
120 µg L ⁻¹			6.3 – 7.0	1525 – 1650
Trial 6 ^a	Continuous	26 ± 2°C		
Control			5.7 – 6.9	1730 – 1950
7.5 µg L ⁻¹			6.2 – 7.0	1801 – 1926
15 µg L ⁻¹			6.5 – 7.0	1770 – 1895
30 µg L ⁻¹			6.5 – 7.0	1730 – 1850
60 µg L ⁻¹			6.6 – 7.0	1660 – 1870
120 µg L ⁻¹			6.7 – 7.2	1420 – 1650
Trial 7 ^a	Continuous	27 ± 3°C		
Control			6.2 – 6.9	1815 – 1961
7.5 µg L ⁻¹			6.3 – 6.9	1810 – 1934
15 µg L ⁻¹			6.3 – 6.9	1801 – 1914
30 µg L ⁻¹			6.4 – 6.9	1772 – 1892
60 µg L ⁻¹			6.4 – 6.9	1726 – 1825
120 µg L ⁻¹			6.9 – 7.1	1606 – 1688

^a using revised experimental method, including the use of modified Hoagland's solution as a diluent and increased frequency of test solution renewal.

Large differences were recorded between nominal and measured CYN concentrations in trials one to four. Some treatments contained as little as 38% of the nominal concentrations, regardless of the time since the last test solution renewal had occurred (Table 4.2). However, CYN loss was not consistent, since some treatments had up to 90% of the toxin remaining (Table 4.2).

Measured CYN concentrations were consistent across trials five to seven (Figure 4.1); based on averages of measured samples, actual test concentrations were:

trial five: 0, 7, 13, 27, 57 and 113 $\mu\text{g L}^{-1}$ CYN_{EXC};

trial six: 0, 8, 13, 30, 50 and 110 $\mu\text{g L}^{-1}$ CYN_{EXC}; and

trial seven: 0, 8, 13, 28, 52 and 117 $\mu\text{g L}^{-1}$ CYN_{EXC}.

Deoxy-CYN concentrations were always less than 10 $\mu\text{g L}^{-1}$ (Figure 4.1). Within-replicate variability was low, with a standard error of 0.07, 0.63 and 1.81 recorded from the 50, 100 and 200 $\mu\text{g L}^{-1}$ CYN treatments in trials five, six and seven, respectively. Results for trials five to seven have been presented using these (measured) test concentrations. However, nominal test concentrations are still reported for trials one to four, since there were not enough samples taken to confidently determine the actual test concentrations.

Table 4.2 Measured CYN concentrations of randomly chosen treatment solutions sampled at completion of trials one to four. Values indicate CYN concentration in three pooled replicates. NA = not applicable.

Trial Number / Type	Days since solution replacement	Nominal concentration ($\mu\text{g L}^{-1}$ CYN _{Exc})	Actual concentration ($\mu\text{g L}^{-1}$ CYN)	Percent (%) remaining
1 (Pilot)	1	100	54	54
1 (Pilot)	1	100	90	90
2	3	350	150	43
2	3	500	220	44
3	3	250	95	38
3	3	350	230	66
4	3	350	289	83
4	1	0 (control)	0.5	NA

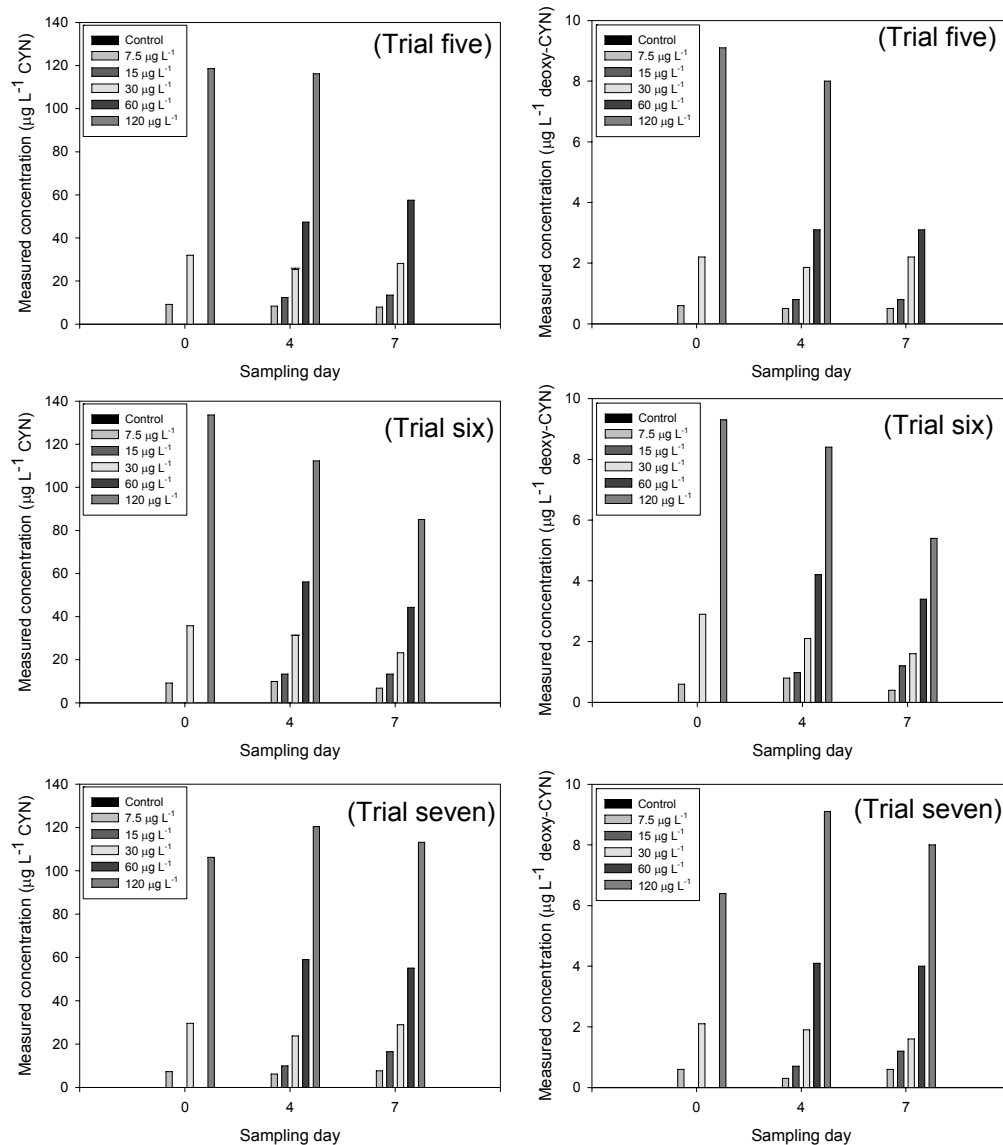


Figure 4.1 Cylindrospermopsin and deoxy-cylindrospermopsin concentrations in treatment solutions sampled during ecotoxicity trials five to seven. Legends indicate nominal test concentrations for cylindrospermopsin. The 400 $\mu\text{g L}^{-1}$ sample collected on day seven in trial five was damaged and has not been shown.

4.3.2 Plant health and relative growth rates

4.3.2.1 Plant growth and changes in frond morphology and colour

Growth of *Spirodela* was highly variable between trials. In trials one to four, plants in control dishes failed to achieve exponential growth after the first four to seven days of incubation (Figure 4.2A - C). In contrast, *Spirodela* grew rapidly throughout trials five to seven, with many dishes numbering at least 250 fronds by the end of the incubation period (Figure 4.2 D – F). This corresponded with doubling times of 1.8 ± 0.16 ; 1.6 ± 0.08 and 1.7 ± 0.13 , for control plants in trials five, six and seven, respectively, which validated the test method (OECD 2002).

Frond chlorosis or necrosis was not recorded in any treatment during trial one. Due to rapid growth of the plants, most test dishes became overcrowded after day seven, causing some plants to become partly submerged. Replenishment of test solutions was also made difficult without accidentally removing small fronds. Consequently, the initial (day zero) percentage cover was reduced to 25 fronds per dish (< 10% coverage) for trials two to four.

Clumping of *S. oligorrhiza* plants occurred in treatments containing high concentrations of *C. raciborskii* and toxin (250, 350 and 500 $\mu\text{g L}^{-1}$ CYN_{EXC}) during trial two, whereas control plants were separated into flat, discrete two- and three-frond units (Plates 4.1, 4.2). Clumping occurred early in high CYN-treatments, although some 100 $\mu\text{g L}^{-1}$ treatments also showed this effect later (day twelve). In some cases, daughter fronds of ‘clumped’ plants senesced and decomposed, forming a dark green-brown scum. Obvious frond deformities were not observed in any treatment, although shortened

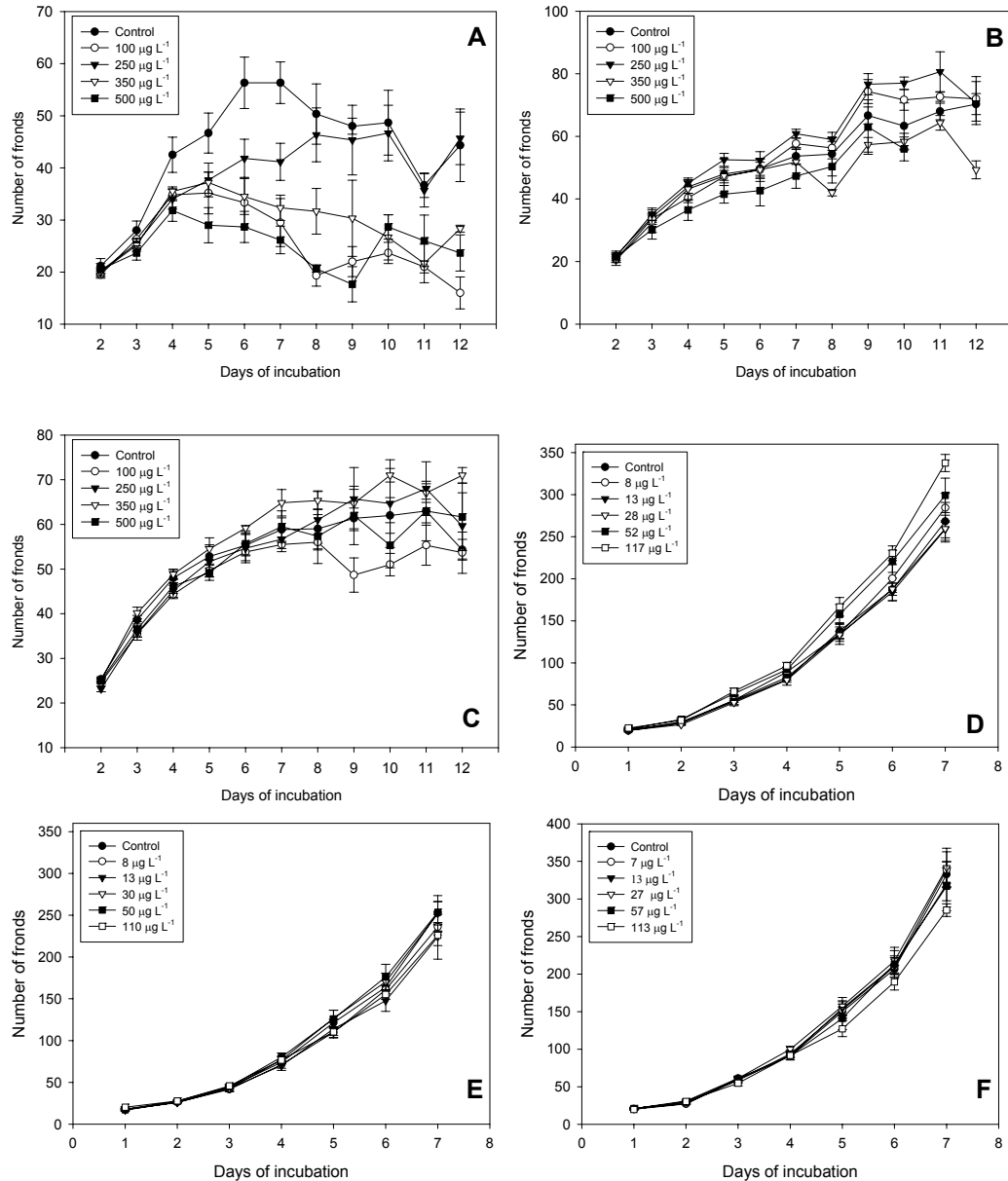


Figure 4.2 Number of *Spirodela oligorrhiza* fronds recorded during incubation with *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin. (A) trial two, (B) trial three, (C) trial four, (D) trial five, (E) trial six and (F) trial seven. Graphs indicate average values ($n = 6$ or 3 , depending on day of incubation) \pm standard error. (Trial one not shown since frond count data not collected).

S. oligorrhiza root lengths were observed in treatments containing high CYN and *C. raciborskii*. The root lengths of plants in controls (approximately 20 – 25 mm) were almost double those in the 250, 350 and 500 $\mu\text{g L}^{-1}$ treatments (10 – 15 mm).

Senescence and necrosis of fronds was dramatic in trial three; but was again restricted to solutions with high CYN (and algal debris) concentrations. Plants succumbed by initially turning black or brown around the outer frond margins; later, entire fronds were affected. A light brown scum and air bubbles were also observed near dead and dying plants (Plates 4.3, 4.4). Pale and necrotic fronds appeared in low-concentration (100 $\mu\text{g L}^{-1}$) and control dishes; however, this occurred only by day fourteen, and affected fewer fronds to a lesser extent compared with toxin-exposed plants. Plants in trial four responded similarly to those in trial two: clumps and decomposing scums were noted in dishes containing *C. raciborskii* and CYN treatments.

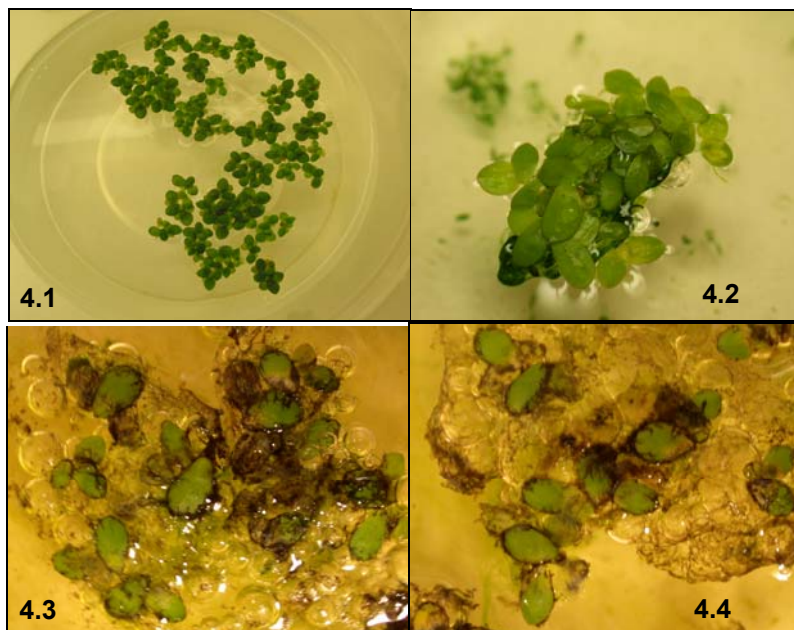


Plate 4.1 *Spirodela oligorrhiza* in a control dish from trial five. **Plate 4.2** Clumping of *S. oligorrhiza* during trial two (250 $\mu\text{g L}^{-1}$ treatment, day eleven). **Plates 4.3 & 4.4** Necrotic and decomposing fronds in *Spirodela oligorrhiza* during trial three. Treatments are 250 and 350 $\mu\text{g L}^{-1}$ extracellular cylindrospermopsin (nominal value), respectively, on day twelve.

Plant abnormalities, leaf discolouration or other signs of stressed plants were not present in any treatment in trial five. In trial six, some plants in the control dishes appeared to have smaller fronds than the toxin-exposed plants, but this was not consistent across all dishes, nor on each incubation day. In trial seven, approximately ten of the thirty test dishes featured plants with yellowed daughter fronds mid-way through the incubation. However, these were not restricted to a certain treatment and the discolouration generally disappeared within two days.

4.3.2.2 Frond percentage coverage

Rapid growth of plants during the first week of the pilot trial caused overcrowding, which rendered percentage coverage analyses impossible. Consequently, analyses were not conducted past day seven. Until this point, treatment (algal and toxin) exposure was associated with growth stimulation compared with the controls (Figure 4.3A). The plants in the 100 $\mu\text{g L}^{-1}$ treatments appeared to be stimulated the least.

In trials two to four, up to 80% inhibition and > 60% stimulation of plant growth was recorded in the treatments, compared with the control plants. *Spirodela* growth was quite variable between trials. In trials two to four, growth inhibition occurred during the first few days of incubation, whereas growth stimulation occurred after incubation periods exceeding one week (Figure 4.3B – D). The strength of inhibition or stimulation depended on both the length of incubation and the CYN concentrations (and cellular debris) in the test solutions (Figure 4.3B – D). For example, in trial three, the first six days of incubation were associated with growth inhibition. However, this was mostly converted to growth stimulation for the last four days of the trial (Figure 4.3C). The exception was the 500 $\mu\text{g L}^{-1}$ treatment, which continued to experience growth inhibition (Figure 4.3C).

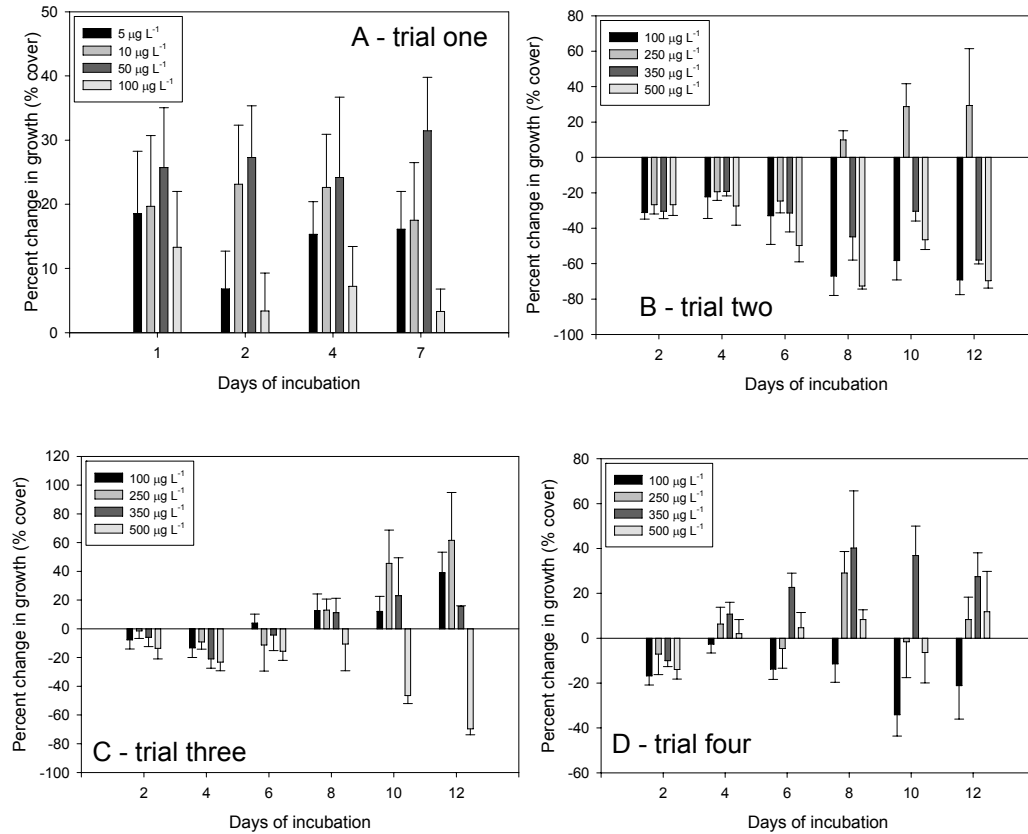


Figure 4.3 Percent (%) change in growth of *Spirodela oligorrhiza* exposed to *Cylindrospermopsis raciborskii* whole cell extracts containing extracellular toxin, based on percentage cover of fronds, compared with controls: (A) trial one, (B) trial two, (C) trial three, (D) trial four. Bars show average values ($n = 9, 6$ or 3 , depending on exposure time) \pm standard error.

Exposures to whole cell extracts containing CYN were also linked with changes in the RGRs of *Spirodela*. However, there did not appear to be a consistent pattern in the response of *Spirodela* to the test solutions. For example, in trial two, the effect of toxin concentrations on the RGR of *S. oligorrhiza* was time-dependent, with increasing exposure times, but not toxin concentrations, increasing the inhibition effect of toxin exposure (Figure 4.3, Table 4.3). In contrast, both increased exposure time and increased toxin concentrations were linked to decreased RGRs in the first six days of trial three (Figure 4.4). Significant effects of exposure concentrations, time or interactions were not detected in any trial during the second incubation phase ($p \leq 0.010$, Table 4.3).

Table 4.3 Summary results for repeated measures ANOVA of relative growth rate data based on percentage coverage of *Spirodela oligorrhiza*. NS = not significant ($p > 0.010$; heterogeneous datasets); NA = not applicable, percentage coverage analyses not conducted.

	First exposure interval ^a	Between-subjects contrasts ^a	Second exposure interval ^b
Trial one			
Mauchly's test for Sphericity	$p < 0.001$; $df=2$; $M=0.438$		
Treatment	NS	Day 2 / Day 4: $p = 0.002$; $F_{1,40}=11.358$	NA
Exposure time ^c	$p = 0.001$; $F_{2,39} = 7.843$		
Interaction (Time x Treatment) ^c	NS		
Trial two			
Mauchly's test for Sphericity	NS		NS
Treatment	NS	Day 4 / Day 6: $p = 0.012$; $F_{1,25}=7.298$	NS
Exposure time ^c	$p = 0.001$; $F_{2,50} = 4.640$		NS
Interaction (Time x Treatment) ^c	NS		NS
Trial three			
Mauchly's test for Sphericity	$p = 0.010$; $df=2$; $M=0.670$		NS
Treatment	$p = 0.000$; $F_{2,24} = 7.538$	Day 2 / Day 4: $p = 0.005$; $F_{1,24}=9.762$	NS
Exposure time ^c	$p < 0.001$; $F_{2,23} = 24.314$		NS
Interaction (Time x Treatment) ^c	$p = 0.001$; $F_{4,24} = 6.746$		NS
Trial four			
Mauchly's test for Sphericity	$p = 0.017$; $df=2$; $M=0.711$		NS
Treatment	NS	Day 4 / Day 6: $p = 0.000$; $F_{1,25}=39.437$	NS
Exposure time ^c	$p < 0.001$; $F_{2,24} = 30.098$		NS
Interaction (Time x Treatment) ^c	$p = 0.002$; $F_{4,25} = 5.629$		NS

^a seven day's exposure (trial 1) or six day's exposure (trials 2 – 4); ^b twelve day's exposure; ^c values from are Sphericity Assumed statistic; or, where Mauchly's test was significant, Roy's Largest Root Statistic.

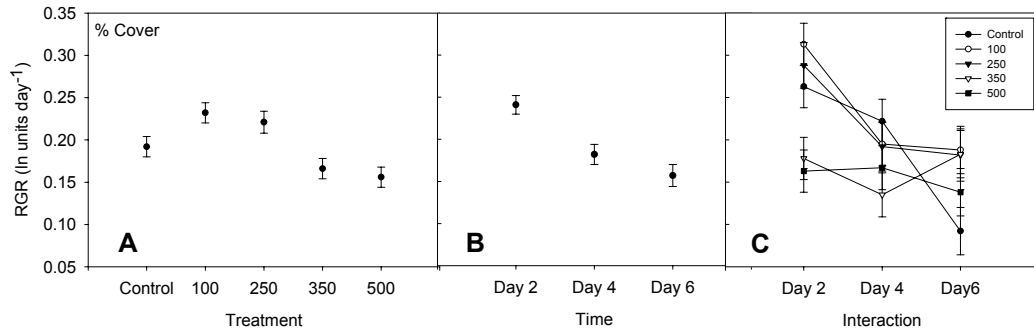


Figure 4.4 Example of *Spirodela oligorrhiza* relative growth rate data calculated from percentage cover analyses, prior to first harvest for trial three: (A) effect of toxin concentration; data pooled within treatment groups; (B) effect of exposure time, data pooled within groups; (C) interaction, showing separate data for each treatment / time combination. Graphs show means \pm standard error.

4.3.2.3 Frond counts

Frond count data generally agreed with patterns seen in the percentage coverage data for the same trials. For example, in trial two, most treated plants experienced reduced growth compared with the controls (Figure 4.5); this trend was also recorded for the percentage cover analyses. In trials three and four, most treatments experienced growth inhibition during early incubation periods. However, plant growth responses were variable after one week, recording both strong inhibition (trial three) and strong stimulation (trial four) (Figure 4.5).

Spirodela plants also recorded variable growth when exposed to *C. raciborskii* whole cell extracts using the revised test method. In trial five, exposure to the treatment solutions generally corresponded with growth stimulation, with the $117 \mu\text{g L}^{-1}$ CYN treatment recording 26% stimulation compared with the controls by the end of the seven day incubation period (Figure 4.5). Plants in some of the dishes containing medium or low-concentration test solutions ($8, 13, 28 \mu\text{g L}^{-1}$ CYN_{EXC}) also experienced up to 10% inhibition, depending on the time of incubation.

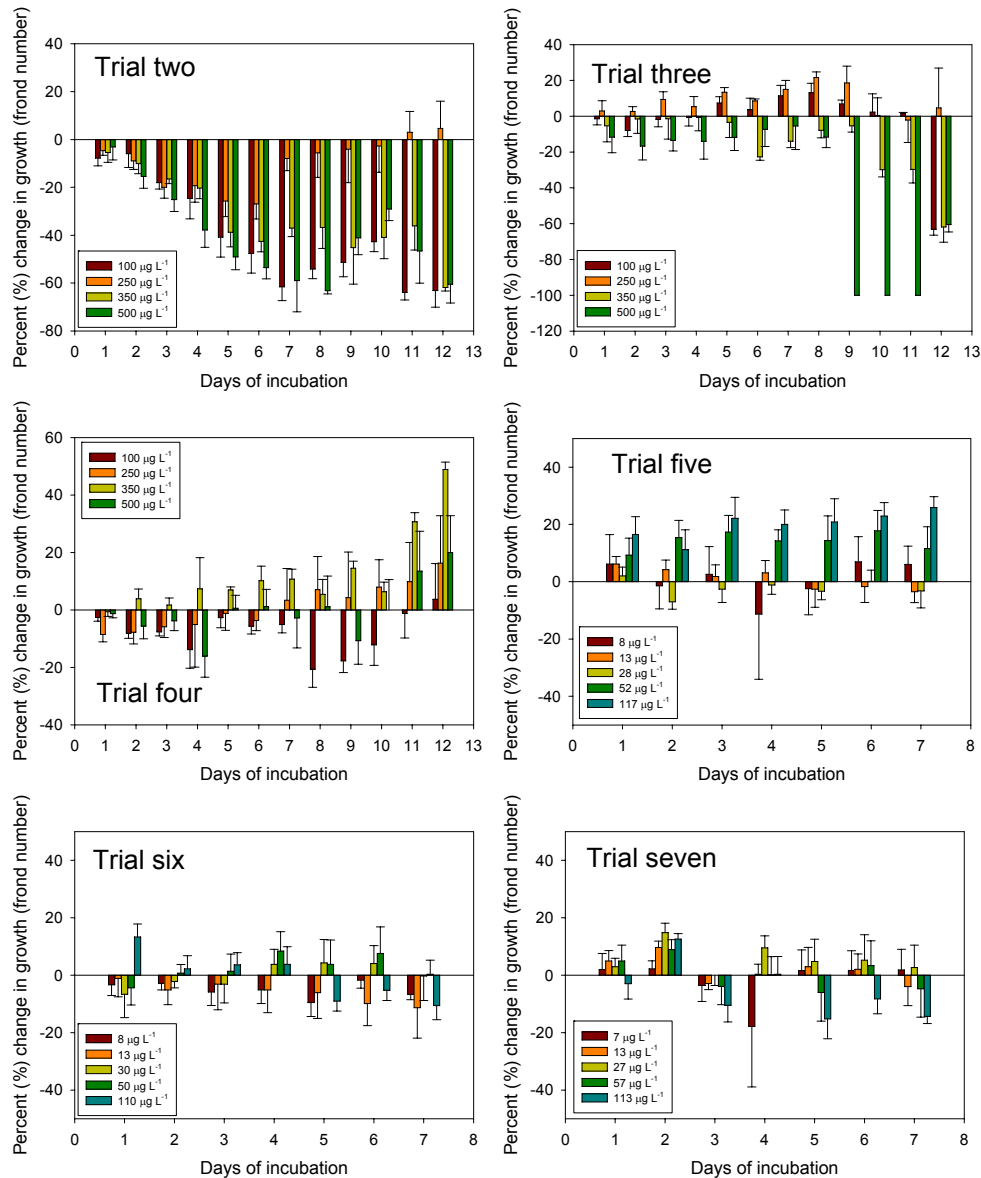


Figure 4.5 Percent (%) change in the frond numbers of *Spirodela oligorrhiza* plants exposed to *Cylindrospermopsis raciborskii* whole cell extract containing extracellular toxin, compared with controls. Bars show average values \pm standard error. Legends are nominal test concentrations (trials two to four) or measured concentrations (trials five to seven). Test methods vary between trials two to four compared with trials five to seven.

Spirodela growth in trials six and seven was different from the earlier trials.

Treatments with 30 – 60 $\mu\text{g L}^{-1}$ CYN recorded growth stimulation of approximately 5 – 15% compared with the controls, depending on the day of incubation (Figure 4.5). In contrast, 10 – 20% growth inhibition was recorded

from treatments containing the least (7 or 8 $\mu\text{g L}^{-1}$) or most (110 or 113 $\mu\text{g L}^{-1}$) toxin, particularly towards the end of the incubation periods (Figure 4.5).

Toxin exposure concentration significantly affected the RGRs of *Spirodela* (based on frond counts) in trials two and three, but this was overshadowed by an interaction effect in trial two. Control or low toxin treatments ($\leq 250 \mu\text{g L}^{-1}$) recorded significantly increased RGRs compared with the highest concentrations (500 $\mu\text{g L}^{-1}$) (post-hoc testing, Table 4.4; Figure 4.6). Significant interactions were present on five of the nine possible occasions, but the nature of these was difficult to discern (Table 4.4, Figure 4.6).

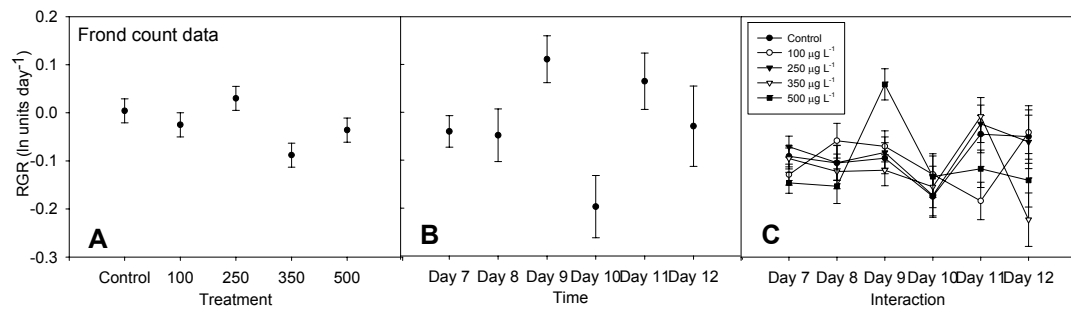


Figure 4.6 Example of *Spirodela oligorrhiza* relative growth rates calculated from frond count data during trial two: (A) effect of toxin concentration; data pooled within treatment groups; (B) effect of exposure time; data pooled within groups; (C) interaction, separate data for each treatment / time combination. Graphs show average values ($n = 3$) \pm standard error.

4.3.2.4 Final biomass

Spirodela biomass appeared to be stimulated by all exposure concentrations in trial five (Figure 4.7A), recording a maximum increase of over 40% in the 117 $\mu\text{g L}^{-1}$ treatment. One-way ANOVA detected a significant difference between the final biomasses of the different treatments ($p = 0.023$, $F = 3.28$, $df = 5$).

Table 4.4 Summarised results from two-way repeated measures ANOVAs for relative growth rate data based on frond counts of *Spirodela oligorrhiza*. NS = not significantly different, $p > 0.01$ for heterogeneous datasets; NA= not applicable, trials lasted only one week.

	Days 0 – 6 or days 0 – 7 ^b	Significantly different treatments ^c	Days 7 – 12	Significantly different treatments ^c
Trial two				
Mauchly's Sphericity test	$p = 0.028$; df=9; M=0.449		$p = 0.021$; df=14; M=0.034	None significant
Treatment	$p = 0.000$; $F_{4, 25} = 8.315$	Not applicable, interaction effects confound any treatment effects	NS	
Exposure time ^a	$p = 0.000$; $F_{4, 22} = 104.406$		NS	
Interaction ^a	$p = 0.001$; $F_{4, 25} = 6.114$		$p = 0.000$; $F_{5, 9} = 14.554$	
Trial three				
Mauchly's Sphericity test	NS	100 / 500 $\mu\text{g L}^{-1}$: $p = 0.030$ 250 / 500 $\mu\text{g L}^{-1}$: $p = 0.038$	NS	None significant
Treatment	$p = 0.001$; $F_{4, 25} = 6.365$		NS	
Exposure time ^a	$p = 0.000$; $F_{5, 25} = 17.305$		$p = 0.000$; $F_{5, 40} = 17.187$	
Interaction ^a	NS		NS	
Trial four				
Mauchly's Sphericity test	$p = 0.015$; df=14; M=0.300	None significant	$p = 0.017$; df=14; M=0.031	None significant
Treatment	NS		NS	
Exposure time ^a	$p = 0.001$; $F_{5, 21} = 67.031$		$p = 0.003$; $F_{5, 6} = 14.866$	
Interaction ^a	NS		$p = 0.000$; $F_{5, 9} = 22.393$	
Trial five				
Mauchly's Sphericity test	$p = 0.000$; df=20; M=0.111	None significant	NA	NA
Treatment	NS			
Exposure time ^a	$p = 0.000$; $F_{6, 19} = 40.598$			
Interaction ^a	NS			
Trial six				
Mauchly's Sphericity test	$p = 0.000$; df=20; M=0.044	None significant	NA	NA
Treatment	NS			
Exposure time ^a	$p = 0.000$; $F_{6, 19} = 33.283$			
Interaction ^a	$p = 0.007$; $F_{6, 23} = 3.978$			
Trial seven				
Mauchly's Sphericity test	$p = 0.003$; df=20; M=0.113	None significant	NA	NA
Treatment	NS			
Exposure time ^a	$p = 0.000$; $F_{6, 18} = 50.795$			
Interaction ^a	$p = 0.001$; $F_{6, 22} = 5.380$			

^atime x treatment, Sphericity Assumed statistic or Roy's Largest Root Statistic; ^bdays 0 – 6 for trials one to four, days 0 – 7 for trials five to seven; ^cDunnett's T3 posthoc test.

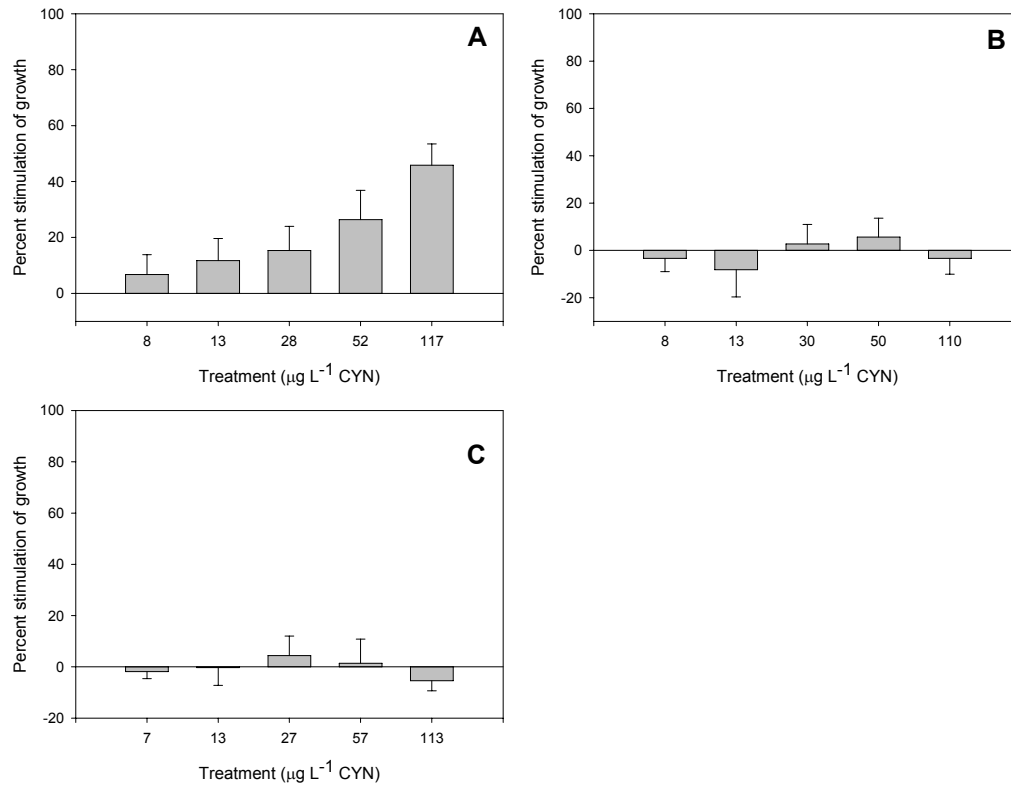


Figure 4.7 Percent change in the growth of *Spirodela oligorrhiza*, based on fresh biomass relative to control plants, following exposure to *Cylindrospermopsis raciborskii* whole cell extracts containing extracellular toxin: (A) trial five (B) trial six and (C) trial seven. Bars show average values ($n = 5$) \pm standard error.

In contrast, biomass changes between treatments were minimal ($< 10\%$ change) in all experimental treatments in trial six and seven (Figure 4.7B, C), and a significant difference was not present between treatments (one-way ANOVA; $p = > 0.050$).

4.3.3 Chlorophyll content

Chlorophyll *b* values from the first week of trial one (the pilot trial) were rejected due to an error in calibration. Values in weeks two and three were generally similar, regardless of treatment concentration (Figure 4.8). Significant differences between treatments were not present in trial one (Table 4.5), and

CYN exposure concentration was not significantly correlated with any of the chlorophyll concentrations (Table 4.6).

Significant differences were not recorded between treatments in trial two (Table 4.5). However, treatments with high toxin concentrations ($\geq 250 \mu\text{g L}^{-1}$) had almost double the total chlorophyll content of those with low CYN (Figure 4.9A-C). This corresponded with significant, positive correlations between CYN exposure concentrations and most chlorophyll values (Table 4.6).

Table 4.5 Summary of multivariate ANOVAs for chlorophyll data from *Spirodela oligorrhiza* exposed to *Cylindrospermopsis raciborskii* whole cell extracts. NS = not significant ($p > 0.010$ for trials one and two or $p > 0.050$ for trials five to seven); NA = not applicable, MANOVA was not significant.

Trial number	MANOVA result	Source of significance (ANOVA)
One ^a	NS	NA
Two ^b	NS	NA
Five	NS	NA
Six	$p = 0.009$; $F_{5,24} = 4.016$	Total chlorophyll $p = 0.043$; $F_{5,24} = 2.728$
Seven	NS	NA

^a weeks two and three combined; ^b weeks one and two combined.

Table 4.6 Pearson Product Moment Correlations between chlorophyll values of *Spirodela oligorrhiza* and CYN exposure concentration. (+) positive; (-) negative; NS = not significant ($p > 0.050$).

Trial number		One ^b	Two ^a	Five	Six	Seven
Toxin concentration ^c	Chlorophyll <i>a</i>	NS	+	-	-	NS
	Chlorophyll <i>b</i>	NS	+	-	NS	NS
	Total chlorophyll	NS	+	-	-	NS
	<i>a:b</i> ratio	NS	-	NS	NS	NS
Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	+	+	+	+	NS
	Total chlorophyll	+	+	+	+	+
	<i>a:b</i> ratio	NS	-	NS	NS	+
	Total chlorophyll	+	+	+	+	NS
Chlorophyll <i>b</i>	<i>a:b</i> ratio	NS	-	-	NS	NS
	Total chlorophyll	NS	-	NS	NS	NS

^a weeks two and three combined; ^b weeks one and two combined; ^c nominal toxin concentrations.

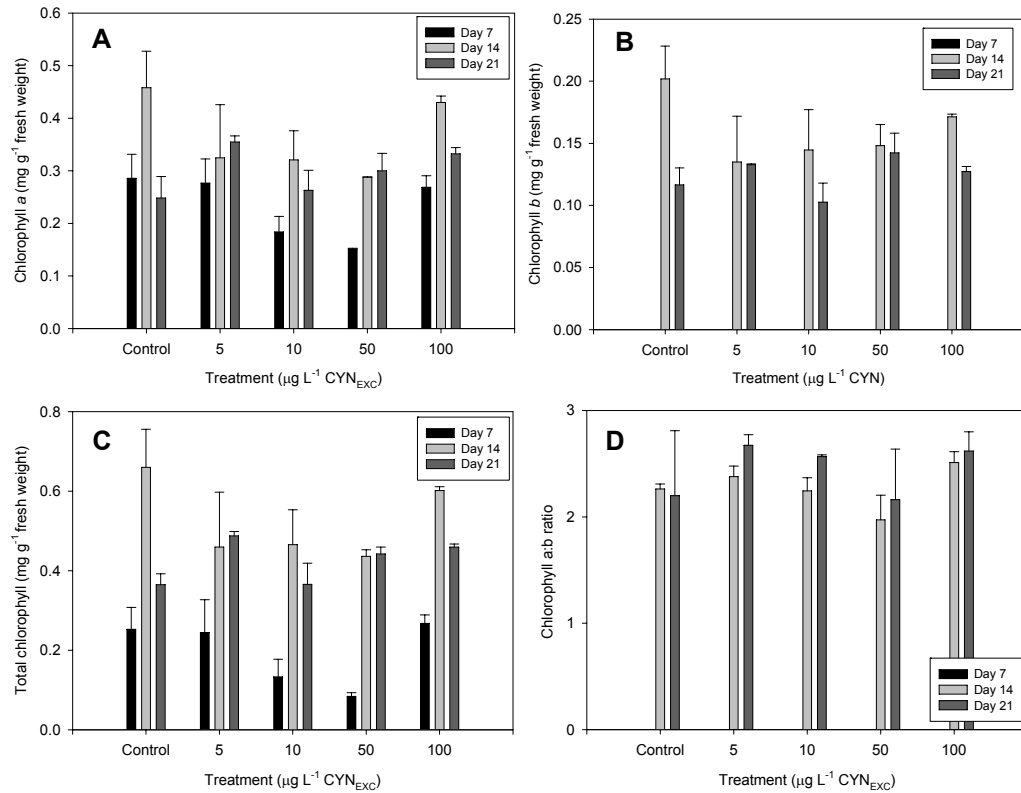


Figure 4.8 Chlorophyll content of *Spirodela oligorrhiza* in trial one: (A) chlorophyll a, (B) chlorophyll b (week one not shown), (C) total chlorophyll (D) chlorophyll a:b ratio. Bars show average ($n = 2$) \pm standard error. CYN_{EXC} = extracellular cylindrospermopsin.

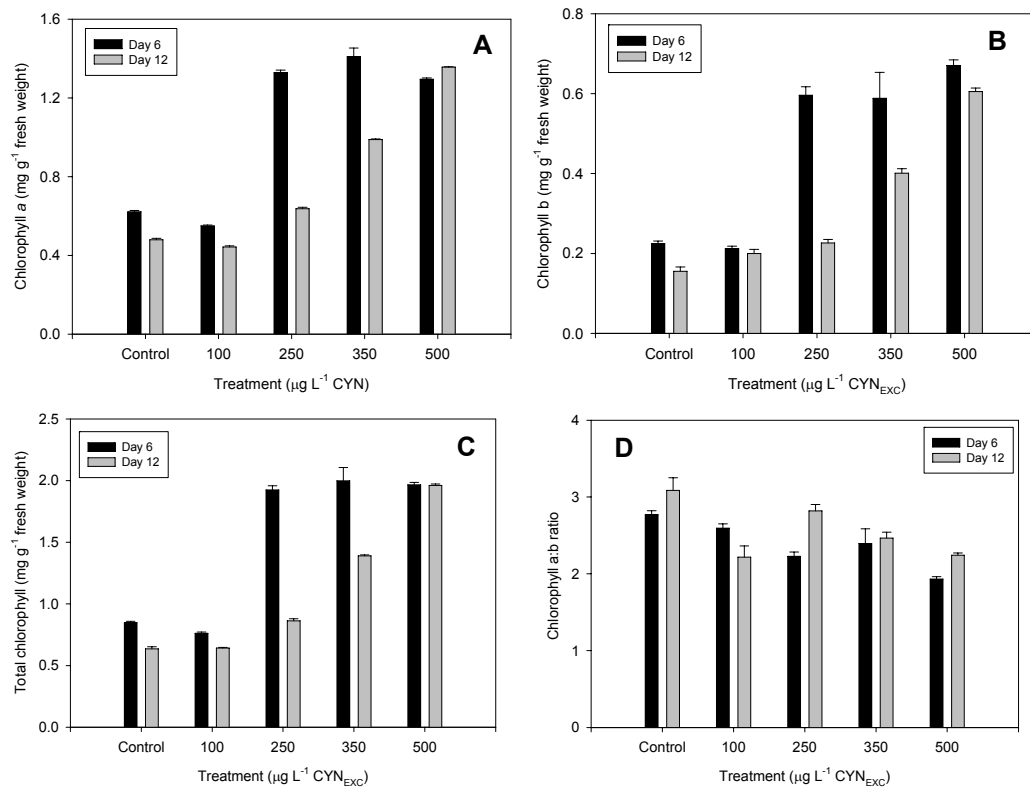


Figure 4.9 Chlorophyll content of *Spirodela oligorrhiza* in trial two: (A) chlorophyll a, (B) chlorophyll b, (C) total chlorophyll, (D) chlorophyll a:b ratio. Bars show average ($n = 2$) \pm standard error. CYN_{EXC} = extracellular cylindrospermopsin.

Chlorophyll values were similar across trials five and six, but were slightly lower overall in trial seven (Figures 4.10A, 4.11A, 4.12A). A significant difference between treatments was recorded only in trial six, where the total chlorophyll value recorded from the 8 $\mu\text{g L}^{-1}$ treatment was significantly higher than that of the 117 $\mu\text{g L}^{-1}$ treatment (Figure 4.11C; Table 4.5). There was also a significantly negative correlation between toxin exposure concentrations and chlorophyll *a*, *b* and total chlorophyll in trial five (Table 4.6). However, post-hoc Tukey tests also recorded significant differences amongst treatments with respect to total chlorophyll in trial six, and chlorophyll *a* in trial five (Figures 4.10, 4.11). In all cases, treatments containing 8 $\mu\text{g L}^{-1}$ recorded higher chlorophyll values than plants in dishes containing $> 110 \mu\text{g L}^{-1}$ toxin.

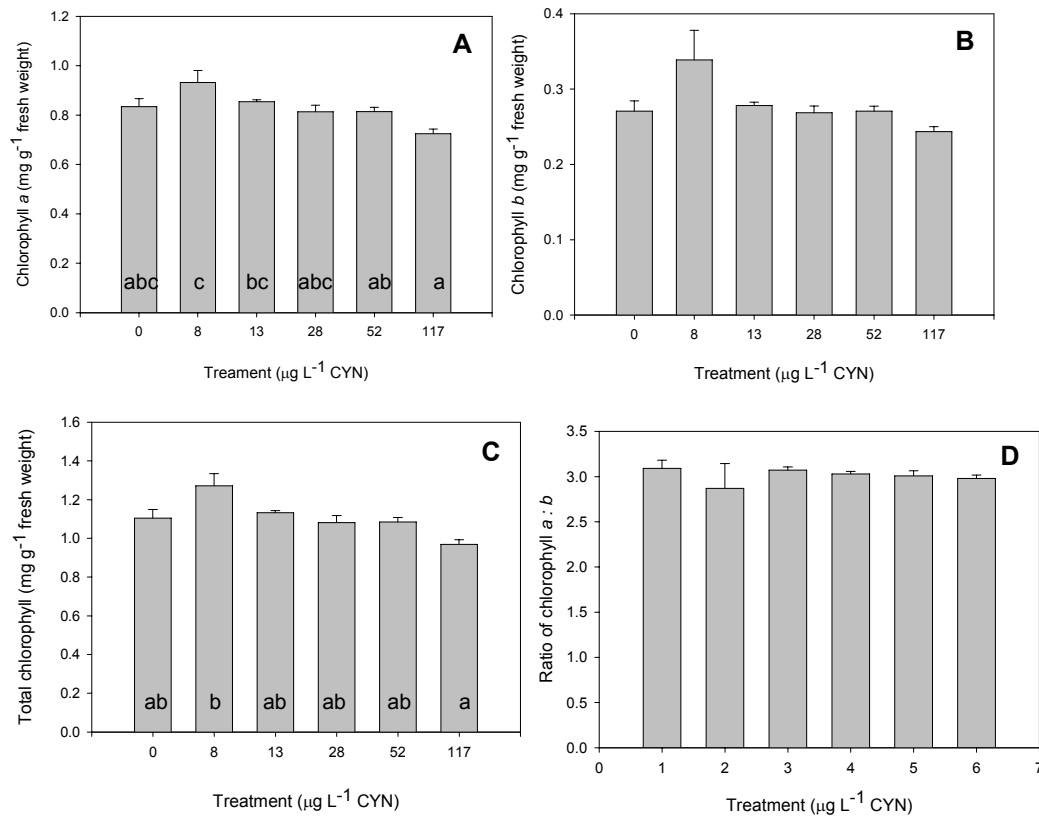


Figure 4.10 Chlorophyll content of *Spirodela oligorrhiza* in trial five: (A) chlorophyll *a*, (B) chlorophyll *b*, (C) total chlorophyll, (D) chlorophyll *a*:*b* ratio. Values show average of duplicate measurements \pm standard error. Graphs sharing the same letter are not statistically different from each other ($p > 0.050$, post-hoc Tukey tests).

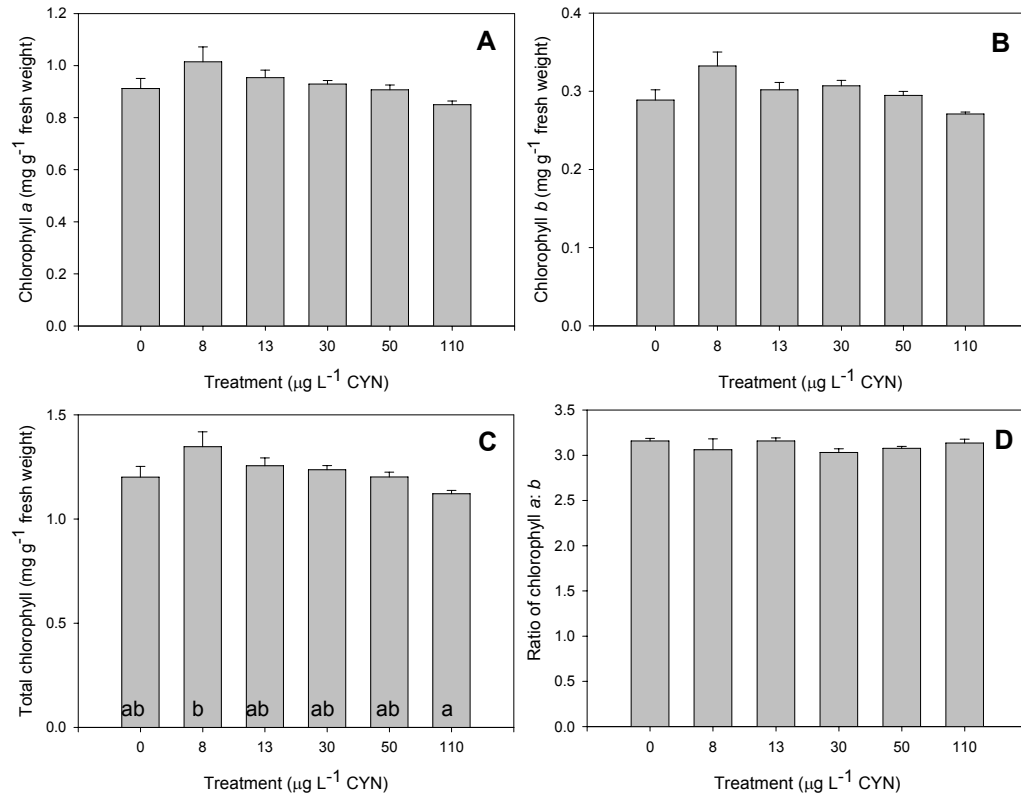


Figure 4.11 Chlorophyll content of *Spirodela oligorrhiza* in trial six: (A) chlorophyll a, (B) chlorophyll b, (C) total chlorophyll, (D) chlorophyll a:b ratio. Values show average of duplicate measurements \pm standard error. Graphs sharing the same letter are not statistically different from each other ($p > 0.050$, post-hoc Tukey tests).

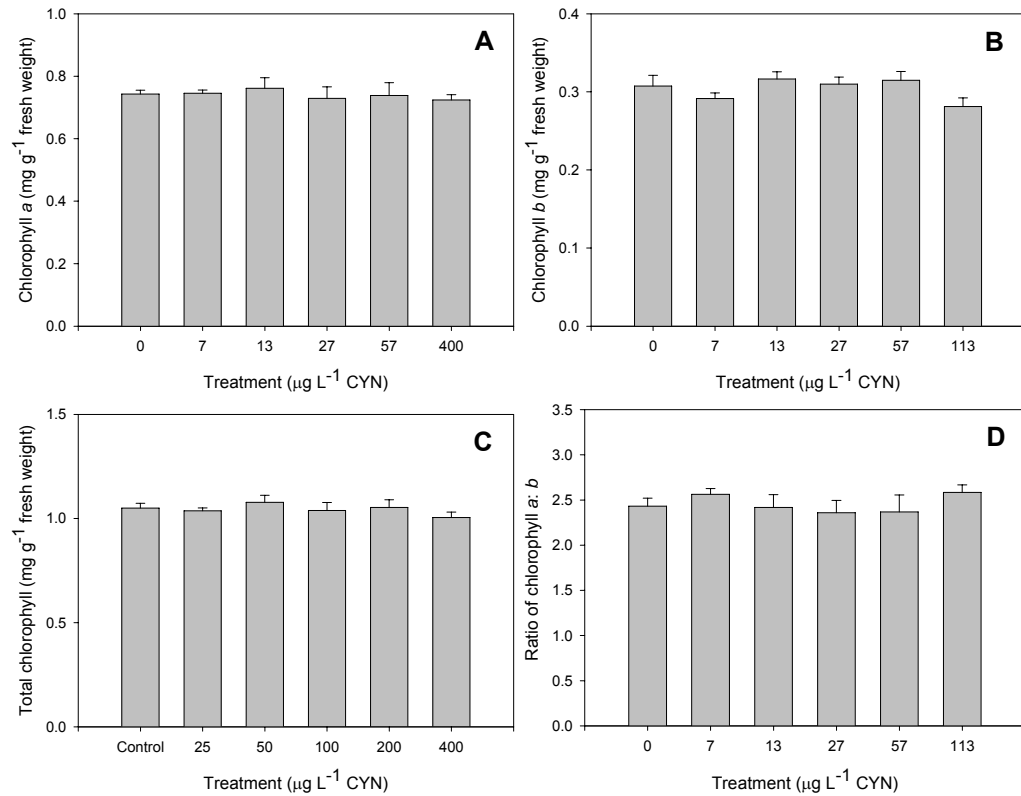


Figure 4.12 Chlorophyll content of *Spirodela oligorrhiza* in trial seven: (A) chlorophyll a, (B) chlorophyll b, (C) total chlorophyll, (D) chlorophyll a:b ratio. Values show average of duplicate measurements \pm standard error.

4.3.4 Free CYN bioconcentration

The maximum average free CYN concentration recorded from *S. oligorrhiza* was approximately $30 \mu\text{g kg}^{-1}$ fresh tissue (Figure 4.13A, B). In trial four, some problems were experienced with freeze-dried weights; CYN values based on these have therefore not been reported. The average bioconcentration factor¹ for *S. oligorrhiza* was 0.044 and 0.038, for six and twelve day's exposure, respectively, assuming 1 gram of tissue = 1mL water (Table 4.7). Bioconcentration did not occur in any treatment ($\text{BCF} < 1$), and was not concentration-dependent. An ANOVA could not be conducted since some treatments had zero standard deviation. However, a highly significant positive correlation was detected between *S. oligorrhiza* free CYN concentrations (reported as fresh weight) and nominal CYN concentrations (Pearson Product Moment Correlation; $p = 0.000$; $r = 0.628$; $n = 56$).

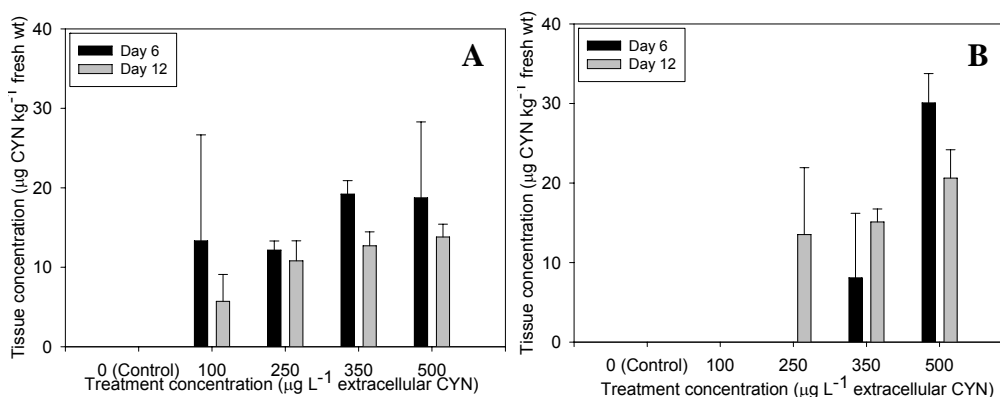


Figure 4.13 Cylindrospermopsin concentrations in the tissues of *Spirodela oligorrhiza* following six and twelve days exposure to extracellular toxin solutions: (A) trial three, (B) trial four. Graphs depict average ($n = 3$) \pm standard error.

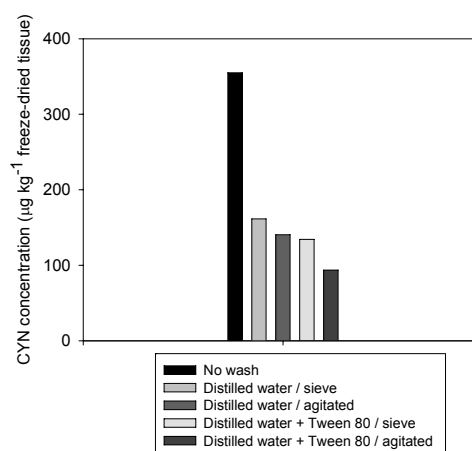
¹ Bioconcentration factor (BCF, fresh weight tissue concentration divided by exposure concentration) used compared with bioaccumulation factor (BAF), as uptake in aquatic plants is considered only possible from extracellular (aqueous, dissolved, non-cell bound) toxins.

Table 4.7 Bioconcentration factors for *Spirodela oligorrhiza* following six and twelve day's exposure to extracellular toxin solutions. CYN_{EXC} = extracellular cylindrospermopsin.

Nominal concentration ($\mu\text{g L}^{-1}$ CYN _{EXC})	Bioconcentration factor (average \pm standard error)	
	6 days exposure	12 days exposure
Trial three		
150	0.13 \pm 0.13	0.06 \pm 0.03
250	0.05 \pm 0.0	0.04 \pm 0.01
350	0.05 \pm 0.0	0.04 \pm 0.01
500	0.04 \pm 0.02	0.03 \pm 0.00
Trial four		
150	0.0 \pm 0.0	0.0 \pm 0.0
250	0.0 \pm 0.0	0.05 \pm 0.03
350	0.02 \pm 0.02	0.04 \pm 0.0
500	0.06 \pm 0.01	0.04 \pm 0.01
Average over both trials	0.044	0.038

4.3.5 Toxin adsorption to plant cell walls

Different rinsing methods resulted in changes to the CYN contents of *S. oligorrhiza* tissues. More rigorous rinsing treatments were generally associated with the smallest toxin quantities (Figure 4.14). Rinsing with distilled water reduced toxin concentrations by 55% compared with unwashed plants. The best reduction in toxin was achieved by agitating the plant material with distilled water and Tween 80 (Figure 4.14).

**Figure 4.14** Toxin concentrations of *Spirodela oligorrhiza* tissues following five different rinsing methods.

4.3.6 Sampling, mixing and analysis of CYN culture strength

Values obtained from remixed samples ranged from 94 - 102 $\mu\text{g L}^{-1}$ below that obtained from the single-mixed sample (Table 4.8). There was also an unexpectedly large variation (10.2%) between the five replicate samples collected after the second mix; although a second analysis reduced this variation to 8.1% (Table 4.8).

Table 4.8 Cylindrospermopsin concentrations of six *Cylindrospermopsis raciborskii* samples after first and second mixing. CYN = cylindrospermopsin.

Sample	CYN concentration ($\mu\text{g L}^{-1}$)	
First mixing	600	
	First integration	Second integration ^a
Second mixing		
# 1	482	484
# 2	482	481
# 3	398	417
# 4	506	515
# 5	418	446
Mean	457.2	468.6
Standard deviation	46.5	37.8
Variation (SD as % of mean)	10.2	8.1

^asame peaks interpreted by different assessor

4.4 Discussion

4.4.1 Water quality

Conductivity and pH variations amongst treatments probably resulted from the presence of the whole-cell extracts: treatments containing higher toxin concentrations also contained larger amounts of source culture, which had different conductivity and pH values than the diluent media. The higher conductivities in trials five to seven, compared with trials one to four, was due to the higher salt concentration in the modified Hoagland's media (see control conductivities in trials five to seven, Table 4.1).

In trials one to four, between-trial pH variation may be explained by the pH of the original culture banks used to prepare treatment solutions: two-to-three month old cultures of *C. raciborskii* (similar age to those used in these trials) have since had pH values measured between 9.26 – 9.68. Other two-month old cultures of other *C. raciborskii* strains grown under similar conditions have also had pH reported at 10.5 (Baker 1998). In addition, the large pH increases recorded with increasing exposure time probably resulted from photosynthetic activity and secretion of hydroxide by *S. oligorrhiza*.

In trials five to seven, the source culture used to prepare test solutions in the ecotoxicity trials had exceptionally high toxin concentration (2,287 $\mu\text{g L}^{-1}$ CYN). Therefore, most of the test solutions comprised the diluent media (modified Hoagland's solution). Consequently, pH variation between treatments was much less compared with that recorded in the earlier trials, since the test solutions for trials one to four were prepared with stock cultures of much reduced CYN concentrations (for example, < 700 $\mu\text{g L}^{-1}$).

4.4.2 CYN treatment concentrations

Explaining CYN losses during the first four trials is difficult: CYN is not known to adsorb to Pyrex glass (Chiswell *et al.* 1999), making losses to test vessels unlikely. Free CYN uptake by *S. oligorrhiza* appears to account for only nanogram quantities of toxin (see 4.3.5). Furthermore, based on available literature, the half-solution renewal at three-day intervals should have been adequate to counteract decomposition. Chiswell *et al.* (1999) reported low decomposition rates in 1 mg L^{-1} CYN extracts exposed to similar experimental

conditions as used in the current tests, with 89% of toxin remaining after one month's exposure at 35°C; 78% remaining after eight weeks at pH 10; and 42% remaining after five weeks under 42 $\mu\text{E m}^{-2} \text{s}^{-1}$. The presence of plant pigments (chlorophylls, carotenoids, xanthophylls) could have promoted rapid CYN decomposition (Chiswell *et al.* 1999). If so, *S. oligorrhiza* may have also experienced additional toxicity from CYN decomposition products. However, consequent testing has since indicated that inadequate mixing of culture banks was likely to be responsible for CYN variability (see 4.4.5).

Trace CYN concentrations (0.5 $\mu\text{g L}^{-1}$) detected in some controls could indicate contamination of the distilled water source used to prepare treatments, especially as the Fitzroy River (Rockhampton's tap water supply source) sometimes experiences *C. raciborskii* blooms. Glassware contamination is possible, but unlikely, since glassware was triple rinsed in tap water. Bleach rinsing of all glassware successfully eliminated CYN from controls during trials five to seven.

4.4.3 Sublethal effects in *S. oligorrhiza*

4.4.3.1 Changes in frond morphology

Clumping of *S. oligorrhiza* has not previously been reported as an indicator of sub-lethal toxicity. Rather, duckweed fronds separate when exposed to heavy metal toxicity (Bengtsson *et al.* 1999; Li & Xiong 2004). Duckweed exhibits many stress-related symptoms, including chlorosis, necrosis, root destruction, loss of buoyancy and protuberances (Wang 1990), and it is possible that this symptom is a unique indicator of cyanotoxin exposure. The reason for this

response can only be speculated: possibly, chemicals released during frond decomposition altered frond surface tension.

Frond necrosis and death in *S. oligorrhiza* was associated with exposures to whole cell extracts containing $\geq 250 \mu\text{g L}^{-1}$ CYN_{EXC}. These symptoms were not noted from plants in trials using the revised methods (trials five to seven); however, the maximum toxin concentrations here were $\leq 120 \mu\text{g L}^{-1}$. Romanowska-Duda and Tarczyńska (2002) observed chlorosis of *S. oligorrhiza* fronds at microcystin-LR (MC-LR) exposures between $86 - 344 \mu\text{g L}^{-1}$. Weiß *et al.* (2000) also reported frond deformations and reduced frond size in *Lemna minor* following three days exposure to $3 - 5 \text{ mg L}^{-1}$ MC. Absence of deformities in the present trial may have resulted from the use of far lower test concentrations ($\leq 500 \mu\text{g L}^{-1}$), despite the possibility of toxic substances other than CYN being present in the *C. raciborskii* cell debris (Hawkins *et al.* 1997; Falconer *et al.* 1999; Norris *et al.* 1999; Froscio *et al.* 2001; Saker *et al.* 2003; Falconer 2005).

Reduced root lengths were also recorded in some, but not all, trials. This may indicate plant stress, evidenced by the redistribution of plant resources (Cedergreen & Madsen 2002). Several studies of other algal toxins have also examined root effects: Kirpenko (1986) reported loss of rootlets and browning of duckweed plants exposed to blue-green algal toxin, although neither the cyanoprokaryote species nor toxin type was reported. Yin *et al.* (2005) reported loss of root hairs in *Vallisneria* after 30 day's exposure to 10 mg L^{-1} MC-RR. In terrestrial plants, MC-RR almost completely blocked root and hair formation in

mustard seedlings at 20 and 40 mg L⁻¹ exposure (Kurki-Helasma & Meriluoto 1998), and roots of runner beans became discoloured and grew poorly following MC-LR exposure (McElhiney *et al.* 2001).

4.4.3.2 Frond percentage coverage and frond count analyses

Both the percentage coverage and frond count data provided similar patterns of growth in *S. oligorrhiza*. Although the growth was variable, overall, exposure to whole cell extracts containing toxin initially caused growth inhibition. However, this response shifted to growth stimulation as incubation times increased (> 3 or 4 days). In many cases, exposure to higher toxin concentrations ($\geq 350 \mu\text{g L}^{-1}$ for trials one to four; $\geq 100 \mu\text{g L}^{-1}$ for trials five to seven) caused persistent growth inhibition, with no evidence of recovery or growth stimulation over a seven-day period. However, some of the plants in trials two, four and five proved to be exceptions to these patterns.

These results appear to suggest that *S. oligorrhiza* may be capable of physiologically responding to the presence of low-concentration CYN (and *C. raciborskii*) in short timeframes (less than seven days). By contrast, plants exposed to higher toxin concentrations either adapt more slowly or, alternatively, cannot adapt due to toxicity. This scenario, where low concentrations of a stressor exert mildly stimulant effects, whereas higher concentrations exert adverse effects, is known to occur with several herbicides and plant growth regulators (Lichtenthaler 1996). The pattern of response for longer incubation times (greater than seven days) is not known, since the responses of the plants in the present work were highly variable after seven days of incubation.

Stimulation of RGRs in low-CYN exposed *S. oligorrhiza* could be interpreted as a mechanism by which the plants attempt to overcome or outcompete a stressor (for example, *C. raciborskii*). For example, increasing the number and coverage of *Spirodela* fronds is likely to result in shading of *Cylindrospermopsis*, possibly leading to bloom collapse.

The trial results also highlight the apparent variability in the response of *Spirodela* to whole cell extracts containing CYN. For example, the 100 µg L⁻¹ treatment was common to all seven experiments (though this test concentration was only nominal, not measured, for trials one to four). At this dose of CYN and whole cell extract, the response of *Spirodela* with respect to frond counts included prolonged inhibition, prolonged stimulation, and phases of stimulation and inhibition together, in the different trials. The source of this variability is unknown: possibly, slight changes in the condition of the inoculum cultures (for example, age of plants) could have contributed to the different responses. However, for trials five to seven at least, inocula were always selected as young, rapidly growing plants of two to four fronds.

Decreases in frond number, particularly during trial two, could have resulted from decomposition, although it is possible that clumping of plants might also have prevented accurate frond counts. The latter is unlikely, though, as clumps were broken apart for recounting at the end of trial four, and subsequent values agreed with original counts. Decreasing growth rates over time, and the appearance of slight frond necrosis noted in the controls may have resulted from nutrient limitation caused by the elevated pH values.

Lastly, significant decreases in plant growth were limited to the first week of exposure for percentage coverage analyses in trials two to four; whereas this was not the case for the frond count data for the same trials. These anomalies may reflect the more ‘instantaneous’ nature of percentage cover analysis, whereas production of entirely new fronds may take several days to occur.

4.4.3.3 Plant biomass

In trials six and seven, the biomass results appeared to strengthen the conclusion that plants experiencing exposures to medium toxin concentrations ($30 - 60 \mu\text{g L}^{-1}$) were capable not only of CYN (and/or *C. raciborskii*) tolerance, but also of growth stimulation, by the end of the incubation period (Figure 4.7). This stimulation was not statistically significant compared with the controls, since there was high variability between replicates. This variability was probably related to the use of fresh biomass; dried samples could not be obtained since the fresh tissues were required for chlorophyll analyses.

4.4.3.4 Chlorophyll content

The chlorophyll values of *Spirodela* were highly variable, especially in trials one and two, compared with the later trials, five to seven. Chlorophyll contents (*a*, *b*, total chlorophyll) recorded during trial one were less than half those obtained in all other trials, suggesting that the illumination levels ($40 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) were too low compared with the later trials ($110 \mu\text{mol photon m}^{-2} \text{s}^{-1}$). The results obtained in the earlier trials may be discarded on this assumption.

In trials five and six, chlorophyll *a* and total chlorophyll values experienced small peaks when *Spirodela* was exposed to whole cell extracts containing $8 \mu\text{g L}^{-1}$ toxin (Figures 4.10, 4.11). In comparison, the remaining treatments (up to $117 \mu\text{g L}^{-1}$ toxin) recorded values comparable with the controls. This directly contrasts with other studies of *S. oligorrhiza* that have indicated that chlorophyll contents generally decrease with cyanotoxin exposure (Weiß *et al.* 2000; Romanowska-Duda & Tarczynska 2002). Nevertheless, the chlorophyll results again appear to reinforce the argument for stimulation of growth under low toxin exposure concentrations.

It seems unusual that stimulation with respect to chlorophyll values was restricted to the $8 \mu\text{g L}^{-1}$ treatments, whereas stimulation of frond numbers and biomass on day seven was recorded in several other treatments (Figure 4.5 D – F; Figure 4.7). However, this could be explained if *Spirodela* is able to rapidly reproduce frond numbers (and hence biomass), whereas rapid chlorophyll synthesis may be delayed. Equally, decreases in chlorophyll could have occurred during the initial incubation period, concomitant with the growth inhibition recorded via the frond counts. Since tissues were harvested and analyses for chlorophyll done only on day seven, this remains unknown.

4.4.4 Bioconcentration and cell wall adsorption

S. oligorrhiza does not bioconcentrate free CYN; moreover, most (or all) free toxin associated with *S. oligorrhiza* tissues is represented by cell wall adsorption, with a seemingly dose-dependent relationship apparent between CYN exposure

concentrations and tissue concentrations (Figure 4.13). This is further supported by the results of the rinsing experiment.

Agitating plants in a beaker containing the Tween 80 removed 43% of toxin present after washing with distilled water; however, this represented a change of less than 5 µg CYN kg⁻¹ fresh *S. oligorrhiza*. The rinsing methods used during the ecotoxicity trials (rinsing with distilled water) were therefore considered adequate, and were continued for all subsequent trials (including those for *Hydrilla verticillata*, see next chapter). More rigorous methods, such as the use of surface sterilisation techniques, were avoided since these could result in CYN denaturation and/or HPLC interferences.

Irrigation of consumable crops with CYN-contaminated water may represent some risk for human intoxication via ingestion of adsorbed toxin, even if this was to occur in small quantities. This issue has previously been studied with MC (Abe *et al.* 1996; Codd *et al.* 1999b).

4.4.5 CYN decomposition rates and CYN sampling accuracy

The CYN mixing experiment results strongly suggest that obtaining CYN culture strength based on a single sample, and a single mixing event, is not adequate. This may also help to explain the ‘loss’ of CYN from culture vessels (see 4.3.1): it is likely that original dilutions were not of the desired concentration initially. In all future trial work, culture banks were frozen, thawed and mixed at least twice before subsampling, and at least two samples taken for determination of CYN content.

The high standard deviation (10.2%; 8.1% on re-interpretation) between the remixed replicate samples is unusual, given that the technique used by QHSS usually obtains approximately 3 – 5% repeatability (samples run on the same day) or slightly higher for reproducibility (same sample in different analytical runs) (Geoff Eaglesham, pers. comm.). However, standards for the method usually run to only 10 $\mu\text{g L}^{-1}$, which represents the higher end CYN concentrations in water samples received by QHSS. Thus, the abnormally high CYN concentrations of the cultures (400 – 500 $\mu\text{g L}^{-1}$ and above) may have increased variation (Geoff Eaglesham, pers. comm.).

4.4.6 Validation of revised test method

The decreased chlorophyll values and slow doubling times recorded from control *Spirodela* in the earlier trials (one and two) suggests that ASM-1 media was not a suitable growth media for *Spirodela*, and/or that illumination levels were too low. It seems likely that a combination of both these contributed to the unreliable results obtained in the earlier trials. Thus, although the use of ASM1 media would have been as a control and diluent in terms of best matching the ASM1 present in the *C. raciborskii* whole cell extracts, future work should use modified Hoagland's media. Accordingly, some of the plant responses recorded in the trials one to four, but not trials five to seven, may have occurred due to the presence of the unsuitable ASM1 media. On the other hand, the toxin concentrations in the earlier trials (0 – 500 $\mu\text{g L}^{-1}$) were far higher compared with those for trials five to seven (0 – 120 $\mu\text{g L}^{-1}$). Nonetheless, there should be some caution in reporting that these symptoms (for example, plant necrosis, frond decomposition and reduced root lengths) are related to CYN exposures.

Chapter five: ecotoxicity and bioconcentration risk in *Hydrilla verticillata*

Note to reader: material in this chapter has been published in part in **White, S.H.**, Duivenvoorden, L. J. & Fabbro, L. D. (2005). Absence of Free-Cylindrospermopsin Bioconcentration in Water Thyme (*Hydrilla verticillata*). *Bulletin of Environmental Contamination and Toxicology* 75 (3): 574-583.

A second manuscript is also currently in submission: **White, S.H.**, Fabbro, L. D. and Duivenvoorden L. J., “Growth responses of water thyme (*Hydrilla verticillata*) to whole cell extracts of *Cylindrospermopsis raciborskii*”.

5.1 Introduction

Hydrilla verticillata (L. fil.) Royle is a rooted aquatic plant that plays numerous important roles in tropical aquatic food webs. The following trials examine exposure risks and bioconcentration potential in *H. verticillata* following exposure to *C. raciborskii* whole cell extracts containing extracellular CYN (CYN_{EXC}) concentrations $\leq 500 \mu\text{g L}^{-1}$ over a fourteen-day period.

5.2 Materials and methods

5.2.1 Culture of test organisms

H. verticillata plants were collected from a private property at Cawarral, north-west of Rockhampton (trials one, four) or an ornamental pond on the CQU campus (trials two, three). Neither location is known to experience blooms of *C. raciborskii* or other CYN-producers. Plants were washed thoroughly in tap water to remove epiphytic growth and other aquatic biota, and kept under experimental conditions for at least one week prior to use.

5.2.2 Test procedures

A preliminary range-finding test (trial one) was conducted using *C. raciborskii* whole cell extracts, prepared at nominal treatment concentrations of 0, 100 and 250 $\mu\text{g L}^{-1}$ CYN_{EXC} with a fourteen-day exposure period. Two definitive trials (trials two, three) tested *H. verticillata* exposure to whole cell extracts containing 0 (control), 25, 50, 100, 200, and 400 $\mu\text{g L}^{-1}$ CYN_{EXC} over fourteen days. In addition, a depuration trial (trial four) examined the effects of seven-day exposure followed by a seven-day ‘depuration’ phase. In the latter, *H. verticillata* fragments were exposed to 0, 100, 250, 350 and 500 $\mu\text{g L}^{-1}$ CYN_{EXC} solutions for seven days, before being transferred into CYN-free ASM1 media for a further seven days. The pilot and depuration trials were done in sequence, followed by the definitive trials.

Treatments ($n = 6$) comprised 150 mL test solutions, prepared by freeze-thawing *C. raciborskii* cultures of known toxin concentration to ensure all toxin was extracellular, and diluting to desired test concentrations using autoclaved ASM1 algal media (Gorham *et al.* 1964). Controls were ASM1 media only. Test sections of *Hydrilla* were obtained by separating lateral stems from mother plants: each fragment comprised an apical tip with no lateral stems or roots (average length $66.5 \text{ mm} \pm 5.78$ standard error). All plants were healthy, with no visible necrosis or chlorosis. Two plant fragments were weighed (fresh weight, to 0.01 g), measured (main stem length; mm) and the number of nodes counted, before each pair were randomly assigned to each test vessel.

Plants were harvested ($n = 3$ test dishes) on days seven and fourteen and the following growth parameters recorded from both fragments in the dish: main stem length;

number of nodes; number and length of lateral stems; number and length of roots and overall plant biomass (fresh weight). Internode distance (mm) was calculated by dividing main stem length by the number of nodes. Cumulative and average root and lateral stem length was calculated by summing individual scores and dividing by n , respectively. Relative growth rates (RGRs) were calculated based on elongation of main stem, increase in node number, and increase in biomass. One fragment of *Hydrilla* per dish (including roots, where present) was used for chlorophyll determination, and the other for CYN determination.

Conductivity and pH were measured at two-day intervals throughout all trials; temperature was continuously monitored at 4h intervals. To achieve constant CYN concentrations, test solutions were replenished by half (75 mL) with fresh solution at three-day intervals throughout all trials (days 0, 3, 6, 9 and 12). Following plant harvest (days seven and fourteen), leftover solutions were reserved, pooled within treatments, frozen, and two randomly selected samples analysed for CYN to check the efficacy of test solution replacement. Toxin analyses were conducted at QHSS (Coopers Plains, Brisbane) using HPLC/Electrospray/Tandem Mass Spectrometry (limit of detection, $0.2 \mu\text{g L}^{-1}$) (Eaglesham *et al.* 1999; Norris *et al.* 1999). Full details of this method are provided in Appendix B.

Plants entering the second phase of the depuration trial (day seven onwards) were transferred, without rinsing, into new dishes containing 100 mL of ASM1 media only. The trial continued as before, with ASM1 solution replaced by half at three-day intervals; dishes were harvested on day fourteen as described above.

5.2.3 Chlorophyll analyses

Chlorophyll determinations were carried out in all trials, using spectrophotometric determination (Hitachi U-2000 spectrophotometer) of chlorophyll *a* and *b*, using the standard method of the USEPA (1994), modified using 80% chilled acetone extraction and the chlorophyll calculation equations described in Inskeep and Bloom (1985). All measurements were taken in duplicate and the average value used.

5.2.4 Bioconcentration analyses

Free CYN analyses were conducted on samples from trials two and three and selected samples from trial four. Fragments (stem, leaves and root/s) were weighed, moistened with distilled water, ground into slurry using a glass rod and washed into pre-weighed tubes. Samples were frozen, freeze-dried (approximately 12 h), brought to room temperature and reweighed. Tubes were then transported to QHSS for analysis of free CYN. There, plant material was homogenised with 5mL HPLC grade water, filtered (0.45 micron, Millex-HV) and analysed using HPLC/Electrospray/tandem Mass Spectrometry (limit of detection, 0.2 $\mu\text{g L}^{-1}$; or 1.0 nanogram per 5 mL tube). Spike recoveries for 5ml of homogenate averaged 97.5% at 3.2 $\mu\text{g L}^{-1}$ and 90% at 6.5 $\mu\text{g L}^{-1}$. Full details of extraction and analysis methods are provided in Appendix C. Samples from trial three were also analysed for (free) deoxy-CYN content.

5.2.5 Statistical analyses

Growth and chlorophyll data were examined using two-way multivariate ANOVAs with Roy's Largest Root Statistic (SPSS for Windows v11.5.0). This method was used in preference to repeated standard-alone univariate ANOVAs, which would have resulted in a high Type 1 error rate (Quinn & Keough 2002). To avoid

pseudoreplication, one *Hydrilla* fragment per dish was eliminated from these analyses (random numbers table, Fowler *et al.* (1998)) leaving $n = 3$ for each treatment/time combination. As data were heterogeneous and could not be suitably transformed, a lower level of significance ($\alpha = 0.01$) was adopted to increase the reliability of significant effects (Underwood 1981). MANOVAs were followed by multiple univariate ANOVAs to identify the source(s) of significance. Dunnett's T3 tests for multiple pairwise comparisons were used to detect significant differences between plants exposed to different CYN_{EXC} test concentrations ($\alpha = 0.01$).

Pearson Product Moment Correlations ($\alpha = 0.050$) were used to examine the relationships between growth parameters, chlorophyll content, free CYN content of tissues and nominal CYN_{EXC} exposure concentrations in trials two to four. Growth parameter data from the first and second exposure weeks were analysed separately since significant differences were present between exposure times. Growth data was also examined using hierarchical clustering based on Bray-Curtis similarities; dendograms and multidimensional scaling plots were generated in Primer v5.0. Data were entered as the natural-log transformed averages for each treatment and time combination (except relative growth rate values, since these were already expressed as \ln units day^{-1}). Bioconcentration data could not be analysed statistically as some treatments had zero standard deviation.

5.3 Results

5.3.1 Water quality and test conditions

Temperature variability was recorded in some trials due to the effects of photoperiod (Table 5.1). High pH values were recorded in all four experimental trials, along with widely ranging conductivity values, especially between treatments (Table 5.1).

Table 5.1 Ranges for water quality parameters recorded in trials using *Hydrilla verticillata*. L:D = light:dark.

Trial number	Photoperiod ^a	Illumination ($\mu\text{mol photon m}^{-2}\text{s}^{-1}$)	Ambient temperature ($^{\circ}\text{C}$) ^b	pH	Conductivity ($\mu\text{S cm}^{-1}$)
One	Continuous	40	27 ± 2	8.6 – 11.0	180 – 490
Two	12:12 L:D	60 – 75	$25 \pm 3^{\text{c}}$	7.3 – 11.0	360 – 770
Three	12:12 L:D	60 – 75	$26 \pm 4^{\text{c}}$	7.0 – 11.1	230 – 720
Four	Continuous	40	27 ± 2	8.3 – 10.3	210 – 470

^atrials done in sequence of one, four, two, three; ^bhourly measurement of ambient air temperature using Hastings StowAway Tidbit loggers; ^c wider temperature range was due to 12:12 photoperiod.

Table 5.2 Cylindrospermopsin (CYN) concentrations of randomly chosen test solutions. Values indicate three pooled replicates of test solution. NA = not applicable, CYN-free phase.

Trial number	Days since last half-solution replacement	Nominal concentration ($\mu\text{g L}^{-1}$ CYN)	Actual concentration ($\mu\text{g L}^{-1}$ CYN)	Percent remaining (%)
One ^a	1	100	45	45
One ^a	2	250	118	47
Two	1	50	41.2	82
Two	2	50	43.3	87
Three	2	50	32	64
Three	5 ^a	400 ^a	138 ^a	35 ^a
Four	1	250 (CYN phase)	229	92
Four	2	0 (CYN-free phase)	5	NA

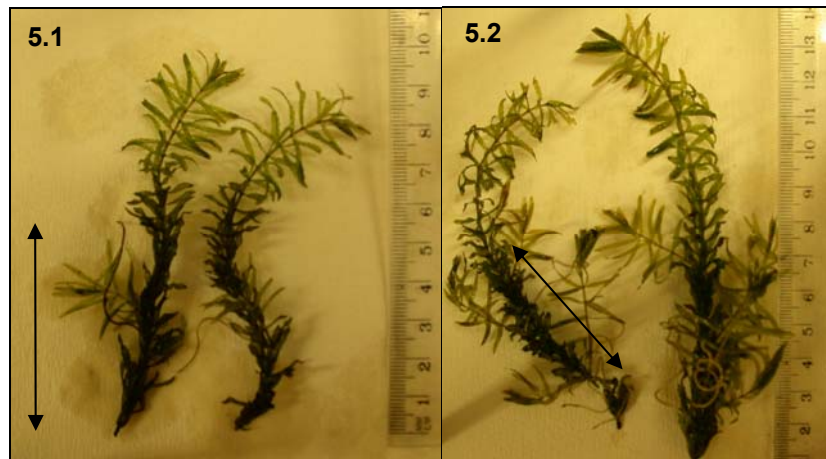
^a last static renewal missed due to lack of available toxin.

5.3.2 CYN treatment concentrations

Some CYN losses were experienced, particularly in trial one, where treatment concentrations were < 50% of desired values (Table 5.2). Concentrations of CYN in trials two to four were more satisfactory: an exception was the 400 $\mu\text{g L}^{-1}$ treatment, which, due to lack of renewal on day twelve, dropped to 35%. In trial four, trace (5 $\mu\text{g L}^{-1}$ CYN) amounts of toxin were present by the end of the second (depuration) phase.

5.3.3 Growth of *H. verticillata*

Chlorosis or necrosis was not recorded in any experiment (Plates 5.1; 5.2); browning off of lateral stems was observed in trial three. In trials one and four, plants exposed



Plates 5.1 & 5.2 Example of *Hydrilla verticillata* plants on day fourteen of trial one (control and 250 $\mu\text{g L}^{-1}$ toxin treatments, respectively). Arrows indicate approximate length of the fragment used to begin the trial.

to extracts containing 100 or 250 $\mu\text{g L}^{-1}$ CYN_{EXC} were brittle and fractured when they were handled. In all trials, plants exposed to extracts containing $\geq 200 \mu\text{g L}^{-1}$ CYN_{EXC} had sticky, elastic roots, with root tips lacking structure.

Significant interactions were detected in every trial except trial four (MANOVA, Table 5.3). In some test chambers, exposure effects were increased by increasing exposure time (see exemplars in Figure 5.1; remaining graphs available in Appendix D). The concentration of CYN_{EXC} (and possibly other *C. raciborskii* cell substances) significantly increased the growth of *Hydrilla* with respect to root and lateral stem production (univariate ANOVA and post-hoc analyses, Figure 5.1A, B; Table 5.3). Several other parameters also recorded increased growth with increasing toxin exposure concentrations (RGR biomass, internode distance), although these were not statistically significant ($\alpha > 0.010$) (Figure 5.1C). Significant differences were present only between the control and highest exposure concentrations, with toxin-exposed plants growing more vigorously. Increased exposure time also corresponded to increased plant growth.

Table 5.3 Summary results for two-way MANOVAs (Roy's Largest Root Statistic) followed by ANOVAs for growth of *Hydrilla verticillata* exposed to whole cell extracts of *Cylindrospermopsis raciborskii* containing cylindrospermopsin. NS = not significant ($p > 0.010$ for heterogeneous datasets).

		Trial one	Trial two	Trial three	Trial four
MANOVA	Treatment	NS	$p < 0.001$; $F_{10,19} = 9.431$	$p < 0.001$; $F_{10,18} = 9.178$	$p = 0.004$; $F_{10,14} = 4.746$
	Time	NS	$p < 0.001$; $F_{10,15} = 10.738$	NS	$p = 0.005$; $F_{10,11} = 5.540$
	Interaction	$p = 0.002$; $F_{10,4} = 31.725$	$p = 0.003$; $F_{10,19} = 4.456$	$p = 0.001$; $F_{10,18} = 45.840$	NS
Univariate ANOVAs					
RGR (main stem length)	Treatment	NS	NS	NS	NS
	Time	NS	$p = 0.000$; $F_{1,24} = 21.868$	NS	NS
	Interaction	NS	NS	NS	NS
RGR (number of nodes)	Treatment	NS	NS	NS	NS
	Time	NS	NS	NS	NS
	Interaction	NS	NS	NS	NS
RGR (biomass increase)	Treatment	NS	NS	NS	NS
	Time	NS	NS	$p = 0.006$; $F_{1,23} = 9.025$	NS
	Interaction	NS	NS	NS	NS
Internode distance	Treatment	NS	NS	NS	NS
	Time	NS	NS	NS	NS
	Interaction	NS	NS	NS	NS
Number of lateral stems	Treatment	NS	NS	NS	NS
	Time	NS	$p = 0.009$; $F_{1,24} = 8.167$	NS	NS
	Interaction	NS	NS	NS	NS
Cumulative length lateral stems	Treatment	$p = 0.009$; $F_{2,12} = 7.195$	NS	NS	NS
	Time	$p = 0.002$; $F_{1,12} = 15.457$	$p < 0.001$; $F_{1,24} = 45.229$	$p = 0.007$; $F_{1,23} = 8.715$	$p < 0.001$; $F_{1,20} = 20.169$
	Interaction	NS	NS	NS	NS
Average length lateral stems	Treatment	NS	NS	NS	NS
	Time	$p < 0.001$; $F_{1,12} = 25.294$	$p < 0.001$; $F_{1,24} = 45.717$	$p = 0.001$; $F_{1,23} = 13.630$	$p < 0.001$; $F_{1,20} = 30.849$
	Interaction	NS	NS	NS	NS
Number of roots	Treatment	NS	$p < 0.001$; $F_{5,24} = 9.064$	NS	NS
	Time	NS	NS	NS	NS
	Interaction	$p = 0.001$; $F_{2,12} = 11.818$	NS	NS	NS
Cumulative length roots	Treatment	NS	$p = 0.001$; $F_{5,24} = 96.524$	NS	NS
	Time	NS	NS	NS	NS
	Interaction	$p = 0.003$; $F_{2,12} = 9.452$	$p = 0.007$; $F_{5,24} = 4.195$	NS	NS
Average length roots	Treatment	NS	NS	NS	NS
	Time	NS	NS	NS	NS
	Interaction	NS	NS	NS	NS

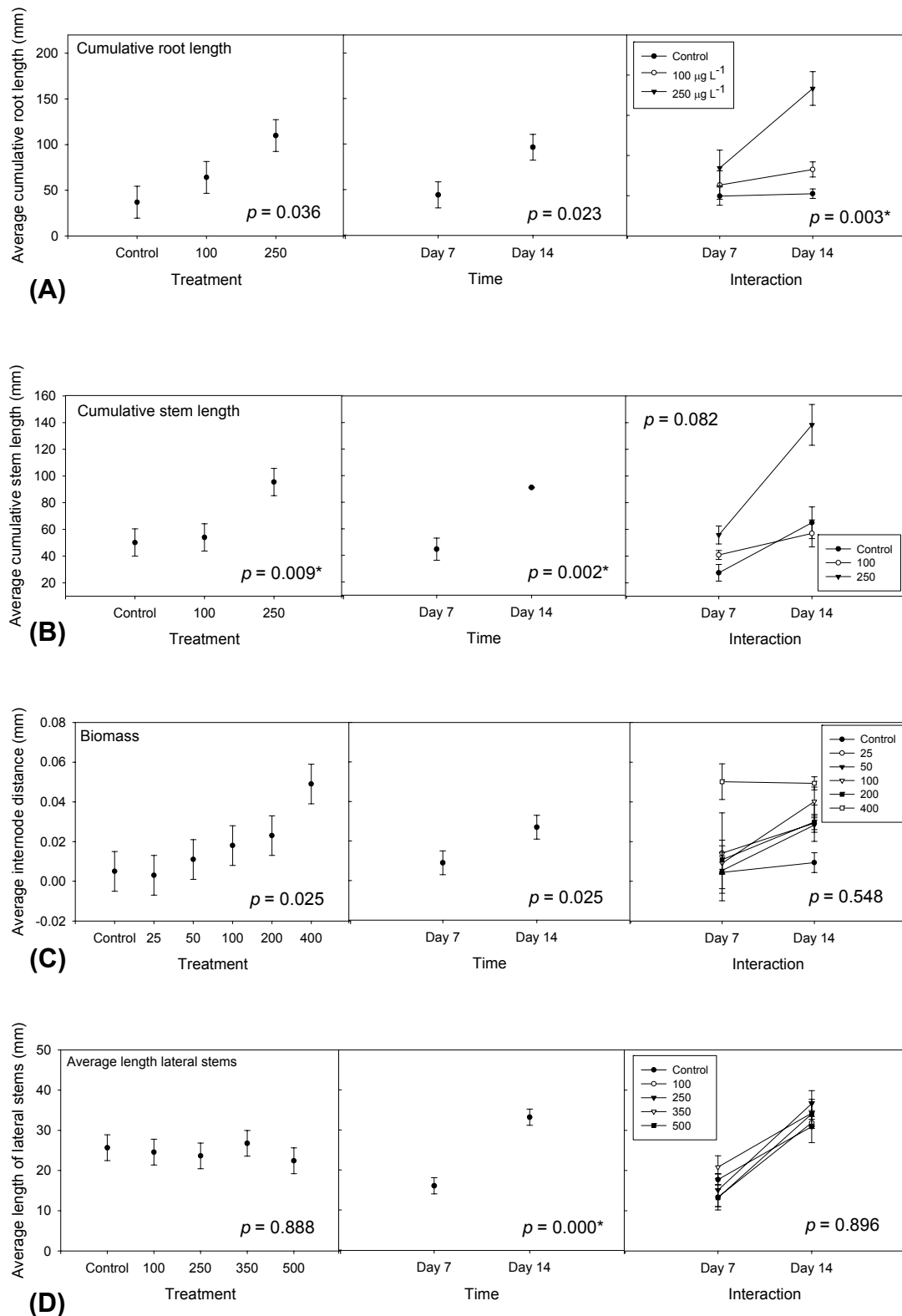


Figure 5.1 Example results for *Hydrilla verticillata* following exposure to *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin. (A) Cumulative root length, trial one; (B) cumulative stem length, trial one; (C) relative growth rate (biomass) trial two; (D) average length lateral stems, trial four. Bars show means (pooled for treatment and time) \pm standard error. * indicates significance at $p = 0.010$. Reader note: remaining graphs available in appendix D.

Exposure effects were variable in trial four; some growth parameters recorded increased growth with increased exposure concentration. Plant growth during the CYN-free phase was usually higher compared with growth during the exposure phase. Significant interactions were not present for any growth parameters (Figure 5.1D, Table 5.3, and see Appendix D).

Increased CYN_{EXC} (and *C. raciborskii* debris) exposure concentrations were positively correlated with both plant biomass and root production (Pearson Product Moment Correlation, Table 5.4). However, higher root production appeared to occur at the expense of main stem elongation, at least in trial three (Table 5.4). In trial four, lateral stem production was negatively correlated with both toxin exposure concentrations and with plant biomass. Finally, exposure treatment/exposure time combinations were clustered using Bray-Curtis similarity analyses. There were no obvious groupings according to toxin concentration nor exposure time during trial one; 88% similarity was attained across all groups (Figure 5.2A, B). In contrast, values were strongly clustered according to exposure phase in trial two, excepting the control/day fourteen data (Figure 5.3A).

Three distinct groupings were evident on the multidimensional scaling (MDS) plot. These comprised groups of data from the first and second weeks of exposure, with the controls forming an isolated group of their own (92% similarity) (Figure 5.3A, B). Treatments containing 400 µg L⁻¹ CYN_{EXC} appeared to be slightly set apart from all other treatment concentrations, in both exposure phases.

Table 5.4 Statistically significant ($p < 0.050$) Pearson Product Moment correlations between *Hydrilla verticillata* growth parameters and nominal cylindrospermopsin exposure concentrations. (+) = positive; (-) = negative; NS = not significant; CYN = cylindrospermopsin.

		Trial two (week 1)	Trial two (week 2)	Trial three (week 1)	Trial three (week 2)	Trial four (week 1)	Trial four (week 2)
CYN Exposure	RGR biomass	+	+	NS	+	NS	
	Number of roots	NS	+	NS	NS	NS	Not applicable ^a
	Average root length	NS	+	NS	NS	NS	
	Cumulative root length	NS	NS	NS	+	NS	
	Number of lateral stems	NS	NS	NS	NS	-	
RGR Main stem	RGR node number	NS	+	NS		NS	+
	RGR biomass	NS	NS	+	+	NS	NS
	Number of roots	NS	NS	NS	-	NS	NS
	Average root length	NS	NS	NS	-	NS	NS
RGR Node number	RGR biomass	+	NS	NS	NS	NS	NS
	Cumulative root length	+	NS	NS	NS	NS	NS
RGR Biomass	Number of roots	NS	+	NS	NS	NS	NS
	Average root length	NS	+	NS	NS	NS	NS
	Cumulative root length	+	NS	+	NS	NS	NS
	Number of lateral stems	NS	NS	NS	NS	NS	-
Internode number	Average root length	-	NS	NS	NS	NS	NS
	Cumulative stem length	NS	-	NS	NS	NS	NS
Number of lateral stems	Average stem length	+	+	+	+	+	+
	Cumulative stem length	NS	NS	NS	NS	+	NS
	Number of roots	+	NS	+	NS	NS	NS
	Average root length	+	NS	+	NS	NS	NS
Average length lateral stems	Number of roots	+	NS	+	NS	NS	NS
	Average root length	+	NS	+	NS	+	+
	Cumulative stem length	NS	+	NS	+	+	+
	Cumulative root length	NS	NS	NS	NS	NS	+
Cumulative length lateral stems	Cumulative root length	NS	NS	NS	NS	NS	+
Number of roots	Average root length	+	+	+	+	+	+
	Cumulative root length	NS	+	NS	+		
Average length roots	Cumulative root length	NS	+	+	+	+	+

^acould not be calculated, CYN not added to test solutions during the depuration phase

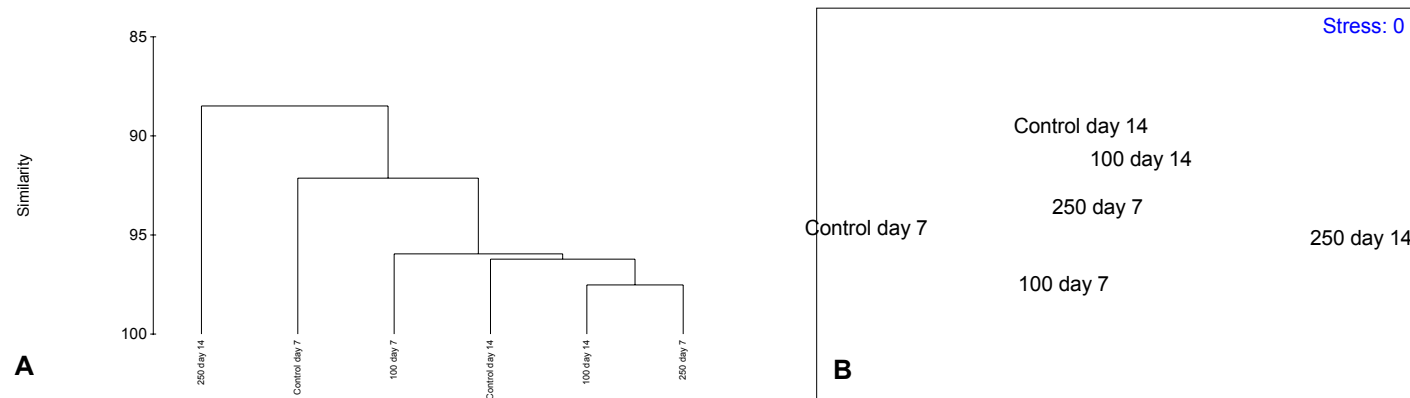


Figure 5.2 Analyses of *Hydrilla* growth parameters from trial one: (A) dendrogram of hierarchical clustering based on Bray-Curtis similarities of natural-log transformed data and group average linkages; (B) multidimensional scaling plot of Bray-Curtis similarity data. Labels show treatment ($\mu\text{g L}^{-1}$ CYN) followed by exposure time.

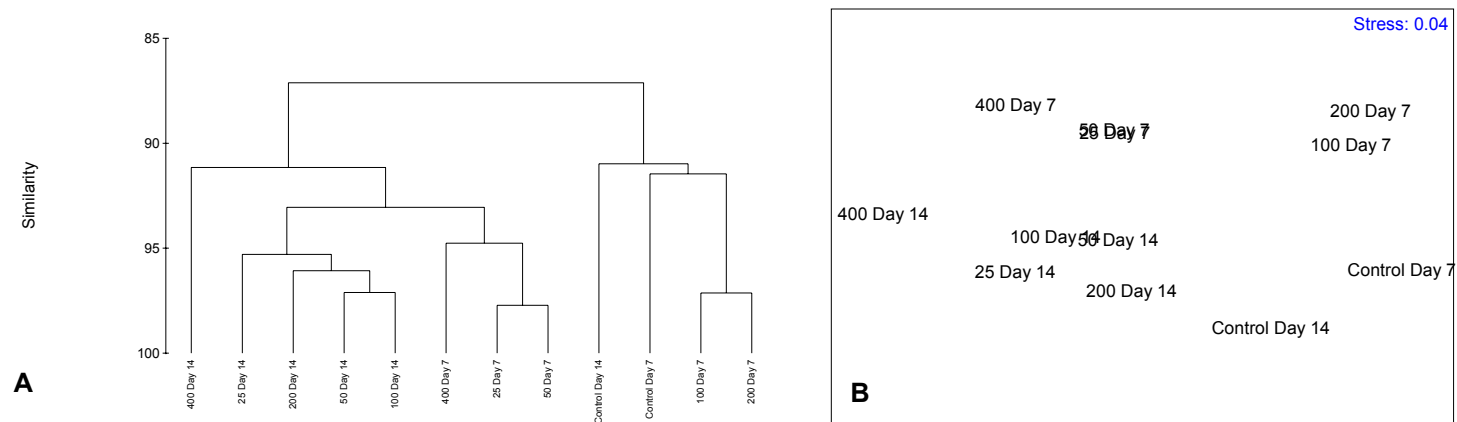


Figure 5.3 Analyses of *Hydrilla* growth parameters from trial two: (A) dendrogram of hierarchical clustering based on Bray-Curtis similarities of natural-log transformed data and group average linkages; (B) multidimensional scaling plot of Bray-Curtis similarity data. Labels show treatment ($\mu\text{g L}^{-1}$ CYN) followed by exposure time.

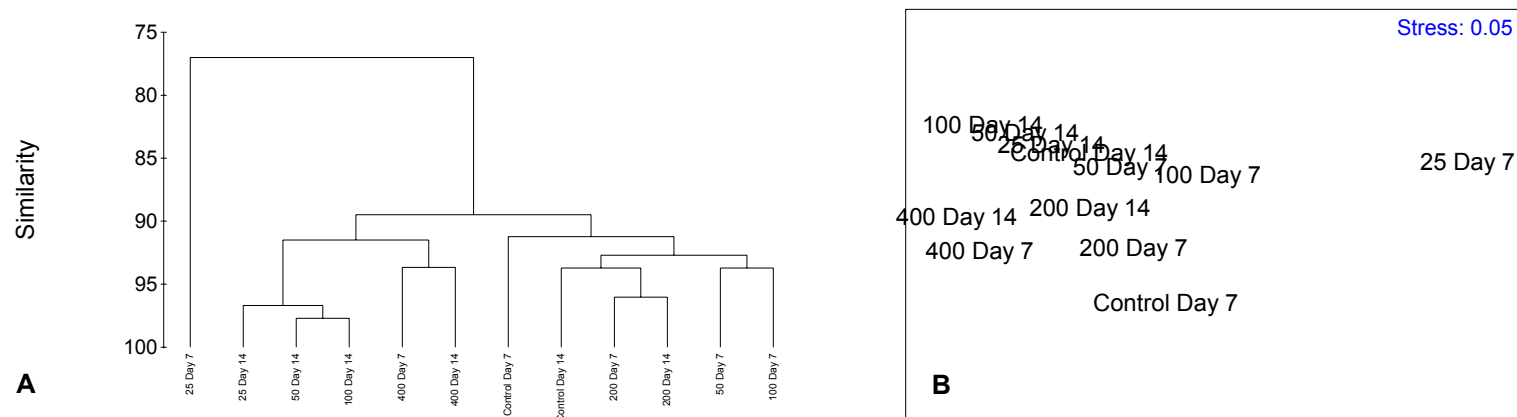


Figure 5.4 Analyses of *Hydrilla* growth parameters from trial three: (A) dendrogram of hierarchical clustering based on Bray-Curtis similarities of natural-log transformed data and group average linkages; (B) multidimensional scaling plot of Bray-Curtis similarity data. Labels show treatment ($\mu\text{g L}^{-1}$ CYN) followed by exposure time.

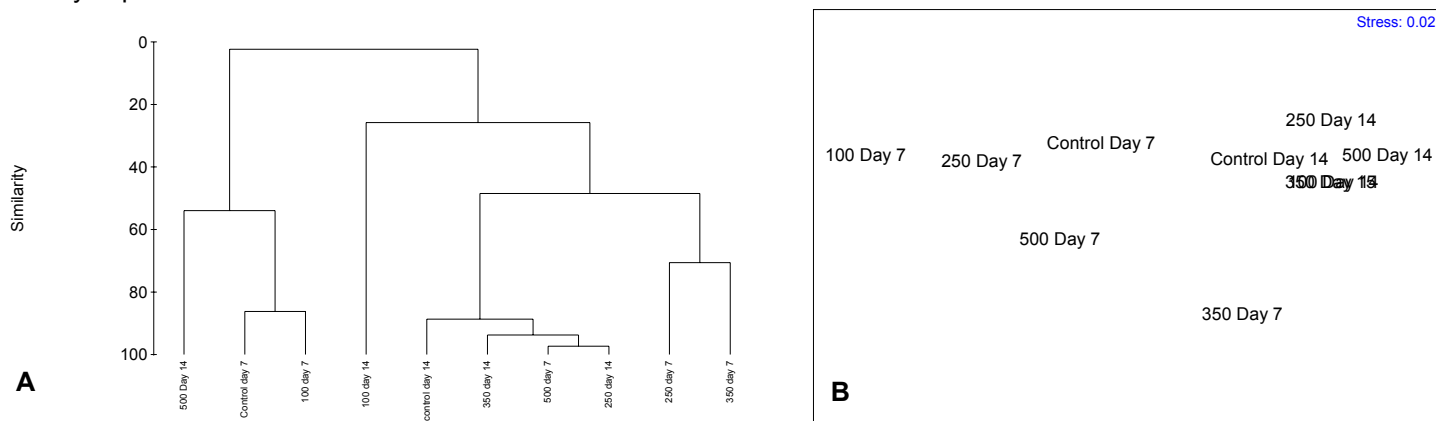


Figure 5.5 Analyses of *Hydrilla* growth parameters from trial four: (A) dendrogram of hierarchical clustering based on Bray-Curtis similarities of natural-log transformed data and group average linkages; (B) multidimensional scaling plot of Bray-Curtis similarity data. Labels show treatment ($\mu\text{g L}^{-1}$ CYN) followed by exposure time.

In trial three, controls again shared 92% similarity (Figure 5.4A). However, the differentiation of groupings based on exposure time were not clear (Figure 5.4A). Tight clustering of the day-fourteen data, at least, was evident on the MDS plot (Figure 5.4B); again, the two treatments containing 400 $\mu\text{g L}^{-1}$ CYN_{EXC} were slightly isolated from all the other exposure regimes. The dendrogram for trial four data grouped samples according to exposure phase (Figure 5.5A). On the MDS plot, treatments were widely spaced during the first (toxin-exposed) week, but strongly clustered (97% similarity) in the CYN-free week (Figure 5.5A, B).

5.3.4 Chlorophyll content

The effects of CYN exposure on *H. verticillata* chlorophyll content were variable. The concentration of CYN_{EXC} was associated with significantly decreased chlorophyll content during trial two, although there was no change to the relative concentrations of chlorophyll *a* and *b* (Table 5.5; Figure 5.6 A-D). In contrast, there was a significant interaction effect present in trial three (Table 5.5). Significant effects resulting from CYN exposure concentration or exposure time were not detected in trial four (Table 5.5).

Increasing CYN_{EXC} exposure concentrations were negatively correlated with chlorophyll *a*, chlorophyll *b* and total chlorophyll values during trial two (Table 5.6). In contrast, significant correlations were not present during trial three, except a positive correlation between the chlorophyll *a:b* ratio and CYN exposure in the first exposure week (Table 5.6).

Table 5.5 Summary results for two-way MANOVAs (Roy's Largest Root Statistic) followed by univariate ANOVAs for chlorophyll data of *Hydrilla verticillata* exposed to whole cell extracts of *Cylindrospermopsis raciborskii* containing cylindrospermopsin. NS = not significant, $p > 0.010$.

	Trial one (0 – 250 µg L ⁻¹ CYN _{EXC})	Trial two (0 – 400 µg L ⁻¹ CYN _{EXC})	Trial three (0 – 400 µg L ⁻¹ CYN _{EXC})	Trial four (0 – 500 µg L ⁻¹ CYN _{EXC})
MANOVA				
Treatment	NS	$p < 0.001$; $F_{5,21} = 15.714$	NS	NS
Exposure time	$p = 0.003$; $F_{4,9} = 8.967$	NS	NS	NS
Interaction	NS	NS	$p = 0.001$; $F_{5,22} = 6.038$	NS
Univariate ANOVA				
Chlorophyll a				
Treatment	NS	$p = 0.003$; $F_{5,21} = 5.156$	NS	NS
Exposure time	NS	NS	NS	NS
Interaction	NS	NS	NS	NS
Chlorophyll b				
Treatment	NS	$p < 0.001$; $F_{5,21} = 9.476$	NS	NS
Exposure time	NS	NS	NS	NS
Interaction	NS	NS	NS	NS
Total chlorophyll				
Treatment	NS	$p < 0.001$; $F_{5,21} = 9.722$	NS	NS
Exposure time	NS	NS	NS	NS
Interaction	NS	NS	NS	NS
Chlorophyll a:b ratio				
Treatment	NS	NS	NS	NS
Exposure time	$p = 0.001$; $F_{1,12} = 2.613$	NS	NS	NS
Interaction	NS	NS	$p = 0.005$; $F_{5,22} = 4.533$	NS

Table 5.6 Statistically significant ($p < 0.050$) Pearson Product Moment Correlations between chlorophyll values of *Hydrilla verticillata* and nominal cylindrospermopsin exposure concentrations. (+) positive; (-) negative; ns = not significant.

		Trial two (week 1)	Trial two (week 2)	Trial three (week 1)	Trial three (week 2)	Trial four (week 1)	Trial four (week 2)
Nominal cylindrospermopsin exposure concentrations	Chlorophyll <i>a</i>	-	-	NS	NS	NS	Not applicable ^a
	Chlorophyll <i>b</i>	-	-	NS	NS	-	
	Total chlorophyll	-	-	NS	NS	NS	
	<i>a:b</i> ratio	NS	NS	+	NS	NS	
Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	+	+	+	+	+	
	Total chlorophyll	+	+	+	+	+	
	<i>a:b</i> ratio	NS	NS	NS	NS	NS	
Chlorophyll <i>b</i>	Total chlorophyll	+	+	+	+	+	
	<i>a:b</i> ratio	-	NS	NS	NS	NS	

^acould not be calculated, toxin not added during depuration phase.

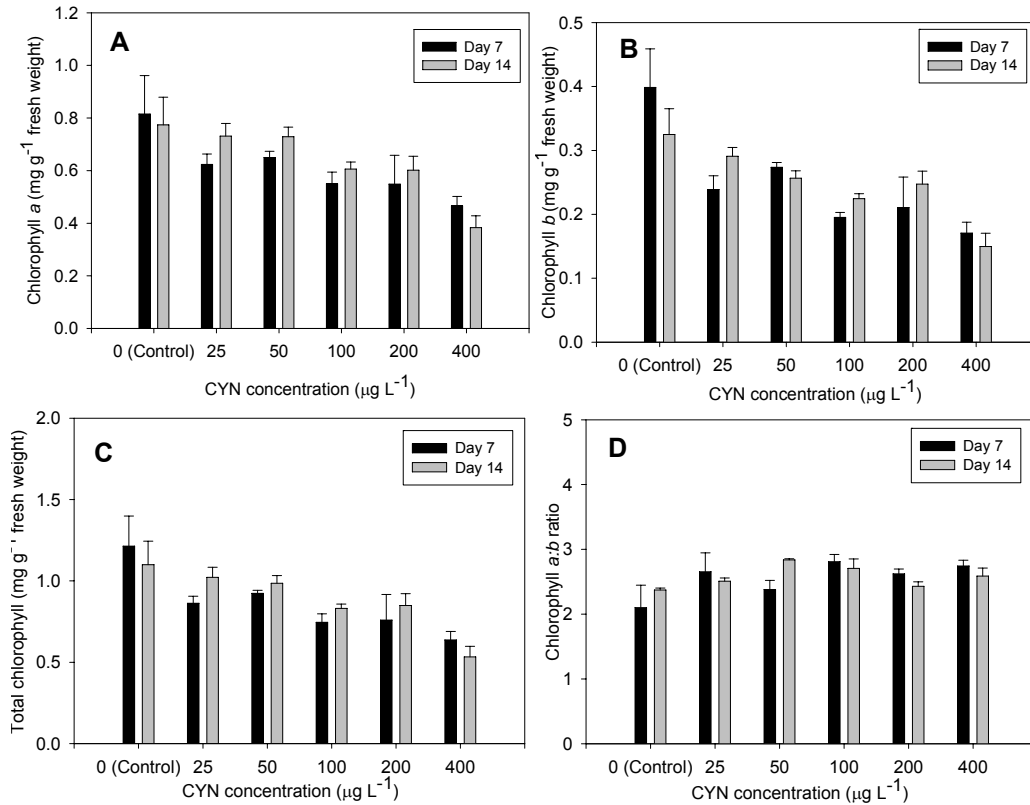


Figure 5.6 Chlorophyll content of *Hydrilla verticillata* during trial two (A) chlorophyll a, (B) chlorophyll b, (C) total chlorophyll, (D) chlorophyll a:b ratio. Graphs show average ($n = 2$) \pm standard error.

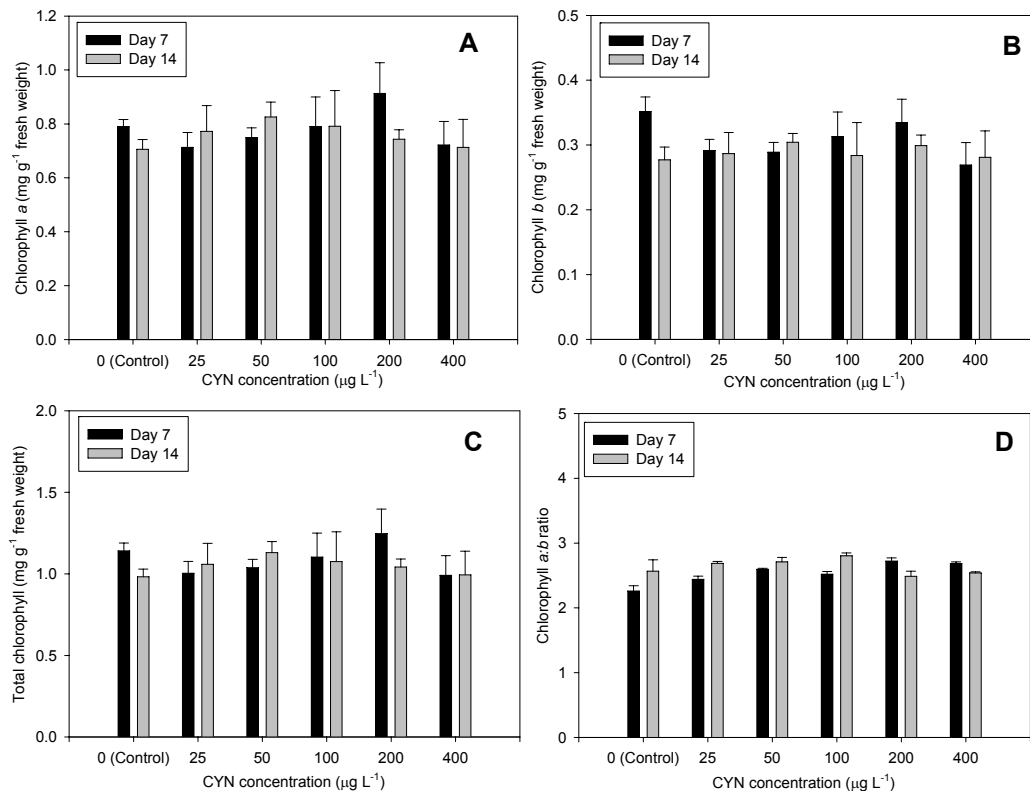


Figure 5.7 Chlorophyll content of *Hydrilla verticillata* during trial three (A) chlorophyll a, (B) chlorophyll b, (C) total chlorophyll, (D) chlorophyll a:b ratio. Graphs show average ($n = 2$) \pm standard error.

5.3.5 Bioconcentration of free CYN

A maximum of 176 µg free CYN kg⁻¹ freeze-dried tissue was recorded from *H. verticillata* (Figure 5.8A, B). Plants in high-exposure treatments ($\geq 100 \mu\text{g L}^{-1}$) recorded the highest tissue toxin concentrations. Exposure concentrations of 50 µg L⁻¹ or below resulted in little or no free CYN in the tissues. Little free CYN was present in most plants by the end of the depuration phase (Figure 5.8C). In trials two and three, tissue toxin concentrations significantly positively correlated with CYN exposure concentrations ($p < 0.001$ for both trials).

Based on a regression equation calculated from the fresh and freeze-dried data from trials two and three, maximum toxin concentrations were equivalent to 15 µg free CYN kg⁻¹ fresh weight ($y = 0.3823 + (0.0825x)$; $r^2 = 0.9140$; SigmaStat 3.0). The average bioconcentration factor¹ (BCF; fresh weight tissue concentration / exposure concentration) over trials two and three was 0.04 and 0.036 for seven and fourteen day's exposure, respectively (Table 5.7). In trial four, BCFs were similar to those obtained in earlier trials, but toxin was not detected during the second phase of the experiment.

Free deoxy-CYN concentrations were similarly low, peaking at approximately 160 µg kg⁻¹ freeze-dried tissue (Figure 5.9), roughly equivalent to 15 µg kg⁻¹ fresh weight ($y = 0.1208 + (0.0908x)$; $r^2 = 0.9517$). The BCF¹ was not calculated since deoxy-CYN exposure concentrations were unknown.

¹ bioconcentration factor (BCF) used compared with bioaccumulation factor (BAF) since uptake in aquatic plants is considered only possible from extracellular (dissolved) toxin sources.

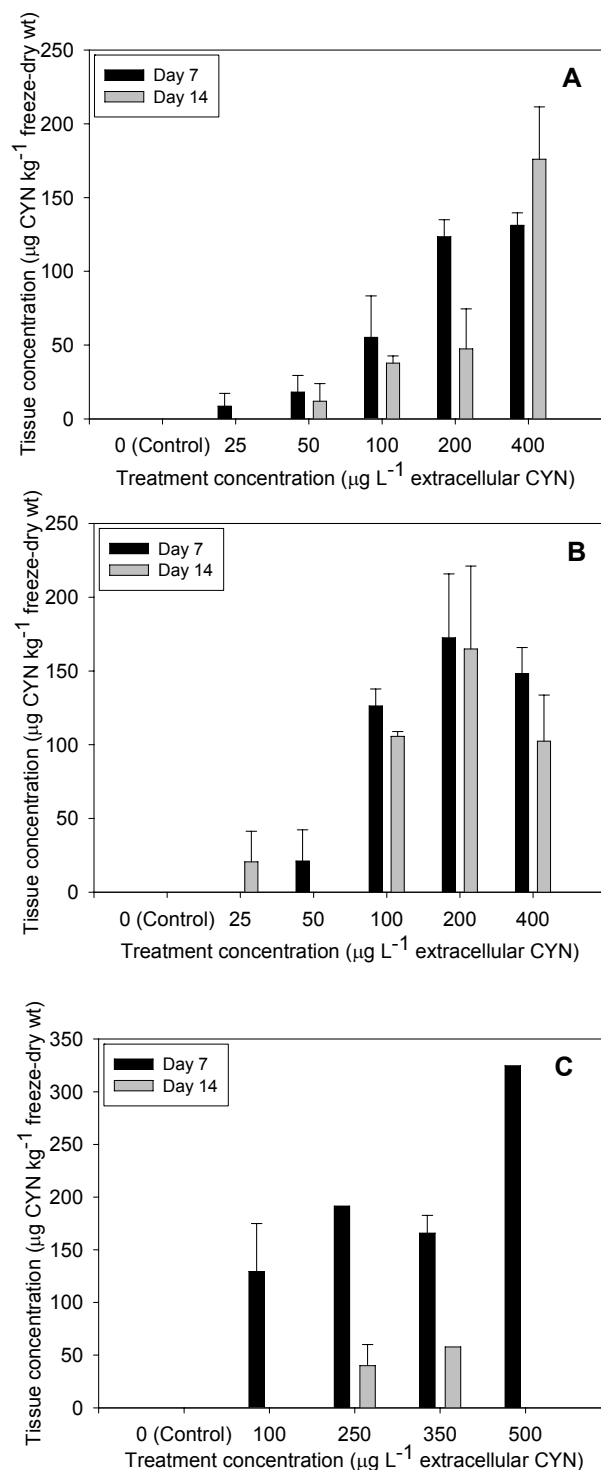


Figure 5.8 Free CYN concentrations in the tissues of *Hydrilla verticillata* following exposure to *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin: (A) trial two, (B) trial three, (C) trial four. Bars depict the average ($n = 3$) \pm standard error. Selected samples only tested in (C).

Table 5.7 Bioconcentration factors for *Hydrilla verticillata* following seven and fourteen days exposure to *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin. CYN_{EXC} = extracellular cylindrospermopsin.

Nominal concentration ($\mu\text{g L}^{-1}$ CYN _{EXC})		Bioconcentration factor (average \pm standard error)	
		7 days exposure	14 days exposure
Trial two			
	25	0.02 ± 0.02	0 ± 0
	50	0.05 ± 0.03	0.01 ± 0.01
	100	0.05 ± 0.03	0.03 ± 0.0
	200	0.05 ± 0.01	0.03 ± 0.01
	400	0.03 ± 0.0	0.03 ± 0.00
Trial three			
	25	0.0 ± 0.0	0.07 ± 0.07
	50	0.05 ± 0.05	0.0 ± 0.0
	100	0.12 ± 0.02	0.11 ± 0.0
	200	0.09 ± 0.02	0.06 ± 0.02
	400	0.04 ± 0.01	0.02 ± 0.00
Average over both trials		0.05	0.036
Trial four			
	150	0.07 ± 0.01	0 ± 0
	250	0.02 ± 0.02	0.01 ± 0
	350	0.03 ± 0.0	0 ± 0
	500	0.01 ± 0.01	0 ± 0

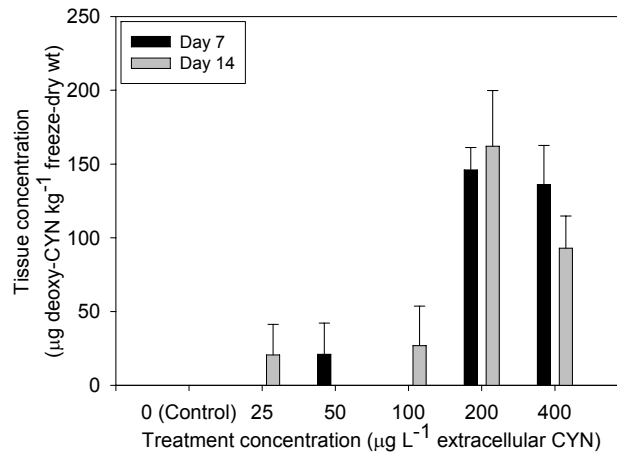


Figure 5.9 Free deoxy-CYN concentrations in the tissues of *Hydrilla verticillata* during trial three. Bars depict the average ($n = 3$) \pm standard error.

5.4 Discussion

5.4.1 Water quality

High pH values in some treatments may be partly explained by the original pH of the algal cultures used to prepare the treatments. However, photosynthetic activity and secretion of hydroxide ions by *H. verticillata* may also have contributed to elevated pH (see 4.4.1). Increased conductivities probably resulted from evaporative concentration.

5.4.2 CYN treatment concentrations

Relying on single CYN values for culture strength may contribute to inaccuracy in CYN test concentrations (see section 4.4.5). Unfortunately, these results were not available prior to conducting trials one and four. Hence, CYN recoveries from test solutions were lower than expected at the end of these two trials. In contrast, the average of five determinations was used to prepare treatments in trials two and three, resulting in CYN values closer to the desired concentrations (Table 5.2). CYN concentrations detected in the second phase of trial four (Table 5.2) may represent CYN introduced by association with the *Hydrilla* plant material, especially as material was not rinsed before transfer into CYN-free dishes in the second week.

5.4.3 Growth of *H. verticillata*

H. verticillata growth was stimulated by exposure to the whole cell extracts containing CYN_{EXC}. The greatest increases in growth – particularly in the roots and lateral stems – were recorded in treatments with maximum toxin concentrations (400 µg L⁻¹). In contrast, similar (stimulant) effects in *S.*

oligorrhiza were observed at far lower concentrations of CYN_{EXC} ($< 100 \mu\text{g L}^{-1}$, see 4.4.3). Possibly, the larger surface area to volume ratio of *S. oligorrhiza* may act to increase available CYN uptake sites, thus increasing toxicity risk. In addition, *H. verticillata* may be a stronger competitor compared with other aquatic macrophytes (Gopal & Goel 1993, p. 166), thus being able to withstand higher toxin exposure concentrations. The brittleness of some plants could conceivably contribute to bloom avoidance by *Hydrilla*: stem fragments which are easily separated from the main stem might increase plant dispersal and subsequent colonisation of other (non-CYN contaminated) environments.

Exposure to CYN-containing solutions appeared to promote the redistribution of plant resources in *H. verticillata*, possibly to achieve maximal plant growth. Root production in trial three, for example, was accelerated at the expense of the main stem, although this did not result in compromised plant biomass (Table 5.4). In contrast, in week one of trial four, lateral stem production decreased with CYN and *C. raciborskii* exposure and was correlated with decreased plant biomass (Table 5.4). Maximised root production together with minimised lateral stem production may be a strategy by which *H. verticillata* maintains or increases plant growth during a toxic bloom. Reduced chlorophyll values recorded in conjunction with CYN exposure further supports this argument, since production of extra stem portions may offer minimal survival advantage because of decreased chlorophyll content.

5.4.2.1 Benefits of increased root production

Increased root production is notable since *Hydrilla* usually invests little in root sections compared to other macrophytes (low root tissue to foliage ratio) (Gopal

& Goel 1993, p. 166). Several benefits may be conferred by additional root biomass. Increasing the relative proportion of roots may decrease CYN concentration and toxicity; especially if root and shoot sections have differential rates of CYN uptake. This occurs with *H. verticillata* and mefluidide (Liu & Lembi 1999). However, investigations of algal toxins other than CYN have conflicting results: *Elodea canadensis* has greater MC concentrations in leaves (Pflugmacher *et al.* 1998), whereas *Vallisneria natans* appears to stockpile toxin in the roots (Yin *et al.* 2005). Moreover, the proportion of total plant biomass represented by the roots of *Hydrilla* is minor compared with the leaves.

The uptake dynamics of root-and-shoot sections are likely to depend on both the species-specific roles of each tissue type and the habit of the plant (submerged, floating or emergent). It is conceivable that macrophytes that take up nutrients via roots and leaves may also experience toxin uptake in these tissues. Furthermore, if roots are normally buried in the substrate (as in *Hydrilla*), bioavailable CYN concentrations may be less than those in the water column, thus minimizing uptake. This benefit would also be compounded where root production occurs at the expense of leaves and stems, thereby further reducing ‘vulnerable’ plant surface area in the water column.

Increased root production may offer the advantage of decreasing CYN detoxification mechanisms. Inhibition of cytochrome P-450 is known to offer some protection from CYN, possibly because P-450 is involved in the production of breakdown metabolites more toxic than the parent molecule (Runnegar *et al.* 1995). If *H. verticillata* roots are located in a waterlogged substrate, oxygen supplies necessary for P-450 reactions may not be available. Consequently,

reduced P-450 activity in the roots of *Hydrilla* may be linked with reduced CYN toxicity. Studies of detoxication enzymes in the reed *Phragmites australis* have revealed that highest enzyme activities occur in the root (Pflugmacher *et al.* 1999b). However, *P. australis* is specially adapted to a submerged life via the use of internal air spaces that allow increased oxygen for P-450 reactions; root peroxidase activity in *P. australis* is reportedly twice that of whole *H. verticillata* (Byl *et al.* 1994; Pflugmacher *et al.* 1999b). Reduced *P. australis* root masses have also been observed in a Japanese lake experiencing perennial toxic *M. aeruginosa* blooms (Yamasaki 1993), further suggesting a relationship between root detoxication mechanisms and algal toxins.

Increased root production in *H. verticillata* may also confer increased plant protection against CYN (or other *C. raciborskii*) toxicity via the production of root compounds. Some suggestion of these compounds was recorded in the present work: plants with increased roots attained higher overall biomass, and roots in high CYN-concentration treatments were sticky and elastic, possibly indicating the excretion of strong compounds. Such root compounds may include plant root exudates, allelochemicals or phytochelatins.

Root exudates are chemicals usually released to increase solubilisation of metals or nutrients, such that maximal absorption in the root zone is achieved (Hall 2002). Root exudates from *H. verticillata* may bind to CYN, thereby preventing the molecule from entering plant cells: this process is known to occur for heavy metals (Samecka-Cymerman & Kempers 1996). However, as root exudates are primarily proteins, their synthesis may be inhibited by CYN exposure. Their production in CYN-stressed plants thus appears unlikely. Furthermore, the

release of such compounds into the sediments may offer little advantage given that most available CYN would be located in the overlying water column.

Allelochemicals are released by plants to achieve a competitive advantage over other plant or phytoplankton species (Gopal & Goel 1993). The role(s) of such chemicals are poorly understood in aquatic plants (Sutton & Portier 1989). Several aquatic macrophytes produce allelochemicals, including fatty acids, which reduce growth of cyanoprokaryotes such as *Microcystis*, *Anabaena* and *Phormidium* (Gopal & Goel 1993; Nakai *et al.* 1999; Körner & Nicklisch 2002; Nakai *et al.* 2005). Whether *Hydrilla* produces allelochemicals effective for *C. raciborskii* is unknown. Moreover, release of chemicals from the roots may not target planktonic *C. raciborskii*: compounds originating from the leaves could better achieve this.

Lastly, phytochelatins (PCs) are low molecular weight, metal-rich complexes, usually associated with plant protection against heavy metals (Stillman *et al.* 1992). PCs may also act as antioxidants by scavenging free radicals (Stillman *et al.* 1992). *Hydrilla* effectively detoxifies both cadmium and lead via the use of phytochelatins (Gupta *et al.* 1995; Tripathi *et al.* 1996). It is possible, therefore, that PCs are also synthesized by *Hydrilla* in response to CYN exposure.

5.4.3 Changes to chlorophyll content

Significant decreases in chlorophyll resulting from CYN and *C. raciborskii* exposure (as recorded in trial two) may indicate the interaction of CYN with enzymes involved in chlorophyll synthesis. This process was recorded in trials of *Hydrilla* with cadmium and fluridone (Netherland & Getsinger 1995; Garg *et*

al. 1997). Decreased chlorophyll content may also result from accelerated pigment decomposition: this has been suggested with MC exposure (Weiß *et al.* 2000).

5.4.4 Free CYN and deoxy-CYN bioconcentration

Free CYN bioconcentration did not occur in *H. verticillata* ($BCF < 1$). Previous work with *S. oligorrhiza* has indicated that any detected free CYN is likely to represent toxin adsorbed to the cell wall of *H. verticillata*, rather than truly intracellular toxin (see 4.4.4). CYN values recorded from the depuration trial are also consistent with adsorption and ‘rinsing’ of toxins from the plant surface area. Free deoxy-CYN concentrations were quite similar to those of CYN: again, bioconcentration was not recorded. Possible effects of the presence of deoxy-CYN in *H. verticillata* tissues are unknown, since there is confusion in the literature regarding deoxy-CYN toxicity (Norris *et al.* 1999; Looper *et al.* 2005).

Chapter six: general discussion of exposure effects and bioconcentration in aquatic plants

6.1 Mechanism of action in aquatic macrophytes

Protein synthesis inhibition (PSI) is thought to be the primary mode of action for CYN toxicity (Runnegar *et al.* 1994; Runnegar *et al.* 1995; Shaw *et al.* 2000). Both *Spirodela* and *Hydrilla* recorded exposure effects that could result from PSI. In both test species, exposure to whole cell extracts of *C. raciborskii* containing low concentrations of CYN ($\leq 400 \mu\text{g L}^{-1}$) resulted in growth stimulation, even when growth inhibition was sometimes recorded beforehand (for example, in the *Spirodela*). Humpage and Falconer (2003) noted that PSI resulting from low dose CYN exposures might ‘cause growth of organs that synthesise proteins required for homeostasis, and that [growth] increases could be compensatory hypertrophy’. Thus, whilst CYN toxicity may cause reduced protein synthesis in selected cells, it is possible that it could also cause a stimulatory effect. Nonetheless, PSI cannot be the only cause of CYN toxicity in plants: Metcalf *et al.* (2004) reported that inhibition of pollen germination in tobacco seedlings was significantly inhibited, but this could not have resulted from PSI; since protein synthesis does not occur in dormant pollen.

An alternate mode of toxic action applicable to plants might be the inhibition of glutathione (GSH) synthesis. Inhibition of GSH synthesis by CYN appears to require bioactivation by cytochrome P-450 enzymes, at least in mammalian hepatocytes (Terao *et al.* 1994; Runnegar *et al.* 1995; Froscio *et al.* 2003; Humpage *et al.* 2005). The cells of *S. oligorrhiza* and *H. verticillata* could have large concentrations of P-450 enzymes, since plants require these for the synthesis of chlorophyll pigments,

growth regulators and plant toxins (Anzenbacher & Anzenbacherová 2001). If so, increased CYN toxicity could result from enzymatic activity: Shaw *et al.* (2000) showed that CYN toxicity in primary rat hepatocytes was significantly higher than that in cultures of HeLa ketone body cells, due to higher P-450 activity in the former. However, since there may be only one P-450 family common to plants and animals (Nelson 1999), the dynamics of CYN toxicity relating to P-450 activity may be entirely different in these groups. For example, if P-450 enzymes that usually regulate growth and chlorophyll production are engaged in CYN processing, they may no longer be available for their usual roles: decreased cell growth and chlorophyll synthesis may result. However, no other studies of CYN have suggested this.

A third possible mechanism of CYN toxicity in plants may include a process unrelated to chlorophyll content. Vasas *et al.* (2002) reported growth inhibition in etiolated mustard seedlings: these plants have no chlorophyll present due to light deprivation. Such effects could result from a general plant response. For example, Pflugmacher (2004) theorised that lysed MC may prompt the oversupply of plant antioxidants, thus promoting the production of harmful, excess reactive oxygen species (ROS). Since CYN toxicity requires biotransformation (Runnegar *et al.* 1995), the formation of ROS by-products is possible (Pflugmacher 2004). However, antioxidant concentrations in conjunction with CYN exposure in aquatic plants have never been measured.

Growth ‘stimulation’ resulting from CYN and *C. raciborskii* exposures may be explained by the toxin exerting mild plant ‘stress’: this can activate cell metabolism and increase physiological activity, and several herbicides and plant growth regulators

have this effect at low concentrations (Lichtenthaler 1996). This is also not a surprising finding given that CYN is an alkaloid: many organic substances belonging to this group are ‘medicinal’ in small quantities whilst being highly toxic at high doses (for example, nicotine, cocaine and morphine).

In many cases, *Spirodela* and *Hydrilla* were both stimulated by low or intermediate toxin (and algal debris) concentrations, rather than high doses of toxin-containing cell extracts. Hawkins *et al.* (1985) have already reported that increased CYN doses do not necessarily result in increased toxicities: better survival times were recorded from IP injected mice when the administered doses of CYN were two to ten-fold higher (Hawkins *et al.* 1985). The reason for this is unclear, but may be linked with the formation of toxic metabolites during metabolism of the parent CYN molecule. For example, if aquatic macrophytes detoxify CYN during low exposure concentrations ($\leq 200 \mu\text{g L}^{-1}$), they may experience toxicity from CYN and its metabolites. In contrast, exposure to high toxin concentrations may retard detoxification mechanisms: this would also prevent formation of toxic metabolites, and hence possibly reduce toxicity.

6.2 Pathways for toxin uptake in aquatic macrophytes

Toxin uptake by both test species seems likely: exposure effects were recorded from both *S. oligorrhiza* and *H. verticillata*, and all the known mode(s) of action for CYN require the toxin to become integrated into the cells of the target organism to proceed. To date, there has not been a study to determine whether active or passive uptake is more likely in aquatic plants. Chong *et al.* (2002) concluded that passive CYN uptake was possible in rat hepatocytes, given the relatively small molecular weight of the toxin. CYN is also highly water soluble (Sivonen & Jones 1999), thereby increasing

the appeal of a diffusive uptake method. Should passive uptake occur, it is possible that differential uptake may occur between root and leaf sections: this has already been shown to occur for MC (Kurki-Helasmo & Meriluoto 1998; Pflugmacher *et al.* 2001; Yin *et al.* 2005).

The issue of cell permeability to CYN has not yet been examined, and cell permeability continues to be debated even for MC (Dawson 1998; Duy *et al.* 2000; Vesterkvist & Meriluoto 2003). In *Phragmites australis*, for example, small-diameter stems had increased MC uptake compared with those having large diameters (Pflugmacher *et al.* 2001), possibly because passive uptake is increased by larger surface area to volume ratios. It is also possible that CYN alters the membrane permeability of aquatic plants; this is the case with several plant hormones (Nilsen & Orcutt 1996). Further studies using radiolabelled toxin would help to determine toxin uptake methods and also if (and where) toxin binding occurs.

6.3 Comparing *Spirodela* with *Hydrilla*

6.3.1 Sublethal effects and susceptibility

The two test species appeared to have different CYN susceptibilities: different *C. raciborskii* (with CYN_{EXC}) exposure concentrations were required to produce similar stimulation effects in *Hydrilla* compared to *Spirodela*. For example, increases in the growth of *S. oligorrhiza* were often recorded from solutions containing 30 – 60 µg L⁻¹ CYN (Figure 4.5), whilst a similar response in *H. verticillata* was recorded at 400 µg L⁻¹. Growth stimulation has been recorded in *Sinapis alba* (white mustard) seedlings after exposure to 1 mg mL⁻¹ dry weight lyophilised *Aphanizomenon ovalisporum*, a cyanoprokaryote species capable of CYN production (Vasas *et al.* 2002).

Redistribution of plant resources was recorded in *H. verticillata* but not in *S. oligorrhiza*. This suggests possible variation in exposure effects due to differences in plant niches. In *Hydrilla*, for example, increased root growth at the expense of main and lateral stems may act to minimise CYN uptake and toxicity. In contrast, this may not hold true for *Spirodela*, since the roots are suspended in the water column. In fact, decreased root production in *Spirodela* may represent a mechanism to minimise toxin uptake: one study has shown that runner beans suffer root growth inhibition due to the presence of MC-LR, and subsequently take up 30% less media (by volume) than non-exposed plants (McElhiney *et al.* 2001). Thus, the niche of different aquatic macrophytes may affect the nature of CYN exposure risk (for example, increased hazards due to water column positioning where toxins may be concentrated).

6.3.2 Bioconcentration

Neither *Spirodela* nor *Hydrilla* bioconcentrated free-CYN; toxin adsorption was demonstrated in *Spirodela* and appeared similarly likely in *Hydrilla*. Maximum tissue CYN values in *S. oligorrhiza* (30 $\mu\text{g kg}^{-1}$ fresh weight) were twice those of *H. verticillata* (15 $\mu\text{g kg}^{-1}$ fresh weight). The adsorptive capacities of plants generally depend on their physiological condition and surface area to volume ratios (Guilizzoni 1991). Higher CYN values in *S. oligorrhiza* could indicate a larger surface area available for toxin adsorption. Yin *et al.* (2005) noted that surface area to volume ratios may be intrinsic in affecting bioaccumulation capabilities. However, Mitrovic *et al.* (2004) suggested *Ceratophyllum demersum* would have greater surface area for anatoxin-a uptake than *Lemna minor*. Dead *Spirodela polyrrhiza* plants have been shown to accumulate twice the cadmium of living plants, as cell permeability increased and intracellular accumulation occurred (Noraho & Gaur 1996). Thus, the

necrotic *S. oligorrhiza* fronds observed in the present work might represent increased opportunity for CYN sorption or accumulation.

Absence of free-CYN bioconcentration indicates either that (a) toxin cannot become intracellular; (b) that intracellular toxin is transported out of the cell at a rate equal to entry; or (c) that toxin, once intracellular, becomes bound or modified within plant tissues and hence cannot be detected via HPLC. If the exposure effects reported from both test plants are considered to relate to toxin exposure, toxin uptake must have occurred, given that the known modes of action for CYN require the molecule to be intracellular (see 6.1). This would discount (a) above; though some treatment effects may also have resulted from small differences in nutrient quantities between the test solutions. The capability for plants to transport CYN out of cells (b) is unknown. Toxin binding and/or modification (c) is known for MC, where covalent binding to protein phosphatases occurs (Williams *et al.* 1997; Kankaanpää *et al.* 2002). Microcystin might also be taken up by plant chloroplasts, be non-enzymatically bound or enzymatically conjugated to GSH, or be bound to protein phosphatases (MacKintosh *et al.* 1990; Pflugmacher 2002). If CYN similarly binds to plant chloroplasts, or forms toxic conjugates, this could explain why CYN bioaccumulation has been recorded from aquatic animals (Saker & Eaglesham 1999; Saker *et al.* 2004), whereas bioconcentration is absent in *S. oligorrhiza* and *H. verticillata*. However, this argument is also limited by those animals being filter feeders, since these could reasonably be expected to ingest greater quantities of toxin.

6.4 Comparative toxicity of CYN and other cyanotoxins

Blue-green algal toxins exert a range of effects on aquatic and terrestrial, floating and rooted, monocotyledonous and dicotyledonous species (Table 6.1). However, toxins

Table 6.1 Effects of algal toxins on aquatic and terrestrial plants, as reported in current literature, compared with effects reported during cylindrospermopsin exposure in the present study^a. CYN = cylindrospermopsin; MC = microcystin. POD = peroxidase enzyme; Chl. = chlorophyll; IC₅₀ = dose required to cause inhibition in 50% of the population; RNase = ribonuclease; GST = glutathione-S-transferase; GI₅₀ = dose required to exert growth inhibition in 50% of the population

Toxin	Test species	Exposure concentration	Effects	Reference
Aquatic species				
CYN (extracellular), with <i>C. raciborskii</i> whole cell extracts	<i>Spirodela oligorrhiza</i>	8 – 100 µg L ⁻¹ ≤ 250 µg L ⁻¹ ≥ 350 µg L ⁻¹	Increased chlorophyll content Mild growth stimulation Necrosis of leaves; clumping	This study; see figures 4.9, 4.10 Section 4.3.2.2/3/4 Section 4.3.2.1
CYN (extracellular), with <i>C. raciborskii</i> whole cell extracts	<i>Spirodela oligorrhiza</i>	≤ 117 µg L ⁻¹	Increased chlorophyll <i>a</i> and total chlorophyll content	This study; (for example, see Figure 4.10)
CYN (extracellular), with <i>C. raciborskii</i> whole cell extracts	<i>Hydrilla verticillata</i>	100-250 µg L ⁻¹ ≥ 200 µg L ⁻¹ 400 µg L ⁻¹	Brittle plants Sticky, elastic roots Increased growth in stems and roots	This study; section 5.2.3 Table 5.3; Figure 5.1
CYN whole cell extracts	<i>Nicotiana tabacum</i> (tobacco)	0 – 400 µg L ⁻¹ 5 – 1000 mg L ⁻¹ 138 mg L ⁻¹	Variable chlorophyll effects Inhibition of pollen germination Reduced incorporation of radiolabelled leucine (= protein synthesis inhibition?)	Tables 5.5 & 6 Metcalf <i>et al.</i> (2004)
CYN pure extract ^c	<i>Sinapis alba</i> (white mustard)	0 - 160 mg L ⁻¹	Growth inhibition (IC ₅₀ 18.2 mg L ⁻¹)	Vasas <i>et al.</i> (2002)
MC-RR	<i>Lemna minor</i> L.	3 – 5 mg L ⁻¹ ; 6 days 5 mg L ⁻¹ ; 6 days	Frond growth inhibition Reduced photosynthetic capability	Weiß <i>et al.</i> (2000)
MC-RR	<i>Vallisneria natans</i>	0.1 µg L ⁻¹ - 10 mg L ⁻¹ for 30 days ≥ 10 µg L ⁻¹ for 30 days	Reduced germination rate; reduced fresh weight and leaf length; decreased root and leaf numbers, root hair loss, yellowed new leaves	Yin <i>et al.</i> (2005)
MC-LR extract	<i>Lepidium sativum</i> (watercress)	10 mg L ⁻¹ 10 µg L ⁻¹	Growth inhibition - lower fresh weights, reduced root and leaf lengths	Gehring <i>et al.</i> (2003)

Cont'd over

Toxin	Test species	Exposure concentration	Effects	Reference
MC-LR	<i>S. oligorrhiza</i>	50 µg L ⁻¹ for 24 h	Reduction in frond numbers	Romanowska-Duda <i>et al.</i> (2002); Romanowska-Duda & Tarczynska (2002)
MC-LR extract	<i>S. oligorrhiza</i>	200 µg L ⁻¹ 86 - 344 µg L ⁻¹ ; 24 h 344 µg L ⁻¹ and above	Reduced frond size Altered frond morphology Reduced frond mass; Decreased chlorophyll a; chlorosis	
MC-LR	<i>Lemna minor</i>	86 – 344 µg L ⁻¹ ; 96h 200 µg L ⁻¹ 10 - 20 µg mL ⁻¹ for 5 days	Stimulated acid phosphatase activity 25% increase in RNase activity Decreased growth rate (frond number, weight); smaller and thinner roots, increased POD activity	Mitrovic <i>et al.</i> (2005)
	<i>Wolffia arrhiza</i>	15 µg mL ⁻¹ for 5 days		
MC-LR	<i>Lemna gibba</i>	≤ 10 µg L ⁻¹ (toxin standard or <i>M. aeruginosa</i> filtrate)	Growth reduction (frond number) No significant change to plant or frond number, dry weight, growth rate or chlorophyll content	LeBlanc <i>et al.</i> (2005)
MC-LR	<i>Scenedesmus armatus</i>	0.25 µg L ⁻¹	Increased GST	Pietsch <i>et al.</i> (2001)
	<i>Ceratophyllum demersum</i>	0.25 µg L ⁻¹	Elevated peroxidase activity Increased GST	
MC-RR	<i>Scenedesmus armatus</i> ; <i>Ceratophyllum demersum</i>	0.25 µg L ⁻¹	Increased GST	Pietsch <i>et al.</i> (2001)
MC (Crude extract; 70% MC-RR)	<i>Scenedesmus armatus</i> ; <i>Ceratophyllum demersum</i>	0.25 µg L ⁻¹ ; 1 h	Elevated peroxidase activity Inhibition of GST	Pietsch <i>et al.</i> (2001)
MC-LR	<i>Ceratophyllum demersum</i>	5 µg L ⁻¹ for 24 h	Inhibition of photosynthesis Increased GST	Pflugmacher (2004)
			Decreased total glutathione Increased hydrogen peroxide levels Increased antioxidative enzyme activity	

Cont'd Over

Toxin	Test species	Exposure concentration	Effects	Reference
MC-LR	<i>Phragmites australis</i>	0.5 µg L ⁻¹ for 24 h	Increased soluble GST activity	Pflugmacher <i>et al.</i> (2001)
MC-LR	<i>Ceratophyllum demersum</i>	5 mg L ⁻¹ for 24 h	Plant death	Pflugmacher (2002)
		1 µg L ⁻¹ for 6 weeks	Growth inhibition	
		0.5 µg L ⁻¹ ; period unspecified	Reduced photosynthetic O ₂ ; Increased chl. <i>b</i> ; decreased chl. <i>a</i>	
	<i>Myriophyllum spicatum</i> , <i>Cladophora</i> sp., <i>Elodea canadensis</i> , <i>Phragmites australis</i>	0.5 µg L ⁻¹	Inhibition of photosynthesis	
Anatoxin-a	<i>Chladophora fracta</i>	25 g L ⁻¹	Increased peroxidase activity	Mitrovic <i>et al.</i> (2004)
	<i>Lemna minor</i>	25 mg L ⁻¹ 5 – 20 mg L ⁻¹	Increased peroxidase activity Increased catalase and GST activity, and reactive oxygen species; reduced photosynthetic O ₂ production	
TERRESTRIAL				
MC-LR (purified extract)	<i>Phaseolus vulgaris</i> (runner bean)	Approx. 20 µg L ⁻¹ ; topical application	Single application ^b : Photosynthesis inhibition (8 hours) post-application; necrotic patches (2 days); Repeated application ^c : Inhibition of photosynthesis	Abe <i>et al.</i> (1996)
MC-LR (purified extract)	<i>Sinapis alba</i> (white mustard)	Test concentrations 3.5 – 30 mg L ⁻¹	Growth inhibition (fresh weight, plant length, lateral root formation, cotyledon formation, anthocyanin accumulation)	M-Hamvas <i>et al.</i> (2003)
Purified MC toxin, crude <i>Microcystis</i> extracts, frozen whole cells, and bloom materials	<i>Sinapis alba</i>	Approx. ≥ 1 mg L ⁻¹	Growth inhibition	Kós <i>et al.</i> (1995)
MC-LR	<i>Ipomea batatas</i> (sweet potato)	Leaf petiole excisions treated with 2 µm	Inhibition of sucrose-inducible gene expression	Takeda <i>et al.</i> (1994)

Cont'd over

Toxin	Test species	Exposure concentration	Effects	Reference
MC-RR	<i>Sinapis alba</i>	Approx $\geq 5 \text{ mg L}^{-1}$	Growth inhibition Malformed stems	Kurki-Helasmo & Meriluoto (1998)
		20 mg L^{-1}	Increased protein content Absence of roots and hairs	
MC-LR	<i>Solanum tuberosum</i> (Irish potato)	$5 \text{ } \mu\text{g L}^{-1}$; 16 days $50 \text{ } \mu\text{g L}^{-1}$; 16 days	Growth inhibition Reduced chlorophyll content	McElhiney <i>et al.</i> (2001)
MC-LF, -LR and – RR	<i>Sinapis alba</i>	$\text{GI}_{50} 1.6 \text{ mg L}^{-1}$ (MC-RR) $\text{GI}_{50} 1.9 \text{ mg L}^{-1}$ (MC-LR) $\text{GI}_{50} 7.7 \text{ mg L}^{-1}$ (MC-YR)	Growth inhibition	
MC-LR	<i>Phaseolus vulgaris</i> (runner bean)	1 mg L^{-1}	Necrosis, loss of leaves	
MC-LR	<i>Spinacia oleracea</i> (spinach)	$0 - 3 \text{ } \mu\text{mol}$; 12 h incubation in the dark	Dose-dependant decrease of 14^{d} incorporation into sucrose Stimulation of starch synthesis Decreased photosynthesis	Siegl <i>et al.</i> (1990)
Other compounds				
<i>Microcystis aeruginosa</i> extract (probably MC-LR)	<i>Chlorella</i> sp. <i>Scenedesmus</i> sp. <i>Anabaena</i> sp. <i>Nostoc muscorum</i>	$25 - 300 \text{ mg L}^{-1}$	Growth inhibition	Singh <i>et al.</i> (2001)
2,5-dimethyldodecanoic acid (Lyngbya aetuarii)	<i>Lemna minor</i>	$200 \text{ } \mu\text{g L}^{-1}$	Growth inhibition	

^aKirpenko (1986) has not been included since the toxin studied was not stated; this is not an exhaustive list; ^btoxin applied at concentrations of $14.8 - 1430 \text{ nmol MC-LR m}^{-2}$; ^cCYN obtained from *Aphanizomenon ovalisporum*; ^dtoxin applied at concentrations of 0.306 and 3.42 nmol m^{-2}

with different mechanisms of action may produce vastly different toxic effects. Moreover, given that CYN has multiple modes of toxicity, whereas other toxins, such as MC, do not, differences in plant responses for each toxin may be expected.

Exposure of *S. oligorrhiza* to the protein phosphatase inhibitor MC at 0 – 344 $\mu\text{g L}^{-1}$ is associated with reduction in frond numbers and frond size, altered frond morphology and decreased chlorophyll *a* (Romanowska-Duda *et al.* 2002; Romanowska-Duda & Tarczynska 2002). In contrast, exposure to whole cell extracts containing CYN at similar concentrations (0 - 500 $\mu\text{g L}^{-1}$) corresponded with stimulated plant growth and increased chlorophyll content. A similarity between these studies was that necrosis of *S. oligorrhiza* occurred during exposure to CYN ($\geq 350 \mu\text{g L}^{-1}$, this study), and MC-LR extract (344 $\mu\text{g L}^{-1}$) (Romanowska-Duda & Tarczynska 2002).

6.5 Comparative bioconcentration of CYN and other cyanotoxins

Microcystin accumulation studies clearly outnumber other investigations of algal toxins in aquatic macrophytes. *Ceratophyllum demersum*, *Elodea canadensis*, *Phragmites australis* and the bryophyte *Vesicularia dubyana* all contained MC after seven days exposure at 2.5 mg L^{-1} (Pflugmacher *et al.* 1998). However, toxin concentrations were only 1 to 120 pg g^{-1} (= ng kg^{-1}) fresh weight. Kurki-Helasmo and Meriluoto (1998) demonstrated uptake of ^3H -dihydro-MC-LR in mustard seedlings, but BCF values could not be calculated since toxin was reported as ng mg^{-1} protein. Pflugmacher *et al.* (2001) reported uptake of ^{14}C MC-LR in the emergent reed *P. australis*, with uptake occurring within 30

minutes of toxin exposure. Yin *et al.* (2005) showed dose-dependent MC-RR uptake to occur in *Vallisneria natans*, but tissue toxin concentrations were not high enough to indicate bioconcentration.

Most recently, Mitrovic *et al.* (2005) examined bioaccumulation of MC-LR in *Lemna minor*, with tissue toxins recorded at 0.046 ng mg^{-1} fresh weight, following MC-LR exposure concentrations of $3 \text{ } \mu\text{g mL}^{-1}$. Since this is equivalent to $46 \text{ } \mu\text{g kg}^{-1}$ fresh weight, bioconcentration did not occur ($46 \text{ } \mu\text{g kg}^{-1} < 3000 \text{ } \mu\text{g L}^{-1}$). Nonetheless, the reported value is strikingly similar to the maximum CYN value reported from *S. oligorrhiza* in the present work ($30 \text{ } \mu\text{g kg}^{-1}$), though this resulted from far lower exposure concentrations ($500 \text{ } \mu\text{g L}^{-1}$).

6.6 Implications for CYN exposure risks to the test species

In general, both *S. oligorrhiza* and *H. verticillata* were able to grow and survive when exposed to *C. raciborskii* whole cell extracts containing approximately $0 - 500 \text{ } \mu\text{g L}^{-1}$ CYN. In contrast, blooms containing MC – which is mostly intracellular – have been associated with decreased diversity and abundance of macrophytes (Abe *et al.* 1996; Casanova *et al.* 1999; Yin *et al.* 2005). The sensitivity of *S. oligorrhiza* to MC-LR exposure has prompted the possibility of this species being used as a bioassay organism for toxic *Microcystis* blooms (Romanowska-Duda & Tarczynska 2002). However, the variability in growth responses recorded from the duckweed in the present work with CYN suggests it would not be a reliable indicator of toxic *Cylindrospermopsis* blooms.

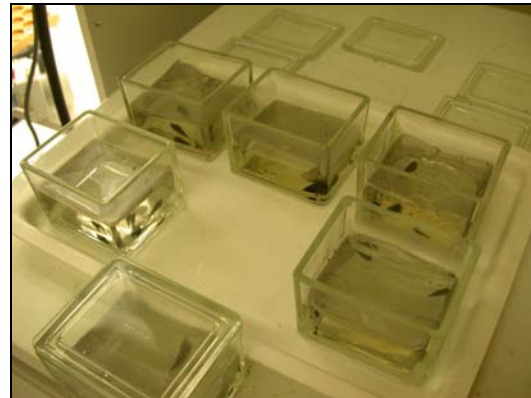
Since both *Spirodela* and *Hydrilla* appeared to tolerate exposure to CYN (and *C. raciborskii* debris), both species may play important roles in the food webs of lakes and reservoirs affected by toxin-producing *Cylindrospermopsis* blooms. Firstly, they may offer nutrient and light competition against the phytoplankton (including toxic blue-greens). This is especially the case for *H. verticillata* which may have the ability to produce root compounds as an adaptive strategy to minimize CYN toxicity. This competitive ability could be exploited: if they have a long-term ability to cope with CYN and *C. raciborskii* toxicity, *Spirodela* and *Hydrilla* may be used as remediation species, for example, to remove nutrients from the water column. Secondly, in the absence of bioconcentration (and with minimal toxin adsorption), they could represent alternate food sources for grazers during blooms. This may be especially important in cases where total domination by blue-greens has meant that toxin laden cyanoprokaryote cells are the only other option for herbivores. Finally, *Spirodela* and *Hydrilla* may also enable recolonisation, increased habitat complexity and increased water quality in affected aquatic systems (for example, Casanova *et al.* 1997).

SECTION III

Bioaccumulation risk and toxicity in aquatic invertebrates and vertebrates



Bufo marinus test flasks



Test chambers with *Melanoides tuberculata*

General introduction to section III

The use of aquatic animals as test organisms introduces an important change to experimentation techniques. In contrast to aquatic plants, aquatic animals may be at risk of multiple modes of toxin exposure. That is, both the intracellular and extracellular components of total CYN concentrations represent a risk for toxin uptake. Hence, to determine the relative importance of each of these, trials using both extracellular-only and intracellular toxin have been included. Aquatic animals also represent new challenges in regards to exposure regimes. These could be dramatically different to those of aquatic plants: due to their mobility, toxin avoidance is possible. In addition, toxin concentrations may differ depending on positioning within the water column. Therefore, the overall nature of the exposure regime may be determined by the animal's habitat requirements (such as benthic habitats of macroinvertebrates).

Chapters seven and eight discuss CYN exposure, bioconcentration and bioaccumulation in the aquatic snail, *Melanoides tuberculata*, and early life stages of the cane toad, *Bufo marinus*. An examination of both lethal and sublethal toxicities is made, including effects ranging from the gross level (mortality, overall growth rates) down to the cellular level (histopathological effects). Chapter nine contains an overall discussion of CYN toxicity, uptake and accumulation in aquatic animals (comparable with chapter six, as done for aquatic macrophytes).

Chapter seven: ecotoxicity, bioconcentration and bioaccumulation risks in *Melanoides tuberculata*

Note to reader: material in this chapter has been published in part in **White, S.H.**, Duivenvoorden, L. J., Fabbro, L. D. & Eaglesham, G. K. (2005). Influence of intracellular toxin concentrations on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata*). *Toxicon* 47(5): 497-509.

A second manuscript is also currently in submission: **White, S.H.**, Duivenvoorden, L. J. and Fabbro, L. D., Sublethal responses in *Melanoides tuberculata* following exposure to *Cylindrospermopsis raciborskii* containing cylindrospermopsin.

7.1 Introduction

Information regarding the sensitivity of aquatic snails to algal toxins is critical. Mass deaths of aquatic snails have been recorded in conjunction with a toxic *Microcystis* bloom (White *et al.* 2005) but limited information is available regarding CYN toxicity to invertebrates. Several gastropod species are known to bioaccumulate MC (Kotak *et al.* 1996; Zurawell 2001; Yokoyama & Park 2002; Ozawa *et al.* 2003; Yokoyama & Park 2003), but no studies have examined CYN bioaccumulation in this group. Multiple modes of CYN uptake are possible in gastropods. Herbivorous gastropods may ingest cellular toxins by grazing (intentionally or accidentally) on toxic blue-green algae. Snails may also be vulnerable to transdermal uptake because of their submerged habit and consequent prolonged contact with dissolved toxins.

Gastropods are vital components of aquatic food webs, providing food for fish, crayfish, birds, turtles, amphibians and insects (Lodge 1986; Dillon 2000). Aquatic snails, especially *Lymnaea stagnalis*, have been widely used as ecotoxicity test

organisms for investigations of pesticide and heavy metal exposures (for example, Klobucar *et al.* 1997; Pyatt *et al.* 2002). Several authors have examined snail activity and behaviour as methods for determining toxic effects (Burris *et al.* 1990; Campbell *et al.* 2000). *Melanoides tuberculata* Müller 1774 is an introduced Asian prosobranch (gill-breathing) snail (Dillon 2000) typically found in stagnant or slow-flowing waters (Rader *et al.* 2003). This chapter examines ecotoxicity, bioconcentration and bioaccumulation in *M. tuberculata*, following exposure to *C. raciborskii* whole cell extracts or live *C. raciborskii* cultures containing CYN.

7.2 Materials and methods

7.2.1 Collection and culture of experimental organisms

M. tuberculata were collected by hand from Moores Creek (Rockhampton, Queensland). Toxin was not present in Moores Creek at time of sampling. Culturing took place in a large plastic tub filled with water from Moores Creek. The tub was housed in a controlled climate room ($24 \pm 2^{\circ}\text{C}$) under 12:12 light:dark photoperiod (to reflect natural photoperiod at time of collection). Snails were provided filamentous green algae (possibly *Chladophora* sp.) obtained from the collection site. Dr. Winston Ponder (Australian Museum, Sydney) provided species confirmation. A subsample ($n = 20$) of the cultured population was monitored for cercarial shedding, which indicates the presence of internal parasites. Cercariae were not recorded during one month's observation (kindly conducted by Miss Leonie Barnett).

7.2.2 Test procedures

7.2.2.1 Pilot trial

Semi-static renewal trials were conducted based on ASTM (2003). A preliminary range-finding test (trial one) was performed using *C. raciborskii* whole cell extracts at

three nominal test concentrations (control, 50 and 100 $\mu\text{g L}^{-1}$ CYN). The trial was performed in a controlled temperature room operating at $24 \pm 2^{\circ}\text{C}$ and 12:12 light:dark photoperiod ($55 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent tubes). Test vessels were 500 mL glass conical flasks, stoppered with a rubber bung to reduce evaporation and prevent snails escaping. All flasks were aerated by glass pipettes, to minimise possible toxin sorption to plastics (Hyenstrand *et al.* 2001).

Treatment solutions (300 mL per test vessel) were prepared by double freeze-thawing well-mixed *C. raciborskii* cultures of known toxin concentration (average value of two culture aliquots), and diluting to the desired CYN concentrations using tap water. Double freeze-thawing ensured all toxin was present in the extracellular form (CYN_{EXC}). Tap water was treated by filtering (Whatman GF/F glass microfibre filters); autoclaving (125°C ; 25 minutes) and buffering to pH 8.0 (using NaOH / HCl).

Trials commenced by randomly assigning five snails per flask. Prior to addition, snails were gently blotted dry on paper towels and the collective fresh weight recorded per flask (to 0.01 g). Snail behaviour was measured at 24 h, 48 h, and every 48 h thereafter, usually less than two hours after the (artificial) onset of daylight. Behaviour was calculated by individually scoring each snail as follows:

- score zero (dead): no response to gentle probing of the operculum; further confirmed by transfer to fresh water and monitoring to see if alive
- score one (alive): tissues retracted into shell but snail not dead, evidenced by tight shutting of operculum when stimulated by gentle probing
- score two (active): snail extending muscular foot

- score three (very active): snail actively mobile (swimming, seeking food), often with tentacles extended.

A total score per flask was calculated by multiplying the number of snails per category with the relevant score, and summing all categories (for example, $\Sigma (3 \times 2)$, (1×1) , (1×0)). Behaviour scores per flask could therefore range from a maximum of fifteen to a minimum of zero.

Some snails climbed out of the water during the experimental period. Since this could have affected exposure to the toxin, the number of 'climbed out' snails was also recorded. A snail was considered 'out of the water' if none of the soft tissue (= muscular foot) was in contact with the treatment solution.

The release of hatchlings during experimental trials was also recorded to study effects of CYN exposure on reproduction. *M. tuberculata* belongs to the Thiariidae, which are characterised by their parthenogenetic and ovoviviparous reproductive styles (Burch 1982). In particular, *Melanoides* feature a cephalic brood pouch that enables fully developed (shelled and free-swimming) young to be released from an opening on the right side of the neck (Burch 1982; Dillon 2000). Cumulative numbers of released hatchlings were recorded at the same time as behavioural observations.

Snails were fed 5 cm² discs of boiled lettuce every 48 h following sublethal effects analyses. Approximately equal portions were made by cutting around lettuce leaves placed on a cardboard template. Uneaten food was removed to prevent fouling; based on the results of aquatic plant trials (see 4.3.5), minimal toxin losses were expected to result from toxin adsorption to lettuce.

Basic water quality parameters were measured at 48 h intervals, including conductivity, pH, dissolved oxygen and total ammonia. Treatment solutions were replaced by half-volume at three-day intervals to maintain constant CYN concentrations. Trial one ran for six days before ammonia concentrations reached unacceptable concentrations and the trial was abandoned. Selected treatment solutions were randomly chosen for CYN determination. Some of these samples were filtered through a sieve prior to analyses, to determine possible CYN uptake and/or adsorption onto uneaten lettuce leaves.

7.2.2.2 Exposure to *C. raciborskii* whole cell extracts

Two definitive trials (trials two, three) examined exposure to freeze-thawed *C. raciborskii* whole cell extracts, using a control plus five treatment concentrations of 25, 50, 100, 200 and 400 $\mu\text{g L}^{-1}$ CYN_{EXC}. Experimental methods were slightly modified from the techniques used in trial one.

Test vessels were rectangular glass dishes with glass lids. Rectangles of fine stainless steel mesh (Termi-mesh commercial-grade termite barrier) were used to prevent snails escaping exposure to the test solutions (Plate 7.1). Small holes were punched through the mesh to accommodate aerators. Test chambers were randomly arranged and half-submersed in a water bath at $26.5 \pm 1.0^{\circ}\text{C}$. Water was kept well circulated by the use of Thermoline temperature and flow regulators (Plate 7.2). Water temperatures were logged half-hourly and individual aeration to each dish provided by a HiBlow electric air compressor (Sakuragawa Pty Ltd, Japan). The trial was run under a 12:12 light:dark photoperiod regime at $80 \mu\text{mol photon m}^{-2} \text{s}^{-1}$.

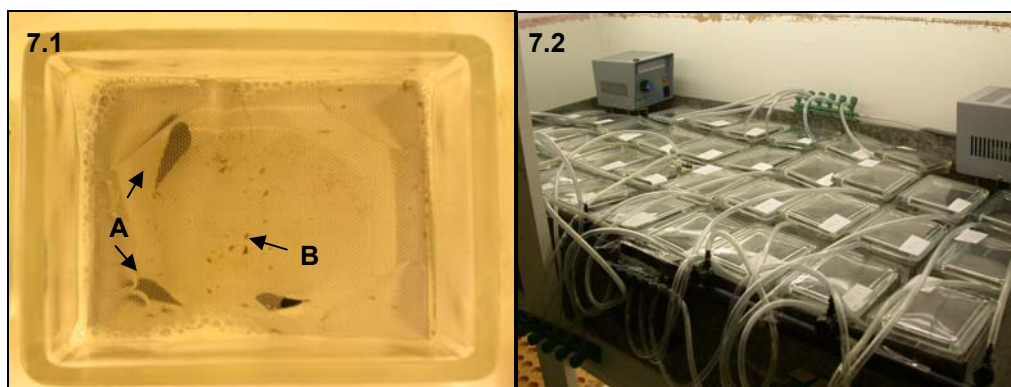


Plate 7.1 Test vessel showing Termi-mesh cage to prevent snail escapees: (A) adult snails and (B) released hatchlings. **Plate 7.2** Experimental setup, showing individually aerated test chambers and water circulation devices.

Test solutions were prepared as for trial one, except that solution renewals were increased to two-day intervals, with the full test volume replaced, to avoid problems with total ammonia concentrations (see 7.3.2). In trial two, the control and dilution waters were filtered, non-sterile (not autoclaved) tap water. Due to problems with this water, however (see 7.3.1, 7.3.4), control and dilution waters in trial three were changed to filtered (Whatman GF/F glass microfibre) Moores Creek water obtained from the specimen collection site.

Test solutions were reduced to 200 mL only, roughly equivalent to a biomass loading of 5 g L^{-1} . The American Society for Testing and Materials guidelines recommends a maximum biomass loading of 0.8 g L^{-1} to ensure adequate aeration and prevent overcrowding (ASTM 2003). However, test volume was limited by CYN availability, and this design ensured the minimum weight of snails required for tissue testing. Regular renewal of treatment solutions and individual aeration prevented poor water quality with respect to ammonia and oxygenation.

In both trials, three snails were weighed collectively and randomly assigned to a flask. Snail behaviour was again recorded at 24 h, 48 h and 48 h thereafter, by removing test chambers from the water bath and allowing settling for two minutes. Afterwards, behaviour was assessed using a refined version of the method used in trial one. Snails were recorded as active (score five; muscular foot extended and/or actively swimming or feeding), alive (score three; operculum closed but snail alive) or dead (score zero, no response to stimulus of the operculum). A single test chamber could therefore receive a score ranging from zero (all dead) to fifteen (three active snails). Climbing out was not recorded since the mesh prevented this. The cumulative number of emerged hatchlings in each test chamber was also recorded, as before.

Snails were not fed during trials two and three since toxin adsorption to food sources may have influenced the bioavailability of extracellular CYN (for example, snails could directly ingest extracellular toxin if adsorbed to food). Furthermore, minimising food sources also reduced faecal matter, which may decrease dissolved oxygen concentrations and hence the biological activity of some materials (ASTM 2003). Other authors have used exposure periods of similar length, without food provision, successfully (Lajtner *et al.* 1996; Klobucar *et al.* 1997).

Water quality data were collected after behavioural observations as described in trial one. Water hardness and alkalinity values were also measured from 150 mL filtered subsamples (0.45 µm Millipore nylon filter) of the control/dilution waters for each trial. Total alkalinity was determined by titrating a 50 mL or 100 mL sample to pH 4.5 using 0.02 molar HCl. Hardness was calculated using atomic absorption

spectroscopy, with lanthanum releasing agent added to acidified samples. Selected treatment solutions were reserved and frozen for CYN analyses.

Snails were harvested ($n =$ three flasks per treatment) on days seven and fourteen. Snails were collectively weighed and relative growth rates (based on biomass increase) were calculated using

$$RGR = \frac{\ln x - \ln o}{E};$$

where ‘o’ represents fresh weight (grams) on day zero, ‘x’ represents fresh weight at harvest, and ‘E’, exposure time in days. Snails were euthanized by freezing and stored frozen until CYN analyses were carried out. Only selected snails were analysed from trial two since the controls had failed.

7.2.2.3 Control waters test

Low snail activity recorded in control chambers during trial two prompted an examination of the most suitable control waters for use in further experimentation. Five different water samples were tested, including filtered and non-filtered solutions from a range of sources (Table 7.1). Autoclaved solutions were not considered as trial one had already shown that this was unsuccessful.

Solutions were tested in chambers ($n = 3$ for each type) holding 200mL test solution and three *M. tuberculata*. The trial ran for six days at $24.5 \pm 1^{\circ}\text{C}$ and with 12:12 light: dark photoperiod at $80 \mu\text{mol photon m}^{-1}\text{s}^{-2}$. Test solutions were renewed every 48 h. Snail behaviour and water quality was recorded as described for trial two.

Table 7.1 Basic properties of solutions trialled for use as control and dilution waters. Values are as measured prior to commencement of trial.

Water Type	Filtration	pH	Conductivity ($\mu\text{S cm}^{-1}$)	Hardness (mg L^{-1} CaCO_3)	Alkalinity (mg L^{-1} CaCO_3)	Ammonia (ppm)
Distilled water	None	7.3	26	<10	<5	0
48h-aged tap water	None	8.0	303	75	86	0
48h-aged tap water	GF/F ^a	8.0	302	69	59	0
ASM-1 ^b	None	6.6	404	15	61	0
Moore's Creek ^c	GF/F ^a	7.7	1049	140	312	0

^aWhatman GF/F glass microfibre filters; ^bnon-autoclaved algal growth medium, see appendix A; ^cat original collection site of *M. tuberculata*.

7.2.2.4 Exposure to live *C. raciborskii* culture

This trial (trial four) was conducted identically to the whole cell extract trials (two and three) with respect to test chamber size and type, number of snails, number of replicates, overall test conditions, and monitoring of water quality and snail behaviour. However, instead of using freeze-thawed whole cell extracts, treatment solutions were prepared from several one-litre live *C. raciborskii* cultures. These live cultures were pooled into a tank on the day prior to trial commencement. Treatments were prepared by thoroughly mixing the tank culture before subsampling and diluting to test concentrations of 10%, 20%, 30%, 40% and 50% live culture, using filtered Moore's Creek water. Controls were filtered Moore's Creek water only.

The different fractions of CYN (CYN_{EXC} , CYN_{INC} and total CYN, (CYN_{TOT})) were monitored closely throughout the trial. On day zero, and every 48 h thereafter, two 50 mL subsamples were collected from the source culture (tank), and two subsamples per treatment concentration (pooled sample of reserved test solutions from all replicates within a given treatment). For each pair, one sample was filtered (Whatman GF/F glass microfibre) and frozen for determination of CYN_{EXC} ; the second was

frozen without filtering for determination of CYN_{TOT}. Total CYN determinations only were done for control samples, since CYN was not expected. Variability within treatment replicates was also tested on random samples.

One treatment was randomly chosen for an examination of CYN concentrations associated with *M. tuberculata* faecal matter. Two 50 mL aliquots were subsampled to determine the toxin components (as described above). A third subsample was also collected per replicate and allowed to sediment for approximately five minutes, such that faeces dropped to the bottom of the collection vessel (gauged visually). The ‘supernatant’ only was collected (unfiltered) and analysed for CYN content.

Cell concentrations of *C. raciborskii* were monitored in selected treatments, including the source culture, by periodically collecting 10 mL aliquots and preserving with Lugol’s iodine. Later, duplicate trichome counts were performed using the Sedgewick-Rafter method: each chamber was counted for a total of 25 squares or 100 trichomes, whichever was reached soonest (Hötzl & Croome 1998). Trichome counts were converted to cell counts by dividing average trichome length ($n = 200$ trichomes; from control and various experimental treatments) by the known cell length of *C. raciborskii* strain FR001 in culture (Fabbro *et al.* 2001). Cell quotas of CYN were calculated from known CYN concentrations and the average of duplicate cell counts.

7.2.3 Analysis of free CYN and free deoxy-CYN from snail tissues

Specimens were thawed and the shell manually dissected from the soft tissues. Bioaccumulation analyses were conducted on adult snails only, since shell dissection

from hatchlings was impractical. Soft tissues were rinsed in distilled water and placed in a centrifuge tube, freeze-dried, brought to room temperature in a desiccator and reweighed. Tissues were homogenised in Milli-Q water and immediately frozen. Samples were sent to QHSS for analysis of free CYN and free deoxy-CYN concentration via HPLC/MS-MS (limit of detection ≤ 3.0 ng per 5 mL sample (equivalent to $0.5 \mu\text{g L}^{-1}$ for both CYN and deoxy-CYN; see Appendix C for full details). Spike recoveries for CYN averaged 87.5% ($n = 4$; range 1.8 to $5.7 \mu\text{g L}^{-1}$).

7.2.3.1 Evaluation of freeze-drying procedure

In trial one, an additional six replicates of the $50 \mu\text{g L}^{-1}$ treatment were prepared. Following snail harvest on day seven, these replicates were treated exactly as the main experimental samples but the freeze-drying step was omitted. This allowed a determination of possible CYN losses resulting from freeze-drying of soft tissues.

7.2.3.2 Evaluation of CYN in the shell

Gastropod shells were retained following snail dissections on day seven of trial three (whole cell extract exposure), to determine the concentration of (free) CYN associated with the shell component. Shells were pooled within treatment groups ($n = 9$ shells per treatment), left unrinsed, weighed (fresh weight) and frozen, before thawing and macerating into a fine slurry using a glass mortar and pestle with 2 mL of Milli-Q water. The slurry was washed into centrifuge tubes and immediately frozen, prior to sending to QHSS for CYN determinations.

7.2.4 Statistical analyses

Relative growth rates (biomass) were examined using two-way ANOVA, with the lower significance level ($\alpha = 0.01$) applied to heterogeneous datasets. Behavioural

data was examined using two-way repeated measures ANOVAs. Sphericity was checked using Mauchly's test: where this value was significant, the MANOVA value (Pillai's Trace statistic) was reported instead of Roy's Largest Root. Dunnett's T3 tests were used to detect significant differences between individual treatment groups ($\alpha = 0.01$). Numbers of hatchlings were analysed using two-way ANOVA ($\alpha = 0.05$, data homogeneous) and post-hoc Tukey testing, using the total number of hatchlings recorded from each test chamber on harvest days. Pearson Product Moment correlations compared relationships between CYN and deoxy-CYN tissue concentrations, the respective bioconcentration and bioaccumulation factors together with exposure regimes (toxin concentration, time).

7.3 Results

7.3.1 Control waters test

Highest snail activity was consistently recorded in the Moores Creek and ASM-1 test chambers, followed by the distilled water (Figure 7.1). Unfiltered aged tap water recorded the lowest snail activity. Moores Creek water was also associated with high numbers of hatchlings, with a complete absence of these occurring in the filtered aged tap water (Figure 7.1).

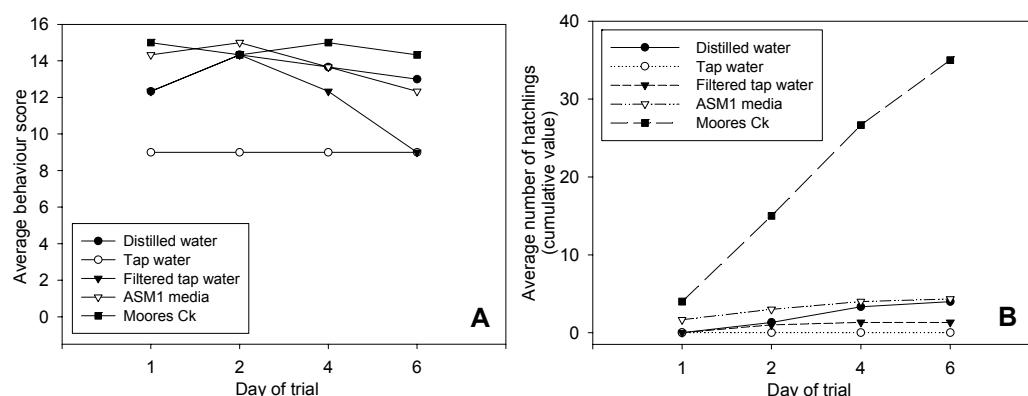


Figure 7.1 Responses of *Melanoides tuberculata* snails in waters from different sources: (A) snail behavioural values and (B) number of hatchlings released. Lines show average values ($n = 3$).

7.3.2 Water quality

Dissolved oxygen levels were generally $\geq 80\%$ saturation; total ammonia concentrations also remained within reasonable limits (≤ 1.0 ppm), except the $400 \mu\text{g L}^{-1}$ treatments in trials two and three (Table 7.2, next page).

7.3.3 CYN, deoxy-CYN and cell concentrations

Measured CYN_{EXC} concentrations met or exceeded nominal concentrations in trials one and two, but were slightly lower in trial three (Table 7.3). In trial one, removal of uneaten lettuce pieces resulted in a toxin decrease of just $1.5 \mu\text{g L}^{-1}$ (equivalent to approximately 5% toxin loss). Deoxy-CYN concentrations relative to total CYN quantities were widely variable (28 – 76%, Table 7.3).

Variability of CYN values between treatments was low ($<10\%$) in trial four, where live *C. raciborskii* was used (Table 7.4). Peaks and troughs of total CYN concentrations in the source culture were not reflected in the toxin concentration of treatments (Figure 7.2A). Average total toxin concentration in the source

Table 7.3 Toxin concentrations of selected treatment solutions from trials one to three. Values represent three pooled replicates. NT = not tested; NA = not applicable; CYN = cylindrospermopsin.

Trial	Day(s) since solution renewal	Nominal CYN ($\mu\text{g L}^{-1}$)	Measured CYN ($\mu\text{g L}^{-1}$)	Percent remaining (%)	Measured deoxy-CYN ($\mu\text{g L}^{-1}$)	Deoxy-CYN (% of CYN)
One	3 ^a	Control	3	NA	2.3	77
One	3 ^a	100	139	139	83.0	60
One	3 ^a	50	66	132	41.0	62
One	3 ^{ab}	50	62	125	37.0	60
Two	1	100	100	100	NT	NA
Two	1	200	250	125	NT	NA
Three	2	50	31	62	9.1	29
Three	1	400	336	84	95.1	28

^atest solutions replaced by half-volume only, at three day intervals; ^bsample filtered through a kitchen sieve to remove lettuce pieces.

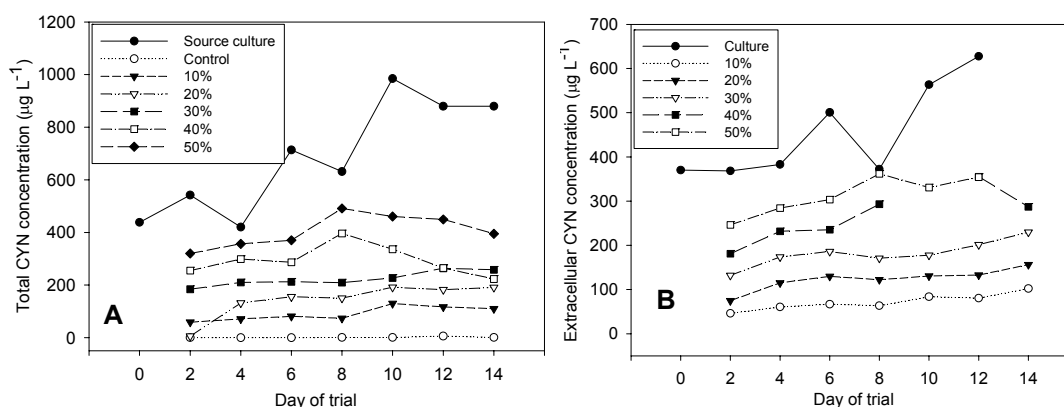
Table 7.2 Ranges for water quality parameters measured throughout the control waters test and trials one to four. NT = not tested.

Trial number	Illumination ($\mu\text{mol photon m}^{-2} \text{s}^{-1}$)	Temperature ($^{\circ}\text{C}$)	Alkalinity ^a (mg/L of CaCo3)	Hardness ^a (mg/L of CaCo3)	pH	Conductivity ($\mu\text{S cm}^{-1}$)	Dissolved oxygen (% Saturation)	Ammonia (ppm)
Control waters trial	60 – 75	25.5 \pm 1 $^{\circ}\text{C}$						
Distilled water			<10	<5	7.2 – 7.7	24 – 44	≥ 87	0 – 0.25
Aged tap water			75	86	8.0 – 8.4	303 – 341	≥ 89	0 – 0
Filtered aged tap			69	59	7.8 – 8.4	302 – 361	≥ 88	0 – 0.25
ASM-1 media			15	61	6.6 – 6.8	404 – 460	≥ 90	0 – 0.5
Moore's Ck water			140 ^d	312 ^d	7.7 – 8.8	1049 – 1095	≥ 88	0 – 0.5
Trial one	55 ^b	24 \pm 2 $^{\circ}\text{C}$	NT	NT	7.92 – 8.46	282 – 500	≥ 75	0 – >5 ppm ^c
Trial two	60 – 75	25.5 \pm 1 $^{\circ}\text{C}$	140 ^d	312 ^d				
Control					8.1 – 8.5	299 – 390	≥ 82	0 – 0.5
25					8.2 – 8.7	279 – 465	≥ 87	0 – 0.1
50					8.2 – 8.8	280 – 452	≥ 81	0 – 0.5
100					8.2 – 8.6	290 – 483	≥ 82	0 – 0.5
200					7.8 – 8.6	305 – 412	≥ 78	0.5 – 1
400					7.6 – 8.6	289 – 396	≥ 73	2 – 5
Trial three	60 – 75	24.5 \pm 1.5 $^{\circ}\text{C}$	119	357				
Control					8.0 – 8.7	117 – 139	≥ 85	0 – 0.5
25					8.0 – 8.7	117 – 132	≥ 80	0.25 – 0.5
50					8.0 – 8.8	116 – 125	≥ 83	0.25 – 0.5
100					8.0 – 8.7	112 – 125	≥ 83	0 – 0.5
200					8.1 – 8.8	101 – 111	≥ 85	0.25 – 1.0
400					8.2 – 8.6	85 – 110	≥ 82	0.25 – 2.0
Trial four	60 – 75	25.0 \pm 1.5 $^{\circ}\text{C}$	110	195				
Control					8.5 – 8.7	625 – 815	≥ 80	0 – 0.25
10%					8.0 – 8.8	607 – 873	$\geq 70^{\text{e}}$	0 – 0.5
20%					8.0 – 8.7	583 – 733	$\geq 77^{\text{e}}$	0 – 0.5
30%					8.0 – 8.6	566 – 694	$\geq 62^{\text{e}}$	0 – 1.0
40%					8.0 – 8.9	527 – 632	≥ 81	0 – 0.5
50%					7.9 – 8.5	536 – 626	≥ 73	0 – 0.5

^acontrol/dilution water, prior to trial; ^blight tubes changed after this trial; ^ctrial subsequently abandoned after six days; ^dsame sample used for determinations of control waters trial and trial three; ^esome outliers, but typically $\geq 80\%$.

Table 7.4 Toxin variability in selected treatments during trial four. CYN = cylindrospermopsin.

	Total CYN concentration ($\mu\text{g L}^{-1}$)	
	20% (day six)	40% (day ten)
Replicate 1	109.3	346.0
Replicate 2	98.5	336.5
Replicate 3	82.5	325.8
Average \pm standard error	96.77 \pm 7.78	336.1 \pm 5.83
% variation	8.03%	1.73%

**Figure 7.2** Cylindrospermopsin concentrations during trial four (A) total cylindrospermopsin (B) extracellular cylindrospermopsin (controls not tested). Toxin concentrations of treatment solutions are as measured after two days residence time in the experimental flasks.

culture was $659 \mu\text{g L}^{-1}$, whilst for the control and culture treatments were 1, 91, 167, 223, 294 and $406 \mu\text{g L}^{-1}$, respectively (Table 7.5). Fortunately, maximum and minimum values were therefore easily comparable with those of the whole cell extract trials ($0 - 400 \mu\text{g L}^{-1}$). One control sample (day 12) had minor CYN concentration (Table 7.5).

Extracellular toxin represented between 72 – 81% of total CYN, with the ratio generally consistent between treatment concentrations (Figure 7.2B, Table 7.5). Average deoxy-CYN concentrations ranged between $3 - 12 \mu\text{g L}^{-1}$ (controls excluded), representing 1.8 – 3.3% relative to average total CYN values in corresponding treatments (Table 7.5).

Table 7.5 Values for cylindrospermopsin fractions in the inoculum culture, control and experimental treatments during trial four. NT = not tested; NA = not applicable; NC = not calculated, ND = not detected ($< 0.5 \mu\text{g L}^{-1}$); CYN_{TOT} = total cylindrospermopsin; CYN_{INC} = intracellular cylindrospermopsin, CYN_{EXC} = extracellular cylindrospermopsin; SE = standard error.

Day of Trial	Source culture	Toxin concentration ($\mu\text{g L}^{-1}$ or % EXC)					
		Control	10%	20%	30%	40%	50%
CYN_{TOT}							
0	438	NT	NT	NT	NT	NT	NT
2	542	ND	57	5.5 ^a	184	255	320
4	420	ND	71	132	210	299	357
6	714	ND	81	155	213	287	370
8	631	ND	73	150	209	396	491
10	985	ND	130	191	227	336	460
12	879	5.8	117	182	264	264	449
14	NT	ND	110	191	258	223	394
Average \pm SE	659 \pm 81	NA	91 \pm 10	167 \pm 9 ^a	223 \pm 11	294 \pm 22	406 \pm 24
CYN_{EXC}							
0	370	NT	NT	NT	NT	NT	NT
2	369	NT	46	75	132	181	246
4	382	NT	61	115	174	232	284
6	501	NT	67	130	186	236	303
8	372	NT	64	122	171	293	362
10	563	NT	84	131	178	NT	331
12	628	NT	81	133	201	355	354
14	NT	NT	102	156	230	287	NT
Average \pm SE		NA	72 \pm 7	123 \pm 9	182 \pm 11	264 \pm 23	313 \pm 17
% EXC							
0	84	NT	NT	NT	NT	NT	NT
2	68	NT	78	NA ^a	72	71	77
4	91	NT	85	87	83	78	80
6	70	NT	83	84	88	82	82
8	59	NT	87	81	82	74	74
10	57	NT	65	68	78	NA	72
12	71	NT	69	73	76	134	79
14	NT	NT	93	82	89	129	NA
Average \pm SE	72 \pm 5	NA	80 \pm 4	79 \pm 3	81 \pm 2	NC ^b	77 \pm 1
INC/EXC ratio (average)	NC	NA	0.28	0.27	0.23	0.30	0.30
DEOXY-CYN_{TOT}							
0	5.6	NT	NT	NT	NT	NT	NT
2	6.2	ND	0.9	0.6	5.4	6.3	8.9
4	11.4	ND	2.3	3.4	5.4	9.8	11.3
6	19.3	ND	2.6	3.8	5.0	9.8	10.7
8	7.5	ND	1.2	1.8	2.4	9.8	12.9
10	26.2	ND	4.2	5.3	7.0	3.5	13.9
12	9.2	ND	3.4	5.7	9.2	8.1	14.4
14	NT	ND	3.3	7.2	7.8	5.3	12.1
Average \pm SE	12 \pm 2.9	NA	3 \pm 0.5	4 \pm 0.8	6 \pm 0.8	7 \pm 1.0	12 \pm 0.7
Deoxy-CYN (% of CYN_{TOT})	1.8	NA	3.3	2.4	2.7	2.4	3.0

^aday two result discarded, given the outlier nature and the result recorded for the day two extracellular component; ^bsome (anomalous) results exceeded 100%.

When calculated from CYN_{INC} concentrations, the approximate toxin quota (Q_{CYN}) for *C. raciborskii* cells in the source culture was $0.09 \text{ pg cell}^{-1}$, whilst experimental treatments recorded an average Q_{CYN} of $0.21 \text{ pg cell}^{-1}$ (Table 7.6). However, when calculated from CYN_{TOT} concentrations, all Q_{CYN} values were up to ten-fold higher than the values calculated from CYN_{INC} (Table 7.6). Too few samples were taken to identify trends in Q_{CYN} over the experimental period.

Table 7.6 Cell quotas of *Cylindrospermopsis raciborskii* in the source culture and treatment solutions, based on total (CYN_{TOT}) and intracellular-only (CYN_{INC}) cylindrospermopsin values. NC = not calculated; SE = standard error.

Treatment	Cells mL^{-1} Average \pm SE	CYN_{TOT} (ng mL^{-1})	Cell quota (pg cell^{-1})	CYN_{INC} (ng mL^{-1})	Cell quota (pg cell^{-1})
Source day 0	1288,950 \pm 365203	438	0.34	68	0.05
Source day 4	2032575 \pm 26440	420	0.21	37	0.02
Source day 10	2118505 \pm 16525	985	0.46	421	0.20
Source day 12	1983 \pm 661	879	NC ^a	251	NC ^a
Average			0.34		0.09
40% day 2	853351 \pm 36355	255	0.30	73	0.09
30% day 4	138149 \pm 7271	210	1.52	36	0.26
40% day 4	521529 \pm 7271	299	0.57	67	0.13
10% day 6	31992 \pm 529	81	2.53	14	0.44
30% day 8	409159 \pm 661	209	0.51	38	0.09
50% day 8	1052642 \pm 47923	491	0.47	129	0.12
20% day 10	295467 \pm 6610	191	0.65	61	0.21
30% day 14	120302 \pm 200	258	2.14	42	0.35
Average			1.09		0.21

^a not calculated due to unexpectedly low cell concentrations.

The effect of faecal removal from samples was variable. Removal of faecal strings corresponded with reduced CYN concentrations only in the 10 and 50% treatments, whereas toxin concentrations were increased by faecal removal in all other treatments (Table 7.7). However, during the trial, more faeces were present in treatments containing higher *C. raciborskii* concentrations ($\geq 30\%$, general observation).

Table 7.7 Toxin concentrations of test solutions on day twelve, before and after the removal of faecal matter by sedimentation.

Treatment	Total CYN concentration ($\mu\text{g L}^{-1}$)			
	Unsedimented sample	Sedimented sample (supernatant)	Change	Change (% of total)
10%	116.9	106.2	-10.7	- 9.2
20%	182.2	189.6	+7.4	+ 4.1
30%	263.6	264.5	+0.9	+ 0.3
40%	264.4	336.3	+71.9	+ 27.2
50%	449.2	437.1	-12.1	- 2.7

7.3.4 Sublethal effects

Results from trial one have not been considered here due to unacceptably high total ammonia concentrations reached during exposure (see 7.3.1).

7.3.4.1 Snail behaviour

The behaviour of *M. tuberculata* in trial two, where tap water was used to dilute the test solutions, was markedly different compared with trials three and four (Figure 7.1), which used filtered Moores Creek water. Treatments containing high concentrations of toxin and whole cell extracts showed the highest levels of snail activity. In contrast, lower test concentrations were associated with immobile or dead snails. A significant interaction between treatment and time was present in both exposure periods (Table 7.8); generally, snail activity in the low-CYN treatments ($\leq 100 \mu\text{g L}^{-1}$) progressively decreased over time (Figure 7.3A).

Control waters were adjusted in trial three; consequently, all snail behavioural scores were ≥ 12 , regardless of treatment and exposure time. Snail deaths were not recorded in this trial in any treatment. Significant differences were not detected between treatments during the trial; a significant effect of exposure time was recorded only during the second week (Table 7.8).

Table 7.8 Summary results for two way repeated measures ANOVA conducted on *Melanoides* behaviour data collected in trials two to four.

	Week one	Week two
Trial two		
Mauchly's test for Sphericity	$p = 0.010$; $df=5$; $M=0.589$	$p < 0.001$; $df=5$; $M=0.089$
Treatment	$p < 0.001$; $F_{5,30} = 80.114$	$p < 0.001$; $F_{5,12} = 89.985$
Exposure time ^a	$p = 0.001$; $F_{3,28} = 7.743$	NS
Interaction ^a	$p < 0.001$; $F_{5,30} = 6.982$	$p < 0.001$; $F_{5,12} = 16.458$
Trial three		
Mauchly's test for Sphericity	NS	$p < 0.001$; $df=5$; $M=0.052$
Treatment	NS	NS
Exposure time ^a	NS	$p = 0.005$; $F_{3,9} = 8.927$
Interaction ^a	NS	NS
Trial four		
Mauchly's test for Sphericity	NS	NS
Treatment	NS	NS
Exposure time ^a	NS	NS
Interaction ^a	NS	NS

^avalues from two-way ANOVAs (Sphericity Assumed statistic); or, where Mauchly's test was significant, two-way repeated measures MANOVAs (Roy's Largest Root Statistic).

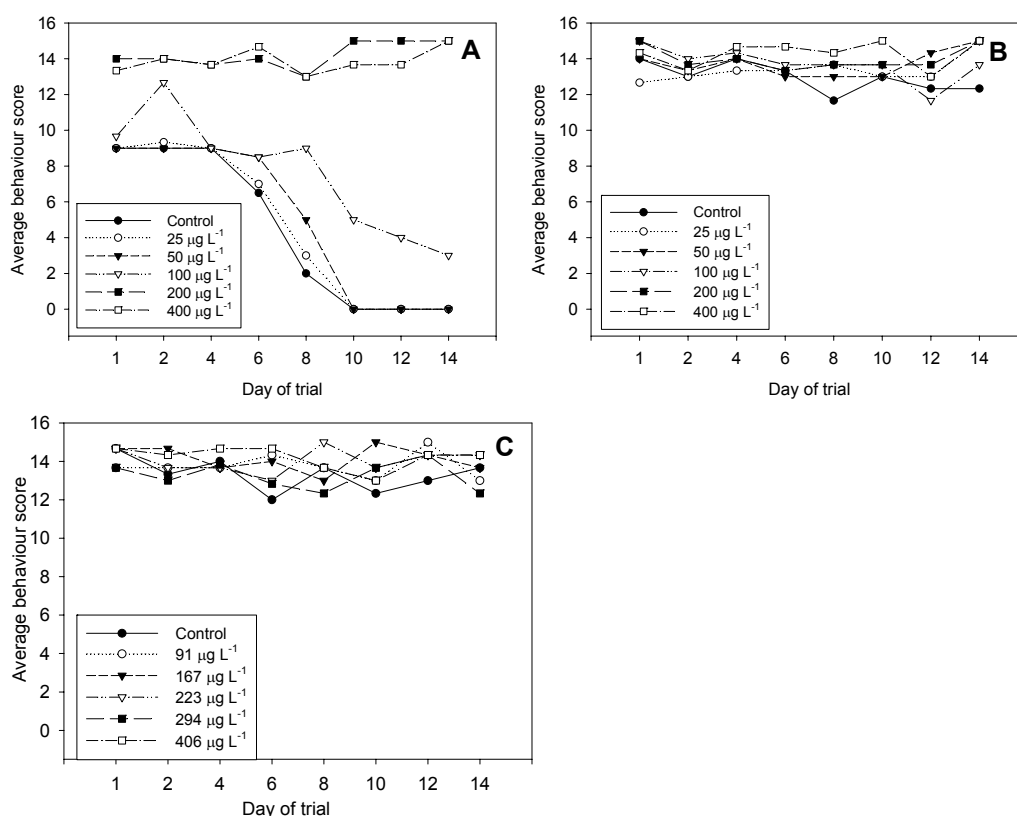


Figure 7.3 Behaviour scores of *Melanoides tuberculata* during exposure to *Cylindrospermopsis raciborskii* whole cell extracts or live cultures containing cylindrospermopsin: (A) trial two, (B) trial three, (C) trial four. Graphs show average ($n = 6$, days one to six; or $n = 3$, days eight to fourteen). Legends indicate nominal extracellular cylindrospermopsin concentrations (A, B), or measured total cylindrospermopsin concentrations (C).

7.3.4.2 Release of hatchlings

Hatchlings of *M. tuberculata* were released during trials two, three and four. All young survived and were apparently healthy (= mobile) despite the presence of CYN (and *C. raciborskii* cells or cellular debris). The pattern of release was generally a gradual one, with one to six new hatchlings appearing during each 48h interval. In trial two, the 200 and 400 $\mu\text{g L}^{-1}$ treatments recorded ≥ 30 hatchlings by day fourteen, whilst all other treatments recorded ≤ 1 (Figure 7.4A). Data from this trial could not be analysed by ANOVA as some samples had zero standard deviation.

In trial three, treatments containing high concentration of CYN and cell debris again recorded the highest number of hatchlings, particularly towards the end of the fourteen-day trial. However, the magnitude of this effect was reduced compared with trial two (Figure 7.4B). Significant effects of treatment, time or interaction were not detected during this trial ($p > 0.050$; two-way ANOVA).

Fewer hatchlings overall (generally, ≤ 10 per treatment) were produced during the live culture trial (trial four). In addition, the pattern of release changed: controls and low-CYN concentration treatments recorded the most hatchlings, whilst the 40% and 50% treatments recorded the least (Figure 7.3C). Time was the only factor to exert a significant effect ($p = 0.005$; $F_{1, 24} = 9.690$, two-way ANOVA).

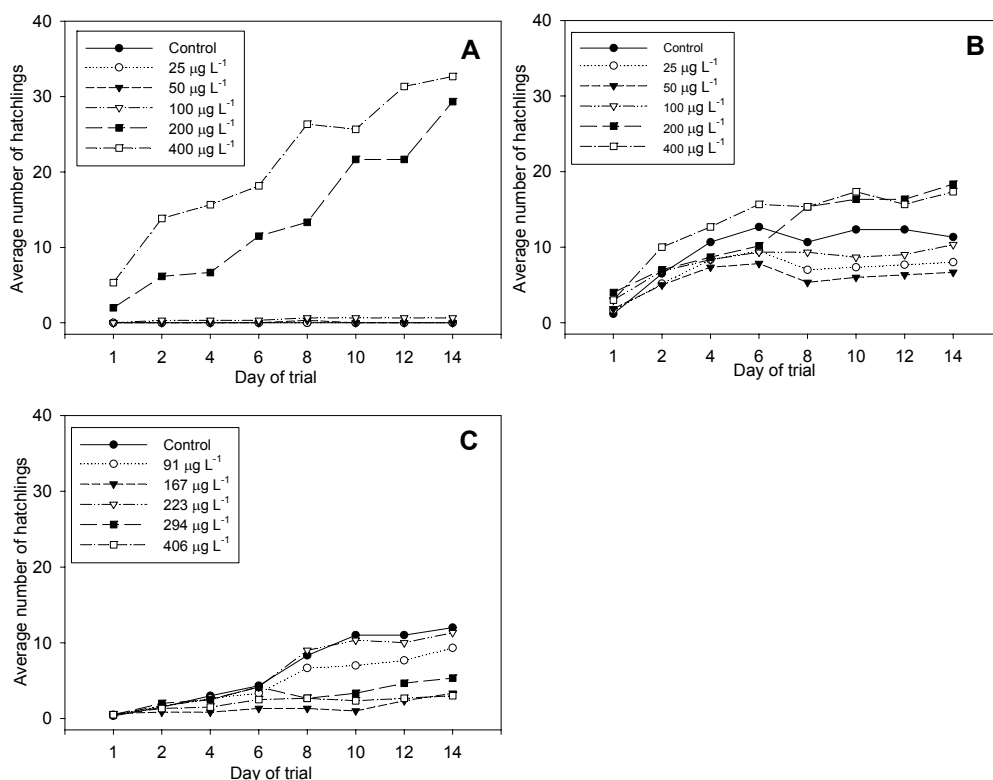


Figure 7.4 Average numbers of hatchlings released by *Melanoides tuberculata*, during exposure to whole cell extracts or live cultures of *Cylindrospermopsis raciborskii* containing cylindrospermopsin: (A) trial two; (B) trial three; (C) trial four. Legends indicate nominal extracellular cylindrospermopsin concentrations (A, B), or measured total cylindrospermopsin concentrations (C).

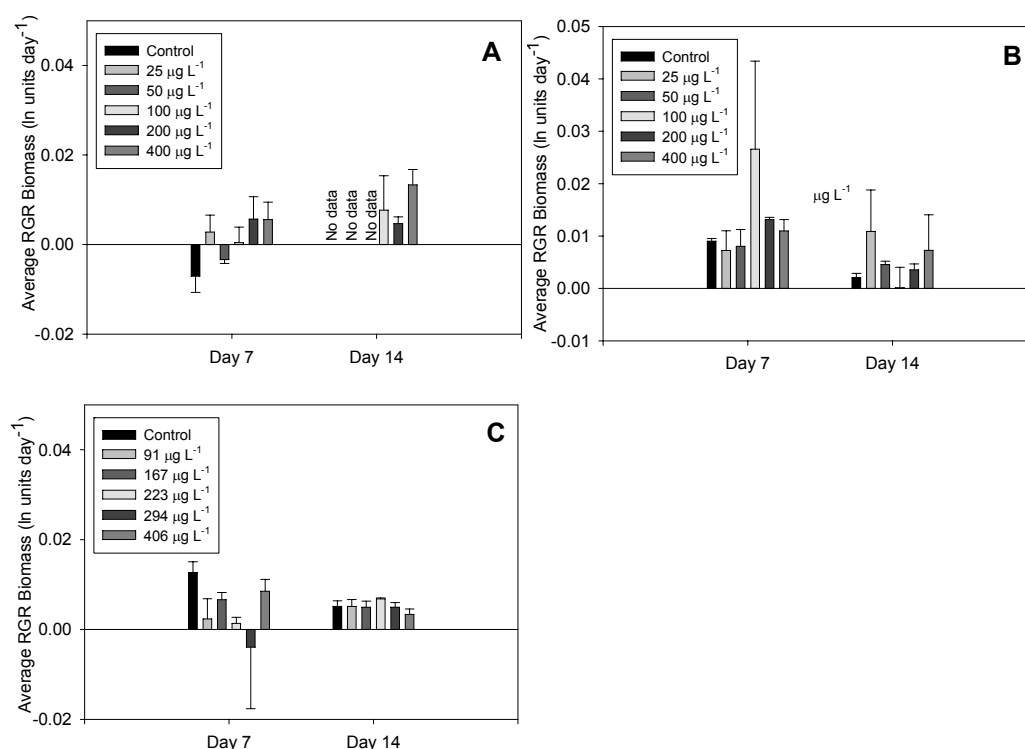
Pearson Product Moment correlations examined the relationships between snail behaviour scores (treatment averages) and the cumulative number of hatchlings released for each exposure phase for each of trials two to four. In half of the tested cases, a strongly significant, positive correlation was found between snail activity and the number of hatchlings (Table 7.9). However, in the remaining half, significant correlations were not present.

Table 7.9 Summary of Pearson Product Moment correlations to compare average activity levels with the cumulative number of hatchlings per treatment for *Melanoides tuberculata* in trials two to four. NS = not significant, $p \geq 0.050$.

Trial	Day 0 – 7	Day 8 – 14
Two (whole cell extract)	$p < 0.001$; $r=0.821$	$p < 0.001$; $r=0.918$
Three (whole cell extract)	NS	$p = 0.007$; $r=0.615$
Four (live <i>C. raciborskii</i>)	NS	NS

7.3.4.3 Relative growth rates

Since some snails died in trial two (Figure 7.3A), this prevented calculations of some treatment RGRs based on changes in fresh weights (Figure 7.5A). Positive growth was generally limited to treatments containing *C. raciborskii* and $\geq 100 \mu\text{g L}^{-1}$ CYN. In contrast, growth rates were usually positive during trials three and four (Figure 7.5B, C). Two-way ANOVA ($\alpha = 0.01$, heterogeneous data) found no significant differences between treatment, time or interaction in any trial ($p > 0.010$).

**Figure 7.5** Average relative growth rates (RGR) in *Melanoides tuberculata*, following exposure to solutions containing *Cylindrospermopsis raciborskii* and cylindrospermopsin: (A) trial two, (B) trial three, (C) trial four. Graphs show averages ($n = 3$) \pm standard error. Legends indicate nominal extracellular cylindrospermopsin concentrations (A, B), or measured total cylindrospermopsin concentrations (C).

7.3.5 Bioaccumulation

7.3.5.1 Toxin losses due to freeze-drying

M. tuberculata samples subjected to freeze-drying recorded strikingly similar tissue toxin concentrations to those that had not undergone the procedure (Figure 7.6). CYN and deoxy-CYN concentrations were approximately $40 \mu\text{g kg}^{-1}$ fresh tissue and $20 - 25 \mu\text{g kg}^{-1}$ fresh tissue, respectively.

7.3.5.2 Free CYN associated with shell

Toxin deposition into the shell appeared to be generally dose-dependant (Figure 7.7). Since shells were not rinsed, CYN may have been related to either adsorbed and/or bioaccumulated CYN. However, since the component of CYN represented by adsorbed toxin is not known, BCFs were calculated using the total CYN recorded from the shell, although this may overestimate bioconcentration. The average BCF calculated for snails exposed to treatment containing $25 - 400 \mu\text{g L}^{-1}$ CYN was 0.12.

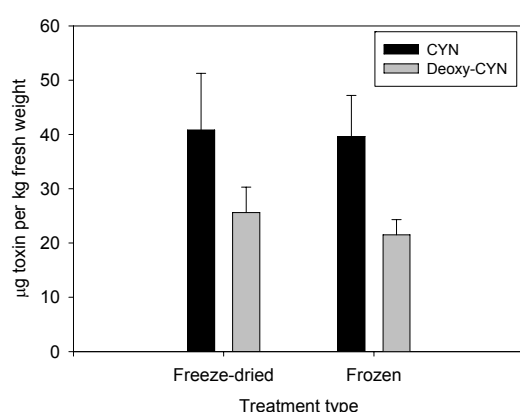


Figure 7.6 Toxin concentrations of frozen and freeze-dried *Melanoides tuberculata* samples. Graphs show average value ($n = 3$) \pm standard error.

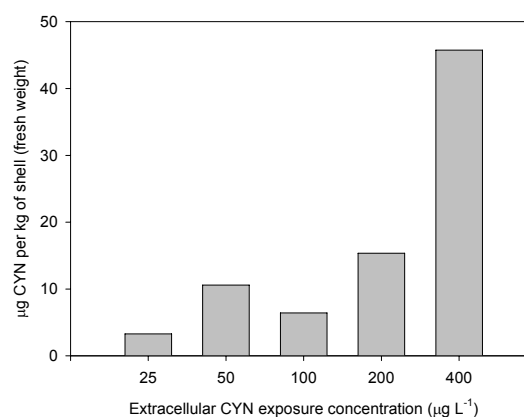


Figure 7.7 Free cylindrospermopsin recorded from the shells of *Melanoides tuberculata* following seven days exposure to *Cylindrospermopsis raciborskii* whole cell extract containing cylindrospermopsin.

7.3.5.3 Free CYN bioconcentration and bioaccumulation

Free CYN concentrations in *M. tuberculata* varied greatly according to exposure concentrations, toxin type, and, to a lesser extent, the length of exposure (Figure 7.8 A-C). In all trials, trends in CYN concentration and accumulation appeared to be similar, regardless of whether CYN was expressed in fresh or freeze-dried equivalents (Figures 7.8, 7.9). Maximum free CYN concentrations corresponded with highest exposure concentrations ($400 \mu\text{g L}^{-1}$) and longest exposure periods (fourteen days) in both trials two and three (Figure 7.8 A, B). However, tissue toxin concentrations recorded from each of these trials were quite variable, despite both trials examining whole-cell extract exposure (Figure 7.8 A, B).

Increased toxin exposure concentrations exerted only a weak effect on tissue toxin concentrations during the live culture trial, compared with the trials using *C. raciborskii* whole cell extracts. However, increasing exposure times (seven days compared with fourteen days) generally resulted in doubling of tissue CYN concentrations (Figure 7.8C). Overall, tissue toxin concentrations were dramatically increased overall compared with those recorded from trials two and three, despite similar total toxin exposure concentrations (approximately $0 - 400 \mu\text{g L}^{-1}$).

Tissue toxin concentrations in trial four were more variable than were those in trial three. When standard errors were expressed as a percentage of data averages, nearly 45% error was recorded in the second week of trial four (Table 7.10). Error calculations were not conducted for trial two since only selected samples were analysed for CYN.

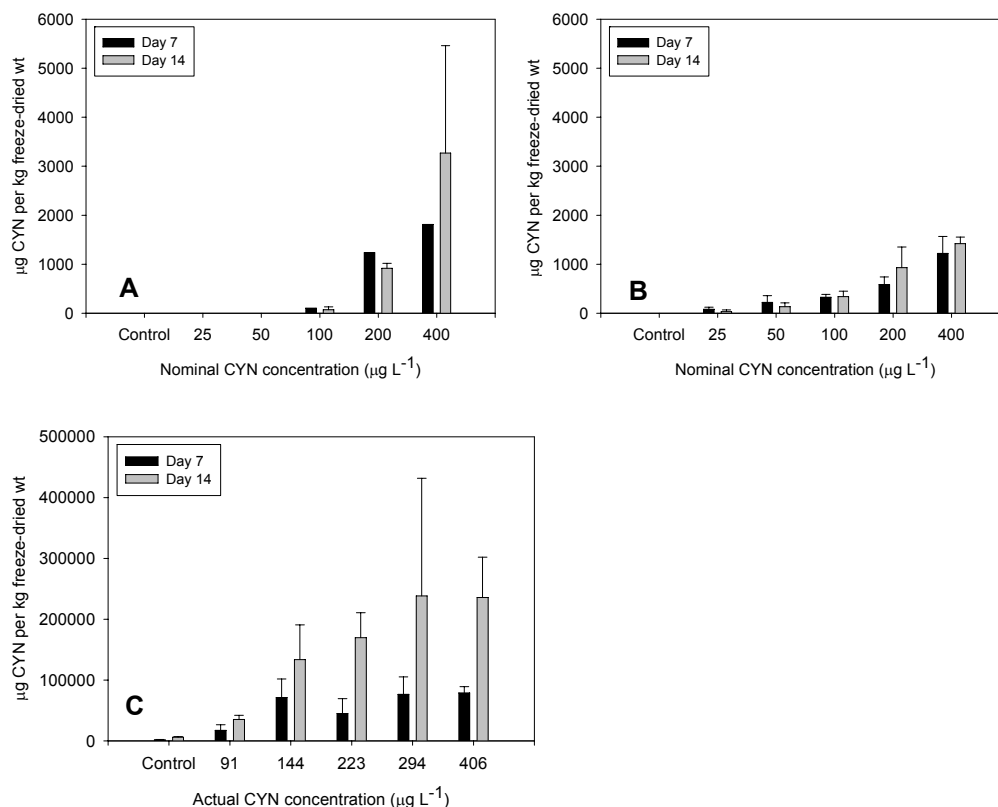


Figure 7.8 Free cylindrospermopsin (dry-weight equivalent) in *Melanoides tuberculata*: (A) trial two, (B) trial three, (C) trial four. Graphs show average ($n = 3$, except trial two where only selected samples tested) \pm standard error.

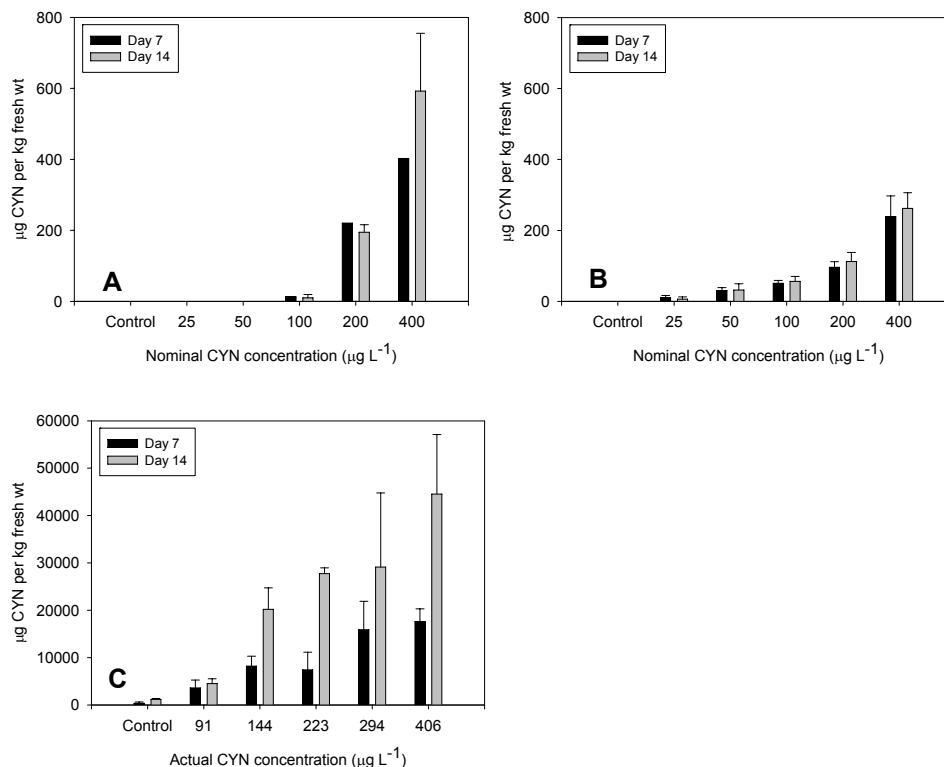


Figure 7.9 Free cylindrospermopsin (fresh-weight equivalent) in *Melanoides tuberculata*: (A) trial two, (B) trial three, (C) trial four. Graphs show average ($n = 3$, except trial one where only selected samples tested) \pm standard error.

Table 7.10 Variability in data recorded from trials three (*Cylindrospermopsis raciborskii* whole cell extract exposure) and four (live *C. raciborskii* exposure). Values were calculated from group averages for each treatment ($n = 6$).

	Trial three		Trial four	
	Week one	Week two	Week one	Week two
Average CYN ($\mu\text{g kg}^{-1}$) ^a	406.1	475.6	48445	136446
Average standard deviation	213.5	225.0	29981.9	105586.1
Average standard error	123.2	129.9	17310	60960
% error (std error/average)	30.3	27.3	35.7	44.7

^afreeze-dried weight**Table 7.11** Pearson Product Moment correlations between the cylindrospermopsin concentrations of *Melanoides tuberculata* tissues and exposure regime. Cells show p value; correlation coefficient; sample size. NS = not significant; $p > 0.050$.

Trial	Exposure concentration	Exposure time
Trial two (CYN _{TOT})	$p = 0.014$; 0.685; 12	NS
Trial three (CYN _{TOT})	$p < 0.001$; 0.865; 36	NS
Trial four		$p = 0.026$; 0.363; 36
CYN _{TOT}	$p = 0.005$; 0.457; 36	
CYN _{EXC}	$p = 0.004$; 0.466; 36	
CYN _{INC}	$p = 0.005$; 0.460; 36	
CYN _{INC} /CYN _{EXC} ratio	$p = 0.035$; 0.352; 36	

A strong, positive correlation was evident between tissue CYN concentrations and CYN exposure concentrations (total or extracellular) in all trials (Table 7.11). In trial four, where live *C. raciborskii* was used, tissue toxin concentrations significantly positively correlated with CYN_{TOT}, CYN_{INC} and CYN_{EXC}, and the ratio of INC:EXC toxin. Exposure time was significantly, positively correlated with absolute tissue toxin concentrations only in trial four (Table 7.11).

Regression analyses examined relationships between fresh and freeze-dried weights, for the whole cell extract and live culture trials, respectively (Figure 7.10A, B). The correlation coefficient was higher in trial four ($r^2 = 0.8272$) than

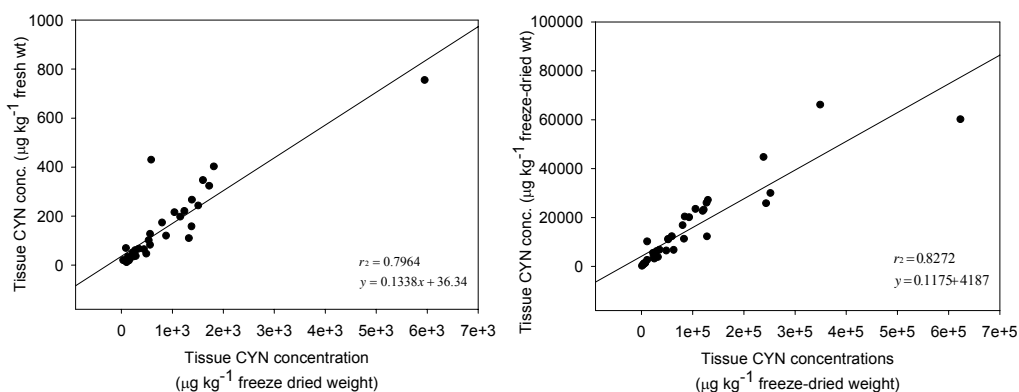


Figure 7.10 Regression of tissue cylindrospermopsin concentrations, expressed as freeze-dried and fresh values: (A) data from trials two and three using *Cylindrospermopsis raciborskii* whole cell extracts (B) data from trial four using live *C. raciborskii*.

the whole cell extract trials ($r^2 = 0.7964$). However, since neither equation closely represented actual fresh-weight data ($r^2 < 0.90$), bioconcentration factors (BCF) and bioaccumulation factors (BAF) were calculated from fresh-weight tissue toxin concentrations as recorded during analyses.

Bioconcentration occurred only in trial two, when toxin concentrations were $\geq 200 \mu\text{g L}^{-1}$ CYN_{EXC} (Table 7.12). The maximum BCF was 1.48, recorded after fourteen days exposure to $400 \mu\text{g L}^{-1}$. Generally, increased exposure times and exposure concentrations resulted in higher BCFs, though these were rarely > 1 (Figure 7.11A). In comparison, bioaccumulation values were strikingly different. Every treatment in trial four (excepting the controls) recorded bioaccumulation by *M. tuberculosis*, with BAF > 100 especially predominant in the second week of exposure (Table 7.12). Again, high BAFs corresponded with increased exposure concentration and exposure time (Figure 7.11B).

The concentration of CYN in the treatment solution significantly positively correlated with the BCF of *M. tuberculata* only in trial two (Table 7.13). Increasing exposure time was significantly positively correlated with BAF values in trial four (Table 7.13).

Table 7.12 Bioconcentration and bioaccumulation factors for *Melanoides tuberculata* following exposure to *Cylindrospermopsis raciborskii* containing cylindrospermopsin. ND = no data due to snail deaths; EXC = extracellular only; total = extracellular and intracellular.

Trial	CYN concentration ($\mu\text{g L}^{-1}$) ^b	Exposure regime Toxin type	Period	BCF or BAF ^a (average \pm standard error)
Trial two	25	EXC	7 days	0 ^c
	50	EXC	7 days	0 ^c
	100	EXC	7 days	0.13 ^c
	200	EXC	7 days	1.10 ^c
	400	EXC	7 days	1.00 ^c
	25	EXC	14 days	ND
	50	EXC	14 days	ND
	100	EXC	14 days	0.10 \pm 0.10
	200	EXC	14 days	0.97 \pm 0.11
	400	EXC	14 days	1.48 \pm 0.41
Trial three	25	EXC	7 days	0.43 \pm 0.23
	50	EXC	7 days	0.62 \pm 0.16
	100	EXC	7 days	0.51 \pm 0.08
	200	EXC	7 days	0.48 \pm 0.08
	400	EXC	7 days	0.60 \pm 0.15
	25	EXC	14 days	0.26 \pm 0.26
	50	EXC	14 days	0.64 \pm 0.35
	100	EXC	14 days	0.56 \pm 0.14
	200	EXC	14 days	0.56 \pm 0.13
	400	EXC	14 days	0.66 \pm 0.11
Trial four	91	Total	7 days	39.52 \pm 18.36
	167	Total	7 days	49.13 \pm 12.45
	223	Total	7 days	33.33 \pm 16.67
	294	Total	7 days	54.10 \pm 20.31
	406	Total	7 days	43.42 \pm 6.53
	91	Total	14 days	49.57 \pm 11.05
	167	Total	14 days	120.97 \pm 27.09
	223	Total	14 days	124.42 \pm 5.34
	294	Total	14 days	98.99 \pm 53.29
	406	Total	14 days	109.73 \pm 30.94
Shells	25 - 400	EXC	7 days	0.12 \pm 0.03

^abioconcentration (whole cell extract trials) or bioaccumulation (live *C. raciborskii* trial);

^bnominal toxin concentrations (trials two, three) or measured toxin concentrations (trial four); ^cselected samples analysed only.

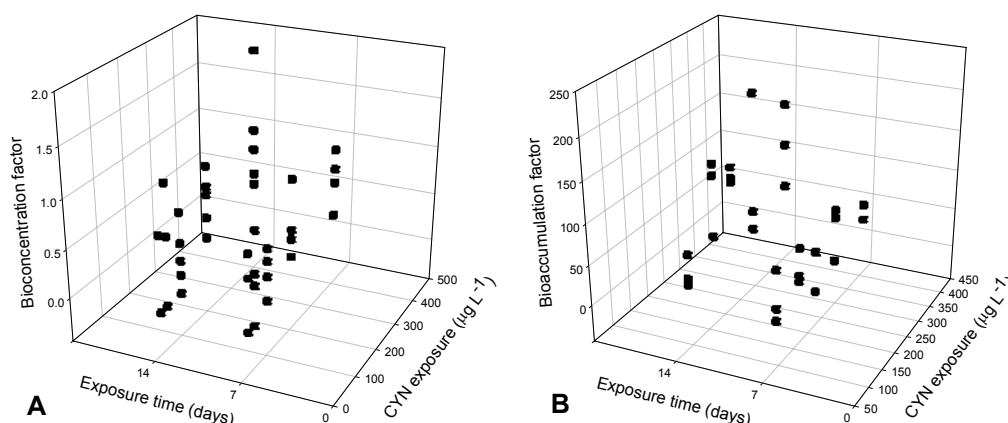


Figure 7.11 Three-dimensional scatterplot comparing exposure concentration and exposure time with (A) bioconcentration factors or (B) bioaccumulation factors for *Melanoides tuberculata*.

Table 7.13 Summary results for Pearson Product Moment correlations between bioconcentration or bioaccumulation values for *Melanoides tuberculata* and exposure regime. NS = not significant ($p > 0.050$). Cells contain p value, correlation coefficient and sample size.

Trial	Exposure concentration	Exposure time
Trial two ^a	$p = 0.001$; 0.861; 11	NS
Trial three ^b	NS	NS
Trial four ^b		$p = 0.001$; 0.582; 30
CYN _{TOT}	NS	
CYN _{EXC}	NS	
CYN _{INC}	NS	
CYN _{INC} /CYN _{EXC} ratio	NS	

^aselected values only; ^bdata for controls omitted.

7.3.5.4 Free deoxy-CYN bioconcentration and bioaccumulation

Deoxy-CYN concentrations were analysed in trials three and four only (Figure 7.12A, B). Values were far lower than CYN tissue concentrations, peaking at 7,113 $\mu\text{g kg}^{-1}$ during trial four. Bioconcentration factors could not be calculated in the whole cell extract trial since deoxy-CYN exposure concentrations were unknown. However, in the live culture trial, average BAFs ranged from 20.4 – 249.3 (Table 7.14). Increased deoxy-CYN BAFs also corresponded with increased deoxy-CYN exposure concentrations and exposure times (Figure 7.13),

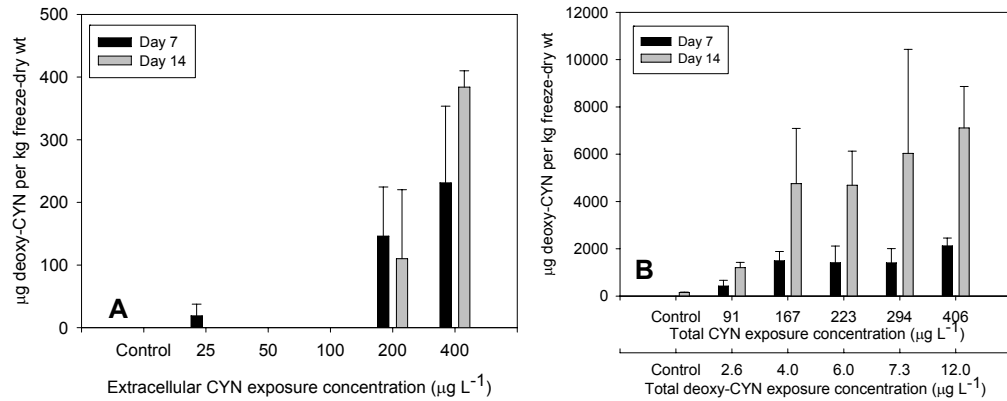


Figure 7.12 Free deoxy-cylindrospermopsin in the soft tissues of *Melanoides tuberculata*: (A) trial three, exposure to *Cylindrospermopsis raciborskii* whole cell extracts containing extracellular toxin; (B) trial four, exposure to live *C. raciborskii* culture. Note: treatment deoxy-cylindrospermopsin values were not known in trial three.

Table 7.14 Deoxy-CYN bioconcentration and bioaccumulation factors for *Melanoides tuberculata* exposed to live *Cylindrospermopsis raciborskii* treatments (controls not included).

Measured concentration (µg L ⁻¹)	Exposure regime Type	Period	BAF (average ± standard error)
3	Total	7 days	29.4 ± 15.85
4	Total	7 days	38.4 ± 1.45
6	Total	7 days	31.3 ± 10.92
7	Total	7 days	99.1 ± 30.94
12	Total	7 days	20.4 ± 9.15
3	Total	14 days	249.3 ± 8.22
4	Total	14 days	73.6 ± 27.64
6	Total	14 days	134.2 ± 27.51
7	Total	14 days	66.7 ± 52.97
12	Total	14 days	112.0 ± 27.69

similarly to the CYN bioaccumulation results. However, the BAFs of CYN and deoxy- CYN were significantly different ($p > 0.050$; one-way ANOVA), with the mean of CYN values higher than that for deoxy-CYN (85.4 and 72.3, respectively).

Both CYN treatment concentration and exposure time had significant impacts on the absolute values of deoxy-CYN in *Melanoides* (Table 7.15). However, only exposure time was significant in influencing the BAF for deoxy-CYN.

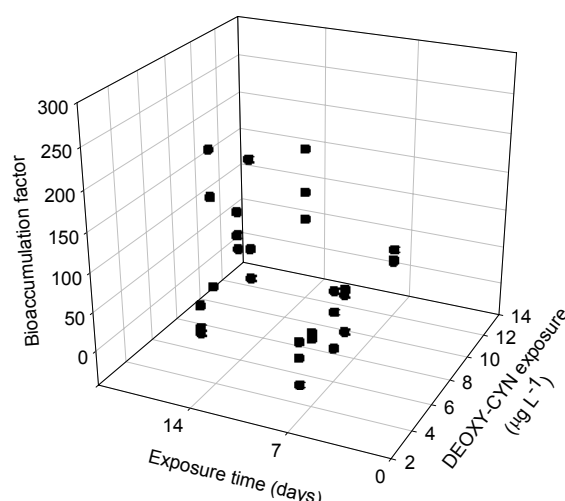


Figure 7.13 Three-dimensional scatterplot comparing exposure concentration and exposure time with bioaccumulation factors for *Melanoides tuberculata* following exposure to deoxy-cylindrospermopsin. Controls not included.

Table 7.15 Pearson Product Moment Correlations between *Melanoides tuberculata* tissue deoxy-cylindrospermopsin concentrations and bioaccumulation factor with exposure regime. Cells contain p value, correlation coefficient and sample size; controls not included. NS = not significant; $p > 0.050$.

	Exposure concentration	Exposure time
Tissue toxin concentration (μg deoxy-CYN kg^{-1} fresh)	$p = 0.040$; 0.377; 30	$p = 0.004$; 0.513; 30
Bioaccumulation factor	NS	$p = 0.001$; 0.556; 30

7.4 Discussion

7.4.1 Control waters test

Moore's Creek water was the most appropriate choice as dilution and control media, given that high activity levels were recorded in these treatment flasks. However, since the behaviour of *M. tuberculata* has never been studied, behaviour scores between fourteen and fifteen were assumed to indicate 'usual' activity levels for this species. It is conceivable that these scores instead signalled abnormally high activity; for example, stressed animals attempting to escape the test environment. This was considered unlikely given that scores less than ten were associated with snail deaths (trial two, Figure 7.3A). Furthermore,

‘natural’ stream sources for diluent and control waters are sometimes recommended in preference to artificial solutions, provided these are adequately filtered and stored prior to use (Riethmuller *et al.* 2003).

7.4.2 Water quality

Differences in conductivity, alkalinity and hardness values of waters may be related to rain periods that occurred between specimen (and hence control water) collections. However, as *M. tuberculata* thrives in these conditions naturally, with large populations evident in local waterways, such variations are expected to have had minimal influence on the snails during experimentation.

The abnormally high ammonia concentrations recorded from the 400 µg L⁻¹ treatments during the whole-cell extract trials probably reflect faecal production by the snails, especially since these treatments also featured large numbers of hatchlings. Faecal production also seemed to be higher in these treatments (general observation). In all other cases, ammonia concentrations seemed to be best overcome by reducing snail biomass loading for each test chamber (three snails per flask) and ensuring regular renewal of test solutions (48h intervals).

7.4.3 CYN, deoxy-CYN and cell concentrations

Loss of CYN from test solutions during trial three probably reflects microbial degradation, since the creek water was not sterile. Conversely, even though tap water used during trial two may contain chlorine residues, CYN was not lost from solutions during this trial. Removal of uneaten lettuce pieces from treatment solutions decreased extracellular toxin concentrations by

approximately 5%; however, this value falls within the expected variation for subsampling and CYN analysis error. Nonetheless, if this value is truly representative of losses resulting from toxin adsorption, addition of food into test chambers could considerably reduce available toxin, particularly at higher ($400 \mu\text{g L}^{-1}$) test concentrations.

Deoxy-CYN production as a percentage of CYN concentrations ranged widely throughout the trials, being approximately 65%, 30% and $< 5\%$ for trials one, two/three and four (Tables 7.3, 7.5). It seems possible that older, more-CYN rich cultures may produce less deoxy-CYN: source cultures used in trials one, two/three and four contained 257, 900 and $659 \mu\text{g L}^{-1}$ total CYN, and were approximately three-month, five month and six-month old cultures, respectively.

Production of the deoxy-CYN analog has been reported at 27 – 200% of the quantity of CYN for an Australian *C. raciborskii* strain grown in Jaworski's media (Norris *et al.* 2001) but $< 10\%$ for a Thailand strain grown in CT media containing nitrogen (Li *et al.* 2001b). Deoxy-CYN production has also been reported from *Raphidiopsis curvata*, where the analog was recorded at concentrations exceeding 2000 times that of CYN (Li *et al.* 2001a). The production of deoxy-CYN relative to CYN may therefore depend on three factors: one, species and/or strain responsible for production; two, the makeup of culture media, particularly with respect to nitrogen availability; and three, the culture age and overall toxin concentration.

Contamination of a control treatment in trial four could indicate glassware contamination, despite glassware being bleached overnight, washed and

thoroughly rinsed prior to next use. The CYN value could not have been an anomaly in the detection technique, since tissue toxins harvested on day fourteen confirmed this increase.

Calculated Q_{CYN} values are difficult to compare with most published data, where values are typically recorded at toxin concentration per dry weight of cells (for example, Saker & Griffiths 2000; Saker & Neilan 2001; Li *et al.* 2001a). Saker *et al.* (2004) reported a Q_{CYN} value of a Townsville *C. raciborskii* strain at $0.03 \text{ pg cell}^{-1}$, similar to the value for the source culture used in the present work. An analysis of natural blooms in Queensland lakes and reservoirs indicated that although highly variable, *C. raciborskii* cell concentrations of approximately $20,000 \text{ cells mL}^{-1}$ generally corresponded with toxin concentrations of $1 \text{ } \mu\text{g L}^{-1}$ CYN (McGregor & Fabbro 2000). Toxin loads recorded during the experimental trials were probably much higher than in natural settings, since culture conditions are designed to promote optimal CYN production.

Average Q_{CYNs} recorded from experimental treatments were nearly triple those of the source culture, regardless of whether these had been calculated from total or intracellular-only toxin determinations. Since treatments represented samples in which snail grazing may have occurred, increased toxin loads per cell may indicate a competitive response by *C. raciborskii*. That is, increased toxin loads may lower the palatability of *C. raciborskii* or increase the alga's toxicity: both could be effective in deterring potential grazers. In direct contrast, Fabbro *et al.* (2001) recorded decreased toxin quotas for both straight and coiled cultured *C. raciborskii* following grazing by the ciliate *Paramecium*, although the authors noted that bacterial decomposition might have contributed to this result.

Absolute toxin concentrations ($\mu\text{g L}^{-1}$) of their straight culture increased after grazing, however, this did not correspond with increased Q_{CYN} since cell concentrations were also higher. The authors suggested that cell quotas were critical in influencing the toxicity of *C. raciborskii*, with lower values corresponding to greater reproduction rates in *Paramecium* (Fabbro *et al.* 2001). The possible effects of Q_{CYNs} on toxin uptake, toxicity and feeding behaviour in *M. tuberculata* are not known. For example, Pereira *et al.* (2004) demonstrated reduced cell clearance of mussels fed *Aphanizomenon issatschenkoi* when cells had high cell toxin content. This suggests that feeding could be inhibited or could cease when cells have high toxin quotas, if preferential selection is used as a mechanism to avoid toxicity.

Toxin detected within the faecal material (Table 7.7) may represent concentrations excreted by *M. tuberculata*, or toxin adsorbed to the material after defecation. Given the variability in toxin concentrations following removal of faeces, it is difficult to determine if any CYN was actually present in the faecal strings. Possibly, the method used to remove faecal matter was inappropriate, especially since rudimentary sedimentation, rather than filtration, was used. Amorim & Vasconcelos (1999) successfully sedimented and filtered faeces from MC-exposed *Mytilus* mussels, showing that the faeces represented approximately 25% of total toxin available. This value, however, represented $< 0.75 \mu\text{g MC}$ in absolute terms. Reingestion of faecal matter was also flagged as a possible reason for increases in tissue toxins during an experimental phase where mussels were placed in toxin-free media (Amorim & Vasconcelos 1999).

7.4.4 Sublethal effects

7.4.4.1 Snail behaviour

The lack of significant treatment effects recorded for *M. tuberculata* suggests that exposure to $\leq 400 \mu\text{g L}^{-1}$ CYN in the form of whole cell extracts, or as live culture, has no effect on adult snail behaviour. Another conclusion could be that the method of measurement used did not discriminate enough to detect such changes. However, similar techniques have been effective in studies of snail behaviour to examine the effects of aluminium and silicic acid (Campbell *et al.* 2000) and the algal toxin MC (White *et al.* 2004).

Another possibility is that CYN (or other cell substances) do not exert toxicity at a gross activity level. This seems unlikely given that CYN has been shown to exert effects on snail neurons by inhibiting acetylcholine responses (Vehovszky *et al.* 1997). However, Terao *et al.* (1994) reported that mice behaviour was completely normal throughout sixteen hours of CYN exposure. This was despite that fact that multiple organ, cellular and subcellular effects had manifested during this time, including the first phase of CYN toxicity at the ultrastructural level (detachment of ribosomes from the rough endoplasmic reticulum).

The lack of mortality in the aquatic snails is curious: as Yokoyama and Park (2002) have already noted, molluscs are particularly susceptible to algal toxicity due to uptake via grazing. Snails could preferentially select against blue-green algal cells that are undesirable due to their low nutritional value or toxin content (Lirås *et al.* 1998; Leonard & Paerl 2005), instead choosing non-toxic, nutritious green algae. However, since *C. raciborskii* cells or cell extracts were the only

food sources available during the trials, preferential selection for other algae was not possible. The consistently high activity levels across all treatment groups could suggest that the snails were lacking food. Poor food availability potentially represents a strong motivator for snail movement (Burris *et al.* 1990; Dillon 2000). High activity could also suggest that snails were trying to escape toxin exposure, but this does not explain similarly high activity in the controls. Furthermore, decreased activity in snails has been noted as a strategy to minimise toxicant uptake and toxicity (Truscott *et al.* 1995). Ultimately, activity levels therefore represent a trade-off between minimising toxin uptake compared with maximising chances of toxin escape and the ability to forage for food.

7.4.4.2 Release of hatchlings

Increased release of hatchlings by *M. tuberculata* could indicate over-reproduction or abortion, possibly as a sign of stress. For example, exposure to CYN (and *C. raciborskii*) concentrations may prompt a shock response in the snails and result in early termination of brooding. If so, released embryos may be too underdeveloped to survive (A/Prof Rob Dillon, Department of Biology, College of Charleston, South Carolina, pers. comm.). However, a toxic shock response would be better evidenced by the sudden release of all hatchlings early in the exposure phase, rather than gradually. Adult *Melanoides* typically release hatchlings at a constant rate, determined by the production and maturation of the embryos (A/Prof Rob Dillon, pers. comm.). Furthermore, all young in the trials appeared to be healthy, surviving offspring, at least from general observation. In oviparous species, the gelatinous capsule surrounding the egg masses may offer some protection to developing embryos, by minimising water (and hence

dissolved toxin) uptake (Singh & Agarwal 1986). In *M. tuberculata*, the protective brood pouch could have limited transdermal exposure.

In the third trial, where intracellular toxin was present, the pattern of release was reversed: fewer hatchlings appeared in treatments containing higher CYN concentrations. This suggests that exposure to live *C. raciborskii* may be more toxic than whole extracts containing extracellular toxin only. For example, whole cell extracts associated with toxin exposures $\leq 400 \mu\text{g L}^{-1}$ could cause a mild, stimulatory toxicity, whilst live exposures of similar total toxin concentrations ($406 \mu\text{g L}^{-1}$) may exert sufficient toxicity to decrease reproduction.

Few papers have dealt with development of young by freshwater snails; those which do have focussed on the hatching and survival of eggs from oviparous species. Pesticide and heavy metal toxicities and acidic pH values are associated with decreases in egg production, delayed hatching or decreased embryonic development and death of young (Holcombe *et al.* 1984; Singh & Agarwal 1986; Gomot 1998; Tripathi & Singh 2002). These results suggest that exposures to hazardous substances are associated with reductions in embryo production, development and survival. In particular, delayed hatching may indicate a strategy to prolong development, since the protective gelatinous capsule can aid in avoiding toxicant exposure. Most recently, Ellis-Tabanor and Hyslop (2005) reported that exposure to the pesticide endosulfan caused *M. tuberculata* to produce fewer, but larger, hatchlings. It was suggested that endosulfan might inhibit calcium metabolism, thus delaying embryonic shell development and

causing the parent snails to retain hatchlings in the brood pouch (Ellis-Tabanor & Hyslop 2005).

The response of *M. tuberculata* to *C. raciborskii* and CYN exposures in regards to hatchling release may therefore represent one of three scenarios. One, toxin exposure is truly beneficial to the species, reflected by increased development and release of hatchlings. However, behavioural results from the extracellular trials do not suggest this is the case. A second scenario is that CYN is mildly stimulatory at low toxin concentrations, or extracellular-only concentrations, evidenced by an increased release of apparently healthy young.

On the other hand, higher exposure concentrations could promote early release of hatchlings (= abortion), causing decreased survivorship. Since CYN primarily exerts toxicity via protein synthesis inhibition, CYN toxicity might particularly target tissues with a fast cell reproduction rate, such as embryonic tissues. The effect of CYN (and/or *C. raciborskii*) exposure on calcium metabolism (and hence shell and muscle development) is unknown.

Finally, release of hatchlings may represent the actual toxic response; specifically, a trade-off between reducing 'brooding' stress on the adult versus increasing the toxin exposure risk to hatchlings once released from the brood pouch. Given the anomalies between the extracellular and live exposure trials, which of these three scenarios is applicable to *C. raciborskii* and CYN remains unresolved. A more thorough examination of released hatchlings (for example,

parameters such as size at birth, long-term survival rates) could provide more answers.

7.4.4.3 Relative growth rates

Significant impacts on RGRs of adult *M. tuberculata* were not recorded during exposure to whole cell extracts or live cultures containing $\leq 400 \mu\text{g L}^{-1}$ CYN. Possibly, biomass measurements may not have been sensitive enough (fresh weights recorded to 0.01 g), especially since adult freshwater gastropods (especially prosobranchs) grow relatively slowly (Dillon 2000). Negative or poor growth rates may also evidence lack of food, especially if *M. tuberculata* is capable of preferentially selecting against toxic *C. raciborskii* algal debris (the only available food source). Zooplankton species are known to avoid toxic (and low-nutritional quality) cyanoprokaryote cells (Reinikainen *et al.* 1994; DeMott 1999; Mohamed 2001) and freshwater gastropods are known to graze selectively, though not specifically regarding cyanoprokaryote species (Lodge 1986). However, faecal strings were present in the higher-end CYN concentrations ($\geq 100 \mu\text{g L}^{-1}$ CYN_{EXC} for trials two and three; or $\geq 30\%$ culture strength for trial four), especially towards the end of trial periods. This appears to indicate the ability of *M. tuberculata* to graze live or dead toxic *C. raciborskii*, although faeces were not examined microscopically for trichome fragments.

7.4.5 Overall CYN toxicity

The toxicity of *C. raciborskii* and CYN towards aquatic macroinvertebrates has been poorly studied. If the effects recorded in this study are assumed to result from CYN exposure (compared with other cellular substances such as LPSs), this indicates the toxic effects of CYN are not limited to vertebrate (particularly

mammalian) species. Crude extracts of *C. raciborskii* containing anatoxin have been shown to exert toxicity on molluscan neurones and neurotransmitter receptors (Kiss *et al.* 2002), whereas CYN-containing extracts reduced acetylcholine responses (Vehovszky *et al.* 1997). Exposure to toxin-producing *C. raciborskii* was associated with mortality, decreased body sizes and increased enzymatic activity in *Daphnia* (Nogueira *et al.* 2004a). Purified CYN causes death of brine shrimp (Metcalf *et al.* 2002b), however redclaw crayfish and rainbowfish survived a *C. raciborskii* bloom containing up to 589 $\mu\text{g L}^{-1}$ CYN, apparently with few ill effects (Saker & Eaglesham 1999). Saker *et al.* (2004) also recorded high CYN bioaccumulation in the mussel *Anodonta*, again with no apparent side effects.

Although toxic effects may occur in freshwater gastropods, it appears that these do not manifest on a gross behavioural (activity) scale, impair reproduction, nor affect RGRs. Most importantly, snail death does not occur at exposure to cell extracts containing $\leq 400 \mu\text{g L}^{-1}$ CYN_{EXC}, nor during exposure to live *C. raciborskii* cultures associated with up to 406 $\mu\text{g L}^{-1}$ CYN_{TOT}.

7.4.6 Bioaccumulation

7.4.6.1 Toxin losses during freeze-drying

Freeze-drying of samples (100°C under vacuum, up to 96 h) did not cause a significant reduction in CYN or deoxy-CYN concentrations of snail tissues (Figure 7.6). This result is important, since it validates the use of freeze-dried weights in reporting tissue toxins, and these may be more accurate than the fresh weight equivalents.

7.4.6.2 CYN associated with shell

M. tuberculata did not bioconcentrate CYN into shell material ($BCF < 1$), with maximum (free) CYN concentrations recorded at $45 \mu\text{g kg}^{-1}$. Potentially, this value could represent toxin adsorbed to the external shell wall, rather than uptake and internal deposition (as noted for aquatic macrophytes in section one). Bioconcentration could occur as snails secrete the shell layer from the mantle during normal growth, but this may require far longer periods of CYN exposure. Interaction(s) between CYN and the proteinaceous and calcareous components of freshwater gastropods shells is/are not known. Thus, CYN may have been deposited into the shell, but could not be extracted via HPLC/MS-MS due to binding processes.

7.4.6.3 Free CYN bioconcentration and bioaccumulation

Values for CYN bioconcentration and bioaccumulation in *M. tuberculata* could have been underestimated. If CYN became bound within the tissues, or was metabolised, these toxin components would have been overlooked using the HPLC technique. A dramatic underestimation of tissue toxins could result: Williams *et al.* (1997) reported that only 0.1% of total MCs were detected from *Mytilus* mussels using protein phosphatase assays. Spike recoveries in the present work also indicated that, on average, 12.5% of CYN could be lost during the extraction and detection process (average spike recovery = 87.5%).

Over-estimation of bioaccumulation may also have occurred if tissue toxin concentrations were artificially inflated by toxin derived from the stomach of *M. tuberculata*. Up to 83% of MC detected in *Lymnaea* snails was shown to

actually originate from the alimentary canal (Eriksson *et al.* 1989). Several other studies have attributed large proportions of toxins to the digestive glands of molluscs, although the concentrations of these are highly variable between species (Vasconcelos 1995; Saker *et al.* 2004). However, to remain consistent with the definition of bioaccumulation, such toxins should not be regarded as tissue toxins until absorption takes place.

Toxin concentrations represented by CYN in the alimentary canal are impossible to determine if whole-animal analyses are undertaken (as done here). Dissection of the alimentary tract from the snails prior to analyses could have reduced or eliminated this problem. However, this was not done since the study was designed to emphasize environmental relevance: toxin uptake was investigated because of possible linkages with increased adverse effects, including implications for toxin uptake by snail predators. For the latter, making the distinction between toxin in the alimentary tract and the rest of the snail is meaningless, since predators typically consume the entire body of snails whole.

Considerable variability in tissue toxin concentrations was recorded between the extracellular trials, despite both having identical exposure regimes. The variability of the data could indicate the impact of stress on toxin uptake, metabolism and depuration. For example, additional or synergistic effects (such as poor water quality combined with CYN toxicity, see section 7.3.1) may have resulted in a reduced ability to metabolize toxin. However, only selected samples were analysed for CYN in trial two. The peak toxin concentration (3,267 $\mu\text{g g}^{-1}$ freeze-dried weight) was the average of two samples that had vastly

different toxin concentrations (5951 and 583 $\mu\text{g g}^{-1}$). If these data are discounted, values for trial two compare favourably with those of trial three.

The high variability in data recorded from trial four (Table 7.10) appears common for studies of algal toxin bioaccumulation. Microcystin uptake in *Sinotaia histrica* reportedly varied from 0.42 – 2.65 μg toxin per snail per day (Ozawa *et al.* 2003). In this work, some variability would have been removed since multiple *Melanoides* were assigned per test chamber ($n = 3$) and then pooled together for CYN determinations. Previous exposure, differential grazing rates, uneven placement of snails and the spatial distribution of toxin-laden phytoplankton cells within the water column could all contribute to inconsistencies in MC concentrations of freshwater pulmonate snails (Prepas *et al.* 1997; Zurawell *et al.* 1999). However, the laboratory setting for *M. tuberculata* trials allowed most of these variables to be controlled, with the notable exception of grazing rates. Hence, grazing rates are probably the primary cause of variability.

Effects of toxin exposure concentrations and exposure time

Significant, positive correlations were detected between all types of exposure concentrations (CYN_{EXC} , CYN_{INC} and CYN_{TOT}) and tissue CYN concentrations in trials two to four (Table 7.11). Positive correlations between tissue toxin concentrations and intracellular toxins have been recorded in several other bioaccumulation studies (Zurawell *et al.* 1999; Yokoyama & Park 2002). However, the present data contrasts the work of Yokoyama and Park (2002), where no significant correlation existed between tissue toxins and the amount of

extracellular MC available. However, their study could have been confounded since only a tiny proportion of MC ($\leq 2\%$) was represented by the dissolved fraction (Yokoyama & Park 2002).

A significant effect of exposure time was present only during trial four. Possibly, correlations for exposure time may have been confounded since tissue harvests only took place on days seven and fourteen. Other authors have examined tissue toxin concentrations at two-day or even hourly intervals (Zurawell 2001; Saker *et al.* 2004). The effects of exposure time deserve close attention, however, because time may also be significant in determining accumulation risk for specific tissues. For example, Saker *et al.* (2004) reported that the relative distribution of CYN between haemolymph, viscera and remaining tissues varied significantly with exposure time.

When tissue contamination is considered relative to bioavailable toxin (that is, BCF or BAF values), the influences of exposure regime are slightly changed. Here, exposure concentrations significantly correlated with BCF values only in trial two (Table 7.13), with higher BCF values associated with higher available CYN in the test media. This contrasts with Streit (1998, p. 378), who concluded that bioaccumulation (really, bioconcentration) factors in invertebrates tend to be higher when exposure concentrations are lower. Moreover, exposure concentration, be it expressed as total, intracellular-only or extracellular-only, did not correlate at all with BAF values in the live culture trial. Exposure time, however, was again positively correlated with tissue toxins and BAF during the live *C. raciborskii* trial.

In plainer terms: high CYN_{TOT} concentrations are likely to result in snails having higher tissue contamination values than those in low CYN_{TOT} concentration environments. However, the overall bioconcentration/bioaccumulation values are likely to be similar in both conditions. In contrast, longer exposure times will result in both higher absolute tissue toxin concentrations *and* significantly increased BAFs, at least where intracellular toxin is present. Presumably, the latter situation results because *M. tuberculata* are unable to maintain constant rates of toxin metabolism or depuration for periods longer than seven days.

Effects relating to the availability of different toxin components

The differences between bioconcentration and bioaccumulation values in *M. tuberculata* were considerable. Exposure to live *C. raciborskii* containing CYN_{INC} resulted in far higher toxin loads in *M. tuberculata* than when exposure was limited to CYN_{EXC} only. This was despite each of the trials measuring a similar range of toxin exposure concentrations (zero to approximately $400 \mu\text{g L}^{-1}$ CYN_{TOT}). Intracellular toxin accounted for less than one-quarter of the total bioavailable toxin in the live trial (Table 7.5), but led to $> 100\%$ increases of BAFs compared to BCFs.

These data indicate that intracellular toxin is critical in elevating CYN bioaccumulation values in *M. tuberculata*. A similar conclusion could be reached after examining the work of Prepas *et al.* (1997). In that study, exposure to $50 \mu\text{g L}^{-1}$ dissolved MC over three days did not allow toxin bioaccumulation in the freshwater clam, *Anodonta*. However, just $8 \mu\text{g L}^{-1}$ total MC exposure (cellular and dissolved fractions present) resulted in tissue concentrations of

$776 \pm 569 \mu\text{g kg}^{-1}$ dry weight (Prepas *et al.* 1997). Since the relative proportions of intracellular and extracellular toxins are dynamic throughout toxic cyanoprokaryote blooms (see chapter two), this concept has critical implications for the bioaccumulation risk of *M. tuberculata* and probably other freshwater mollusc species.

Clearly, CYN exposure concentration strongly influences the potential for tissue contamination (absolute toxin concentrations; $\mu\text{g kg}^{-1}$) in *M. tuberculata*. However, since the issues of exposure time and toxin fraction availability must also be considered, toxin contamination cannot accurately be predicted from exposure concentration alone. Values for BCF and BAF seem to depend more closely on exposure time and, more importantly, the presence of intracellular toxin. In natural blooms, proportions of dissolved and cell-bound toxins (CYN and others) may be highly variable due to algal growth phase, degradation and dilution (Zurawell *et al.* 1999). Thus, accurate predictions of bioaccumulation risk are not possible unless these ratios are quantified properly and regularly.

Comparisons with other test species and toxins

To date, CYN accumulation has been studied in only one other freshwater mollusc. Saker *et al.* (2004) reported CYN values of up to $61,500 \mu\text{g kg}^{-1}$ dry weight from the haemolymph of *Anodonta* mussels, following maximum total CYN exposure concentrations of $90 \mu\text{g L}^{-1}$ over 2 – 18 days. Values for the whole mussel tissue were considerably lower at $2,900 \mu\text{g kg}^{-1}$ dry weight (Table 7.16). In comparison, toxin concentrations detected from whole *M. tuberculata* tissues peaked at 17,260 and $35,260 \mu\text{g CYN kg}^{-1}$ dry weight following seven and fourteen days exposure to $91 \mu\text{g L}^{-1}$ CYN_{TOT}.

Table 7.16 Some values for bioaccumulation^a of algal toxins in molluscan species. Data are maxima reported in the literature. NR = not recorded; HPLC = high performance liquid chromatography; PP = protein phosphatase; MC = microcystin; CYN = cylindrospermopsin; hp = hepatopancreas; GC/MS= gas chromatography / mass spectrometry. Total = cellular and dissolved components together.

Species	Study type	Toxin type	Max. exposure concentration	Max. exposure	Max. tissue toxin $\mu\text{g kg}^{-1}$ DW	Analysis method	Reference
Freshwater gastropods							
<i>Helisoma trivolis</i>	Field	MC-LR; dissolved and cellular	Up to 11 $\mu\text{g L}^{-1}$ cellular, plus 0.3 $\mu\text{g L}^{-1}$ dissolved	NR	11,000 (whole)	HPLC	Kotak <i>et al.</i> (1996)
<i>Lymnaea stagnalis</i>	Field	MC	Up to 11 $\mu\text{g L}^{-1}$	NR	96, 000 (whole)	HPLC	Kotak <i>et al.</i> (1996)
<i>Melanooides tuberculata</i>	Laboratory	CYN; dissolved CYN; total Deoxy-CYN; dissolved Deoxy-CYN; total	400 $\mu\text{g L}^{-1}$ ^b 406 $\mu\text{g L}^{-1}$ ^c NR 12 $\mu\text{g L}^{-1}$ ^c	14 days 14 days 14 days 14 days	1,420 ^d (whole) 238,248 (whole) 384 (whole) 7,113 (whole)	HPLC	This study
<i>Physa gyrina</i>	Field	MC	Up to 11 $\mu\text{g L}^{-1}$	NR	121,000 (whole)	HPLC	Kotak <i>et al.</i> (1996)
<i>Sinotaia histrica</i>	Field	MC (RR & LR); total	0.476 $\mu\text{g L}^{-1}$	NR	19,500 (intestine) 3,200 (hp)	HPLC	Ozawa <i>et al.</i> (2003)
	Laboratory	MC; total	20.1 \pm 6.1 $\mu\text{g L}^{-1}$	1-15 days	436,000 (hp)		
Freshwater bivalves							
<i>Anodonta cygnea</i>	Laboratory	<i>Oscillatoria</i> toxin; dissolved and cellular	40 – 60 $\mu\text{g L}^{-1}$	15 days	70, 000 (whole)	HPLC	Eriksson <i>et al.</i> (1989)
<i>Anodonta cygnea</i>	Laboratory	CYN; total	14 – 90 $\mu\text{g L}^{-1}$	2 – 16 days	61,500 (haemolymph) 5,900 (viscera) 2,900 (whole)	HPLC	Saker <i>et al.</i> (2004)
<i>Anodonta grandis simpsoniana</i>	Laboratory	MC-LR; dissolved	51 – 55 $\mu\text{g L}^{-1}$	3 days	\leq detection (whole)	PP assay	Prepas <i>et al.</i> (1997)
	Field	MC-LR; total	0 – 8.3 $\mu\text{g L}^{-1}$	21 – 28 days	776 \pm 569 (whole)		
<i>Anodonta woodiana</i>	Field	MC (RR& LR); total	36 $\mu\text{g L}^{-1}$ cellular and 0.211 $\mu\text{g L}^{-1}$ dissolved	Monthly collections (two years)	12,600 (hp)	HPLC	Yokoyama & Park (2002)

Species	Study type	Toxin type	Max. exposure concentration	Max. exposure period	Max. tissue toxin $\mu\text{g kg}^{-1}$ DW (tissue type)	Analysis method	Reference
<i>Anodonta woodiana</i>	Field study	MC LR & RR; total	NR	NR	2710 (hp) ^f 2000 (gills/muscle) ^f 1190 (gonad) ^f 1310 (gut) ^f	HPLC/LC-MS	Watanabe <i>et al.</i> (1997)
<i>Corbicula sandai</i>	Field	MC (RR & LR); total	0.476 $\mu\text{g L}^{-1}$	NR	\leq detection (hp)	HPLC	Ozawa <i>et al.</i> (2003)
<i>Cristaria plicata</i>	Field	MC (RR& LR); total	36 $\mu\text{g L}^{-1}$	Monthly collections over two years	297,000 (hp)	HPLC	Yokoyama & Park (2002)
<i>Cristaria plicata</i>	Field study	MC LR & RR; total	NR	NR	Nil (hp) ^f	HPLC/LC-MS	Watanabe <i>et al.</i> (1997)
<i>Mytilus galloprovincialis</i>	Laboratory	all MC variants; total	not clear	4 days	10,700 (whole)	ELISA	Amorim & Vasconcelos (1999)
<i>Mytilus galloprovincialis</i>	Laboratory	MC-LR; total	153 \pm 73.79 μg	16 days	Nil (foot) 290 (gills) 230 (muscle) 27, 600 (gut) 460 (all other)	HPLC	Vasconcelos (1995)
<i>Mytilus edulis</i>	Field study	all MC variants; dissolved	one dose of 16.165 mg toxin in freeze-dried cells	53 days	336, 900 wet wt, equivalent to 100% of available MC (whole)	PP assay; Lemieux oxidation and GS/MS	Williams <i>et al.</i> (1997)
<i>Unio douglasiae</i>	Laboratory	MC; total	27 \pm 4.27 $\mu\text{g L}^{-1}$ 50 \pm 7.52 $\mu\text{g L}^{-1}$	15 days	\geq 200, 000 (hp)	HPLC	Yokoyama & Park (2003)
<i>Unio douglasiae</i>	Field	MC (RR& LR); total	and 0.211 $\mu\text{g L}^{-1}$	15 days Monthly collections over two years	630, 000 (hp) 420,000 (hp)	HPLC	Yokoyama & Park (2002)
<i>Unio douglasiae</i>	Field study	MC LR & RR; total	NR	NR	205 (hp, wet wt) ^h	HPLC/LC-MS	Watanabe <i>et al.</i> (1997)

^abioaccumulation and bioconcentration; ^bextracellular exposure; ^ccombined intracellular and extracellular (live *C. raciborskii*) exposure; ^dmaximum value for trial three (maximum value in trial two considered an outlier); ^eshells removed; ^fthese values appear to be wet weight.

An easier comparison between these data can be made following conversion into bioaccumulation factors. *Anodonta cygnea* mussels recorded a bioaccumulation factor of 4.53 when maximum CYN values in the haemolymph ($408 \mu\text{g L}^{-1}$) were compared with the total CYN of the surrounding media ($90 \mu\text{g L}^{-1}$) after fourteen days exposure (Saker *et al.* 2004). In contrast, *M. tuberculata* recorded an average BAF value of 49.57 from the whole tissues in similar exposure conditions (Table 7.12). The reason for the much higher accumulation in *M. tuberculata* could be attributed to multiple factors: the studies differed in terms of species studied, temperature, *C. raciborskii* strain, size of test species and toxin extraction methods.

Direct comparisons between the bioaccumulative potential of CYN and other algal toxins are difficult. Most work has been conducted on MC, which can accumulate in freshwater molluscs at concentrations ranging from 136 – 630,000 $\mu\text{g g}^{-1}$ dry weight, depending on test species and the tissues in question (Table 7.16). Certainly, values for CYN accumulation fall within the recorded range for MC. However, MC is mostly intracellular: Yokoyama and Park (2002) reported 2% of toxin to be extracellular during a *Microcystis* bloom that caused up to 420,000 $\mu\text{g kg}^{-1}$ MC contamination of bivalve tissues. Considering the importance attached to the relative abundances of toxin components in terms of bioaccumulation, comparing these toxins seems inappropriate.

Data recorded from MC bioaccumulation studies may still be useful in identifying general trends that could apply to CYN. For instance, there is considerable disparity in the maximum reported values of MC accumulation,

even in closely related organisms (Table 7.16). This variability could relate to a number of issues, but especially that several different toxin extraction and detection methods were employed. These may lead to inconsistencies between studies, given that some techniques are more suited for detection of cell-bound toxins.

Some studies have reported values based on different tissues types: if toxin deposition is not equal in all organs, this may considerably affect BAF values. In fact, it seems that the hepatopancreas is a definite target for MC deposition (Eriksson *et al.* 1989), so much so that one author was prompted to call the toxin 'hepatotropic' (Zurawell 2001). Again, however, this must be viewed with consideration to environmental relevance: soft tissues are usually consumed whole. The real value of identifying organ-specific toxin deposition may be in helping to determine the mechanism of action of CYN in invertebrates. For example, toxicity could be minimised or exacerbated via compartmentalisation of toxins away from, or into, target tissues.

Issues of different experimental approaches and reporting styles aside, it appears that high interspecific variability is common in toxin bioaccumulation (Table 7.16). Numerous parameters could affect individual accumulation rates, including (but not limited to) variation in functional feeding groups (herbivore/omnivore grazers compared with filter feeders); the ability to preferentially select against ingesting toxin-laden cells; particle rejection and consequent production of pseudofaeces (Babcock-Jackson 2000); specific feeding, assimilation, metabolism and depuration rates; and varying surface area

to volume ratios. The latter may be especially important when comparing large bivalves to the much smaller gastropods. Yokoyama and Park (2002) also provided data that showed different bivalve species varied even in the uptake of MC variants.

Depuration of algal toxins has been closely studied in several freshwater molluscs, with toxin clearance generally proceeding in a bi-phasic pattern. Toxin concentrations generally decrease rapidly within days of transfer into clean media, but this is followed by a prolonged period of gradual clearance (Amorim & Vasconcelos 1999; Zurawell 2001; Yokoyama & Park 2003; Saker *et al.* 2004). This could result either from the release of previously bound toxins, or from toxin metabolism occurring within the tissues (Saker *et al.* 2004). Most importantly, these studies highlight that toxins may persist in the tissues for long periods. Even after transfer into toxin-free water, toxins have been detected from organisms some 30 to 65 days later (Eriksson *et al.* 1989; Saker *et al.* 2004). This is critical information in showing that tissue contamination may be evident even when the water column is free of toxins (Yokoyama & Park 2002).

7.4.6.4 Possible methods of uptake

During trials two and three, the only possible methods of toxin uptake were transdermal uptake (active or passive transport) or accidental drinking of aqueous toxin. The relative contribution of each of these pathways with respect to final tissue toxin concentrations can only be speculated.

Transdermal uptake could result from simple diffusion, where CYN may pass through the cell membranes of the dermis or gill epithelia. Whether this is

possible is not known. Possibly, the protective shell layer may limit transdermal uptake by shielding the fleshy tissues from contact with the toxin.

Equally, however, uptake could result from active transport: freshwater snails actively transport amino acids, sugars and even particulate matter across their general body surfaces as part of normal respiratory, osmoregulatory and digestive processes (Dillon 2000, p. 61). Amino acids may facilitate active transport in the foot (Lam *et al.* 1997). *Lymnaea stagnalis* uses the non-specialised epidermal layers of the dorsal head and mantle for uptake of large particles, via the use of vacuoles, lysosomes and vesicles (Zylstra 1972). However, this uptake appears to be selective, based on the charge, size and type of compounds. The expenditure of energy to take up a toxic molecule seems unlikely.

Accidental ingestion of aqueous toxin via drinking or feeding could allow CYN to enter the alimentary tract. Accidental drinking is a normal part of the bite-and-swallow cycle used by aquatic gastropods (De With 1996). Truly accumulated toxin would then need to cross the epithelial layers of the digestive tract. This echoes the argument above: does this proceed by simple diffusion or active transport molecules? Is CYN able to bind to surface molecules and change the permeability of cell membranes?

Uptake of extracellular toxins must occur, given that bioconcentration was evident in at least one of the whole cell extract trials. The variability in tissue toxin concentrations recorded in trials two to four could provide evidence for a

transdermal uptake method (passive or active). For example, different surface area to volume ratios in these trials could have moderated transdermal uptake rates. However, test organisms were of mostly uniform size, and accidental drinking or grazing rates could also contribute to variability. Thus, the mode of uptake of dissolved toxins remains unknown.

On the other hand, exposure to live *C. raciborskii* in trial four introduced an entirely new route of potential uptake. It has already been suggested that *M. tuberculata* could graze on *C. raciborskii*, although faecal strings were not examined for trichomes. Increased variability of the trial four data set (Table 7.10) was considered to result from differential grazing rates. It seems natural, then, to conclude that direct ingestion of toxin-laden cells during grazing is the probable route of toxin uptake. However, there are some problems inherent with snail grazing on *C. raciborskii*.

Grazing represents an obvious opportunity to ingest large volumes of toxin-laden cells. However, in the natural environment, planktonic *C. raciborskii* trichomes may never exist in habitat of *M. tuberculata*, unless the trichomes sink during adverse conditions, or benthic forms are possible. Gastropods usually graze on attached periphyton, or, conceivably, cyanoprokaryote colonies or filaments that settle out of the water. *Microcystis* is also a primarily planktonic species, but Zurawell *et al.* (1999) suggested that this could be a viable food source if settling-out occurred during the progression of a toxic bloom. A similar argument has been ventured for the planktonic alga *Nodularia* and its associated toxin, nodularin (Sipiä *et al.* 2001b).

Assuming grazing is possible, trichomes or cells of *C. raciborskii* need to unload their cellular toxin during their passage through the alimentary canal, if uptake is to occur. Digestion by freshwater gastropods involves first encountering the scraping radula, followed by tituration (= grinding up) of material in the gizzard, possibly aided by sand grains (Dillon 2000). According to Dillon (2000), only tiny particles $\leq 0.4 \mu\text{m}$ are able to pass into the digestive diverticula, ready for phagocytosis and/or extracellular digestion.

Based on this evidence, *C. raciborskii* cells could not leave the alimentary canal without being lysed. Thus, any toxin present in the cells could become liberated in the alimentary tract. However, intact *Microcystis* colonies have been reported from the faecal strings of *Lymnaea stagnalis*, possibly indicating that cyanoprokaryote cells could escape the mechanical breakdown performed by the gizzard (Zurawell *et al.* 1999). Most notable here, however, is that *Microcystis* colonies are typically encapsulated in mucilaginous sheaths that could be crucial in preventing cell (and colony) lysis (Zurawell 2001).

Assuming cells were lysed within the gut, secretions used to facilitate breakdown of food for later absorption (extracellular digestion) could break apart the CYN molecule. However, the digestive enzymes of snails are not well known, excepting cellulase (Dillon 2000). Furthermore, breakdown processes could prevent toxin detection via HPLC. Thus, it appears that following grazing, simple diffusion or active transport of the entire CYN molecule must occur

within the gastrointestinal tract. Further details of this process (how, where) cannot reasonably be determined without further experimentation.

M. tuberculata had the opportunity for both CYN_{EXC} and CYN_{INC} uptake during trial four. The proportion of toxin uptake represented by dissolved toxin – whatever the uptake route - cannot be clearly quantified. The importance of intracellular toxin in influencing CYN bioaccumulation is inextricably linked with the identification of primary uptake routes. Given that dramatically increased bioaccumulation values correspond with the introduction of (comparatively) small quantities of cell-bound toxin, grazing probably represents the major toxin uptake route.

Conversely, extracellular toxin is likely to contribute to only minor tissue contamination. This conclusion is corroborated by many other studies of algal toxin accumulation, although few physically tested for differences in accumulation resulting from extracellular-only or total toxin (intracellular plus extracellular) exposure (Kotak *et al.* 1996; Prepas *et al.* 1997; Zurawell *et al.* 1999; Ozawa *et al.* 2003; Saker *et al.* 2004).

7.4.6.5 Deoxy-CYN bioconcentration and bioaccumulation

This is the first study to report deoxy-CYN values from the tissues of an aquatic animal. Patterns of deoxy-CYN bioconcentration and bioaccumulation were similar to those of CYN, albeit with much lower final tissue concentrations. Bioaccumulation factors for deoxy-CYN were also significantly less than those for CYN; however, this may be confounded by the exposure concentrations of

deoxy-CYN ($0 - 12 \mu\text{g L}^{-1}$) being less compared with those for CYN ($0 - 406 \mu\text{g L}^{-1}$).

Previously, deoxy-CYN is not thought to contribute to the toxicity of *Cylindrospermopsis*, given the result of mouse bioassays (Norris *et al.* 1999). However, more recent research has contrasted this study (Looper *et al.* 2005). Hence, it is difficult to consider the importance of exposure to, and accumulation of, deoxy-CYN in freshwater aquatic organisms compared with that of CYN.

7.5 Areas of further study

Opportunities abound for further work concerning CYN bioconcentration and bioaccumulation in *M. tuberculata* and other freshwater molluscs. An important sampling design issue highlighted during these trials was that where grazer species are used as test organisms, researchers should anticipate high individual variability and hence increase the number of replicates to counteract this problem. There is a clear need to determine if bioaccumulation values are being underestimated, due to detection methods being unable to account for tissue-bound toxins. Improvements in analytical techniques and/or radiolabelling work may enable progress in this area. Determining the exact method/s of toxin uptake is also vital, so that appropriate risk minimisation procedures can be developed. Finally, a more comprehensive examination of the possible sublethal effects of exposure is also required, especially given that sublethal effects evident on much more subtle scales (immunological, histological) have not yet been explored.

Chapter eight: ecotoxicity, histopathology, bioconcentration and bioaccumulation risks in *Bufo marinus* tadpoles

Note to reader: part of the material in this chapter has been submitted for publication as **White, S.H.**, Duivenvoorden, L. J., Fabbro, L. D. & Eaglesham, G. K.. Mortality and toxin bioaccumulation in *Bufo marinus* following exposure to *Cylindrospermopsis raciborskii* cell extracts and live cultures.

8.1 Introduction

The anuran amphibians are one of the most understudied groups of aquatic organisms with respect to cyanotoxin exposure. A recent review of ecotoxicological effects of algal toxins failed to mention frogs and tadpoles (Wiegand & Pflugmacher 2005). No published works have examined toxin bioaccumulation in this group. However, these animals are generally regarded as useful and sensitive as aquatic toxicity test organisms (Ferrari *et al.* 1997; Bridges 1999; Tyler 1999). Moreover, bioaccumulation in amphibians may be critical, since these animals represent clear linkages to terrestrial environments: individuals that survive CYN exposure during pre-metamorphic stages may grow into adults that enter terrestrial food webs.

In ecotoxicity tests, tadpoles are preferred to adult specimens for a number of reasons. Pre-exposure (and thus potential tolerance) to pollutants is unlikely, given the short timeframes available. Larval stages are also smaller, thus allowing for relatively small resource requirements. Also, the pre-metamorphic stages – embryos, hatchlings and tadpoles – appear to be more susceptible to aqueous toxins than their adult counterparts (Bishop & Martinovic 2000).

Tadpoles, like aquatic snails, have several potential routes for uptake of algal toxins. Tadpoles may experience a high risk of transdermal uptake due to being entirely

submerged, and having greater and more permeable surface areas (such as the gills) than adults. They also have limited options for escaping exposure. The diet of tadpoles allows for the possible ingestion of toxin-laden cells (uptake via food sources), in contrast to that of adult frogs, where toxin ingestion is perhaps less likely.

The cane toad *Bufo marinus* (Linnaeus) (Anura: Bufonidae) was introduced into Australia in 1935 to control the cane beetle *Phyllophaga vandinei* (Tyler 1999). *B. marinus* tadpoles have an average time to metamorphosis of about one month. Since *B. marinus* adults typically choose warm, stagnant waters for spawning, there is a high likelihood for tadpole grow-out to occur in waterways experiencing toxic *C. raciborskii* blooms. The following chapter examines toxicity (including histopathology), bioconcentration and bioaccumulation of CYN in *B. marinus* during exposures to freeze-thawed *C. raciborskii* whole cell extracts and live *C. raciborskii* cultures.

8.1 Materials and methods

8.1.1 Collection and culture of experimental organisms

Specimen collections and all experimental trials were carried out under CQU Animal Experimentation Ethics Committee certificate number A04/03-158. *B. marinus* hatchlings were collected from local waterways in central Queensland (Table 8.1). Cylindrospermopsin analyses indicated that this toxin was not present in the water at the time of specimen collection. Care was taken to collect tadpoles of similar age (Gosner development stage), however, tadpoles may not have come from a single egg string, since ponds typically contained hundreds of conspecifics. Water from each site was also collected for use in culturing and as a diluent in the test solutions. A subsample was also taken and filtered for determinations of water hardness and alkalinity, as described in section 7.2.2.2.

Table 8.1 Details of *Bufo marinus* tadpole collections used in ecotoxicity testing. CQU = Central Queensland University, Rockhampton.

Trial	Collection site	Approximate Gosner stage (at time of collection)
Trial one	Ornamental pond, CQU	22 - 24
Trial two	Ornamental pond, CQU	22 - 24
Trial three (whole cell extracts of <i>C. raciborskii</i>)	Ornamental pond, CQU	24 - 26
Trial four (live <i>C. raciborskii</i>)	Moore's Creek, Rockhampton	24 - 26
Trial five (live <i>C. raciborskii</i>)	Moore's Creek, Rockhampton	24 - 26
Trial six (histology; whole cell extracts of <i>C. raciborskii</i>)	Moore's Creek, Rockhampton	24
Trial seven (histology; live <i>C. raciborskii</i>)	Moore's Creek, Rockhampton	24 - 26

Hatchlings were transferred to a controlled climate facility (30°C) and acclimatised for at least 48 h. As recommended by McDiarmid and Altig (1999), hatchlings were kept in the water from the collection point. Hatchlings were provided with Wardley's *Spirulina* blue-green algal discs (primary constituent *Spirulina* algae meal, minimum protein 32%).

8.1.2 Test procedures

Trials were conducted in an air-conditioned laboratory, with windows blacked out with plastic to minimise light fluctuations from adjoining laboratories. Light (14:10 light:dark photoperiod at approximately 35 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$) was provided by cool white fluorescent tubes (Osram Lumilux) mounted approximately 40 cm from the water surface.

Test chambers were 500 mL glass conical flasks stoppered with rubber bungs to reduce evaporation; chambers were partly submerged in a water bath in which the temperature was maintained by circulation and temperature regulation devices. Test

solutions (300 mL) were prepared by diluting freeze-thawed or live *C. raciborskii* cultures (depending on trial type) to achieve the desired concentrations. Control and dilution waters were filtered water (Whatman GF/F glass microfibre) from the tadpole collection site. Test solutions were renewed every 48 h to maintain constant CYN concentrations; solutions were also reserved and frozen at the end of harvest periods and two randomly chosen samples analysed for actual CYN concentrations.

During experimentation, tadpoles were fed Wardley's *Spirulina* blue-green algal discs; other food such as boiled lettuce and higher-protein mixes could have caused fouling. Since *Spirulina* is a cyanoprokaryote, the discs also represented a non-toxic blue-green control, thus allowing any toxic effects to be attributed to CYN or *C. raciborskii*-derived substances, rather than from common blue-green algal components (such as lipopolysaccharides). Uneaten food was removed during test solution replacement (every 48 h).

Basic water quality parameters (total ammonia, pH, dissolved oxygen and conductivity) were checked every 48 h prior to test solution renewal. Care was taken to minimise tadpole disturbance.

8.1.2.1 Trial one (pilot)

A range-finding test was conducted over six days, using a control and two treatment concentrations (50 and 200 $\mu\text{g L}^{-1}$ CYN_{EXC}). Each treatment was prepared with three replicates (total $n = 9$). Three tadpoles, all at approximate Gosner development stages 24 – 26 (Gosner 1960), were randomly assigned to each test chamber. Each flask had one-quarter of a *Spirulina* disc (< 0.1 g) added every 48 h.

Tadpole behaviour, disturbance response, percentage mortality and abnormalities were recorded daily. Behaviour was measured by recording the number of seconds a single tadpole spent swimming during 30 seconds of observation. Values ($n = 3$) were then averaged to obtain a score for each flask. The ability of tadpoles to respond to a disturbance was measured by gently probing each individual with a glass rod, and assigning a score of zero to three. Values were assigned as follows: zero, no movement (usually indicating death); one, slight motion (tail wriggle) but not away from source of disturbance; two, slow swimming away from rod; and three, an immediate burst of swimming away from rod. Treatment values were then normalised by subtracting the average value recorded from controls.

To satisfy ethical guidelines, moribund animals had to be euthanized: these were recorded as dead. Tadpoles were considered dead or moribund if they showed no movement after gentle probing with a glass rod. Dead carcasses were removed daily but the tadpole(s) were not replaced with live animals. At the end of six days, surviving tadpoles were euthanized by immersion in ice-cold distilled water, followed by freezing.

Flasks were not individually aerated (as done in *M. tuberculata* trials) since the aerator tips might have affected swimming and behaviour, and since the test species is accustomed to survival in warm, shallow ponds where dissolved oxygen concentrations are low.

8.1.2.2 Trial two

The second (pilot) trial improved on the experimental techniques used above. Test concentrations were again 0, 50 and 200 $\mu\text{g L}^{-1}$ CYN_{EXC}. Replication was increased

to six. To prevent fouling, the amount of food in each chamber was reduced: the algal discs were crushed using a mortar and pestle, and a measure of feed placed into each flask every 48 h (< 0.1 g). Individual aeration was introduced into each test flask to ensure adequate ($> 75\%$) oxygenation. Since live *C. raciborskii* trichomes are buoyant, aeration also prevented the algae from accumulating at the surface in later trials. This may have altered the likelihood of ingestion.

Tadpole behaviour was measured using a modification of the technique used by Bridges (1999). After entering the laboratory, several minutes were allowed for the tadpoles to acclimatise to the observer. Each flask in the trial was observed for a three-second period, the number of tadpoles swimming was recorded, and the assessor moved on to the next flask. Swimming was defined as any tail movement. This was repeated until ten observations were done on each flask, each spaced three minutes apart (total, 30 minutes). The scores for each flask ($n = 10$) were then averaged to obtain a final score for that day. Observations were repeated daily, typically two to three hours after onset of (artificial) photoperiod. Labels on flasks were not visible to the assessor prior to making the observations, flasks were repositioned every second day, and the same assessor was used throughout the trial. Following behavioural observations, each tadpole was gently tapped on the tail before the disturbance response was recorded (as described above); tadpoles were stationary before ‘tapping’ to ensure consistency in recording results.

Tadpoles were harvested ($n =$ three flasks) on days seven and fourteen. Tadpoles were euthanized by immersion in ice water, blotted dry with paper towels, Gosner development stage recorded, weighed (fresh biomass, to 0.01 g) and measured for

total tadpole length (snout to tail) and total width (widest body part) using vernier callipers. Tadpoles were then placed in centrifuge tubes and frozen until CYN analyses were carried out.

8.1.2.3 Trial three: whole cell extract (extracellular toxin) exposure

In trial three, the toxicity and bioconcentration of CYN was measured in *B. marinus* using nominal treatment concentrations of 0, 25, 50, 100, 200 and 400 $\mu\text{g L}^{-1}$ CYN_{EXC}, administered as whole-cell extracts of a freeze-thawed *C. raciborskii* culture. The trial lasted 14 days. Each treatment was prepared with six replicates (total $n = 36$). Test chambers were individually aerated throughout experimentation. Three tadpoles were assigned per chamber. The tadpoles were fed one half-measure of crushed algae discs during the first week (< 0.05 g); this increased to one full measure (< 0.1 g) during the second week due to tadpole growth.

Tadpole behaviour was recorded as for the second pilot trial, except that the interval between flask observations was increased to four minutes (total period, 40 min). Disturbance response and mortality in all trials were recorded as for the second pilot trial. Tadpole biomass was also recorded prior to trial commencement, to allow relative growth rates (RGR) to be calculated. Tadpole(s) were added to a tared beaker of control water and the change in weight was recorded (to 0.01 g). This method of determining biomass was found to have an average of 19% error ($n = 50$; ten tadpoles each weighed five times), but was the only method of weighing the tadpoles without causing harm (for example, drying each specimen with paper towel). Tadpoles were harvested at days seven and fourteen, analysed and stored as described in the second pilot trial.

8.1.2.4 Trials four and five: exposure to live *C. raciborskii*

Trials four and five were conducted to examine exposure effects and bioaccumulation of CYN following exposure to live *C. raciborskii*. The trials lasted only seven days each, due to rapid death of tadpoles. Trials were conducted using treatment concentrations of 0, 10%, 20%, 30%, 40% and 50% strength of a live *C. raciborskii* culture. The culture was obtained by pooling together multiple smaller (one-litre) cultures in a fifteen-litre glass fish tank, approximately one week prior to commencement of trial. Treatments were prepared by diluting the source culture to the required concentrations using filtered (Whatman GF/F glass microfibre) Moores Creek water. Treatments were prepared with six replicates.

For these trials, only one tadpole was assigned per chamber, since chemical cues produced from dead or dying organisms in the same flask could have affected the behaviour of remaining organisms. For example, decreases in swimming activity are known to occur with chemical cues from crushed conspecifics (Marquis *et al.* 2004). Reduction in tadpole numbers also helped to decrease ammonia production. Tadpoles were fed one half-measure (< 0.05 g) of crushed *Spirulina* discs throughout the trials.

Behavioural responses, disturbance response and biomass were recorded as for trial three (whole-cell extract exposure). Tadpole deaths (percentage mortality) were also recorded for each flask where applicable. Flasks containing dead tadpoles were removed from the trial. Further analyses were not conducted on these specimens. Surviving tadpoles were harvested on day seven and weight, length, width and Gosner stage analyses done as for previous trials. Specimens were frozen until CYN analyses could be carried out.

Concentrations of CYN_{EXC}, CYN_{INC} and CYN_{TOT} were monitored closely during trials four and five, as described in the *Melanoides* trials (see 7.2.2.4). Solutions from flasks containing dead tadpoles were not added to the pooled samples, since these may have experienced reduced grazing rates and thus different toxin concentrations. The cell concentrations of *C. raciborskii* were examined in selected treatments using the Sedgewick-Rafter count method (see 7.2.2.4).

8.1.3 Toxin analyses

Tissue analyses for free CYN and deoxy-CYN were carried out on specimens from trials two to five. Tadpoles were frozen immediately following euthanasia, freeze-dried, and brought to room temperature before being reweighed. Freeze-dried tissues were homogenised in Milli-Q water using an Ultraturrax (24,000 rpm for approximately 1 minute) and frozen. Samples were then sent to QHSS for analysis of free CYN and deoxy-CYN concentration using HPLC/MS-MS (limit of detection, equivalent to 0.3 µg L⁻¹ for both CYN and deoxy-CYN; see Appendix C for full details). Spike recoveries for CYN averaged 78% ($n = 9$; sample concentrations ranged from 0.6 – 3.3 µg L⁻¹).

8.1.4 Trials six and seven: histopathological effects of exposure

Two further trials (six and seven) examined the histopathological changes in *B. marinus* tadpoles when exposed to freeze-thawed *C. raciborskii* whole cell extracts (histology trial one) or live *C. raciborskii* cultures (histology trial two). Both trials were prepared using six flasks: three contained Moores Creek water (controls) and three contained the treatment solutions. In the first trial, treatments were freeze-thawed *C. raciborskii* whole cell extract containing 200 µg L⁻¹ CYN_{EXC}. In the

second trial, treatments comprised a live *C. raciborskii* culture containing $107 \mu\text{g L}^{-1}$ CYN_{TOT} (averaging 81% in the extracellular form). These treatments were sourced from the tank used in trial four. Tadpoles were fed crushed *Spirulina* algal discs as for the main experimental trials.

Both trials commenced with the addition of two tadpoles per flask (day zero). A control and treatment flask was removed at 48h, 96h and 168h. Tadpoles were anaesthetized in ice water before euthanizing by immersion in 10% neutral buffered formalin (one part tissue to ten parts fixative). Specimens were stored for approximately three months prior to tissue processing. For histological analyses, fixed specimens were paraffin embedded, serially sectioned (4 – 10 μm transverse sections), and stained using Harris' modified haematoxylin and eosin (full details of these techniques are provided in Appendix E). These methods were validated using field-collected *B. marinus* prior to the use of experimental specimens. Stained specimens were examined and photographed under bright-field microscopy (Zeiss Axioskop microscope equipped with MC80 photomicroscope, Neofluar and Achrostat lenses; maximum magnification x1600).

8.1.5 Trial eight: the influence of added food on bioavailable CYN

Trial eight was conducted to examine the extent, if any, of toxin adsorption onto crushed *Spirulina*, since this may have altered the bioavailability of CYN. Nine flasks were prepared with a 300 mL solution containing $200 \mu\text{g L}^{-1}$ CYN_{EXC} (as whole cell extracts of *C. raciborskii*). One full measure or half-measure of crushed *Spirulina* was added to the flasks ($n = 3$ per treatment). The last three flasks had no *Spirulina* added. All flasks were placed in the water bath for 48 h under the same experimental

conditions as the definitive trials, except that flasks were not aerated. After 48 h, solutions from each flask were filtered (Whatman GF/F glass microfibre filters), frozen, and analysed for CYN (including deoxy-CYN) concentration.

8.1.6 Statistical analyses

Statistical analyses were not performed on data from trial one. For the remaining trials, behaviour was analysed using one- or two-way repeated measures ANOVA, with between subjects-effects referring to 'toxin exposure concentration' and within-subjects effects referring to 'exposure time in days'. Sphericity was checked using Mauchly's test: where this value was significant, multivariate p values (Roy's Largest Root) for time and interaction were reported instead of the Sphericity Assumed Statistic. For post hoc testing, within-subjects contrasts compared values from different exposure times; Tukey tests (for homogeneous data) or Dunnett's T3 tests (for heterogeneous data) were used to detect significant differences between toxin exposure concentrations. Length, width, Gosner development stage and RGR (biomass) were compared with CYN concentrations using Pearson Product Moment correlations.

Mortality data from trials four and five were acceptable for calculation of LC_{50} values (ASTM 2003). It should be noted that LC_{50} values are not normally calculated for mortality results pertaining to mixtures of chemicals, and live cultures of *C. raciborskii* may contain more than one toxin (CYN, deoxy-CYN, other). However, since the toxicity of CYN is hardly known for amphibians, approximate LC_{50} values were estimated using the mortality results of the live culture trials. Seven-day LC_{50} values were calculated using the Trimmed Spearman-Kärber method in SPEARMAN

version 1.5 (Hamilton *et al.* 1977). This is a modification of the popular Spearman-Kärber that allows calculation of LC_{50} s in cases where zero and 100% mortalities are not recorded within the treatment concentrations. Calculations were not done using the Probit model since this has low tolerance to unusual datasets (for example, medium exposure concentrations recording greater mortalities than those of high exposures) (Hamilton *et al.* 1977).

Bioconcentration and bioaccumulation data were also analysed using Pearson Product Moment correlations.

8.2 Results

8.2.1 Water quality

Temperature and pH values were similar across all trials, although conductivity was more variable (Table 8.2). Dissolved oxygen concentrations in trial one were extremely low, but achieved $\geq 75\%$ saturation following the introduction of individual aerators (Table 8.2). Total ammonia concentrations were high in trials one to three, with values typically approaching 3 – 4 ppm. However, these were reduced in later trials since tadpole biomass loading per flask was decreased.

8.2.2 CYN, deoxy-CYN and cell quotas

Measured CYN_{EXC} concentrations varied 68 – 93% from nominal values during one extracellular trial (Table 8.3). Deoxy-CYN concentrations ranged from above 30% to less than 10% of total CYN values. In the live trials, within-replicate variability was less than 2% (Table 8.4).

Table 8.2 Ranges for water quality parameters during ecotoxicity tests with *Bufo marinus* tadpoles. NT = not tested.

	Temperature (°C)	Alkalinity (mg L ⁻¹ CaCO ₃)	Hardness (mg L ⁻¹ CaCO ₃)	pH	Conductivity (µS cm ⁻¹)	DO (% Saturation)	Total ammonia (mg L ⁻¹)
Trial one	24 ± 2	NT	NT	7.2 - 7.4	323 - 432	≤ 25 ^a	0 - 1.0
Trial two	23.5 ± 1	96	81	8.0 - 8.3	313 - 382	≥ 75	0.5 - 4.0 ^b
Trial three (cell extract)	23.5 ± 1	72	63			≥ 75	
Control				8.0 - 9.4	210 - 264		≤ 4
25				8.1 - 9.1	214 - 274		≤ 4
50				8.0 - 9.3	228 - 275		≤ 4
100				8.1 - 9.3	231 - 319		≤ 4
200				8.0 - 9.4	276 - 327		≤ 4
400				8.0 - 8.2	256 - 374		≤ 8
Trial four (live culture)	23.5 ± 0.5	146	267			≥ 80	
Control				8.6 - 8.9	976 - 991		≤ 1
10				8.6 - 8.8	885 - 956		≤ 1
20				8.6 - 8.8	877 - 940		≤ 0.5
30				8.6 - 8.8	778 - 899		≤ 4 ^b
40				8.5 - 8.8	739 - 871		≤ 1
50				8.5 - 8.6	687 - 838		≤ 2
Trial five (live culture)	23.5 ± 1	146	250			≥ 82	
Control				8.5 - 8.7	780 - 763		≤ 0.5
10				8.2 - 8.7	730 - 843		≤ 0.5
20				8.1 - 8.7	698 - 801		≤ 1
30				8.0 - 8.7	695 - 774		≤ 2
40				7.8 - 8.7	650 - 750		≤ 1
50				7.7 - 8.5	620 - 703		≤ 2

^aaeration not provided to test chambers; ^b4 mg L⁻¹ recorded in one flask only, values typically 1 mg L⁻¹.

Table 8.3 Toxin concentrations of test solutions sampled on harvest days in trials two and three. NA = not applicable; ND = not detected, CYN = cylindrospermopsin. Solutions were not tested in trial one due to high total ammonia concentrations.

Trial	Day(s) since solution renewal	Nominal CYN ($\mu\text{g L}^{-1}$)	Actual CYN ($\mu\text{g L}^{-1}$)	Percent remaining (%)	Deoxy-CYN ($\mu\text{g L}^{-1}$ CYN)	Percent deoxy compared with CYN
Trial two	1	Control	ND	NA	ND	NA
Trial two	2	50	46.7	93.4	14.5	31
Trial three	1	50	38.9	77.8	3.5	8.9
Trial three	1	400	273.5	68.3	20.1	7.3

Table 8.4 Variability of cylindrospermopsin concentrations in selected treatments during trials four and five. CYN = cylindrospermopsin.

	Total CYN concentration ($\mu\text{g L}^{-1}$)	
	Trial four 20%; day six	Trial five 30%; day six
Replicate 1	86.9	102.6
Replicate 2	86.1	104.7
Replicate 3	89.3	108.5
Average \pm standard error	87.43 \pm 0.96	105.27 \pm 1.73
% variation	1.09	1.64

Test concentrations in trial four were higher than those in trial five, ranging from 0 – 232 $\mu\text{g L}^{-1}$ and 0 – 180 $\mu\text{g L}^{-1}$ CYN_{TOT}, respectively (Tables 8.5, 8.6; Figures 8.1, 8.2). There was some variability in the toxin concentration of the source culture during trial four (Figure 8.1A, B). A greater percentage of the available toxin was extracellular during trial five, averaging 78.3% (trial five) compared with 71.5% (trial four). Deoxy-CYN concentrations were also marginally higher in trial five, but were < 5% relative to the quantity of CYN (Tables 8.5, 8.6).

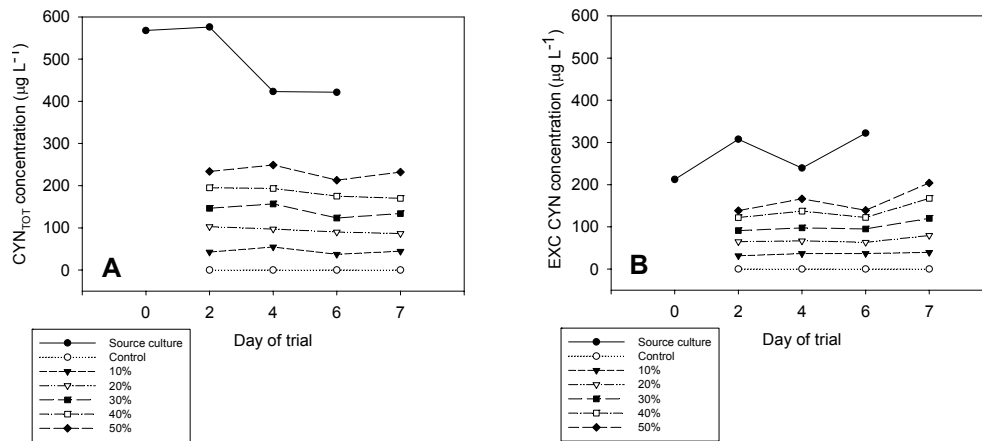


Figure 8.1 Measured toxin concentrations of source and treatment solutions in trial four: (A) total cylindrospermopsin (CYN_{TOT}), (B) extracellular cylindrospermopsin (CYN_{EXC}). Toxin concentrations of treatment solutions are as measured after two day's residence time in the experimental flasks.

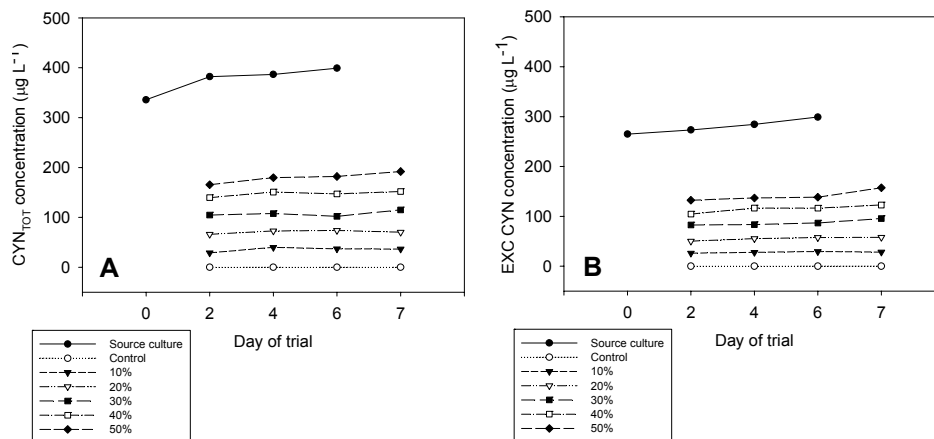


Figure 8.2 Measured toxin concentrations of source and treatment solutions in trial five: (A) total cylindrospermopsin (CYN_{TOT}), (B) extracellular cylindrospermopsin (CYN_{EXC}). Toxin concentrations of treatment solutions are as measured after two day's residence time in the experimental flasks.

Table 8.5 Toxin concentrations in the original culture and treatment solutions during trial four. NT = not tested; NC = not calculated; CYN_{TOT} = total cylindrospermopsin (intracellular plus extracellular); CYN_{EXC} = extracellular cylindrospermopsin; SE = standard error.

Day of Trial	Toxin concentrations ($\mu\text{g L}^{-1}$ or % EXC) in experimental treatments						
	Source culture	Control	10%	20%	30%	40%	50%
CYN_{TOT}							
0	568	NT	NT	NT	NT	NT	NT
2	576	0	43	103	146	195	234
4	423	0	55	97	157	193	249
6	421	0	37	90	123	175	213
7	NT	0	45	86	134	170	232
Average \pm SE	497 \pm 37	0 \pm 0	45 \pm 3	94 \pm 3	140 \pm 6	183 \pm 6	232 \pm 6
CYN_{EXC}							
0	212	NT	NT	NT	NT	NT	NT
2	308	NT	31	65	91	122	138
4	239	NT	37	67	98	137	166
6	322	NT	37	63	95	122	139
7	NT	NT	39	79	120	167	204
Average \pm SE	270 \pm 23	NT	36 \pm 2	69 \pm 3	101 \pm 6	137 \pm 9	162 \pm 13
% EXC							
0	37	NC	NC	NC	NC	NC	NC
2	53	NC	74	63	62	63	59
4	57	NC	67	69	62	71	67
6	77	NC	99	70	77	70	65
7	NC	NC	88	92	90	98	88
Average \pm SE	56 \pm 7	NC	82 \pm 6	74 \pm 6	73 \pm 6	75 \pm 7	70 \pm 5
INC/EXC ratio	0.91	0	0.25	0.39	0.41	0.36	0.46
DEOXY-CYN_{TOT}							
0	27	NT	NT	NT	NT	NT	NT
2	23	0	2	4	5	6	8
4	10	0	2	3	5	5	8
6	12	0	1	4	3	5	8
7	NT	0	1	3	3	6	6
Average \pm SE	18 \pm 4	0 \pm 0	1 \pm 0.2	3 \pm 0.1	4 \pm 0.4	6 \pm 0.3	7 \pm 0.5
Deoxy-CYN (% of total CYN)	3.6	0	2.2	3.1	2.8	3.2	3.0

Table 8.6 Toxin concentrations in the original culture and treatment solutions during trial five. NT = not tested; NC = not calculated; CYN_{TOT} = total cylindrospermopsin (intracellular plus extracellular); CYN_{EXC} = extracellular cylindrospermopsin; SE = standard error.

Day of Trial	Toxin concentrations ($\mu\text{g L}^{-1}$ or % EXC) in experimental treatments						
	Source culture	Control	10%	20%	30%	40%	50%
CYN_{TOT}							
0	336	NT	NT	NT	NT	NT	NT
2	382	0	29	66	105	140	166
4	387	0	40	73	108	151	180
6	399	0	37	74	102	147	182
7	NT	0	37	70	115	152	192
Average \pm SE	376 \pm 12	0 \pm 0	36 \pm 2	71 \pm 2	107 \pm 2	147 \pm 2	180 \pm 5
CYN_{EXC}							
0	265	NT	NT	NT	NT	NT	NT
2	273	0	26	50	83	105	132
4	284	0	28	55	84	117	137
6	299	0	29	57	87	116	138
7	NT	0	28	58	96	123	157
Average \pm SE	280 \pm 6	0 \pm 0	28 \pm 1	55 \pm 2	87 \pm 3	115 \pm 3	141 \pm 5
% EXC							
0	79	NC	NC	NC	NC	NC	NC
2	72	NC	90	76	79	75	80
4	74	NC	70	76	78	77	76
6	75	NC	80	78	85	79	76
7	NC	NC	78	83	83	81	82
Average \pm SE	75 \pm 1	NC	79 \pm 4	78 \pm 1	81 \pm 2	78 \pm 1	79 \pm 1
INC/EXC ratio	0.34	0	0.27	0.28	0.23	0.28	0.28
DEOXY-CYN_{TOT}							
0	6	NT	NT	NT	NT	NT	NT
2	7	0	1	1	3	4	5
4	9	0	1	2	2	4	5
6	6	0	1	2	2	3	4
7	NT	0	0	1	1	2	3
Average \pm SE	7 \pm 0.6	0 \pm 0	1 \pm 0.1	1 \pm 0.3	2 \pm 0.4	3 \pm 0.4	4 \pm 0.5
Deoxy-CYN (% of total CYN)	1.8	0	2.7	1.4	1.8	2.0	2.2

Toxin cell quotas (Q_{CYN}) for *C. raciborskii* were variable, depending on whether they were calculated from total toxin concentrations or the intracellular-only toxin fraction (Table 8.7). Cell quotas were generally up to five-fold higher when calculated from total toxin, rather than intracellular-only toxin, values. The presence of *B. marinus* did not appear to affect cell quotas, with source and treatment samples recording similar Q_{CYN} values (Table 8.7).

Table 8.7 Cylindrospermopsin cell quotas for *Cylindrospermopsis raciborskii*, as calculated from selected cell counts and total and intracellular-only CYN values. CYN_{TOT} = total cylindrospermopsin (intracellular plus extracellular); CYN_{INC} = intracellular cylindrospermopsin.

Treatment	Cells mL^{-1} (average \pm standard error)	CYN_{TOT} (ng mL^{-1})	Cell quota (pg cell^{-1})	CYN_{INC} (ng mL^{-1})	Cell quota (pg cell^{-1})
Trial Four					
Source day 0	3473555 \pm 49500	567.7	0.16	355.4	0.10
Source day 2	2819165 \pm 2000	576.1	0.20	268.4	0.10
Source day 6	1233862 \pm 9000	421.3	0.34	99.2	0.08
Average			0.24		0.09
40% Day 2	1255900 \pm 5500	195.1	0.16	73.1	0.06
10% Day 4	165250 \pm 600	54.7	0.33	18.1	0.11
30% Day 4	710575 \pm 260	156.8	0.22	59.3	0.08
50% Day 6	1007364 \pm 600	212.9	0.21	73.7	0.07
10% Day 7	62927 \pm 2000	44.8	0.71	5.3	0.08
30% Day 7	112370 \pm 3600	140.1	1.25	13.7	0.12
Average			0.48		0.09
Trial five					
Source day 0	1070820 \pm 5400	335.8	0.31	71	0.07
Source day 2	1707363 \pm 3300	382.1	0.22	108.9	0.06
Source day 4	1090650 \pm 5625	386.6	0.35	102.2	0.09
Source day 6	2198651 \pm 800	399.3	0.18	100.2	0.05
Average			0.27		0.07
20% Day 4	375448 \pm 1800	72.7	0.19	17.3	0.05

8.2.3 Mortality and LC_{50}s

In trial one, *B. marinus* mortality rates averaged 11, 78 and 89% in the control, 50 $\mu\text{g L}^{-1}$ and 200 $\mu\text{g L}^{-1}$ treatments respectively, within the first 24 h of exposure. However, tadpoles surviving past the 24 h appeared to tolerate CYN (and *C. raciborskii*), since only two further deaths occurred within 144 h.

Tadpoles did not die in trials two or three, even in treatments containing the highest algal and toxin concentrations ($400 \mu\text{g L}^{-1} \text{CYN}_{\text{EXC}}$). In contrast, *B. marinus* recorded average mortality rates of up to 66% during exposure to the live *C. raciborskii* cultures in trials four and five (Figure 8.3A, B). Highest mortalities were associated with test solutions containing the highest toxin concentrations (232 or $180 \mu\text{g L}^{-1} \text{CYN}_{\text{TOT}}$), and mortalities increased with increasing exposure times (Figure 8.3A, B). Mortality was also highly variable within treatments. One death was recorded from a control during trial five (Figure 8.3B), probably due to mishandling (the specimen was dropped during test solution replacement).

Generally, tadpoles that died early into the trial did so unexpectedly, with no change in behaviour prior to death. In contrast, from approximately day five onwards, the health of the tadpoles progressively worsened: individuals that appeared lethargic or struggled to swim away when probed (see section 8.3.4.2) generally died the next day.

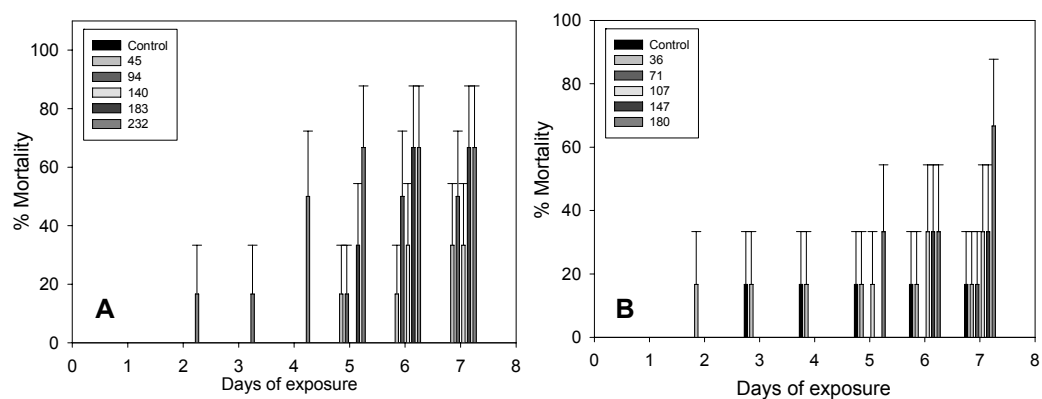


Figure 8.3 Average percent mortality of *Bufo marinus* tadpoles during exposure to live *Cylindrospermopsis raciborskii* cultures containing cylindrospermopsin: (A) trial four, (B) trial five. Legends indicate average total cylindrospermopsin concentrations ($\mu\text{g L}^{-1}$) as calculated in tables 8.4 and 8.5. Bars show average percent mortality \pm standard error.

According to one-way ANOVAs, significant differences in percentage mortality at the end of each trial were not present between treatments in either trial ($p = 0.659$ and $p = 0.377$, for trials four and five, respectively). It is likely that the large variation within treatments prevented the detection of significant effects.

Estimates of the LC_{50} values for trials four and five differed by approximately $44 \mu\text{g L}^{-1}$ (Table 8.8). Furthermore, to check the validity of this method, LC_{50} values were also calculated using the Probit Model within SPSS: values were approximately $150 \mu\text{g L}^{-1}$ (74 – 388 for 95% CI) and $165 \mu\text{g L}^{-1}$ (105 – 436 for 95% CI) for trials four and five, respectively.

Table 8.8 Seven-day (168h) LC_{50} values for trials four and five, calculated via the Trimmed Spearman-Kärber method.

	Trial four^a	Trial five^b
168h LC_{50} ($\mu\text{g L}^{-1}$ CYN)	127.79	171.11
95% CI (upper)	51.21	146.19
95% CI (lower)	318.90	250.29

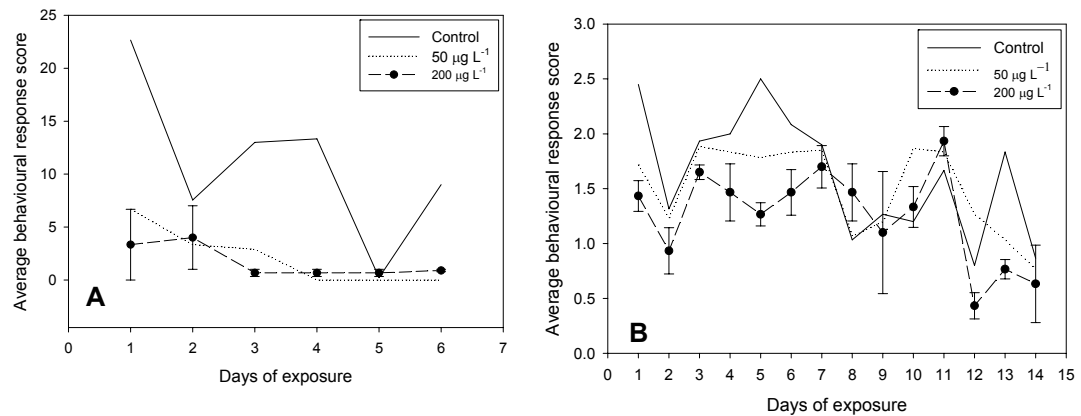
^aSpearman-Kärber trim was 33.33%; ^bSpearman-Kärber trim was 40%; both these are minimum trim required.

8.2.4 Sublethal effects

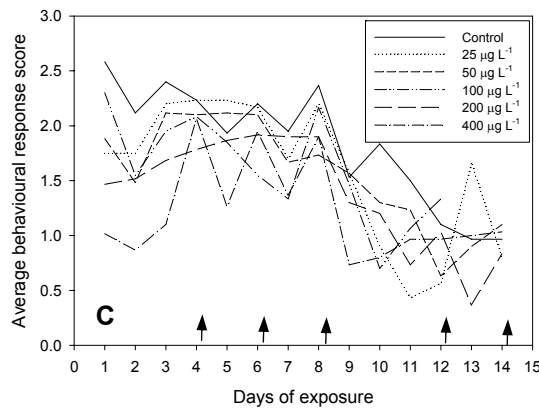
8.2.4.1 Tadpole behaviour

Tadpoles in control treatments received the highest behavioural scores during trial one (Figure 8.4A). However, since deaths in controls and low oxygenation occurred in this trial, further analyses have not been conducted on these results. In trial two, a trough of low activity was recorded across all flasks between exposure days seven and ten (Figure 8.4B). This period coincided with the commencement of road works within 100 m of the testing laboratory; particularly the use of hammering and compacting equipment.

Pilot trials



Exposure to *C. raciborskii* whole cell extract



Exposure to live *C. raciborskii*

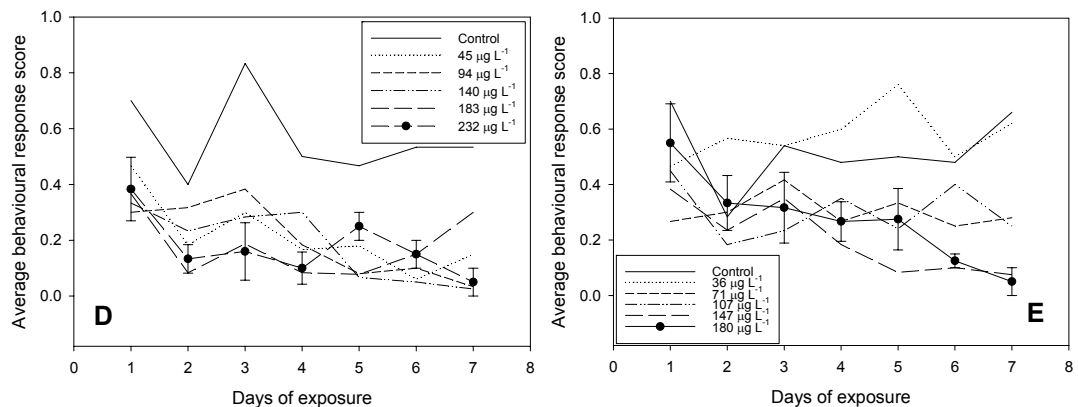


Figure 8.4 Average behavioural scores of *Bufo marinus* tadpoles exposed to *Cylindrospermopsis raciborskii* containing cylindrospermopsin: (A, B) trials one and two, respectively; (C) trial three; (D, E) trials four and five, respectively. Increasing scores indicate more tadpoles swimming and/or more time spent swimming. Arrowheads indicate peaks in activity in 400 & 200 $\mu\text{g L}^{-1}$ treatments. Bars show average \pm standard error (selected treatments only for ease of reading).

In trials three to five, increasing treatment concentrations generally resulted in decreased tadpole behaviour scores (Figure 8.4C – E). Tadpoles exposed to both whole-cell extracts and live *C. raciborskii* cultures received lower behavioural response scores than tadpoles in CYN-free flasks (Figure 8.4C – E). The only exception was the 36 $\mu\text{g L}^{-1}$ treatment in trial five. Tadpoles in solutions containing toxin also appeared to exhibit occasional hyperactivity, such as rapid burst swimming.

In trial three (whole cell extract), a distinct pattern of behaviour emerged in the 400 $\mu\text{g L}^{-1}$ treatments between days three and nine (Figure 8.4C). Tadpoles received low behavioural scores in the 24h immediately following test solution replacement (odd -numbered exposure days). Conversely, peaks in tadpole mobility scores appeared to be recorded simultaneously with test solution renewals (even-numbered exposure days). A similar pattern was evident in the 200 $\mu\text{g L}^{-1}$ treatment four days later (days 12 - 14, Figure 8.4C), and in the control treatments, though at a much smaller amplitude.

Behavioural data were analysed using two-way or one-way repeated measures ANOVA. A significant effect of treatment, without an accompanying interaction, was evident in trials two and five, only. Significant interactions were recorded on two of six possible occasions (Table 8.9).

Table 8.9 Results of two- and one-way repeated measures ANOVAs for *Bufo marinus* behaviour scores. NS = not significant, $p > 0.010$ for heterogeneous datasets. NA = not applicable, trial length only seven days.

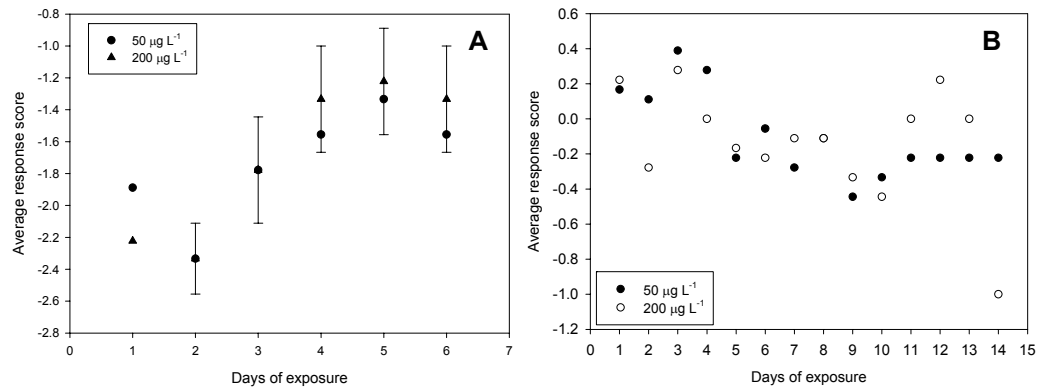
	Week one	Week two
Trial two (pilot)		
Mauchly's test for Sphericity	NS	NS
Treatment	$p < 0.001$; $F_{6, 90} = 7.801$	NS
Exposure time	$p = 0.002$; $F_{2, 15} = 9.440$	$p < 0.001$; $F_{6, 36} = 6.379$
Interaction ^a	NS	NS
Trial three (whole cell extract exposure)		
Mauchly's test for Sphericity	NS	NS
Treatment	$p < 0.001$; $F_{5, 30} = 9.274$	NS
Exposure time	$p < 0.001$; $F_{6, 180} = 5.612$	$p < 0.001$; $F_{6, 60} = 37.650$
Interaction ^a	$p = 0.006$; $F_{30, 180} = 1.878$	NS
Trial four (live <i>C. raciborskii</i> culture)		
Mauchly's test for Sphericity	$p = 0.002$; $M=0.030$; $df=20$	NA
Treatment	NS	
Exposure time ^a	NS	
Interaction ^a	$p < 0.001$; $F_{6, 14} = 14.967$	
Trial five (live <i>C. raciborskii</i> culture)		
Mauchly's test for Sphericity	NS	NA
Treatment	$p = 0.009$; $F_{5, 19} = 4.282$	
Exposure time ^a	NS	
Interaction ^a	NS	

^avalues from two-way ANOVA (Sphericity Assumed statistic); or, where Mauchly's test was significant, two-way repeated measures MANOVAs (Roy's Largest Root Statistic).

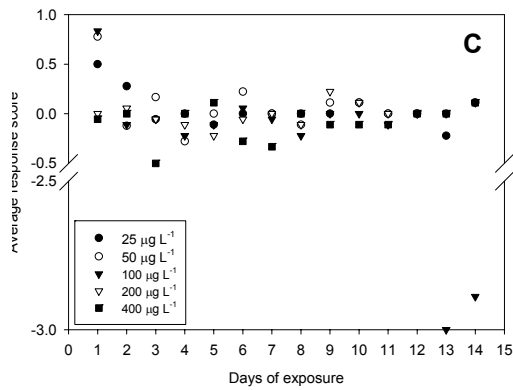
8.2.4.2 Disturbance response

Toxin-exposed tadpoles performed poorly in relation to disturbance response compared with those in control flasks (Figure 8.5). In trials four and five, particularly, some tadpoles were so weak that currents created by the probe (and aerators) caused the tadpoles to be swept about in the flasks. Since values for disturbance responses were assigned differently in different trials, statistics have not been applied to these data.

Pilot trials



Exposure to *C. raciborskii* whole cell extract



Exposure to live *C. raciborskii* cultures

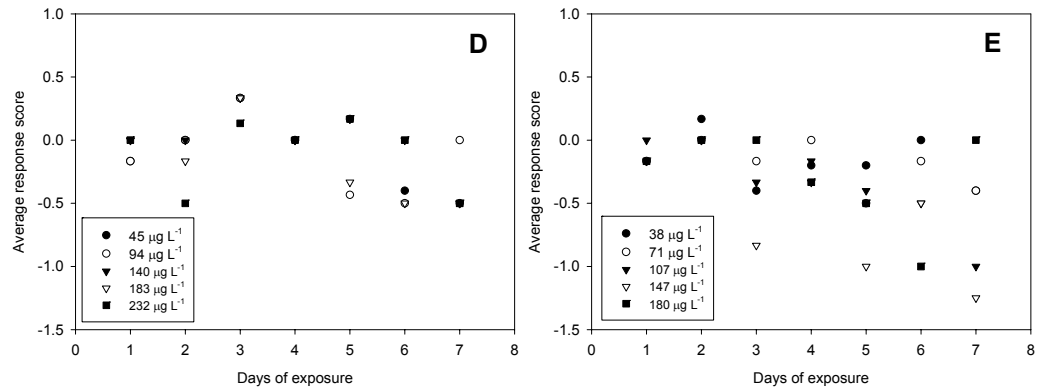


Figure 8.5 Average responses of *Bufo marinus* tadpoles to disturbances following exposure to *Cylindrospermopsis raciborskii* containing cylindrospermopsin: (A, B) trials one and two; (C) trial three; (D, E) trials four and five. All treatments are normalised against control values. Symbols show average \pm standard error (selected trial/treatment only for ease of reading).

8.2.4.3 Tadpole growth and development

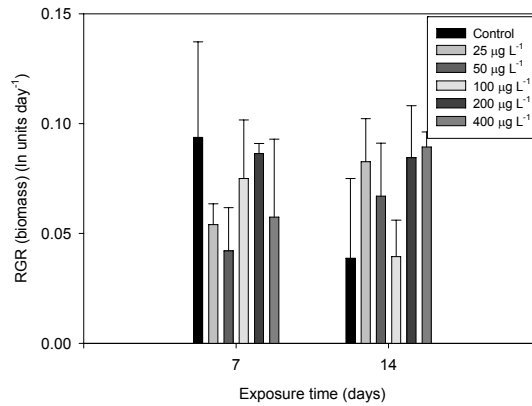
B. marinus recorded positive RGR during exposure to *C. raciborskii* whole cell extracts in trial three; obvious changes in growth patterns were not evident between treatments (Figure 8.6A). In direct contrast, all tadpoles exposed to live *C. raciborskii* recorded negative growth rates, whereas controls always recorded positive growth (Figure 8.6B, C).

Significant correlations were not present between average RGR (biomass), Gosner stage, length and width of tadpoles and CYN_{EXC} exposure concentrations in trial three (Table 8.10). A positive relationship was recorded between tadpole width and CYN_{EXC}. In contrast, tadpole development was usually negatively correlated with CYN_{TOT} concentrations in the live *C. raciborskii* trials (Table 8.10).

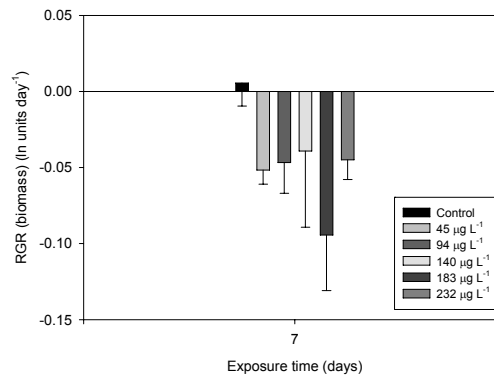
Table 8.10 Pearson Product Moment correlations between *Bufo marinus* growth parameters and cylindrospermopsin concentrations. + significant positive correlation; - significant negative correlation; NS = no significant correlation ($p > 0.050$); NA = not available.

Trial	Exposure period (days)	RGR (biomass) * CYN	Gosner stage * CYN	Width * CYN	Length * CYN
Trial two ^a	7	NA	NS	NS	NS
	14	NA	NA	-	NS
Trial three ^a	7	NS	NS	+	NS
	14	NS	NS	NS	NS
Trial four ^b	7	NS	-	-	-
Trial five ^b	7	-	-	NS	-

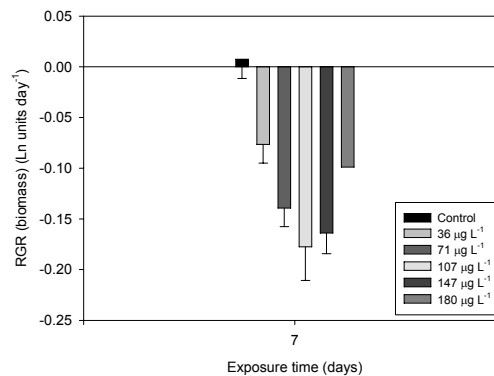
^acorrelations determined using nominal cylindrospermopsin value; ^bcorrelations determined using measured total cylindrospermopsin values in the live cultures.



(A) Trial three (whole cell extract exposure)



(B) Trial four (exposure to live *C. raciborskii*)



(C) Trial five (exposure to live *C. raciborskii*)

Figure 8.6 Average relative growth rates (calculated from biomass) of *Bufo marinus* exposed to *Cylindrospermopsis raciborskii* containing cylindrospermopsin.

8.2.4.4 Abnormalities

Several abnormalities were observed in treatment-exposed tadpoles. Moribund animals typically appeared pale at or following time of death. Many tadpoles also featured a bent tail and/or spine curvature. In trial two, some of the tadpoles exposed to test solutions containing toxin were noted to have unusual body shapes. For example, the abdomen of a tadpole from a $200 \mu\text{g L}^{-1}$ treatment showed a distinct side bulge (Plate 8.1). Other tadpoles from treatments containing the algae and CYN also appeared to have distended abdomens. These abnormalities were not seen in the control tadpoles. In trial four, some CYN-exposed tadpoles appeared to have an enlarged red area on the underside of the belly. These tadpoles were typically dead or moribund; dissection was not attempted due to the small specimen size and since decomposition had already begun. Since tadpoles generally reached Gosner stages 28 – 34 by harvest, no description could be made of abnormalities that present with the emergence of limbs (for example, polydactylism, toe and leg deformities).



Plate 8.1 (A) *Bufo marinus* tadpoles from (left) a $200 \mu\text{g L}^{-1}$ treatment showing side bulge compared with (right) that of a control treatment. (B) Approximate change in *Bufo marinus* development during a fourteen day exposure trial: size at day fourteen (top) compared with size at day zero (bottom).

8.2.5 Bioconcentration and bioaccumulation of free CYN

The maximum average free CYN concentration recorded from *B. marinus* exposed to whole cell extracts was slightly above 60 $\mu\text{g CYN kg}^{-1}$ fresh tissue (Figure 8.7). Tissue toxin concentrations appeared to decrease with longer exposure periods (Figure 8.7): but whether or not this was significant is unknown, since ANOVAs could not be conducted on data with zero variance. Bioconcentration factors (BCFs) were calculated from fresh weight values; bioconcentration was not evident since BCFs were never > 1 (Table 8.11).

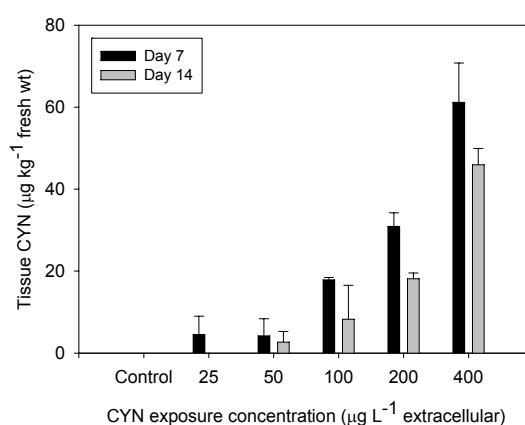


Figure 8.7 Bioconcentration of free cylindrospermopsin in *Bufo marinus* following exposure to *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin in trial three.

Table 8.11 Bioconcentration factors for *Bufo marinus* tadpoles exposed to whole-cell extracts of *Cylindrospermopsis raciborskii* containing cylindrospermopsin. CYN_{EXC} = extracellular cylindrospermopsin.

Concentration ($\mu\text{g L}^{-1}$ CYN _{EXC})	Bioconcentration factor (average \pm standard error)	
	7 days exposure	14 days exposure
25	0.18 \pm 0.18	0 \pm 0
50	0.08 \pm 0.08	0.05 \pm 0.05
100	0.18 \pm 0.01	0.08 \pm 0.08
200	0.15 \pm 0.02	0.09 \pm 0.01
400	0.15 \pm 0.02	0.11 \pm 0.01

Exposure to live *C. raciborskii* cultures resulted in maximum average tissue CYN concentrations of 895 $\mu\text{g kg}^{-1}$ (Figure 8.8). These tissue concentrations were almost 15 times higher than those recorded from trial three, despite total toxin exposures for trial four being almost half the concentration in trial three (232 $\mu\text{g L}^{-1}$ compared with 400 $\mu\text{g L}^{-1}$). In addition, there was a considerable difference in the tissue toxin concentrations of *B. marinus* in trials four and five, despite similar ranges of total toxin exposures. Tissue concentrations were also highly variable within replicates: often, one of the three samples would have no toxin detected whereas the others had high concentrations. Total toxin exposure concentration did not exert a significant effect on tissue toxin concentrations in either trial four or five (one-way ANOVA; $p = 0.056$, $F_{4, 12} = 3.656$ and $p = 0.504$; $F_{4, 13} = 0.898$, respectively).

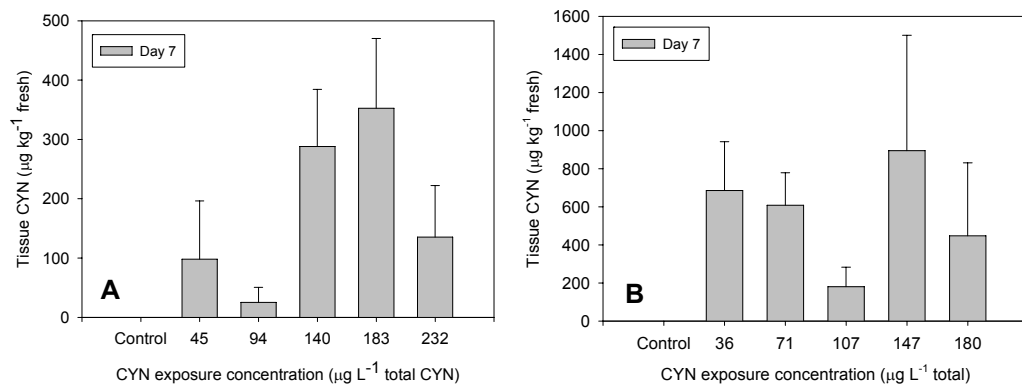


Figure 8.8 Bioaccumulation of free cylindrospermopsin in *Bufo marinus* following exposure to live *Cylindrospermopsis raciborskii* cultures: (A) trial four and (B) trial five.

A poor linear regression existed between fresh and freeze-dried values for tissue toxins ($r^2 = 0.6244$; $y = 0.03x + 119.16$). Hence, bioaccumulation factors (BAFs) were calculated on fresh weight values as recorded during the trials. The resulting BAFs were highly variable, but generally far higher during trial five

(Table 8.12). There did not appear to be a dose-dependent response for BAFs. One-way ANOVA failed to detect a significant influence of exposure concentration on BAF values in either trial ($p = 0.646$, $F_{4, 12} = 0.645$; and $p = 0.076$, $F_{4, 13} = 3.053$).

Nominal CYN_{EXC} exposure concentrations correlated positively with tissue toxin concentrations in the whole extract trial (Table 8.13). However, neither total toxin concentrations nor the intracellular or extracellular fractions were significantly correlated with tissue toxin concentrations in the live culture trials (Table 8.13). Similarly, toxin exposure concentrations and the abundance of the different toxin fractions had no correlation with BCFs or BAFs in trials three and four. Curiously, toxin concentrations were significantly negatively correlated with BAFs in the trial five (Table 8.13).

Table 8.12 Bioaccumulation factors for *Bufo marinus* following seven days exposure to live *Cylindrospermopsis raciborskii* cultures containing cylindrospermopsin. CYN_{TOT} = total cylindrospermopsin; CYN_{EXC} = extracellular cylindrospermopsin.

Trial number	Toxin concentration $\mu\text{g L}^{-1} CYN_{TOT}$ (% CYN_{EXC})	Bioaccumulation factor (average \pm standard error)
Trial four	45 (82)	2.19 ± 2.19
	94 (74)	0.27 ± 0.27
	140 (73)	2.06 ± 0.69
	183 (75)	2.43 ± 0.24
	232 (70)	0.52 ± 0.52
Trial five	36 (79)	19.27 ± 7.19
	71 (78)	8.6 ± 2.41
	107 (81)	1.68 ± 0.95
	147 (78)	6.07 ± 4.11
	180 (79)	0.82 ± 0.82

Table 8.13 Summary results for Pearson Product Moment correlations between tissue cylindrospermopsin concentrations, *Bufo marinus* bioconcentration or bioaccumulation factors, and toxin exposure concentrations. NS = not significant, $p > 0.050$; BCF = bioconcentration factor; BAF = bioaccumulation factor.

Trial	Tissue toxin concentration	BCF or BAF
Trial three (cell extract)		
CYN _{EXC}	$p < 0.001$; $r = 0.910$; $n = 30$	NS
Trial four		
CYN _{TOT}	NS	NS
CYN _{EXC}	NS	NS
CYN _{INC}	NS	NS
CYN _{INC} /CYN _{EXC} ratio	NS	NS
Trial five		
CYN _{TOT}	NS	$p = 0.018$; $r = -0.621$; $n = 14$
CYN _{EXC}	NS	$p = 0.033$; $r = -0.570$; $n = 14$
CYN _{INC}	NS	$p = 0.015$; $r = -0.632$; $n = 14$
CYN _{INC} /CYN _{EXC} ratio	NS	NS

8.2.6 Bioaccumulation of free deoxy-CYN

Deoxy-CYN was not detected from any tadpole tissues sampled during any trials. The limit of detection for this method was $0.3 \mu\text{g L}^{-1}$.

8.2.7 Histopathological effects

Tadpoles did not die during the exposure phases of the histological trials, although one specimen in the extracellular exposure trial was moribund when sampled at 96 h, and several showed signs of lethargic or uncoordinated swimming, as noted in the definitive trials. Exposure to CYN in whole cell extracts and in live *C. raciborskii* culture resulted in major tissue injuries to multiple organs in *B. marinus*. The most dramatic effects were noted in the liver, kidney, and gastrointestinal tract, and all epithelial layers including those lining the external dermis, gills and oral cavity. The severity of effects appeared to be enhanced with increasing exposure times of 48 h, 96 h and 168 h, but the low number of samples prevented a detailed study of the progression of toxicity over this period. Also, exposure to the live *C. raciborskii* culture (trial seven) seemed to produce more dramatic effects than the extracellular solution (trial six), despite

the toxin concentrations being almost double in the latter (200 $\mu\text{g L}^{-1}$ compared with 107 $\mu\text{g L}^{-1}$).

The external dermis of tadpoles was characterised by thickening of the epidermis, with sloughing appearing in several cases (Plate 8.2A – E). The dermal layers were unstructured, and the cells themselves were amorphous, compared with neat and compact layering in control specimens. The nuclear material of toxin-exposed cells was degenerated and grainy, with cells apparently apoptotic. The tadpoles exposed to extracellular toxin and cell debris also featured ‘bubbling’ or swelling of cells and changes in the size of cells comprising the basal layer (Plate 8.2C, D). Toxin-exposed tadpoles also featured an increase in the number and size of clear, gland-like cells in the dermis (possibly, mucous glands).

The epithelial layers lining the roof and floor of the buccal cavity presented with similar effects. Again, the layers were thickened, with disintegration and sloughing of cells, sometimes accompanied by foamy layers, especially on the roof of the buccal cavity (Plate 8.3A - F). Some layers were discontinuous, and featured increased intracellular spaces, rupturing from the basement membrane and increased cell vacuolation (Plate 8.3C – F).

Toxin exposure was associated with disintegration of the cartilaginous framework of the gills, resulting in changes to the gross shape of the gills, which appeared loose and limp (Plate 8.4A – F). The epithelial layers were also disintegrated, with decreased overall cell density. Small epithelial fragments were sometimes seen floating in the branchial spaces (Plate 8.4E).

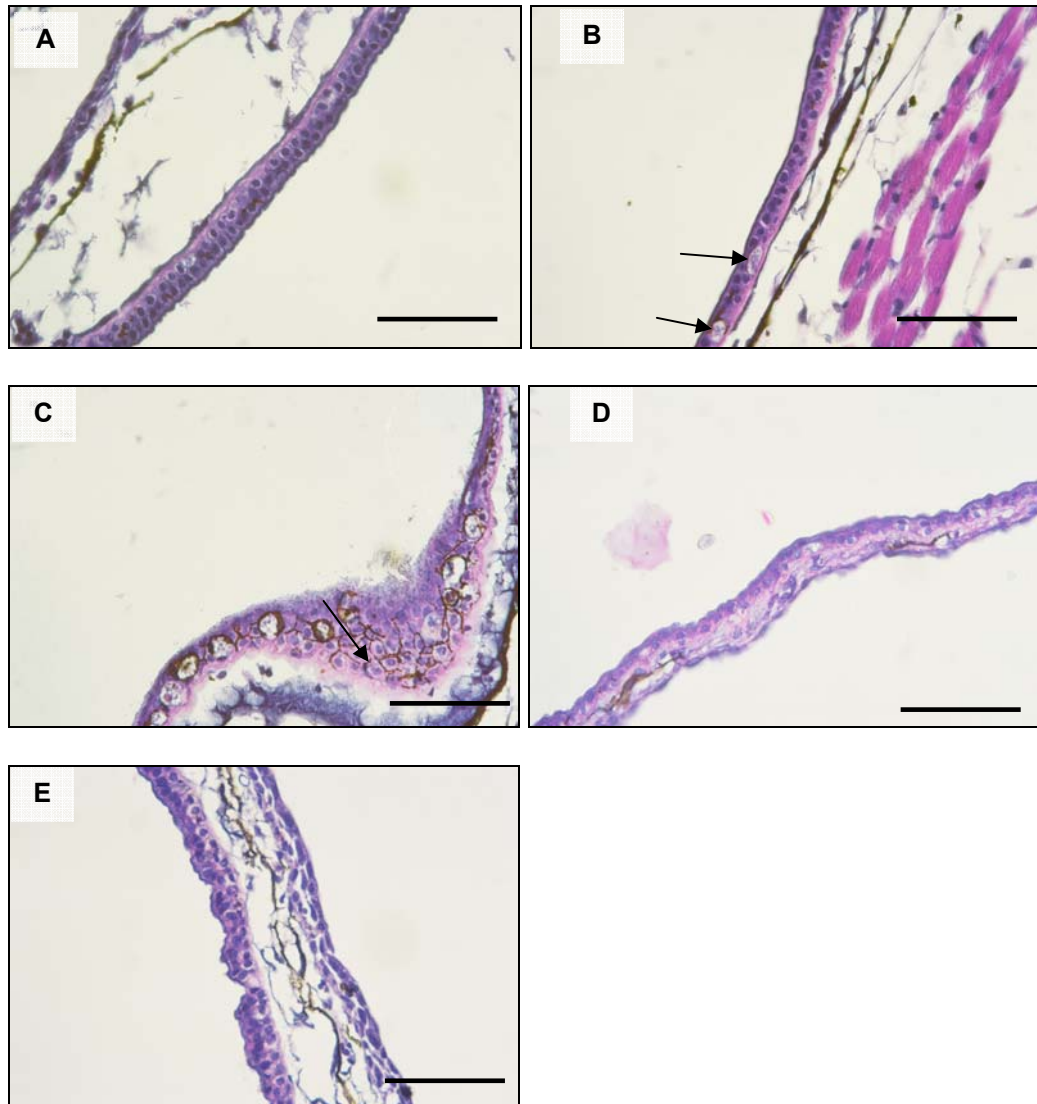


Plate 8.2 External dermis of *Bufo marinus*: (A, B) controls, 8 μm sections; (C, D) tadpoles exposed to freeze-thawed *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin, 8 μm and 6 μm sections; (E) tadpole exposed to live *C. raciborskii*, 6 μm section. Harris' modified haematoxylin and eosin; scale bar is approximately 10 μm . Arrowheads: (B) gland-like cells; (C) swelled cells with changed nuclei.

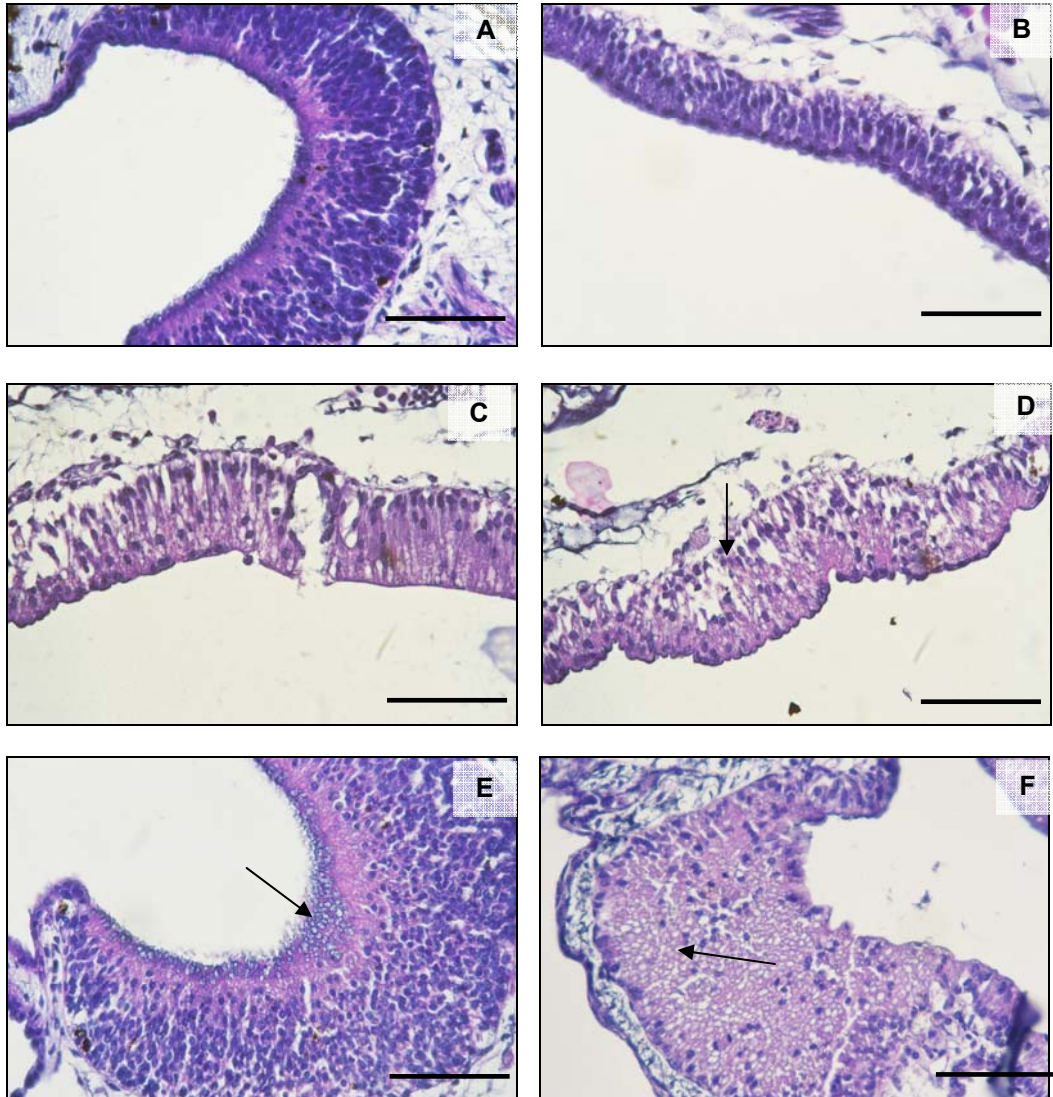


Plate 8.3 Epithelium of the buccal cavity in *Bufo marinus*: (A, B) controls, 8 μm sections; (C-F) toxin-exposed tadpoles, 8, 4, 6 and 4 μm sections, respectively. Harris' modified haematoxylin and eosin; scale bar is approximately 10 μm . Arrowheads: (D) rupturing from the basement membrane; (E) foamy layers and (F) increased cell vacuolation.

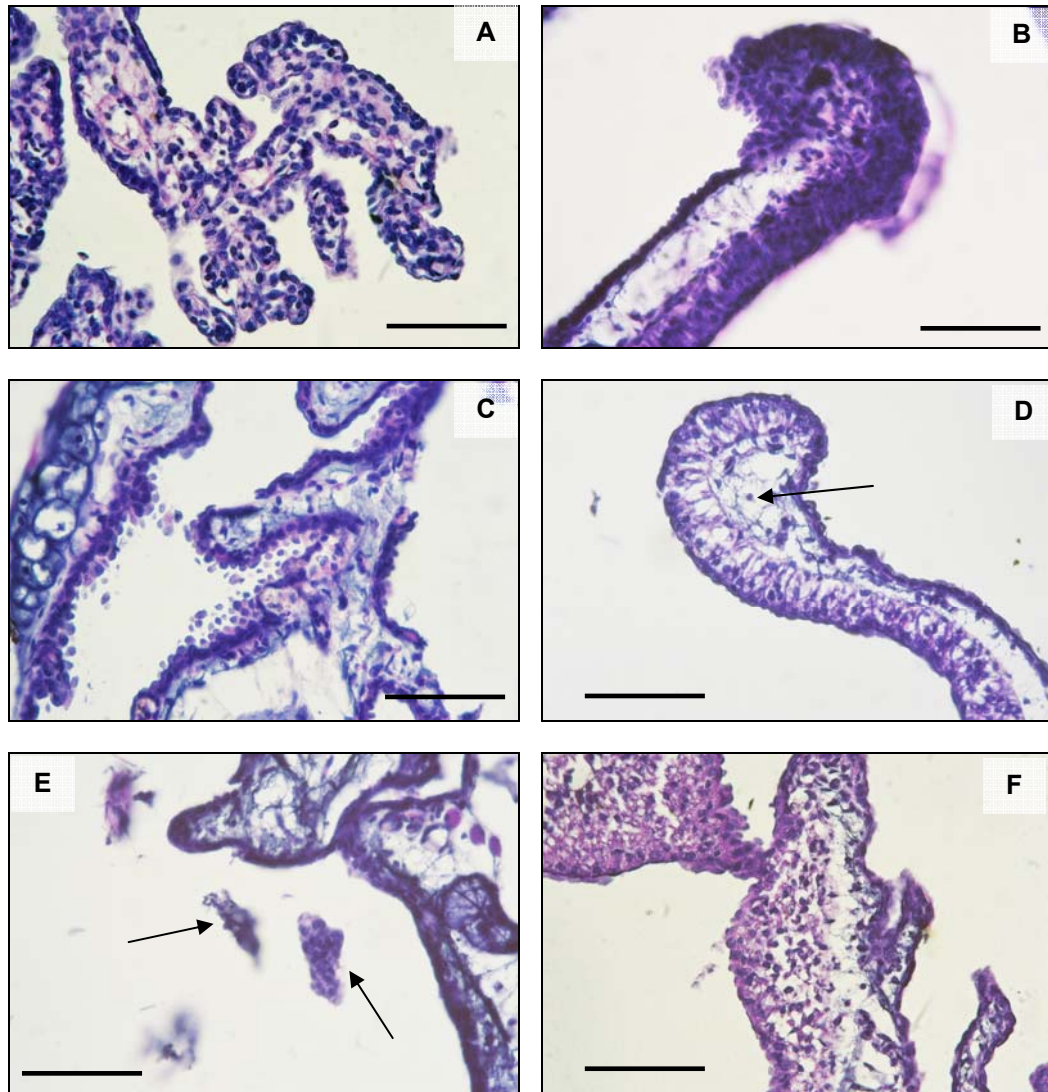


Plate 8.4 Gill epithelia of *Bufo marinus*: (A, B) controls, 10 μm sections; (C-F) toxin-exposed, 8, 10, 8 and 8 μm sections. Harris' modified haematoxylin and eosin; scale bar is approximately 10 μm . Arrowheads: (D) degeneration of the cartilaginous framework; (E) floating gill fragments.

The effects of toxin exposure in the intestine were particularly striking. The intestinal walls appeared extremely grossly irritated, with swollen, broken and discontinuous epithelia featuring large degrees of vacuolation (Plate 8.5A – H). Some of the epithelial layers had detached from the intestinal walls and drifted into the lumen. Blood cells infiltrated the epithelium, and, in one case, a growth filled with blood cells protruded from an intestinal loop (Plate 8.5E). Fat deposits were also common. Minor irritation of the intestinal epithelium was also noted in some controls, particularly in the live culture trial.

Exposure to whole cell extracts of *C. raciborskii* containing CYN resulted in haemorrhaging in the liver, evidenced by the presence of infiltrated blood cells, and in the hepatocytes appearing atrophied, degenerated and disorganised (Plate 8.6A - D). The hepatocytes also featured increases in the number of lipid droplets, and the tubules sometimes contained debris (Plate 8.6D). Exposure to the live *C. raciborskii* solutions resulted in identical effects but with concomitant necrotic tissue (Plate 8.6E – F).

In the nephric ducts of toxin-treated tadpoles, debris was present in the tubules, and the tubules were crowded, collapsed and disintegrated (Plate 8.7A – H). Sometimes, the degree of disintegration was so great that it was difficult to distinguish between tubules. There was also haemorrhaging present, with blood cells filling the spaces between the tubules. One set of tubules also featured an anomalous growth (Plate 8.7H).

Toxic effects towards the heart, pancreas and brain were also present, although less distinctive. In toxin-treated tadpoles, the walls of the heart seemed

thickened, with clumping of blood cells and the presence of smaller, pink casts of cells, possibly cellular or proteinaceous material (Plate 8.8A – H). The pancreas appeared disintegrated and loosely, rather than compactly, arranged and some slides showed necrotic cells (Plate 8.9A – F). In the encephalon (brain), the pink, outer matrix was loosely arranged, whereas cells in the centre of the brain were in clumped associations and appeared disintegrated and, sometimes, necrotic (Plate 8.10A – F). This contrasted with the appearance of control tissues, where the centre was neat and compactly arranged with evenly dispersed cells. Moreover, toxin-exposed tadpoles showed mixing of the outer matrix and inner cells, rather than the distinct boundaries as noted in the controls.

The morphology of the eyes of both control and toxin-exposed tadpoles was extremely variable, and no firm conclusions could be drawn about the effects of CYN (or *C. raciborskii*) on this organ. The gut of several tadpoles contained foodstuffs that were not of algal origin, even in specimens sampled after seven days. This is curious since tadpoles were acclimatised for at least two to three days prior to use in the experiments, and were fed only *C. raciborskii* cells, *C. raciborskii* debris and crushed *Spirulina* discs. Finally, in selected slides from the live culture trial, sections featured intestinal ‘organisms’ that could not be identified (Plate 8.5E), even under oil immersion: these were too small to be either platyhelminths or protozoa.

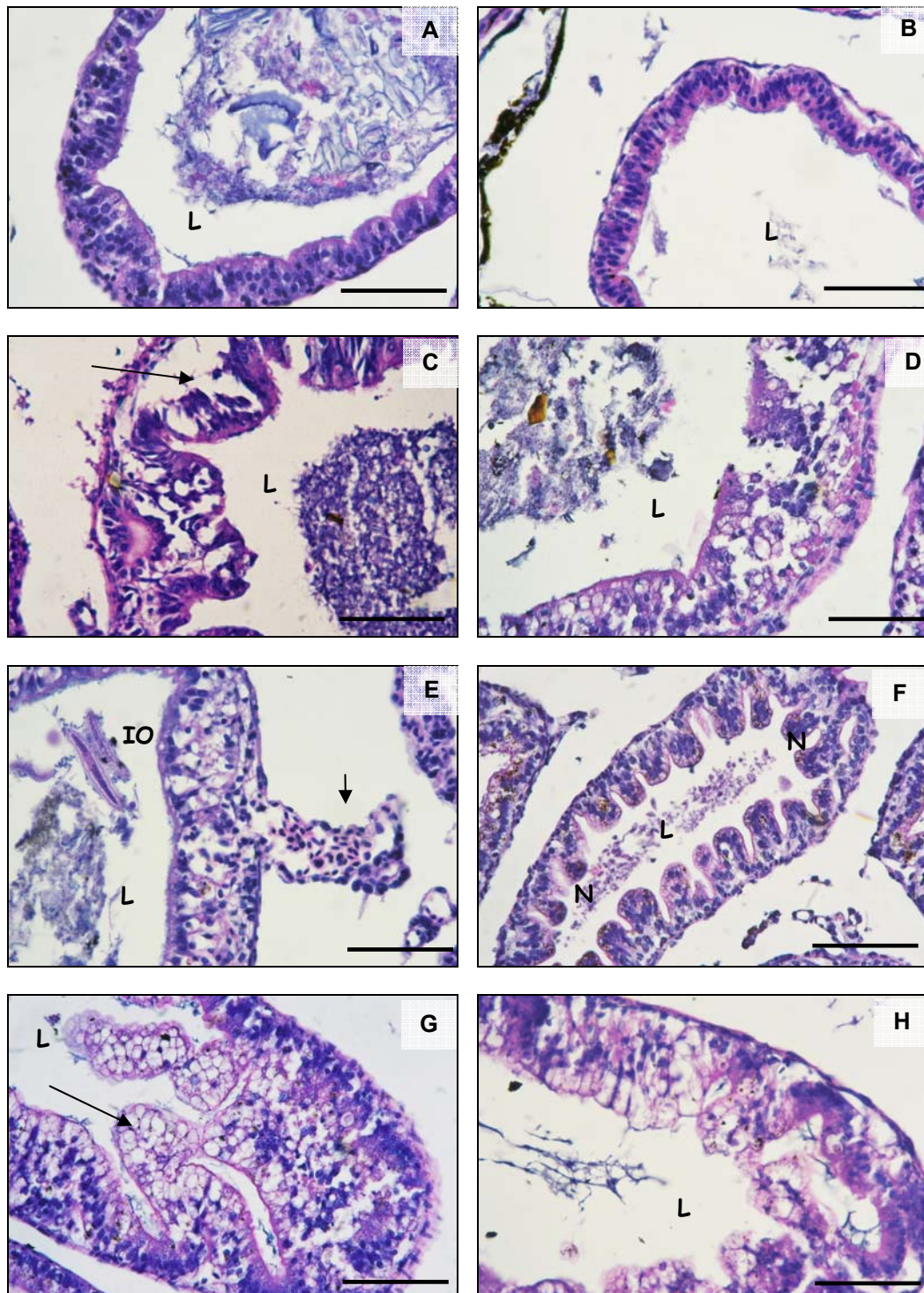


Plate 8.5 Intestinal epithelia of *Bufo marinus*: (A, B) controls, 6 and 8 μm sections; (C-E) tadpoles exposed to freeze-thawed *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin, 8, 7 and 8 μm sections; (F-H) live *C. raciborskii* exposed tadpoles, 6, 8 and 10 μm sections. Harris' modified haematoxylin and eosin; scale bar is approximately 10 μm . Arrowheads: (C) rupturing from the basement membrane; (E) a protruding growth filled with blood cells; (G) large degrees of vacuolation and fat deposits. L = lumen; IO = intestinal organisms; N = necrotic cells.

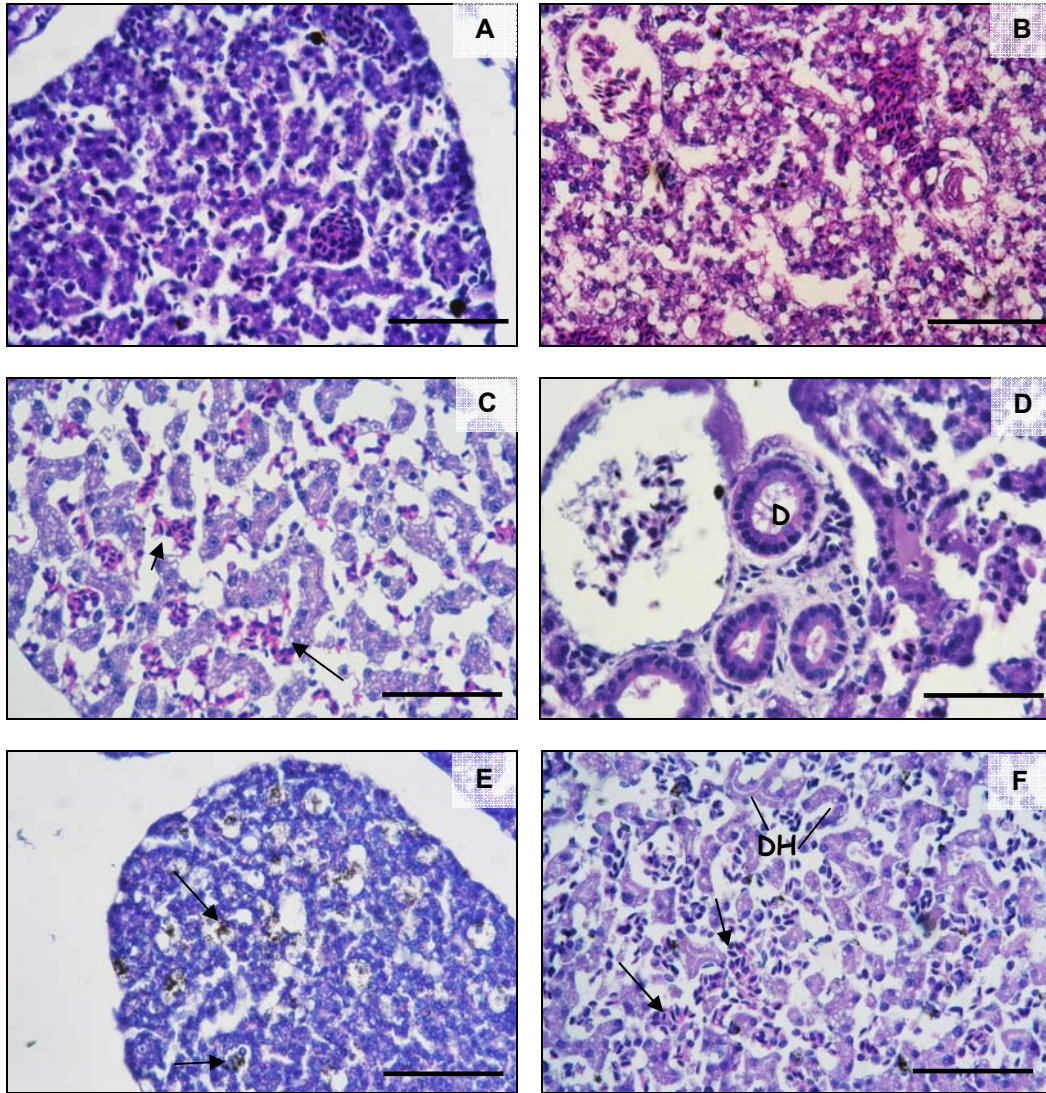


Plate 8.6 Liver and hepatocyte cells of *Bufo marinus*: (A) control, 6 µm section; (B-D) tadpoles exposed to freeze-thawed *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin, 8, 4 and 8 µm sections; (E-F) tadpoles exposed to live *C. raciborskii*, 8 and 4 µm sections. Harris' modified haematoxylin and eosin; scale bar is approximately 10 µm. Arrowheads: (C, F) infiltrated blood cells; (E) necrotic cells. D = debris; DH = damaged hepatocytes.

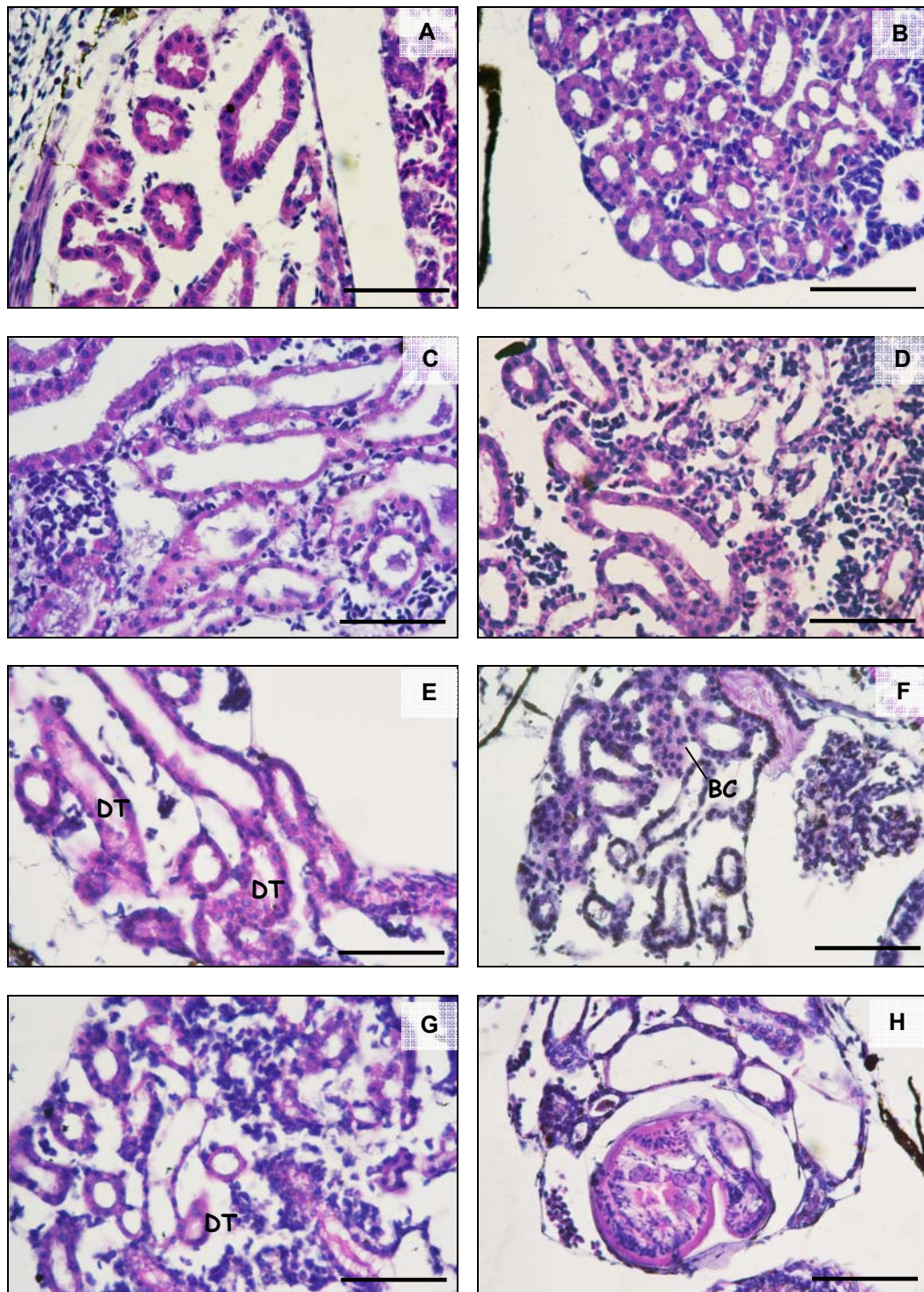


Plate 8.7 Nephric tissues of *Bufo marinus*: (A, B) control, 8 µm sections; (C, D) tadpoles exposed to freeze-thawed *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin, 8 µm sections; (E-H) tadpoles exposed to live *C. raciborskii*, 8 µm sections. Harris' modified haematoxylin and eosin; scale bar is approximately 10 µm. DT = damaged, collapsed tubules; BC = blood cells.

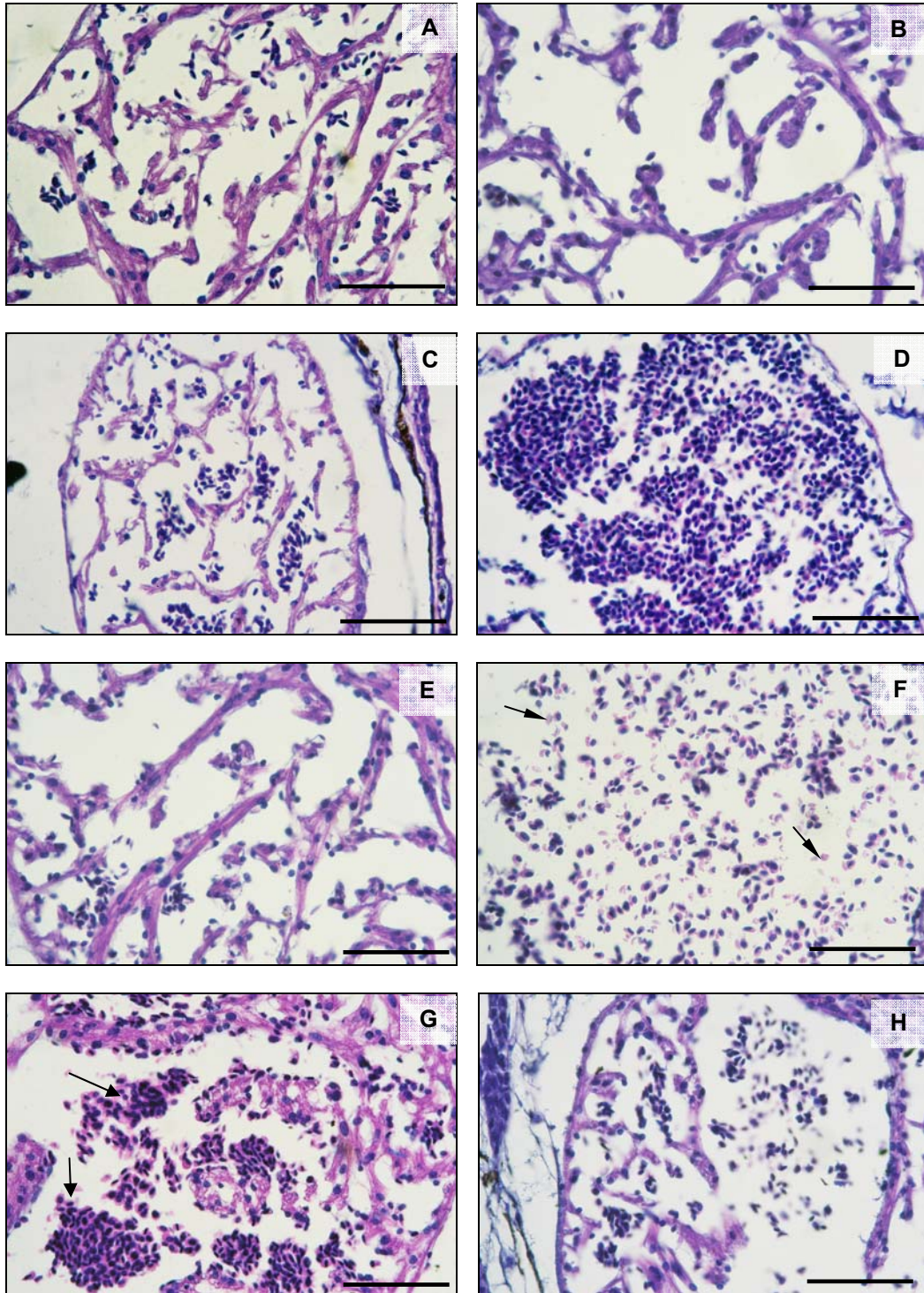


Plate 8.8 Heart and blood cells of *Bufo marinus*: (A - D) control, 8 µm sections; (E-G) tadpoles exposed to freeze-thawed *Cyindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin, 8 µm sections; (H) live *C. raciborskii* exposed, 8 µm section. Harris' modified haematoxylin and eosin; scale bar is approximately 10 µm. Arrowheads: (G) clumped cells; (F) pink casts of cells.

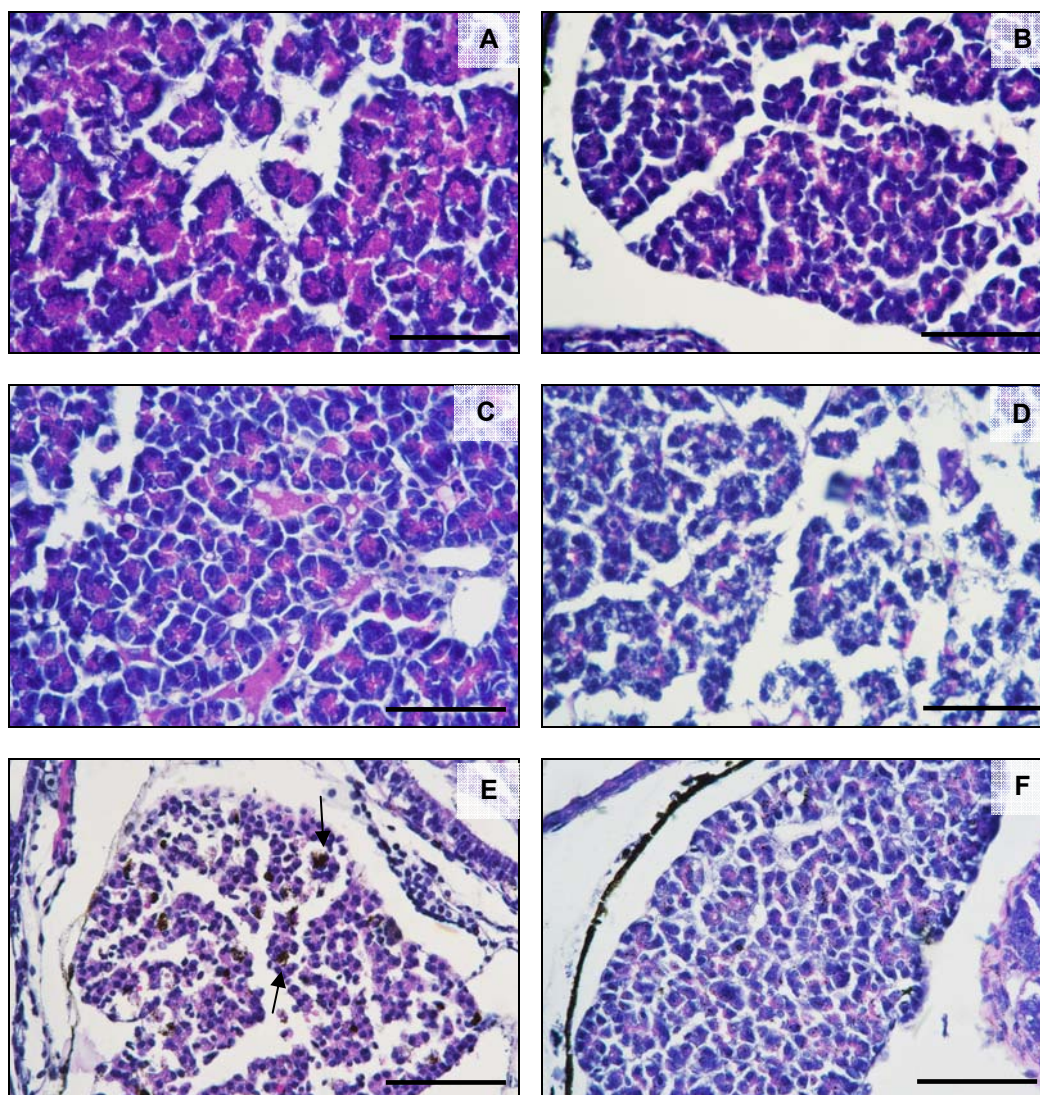


Plate 8.9 Pancreatic tissue of *Bufo marinus*: (A, B) control, 8 µm sections; (C, D) tadpoles exposed to freeze-thawed *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin, 8 and 6 µm sections; (E, F) tadpoles exposed to live *C. raciborskii*, 6 and 5 µm sections. Harris' modified haematoxylin and eosin; scale bar is approximately 10 µm. Arrowheads on plate (E) indicate necrotic cells.

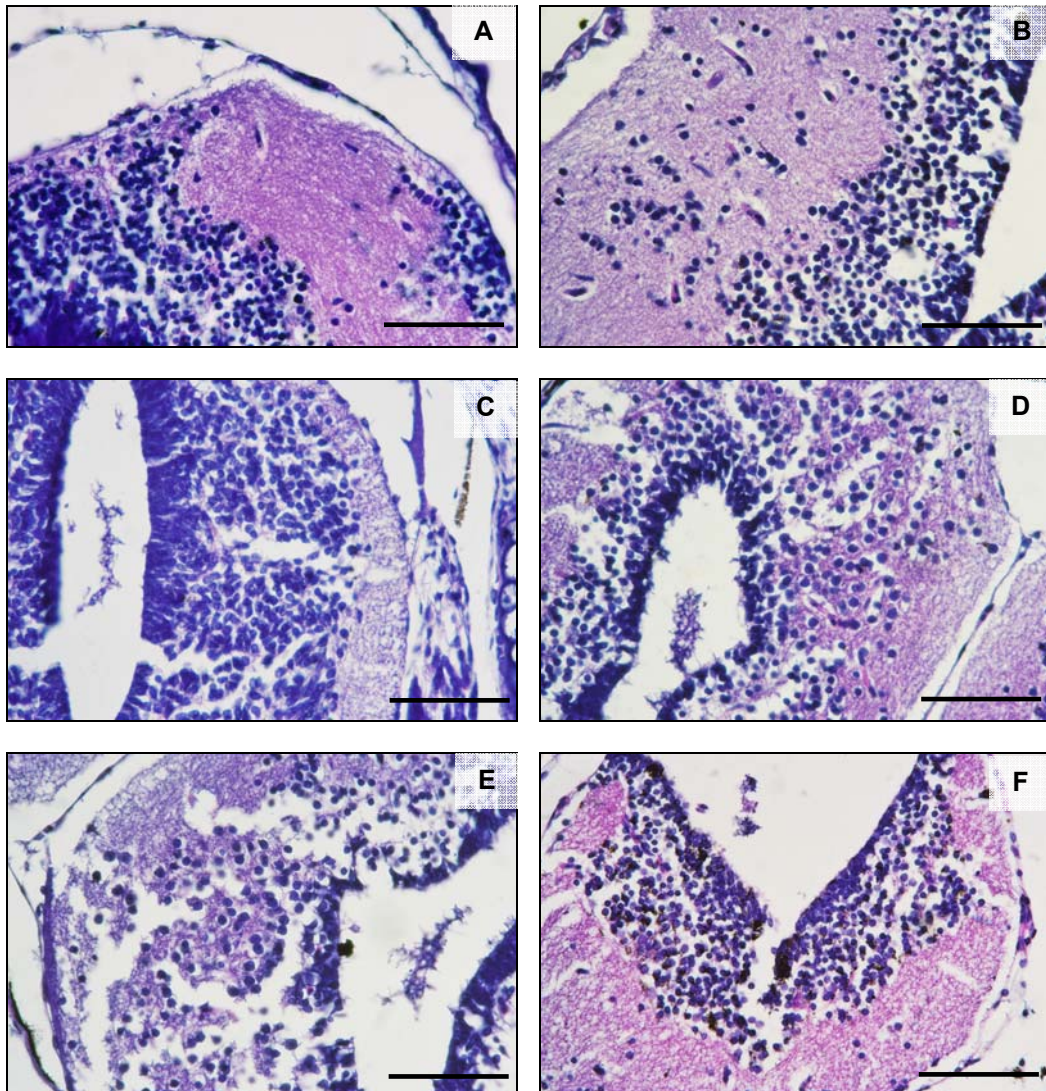


Plate 8.10 Encephalon (brain) of *Bufo marinus*: (A, B) controls, 8 μm sections; (C - E) tadpoles exposed to freeze-thawed *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin, 8 μm sections; (F) tadpoles exposed to live *C. raciborskii*, 6 μm section. Harris' modified haematoxylin and eosin; scale bar is approximately 10 μm .

8.2.8 Food addition and its influence on bioavailable CYN fractions

The addition of crushed *Spirulina* meal influenced the CYN_{EXC} concentrations of test solutions. With no *Spirulina* present, CYN_{EXC} concentrations decreased by 38% in 48h, under conditions identical to those used in the toxicity trials. Addition of one half-measure and one full measure of crushed *Spirulina* resulted in losses of 37% and 45% of available toxin, respectively, over the same exposure period (Figure 8.9). This decrease was significant according to one-way ANOVA ($p = 0.007$; $F_{2,8} = 12.489$). Post-hoc Tukey tests showed the ‘full measure’ (< 1.0 g) treatment significantly differed from the ‘no food’ and ‘half-measure’ (< 0.5 g) treatments; approximately $15 \mu\text{g L}^{-1}$ less CYN was present in this treatment. Deoxy-CYN concentrations were similarly decreased (Figure 8.14), again with ‘full measure’ being significantly less than the remaining two treatments ($p = 0.013$; $F_{2,8} = 9.733$).

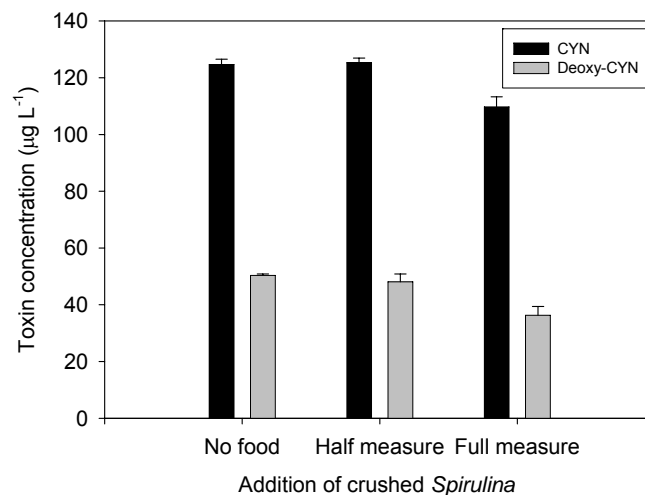


Figure 8.9 Toxin concentrations of treatment flasks after 48 h, with and without the addition of crushed *Spirulina* algal discs. Dotted line shows nominal toxin concentration as prepared on day zero. Bars show average ($n = 3$) \pm standard error.

8.3 Discussion

8.3.1 Water quality

Variation in conductivity values, particularly those of Moores Creek samples, is likely to be related to rainfall events that occurred in between sample collections. Some water quality characteristics exceeded those considered acceptable for larval amphibians: water hardness values should fall within the range 75 – 150 mg L⁻¹, whereas alkalinity should be 15 – 50 mg L⁻¹ (Whitaker 2001, p. 148). However, *B. marinus* may be generally more tolerant than many native species, since tadpole populations were thriving in the water collected at both the ‘Moores Creek’ and ‘library ponds’ sites. High total ammonia concentrations (trials two, three) were likely to be related to production of faecal matter. However, other studies have successfully used higher biomass loadings (for example, ten hatchlings in 350 mL solution (Crossland 1998)) without excess ammonia production.

8.3.2 CYN, deoxy-CYN and cell concentrations

The extracellular fraction abundance (70 – 80%) and the trend for low deoxy-CYN concentrations (< 10 µg L⁻¹) in the trials using live *C. raciborskii* cultures compared favourably with values obtained from a similar trial with *M. tuberculata* (see table 7.5). The cell quotas for the *C. raciborskii* culture (0.09 pg cell⁻¹) were also remarkably similar to those of the snail study (see table 7.6). However, whilst treatment solutions exposed to grazing by *Melanoides* snails recorded almost three-fold increases in Q_{CYN}, this pattern was not recorded from solutions exposed to the *Bufo* grazing. This questions the function of cell quotas in decreasing grazing potential via feeding inhibition (toxicity) or lowered palatability, as suggested in the previous chapter. However, since *Bufo* recorded

high mortality rates during exposure, the possible importance of cell quotas in reduced grazing pressures seems to be of little consequence.

8.3.3 Mortality and LC₅₀s

The presence of live *C. raciborskii* cells containing CYN_{INC} had a dramatic effect on mortality rates in *B. marinus*. Probably, the ingestion of higher toxin loads, and consequent deaths, occurred during trials four and five since ingestion of intact, live cells would include consumption of toxin. By comparison, grazing on cellular debris in the whole cell extract trial (trial three) may not necessarily have involved toxin uptake, since cell lysis probably liberated most toxin. This argument is further supported by the tissue contamination results. The highly variable mortality rates suggest that *B. marinus* tadpoles have different individual susceptibilities with respect to *C. raciborskii* and CYN (including deoxy-CYN), possibly as a function of grazing rates or size.

The significance of the 168 h LC₅₀ values calculated for *B. marinus* is difficult to ascertain, given that the treatment solutions, being whole cell extracts, contained a mix of CYN, deoxy-CYN, toxic cell wall components and potentially other unknown toxins. Thus, LC₅₀ values can be viewed only as indicative of the general toxicity associated with CYN exposures. Differences in the LC₅₀ values of trials four and five could result from differences in the relative abundance of CYN_{INC}: CYN_{EXC}. The *C. raciborskii* solution used in trial four was the most toxic (corresponding with a lower LC₅₀ value), and featured a higher proportion of intracellular CYN (average 25.2% intracellular, compared with 21% intracellular for trial two). This suggests that even small changes in the relative

abundance of intracellular and extracellular toxin could be critical in affecting mortality.

Few studies have been conducted on the mortality rates of amphibians in relation to algal toxin exposure. Gromov *et al.* (1997) found that the 48 h LC₅₀ of lyophilised *M. aeruginosa* biomass was 3.0 mg mL⁻¹ for larval forms of the common frog, *Rana temporaria*. Importantly, the authors noted that the larval forms were so young that they would not have been feeding during the experimental period; thus, only transdermal uptake of extracellular toxin was possible. However, after the 48 h period, the authors also claimed that the larvae became ‘insensitive’ and were ‘able to use the algae as a food source’ (Gromov *et al.* 1997).

For CYN, LC₅₀ values have been calculated for brine shrimp: values decreased with time 24 h to 72 h, but again, even the lowest value (710 µg L⁻¹ purified toxin) was far higher than that reported in this study for one-week exposures in *B. marinus* (127 – 177 µg L⁻¹). Purified toxin is likely to be less toxic than crude or whole-cell extracts since *C. raciborskii* is suspected to possess toxic compounds other than CYN (and deoxy-CYN) present (Hawkins *et al.* 1997; Carmichael 2001b). For *Microcystis*, crude cyanobacterial cell extracts were shown to cause ‘much more pronounced’ effects than did purified MC, across a range of species including fish and amphibian embryos (Oberemm *et al.* 1999). Thus, though the use of whole cell extracts in the present work has direct environmental relevance, this may have resulted in much lower LC₅₀ values than found in other (purified) CYN studies.

Comparisons of several other LC₅₀ values calculated for CYN are not appropriate since most have used injection dosing, or have been carried out on laboratory mice (Ohtani *et al.* 1992; Hiripi *et al.* 1998; Seawright *et al.* 1999; Shaw *et al.* 2000).

8.3.4 Sublethal effects

8.3.4.1 Tadpole behaviour

Vibrations from nearby road works may have influenced tadpole behaviour during trial two. It is also conceivable that elevated total ammonia levels in trials one to three could have influenced tadpole behaviour, especially since high pH values will have shifted the balance between ionised (NH₄) and free (NH₃) molecular forms of ammonia.

Regular patterns of peaks and troughs in activity were associated with exposure times of 48 h and 24 h to fresh test solutions. Since tadpole behaviour was scored prior to the replacement of test solutions at 48 h intervals, disturbance by the assessor was unlikely to have impacted the results. The addition of fresh test solutions, causing an influx of bioavailable toxin, could have resulted in reduced tadpole activity. Following this, filtering, bioconcentration, or metabolism of toxins may have taken place, thereby reducing toxin concentrations and allowing increased tadpole activity. Equally, this could also result from changed water chemistry with the addition of fresh solution.

Decreased activity levels in *B. marinus*, as witnessed in two of the trials (Figure 8.4), may be linked with poor competitive ability, including ability to forage for

limited food resources (Lawler 1989; Bridges 1999). Contrarily, decreased activity may offer reduced risk from predators and can be viewed as a avoidance strategy from visually-oriented hunters (Bridges 1999; Fraker & Smith 2004), and may also afford benefits such as energy conservation (possibly, followed by weight increase and more rapid time to metamorphosis). In addition, decreased activity could allow for decreased toxin uptake via grazing, since feeding levels may also be depressed. Whether or not low activity levels are advantageous or otherwise cannot be judged solely from experiments within a laboratory setting: ultimately, it may be that CYN (or *C. raciborskii*) alters the balance between active and restive periods, each of which afford their own benefits.

Physiologically, decreased activity levels in *B. marinus* may indicate effects on the central nervous system, or, equally likely, that overall cytotoxicity is so potent as to cause general failure of most organ systems. *C. raciborskii* has been linked with toxicity to molluscan neurones, but here the toxin of interest was anatoxin-a (Kiss *et al.* 2002). Since animal behaviour is a mix of sensory, hormonal, neurological and metabolic systems (Scott & Sloman 2004), changes to tadpole activity levels do not necessarily provide information on the mechanism of action for CYN. However, changes in behaviour are often the first indication of sublethal exposure in amphibians (Henry 2000), thus, with further studies, this could be a useful early warning indicator for CYN toxicity, provided that variability between trials is low.

8.3.4.2 Disturbance response

Disturbance response (sometimes known as ‘startle response’) observations provide some insight into the effects of *C. raciborskii* and CYN exposure on complex ecological relationships, such as predator-prey interactions. For example, some moribund tadpoles were swimming sideways: failure to properly orientate the body could render these more vulnerable, especially since the ventral side is typically paler and may attract predators (Fordham *et al.* 2001).

The method of determining disturbance response could be improved for future work: it was sometimes difficult to assign a score to tadpoles, since many would show no immediate response on touching with the probe, but seconds later would swim strongly around the flasks, and not necessarily away from the probe itself. Such problems could be overcome by using a similar technique to that described by Mann and Bidwell (2001). In that study, narcosis was assessed by measuring flight response on a scale of mild or full narcosis, judged as the ability to show coordinated effort to swim, in the opposite direction, for at least one second (Mann & Bidwell 2001).

8.3.4.3 Tadpole growth and development

B. marinus tadpoles experienced significant, negative correlations between growth (RGR calculated from wet biomasses) and development in conjunction with exposure to live *C. raciborskii* cultures containing CYN (and deoxy-CYN) (Table 8.6). A similar pattern was absent in trial three, where whole cell extracts of *C. raciborskii* were used. Decreased tadpole biomass in conjunction with live *C. raciborskii* exposure may cause several adverse effects. There may be an increased risk of pond deaths, as delayed growth and development in temporary

ponds may mean that dry-out occurs before metamorphosis is reached (Lawler 1989). Generally, individuals that are larger at time of metamorphosis have a higher survival rate, may mature earlier, and may continue to enjoy a larger body size, possibly indicating higher fecundity (Wilbur 1997, p. 2288; Bridges 2000). Contrarily, smaller metamorphs have lower locomotory capacity, lower tolerance to dehydration, lower survival until maturity, and most likely lowered future reproductive output (Johansson *et al.* 2001).

Decreased food availability may also be a problem, since these earlier toadlets could deplete resources. On the other hand, tadpoles that stay in the larval stage longer may have increased weight (Bridges 2000). However, biomass data for CYN-exposed *B. marinus* has shown this is not the case.

Decreased bodyweight has been observed in the larvae of both smooth newts and marsh frogs, following exposure to MC-YR and -RR at 50 $\mu\text{g L}^{-1}$ (Oberemm *et al.* 1999). However, it would also have been informative to know the dry weights for RGR analyses in trials three to five, since tadpole body masses may be affected by oedema or water retention when placed under stress (Schuytema & Nebeker 1998).

Biomass decrease in conjunction with CYN (and *C. raciborskii*) exposure may be linked with food consumption, or with changes to metabolic rates. Growth of cane toad larvae is known to be food limited (Hearnden 1991). Cane toads tadpoles may be capable of preferential selection against toxic *C. raciborskii* trichomes (for example, using chemical cues): but this still does not explain

decreased growth in treatment flasks compared with controls, since each also had equal amounts of *Spirulina* meal provided. Possibly, CYN intoxication is linked with reduced overall food intake.

8.3.4.4 Abnormalities

Occasionally, distended abdomens were noted from *C. raciborskii*- and CYN-exposed tadpoles, but this did not feature consistently amongst treatments. Swelled bodies have been observed in conjunction with nitrate experiments, suggesting that tadpole bodies swell due to nitrate toxicity to osmoregulatory mechanisms (Hecnar 1995; Johansson *et al.* 2001). Since elevated ammonia levels were recorded in trials one to three, nitrate toxicity could have contributed at least partially to these effects. However, swollen abdomens have also been recorded in several other cases of stressed tadpoles, including in relation to pesticide and hormone exposure (Nishimura *et al.* 1997; Schuytema & Nebeker 1998). Moreover, bulging of the abdomen as a result of swelling of the liver and gastrointestinal tract cannot be discounted, since this is characteristic of CYN intoxication and corresponds to lipid vacuolation (Shaw *et al.* 2000), and the latter was noted in the liver and intestines of the tadpoles (see plates 8.5 and 8.6).

8.3.5 Histopathological effects of CYN exposure

The impacts recorded in *B. marinus* compare favourably with other histological studies of CYN toxicity, though almost all work to date has been conducted using laboratory mice. A common trend is that the most severe effects are noted in the kidneys and liver, and typically characterised by necrosis, disintegration, loss of overall structure and damage to key cells (Falconer *et al.* 1999; Saker *et*

al. 1999; Seawright *et al.* 1999; Shaw *et al.* 2000; Bernard *et al.* 2003). In particular, the cellular damage noted in *B. marinus* closely approximates the third and fourth stages of CYN intoxication in mice, characterised by fat droplet accumulation and cell death (Terao *et al.* 1994). The characteristic swelling of the liver is also consistent with the present study, since this change usually results from lipid vacuolation of the hepatocytes (Shaw *et al.* 2000) due to protein synthesis inhibition and, possibly, injuries related to the presence of free radicals (Terao *et al.* 1994).

Although CYN-induced necrosis of the hepatocytes is commonly focal and centrilobular (occurring in the cells surrounding central hepatic veins) (Bernard *et al.* 2003), this was not the case in *B. marinus*, where tissue effects seemed consistent across the entire organ. Given the severity of gill damage, it seems likely that broad-scale tissue necrosis could result from respiratory distress, and tissue anoxia (Seawright *et al.* 1999). It has been suggested that CYN effects are more severe in some organs, such as the liver, because these have higher cytochrome P-450 activity, which is implicated in the production of highly toxic CYN metabolites (Runnegar *et al.* 1994; Shaw *et al.* 2000). Other histological studies have reported progressive severity of tissue damage in relation to exposure time, consistent with delayed effects, time-dependency and consecutive stages of CYN intoxication (Hawkins *et al.* 1985; Terao *et al.* 1994; Falconer *et al.* 1999; Chong *et al.* 2002). The small sample size in the present work prevented an investigation of such effects. However, changes to the overall shape of cells, particularly noticeable in the dermal layers, agree with changes

seen in other CYN-treated hepatocytes: these also became flattened and had irregular outlines (Froscio *et al.* 2003).

A notable difference between this study and others examining CYN was the severity of effects resulting from different CYN exposure regimes. In mouse studies, Falconer *et al.* (1999) compared intraperitoneal injection with oral gavage, and reported that the route of administration of toxic extracts did not influence the extent of histological damage. However, in this (present) work, the effects associated with intracellular toxin exposure matched or exceeded those recorded for extracellular exposure, despite the latter solutions having almost twice the total CYN concentrations of the former. Since 81% of the toxin in the live culture was extracellular, this suggests that the presence of cell-bound toxin is critically important in affecting the toxicity of CYN to *B. marinus* tadpoles. Nonetheless, dissolved toxin uptake also appears to contribute to toxicity, and it appears that both oral and transdermal uptake of CYN is likely.

This study also contrasts with current knowledge of CYN. CYN inhibits protein synthesis and reduces cell division rates (Terao *et al.* 1994; Fessard & Bernard 2003). However, some cells of *B. marinus* appeared to proliferate following exposure to *C. raciborskii* and CYN (for example, thickening of the dermal layers and buccal cavity epithelium). The reason for this is not known. It could be speculated that the expansion of these tissues acts to reduce the transdermal uptake of CYN.

Although the preparation of sections did not allow a detailed study of nuclear material, cells were deemed to be apoptotic since nuclear material showed degeneration. Micronucleus induction, cell necrosis and chromosome loss resulting from CYN intoxication at 1 – 10 $\mu\text{g mL}^{-1}$ has been already reported *in vitro* from a human lymphoblastoid cell line (Humpage *et al.* 2000). Humpage *et al.*'s (2000) study suggested possible carcinogenic activity of CYN, although the mechanism for chromosomal damage was unclear.

The casts present in the blood of *B. marinus* are unusual. Kidney injury by CYN exposure is known to produce proteinaceous casts in the urine; in severe cases, these casts can block the distal tubules of mice (Falconer *et al.* 1999). However, the presence of debris was also noted in the nephric tubules of the tadpoles.

The histological effects resulting from CYN exposure also compare with those reported for insecticide exposure in amphibians, including degeneration of gill epithelium, loose appearance of liver and increases in lipid inclusions (Honrubia *et al.* 1993); and fragmentation of gill lining, loss of compactness in the hepatic mass, disintegration of hepatic cells, ruptured blood vessels, and shattered intestinal cells (Noor Alam & Shafi 1999). This indicates that the toxicity risks associated with CYN could be equal to those of agricultural chemicals in terms of effects on natural amphibian populations.

Organ injuries in *B. marinus* were consistent with behavioural effects and tadpole mortality noted during the earlier ecotoxicity trials (extracellular, live culture exposure). For example, reduced activity of tadpoles appears to result from

suffocation, owing to the degeneration of the gill epithelia. Similarly, reduced swimming ability and un-coordination could correspond with the disintegration of the brain tissues and general organ failure, especially in the liver and kidneys. Clumping of blood cells within the heart chambers may also suggest the presence of antibodies, with the organism raising an immune-like reaction to combat a perceived 'infection', though this has never been reported for algal toxins. The formation of fibrin thrombi has also been noted in the portal veins, lungs and kidneys of CYN-dosed mice, in addition to embolic material in the blood vessels of the lungs (Hawkins *et al.* 1985).

The presence of non-algal foodstuffs in the gastrointestinal tracts of some tadpoles is curious, given that tadpoles were acclimatised for two to three days and then used in the experimental trials. During this entire time (maximum ten days), tadpoles were fed only *C. raciborskii* cells or cellular debris, and crushed *Spirulina* discs. It is conceivable the tadpoles retained food in the gut and/or reduced active feeding as part of a toxin avoidance strategy. Heavy metal exposure is associated with shrunken, distorted and empty intestines in *Rana tigrina* (Kumar 1999) and feeding inhibition has already been shown to occur with CYN and other toxins in zooplankton (Boon *et al.* 1994; Nogueira *et al.* 2004a). Decreased activity, depressed appetites (anorexia) and weight loss have also been reported from CYN-exposed mice (Li *et al.* 2001b; Saker *et al.* 2003). In one study, mice contained food from a single administration of freeze-dried *C. raciborskii* some 24 – 72 h post dosing (Seawright *et al.* 1999). Exposure to toxic *Aphanizomenon* cells also resulted in reduced clearance rates in some mussels (Pereira *et al.* 2004). The severe cell damage in the gastrointestinal tract

could thus be caused by long contact times with CYN (Seawright *et al.* 1999; Shaw *et al.* 2000). In contrast, an inflammatory response in the alimentary canal of *Microcystis*-exposed carp was accompanied by increased peristaltic activity and faster gut clearance times (Carbis *et al.* 1997).

Minor irritation in the gastrointestinal tracts of control tadpoles could have resulted from lipopolysaccharide (LPS) contact. *Spirulina* sp. algal discs were provided as a food source, and *S. platensis* (a species cultured commercially) has been known to contain LPS at concentrations of up to 1.5% dry weight (Cohen 1997).

8.3.6 Comparative toxicity

There are no published works available on CYN or *C. raciborskii* toxicity in amphibians, but several studies have examined toxicity of MC in frogs and toads. Oberemm *et al.* (1999) reported no sublethal nor lethal effects in marsh frogs (*Rana ridibunda*) following exposure to 0.5, 5 and 50 $\mu\text{g L}^{-1}$ MC-LR, -RR and -YR. In comparison, concentrations of 45 $\mu\text{g L}^{-1}$ and 36 $\mu\text{g L}^{-1}$ CYN in the live *C. raciborskii* trials were associated with 33 and 16% mortality, respectively.

Purified MC-LR was shown to cause only weak lethality of African clawed frog (*Xenopus laevis*) embryos at 250 $\mu\text{g L}^{-1}$; but approximately 30% embryo mortality at exposure of 500 $\mu\text{g L}^{-1}$ over 96h, equating to a LC_{25} value of 380 $\mu\text{g L}^{-1}$ (Dvoráková *et al.* 2002). However, cyanobacterial biomass (with and without toxin) was less toxic (Dvoráková *et al.* 2002). Toxin exposures $\leq 250 \mu\text{g L}^{-1}$ were not linked with significant growth inhibition. However, frequent and multiple developmental abnormalities of the backbone, tail, brain

and eyes and malformation of head were recorded with purified MC-LR at concentrations as low as $25 \mu\text{g L}^{-1}$, and with MC-containing and MC-free algal biomass (Dvoráková *et al.* 2002). Mortality, malformation or growth inhibition was not recorded in the early life stages of *Xenopus laevis* exposed to $1 - 2,000 \mu\text{g L}^{-1}$ MC-LR or -RR over a maximum of five days exposure (Fischer & Dietrich 2000).

8.3.7 Bioconcentration and bioaccumulation of free CYN

B. marinus tadpoles bioaccumulated, but did not bioconcentrate, free CYN. Absence of bioconcentrated toxin suggests that uptake of extracellular toxin via transdermal uptake or accidental drinking does not occur. Equally, toxin may also be able to be metabolised within the tissues at a rate sufficient to prevent bioconcentration. The amount of toxin present in the tissues actually decreased with exposure time: this could indicate either that depuration occurred, or, alternately, that a reduction in grazing on toxic cells over time caused reduced toxin uptake. Possible reduction in appetites and grazing in the presence of CYN or *C. raciborskii* is discussed above (section 8.3.5).

Apparent increase in the number of gland-like cells in the external dermis (see 8.3.7) could act to inhibit transdermal toxin uptake in *B. marinus*. This corroborates the findings of Fischer and Dietrich (2000), who reported that *Xenopus laevis* (African clawed frog) larvae could not take up MC transdermally, and that the MC toxicity required oral ingestion of toxin.

In the presence of intracellular toxin – even at comparatively smaller quantities – *B. marinus* tadpoles were capable of accumulating toxin to a maximum of 19

times the concentrations available in the test solutions. This is consistent with large quantities of toxin uptake via the oral (grazing) route. Differences in individual grazing rates and differences in the percentage of extracellular toxin between treatments and between trials could explain the variability in BAFs between and within treatments.

Few studies have examined bioaccumulation of algal toxins in amphibians, making comparisons with CYN and other toxins difficult. *X. laevis* larvae were found to contain high concentrations of radioactive MC-LR following exposures of 450 µg L⁻¹, but concentrations were highest in the visceral area and whole-animal samples (Fischer & Dietrich 2000), possibly suggesting that toxin was lying in the gut and thus was not truly accumulated. One environmental study also found frog muscle tissue to contain, on average, 125 µg MC-LR g⁻¹ dry weight tissue (Gkelis *et al.* 2002).

8.3.7.1 Possible methods of uptake

The dramatic increase in tissue toxin concentrations with the introduction of small concentrations of intracellular toxin indicates the ability to bioaccumulate toxin via grazing. Certainly, gross histological injuries to the gastrointestinal tract and liver appear consistent with CYN uptake via oral ingestion, with toxin transported from intestines to liver via the portal vein. This route has already been suggested for MC (Fischer & Dietrich 2000).

Whether *C. raciborskii* is a component of the natural diets of *B. marinus* is not known. Intact trichomes were not observed in the gut during histological examinations. Hoff *et al.* (1992) reported many algal groups from the guts of

tadpoles, including diatoms, filamentous greens and euglenophytes, but cyanoprokaryotes were not included. There was no evidence that *B. marinus* tadpoles actively avoided *C. raciborskii* cells, rather, it appears they simply ingested whatever they encountered, including the *Spirulina* algal discs provided and any settled-out trichomes or (in the case of extracellular trials) algal debris. In addition, the extensive damage to the lining of the gastrointestinal tract noted during histological examination also represents a chance for greater CYN adsorption and absorption rates. This possibility has already been highlighted in studies of mice (Shaw *et al.* 2000).

B. marinus tadpoles may also have a second method of intracellular toxin uptake. In addition to actively foraging for food (grazing), tadpoles utilise a passive ingestion process, where mucus cords secreted within the internal gills are able to pass entrapped particles backwards to the oesophagus (Tyler 1999). In high cell concentration solutions such as those used in the present work (generally in excess of 1,000,000 cells mL⁻¹), toxin-laden *C. raciborskii* cells may be captured by this method and passed into the alimentary tract, so representing a further mode of toxin uptake. However, this uptake route appears unlikely since *B. marinus* tadpoles did not bioconcentrate toxin.

8.3.8 Bioaccumulation of free deoxy-CYN

Deoxy-CYN was not reported from the tissues of *B. marinus*, though at similarly low exposure concentrations, *M. tuberculata* recorded deoxy-CYN BAFs of 30 to 100 (see previous chapter). Thus, it would appear that at comparable toxin exposure concentrations, *Bufo* are unable to accumulate deoxy-CYN, whereas *Melanoides* takes up enough of the analog that toxin detection is possible.

8.3.9 Food addition and its influence on bioavailable CYN fractions

The loss of CYN from flasks containing *Spirulina* suggests that toxin sorption to the algal crush may have occurred. Alternatively, other additives in the *Spirulina* meal may have accelerated the decomposition of CYN. If adsorption occurred, toxin uptake in trial three could have resulted from grazing, rather than transdermal uptake, since sorbed toxin may have been accidentally ingested along with the *Spirulina* crush. Voutsas *et al.* (2002) considered that sorbed substances may have different bioavailability to aquatic organisms, if these form chemical aggregates that are too large to cross biological membranes.

Sorbed toxin may be important in *C. raciborskii* blooms, since most toxin is extracellular, and therefore likely to adsorb to particles. This could have important implications for grazers that use submerged plants as a food source. For example, Kotak *et al.* (1996) reported that gastropods with high accumulated MC values were likely to have ingested sorbed toxin since *Microcystis* forms dense films near the surface of plants such as *Potamogeton* and *Myriophyllum*. However, since bioconcentration did not occur in the tadpoles, the contribution of sorbed CYN (rather than true CYN_{EXC}) appears to be of little importance.

8.5 Areas of further study

There are several avenues available for further study regarding amphibian exposures to CYN. Clearly, reasons for the lack in CYN bioconcentration and deoxy-CYN bioaccumulation should be identified. In addition, studies using adult *B. marinus* may indicate whether effects are short lived, and whether bioaccumulation rates are indeed high due to transdermal uptake and grazing.

Chapter nine: general discussion for aquatic animals

9.1 Comparing *M. tuberculata* with *B. marinus*

9.1.1 Responses to exposure

B. marinus tadpoles were more susceptible to *C. raciborskii* and CYN exposures than were *M. tuberculata* snails, regardless of whether whole cell extracts or live algal cultures were used. *B. marinus* tadpoles featured abnormalities, grew slowly, swam less and died when exposed to the test solutions, particularly in trials using live *C. raciborskii*. By contrast, *M. tuberculata* experienced few adverse effects of exposure. Several authors have concluded that invertebrates, in general, are more tolerant to cyanotoxins than are vertebrates (Pietsch *et al.* 2001; Karjalainen *et al.* 2005). The current work appears to support this conclusion. One limitation, however, was that the *Melanoides* trials examined adults whereas the *Bufo* trials used juvenile organisms: the use of different life stages may have contributed to the different susceptibilities.

Differences in *C. raciborskii* and/or CYN susceptibility in the two test species could reflect dissimilar abilities for toxin uptake and/or metabolism. Both *B. marinus* tadpoles and *M. tuberculata* are grazers and have grossly similar surface area to volume ratios: similar rates of CYN uptake might therefore occur in each. In contrast, toxin metabolism, which usually takes place within the liver (or an analogous organ), could be completely different in the snails when compared with the tadpoles.

It is conceivable that organisms with less evolved liver apparatus have some protection from CYN toxicity. The hepatopancreas found in *Melanoides* snails is likely to provide only poor toxin metabolism capability compared with *Bufo* tadpoles,

which have a true liver. On the other hand, cytochrome P-450 enzymes are especially concentrated in vertebrate livers. These enzymes appear to be responsible for CYN bioactivation (Runnegar *et al.* 1994; Shaw *et al.* 2000); inhibition of cytochrome P-450 affords partial protection from CYN toxicity (Runnegar *et al.* 1995). Therefore, since CYN breakdown products may be more toxic than the parent molecule (Runnegar *et al.* 1994; Norris *et al.* 2002), this reduced toxin metabolism may be beneficial, since production of toxic metabolites would be also reduced.

Whether there is a trend for biologically advanced animals to experience greater toxicity from CYN due to the functioning of the liver (or other organs responsible for toxin metabolism) remains unresolved. Toxic effects of CYN have been demonstrated on several invertebrates (Hiripi *et al.* 1998; Metcalf *et al.* 2002b). In contrast, Saker *et al.* (2004) and Saker and Eaglesham (1999) recorded high levels of CYN from quite highly evolved organisms (mussels, crayfish, rainbow fish) with no mortality. There is considerable variability in the toxicity of CYN amongst mammalian subjects such as mice and cattle (Saker *et al.* 1999), and even between individuals of the same species (Seawright *et al.* 1999). Taken together, these studies suggest that the mode and extent of CYN toxicity is complex and involves more than the toxin metabolism capacity of the liver (or equivalent organ). Further studies involving a broader range of test species may elucidate better the metabolism of CYN.

9.1.2 Specific toxin uptake routes

Determining exact method(s) of toxin uptake in *Melanoides* and *Bufo* is difficult. In *Melanoides*, both bioaccumulation and bioconcentration were recorded. Uptake of CYN_{EXC} caused minimal bioconcentration. Whether this occurred across the foot, via

accidental drinking, or by another process, is unknown. Bioconcentration may also have been reduced in the snails due to the protective layer of the shell. In comparison, CYN bioconcentration was not recorded from *B. marinus* tadpoles, indicating either that extracellular toxin uptake was not possible, or, alternately, that toxin was metabolised following entry into the cells.

The presence of intracellular toxin dramatically increased tissue toxin accumulation in both the snails and the tadpoles, indicating that uptake via grazing was almost certainly a major source of toxin. Consequently, individual variability in grazing rates and the cell quotas of *C. raciborskii* cells are likely to be influential on the final tissue toxin concentrations of both species. It seems likely that *C. raciborskii* filaments were lysed within the gastrointestinal tract following grazing, allowing the toxin to become liberated and available for uptake via active or passive processes. Histological studies indicated major injuries to the gastrointestinal tract, liver and gill tissue of the tadpoles. Again, this argues for uptake via the mouth and/or intestine. Whether uptake occurs across the gill epithelia remains unclear: since the gill epithelium is blood rich, histological damage might have been caused by transportation of CYN (or its metabolite(s)) into the tissues, rather than by direct uptake.

A striking difference between the two species was the complete absence of deoxy-CYN detected from the tissues of *B. marinus*, whilst *M. tuberculata* was capable of considerable deoxy-CYN bioaccumulation (BAFs exceeding 100). Both test species were exposed to similar concentrations of deoxy-CYN, particularly in the live *C. raciborskii* exposure trials. The reason for this difference is unclear.

9.1.3 Mechanism of action of CYN

It is difficult to speculate a possible mode of toxic action for CYN (if any) in *M. tuberculata*, since minimal toxic effects were recorded from this species.

In *B. marinus* tadpoles, the tissue damage noted in the histological studies was consistent with PS inhibition: Shaw *et al.* (2000) have already concluded that lipid vacuolation, a common feature of CYN toxicity, results from PS inhibition, since PSI prevents triglyceride conversion to lipoproteins. The presence of apparently apoptotic cells may also result from DNA effects. Since apoptosis is used to help regulate cell proliferation, changes to DNA structure resulting from CYN-induced micronuclei induction, strand breakage or fragmentation (Humpage *et al.* 2000; Shen *et al.* 2002; Humpage *et al.* 2005) could cause increased numbers of apoptotic cells.

There is reason to suggest that CYN is especially toxic to developing or rapidly growing tissues. Mortality was recorded from the *Bufo* tadpoles but not for any of the other test organisms. In addition, toxic effects were suggested for *Melanoides* hatchlings, but not for the adult snails. Juvenile organisms may be more susceptible to CYN toxicity due to PSI: since developing tissues need to synthesise proteins rapidly for the next phase of cell division, exposure to CYN may prevent normal cell growth. Alternately, manufacture of hormones (also proteins) could be prevented by CYN toxicity. This concept has already been explored in human ovaries, where progesterone production is arrested, even after short exposure times to CYN (Humpage & Young 2005).

A further concept highlighted by these studies is that CYN intoxication appears to be characterised by increased retention of food in the gastrointestinal tract, possibly accompanied by, or resulting from, anorexic tendencies. This feature has been commonly reported in other studies (Hawkins *et al.* 1985; Seawright *et al.* 1999; Li *et al.* 2001b; Saker *et al.* 2003) and was suggested by the histological work with *B. marinus* tadpoles. Possibly, major tissue damage in the gastrointestinal tract prevents animals from wanting to feed further.

9.1.4 Bioconcentration and bioaccumulation

Bioconcentration was hardly recorded from either test organism, whilst tissue bioaccumulation occurred at 120 (snails) or 19 (tadpoles) times the bioavailable CYN concentrations. The contribution of dissolved toxin in causing tissue contamination thus seems minor compared with intracellular toxin. However, in both the *M. tuberculata* and *B. marinus* trials, the quantification of CYN_{EXC} and CYN_{TOT} fractions during live *C. raciborskii* exposure trials was done by determining the CYN concentrations of vacuum-filtered and non-filtered test solutions. It is possible that filtering itself caused rupture of some intact *C. raciborskii* cells, thus inflating the relative abundance of CYN_{EXC} compared with CYN_{INC}. If so, the importance of CYN_{INC} in contributing to CYN accumulation could have been overestimated.

The absence of toxic effects in conjunction with CYN bioaccumulation in *M. tuberculata* is unexpected and difficult to explain. Internal tissue loads resulting from bioaccumulation usually magnify whatever toxic effects are already present, especially if target tissues coincide with the site of toxic action. Even more curious is that few adverse effects occurred despite the treatment solutions containing whole-cell

extracts of live *C. raciborskii* cells. Toxic responses would have been expected to result from lipopolysaccharide components and possibly other toxic substances within *C. raciborskii*. Such complications represent avenues for further studies.

9.2 Exposure implications in the test species and aquatic community

Toxin bioaccumulation has important implications for the food webs of water bodies affected by toxic algal blooms. Many freshwater and terrestrial species (waterfowl, turtles, water rats and fish) consume snails; sometimes these represent the primary diet component (for example, in fish) (Dillon 2000). High CYN concentrations in *M. tuberculata* may therefore indicate strong potential for biomagnification of CYN into higher trophic levels. Similarly, toxin accumulation in *Bufo* tadpoles also represents the potential for toxin uptake via predators. Whether toxin can persist in tadpole tissues throughout metamorphosis is unknown: if so, predation on adult toads may cause contamination in terrestrial food webs. In addition, *C. raciborskii* and/or CYN toxicity may indirectly influence trophic relationships: dead or dying species may attract entirely different predators than those in good health. For example, swimming tadpoles may normally be eaten by visually based predators (birds, fish), whereas a carcass is likely to be grazing upon by omnivorous or detritivorous species.

Declines in frog populations have been noted worldwide, with a multitude of reasons being cited, including habitat fragmentation, global warming, and prolonged and indiscriminate use of pesticides (Kiesecker *et al.* 2001). The results of the present work show that toxic algal blooms could also contribute to reductions in frog and toad communities. However, this extrapolation should be used with caution: the trials only examined one life stage of *Bufo*. Survival rates of frogs and toads differ substantially

according to the developmental stage at which they are tested (Nebeker *et al.* 1998; Harris *et al.* 2000). It is possible that amphibian eggs could receive protection from CYN since these are often accompanied by foamy or jelly coatings. Terrestrial or semi-aquatic adult amphibians are also likely to experience far less exposure risk from toxic algal blooms than fully aquatic juveniles.

SECTION IV

Understanding and managing toxicity and bioaccumulation risks associated with cylindrospermopsin



A recreational hazard sign erected at Lake Elphinstone in central Queensland to warn the public of blue-green algae risks.

Chapter ten: understanding ecotoxicity and bioaccumulation risks associated with CYN and the implications for ecological health

10.1 Toxicity of CYN from *C. raciborskii*

In the previous chapters, influences on *C. raciborskii* and CYN toxicity to each of the test organisms were considered in isolation. However, some general trends regarding CYN toxicity are also evident when results from the present studies are combined with those in the available literature. These concepts are summarised in Figure 10.1 and are discussed briefly below. Key aspects of exposure regimes that are influential to CYN bioaccumulation are also examined. The potential risks of toxin-producing *C. raciborskii* blooms are discussed with respect to both aquatic ecosystem and human health. Finally, the possible evolutionary origin and benefit of toxin production by cyanoprokaryotes (especially in *C. raciborskii*) is investigated.

10.1.1 Dynamics of toxin production by *C. raciborskii*

Production of CYN by *C. raciborskii* (and other species) is essential for toxin-mediated exposure effects to occur. Much is still to be learned about the ecology of *C. raciborskii* and influences on toxin production (Falconer 2005).

10.1.1.1 Toxin content per cell (Q_{CYN})

There is limited information relating to how grazing pressure may affect or trigger toxin production and cell quotas (Fabbro *et al.* 2001). However, if cell quotas are inflated because of grazing (or filter feeding), the toxin concentrations that are ingested by herbivorous organisms may be greatly increased.

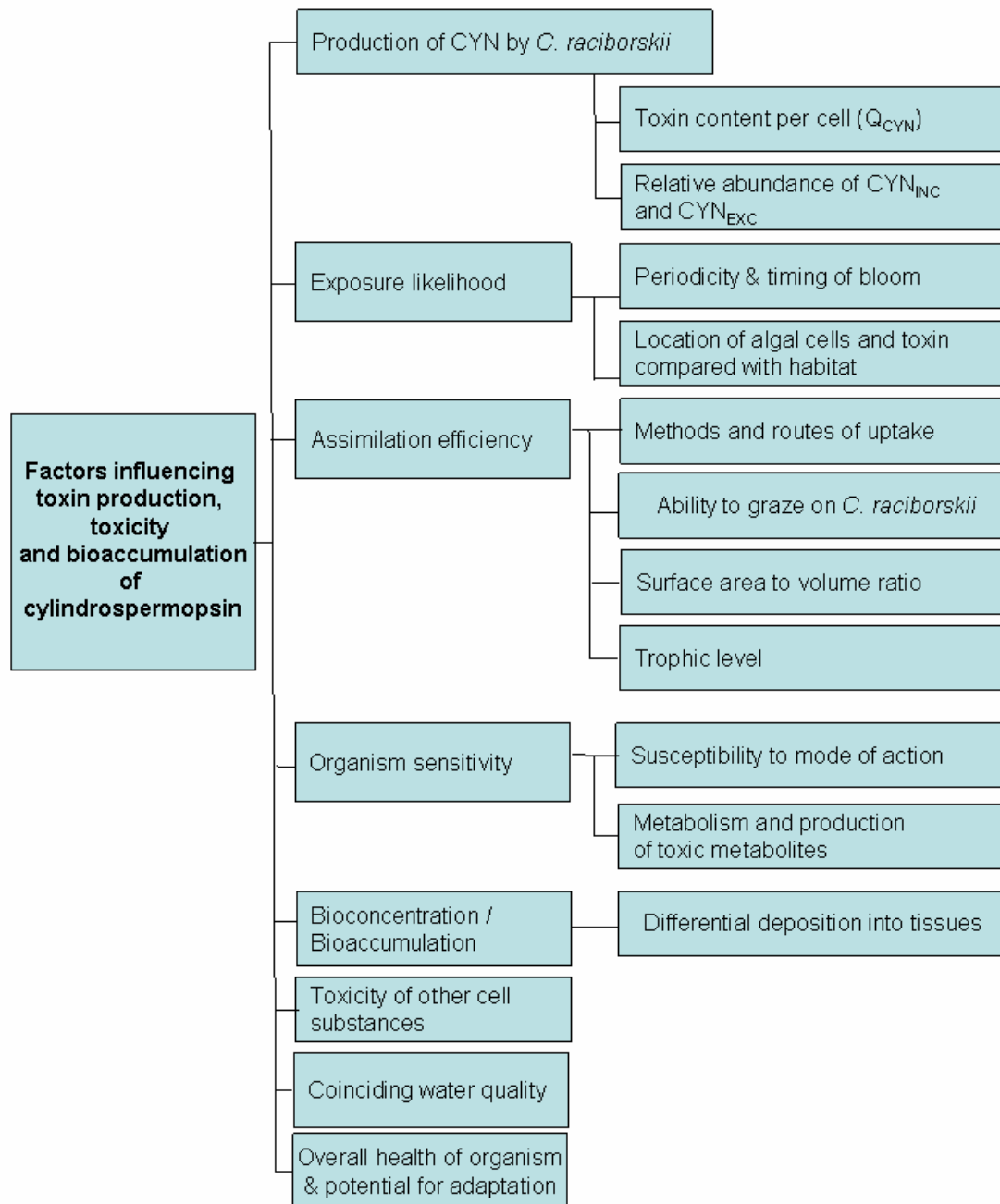


Figure 10.1 Examples of factors influencing toxin production, toxicity and bioaccumulation of cylindrospermopsin from *Cylindrospermopsis raciborskii* in aquatic ecosystems. This is not an exhaustive list.

The toxin producing capability of vegetative cells compared with specialised cells, such as akinetes and heterocytes, has not been examined. Orr and Jones (1998) concluded that each *Microcystis* cell ‘has a genetically fixed quota of toxin’. Since akinetes and heterocytes in *C. raciborskii* begin life vegetatively (Chiswell *et al.* 1997), their genetic capability for toxin production could equal that of vegetative cells. On the other hand, the resting stages and cysts of some algae, such as *Alexandrium*, are many times more toxic than their vegetative cells (Landsberg 2002). The possibility for akinetes to produce high CYN concentrations may be particularly important during bloom senescence, where they are often produced in large numbers (Chiswell *et al.* 1999) and drift into the sediments. This could signal potential toxicity to the benthic (and possibly even hyporheic) fauna that might otherwise escape toxin, since it is usually associated with planktonic algae.

With respect to bioaccumulation, both cell concentrations and cell quotas play important roles in determining risks during toxic algal blooms, since intracellular toxin uptake contributes substantially to CYN tissue contamination (at least in snails and tadpoles). Different algal blooms may represent different bioaccumulation risks, depending on their cell concentration and cell quota characteristics. For example, consider two blooms of equal toxin concentration, and equal CYN_{INC} : CYN_{EXC} . The bloom with high cell density (but low cell quota) may present less bioaccumulation risk than one with low cell density (but high cell quota). However, this is only true where grazers consume equal quantities of cells in each scenario: it is possible that cells with higher toxin quotas cause preferential selection to occur. The role of other food sources should not be discounted, either, since the availability of non-toxic alternative feed might also cause reduced grazing on toxic cells (Soares *et al.* 2004).

10.1.1.2 Relative abundance of CYN_{INC} and CYN_{EXC}

Christoffersen (1996) theorised that toxicological effects will depend on the toxicity (strictly, toxin concentration) and duration of an algal bloom. However, the present work has shown that absolute and relative abundances of toxin fractions (cellular, dissolved) are pivotal in affecting toxin uptake and toxicity to aquatic organisms. Unfortunately, changes in CYN_{INC}: CYN_{EXC} ratios have yet to be closely mapped in naturally occurring blooms. Hawkins *et al.* (2001, p. 466) concluded that a ‘slow growing and persistent bloom of *C. raciborskii* will yield the highest levels of CYN’. However, this does not necessarily mean that such blooms will be the most toxic, especially if large proportions of the toxin are extracellular.

10.1.2 Exposure likelihood

10.1.2.1 Periodicity and timing of bloom

Cylindrospermopsis is a subtropical to tropical species that forms blooms year-round (Padisák 1997). Hence, other aquatic species living in tropical environments are likely to encounter toxic blooms. Furthermore, since *C. raciborskii* cell quotas may actually be higher in cooler temperatures (McGregor & Fabbro 2000), different life stages may be more susceptible than might others. For example, *Melanoides* and *Bufo* both reproduce in summer: early life stages of these species may be exposed to high cell concentrations of *C. raciborskii*, but not necessarily high CYN concentrations. The opposite problem has been identified for tropical *Microcystis* blooms, which are highly toxic in the summer months that coincide with critical breeding periods for many fish species (Wiegand & Pflugmacher 2001).

10.1.2.2 Location of algal cells and toxin in relation to habitat

The positioning of algal cells within an aquatic habitat may be critical in affecting toxin exposure and uptake rates (Figure 10.1). The spatial variation of *Cylindrospermopsis* was discussed in chapter two, with respect to different habitats (planktonic, benthic) experiencing different cell and toxin concentrations (surface scums and intracellular toxin; senescent cells and extracellular toxin). Importantly, however, once in the extracellular form, toxin may diffuse into almost all aquatic habitats (Christoffersen 1996). This again highlights the importance of toxin concentrations overall, but also the importance of the proportion of CYN present in the dissolved form.

10.1.3 Assimilation efficiency

10.1.3.1 Method(s) of uptake

In the absence of radiolabelling work, few conclusions can be reached about the specific pathways of CYN uptake in aquatic organisms. Transdermal uptake, accidental drinking and grazing probably all contribute to tissue contamination, though uptake via grazing was linked with the highest concentrations of tissue toxins in gastropods and tadpoles. Changes to the lining of the gastrointestinal tract resulting from CYN-mediated tissue injuries may also allow greater CYN adsorption/absorption rates across the digestive epithelia. This scenario has already been highlighted in studies using mice. Specialised regions of the gastrointestinal tract may have different toxin uptake capability compared with other tissues. Falconer *et al.* (1999) reported that oral dosing of mice resulted in a 25-fold lower toxic effect than that achieved with intraperitoneal injection, demonstrating that gastrointestinal uptake is not as efficient as that of the parenteral route. Possibly, the

acidity of the stomach decreases CYN uptake rates: Carbis *et al.* (1997) suggested that MC uptake efficiency in feral carp was linked with stomach pH, because MC requires an acidic environment for optimal resorption. Whether CYN resorption is increased by alkaline or basic conditions is not known.

10.1.3.2 Ability to graze on toxic *C. raciborskii*

In both *Melanoides* and *Bufo*, grazing on toxin-laden *C. raciborskii* cells dramatically increased either CYN toxicity, CYN bioaccumulation, or both. Hence, factors that affect grazing rates, such as organism size, metabolism and nutritive needs, are likely to affect CYN toxicity indirectly, via their influence on toxin uptake rates. The possibility for preferential selection of cells with reduced cell quotas to reduce or avoid toxicity has already been noted (see section 7.4.3).

Some authors have concluded that the effects of cyanotoxins on aquatic plants would be minimal and short lived, given that plant-bioavailable toxin (extracellular fractions) are generally low in concentration and transient during a bloom (Jones *et al.* 1994; Casanova *et al.* 1999). However, CYN is usually extracellular (Saker & Griffiths 2000; Norris *et al.* 2001; Metcalf *et al.* 2002a), so plants may experience high CYN uptake risk. In actuality, neither *S. oligorrhiza* nor *H. verticillata* were shown to take up CYN, however, this result could be confounded by the HPLC detection technique, since it cannot detect bound toxin. Radiolabelling techniques may assist in further investigations of CYN uptake in aquatic macrophytes.

10.1.3.3 Size and surface area to volume ratios

Toxin assimilation efficiency is also likely to depend on the size of the organism affected. Both Christoffersen (1996) and Carmichael (1996) concluded that there is a

relationship between organism size and algal toxin sensitivity, with smaller organisms (for example, zooplankters) at greater risk. This scenario probably results from an association between surface area to volume ratios and transdermal toxin uptake. However, given that grazing, not transdermal uptake, was the primary source of toxin contamination in the present work, it seems unlikely that grazer organisms would experience increased toxicities simply because of their size. Thus, surface area to volume ratios may be significant only in organisms that use transdermal uptake as the primary route: in grazers, direct ingestion of toxin is likely to overshadow any minor toxin quantities represented by transdermal uptake.

10.1.3.4 Trophic level

The contribution of trophic level was minimally studied in the present work. Trophic level studies could be confounded by organism size, grazing ability and uptake route(s). Furthermore, since the only trophic level with more than one test organism was that of the primary producers (aquatic macrophytes) comparisons within trophic levels are difficult. One possible influence on toxicity resulting from position within the aquatic food web could be biomagnification. For example, consumption of contaminated tissues from lower trophic levels may contribute to tissue toxin loads, even if ingestion of toxin-laden *C. raciborskii* cells does not occur.

10.1.4 Organism sensitivity

10.1.4.1 Mechanism of action

Insights into the mechanism(s) of toxic action for CYN in mammalian species and/or their cell lines were recently summarised in Falconer (2005). The present work has demonstrated that CYN toxicity is also applicable to *B. marinus* tadpoles, *Melanoides* snails and the aquatic plants *Spirodela* and *Hydrilla*, during environmentally relevant

exposure scenarios. However, one aspect of the present work contrasts the existing knowledge regarding CYN's mechanism of toxicity. In *Bufo* tadpoles, exposure effects included proliferation of some cells, including the dermis of the skin and buccal cavity. This differs from Fessard and Bernard (2003), who reported decreased cell numbers and decreased mitosis rates in the ovary cells of CYN-exposed Chinese hamsters. However, since their study examined purified CYN and the present work used whole cell extracts or live cultures, it is possible that the additional toxic cell substances of *C. raciborskii* could be responsible for the conflicting result.

10.1.4.2 Metabolism and production of toxic metabolites

The relative susceptibility of *Melanoides* and *Bufo* to the mode of action of CYN has already been discussed with respect to the nature of the liver (or analogous organ) and the possession of toxin detoxication systems (see 9.1.1). The susceptibility of aquatic plants to CYN toxicity via protein synthesis inhibition and the production of toxic metabolites in these species remains poorly understood.

10.1.5 Bioconcentration and bioaccumulation

10.1.5.1 Differential deposition into tissues

Organotropy is a situation where toxic substances are deposited preferentially into certain tissues. Saker and Eaglesham (1999) reported that crayfish hepatopancreas tissue contained five times the toxin concentration of crayfish muscle tissues. Cylindrospermopsin may also have an affinity with blood or lymph: Saker *et al.* (2004) reported that 68.1% of the total CYN load in swan mussels came from the haemolymph. However, in both studies, large quantities of toxin were also reported from other areas, including the gonads, viscera, mantle and foot. With respect to MC,

organ specific deposition is thought to be a consequence of specific bile carriers being used for transport (Bischoff 2001). For CYN, however, toxin uptake involves the use of bile acid transporters initially, followed by a secondary, unknown system after 72 h (Chong *et al.* 2002). Thus, CYN organotropy may occur in the early phases of toxin uptake, whereas extended exposure times could be associated with, for example, passive toxin uptake (Chong *et al.* 2002), that causes widespread tissue contamination.

Toxin partitioning of CYN uptake might play an important role in influencing the ecological effects of bioaccumulation. Differential toxin deposition could allow toxin to be stored away from organs that are targets for toxicity, thus reducing the impact of CYN intoxication. However, this is probably unlikely, given that CYN has dramatic effects on the liver and the liver is likely to be the primary organ for toxin deposition.

10.1.6 Toxicity resulting from other cell substances

The use of whole cell extracts or live *C. raciborskii* cultures throughout the present work was done to maximise environmental relevance, although this limited the conclusions that could be drawn about the toxicity of CYN compared with other cell substances produced by *C. raciborskii*. *C. raciborskii* is known to produce saxitoxin, although this usually does not occur in conjunction with CYN production (Pomati *et al.* 2003; Castro *et al.* 2004; Pomati *et al.* 2004a; Pomati *et al.* 2004b), and the strain used in the present work was shown to have no saxitoxin present.

Additional toxicities could relate to unidentified *C. raciborskii* cell substances (Hawkins *et al.* 1997; Falconer *et al.* 1999; Norris *et al.* 1999; Froscio *et al.* 2001;

Saker *et al.* 2003; Falconer 2005). Most studies suggest than synergism between these cell components and CYN would increase toxicity (Norris *et al.* 1999). However, Metcalf *et al.* (2002b) speculated that cellular substances could reduce CYN bioavailability and hence its toxicity. Also, lipopolysaccharides (LPSs) could also prevent effective detoxication of CYN: this has already been suggested for *Microcystis*, where LPSs reduce glutathione enzyme activity and thus also MC detoxication (Best *et al.* 2002). If true for CYN, an LPS-induced reduction in glutathione-S-transferase (GST) could be partly beneficial with respect to reduced production of toxic CYN metabolites. Alternatively, reduced GST could also predispose cells to further CYN toxicity (Runnegar *et al.* 1995). The LPS content of *Cylindrospermopsis* has never been studied.

10.1.7 Water quality

Perhaps the most difficult issue to address is the confounding of algal toxicity results by the coinciding drop in water quality that often occurs in conjunction with cyanoprokaryote blooms. Alkaline pH values, oxygenation supersaturation and low light conditions may occur during the progression of a bloom; decomposition of blooms may lead to deoxygenation and the production of nitrogenous compounds (Carmichael 1996; Oberemm 2001). Thus, additive or synergistic effects in field environments may mean that the true environmental risks of CYN are in fact greater than these (laboratory) trials suggest.

10.1.8 Organism health and prior adaptation

Many factors could influence the responses of aquatic organisms to CYN exposure, including sex, size, life cycle stage, disease, degree of parasitism, nutritional status

and history. With the exception of life history, few studies have examined any of these with respect to toxicity.

Evolutionary adaptation to CYN toxicity by aquatic organisms is likely. Both Ressom *et al.* (1994) and Lirås *et al.* (1998) suggested that the shared environment between cyanoprokaryotes and aquatic organisms is likely to have encouraged the development of coexistence strategies. Recently, Gustafsson *et al.* (2005) demonstrated that increased tolerance to toxic *Microcystis* could be induced in *Daphnia magna*, and that this tolerance could be passed on to successive generations. Allelopathy from adapted aquatic macrophytes could lessen the dominance of toxic phytoplankton species via changes in successional cycles (Nakai *et al.* 1999; Körner & Nicklisch 2002).

In the present work with *Spirodela*, the switch from growth inhibition to growth stimulation that was recorded over time could indicate the ability of duckweed to respond physiologically to CYN. Shaw *et al.* (2000) speculated that tolerance in mice repeatedly exposed to CYN for 90 days could result from the inhibition of enzymes required for metabolic activation of CYN, or from induction of enzymes capable of CYN degradation. Such enzymes may be either constantly expressed or switched on and off in response to toxin exposures (Beattie *et al.* 2003). It is possible that such a similar mechanism occurred via enzyme inhibition or induction in *Spirodela*.

10.2 Focus on bioconcentration and bioaccumulation

10.2.1 Toxin accumulation in different aquatic organisms

A range of values for CYN bioconcentration (toxin sourced from extracellular fraction only) and CYN bioaccumulation (toxin sourced from intracellular and extracellular

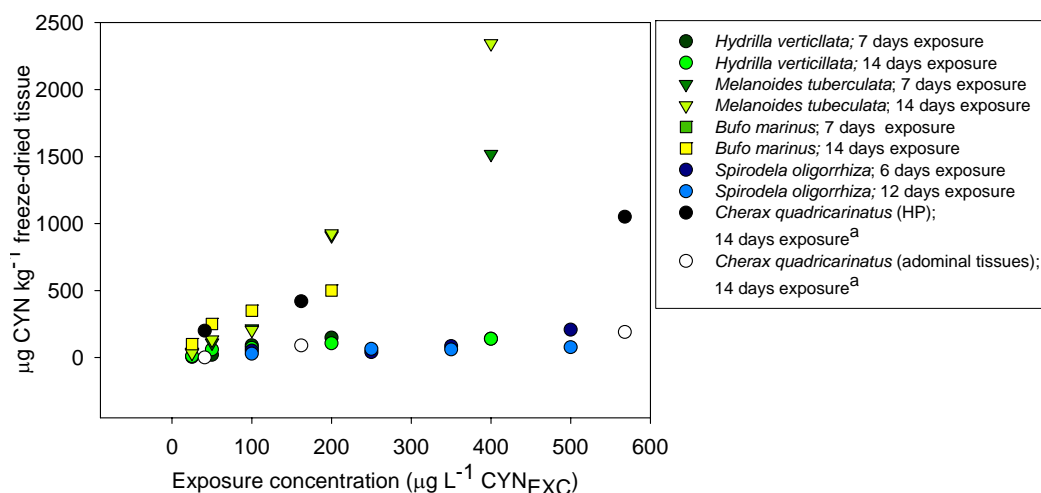


Figure 10.2 Relationship between tissue cylindrospermopsin concentrations (bioconcentration) and extracellular cylindrospermopsin (CYN_{EXC}) exposures, as reported in the present studies and published literature. HP=hepatopancreas tissues; ^a Saker and Eaglesham (1999). Values from published literature are as reported or closest approximation from figures provided. Values from present studies are the average of all trials conducted (where applicable).

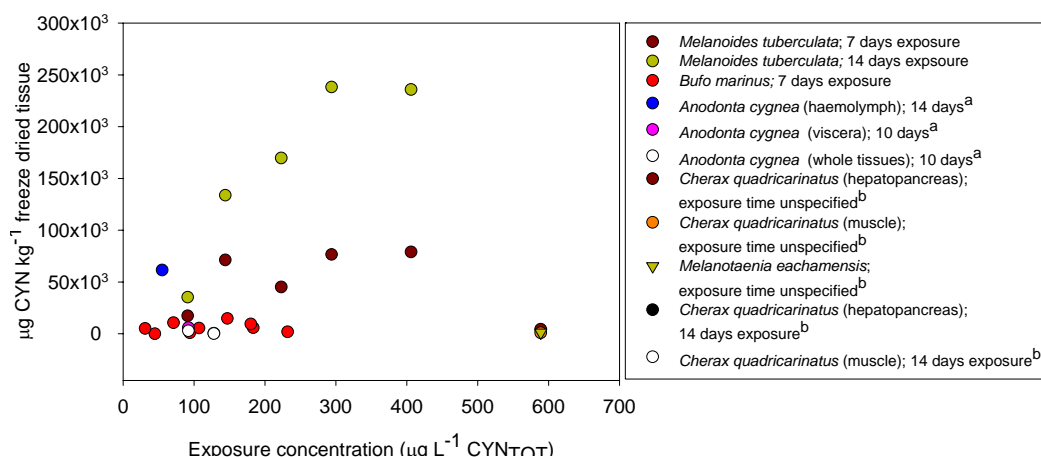


Figure 10.3 Relationship between tissue cylindrospermopsin concentrations (bioaccumulation) and total cylindrospermopsin (CYN_{TOT}) exposures, as reported in the present studies and published literature. ^aSaker *et al.* (2004); ^bSaker and Eaglesham (1999). Values from published literature are as reported or closest approximation from figures provided. *Note:* Data from Nogueira *et al.* (2004a) not included since values were reported as fresh weights. Values from present studies are the average of all trials conducted (where applicable).

fractions) have been reported for aquatic organisms (Figures 10.2, 10.3). For bioconcentration, *Melanoides* snails from the present work were best able to bioconcentrate CYN, followed by *Bufo* tadpoles and aquatic plants (Figure 10.2). It is difficult to compare the *Cherax* data with that of the other species, since values for specialised tissues were reported instead of whole-animal data. For bioaccumulation, the *Melanoides* snails again excelled, followed by *Anodonta* mussels, *Cherax* crayfish, *Bufo* tadpoles and *Melanotaenia* fish (Figure 10.3). This strengthens the argument for the predominance of toxin uptake via grazing (or filter feeding).

There appears to be a relationship between the bioaccumulation ability of each of the organisms and their biological complexity. The simplest organisms, the molluscs, rank first, followed by the crustacean, and then the more advanced amphibian and fish species. One possible explanation for this is that the level of biological advancement of a species indicates the likely ability to metabolise CYN (see section 9.1.1).

10.2.2 Influence of exposure regime on bioaccumulation

10.2.2.1 Length of exposure

The role of time in allowing for toxin metabolism or excretion (depuration) cannot be determined from the present trials, since tissue harvests were undertaken only at weekly intervals. In other studies, uptake of both CYN and MC has been shown to be bi-phasic (Amorim & Vasconcelos 1999; Ozawa *et al.* 2003; Yokoyama & Park 2003; Saker *et al.* 2004). However, such results could be confounded by bound toxins becoming released during detoxification, and hence becoming able to be detected, at different points during each study.

10.2.2.2 Toxin exposure concentrations

Extracellular CYN concentrations clearly influence the extent of tissue contamination: CYN_{EXC} concentrations always positively correlated with tissue toxin concentrations (bioconcentration) in both *Melanoides* and *Bufo*. This was also true for the aquatic plants *Spirodela* and *Hydrilla*, despite their minimal toxin uptake. In contrast, the relationship between intracellular or total toxin exposure concentrations and tissue contamination levels (bioaccumulation) was not consistent. For example, all toxin concentration fractions (CYN_{TOT}, CYN_{EXC}, CYN_{INC}, CYN_{EXC}: CYN_{INC} ratio) correlated positively with tissue toxin concentrations in the snails, whereas none of these significantly correlated with tissue toxin concentrations in the tadpoles. However, it is possible that significant correlations were not detected for the tadpole data because of the variability in tissue toxin concentrations: further studies using more replicates may solve this problem.

The presence of CYN_{INC}, appears to strongly influence the likelihood of CYN deposition into the tissues of both the snails and tadpoles. Exposure to test solutions containing only CYN_{EXC} barely resulted in bioconcentration in any test organism. In contrast, bioaccumulation usually occurred in organisms exposed to CYN_{INC}, even at low exposure concentrations (36 µg L⁻¹ CYN_{TOT}). This agrees with the results of Saker and Eaglesham (1999), who reported that tissue toxin concentrations of redclaw crayfish were considerably lower in laboratory-based, extracellular-only exposure experiments, compared with those recorded from an aquaculture pond containing live *C. raciborskii*, even at comparable total toxin concentrations.

The present work has indicated that tissue contamination in aquatic organisms is likely to occur during natural blooms of *C. raciborskii* if these produce CYN. Blooms containing higher quantities of CYN also represent greater risk of tissue bioaccumulation. Furthermore, if CYN_{INC} is present, tissue toxin concentrations are likely to increase. However, the variability in the present test results makes it difficult to pinpoint the importance of each toxin fraction concentration (CYN_{EXC}, CYN_{INC}, CYN_{TOT}) in influencing tissue toxin concentrations. With future study, it is likely that relationships between internal tissue CYN concentrations and the corresponding adverse effects in aquatic organisms will become clear. This information could be used in ecological risk assessments (Connell *et al.* 1999). However, if the influence of toxin fractions on tissue toxin concentration is inconsistent, as suggested by the present data, it will remain difficult to judge environmental effects solely from environmental (compared with tissue) toxin concentrations.

10.3 Toxic *C. raciborskii* – implications for aquatic ecosystem health

The possible ecological consequences of toxic cyanoprokaryote blooms were first highlighted over a decade ago: Christoffersen (1996) noted that ‘toxin concentrations have potentially negative effects on organisms in nature’ and ‘species from all trophic levels seem susceptible . . . since most organisms may come into contact with cells or released toxin’. Later, Landsberg (2002) noted that harmful algal blooms could include impacts on aquatic species interactions, health, population growth, ecology and ecosystem integrity. In addition, blooms could represent potential vectors for pathogens, since bacteria may be associated with dense algal blooms, and algal toxicity could cause weakened animals to succumb to disease (Landsberg 2002). Most recently, Wiegand and Pflugmacher (2005) summarised the adverse effects that

occur in conjunction with exposure to cyanotoxins, however, few studies of CYN impacts on aquatic organisms were mentioned.

Cylindrospermopsin is of particular concern since its primary mechanism of action – protein synthesis inhibition – applies to all aquatic species. The present studies have shown that when combined with whole cell extracts or live *C. raciborskii* cultures, the toxic effects of CYN may include changes to growth, resource distribution, behaviour, hatching rates, survivorship and evidence of cell and tissue injury. Moreover, uptake is possible via multiple routes, and substantial bioaccumulation occurs in some organisms. These results suggest that CYN toxicity could be far-reaching, even extending to organisms that are not in direct contact with toxin or the algal cells (for example, organisms that ingest contaminated prey).

Data from the present work and published studies showed that *C. raciborskii* is likely to cause changes to organisms from all trophic levels (phytoplankton, zooplankton, macrophytes, invertebrates and vertebrates). Local, transient and episodic losses in zooplankton and phytoplankton diversity, abundance, community composition, and size classes have been recorded or implicated in conjunction with toxic *C. raciborskii* blooms (Bouvy *et al.* 2000; Bouvy *et al.* 2001; Fabbro *et al.* 2001; Leonard & Paerl 2005). Bouvy *et al.* (2001) also reported an increase in the diversity of bacterial size classes during the progression of a *C. raciborskii* bloom, possibly because of grazing pressure modifications, with larger, grazing resistant bacteria predominating. These effects may also manifest at higher levels to cause ecosystem responses, especially in cases of long-term bloom periodicity and/or repeated exposure events, where aquatic organisms cannot avoid exposure to *C. raciborskii*. The potential for cumulative effects resulting from repeated toxin exposures remains entirely unknown for CYN.

10.4 Toxic *C. raciborskii* and human health

Human health risks associated with toxin-contaminated drinking and recreational water have already been discussed at length (Chorus & Bartram 1999; Falconer 2005). However, bioaccumulation of CYN has now been shown in several organisms: *Melanoides* and *Bufo* in the present work; *Anodonta* mussels (Saker *et al.* 2004); and *Cherax* crayfish and *Melanotaenia* rainbowfish (Saker & Eaglesham 1999). Therefore, it seems that bioaccumulation is likely throughout aquatic food webs. Hence, significant risks could also relate to the consumption of contaminated plants, animals and their products. This problem has already been noted for other algal toxins (Pereira *et al.* 2004; Soares *et al.* 2004; Chen & Xie 2005; Kankaanpää *et al.* 2005) but only briefly for CYN (Saker *et al.* 2004). As one example, crops irrigated with CYN-contaminated water may experience toxin sorption, since adsorption occurred in both *Spirodela* and *Hydrilla*. This has already been highlighted in studies of microcystin (Abe *et al.* 1996; Codd *et al.* 1999b), and for repeated doses of CYN, where toxin might accumulate over time (Metcalf *et al.* 2004).

10.5 Evolution and production of CYN and its function(s)

The evolutionary origin of toxin production by cyanoprokaryotes remains unknown (Carmichael 2001b; Wiegand & Pflugmacher 2005). There is considerable variation in the genetic information that codes for toxin production in cyanoprokaryotes, even within the same species. Different *Cylindrospermopsis* strains may have all, part or none of the complement of genes required for CYN production (Paul Rasmussen, Australian Water Quality Centre, pers. comm.), but how and why different strains have different genetic makeup remains unresolved. If such genes were inherited, and then consequently deleted or mutated, all *Cylindrospermopsis* must have radiated from the same evolutionary ancestor. However, this opposes Padisák (1997), who

suggested that *Cylindrospermopsis* had two independent radiation points (one African and one Australian, based on morphological diversity, shade tolerance, salinity tolerance and akinete production).

A further aspect of toxin production by *C. raciborskii* that is not well understood is the predominance of extracellular toxin during bloom progression. This pattern contrasts that of almost all other algal toxins, which are mostly intracellular (excepting anatoxin-a (Bumke-Vogt *et al.* 1996)). Currently, extracellular toxins are believed to arise strictly from cell lysis (Codd *et al.* 1999a). However, Metcalf *et al.* (2002a) and Hawkins *et al.* (2001) showed that 60 – 80% of CYN was in the extracellular form in actively growing cultures, where cell lysis would be minimal. In addition, Shaw *et al.* (1999) suggested that dominance of CYN_{EXC} during *Aphanizomenon ovalisporum* blooms resulted from continual toxin production and toxin release, or from the persistence of toxin in the environment (thus allowing large extracellular concentrations to accumulate). Certainly, CYN has been shown to be remarkably stable in a range of conditions (Chiswell *et al.* 1999). Hawkins *et al.* (2001) speculated that the reason for CYN_{EXC} dominance in older *C. raciborskii* cells was increased cell wall permeability. However, it is conceivable that *C. raciborskii* could also transport CYN out of the cell: this has the advantage of allowing producer cells to exert toxicity without being consumed. Since CYN is a small and hydrophilic molecule, passive transport across the cytoplasmic membrane could be possible.

Finally, what is the ultimate role of toxin production by *C. raciborskii*? Does CYN production contribute to overall ecological fitness of the species? Since cyanoprokaryote toxins can represent high proportions of overall biomass, and toxins have been retained over long evolutionary periods, it seems likely that these have

important biological function(s) (Codd 1995; Young *et al.* 2005). However, such functions may not necessarily be related to their toxic properties (Oulette & Wilhelm 2003). Since CYN affects numerous aquatic and terrestrial organisms, it is tempting to conclude that toxin production offers defence against grazing, and/or reduces resource competition. However, many other roles have been speculated for cyanotoxins, including cell protection, iron chelation, transmembrane transporters, allelopathy, antibiotic or antifungal properties and as cell signalling messengers (Codd 1995; Utkilen & Gjølme 1995; Orr & Jones 1998; Codd 2001; Babica *et al.* 2006; Dittman & Wiegand 2006).

10.6 Limitations of this study

The constraints to this work should not be overlooked. Trials were carried out using only one strain of *C. raciborskii*, and there is evidence that toxicity varies between strains, particularly in those that produce more than one toxin or analog (Falconer 2005, p. 96). Furthermore, whole cell extracts and live cultures may contain other bioactive compounds (Sivonen & Jones 1999; Saker *et al.* 2003; Nogueira *et al.* 2004a). Thus, the present ecotoxicity tests do not allow toxic effects to be ascribed solely to the presence of CYN (including deoxy-CYN). Nonetheless, this approach has greater environmental relevance in terms of naturally toxic blooms compared with studies using purified toxin. The inability of current detection techniques to detect bound CYN is considerably important given the likelihood of toxin binding, and consequent underestimation of tissue toxin concentrations. Presently, radioactive labelling represents the only method of detecting bound CYN: this was not pursued in the current project due to time and equipment constraints.

Chapter eleven: managing cylindrospermopsin and toxic *C. raciborskii* blooms – challenges and opportunities

11.1 Current approaches to management of cyanoprokaryote blooms

11.1.1 Managing human health risks

An increasing reliance on surface water storages worldwide has thrust the management of toxin-producing algal blooms into the public arena (Bormans *et al.* 2004). Recently, the World Health Organisation (WHO) developed drinking water standards for one of the most common algal toxins, MC (WHO 2004). These guidelines are based on tolerable daily intake calculations and have been adopted by at least seven nations. In contrast, a WHO drinking water guideline does not exist for CYN; the Chemicals Working Group which reports to the WHO currently has the derivation of a provisional CYN guideline on its agenda (Chorus 2005b).

Currently, only two countries have CYN drinking water guidelines: these are currently 15 $\mu\text{g L}^{-1}$ in Brazil (recommended, not mandatory) and 1 $\mu\text{g L}^{-1}$ in Australia (Chorus 2005a). The Australian value was proposed by Humpage and Falconer (2003) after consideration of mouse data, but was not adopted in the 2004 version of the Australian Drinking Water Guidelines due to lack of research data (NHMRC & NRMCC 2004). There also exists a debate regarding whether to lower the CYN guideline due to carcinogenicity concerns. Based on the current (and limited) knowledge of CYN toxicity, the 1.0 $\mu\text{g L}^{-1}$ value as daily intake in drinking water results in a theoretical risk of 1 in 20,000 excess cancers in a population (Falconer 2005). However, the WHO usually derive guidelines that result in no greater than 1 in 100,000 excess cancers: if aligned with this, the CYN value could be set at only 0.2 $\mu\text{g L}^{-1}$ (Falconer 2005), which is close to the level of detection.

Recreational water guidelines, unlike drinking water values, are usually based on cell, rather than toxin, concentrations. For example, Queensland, Australia uses ‘increased vigilance’ when cell counts exceed 2000 cells mL⁻¹, and alert levels one and two correspond with 20,000 or 100,000 cells mL⁻¹ (or their equivalent chlorophyll values) (Environmental Health Unit of Queensland Health 2001). A similar approach has been adopted in the WHO blue-green algal guidelines for recreational waters; here, the likelihood of adverse health effects is derived from cell concentrations of 20,000 cells mL⁻¹ (low risk); 100,000 cells mL⁻¹ (moderate risk) and the formation of scums (high risk)(WHO 2004). Inclusion of cell concentrations in such guidelines is important since the toxicities associated with blooms are not necessarily restricted to toxin concentrations. For example, Pilotto (1997) found that health effects in humans were significantly related to exposure time and *Microcystis* cell density, but not MC concentrations.

11.1.2 Managing aquatic ecosystem health

Ressom *et al.* (1994, p. 69) claimed that ‘much more is known about the effects of cyanobacteria and their toxins on animals than humans’. The problem, however, is that such data have not been critically reviewed to assess the possible impacts of cyanotoxins on ecosystem health. Evaluations of the ecological impacts of toxin-containing blooms have been included in only a few scientific papers, and the resultant knowledge kept within scientific circles (Azevedo 2005). In comparison, studies linking cyanotoxins to human illnesses (Hawkins *et al.* 1985; Azevedo *et al.* 2002) have been well-publicised. However, considering the evidence for the toxicity of blue-green algae and their toxins on aquatic organisms (especially the data reported in previous chapters), there is an urgent need to develop suitable management

strategies to address the multidimensional environmental effects associated with toxic blooms.

Management approaches that address the environmental effects of cyanoprokaryote blooms – and not just those containing CYN – are poorly developed. It appears that few nations have regulatory strategies that specifically target environmental impacts or bioaccumulation; although some countries rely on basic catchment management plans to manage such risks (Table 11.1). For example, blooms are managed indirectly via eutrophication action plans in the UK and South Africa (Ferguson 1997, Table 11.1). In the United States, total maximum daily loads are being developed for priority water bodies: these refer to the ‘maximum amount of a pollutant that a waterbody can receive whilst still meeting water quality standards’. Currently, greater than one hundred pollutants are recognised; there are no provisions specifically for algal toxins but several algal-related parameters (abundance, bloom frequency, nutrients and chlorophyll *a*) are included (Burns 2005).

11.1.3 Managing environmental compliance

Possible environmental harm resulting from toxin-producing cyanoprokaryote blooms also has important implications for compliance with environmental law. In Queensland (Australia), industrial activities such as mining and power generation are termed ‘environmentally relevant activities’ (Queensland Environmental Protection Act 1994). It is currently an offence under this act for such industries to release prescribed contaminants, including chemicals, odours and living organisms, into waterways. One such incident has already occurred in Tasmania, Australia, during

Table 11.1 Current approaches to management of cyanoprokaryote blooms that specifically relate to environmental impacts.

Country	Current position	Communicated by
Australia	<ul style="list-style-type: none"> No environmental guidelines exist Microcystin included in the ANZECC guidelines for livestock protection (ANZECC 2000) 	Burch & Humpage (2005)
Canada	<ul style="list-style-type: none"> No protection of aquatic ecosystem health from cyanobacteria 	Michèle Giddings, Health Canada
Germany	<ul style="list-style-type: none"> No environmental guidelines exist 	Dr. Ingrid Chorus (Federal Environment Agency, Berlin)
New Zealand	<ul style="list-style-type: none"> No environmental guidelines currently exist; but the Ministry for the Environment together with the Ministry of Health are currently working on development of environmental standards Extensive marine biotoxin monitoring programs in place, but only with respect to shellfish contamination for commercial, recreational and traditional take and consumption 	Dr. Susie Wood (Cawthron Institute) Dr Alexander Kouzminov (NZ Ministry of Health)
South Africa	<ul style="list-style-type: none"> No formal policy regarding cyanoprokaryotes exists (for human or environmental risk) Environment and sustainable future are highest priority issues within the South African Water Act 1998 Eutrophication standards currently being developed Current research aims to improve protection for humans, cattle and wild animals 	Carin Van Ginkel (Department of Water Affairs and Forestry, South Africa)
Spain	<ul style="list-style-type: none"> No regulations dealing with non-human risks 	Dr Antonio Quesada (Universidad Autónoma de Madrid)
U.S.A.	<ul style="list-style-type: none"> US Environmental Protection Agency currently setting total maximum daily load limits for ecological impairment of priority water bodies 	John Burns (PBS&J Jacksonville, Florida, USA)

Note: information in this table was obtained via personal email communication with each of the listed persons, unless where otherwise referenced. Information correct as at December 2005.

1996, where subsidence of the retaining wall of a sewage pond resulted in the discharge of toxic *Microcystis* into a nearby recreational lake (Bartram *et al.* 1999, p. 229). The local council was prosecuted by Environment Tasmania on a charge of material environmental harm (originally, serious environmental harm) and ordered to pay fines totalling \$30,000 (Bartram *et al.* 1999).

11.2 Future management options for environmental risks

11.2.1 Monitoring and early warning systems

Regular monitoring plays an important role in gathering knowledge about the ecology, periodicity, toxicity and environmental effects associated with *C. raciborskii* blooms. However, the type and scale of monitoring needs careful consideration: which features of blooms require monitoring? In marine algal blooms, cell concentrations (or cell biomasses) and environmental and tissue toxin concentrations (such as paralytic shellfish poisons) are quantified regularly, especially in studies to determine the suitability of flesh for harvest (consumption). Studies of similar intensity are rarely conducted during freshwater blooms; consequently, records of animal and plant health are lacking. Comprehensive monitoring could allow linkages to be drawn between toxin-producing blooms and effects on susceptible animals. In particular, one improvement to toxin sampling could be to quantify both intracellular and extracellular toxin components: this has only recently been included in surveys for MC (Chorus 2001).

11.2.1.1 Rapid toxin detection

Given that ecological impacts are likely to occur in tandem with toxin production in cyanoprokaryote blooms, early warning systems such as rapid-test techniques are critical in triggering effective and timely management responses (Oulette & Wilhelm 2003; Codd *et al.* 2005a). Enzyme-linked immunosorbent assays (ELISA), for example, are particularly sensitive methods that detect toxins early in bloom development. In Australia, preliminary work has been done to develop a CYN-specific ELISA (Baker 1998).

Polymerase chain reaction (PCR) and DNA microarray tests offer some ability to identify blooms that are potentially toxic because of their genetic makeup (Fergusson & Saint 2003). The PCR technique can now detect genes in *C. raciborskii* and *Aphanizomenon* responsible for CYN production (Rasmussen *et al.* 2005). Currently, however, this technique is only semi-quantitative for cell numbers, since whether or not these species carry multiple copies of genetic information is unknown. Reverse transcriptase ‘real-time’ (RT-PCR) could allow an indication of not only the toxigenicity of certain strains, but also the level of expression for toxin-coding genes. Thus, in the future, RT-PCR may offer a better understanding of environmental triggers for gene expression.

Use of solid-phase adsorption toxin tracking techniques for the detection of algal toxins over extended time periods have recently been investigated (MacKenzie *et al.* 2004; Takahashi *et al.* 2005), although techniques to capture the highly hydrophilic CYN have not been developed. Similar devices (semi-permeable membranes) have been successfully used for pesticide and other pollutant monitoring (Petty *et al.* 2000). These samplers are useful because they are relatively cheap and avoid the difficulties of phytoplankton sampling, identification and enumeration (MacKenzie *et al.* 2004) and are capable of detecting pulse events that may be missed by intermittent sampling regimes. On the other hand, such devices are also retrospective: their main contribution to managing environmental effects may be simply to further understanding of ecological processes that occur during toxic blooms.

11.2.1.2 Bioindicators and biomarkers

Spirodela, *Hydrilla*, *Melanoides* and *Bufo* had different sensitivities when these species were exposed to toxin-containing *C. raciborskii*. This indicates that bioindicator species could be used to aid the detection and management of algal toxicity. Bioassays using *S. oligorrhiza* have been flagged for use in MC detection (Romanowska-Duda & Tarczynska 2002). However, inconsistent results were obtained from trials with *Spirodela* (chapter four): it therefore seems unlikely that this species could be confidently used to indicate toxin-producing *C. raciborskii* blooms.

B. marinus tadpoles appeared to be highly susceptible to CYN exposure; in particular, their histology provided consistent results and an easy comparison between CYN-exposed and control specimens. Unfortunately, several problems preclude *B. marinus* tadpoles being suitable for use as bioindicators in Australia: frog and toad species often exhibit highly variable susceptibilities to pollutants; the cane toad is not native to Australia; and tadpoles may be present only in selected water bodies, and only for a short period (Norris & Norris 1995; Cowman & Mazanti 2000).

The zooplankter *Daphnia* has been quite extensively studied for its responses to toxic algal blooms (Reinikainen *et al.* 1994; Hietala *et al.* 1997; Lauren-Maatta *et al.* 1997; Kyselková & Marsalek 2000; Tarczynska *et al.* 2001; Lürling 2003), though only one study has examined CYN toxicity (Nogueira *et al.* 2004a). *Daphnia*, or other zooplankton such as *Paramecium* (Fabbro *et al.* 2001), may therefore be promising as bioindicators of toxic *C. raciborskii* blooms. A successful bioassay for CYN has also been developed using *Artemia salina* (Metcalf *et al.* 2004), but brine shrimp are unlikely to coexist with freshwater *C. raciborskii* blooms.

The variable responses to *C. raciborskii* exposures recorded from the four test species suggests that using a set of representative organisms, rather than simply one species, may be more desirable to accurately evaluate ecosystem level effects caused by toxic algal blooms. The Australian River Assessment Scheme has adopted a similar approach for monitoring water quality, using groups of macroinvertebrates (Smith *et al.* 1999). However, to interpret such results accurately, large volumes of laboratory and field-based data are needed: this would allow responses to be assessed under varying water quality, water chemistry and temperature conditions.

Specific physiological responses are also being increasingly examined for use as biomarkers of algal toxicity (Handy & Depledge 1999). To date, studies of algal toxins have focussed on the potential use of biochemical responses; for example, protein synthesis inhibition assays (Froschio *et al.* 2001) and the presence of oxygen stress, detoxication or ion regulation enzymes (Fastner *et al.* 2001; Vinagre *et al.* 2002; Wiegand *et al.* 2002; Pflugmacher 2004). Presently, however, there is not enough known about the toxicity of CYN on aquatic organisms to identify a suitable biomarker. Future studies of biochemistry, histology and immunology may provide a reliable assay.

With respect to monitoring bioaccumulation, Zurawell (2001) noted the potential of freshwater Lymnaeids (gastropods) as bioindicator organisms for MC, since these can accumulate large amounts of toxins with no side effects and have been used as such successfully in heavy metal studies. A similar conclusion might be reached for *M. tuberculata*, since these survive CYN exposure and accumulate the toxin well (see

chapter seven). Furthermore, the species is relatively common, especially in central Queensland.

11.2.2 Setting guidelines

Many management and regulatory options relate to toxic algal bloom management. The nature of these and how best to apply them has already been comprehensively discussed with respect to human health protection (Chorus & Bartram 1999). However, given the range of toxic effects demonstrated in the previous chapters, management strategies now need to be evaluated for their relevance to ecosystem health risks. Many of the problems faced when trying to set suitable regulatory standards for ecological risks are the same as those facing the management of human risks. The main issues involved include setting guideline values or standards, choosing the values that such standards are based on, and ensuring these effectively cover all risk scenarios such as different producer species and different toxins, different water bodies and different target species.

11.2.2.1 Guidelines based on toxin concentrations

The existing WHO drinking water guidelines are based on toxin concentrations. Since toxin exposure concentration clearly influences the extent and nature of toxic response in aquatic organisms (as shown in previous chapters), it seems appropriate that environmental guidelines could also be based on toxin values. However, both human health guidelines (for example, WHO 2004) and environmental health guidelines (for example, ANZECC 2000) rely on huge quantities of laboratory and field-based ecotoxicity data. To date, few studies have examined the long-term impacts and potential cumulative effects associated with cyanoprokaryote blooms,

particularly with respect to CYN contamination. Moreover, derivation of drinking water guidelines usually involves calculation of tolerable daily intake (TDI) values for lifelong exposures: these must be calculated separately for different age (infant, adult) and exposure scenarios. How could TDIs be set for aquatic ecosystems that may contain tens or hundreds of different species, often with hugely different risk scenarios and life spans? This problem is highlighted by Duy *et al.* (2000), who calculated separate TDIs for CYN contamination of livestock drinking water: sixteen different derivations were completed and the consequent guideline values ranged from 0.6 – 9.1 $\mu\text{g L}^{-1}$.

Drinking water standards for MC are currently set using total MC values, as MC-LR equivalents. This is already causing problems since it now appears that MC-LR is not the most common, nor most potent, of the microcystin variants (Chorus 2005b). Thus, the possibility of more than one CYN analog must be considered (for example, CYN, deoxy-CYN), in addition to other toxins such as anatoxin (see 10.1.6). Furthermore, the present work with CYN has shown that toxin concentrations, particularly intracellular toxin, are critical in influencing toxic response and bioaccumulation potential associated with exposure. Since environmental risk scenarios may vary according to the relative abundance of CYN_{INC}: CYN_{EXC}, guideline values should not simply be based on total toxins (as currently done for drinking water), but on all available fractions. This, however, may result in standards that are costly to enforce. A similar problem exists with heavy metals, for example: only certain metal speciations represent risk, but trigger values are based on the sum of all chemical forms (total concentrations), since quantifying each separately would be too costly.

If environmental standards are set using toxin concentrations, repeated toxin testing may be required to ensure compliance. Since *Cylindrospermopsis* prefers low-light environments (Padisák 1997), there is considerable difficulty in visually detecting subsurface blooms (McGregor & Fabbro 2000). Occasional, random sampling is unlikely to capture the peaks and troughs in toxin concentrations throughout a bloom, particularly in the case of short bloom intervals. An additional problem is that official methods for CYN detection occur only in Australia, where HPLC-MS is currently the method of choice (Nicholson & Burch 2001; NHMRC & NRMCC 2004), although the New Zealand draft guidelines also nominate LC-MS for use (Wood & Holland 2005). Certified quantitative analytical standards do not currently exist, which prevents interlaboratory calibrations (Chorus 2005b). Nevertheless, it appears that currently used techniques are generally reproducible and comparable (Törökne *et al.* 2004). Lastly, the development of a reliable and accurate method suitable for the detection of bound toxin remains an important challenge in studies of algal toxin accumulation.

11.2.2.2 Guidelines based on cell concentrations

Cell concentrations (or cell biomass) are a valuable way to measure exposure risks associated with a bloom because these allow a rough assessment of toxicities related to *C. raciborskii* cell substances other than CYN (see 10.1.6). Since cell concentrations are necessary to determine cell quotas (Q_{CYNs}), they may also help determine the likely toxin loads ingested by grazer species. In the case of *Cylindrospermopsis*, however, basing an environmental standard solely on cell counts is not appropriate. Different strains of *C. raciborskii* can have very different toxicities

(Bernard *et al.* 2003; Fastner *et al.* 2003; Fergusson & Saint 2003; Saker *et al.* 2003); even genetically similar strains are known to express toxicity differently depending on nutrient availability and temperature (Saker *et al.* 1999; Saker & Griffiths 2000; Saker & Neilan 2001).

The relationship(s) between *C. raciborskii* cell concentrations and toxin concentration are not consistent (Eaglesham *et al.* 1999; McGregor & Fabbro 2000; Griffiths & Saker 2003). For example, McGregor and Fabbro (2000) suggested that 20,000 *C. raciborskii* cells mL⁻¹ could be considered roughly equivalent to 1 µg L⁻¹ CYN in Queensland lakes and reservoirs. However, cell count and toxin data provided for a different Queensland storage (Chiswell *et al.* 1999) agreed with this value on only two of eight occasions. The difference in these studies probably arose because toxin concentrations were measured during different bloom phases: the latter study measured toxin production over four months. However, even if a reliable toxin quota could be calculated per 'normal' *C. raciborskii* cell, cell counts would still omit the toxicity contributed by the extracellular toxin fraction, which may be significant (Griffiths & Saker 2003).

11.2.2.3 A possible solution

How can the extent of ecological risks posed by toxin-producing *Cylindrospermopsis* blooms be best measured? Measurement of the following factors is necessary to quantify environmental risks related to a *C. raciborskii* bloom: the concentration of CYN, the relative abundance of CYN_{INC}: CYN_{EXC}, the presence of analogs (deoxy-CYN) or other toxins (for example, saxitoxin); and the presence and concentration of any additional cellular substances (endotoxins, LPSs) within the cell biomass, and

how these vary with time. At present, quantification of cellular substances is best approached via measurements of cell concentrations or biovolume. A further issue is the aspect of exposure time, including how rapidly the blooms may flux between intracellular and extracellular toxin dominance. It therefore appears that management of ecological risks is likely to be best approached by combining standards used for both recreational (cell concentrations) and drinking water (toxin concentrations), and by applying these repeatedly during a bloom.

Calculating environmental risks could be done by combining an evaluation of both total toxin present and the number of toxin-producing cells (Table 11.2). The importance of CYN (particularly CYN_{INC}) in comparison to *C. raciborskii* cells could be highlighted via the use of a weighting factor. The particular significance of intracellular toxin is addressed by the addition of another risk factor, based on the % of CYN_{INC} in the bloom. Bloom density (and, by association, the likely extent of endotoxins or LPSs) is included by a third risk factor. Multiplication of the three factors A, B and C gives a final risk value (Table 11.2). Deoxy-CYN has not been addressed, since there are contrasting reports of its toxicity (Norris *et al.* 1999; Neumann *et al.* 2005).

Cell biomass or equivalent chlorophyll *a* could replace cell concentration data: these have been used in regulatory approaches in the past, but there is some doubt as to their accuracy, particularly where sample preservatives cause inaccurate measurements (Hawkins *et al.* 2005). In addition, the use of chlorophyll *a* units to characterise risks associated with a bloom must assume *C. raciborskii* dominance; and this may not always be the case. Furthermore, for a full estimation of ecological risks, an

Table 11.2 Possible method for calculating and interpreting an ecological risk value for blooms of *Cylindrospermopsis raciborskii*. Shaded cells show an example.

Toxin concentration ($\mu\text{g L}^{-1}$) CYN _{TOT}	Risk factor (A)
≤ 0.5	1
0.6 – 1	5
1.1 – 10	10
11 – 50	20
51 – 99	30
≥ 100	40
% of toxin as CYN _{INC}	Risk factor (B)
$\leq 25\%$	1
26 – 50 %	5
51 – 75%	10
$\geq 76\%$	20
<i>C. raciborskii</i> (cells mL ⁻¹) ^a	Risk factor (C)
$\leq 20,000$	1
20,001 – 50,000	2
50,001 – 100,000	3
100,001 – 200,000	4
$\geq 200,001$	5
FINAL RISK VALUE ((A*B*C)/100)	3
Final Risk value	Ecological Risk Rating
≤ 0.5	Negligible
0.6 – 1.0	Low
1.1 – 10.0	Medium
10.1 – 25.0	High
25.1 – 40	Extreme

^aor other measure of cellular mass such as biovolume or chlorophyll units (see text)

indication of water quality characteristics (pH, alkalinity, temperature) and potential synergies between toxins, cell components and other toxicants likely to be present in natural water bodies (pesticides, water quality) must also be addressed.

The values in this table are currently arbitrary, although the magnitude of each of the risk factors has some scientific basis. For example, as the results of previous chapters have shown, medium or high concentrations of CYN_{INC} (risk factors 10 – 20) may have equal importance to overall toxin concentrations (risk factors 10 – 40) with respect to final toxicities (Table 11.2). In comparison, the contribution of *C. raciborskii* cell masses to sublethal and lethal toxicities is likely to be comparatively

less (risk factors 1 – 5). The values have also been designed to ensure final risk values are practical, rather than large, unwieldy figures.

Once an ecological risk rating has been interpreted (Table 11.2), management actions could be triggered based on the severity of the environmental risk. This is where environment guidelines are confronted by a huge problem: guidelines are reactive in nature. Drinking water guidelines (in theory) work because they prevent unsuitable raw or treated water supplies reaching a human population. In contrast, in the natural environment, exposure occurs simultaneously with bloom development and the production of toxins: assigning a risk rating (Table 11.2) therefore means that effects are already occurring. What kind of action can be taken to reduce environmental risk? It is impractical (in most circumstances) to remove aquatic organisms from a water body in the event of a toxic bloom. However, there is still value in assigning an ‘ecological risk rating’ to a water body: benefits may include triggering further environmental studies or management actions and the forewarning of human consumers regarding edible species (crayfish, fish, mussels).

11.2.3 An alternative to guidelines

A relatively new approach to toxic algal bloom management, introduced by Chorus (2005b), is that known as a Water Safety Plan (WSP). This can be likened to the ‘hazard analysis and critical control point’ (HACCP) approach that has been adopted in many food industries with respect to controlling microbiological contamination. A management ‘loop’ enables users to define the problem, configure a solution and check the success of the solution.

Unlike standards or guidelines, which encourage compliance but not necessarily an understanding of hazards (Chorus 2005b), WPSs represent a wider approach that entices water managers to look at management approaches to minimise toxic bloom occurrences overall. Furthermore, since many of the factors contributing to toxic algal blooms relate to water quality (for example, nutrients), minimisation of such hazards is likely to reduce not only toxic blooms, but also other contributors to poor ecosystem health (such as eutrophication).

Despite their merits, WSPs are yet to be implemented for water bodies experiencing toxic algal blooms. A possible reason is that most other hazards (pesticides, microbial contaminants) are still managed via guideline compliance. It may be difficult to convert managers to the new approach, which may be more appropriate but also requires a greater time and funding commitment to achieve success. A further difficulty is finding suitable endpoints for determining success: are environmental risks managed successfully when there is an absence of toxicity, absence of blooms, or complete absence of blue-green species in the water column? Internal effect concentrations can link tissue concentrations with the extent of adverse effects, but these may be confounded by sex and age sensitivities, seasonal variation, nutritional and health status of organisms (Connell *et al.* 1999). Furthermore, in the case of CYN, the use of such endpoints is hampered by a lack of data. There is the potential, however, for ecological risk values to identify water bodies that could consider implementing WSPs, or other management options in the future.

11.2.4 Bloom control

In the short term, bloom prevention, control and treatment methods are likely to offer the most achievable outcomes in terms of minimising future environmental effects.

Numerous control and treatment options have been forwarded for effective management of toxic algal blooms (Carmichael & Falconer 1993; Chiswell *et al.* 1997; Fabbro *et al.* 2001; Senogles *et al.* 2001; Griffiths & Saker 2003; Haider *et al.* 2003; Falconer 2005). For example, blooms may be discouraged by destratification, release of stored waters, catchment management to reduce eutrophication (including via phosphorus removal), copper sulfating and grazing (biological control). Toxin removal may proceed via filtration, chlorination, activated carbon, ozonation, UV irradiation and photocatalysis. However, the efficacy of some of these methods is questionable depending on water chemistry (Sivonen & Jones 1999). The management of blooms using biomanipulation or top-down approaches has been mooted within the scientific literature but these seem generally focussed on changes to successional cycles and the removal of algal cells (Brock 1998; Meijer *et al.* 1999; Hehmann *et al.* 2001), not their toxins.

The treatment of CYN-containing blooms poses a special problem for water bodies that have dual purposes. For example, water storages may be important in providing both reliable drinking water supplies and habitats for ecologically or commercially important species (for example, fish nurseries). Currently, drinking water guidelines discourage the use of algicides (for example, copper sulfate) to control blooms, since these cause cell lysis and release of toxins. Extracellular toxins are more difficult to remove via water treatment processes such as flocculation and filtration (Hoeger *et al.* 2004). In fact, Hawkins *et al.* (2001) noted that blooms containing high levels of extracellular CYN pose the highest risk to (human) consumers.

In stark contrast, the present work has suggested that intracellular toxin fraction is the most dangerous in terms of environmental effects, because of the association with

uptake routes, especially grazing. Furthermore, there has been the suggestion, at least for MC, that some ‘product’ molecules are actually more toxic than their ‘parent’ molecules, once released from the cells (Jones & Orr 1994). Thus, minimisation of environmental risks could be achieved by treating an algal bloom with an appropriate algicide, thus allowing the toxin to convert to mostly extracellular fraction. However, algicide use is, in many other respects, an ecological disaster, and increases the cost associated with treating potable water supplies. Clearly, this is one example where management of human health risks and environmental health risks collide.

11.2.5 Remedial measures

Mitigation of toxic blooms in regard to human health effects have already been extensively discussed in Falconer (2005) and in various chapters within Chorus and Bartram (1999). Strategies that minimise future environmental risks, however, are more difficult to identify. For example, if algal blooms are capable of causing losses in biodiversity, species abundance and species richness, the reintroduction of susceptible species (for example, restocking of fish populations) could be required after a toxic bloom. To minimise further environmental problems caused by toxin bioaccumulation and trophic transfer, removal of contaminated species may be needed.

11.3 Special focus: management of bioaccumulation

11.3.1 The predictive model revisited

The predictive management model (chapter two) examined the bioavailability of different toxin fractions (CYN_{INC} , CYN_{EXC}) to different food web compartments (producers, grazers, predators) so that bioaccumulation risk could be assessed during

toxic algal blooms. The model may also be used with respect to human health risk assessment, particularly for decision-making regarding the suitability of aquatic organisms for consumption. Whilst the predictive model lacks the complexity of other commonly used predictive approaches (for example, quantitative structure activity relationships and other mechanistic models), it also lacks specificity and thus can be used in any food web to characterize risk to any component of an aquatic system, from grazers to top-level predators.

The three main issues addressed by the model were fraction availability, toxin thresholds and length of exposure. The results of the laboratory trials appeared to validate the assumptions made in the model. Fraction availability (*C. raciborskii* whole cell extracts compared with live cultures) affected the extent of toxic effects and the potential for bioaccumulation in both *Melanoides* snails and *Bufo* tadpoles. Despite this, changes in the relative abundance of CYN_{INC} and CYN_{EXC} during natural *C. raciborskii* blooms have yet to be closely studied.

Toxin threshold values and caution factors were proposed as a method by which bioaccumulation risk could be determined for different aquatic species. The potential for toxin accumulation was assumed to be variable between different organisms, according to their toxin regulation capabilities. The laboratory work partly supported this argument, since different BCFs/BAFs were recorded from all four of the test species, even though each were tested at similar exposure scenarios (0 – 400 µg L⁻¹). However, this does not necessarily confirm that certain species have regulatory capacities, since they may also use different uptake pathways for toxin.

11.3.2 A revised table for calculating bioaccumulation risk

Toxin exposure concentrations and the proportions of CYN_{INC} or CYN_{EXC} fractions are generally likely to influence tissue toxin accumulation in aquatic organisms, although the exact relationships between these are not yet clear (see 10.2.2). When the model was originally proposed, the influence of toxin fractions and total toxin concentrations on bioaccumulation was anticipated. This resulted in the use of separate intracellular and extracellular thresholds being set in the risk prediction table (Table 2.1). However, that calculation table can now be simplified: data for the four test species studied in the previous chapters has been entered below (Table 11.3). This has been done whilst acknowledging that data from some of the tests were highly variable: nevertheless, this is the only information available.

Table 11.3 Revised table for calculating cylindrospermopsin bioaccumulation risk in the four test species. Note: original table with arbitrary values was provided in chapter two. CYN_{TOT} = total cylindrospermopsin; CYN_{INC} = intracellular cylindrospermopsin.

Species	Exposure route(s)	CYN _{TOT} threshold ^a
Floating aquatic plant <i>Spirodela oligorrhiza</i>	Transdermal	> 500 µg L ⁻¹
Submerged aquatic plant <i>Hydrilla verticillata</i>	Transdermal	> 400 µg L ⁻¹
Herbivorous snail <i>Melanoides tuberculata</i>	Oral and transdermal ^b	Threshold A: 200 µg L ^{-1c} Threshold B: < 91 µg L ⁻¹
Omnivorous tadpole <i>Bufo marinus</i>	Oral and transdermal ^b	Threshold A: ≥ 400 µg L ⁻¹ Threshold B: < 31 µg L ⁻¹

^aapply threshold A where CYN_{INC} represents < 15% of total toxin concentrations and apply threshold B where CYN_{INC} exceeds 15% of total toxin concentrations; ^bsince bioconcentration values were less than bioaccumulation values it appears that uptake via this route is minimal; ^cbioconcentration barely recorded for 200 and 400 µg L⁻¹ exposures (see table 7.12).

Since bioconcentration did not occur during any of the experimental trials with the aquatic macrophytes (*Spirodela*, *Hydrilla*), the thresholds for the aquatic plants are set at the highest toxin concentrations that were tested. However, this assumes HPLC analysis could detect any tissue toxins. It is possible that CYN bioconcentration in aquatic plants occurs at lower exposure concentrations, but that this results in

modified or bound forms of CYN being stored in the plant tissues, which cannot be detected. It also assumes that intracellular toxin availability has no influence on the bioconcentration of CYN in aquatic plants.

The extent of tissue toxin accumulation in both the aquatic snails and tadpoles appears likely to be dependent on the percentage of toxin present in the intracellular form. Both species recorded higher BAFs than BCFs, even though similar total toxin concentrations were available during ‘whole cell extract’ and ‘live culture’ trials. Two thresholds have thus been calculated: one for blooms that contain little or no CYN_{INC}, and those where intracellular toxin represents a significant fraction of total toxin. Each threshold has been set at the lowest experimental treatment concentration from which bioconcentration or bioaccumulation was recorded (Table 11.3). The percentage of intracellular toxin deemed suitable to cause a switch to threshold B was also the lowest reported from the studies (15%, for *M. tuberculata*), but may change depending on data collected in future studies.

To simplify the process further, caution factors can be removed entirely, since it appears that species with multiple uptake routes (for example, transdermal plus grazing) depend principally on the presence of intracellular toxin. For example, snails were capable of both CYN bioconcentration and bioaccumulation, but toxins gathered via the grazing route probably far outweighed any contribution by transdermal uptake.

Apart from changes to the format of the risk prediction table, most of the stepwise framework (proposed in chapter two) can be retained. The contribution of cell quotas in influencing grazing, toxin uptake and toxin accumulation, is not clear (see

10.1.1.1). Saker *et al.* (2004) noted that both exposure time and exposure concentration could be used to predict the amount of CYN present in *Anodonta*. However, naturally high individual variability between organisms limits such predictions. For example, in grazing species such as *M. tuberculata*, different grazing rates and habitats might cause large variations in tissue toxin concentrations. Cell quotas may help to remove some of this variation, and future improvements to the model could include exposure thresholds for Q_{CYNs} . Should these need to be incorporated into the model in future, this could easily be done at steps four and five: a cell count could be performed as an adjunct to toxin testing (step four), and Q_{CYNs} could be calculated from CYN_{INC} toxin concentrations during step five.

11.4 Where to from here?

Recently, Chorus (2005b) pointed out that whilst the management of water supplies in regard to algal toxins may have increased the suitability of drinking water globally, it has also resulted in an ever-growing list of pollutants and organisms that must be eliminated for standards to be met. The introduction of environmental standards faces a similar problem. Trying to eliminate all environmental risks associated with toxic blooms is not viable in the short term; at regional, national or international scales. Some could argue that blooms are natural occurrences and hence their toxic effects are natural. Accordingly, management approach could instead focus on reducing the factors that contribute to the ‘unnatural’ frequency in the occurrence and toxicity of blooms, such as global warming and the ongoing trend for eutrophication of water storages (Garnett *et al.* 2003).

The management of ecological risks is a complex issue that requires proactive, not reactive, strategies to be effective. Future studies should focus on providing the correct information needed to progress these guidelines for environmental protection of water bodies affected by toxic *C. raciborskii* blooms. Guidelines for environmental risks should combine both cell concentrations and toxin concentrations. However, these are only effective where efficient and timely bloom control strategies are in place. The real value of such guidelines may be to trigger the use of wider approaches, such as WSP, to safeguard water bodies against future blooms. Given the lack of data regarding long-term effects of toxic algal blooms on ecosystem health, and the current focus on resolving issues regarding public health, it may be some time before regulatory standards address problems of ecosystem health risks. However, appropriate environmental management of CYN cannot occur until two issues are resolved: one, ways to monitor both intracellular and extracellular fractions of toxins throughout a bloom; and two, to develop the ability to detect bound toxins present within the tissues of aquatic organisms, so that the full extent of toxin accumulation can be determined.

Chapter twelve: conclusions and areas for further study

12.1 Research summary

This research has described the toxic effects and bioaccumulation in four aquatic species exposed to environmentally relevant concentrations of CYN. The core research questions posed in the review of literature and in the development of the management model have all been addressed (Table 12.1). Each of the test species recorded different responses to CYN exposure, including differences between toxin administered with *C. raciborskii* whole cell extracts and with live *C. raciborskii* cultures. Several influences on the toxicity of CYN were identified, including different exposure regimes, different uptake modes, and the ability of individuals to metabolise toxin. Toxin accumulation was shown in only two of the four test species (Table 12.1), although the limitations of the HPLC technique may have prevented toxin detection in the aquatic plants.

This study has demonstrated that it is likely that toxin-producing *C. raciborskii* blooms pose multiple risks to populations of aquatic macrophytes, macroinvertebrates and vertebrates, including the threat of toxin bioaccumulation. The final research question, ‘how can bioaccumulation of cylindrospermopsin in tropical freshwater systems be best managed?’ was considered in chapters two and eleven. Since CYN production is most commonly known from *C. raciborskii*, an understanding of the biology and ecology of this species is essential to proper management of toxic blooms. Furthermore, a predictive management approach appears to be most suited to minimising environmental effects and bioaccumulation.

Table 12.1 Consideration of core research questions (experimental hypotheses). CYN = cylindrospermopsin; INC = intracellular; EXC = extracellular.

Hypotheses tested	<i>S. oligorrhiza</i>	<i>H. verticillata</i>	<i>M. tuberculata</i>	<i>B. marinus</i>
Arising from the review of literature:				
1. H ₀ : CYN does not bioconcentrate in any of the test species	Accept	Accept	Reject	Accept
H ₀ : CYN does not bioaccumulate in any of the test species	Not applicable	Not applicable	Reject	Reject
2. H ₀ : Lethal effects do not occur in conjunction with CYN exposure and bioaccumulation ^a	Accept	Accept	Accept	Reject
H ₀ : Sublethal effects do not occur in conjunction with CYN exposure and bioaccumulation ^a	Not clear	Not clear	Not clear ^b	Reject
3. H ₀ : Changes in exposure regimes (toxin concentration, time) have no impact on adverse effects	Reject	Reject	Reject ^d	Reject
H ₀ : Changes in exposure regimes (toxin concentration, time) have no effect on bioaccumulation ^a	Not applicable ^c	Not applicable ^c	Reject	Reject
Arising from management model analyses:				
1. H ₀ : CYN uptake will be identical at identical toxin exposure concentrations, regardless of relative abundance of CYN _{INC} or CYN _{EXC} fractions	Not tested	Not tested	Reject	Reject

^abioconcentration for aquatic macrophytes; ^bminimal effects apparent in adult snails, but hatchlings may have been affected; ^cbioconcentration was not present in any treatment/time combination; ^dpattern of hatchling release differed between whole cell extract trials and the live trial.

12.2 Areas for further study

Increases in the frequency and toxicity of blue-green algal blooms are being reported on a global basis. This situation may have arisen from better monitoring and reporting efforts (Ressom *et al.* 1994), global warming (Garnett *et al.* 2003; Briand *et al.* 2004), habitat adaptation by certain species (Briand *et al.* 2004), and/or the ongoing eutrophication of surface waters on a worldwide scale (Chorus 2005b). Nevertheless, understanding toxic blue-green algal blooms is clearly important to the ongoing management of surface water resources worldwide.

Further work is still required to broaden our understanding of the ecology and toxicity of *C. raciborskii* and its toxin, CYN (including deoxy-CYN). The following specific studies are recommended:

1. optimisation of current detection techniques to enable the detection of bound or metabolically changed toxin (CYN, deoxy-CYN) within the tissues of aquatic organisms, and radiolabelling studies to examine toxin uptake;
2. confirmation of laboratory studies (the present work) with field investigations, with respect to both ecotoxicity and bioaccumulation, and/or further laboratory work examining other strains of *C. raciborskii*, including non-toxin producing varieties;
3. investigations of the triggers for, and influences on, toxin production by *C. raciborskii*, especially with respect to the relative abundances of extracellular and intracellular toxin fractions; and
4. further comprehensive studies investigating dose-response curves in aquatic organisms, including whole-of-life studies and/or long-term studies examining

intergenerational effects, to determine effects of repeated exposures to toxic blooms.

Other, less urgent studies may also include:

5. the identification of species or cellular processes that show potential for use as bioindicators or biomarkers of toxic *C. raciborskii* blooms;
6. investigations of histological and immunological effects resulting from toxicity, using environmentally relevant exposure scenarios;
7. depuration studies, particularly with respect to treating of tissues prior to human consumption; and
8. descriptions of the toxicity of deoxy-CYN, given that conflicting reports currently exist in the scientific literature.

Chapter thirteen: references cited

- Abe T., Lawson T., Weyers J.D.B., Codd G.A., 1996. Microcystin-LR inhibits photosynthesis of *Phaseolus vulgaris* primary leaves: implications for current spray irrigation practice. *New Phytologist* 133, 651-658.
- Aboal M., Puig M.-A., 2005. Intracellular and dissolved microcystin in reservoirs of the river Segura basin, Murcia, SE Spain. *Toxicon* 45, 509-518.
- Amorim A., Vasconcelos V.M., 1999. Dynamics of microcystins in the mussel *Mytilus galloprovincialis*. *Toxicon* 37, 1041-1052.
- ANZECC, 2000. Australian and New Zealand guidelines for fresh and marine water quality, Australian and New Zealand Conservation Council, Canberra.
- Anzenbacher P., Anzenbacherová E., 2001. Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences* 58, 737-747.
- ASTM (American Society for Testing and Materials) 2003. E 1192-97 Standard guide for conducting tests on aqueous effluents with fishes, macroinvertebrates, and amphibians. American Society for Testing and Materials International, West Conshohocken, Philadelphia.
- Aston H., 1973. Aquatic plants of Australia: a guide to the identification of aquatic ferns and flowering plants of Australia, both native and naturalized, Melbourne University Press, Carlton.
- Azevedo S.M., 2005. 'Management and regulatory approaches for cyanobacteria and cyanotoxins' in I. Chorus, (ed.). Current approaches to cyanotoxin risk assessment, risk management and regulations in different countries, WaBoLu 02/05, Federal Environment Agency, Umweltbundesamt, Dessau, Germany.
- Azevedo S.M.F.O., Carmichael W.W., Jochimsen E.M., Rinehart K.L., Lau S., Shaw G.R., Eaglesham G.K., 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. *Toxicology* 181-182, 441-446.
- Babcock-Jackson L., 2000. Toxic *Microcystis* in Western Lake Erie: Ecotoxicological relationships with three non-indigenous species increase risks to the aquatic community. Ph D thesis. Graduate School, Ohio State University, Ohio.
- Babica P., Bláha L., Maršálek B., 2006. Exploring the natural role of microcystins - a review of effects on photoautotrophic organisms. *Journal of Phycology* 42, 9-20.
- Baker M.L., 1998. Development of immunoassays for the detection of cylindrospermopsin, produced by *Cylindrospermopsis raciborskii*. Master of Applied Science Thesis. Chemistry Department, Central Queensland University, Rockhampton.

- Baker P.D., 1996. Occurrence of *Cylindrospermopsis* in South-Eastern Australia. *Cylindrospermopsis* - A new toxic algal bloom challenge for Australia, Brisbane, Agricultural and Resource Management Council of Australia and New Zealand, Canberra.
- Baker P.D., Humpage A.R., 1994. Toxicity associated with commonly occurring cyanobacteria in surface waters of the Murray-Darling Basin, Australia. *Australian Journal of Marine and Freshwater Research* 45, 773-786.
- Banker R., Carmeli S., Werman M., Telsch B., Porat R., Sukenik A., 2001. Uracil moiety is required for toxicity of the cyanobacterial hepatotoxin cylindrospermopsin. *Journal of Toxicology and Environmental Health Part A* 62, 281-288.
- Bartram J., Vapnek J.C., Jones, G. J., Bowling L., Falconer I., Codd G.A. (1999). 'Chapter 7. Implementation of management plans' in I. Chorus, J. Bartram, (eds) Toxic Cyanobacteria in Water. A guide to their public health consequences, monitoring and management. E and FN Spoon (on behalf of the World Health Organization), London, pp. 179-209.
- Beasley V.R., Cook W.O., Dahlem A.M., Hooser S.B., Lovell R.A., Valentine W.M., 1989. Algae intoxication in livestock and waterfowl. *Veterinary Clinics of North America: Food Animal Practice* 5, 345-361.
- Beattie K.A., Ressler J., Wiegand C., Krause E., Codd G.A., Steinberg C., Pflugmacher S., 2003. Comparative effects and metabolism of two microcystins and nodularin in the brine shrimp *Artemia salina*. *Aquatic Toxicology* 62, 219-226.
- Beek B., Böhling S., Bruckmann U., Franke C., Jöhncke U., Studinger G., 2000. 'The assessment of bioaccumulation' in B. Beek, (ed.). Bioaccumulation new aspects and developments. Berlin, Springer.
- Bengtsson B., Bongo J.P., Eklund B., 1999. Assessment of duckweed *Lemna aequinoctialis* as a toxicological bioassay for tropical environments in developing countries. *Ambio* 28, 152-155.
- Berg K., Skulberg O.M., Skulberg R., 1987. Effects of decaying toxic blue-green algae on water quality - a laboratory study. *Archiv für Hydrobiologie* 108, 549-563.
- Bernard C., Harvey M., Biré R., Krys S., Fontaine J.J., 2003. Toxicological comparison of diverse *Cylindrospermopsis raciborskii* strains: evidence of liver damage caused by a French *C. raciborskii* strain. *Environmental Toxicology* 18, 176-186.
- Best J.H., Pflugmacher S., Wiegand C., Eddy F.B., Metcalf J.S., Codd G.A., 2002. Effects of enteric bacterial and cyanobacterial lipopolysaccharide, and of microcystin-LR, on glutathione S-transferase activities in zebra fish (*Danio rerio*). *Aquatic Toxicology* 60, 223-231.

- Bickel H., Lyck S., Utkilen H., 2000. Energy state and toxin content - experiments on *Microcystis aeruginosa* (Chroococcales, Cyanophyta). *Phycologia* 39, 212-218.
- Bischoff K., 2001. The toxicology of microcystin-LR: occurrence, toxicokinetics, toxicodynamics, diagnosis and treatment. *Veterinary and Human Toxicology* 43, 294-297.
- Bishop C.A., Martinovic B., 2000. 'Guidelines and procedures for toxicological field investigations using amphibians and reptiles' in D. W. Sparling, G. Linder, C. A. Bishop., (eds). *Ecotoxicology of amphibians and reptiles*. Society for Environmental Toxicology and Chemistry, Pensacola, Florida, pp. 697-725.
- Boon P.I., Bunn S.E., Green J.D., Shiel R.J., 1994. Consumption of cyanobacteria by freshwater zooplankton: implications for the success of 'top-down' control of cyanobacterial blooms in Australia. *Australian Journal of Marine and Freshwater Research* 45, 875-887.
- Bormans M., Ford P.W., Fabbro L.D., Hancock G., 2004. Onset and persistence of cyanobacterial blooms in a large impounded tropical river, Australia. *Marine and Freshwater Research* 55, 1-15.
- Bouaïcha N., Nasri A.-B., 2004. First report of cyanobacterium *Cylindrospermopsis raciborskii* from Algerian freshwaters. *Environmental Toxicology* 19, 541-543.
- Bouvy M., Falcão D., Marinho M., Pagano M., Moura A., 2000. Occurrence of *Cylindrospermopsis* (Cyanobacteria) in 39 Brazilian tropical reservoirs during the 1998 drought. *Aquatic Microbial Ecology* 23, 13-27.
- Bouvy M., Pagano M., Troussellier M., 2001. Effects of a cyanobacterial bloom (*Cylindrospermopsis raciborskii*) on bacteria and zooplankton communities in Ingazeira reservoir (northeast Brazil). *Aquatic Microbial Ecology* 25, 215-227.
- Bowling L., 1994. 'Occurrence and possible causes of a severe cyanobacterial bloom in Lake Cargelligo, New South Wales' in G. Jones, (ed.). *Cyanobacterial research in Australia*, *Australian Journal of Marine and Freshwater Research* 45, 737-745.
- Branco C.W., Senna P.A., 1994. Factors influencing the development of *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa* in the Paranoá Reservoir, Brasília, Brazil. *Algological Studies* 75, 85-96.
- Briand J.F., Robillot C., Quiblier-Llobéras C., Humbert J.F., Couté A., Bernard C., 2002. Environmental context of *Cylindrospermopsis raciborskii* (Cyanobacteria) blooms in a shallow pond in France. *Water Research* 36, 3183-3192.
- Briand J.F., Leboulanger C., Humbert J.-F., Bernard C., Dufour P., 2004. *Cylindrospermopsis raciborskii* (Cyanobacteria) Invasion at Mid-Latitudes: selection; wide physiological tolerance, or global warming? *Journal of Phycology* 40, 231-238.

- Bridges C.M., 1999. Effects of a pesticide on tadpole activity and predator avoidance behaviour. *Journal of Herpetology* 33, 303-306.
- Bridges C.M., 2000. Long-term effects of pesticide exposure at various life stages of the Southern Leopard Frog (*Rana sphenoccephala*). *Archives of Environmental Contamination and Toxicology* 39, 91-96.
- Brock M.A., 1998. Biomanipulation as a potential control of algal blooms: 'whole lake' manipulation of water regime to promote or reduce macrophytes. Research report. Department of Botany, University of New England, Armidale, NSW.
- Bumke-Vogt C., Mailahn W., Rotard W., Chorus I., 1996. A highly sensitive analytical method for the neurotoxin anatoxin-a using GC-ECD, and first application to laboratory cultures. *Phycologia* 35, 51-56.
- Burch J.B., 1982. Freshwater Snails (Mollusca: Gastropoda) of North America, United States Environmental Protection Agency, Cincinnati, Ohio.
- Burch M., Humpage A.R., 2005. 'Australia: regulation and management of cyanobacteria' in I. Chorus, (ed.). Current approaches to cyanotoxin risk assessment, risk management and regulations in different countries, WaBoLu 02/05, Federal Environment Agency, Umweltbundesamt, Dessau, Germany.
- Burkhard L.P., Cook P.M., Mount D.R., 2003. The relationship of bioaccumulative chemicals in water and sediment to residues in fish: a visualisation approach. *Environmental Toxicology and Chemistry* 22, 2822-2830.
- Burns J., 2005. 'United States of America: Cyanobacteria and the status of regulatory approaches' in I. Chorus, (ed.). Current approaches to cyanotoxin risk assessment, risk management and regulations in different countries, WaBoLu 02/05, Federal Environment Agency, Umweltbundesamt, Dessau, Germany.
- Burris J.A., Bamford M.S., Stewart A.J., 1990. Behavioural responses of marked snails as indicators of water quality. *Environmental Toxicology and Chemistry* 9, 69-76.
- Bury N.R., Eddy F.B., Codd G.A., 1995. The effects of the cyanobacterium *Microcystis aeruginosa*, the cyanobacterial hepatotoxin microcystin-LR and ammonia on growth rate and ionic regulation of brown trout. *Journal of Fish Biology* 46, 1042-1054.
- Byl T.D., Sutton D.L., Klaine S.J., 1994. Evaluation of peroxidase as a biochemical indicator of toxic chemical exposure in the aquatic plant *Hydrilla verticillata*, Royle. *Environmental Toxicology and Chemistry* 13, 509-515.
- Byth S., 1980. Palm Island Mystery Disease. *Medical Journal of Australia* 2, 40-42.

- Campbell M.M., White K.N., Jugdaohsingh R., Powell J.J., McCrohan C.R., 2000. Effect of aluminum and silicic acid on the behaviour of the freshwater snail *Lymnaea stagnalis*. *Canadian Journal of Fisheries and Aquatic Sciences* 57, 1151-1159.
- Carbis C.R., Rawlin G.T., Grant P., Mitchell G.F., Anderson J.W., McCauley I., 1997. A study of feral carp, *Cyprinus carpio* L., exposed to *Microcystis aeruginosa* at Lake Mokoan, Australia, and possible implications for fish health. *Journal of Fish Diseases* 20, 81-91.
- Carmichael W.W., 1996. 'Toxic *Microcystis* and the environment' in M. Watanabe, K. I. Harada, W. W. Carmichael, H. Fujiki, (eds). *Toxic Microcystis*. CRC Press, Boca Raton, pp. 1-12.
- Carmichael W.W., 2001a. Health effects of toxin-producing cyanobacteria: 'The CyanoHABs'. *Human and Ecological Risk Assessment* 7, 1393-1407.
- Carmichael W.W., 2001b. The Cyanotoxins - Bioactive metabolites of cyanobacteria: occurrence, ecological Role, taxonomic concerns and effects on humans. *Journal of Phycology* 37, 9.
- Carmichael W.W., Azevedo S.M., Molica R., Jochimsen E.M., Lau S., Rinehart K.L., Shaw G., Eaglesham G., 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environmental Health Perspectives* 109, 663-668.
- Carmichael W.W., Falconer I.R., 1993. 'Diseases related to freshwater blue-green algal toxins, and control measures' in I. R. Falconer, (ed.). *Algal toxins in seafood and drinking water*. Academic Press, Sydney, pp. 187-209.
- Carson B., 2000. Cylindrospermopsin - A review of toxicological literature. National Institute of Environmental Health Services, North Carolina, USA.
- Casanova M.T., Douglas-Hill A., Brock M.A., Muschal M., Bales M., 1997. Farm ponds in New South Wales, Australia: relationship between macrophyte and phytoplankton abundances. *Marine and Freshwater Research* 48, 353-60.
- Casanova M.T., Burch M.D., Brock M.A., Bond P.M., 1999. Does toxic *Microcystis aeruginosa* affect aquatic plant establishment? *Environmental Toxicology* 14, 97-109.
- Castro D., Vera D., Lagos N., García C., Vásquez M., 2004. The effect of temperature on growth and production of paralytic shellfish poisoning toxins by the cyanobacterium *Cylindrospermopsis raciborskii* C10. *Toxicon* 44, 483-489.
- Cedergreen N., Madsen T.V., 2002. Nitrogen uptake by the floating macrophyte *Lemna minor*. *New Phytologist* 155, 285-292.

- Chapman A.D., Schelske C.L., 1997. Recent appearance of *Cylindrospermopsis* (cyanobacteria) in five hypereutrophic Florida lakes. *Journal of Phycology* 33, 191-195.
- Chen J., Xie P., 2005. Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in two freshwater shrimps, *Palaemon modestus* and *Macrobrachium nipponensis*, from a large, shallow, eutrophic lake of the subtropical China. *Toxicon* 45, 615-625.
- Chiswell R.K., Smith M., Norris R., Eaglesham G., Shaw G., Seawright A.A., Moore M., 1997. The cyanobacterium, *Cylindrospermopsis raciborskii*, and its related toxin, cylindrospermopsin. *Australasian Journal of Ecotoxicology* 3, 7-23.
- Chiswell R.K., Shaw G.R., Eaglesham G., Smith M.J., Norris K.R., Seawright A.A., Moore M.R., 1999. Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: effect of pH, temperature and sunlight on decomposition. *Environmental Toxicology* 14, 155-161.
- Chong M.W.K., Wong B.S.F., Lam P.K.S., Shaw G.R., Seawright A.A., 2002. Toxicity and uptake mechanism of cylindrospermopsin and lophytotoxin in primary rat hepatocytes. *Toxicon* 40, 205-211.
- Chonudomkul D., Yongmanitchai W., Theeragool G., Kawachi M., Kasai F., Kaya K., Watanabe M.M., 2004. Morphology, genetic diversity, temperature tolerance, and toxicity of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) strains from Thailand and Japan. *FEMS Microbiology Ecology* 48, 345-355.
- Chorus I., (ed.) 2001. Cyanotoxins occurrence, causes, consequences. Springer-Verlag, Berlin.
- Chorus I., (ed.) 2005a. Current approaches to cyanotoxin risk assessment, risk management and regulations in different countries. Publication WaBoLu 02/05. Federal Environmental Agency, Umweltbundesamt, Dessau, Germany.
- Chorus I., 2005b. 'Water Safety Plans A better regulatory approach to prevent human exposure to harmful cyanobacteria' in J. Huisman, H. C. P. Matthijs, P. M. Visser, (eds). Harmful Cyanobacteria. Dordrecht, Springer, The Netherlands, pp. 201-226.
- Chorus I., Bartram J., (eds). 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. Published on behalf of the World Health Organisation by E & FN Spon, London.
- Chorus I., Neisel V., Fastner J., Wiedner C., Nixdorf B., Lindenschmidt K., 2001. 'Environmental factors and microcystin levels in waterbodies' in I. Chorus, (ed.). Cyanotoxins occurrence, causes and consequences. Springer-Verlag, Berlin, pp. 159-177.
- Christoffersen K., 1996. Ecological implications of cyanobacterial toxins in aquatic food webs. *Phycologia* 35, 42-50.

- Clements W.H., Newman M.C., 2002. Community ecotoxicology, John Wiley & Sons, Chichester.
- Codd G.A., 1995. Cyanobacterial toxins: occurrence, properties and biological significance. *Water Science Technology* 32, 149-156.
- Codd G.A., 2001. Cyanobacterial toxins: their actions and multiple fates in microbes, animals and plants. *Journal of Phycology* 37, 13.
- Codd G.A., Bell S.G., Kaya K., Ward C.J., Beattie K.A., Metcalf J.S., 1999a. Cyanobacterial toxins, exposure routes and human health. *European Journal of Phycology* 34, 405-15.
- Codd G.A., Metcalf J.S., Beattie K.A., 1999b. Retention of *Microcystis aeruginosa* and microcystin by salad lettuce (*Lactuca sativa*) after spray irrigation with water containing cyanobacteria. *Toxicon* 37, 1181-1185.
- Codd G.A., Lindsay J., Young F., Morrison L.F., Metcalf J.S., 2005a. 'Harmful cyanobacteria from mass mortalities to management measures' in J. Huisman, H. C. P. Matthijs, P. M. Visser, (eds). Harmful Cyanobacteria. Springer, Dordrecht, The Netherlands, pp. 1-23.
- Codd G.A., Morrison L.F., Metcalf J.S., 2005b. Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology* 203, 264-272.
- Cohen Z., 1997. 'The Chemicals of *Spirulina*' in A. Vonshak, (ed.). *Spirulina platensis (Arthrospira): Physiology, cell biology and biotechnology*. Taylor and Francis, London, pp. 175-204.
- Connell D.W., Chaisuksant Y., Yu J., 1999. Importance of internal biotic concentrations in risk evaluations with aquatic systems. *Marine Pollution Bulletin* 39, 54-61.
- Cowman D.F., Mazanti L.E., 2000. 'Ecotoxicology of "new generation" pesticides to amphibians' in D. W. Sparling, G. Linder, C. A. Bishop, (eds). Ecotoxicology of amphibians and reptiles. Society for Environmental Toxicology and Chemistry, Pensacola, Florida, pp. 233-268.
- Crossland M.R., 1998. A comparison of cane toad and native tadpoles as predators of native anuran eggs, hatchling and larvae. *Wildlife Research* 25, 373-381.
- Cyanosite, 2005, <http://www-cyanosite.bio.purdue.edu/cyanotox/toxins/cylindro.html>, accessed on 6th September 2005.
- Dawson R.M., 1998. The toxicology of microcystins. *Toxicon* 37, 953-962.

- De With N.D., 1996. Oral water ingestion in the pulmonate freshwater snail *Lymnaea stagnalis*. *Journal of Comparative Physiology - Part B Biochemical, Systematic and Environmental Physiology* 166, 337-343.
- DeMott W.R., 1999. Foraging strategies and growth inhibition in five daphnids feeding on mixtures of a toxic cyanobacterium and a green alga. *Freshwater Biology* 42, 263-274.
- Dietrich D.R., 2005. Risk cyanobacterial toxins: occurrence, ecology, detection, toxicology, and health effects assessment. *Toxicology and Applied Pharmacology* 203, 191.
- Dillon R.T., 2000. The ecology of freshwater molluscs, Cambridge University Press, Cambridge, UK.
- Dittman E., Wiegand C., 2006. Cyanobacterial toxins - occurrence, biosynthesis and impact on human affairs. *Molecular nutrition and food research* 50, 7-17.
- Duy T.N., Lam P.K.S., Shaw G.R., Connell D.W., 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. *Review of Environmental Contamination and Toxicology* 163, 113-186.
- Dvoráková D., Dvoráková K., Bláha L., Maršálek B., Knotková Z., 2002. Effects of cyanobacterial biomass and purified microcystins on malformations in *Xenopus laevis*: teratogenesis assay (FETAX). *Environmental Toxicology* 17, 547-555.
- Eaglesham G., Norris K.R., Shaw G.R., Smith M.J., Chiswell R.K., Davis B.C., Neville G.R., Seawright A.A., Moore B.S., 1999. Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. *Environmental Toxicology* 14, 151-154.
- Eberuis M., Mennicken G., Reuter I., Vandenhirtz J., 2002. Sensitivity of different growth inhibition tests - just a question of mathematical calculation? *Ecotoxicology* 11, 293-297.
- Ellis-Tabanor M., Hyslop E., 2005. Effect of sublethal concentrations of endosulfan on growth and fecundity of two species of snails. *Bulletin of Environmental Contamination and Toxicology* 74, 1173-1178.
- Entzeroth M., Mead D.J., Patterson G.M.L., Moore R.E., 1985. A herbicidal fatty acid produced by *Lyngbya aestuarii*. *Phytochemistry* 24, 2875-2876.
- Environmental Health Unit of Queensland Health, 2001. Environmental health assessment guidelines: cyanobacteria in recreational and drinking waters. Queensland Health, Brisbane.
- Eriksson J.E., Meriluoto J., Lindholm T., 1989. Accumulation of a peptide toxin from the cyanobacterium *Oscillatoria agardhii* in the freshwater mussel *Anadonta cygnea*. *Hydrobiologia* 183, 211-216.

- Fabbro L.D., 1999. Phytoplankton ecology in the Fitzroy River at Rockhampton, central Queensland. Ph D thesis. School of Biological and Environmental Sciences, Central Queensland University, Rockhampton, Australia.
- Fabbro L.D., Baker M., Duivenvoorden L.J., Pegg G., Shiel R., 2001. The effects of the ciliate *Paramecium* cf. *caudatum* Ehrenberg on toxin producing *Cylindrospermopsis* isolated from the Fitzroy River, Australia. *Environmental Toxicology* 16, 489-497.
- Fabbro L.D., Duivenvoorden L.J., 1996. Profile of a bloom of the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju in the Fitzroy River in tropical central Queensland. *Marine and Freshwater Research* 30, 579-595.
- Falconer I.R., 1993. 'Mechanism of toxicity of cyclic peptide toxins from blue-green algae' in I. R. Falconer, (ed.) *Algal toxins in seafood and drinking water*. Academic Press, Sydney, pp. 177-186.
- Falconer I.R., 1999. An overview of problems caused by toxic blue-green algae (Cyanobacteria) in drinking and recreational water. *Environmental Toxicology* 14, 5-11.
- Falconer I.R., 2001. Toxic cyanobacterial bloom problems in Australian waters: Risks and impacts on human health. *Phycologia* 40, 228-233.
- Falconer I.R., 2005. Cyanobacterial toxins of drinking water supplies: cylindrospermopsins and microcystins, CRC Press, Boca Raton.
- Falconer I.R., Hardy S.J., Humpage A.R., Froscio S.M., Tozer G.J., Hawkins P.R., 1999. Hepatic and renal toxicity of the blue-green alga (Cyanobacterium) *Cylindrospermopsis raciborskii* in Male Swiss Albino Mice. *Environmental Toxicology* 14, 143-150.
- Falconer I.R., Humpage A.R., 2001. Preliminary evidence for *In Vivo* tumour initiation by oral administration of extracts of the blue green alga *Cylindrospermopsis raciborskii* containing the toxin cylindrospermopsin. *Environmental Toxicology* 16, 192-195.
- Fastner J., Wirsing B., Wiedner C., Heinze R., Neumann U., Chorus I., 2001. 'Microcystins and hepatocyte toxicity' in I. Chorus, (ed.). *Cyanotoxins occurrence, causes, consequences*. Springer-Verlag, Berlin.
- Fastner J., Heinze R., Humpage A.R., Mischke U., Eaglesham G.K., Chorus I., 2003. Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates. *Toxicon* 42, 313-321.
- Feijtel T., Kloepper-Sams P., den Haan K., van Egmond R., Comber M., Heusel R., P. W., Ten Berge W., Gard A., de Wolf W., Niessen H., 1997. Integration of

- bioaccumulation in an environmental risk assessment. *Chemosphere* 34, 2337-2350.
- Ferguson A.J.D., 1997. The role of modelling in the control of toxic blue-green algae. *Hydrobiologia* 349, 1-4.
- Fergusson K.M., Saint C.P., 2003. Multiplex PCR assay for *Cylindrospermopsis raciborskii* and cylindrospermopsin-producing cyanobacteria. *Environmental Toxicology* 18, 120-125.
- Ferrão-Filho A., Domingos P., Azevedo M.T.P., 2002a. Influences of a *Microcystis aeruginosa* Kützinger bloom on zooplankton populations in Jacarepaguá Lagoon (Rio de Janeiro, Brazil). *Limnologica* 32, 295-308.
- Ferrão-Filho A., Kozłowsky-Suzuki B., Azevedo S.M.F.O., 2002b. Accumulation of microcystins by a tropical zooplankton community. *Aquatic Toxicology* 59, 201-208.
- Ferrari L., Demichelis S.O., Garcia M.E., de la Torre F.R., Salibian A., 1997. Premetamorphic anuran tadpoles as test organism for an acute aquatic toxicity assay. *Environmental Toxicology and Water Quality* 12, 117-121.
- Fessard V., Bernard C., 2003. Cell alterations but no DNA strand breaks induced *in vitro* by cylindrospermopsin in CHO K1 cells. *Environmental Toxicology* 18, 353-359.
- Fischer W.J., Dietrich D.R., 2000. Toxicity of the cyanobacterial cyclic heptapeptide toxins microcystin-LR and -RR in early life-stages of the African clawed frog (*Xenopus laevis*). *Aquatic Toxicology* 49, 189-198.
- Fordham C.L., Tessari J.D., Ramsdell H.S., Keefe T.J., 2001. Effects of malathion on survival, growth, development, and equilibrium posture of bullfrog tadpoles (*Rana catesbeiana*). *Environmental Toxicology and Chemistry* 20, 179-184.
- Fowler J., Cohen L., Jarvis P., 1998. Practical statistics for field biologists, John Wiley & Sons, Chichester.
- Fraker S.L., Smith G.R., 2004. Direct and interactive effects of ecologically relevant concentrations of organic wastewater contaminants on *Rana pipiens* tadpoles. *Environmental Toxicology* 19, 250-256.
- Frankart C., Eullaffroy P., Vernet G., 2002. Photosynthetic responses of *Lemna minor* exposed to xenobiotics, copper, and their combinations. *Ecotoxicology and Environmental Safety* 53, 439-445.
- Franke C., 1996. How meaningful is the bioconcentration factor for risk assessment? *Chemosphere* 32, 1897-1905.
- Franke C., Studinger G., Berger G., Böhling S., Bruckman U., Cohors-Fresenborg D., Jöhncke U., 1994. The assessment of bioaccumulation. *Chemosphere* 29, 1501-1514.

- Froschio S.M., Humpage A.R., Burcham P.C., Falconer I.R., 2001. Cell-free protein synthesis inhibition assay for the cyanobacterial toxin cylindrospermopsin. *Environmental Toxicology* 16, 408-412.
- Froschio S.M., Humpage A.R., Burcham P.C., Falconer I.R., 2003. Cylindrospermopsin-induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes. *Environmental Toxicology* 18, 243-251.
- Garg P., Tripathi R.D., Rai U.N., Sinha S., Chandra P., 1997. Cadmium accumulation and toxicity in submerged plant *Hydrilla verticillata* (L.F.) Royle. *Environmental Monitoring and Assessment* 47, 167-173.
- Garnett C., Shaw G., Moore D., Florian P., Moore M., 2003. Impact of climate change on toxic cyanobacterial (blue-green algal) blooms and algal toxin production in Queensland. Queensland Department of Natural Resources and Mines, the National Research Centre for Environmental Toxicology and the Environmental Health Unit of Queensland Health, Rocklea, Queensland.
- Gehring M.M., Kewada V., Coates N., Downing T.G., 2003. The use of *Lepidium sativum* in a plant bioassay system for the detection of microcystin-LR. *Toxicon* 41, 871-876.
- Gkelis S., Vardaka E., Lanaras T., Sivonen K., 2002. The presence of microcystins in aquatic fauna collected from Greek lakes. Abstracts of the International Conference on Advances in the Understanding of Cyanobacterial Toxins; Occurrence, Controlling Factors and Analysis, Porto, Portugal, March 10-11, 2002.
- Gopal B., Goel U., 1993. Competition and Allelopathy in Aquatic Plant Communities. *The Botanical Review* 59, 155-210.
- Gomot A., 1998. Toxic effects of cadmium on reproduction, development and hatching in the freshwater snail *Lymnaea stagnalis* for water quality monitoring. *Ecotoxicology and Environmental Safety* 41, 288-297.
- Gorham P.R., Mc Lachlan J., Hammer U.T., Kim W.K., 1964. Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Breb. *Verhandlungen. Internationale Vereinigung für Theoretische und Angewandte Limnologie* 15, 796-804.
- Gosner K.L., 1960. A simplified table for staging Anuran embryos and larvae with notes on identification. *Herpetologica* 16, 183-190.
- Griffiths D.J., Saker M.L., 2003. The Palm Island mystery disease 20 years on: a review of research on the cyanotoxin cylindrospermopsin. *Environmental Toxicology* 18, 78-93.
- Gromov B., Mamkaeva K., Filatova E., 1997. Effect of toxigenic strains of cyanobacterium *Microcystis aeruginosa* on the larvae of frog *Rana temporaria*.

- Doklady Akademii Nauk (Proceedings of the Russian Academy of Science)* 356, 422-423. [Original article in Russian].
- Guilizzoni P., 1991. The role of heavy metals and toxic material in the physiological ecology of submersed macrophytes. *Aquatic Botany* 41, 87-109.
- Gupta M., Rai U.N., Tripathi A.M., Chandra P., 1995. Lead induced changes in glutathione and phytochelatin in *Hydrilla verticillata* (l. f.) Royle. *Chemosphere* 30, 2011-2020.
- Gustafsson S., Rengefors K., Hansson L.-A., 2005. Increased consumer fitness following transfer of toxin tolerance to offspring via maternal effects. *Ecology* 86, 2561-2567.
- Haider S., Naithani V., Viswanathan P.N., Kakkar P., 2003. Cyanobacterial toxins: a growing environmental concern. *Chemosphere* 52, 1-21.
- Hall J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* 53, 1-11.
- Hamilton D.R., Russo R.C., Thurston R.V., 1977. Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environmental Science and Technology* 11, 714-719; including correction in *Environmental Science and Technology* 12: 417.
- Handy R., Depledge M.H., 1999. Physiological responses: their measurement and use as environmental biomarkers in ecotoxicology. *Ecotoxicology* 8, 329-349.
- Harada K.I., Ohtani I., Iwamoto K., Suzuki M., Watanabe M.F., Watanabe M., Terao K., 1994. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. *Toxicon* 32, 73-84.
- Harris M.L., Chora L., Bishop C.A., Bogart J.P., 2000. Species- and age-related differences in susceptibility to pesticide exposure for two amphibians, *Rana pipiens* and *Bufo americanus*. *Bulletin of Environmental Contamination and Toxicology* 64, 263-270.
- Hawkins P.R., Chandrasena N.R., Jones G.J., Humpage A.R., Falconer I.R., 1997. Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. *Toxicon* 35, 341-346.
- Hawkins P.R., Holliday J., Kathuria A., Bowling L., 2005. Change in cyanobacterial biovolume due to preservation by Lugol's Iodine. *Harmful Algae* 4, 1033-1043.
- Hawkins P.R., Putt E., Falconer I.R., Humpage A.R., 2001. Phenotypical variation in a toxic strain of the phytoplankter, *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) during batch culture. *Environmental Toxicology* 16, 460-467.

- Hawkins P.R., Runnegar M.T.C., Jackson A.R.B., Falconer I.R., 1985. Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated from a domestic water supply reservoir. *Applied and Environmental Microbiology* 50, 1292-1295.
- Hearnden M. 1991. The reproductive and larval ecology of *Bufo marinus* (Anura: Bufonidae). Ph D thesis. Department of Zoology, James Cook University of Northern Queensland, Townsville.
- Hecnar S.J., 1995. Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environmental Toxicology and Chemistry* 14, 2131-2137.
- Hehmann A., Krienitz L., Koschel R., 2001. Long-term phytoplankton changes in an artificially divided, top-down manipulated humic lake. *Hydrobiologia* 448, 83-96.
- Henry P.F.P. 2000. 'Aspects of amphibian anatomy and physiology' in D. W. Sparling, G. Linder, C. A. Bishop, (eds) *Ecotoxicology of amphibians and reptiles*. Society for Environmental Toxicology and Chemistry, Pensacola, Florida, pp. 71-110.
- Hesse K., Kohl J. 2001. 'Effects of light and nutrient supply on growth and microcystin content of different strains of *Microcystis aeruginosa*' in I. Chorus, (ed.). *Cyanotoxins Occurrence, Causes, Consequences*. Springer-Verlag, Berlin.
- Hietala J., Laurén-Määttä C., Walls M., 1997. Sensitivity of *Daphnia* to toxic cyanobacteria: effects of genotype and temperature. *Freshwater Biology* 37, 299-306.
- Hiripi L., Nagy L., Kalmar T., Kovacs A., Voros L., 1998. Insect (*Locusta migratoria migratorioides*) test monitoring the toxicity of cyanobacteria. *Neurotoxicology* 19, 605-608.
- Hoeger S.J., Shaw G.R., Hitzfield B.C., Dietrich D.R., 2004. Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants. *Toxicon* 43, 639-649.
- Hoff K.S., Blaustein A.R., McDiarmid R.W., Altig R. 1992. 'Chapter 9. Behaviour interactions and their consequences' in R. W. McDiarmid, R. Altig, (eds). *Tadpoles: the biology of Anuran larvae*. University of Chicago Press, Chicago, pp. 215-239.
- Holcombe G.W., Phipps G.L., Marier J.W., 1984. Methods for conducting snail (*Aplexa hypnorum*) embryo through adult exposures: effects of cadmium and reduced pH Levels. *Archives of Environmental Contamination and Toxicology* 13, 627-634.
- Honrubia M.P., Herráez M.P., Alvarez R., 1993. The carbamate insecticide ZZ-Ahpox induced structural changes of gills, liver, gall-bladder, heart, and notochord

- of *Rana perezi* tadpoles. *Archives of Environmental Contamination and Toxicology* 25, 184-191.
- Hötzel G., Croome R., 1998. A phytoplankton methods manual for Australian rivers Occasional Paper No 18/98. Land and Water Resources Research and Development Corporation, Canberra.
- Hsiung T.H., Olejnik S., 1994. Power of pairwise multiple comparison procedures in the unequal variance case. *Communications in Statistics: Simulations* 23, 691-710.
- Humpage A.R., Falconer I.R., 2003. Oral toxicity of the cyanobacterial toxin Cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline. *Environmental Toxicology* 18, 94-103.
- Humpage A.R., Young F., 2005. Cylindrospermopsin: potential reproductive toxicity. Abstracts of the 5th Workshop of the Australian Research Network on Algal Toxins, 9 - 11 July 2005, Moreton Bay Research Station, Queensland, Australia.
- Humpage A.R., Fenech M., Thomas P., Falconer I.R., 2000. Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. *Mutation Research* 472, 155-161.
- Humpage A.R., Fontaine F., Froscio S.M., Burcham P.C., Falconer I.R., 2005. Cylindrospermopsin genotoxicity and cytotoxicity: role of cytochrome P-450 and oxidative stress. *Journal of Toxicology and Environmental Health Part A* 68, 739-753.
- Hunt R., 1990. Basic growth analysis, Unwin Hyman, London.
- Hyenstrand P., Metcalf J.S., Beattie K.A., Codd G.A., 2001. Losses of the cyanobacterial toxin microcystin-LR from aqueous solution by adsorption during laboratory manipulations. *Toxicon* 39, 589-594.
- Inskeep W.P., Bloom P.R., 1985. Extinction coefficients of Chlorophyll *a* and *b* in N,N-Dimethylformamide and 80% acetone. *Plant Physiology* 77, 483-485.
- Jacoby J.M., Collier D.C., Welch E.B., Hardy F.J., Crayton M., 2000. Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. *Canadian Journal of Fisheries and Aquatic Sciences* 57, 231-240.
- Johansson M., Räsänen K., Merilä J., 2001. Comparison of nitrate tolerance between different populations of the common frog, *Rana temporaria*. *Aquatic Toxicology* 54, 1-14.
- Jones G.J., Orr P.T., 1994. Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Research* 28, 871-876.

- Jones G.J., Bourne D.G., Blakely R.L., Doelle H., 1994. Degradation of the cyanobacterial hepatotoxin microcystin by aquatic bacteria. *Natural toxins* 2, 228-235.
- Jones G., Baker P.D., Burch M.D., Harvey F.L. 2002. National protocol for the monitoring of cyanobacteria and their toxins in surface waters. Draft version 5.0 (July 2002). Agriculture and Resource Management Council of Australia and New Zealand National Algal Management, Canberra.
- Kankaanpää H., Vuorinen P.J., Sipilä V., Keinänen M., 2002. Acute effects and bioaccumulation of nodularin in sea trout (*Salmo trutta* m. *trutta* L.) exposed orally to *Nodularia spumigena* under laboratory conditions. *Aquatic Toxicology* 61, 155-168.
- Kankaanpää H.T., Holliday J., Schröder H., Goddard T.J., von Fister R., Carmichael W.W., 2005. Cyanobacteria and prawn farming in northern New South Wales, Australia - a case study on cyanobacteria diversity and hepatotoxin bioaccumulation. *Toxicology and Applied Pharmacology* 203, 243-256.
- Karjalainen M., Reinikainen M., Lindvall F., Spoof L., Meriluoto J., 2003. Uptake and accumulation of dissolved radiolabelled nodularin in Baltic Sea zooplankton. *Environmental Toxicology* 18, 52-60.
- Karjalainen M., Reinikainen M., Spoof L., Meriluoto J.A.O., Sivonen K., Viitasalo M., 2005. Trophic transfer of cyanobacterial toxins from zooplankton to planktivores: consequences for Pike larvae and Mysis shrimps. *Environmental Toxicology* 20, 354-362.
- Kiesecker J.M., Blaustein A.R., Belden L.K., 2001. Complex causes of amphibian population declines. *Nature* 410, 681-684.
- Kirpenko N.I., 1986. Phytopathic properties of blue-green algae toxin. *Hydrobiological Journal* 22, 44-47.
- Kiss T., Vehovsky Á., Hiripi L., Kovács M., Vörös L., 2002. Membrane effects of toxins isolated from a cyanobacterium, *Cylindrospermopsis raciborskii*, on identified molluscan neurones. *Comparative Biochemistry and Physiology Part C* 131, 167-176.
- Klobucar G.I.V., Lajtner J., Erben R., 1997. Lipid peroxidation and histopathological changes in the digestive gland of freshwater snails *Planorbis corneus* L. (Gastropoda, Pulmonata) exposed to chronic and sub-chronic concentrations of PCP. *Bulletin of Environmental Contamination and Toxicology* 58, 128-134.
- Komárek J., Anagnostidis K., 1986. Modern approach to the classification system of cyanophytes. *Archiv für Hydrobiologie Supplement* 73 (2), Algological Studies 43, 157-226.

- Komárek J., Anagnostidis K., 1989. Modern approach to the classification system of cyanophytes 4 - Nostocales. *Archiv für Hydrobiologie Supplement* 82, 3 Algological Studies 56, 247-345.
- Körner S., Nicklisch A., 2002. Allelopathic growth inhibition of selected phytoplankton species by submerged macrophytes. *Journal of Phycology* 38, 862-871.
- Kós P., Gorzó G., Surányi G., Borbély G., 1995. Simple and efficient method for isolation and measurement of cyanobacterial hepatotoxins by plant tests (*Sinapis alba* L.). *Analytical Biochemistry* 225, 49-53.
- Kotak B.G., Zurawell R., Prepas E., Holmes C.F., 1996. Microcystin-LR concentration in aquatic food web compartments from lakes of varying trophic status. *Canadian Journal of Fisheries and Aquatic Sciences* 53, 1974-1985.
- Kromrey J.D., La Rocca M.A., 1995. Power and Type I error rates of new pairwise multiple comparison procedures under heterogenous variances. *The Journal of Experimental Education* 63, 634-362.
- Krzyżanek E., Kasza H., Pajak G., 1993. The effect of water blooms caused by blue-green algae on the bottom macrofauna in the Gocalkowice Reservoir (southern Poland) in 1992. *Acta Hydrobiologica* 35, 221-230.
- Kubo T., Sano T., Hosoya K., Tanaka N., Kaya K., 2005. A new simply (sic) and effective fractionation method for cylindrospermopsin analyses. *Toxicon* 46, 104-107.
- Kumar S., 1999. Morphological and anatomical alterations in tadpole larvae of frog, *Rana tigrina* Daudin, 1802 due to toxic effect of copper, lead and zinc. *Journal of Advanced Zoology* 20, 90-94.
- Kurki-Helasmo K., Meriluoto J., 1998. Microcystin uptake inhibits growth and protein phosphatase activity in mustard (*Sinapis alba* L.) seedlings. *Toxicon* 36, 1921-1296.
- Kyselková I., Marsalek B., 2000. Using of *Daphnia pulex*, *Artemia salina* and *Tubifex tubifex* for cyanobacterial microcystins toxicity detection. *Biologia* 55, 647-653.
- Lagos N., Onodera H., Zagatto P.A., Andrinolo D., Azevedo S., Oshima Y., 1999. The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil. *Toxicon* 37, 1359-1373.
- Lahti K., Niemi M., Rapala J., Sivonen K., 1997a. Biodegradation of cyanobacterial hepatotoxins - characterisation of toxin degrading bacteria, in B. Reguera, J. Blanco, M. Fernandez, T. Watt, (eds). Harmful Algae (Algas Nocivas) Proceedings of the VIII International Conference on Harmful Algae, Vigo, Spain, 25 -29 June 1997. Intergovernmental Oceanographic Commission of UNESCO, Santiago de Compostela, Spain, pp. 363-365.

- Lahti K., Rapala J., Färdig M., Niemelä M., Sivonen K., 1997b. Persistence of cyanobacterial hepatotoxin, Microcystin-LR in particulate material and dissolved in lake water. *Water Research* 31, 1005-1012.
- Lajtner J., Erbern R., Klobucar G.I.V., 1996. Histopathological effects of phenol on the digestive gland of *Amphimelania holandri* Fér. (Gastropoda, Prosobranchia). *Bulletin of Environmental Contamination and Toxicology* 57, 458-464.
- Lam P.K.S., Yu K.N., Ng K.P., Chong M.W.K., 1997. Cadmium uptake and depuration in the soft tissues of *Brotia hainanensis* (Gastropoda: Prosobranchia: Thiariidae): a dynamic model. *Chemosphere* 35, 2449-2461.
- Landsberg J.H., 2002. The effects of harmful algal blooms on aquatic organisms. *Reviews in Fisheries Science* 10, 113-390.
- Lauren-Maatta C., Hietala J., Walls M., 1997. Responses of *Daphnia pulex* to toxic cyanobacteria. *Freshwater Biology* 37, 635-647.
- Lawler S.P., 1989. Behavioural responses to predators and predation risk in four species of larval anurans. *Animal Behaviour* 38, 1039-1047.
- LeBlanc S., Pick F.R., Aranda-Rodriguez R., 2005. Allelopathic effects of the toxic cyanobacterium *Microcystis aeruginosa* on duckweed, *Lemna gibba* L. *Environmental Toxicology* 20, 67-73.
- Lehtonen K.K., Kankaanpää H., Leiniö S., Sipiä V., Pflugmacher S., Sandberg-Kilpi, E., 2003. Accumulation of nodularin-like compounds from the cyanobacterium *Nodularia spumigena* and changes in acetylcholinesterase activity in the clam *Macoma balthica* during short term laboratory exposure. *Aquatic Toxicology* 64, 461-476.
- Leonard J.A., Paerl H.W., 2005. Zooplankton community structure, micro-zooplankton grazing impact, and seston energy content in the St. Johns river system, Florida as influenced by the toxic cyanobacterium *Cylindrospermopsis raciborskii*. *Hydrobiologia* 537, 89-97.
- Li R., Carmichael W.W., Brittain S., Eaglesham G., Shaw G., Liu Y., Watanabe M., 2001a. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata* (Cyanobacteria). *Journal of Phycology* 37, 1121-1126.
- Li R., Carmichael W.W., Brittain J.E., Eaglesham G.K., Shaw G.R., Mahakhant A., Noparatnaraporn N., Yongmanitchai W., Kaya K., Watanabe M.M., 2001b. Isolation and identification of the cyanotoxin cylindrospermopsin and deoxycylindrospermopsin from a Thailand strain of *Cylindrospermopsis raciborskii* (Cyanobacteria). *Toxicon* 39, 973-980.

- Li T., Xiong Z., 2004. A novel response of wild-type duckweed (*Lemna paucicostata* Hegelm.) to heavy metals. *Environmental Toxicology* 19, 95-102.
- Lichtenthaler H.K., 1996. Vegetation stress: an introduction to the stress concept in Plants. *Journal of Plant Physiology* 148, 4-14.
- Lirås V., Lindberg M., Nystrom P., Annadotter H., Lawton L., Graf B., 1998. Can ingested cyanobacteria be harmful to the signal crayfish (*Pacifastacus leniusculus*)? *Freshwater Biology* 39, 233-242.
- Liu X.-X., Lembi C.A., 1999. Laboratory evaluation of mefluidide effects on elongation of Hydrilla and Eurasian Watermilfoil. *Journal of Aquatic Plant Management* 37, 55-60.
- Llewellyn L.E., Negri A.P., Doyle J., Baker P.D., Beltran E.C., Neilan B.A., 2001. Radioreceptor assays for sensitive detection and quantitation of saxitoxin and its analogues from strains of the freshwater cyanobacterium, *Anabaena circinalis*. *Environmental Science and Technology* 35, 1445-1451.
- Lodge D.M., 1986. Selective grazing on periphyton: a determinant of freshwater gastropod microdistributions. *Freshwater Biology* 16, 831-841.
- Long B.M., Jones G.J., Orr P.T., 2001. Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. *Applied and Environmental Microbiology* 67, 278-283.
- Looper R.E., Runnegar M.T.C., Williams R.M., 2005. Synthesis of the putative structure of 7-deoxycylindrospermopsin: C7 oxygenation is not required for the inhibition of protein synthesis. *Angewandte Chemie International Edition* 44, 3879-3881.
- Lüring M., 2003. Effects of microcystin-free and microcystin-containing strains of the cyanobacterium *Microcystis aeruginosa* on growth of the grazer *Daphnia magna*. *Environmental Toxicology* 18, 202-210.
- Mackay D., Fraser A., 2000. Bioaccumulation of persistent organic chemicals: mechanisms and models. *Environmental Pollution* 110, 375-391.
- MacKenzie L., Beuzenberg V., Holland P., McNabb P., Selwood A., 2004. Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. *Toxicon* 44, 901-918.
- MacKintosh C., Beattie K.A., Klumpp S., Cohen P., Codd G.A., 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Letters* 264, 187-192.

- Magalhães V.F., Soares R., Azevedo S., 2001. Microcystin contamination in fish from the Jacarepaguá Lagoon (Rio de Janeiro, Brazil): ecological implication and human health risk. *Toxicon* 39, 1077-1085.
- Mann R.M., Bidwell J.R., 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and two exotic frogs. *Environmental Pollution* 114, 195-205.
- Markich S.J., Camilleri C., 1996. Investigation of metal toxicity to tropical biota: Recommendations for revision of the Australian Water Quality Guidelines. Office of the Supervising Scientist of the Alligator Rivers Region, Jabiru.
- Marquis O., Saglio P., Neveu A., 2004. Effects of predators and conspecific chemical cues on the swimming activity of *Rana temporaria* and *Bufo bufo* tadpoles. *Archiv für Hydrobiologie* 160, 153-170.
- Matsunaga H., Harada K.I., Senma M., Ito Y., Yasuda N., Ushida S., Kimura Y., 1999. Possible cause of unnatural mass death of wild birds in a pond in Nishinomiya, Japan: sudden appearance of toxic cyanobacteria. *Natural toxins* 7, 81-84.
- Matveev V., Matveev L., Jones G.J., 1994. A study of the ability of *Daphnia carinata* King to control phytoplankton and resist toxicity: Implications for biomanipulation in Australia. *Australian Journal of Marine and Freshwater Research* 45, 889-904.
- McCarty L.S., Mackay D., 1993. Enhancing ecotoxicological modelling and assessment. *Environmental Science and Technology* 27, 1719-1728.
- McDiarmid R.W., Altig R., 1999. 'Chapter 2. Research materials and techniques' in R. W. McDiarmid, R. Altig, (eds). *Tadpoles the biology of Anuran larvae*. The University of Chicago Press, Chicago, pp. 7-23.
- McElhiney J., Lawton L., Leifert C., 2001. Investigations into the inhibitory effects of microcystins in plant growth, and the toxicity of plant tissues following exposure. *Toxicon* 39, 1411-1420.
- McGregor G.B., Fabbro L.D., 2000. Dominance of *Cylindrospermopsis raciborskii* (Nostocales, Cyanoprokaryota) in Queensland tropical and subtropical reservoirs: Implications for monitoring and management. *Lakes & Reservoirs: Research and Management* 5, 195-205.
- Mebis D., 1998. Occurrence and sequestration of toxins in food chains. *Toxicon* 36, 1519-1522.
- Meijer M., de Boois I., Scheffer M., Portielje R., Hosper H., 1999. Biomanipulation in shallow lakes in the Netherlands: an evaluation of 18 case studies. *Hydrobiologia* 408/409, 13-30.

- Metcalf J.S., Barakate A., Codd G.A., 2004. Inhibition of plant protein synthesis by the cyanobacterial hepatotoxin, cylindrospermopsin. *FEMS Microbiology Letters* 235, 125-129.
- Metcalf J.S., Beattie K.A., Saker M.L., Codd G.A., 2002a. Effects of organic solvents on the high performance liquid chromatographic analysis of the cyanobacterial toxin cylindrospermopsin and its recovery from environmental eutrophic waters by solid phase extraction. *FEMS Microbiology Letters* 216, 159-164.
- Metcalf J.S., Lindsay J., Beattie K.A., Birmingham S., Saker M.L., Törökné A.K., Codd G.A., 2002b. Toxicity of cylindrospermopsin to the brine shrimp *Artemia salina*: comparisons with protein synthesis inhibitors and microcystins. *Toxicon* 40, 1115-1120.
- M-Hamvas M., Máthé C., Molnár E., Vasas G., Grigorszky I., Borbély G., 2003. Microcystin-LR alters the growth, anthocyanin content and single-stranded DNase enzyme activities in *Sinapis alba* L. seedlings. *Aquatic Toxicology* 62, 1-9.
- Mitrovic S.M., Pflugmacher S., James K.J., Furey A., 2004. Anatoxin-a elicits an increase in peroxidase and glutathione S-transferase activity in aquatic plants. *Aquatic Toxicology* 68, 185-192.
- Mitrovic S.M., Allis O., Furey A., James K.J., 2005. Bioaccumulation and harmful effects of microcystin-LR in the aquatic plants *Lemna minor* and *Wolffia arrhiza* and the filamentous alga *Chladophora fracta*. *Ecotoxicology and Environmental Safety* 61, 345-352.
- Mohamed Z.A., 2001. Accumulation of cyanobacterial hepatotoxins by *Daphnia* in some Egyptian irrigation channels. *Ecotoxicology and Environmental Safety* 50, 4-8.
- Mohamed Z.A., Carmichael W.W., Hussein A., 2003. Estimation of microcystins in the freshwater fish *Oreochromis niloticus* in an Egyptian fish farm containing a *Microcystis* bloom. *Environmental Toxicology* 18, 137-141.
- Molica R., Onodera H., Garcia C., Rivas M., Andrinolo D., Nascimento S.M., Meguro H., Oshima Y., Azevedo S., Lagos N., 2002. Toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii* (Cyanophyceae) isolated from Tabocas Reservoir in Caruaru, Brazil, including demonstration of a new saxitoxin analogue. *Phycologia* 41, 606-611.
- Nakai S., Inoue Y., Hosomi M., Murakami A., 1999. Growth inhibition of blue-green algae by allelopathic effects of macrophytes. *Water Science Technology* 39, 47-53.
- Nakai S., Yamada S., Hosomi M., 2005. Anti-cyanobacterial fatty acids released from *Myriophyllum spicatum*. *Hydrobiologia* 543, 71-78.

- Nebeker A.V., Schuytema G.S., Griffis W.L., Cataldo A., 1998. Impact of Guthion on survival and growth of the frog *Pseudacris regilla* and the Salamanders *Ambystoma gracile* and *Ambystoma maculatum*. *Archives of Environmental Contamination and Toxicology* 35, 48-51.
- Neilan B.A., Saker M.L., Fastner J., Törökné A.K., Burns B.P., 2003. Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. *Molecular Ecology* 12, 133-140.
- Nelson D.R., 1999. Cytochrome P450 and the individuality of species. *Archives of Biochemistry and Biophysics* 369, 1-10.
- Netherland M.D., Getsinger K.D., 1995a. Laboratory evaluation of threshold fluridone concentrations under static conditions for controlling *Hydrilla* and Eurasian Watermilfoil. *Journal of Aquatic Plant Management* 33, 33-36.
- Neumann C., Bain P., Shaw G., 2005. The *in vitro* toxicology of deoxycylindrospermopsin. Abstracts of the Australian Research Network for Algal Toxins Workshop, Moreton Bay Research Station, Queensland, 9 - 11th July 2005.
- NHMRC & NRMCM (National Health and Medical Resources Council & National Resource Management Ministerial Council), 2004. Australian Drinking Water Guidelines, National Health and Medical Resources Council, Canberra.
- Nicholson B.C., Burch M.D., 2001. Evaluation of analytical methods for detection and quantification of cyanotoxins in relation to Australian drinking water guidelines, National Health and Medical Research Council of Australia, the Water Services Association of Australia and the Cooperative Research Centre for Water Quality and Treatment, Canberra.
- Nilsen E.T., Orcutt D.M., 1996. The physiology of plants under stress: Vol 1 Abiotic Factors, John Wiley & Sons, Inc, New York.
- Nishimura N., Fukazawa Y., Uchiyama H., 1997. Effects of estrogenic hormones on early development of *Xenopus laevis*. *Journal of Experimental Zoology* 278, 221-233.
- Nogueira I.C.G., Saker M.L., Pflugmacher S., Wiegand C., Vasconcelos V.M., 2004a. Toxicity of the cyanobacterium *Cylindrospermopsis raciborskii* to *Daphnia magna*. *Environmental Toxicology* 19, 453-459.
- Nogueira I.C.G., Pereira P., Dias E., Pflugmacher S., Wiegand C., Franca S., Vasconcelos V.M., 2004b. Accumulation of paralytic shellfish toxin (PST) from the cyanobacterium *Aphanizomenon issatschenkoi* by the cladoceran *Daphnia magna*. *Toxicon* 44, 773-780.
- Noor Alam M.D., Shafi M.D., 1999. Histopathological lesions induced by the insecticide Metacid 50 in tadpole larvae of *Rana cyanophylctis*. *Environment and Ecology* 17, 392-394.

- Noraho N., Gaur J.P., 1996. Cadmium adsorption and intracellular uptake by two macrophytes, *Azolla pinnata* and *Spirodela polyrhiza*. *Archiv für Hydrobiologie* 136, 135-144.
- Norris R.H., Norris K.R., 1995. The need for biological assessment of water quality: An Australian perspective. *Australian Journal of Ecology* 20, 1-6.
- Norris R. L., Eaglesham G., Pierens G., Shaw G., Smith M.J., Chiswell R.K., Seawright A.A., Moore M.R., 1999a. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 14, 163-165.
- Norris R.L., Eaglesham G.K., Shaw G.R., Senogles P., Chiswell R.K., Smith M.J., Davis J.A., Seawright A.A., Moore M.R., 2001a. Extraction and purification of the zwitterions cylindrospermopsin and deoxycylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 16, 394-396.
- Norris R.L., Seawright A.A., Shaw G.R., Smith M.J., Chiswell R.K., Moore M.R., 2001b. Distribution of ¹⁴C cylindrospermopsin *in vivo* in the mouse. *Environmental Toxicology* 16, 498-505.
- Norris R. L., Seawright A.A., Shaw G., Senogles P., Eaglesham G., Smith M.J., Chiswell R.K., Moore M.R., 2002. Hepatic xenobiotic metabolism of cylindrospermopsin *in vivo* in the mouse. *Toxicon* 40, 471-476.
- Oberemm A. 2001. 'Effects of cyanotoxins on early life stages of fish and amphibians' in I. Chorus, (ed.) Cyanotoxins: Occurrence, Causes, Consequences. Berlin, Springer-Verlag, pp. 240-248.
- Oberemm A., Becker J., Codd G.A., Steinberg C., 1999. Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. *Environmental Toxicology* 14, 77-88.
- OECD (Organisation for Economic Cooperation and Development) 2002. Draft guideline 221: *Lemna* sp. growth inhibition test. Guidelines for the Testing of Chemicals Series, OECD, France.
- Ohtani I., Moore R.E., Runnegar M.T.C., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. *Journal of the American Chemical Society* 114, 7941-7942.
- Orr P., Jones G., 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. *Limnology and Oceanography* 43, 1604-1614.
- Oulette A.J.A., Wilhelm S.W., 2003. Toxic cyanobacteria: the evolving molecular toolbox. *Frontiers in Ecology and the Environment* 1, 359-366.

- Ozawa K., Yokoyama A., Ishikawa K., Kumagi M., Watanabe M., Park H.-D., 2003. Accumulation and depuration of microcystin produced by the cyanobacterium *Microcystis* in a freshwater snail. *Limnology* 4, 131-138.
- Padisák J., 1997. *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding highly adaptive cyanobacterium: worldwide distribution and review of its ecology. *Archiv für Hydrobiologie. Supplement 107 (Monographic Studies)* 4, 563-593.
- Park H.-D., Iwami C., Watanabe M.F., Harada K.I., Okino T., Hayashi H., 1998. Temporal variabilities of the concentrations of intra- and extracellular microcystin and toxic *Microcystis* species in a hypertrophic lake, Lake Suwa, Japan (1991-1994). *Environmental Toxicology and Water Quality* 13, 61-72.
- Pastore A., Federici G., Bertini E., Piemonte F., 2003. Analysis of glutathione: implication in redox and detoxification. *Clinica Chimica Acta* 333, 19-39.
- Pereira P., Dias E., Franca S., Pereira E., Carolino M., Vasconcelos V.M., 2004. Accumulation and depuration of cyanobacterial paralytic shellfish toxins by the freshwater mussel *Anodonta cygnea*. *Aquatic Toxicology* 68, 339-350.
- Petty J.D., Jones S.B., Huckins J.N., Cranor W.L., Parris J.T., McTague T.B., Boyle T.P., 2000. An approach for assessment of water quality using semipermeable membrane devices (SPMDs) and bioindicator tests. *Chemosphere* 41, 311-321.
- Pflugmacher S., 2002. Possible allelopathic effects of cyanotoxins, with reference to Microcystin-LR, in Aquatic Ecosystems. *Environmental Toxicology* 17, 407-413.
- Pflugmacher S., 2004. Promotion of oxidative stress in the aquatic macrophyte *Ceratophyllum demersum* during biotransformation of the cyanobacterial toxin microcystin-LR. *Aquatic Toxicology* 70, 169-178.
- Pflugmacher S., Wiegand C., 2001. 'Metabolism of microcystin-LR in aquatic organisms' in I. Chorus, (ed.) Cyanotoxins occurrence, causes, consequences. Berlin, Springer-Verlag, pp. 257-260.
- Pflugmacher S., Wiegand C., Beattie K.A., Codd G.A., Steinberg C.E.W., 1998. Uptake of the cyanobacterial hepatotoxin microcystin-LR by aquatic macrophytes. *Journal of Applied Botany* 72, 228-232.
- Pflugmacher S., Codd G.A., Steinberg C., 1999a. Effects of the cyanobacterial toxin microcystin-LR on detoxication enzymes in aquatic plants. *Environmental Toxicology* 14, 111-115.
- Pflugmacher S., Geissler K., Steinberg C., 1999b. Activity of Phase I and Phase II detoxication enzymes in different cormus parts of *Phragmites australis*. *Ecotoxicology and Environmental Safety* 42, 62-66.
- Pflugmacher S., Wiegand C., Beattie K.A., Krause E., Steinberg C., Codd G.A., 2001. Uptake, effects, and metabolism of cyanobacterial toxins in the emergent reed

- plant *Phragmites australis* (Cav.) Trin. Ex Steud. *Environmental Toxicology and Chemistry* 20, 846-852.
- Pietsch C., Wiegand C., Amé M.V., Nicklisch A., Wunderlin D., Pflugmacher S., 2001. The effects of a cyanobacterial crude extract on different aquatic organisms: evidence for cyanobacterial toxin modulating factors. *Environmental Toxicology* 16, 535-542.
- Pilotto L.S., Douglas R.M., Burch M.D., Cameron S., 1997. Health effects of exposure to cyanobacteria (blue green algae) during recreational water-related activities. *Australian and New Zealand Journal of Public Health* 21, 562-566.
- Pomati F., Neilan B.A., Suzuki T., Manarolla G., Rossetti C., 2003. Enhancement of intracellular saxitoxin accumulation by lidocaine hydrochloride in the cyanobacterium *Cylindrospermopsis raciborskii* T3 (Nostocales). *Journal of Phycology* 39, 535-542.
- Pomati F., Moffitt M.C., Cavaliere R., Neilan B.A., 2004a. Evidence for differences in the metabolism of saxitoxin and C1+2 toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii* T3. *Biochimica et Biophysica Acta* 1674, 60-67.
- Pomati F., Rossetti C., Manarolla G., Burne B.P., Neilan B.A., 2004b. Interactions between intracellular Na⁺ levels and saxitoxin production in *Cylindrospermopsis raciborskii* T3. *Microbiology* 150, 455-461.
- Prepas E.E., Kotak B.G., Campbell L.M., Evans J.C., Hrudey S.E., Holmes C.F., 1997. Accumulation and elimination of cyanobacterial hepatotoxins by the freshwater clam *Anodonta grandis simpsonia*. *Canadian Journal of Fisheries and Aquatic Sciences* 54, 41-46.
- Preußel K., Stüken A., Wiedner C., Chorus I., Fastner J., 2006. First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. *Toxicon* 47, 156-162.
- Pyatt A.J., Pyatt F.B., Pentreath V.W., 2002. Lead toxicity, locomotion and feeding in the freshwater snail, *Lymnaea stagnalis* (L.). *Invertebrate Neuroscience* 4, 135-140.
- QDNR (Queensland Department of Natural Resources), 2003. Queensland harmful algal bloom operational procedures, Harmful Algal Blooms Steering Committee, Queensland Department of Natural Resources and Mines., Rocklea, Queensland.
- Queensland Environmental Protection Act, 1994. Reprint number three, incorporating amendments as at 3rd March 2005. Legislative Assembly of Queensland, Australia.
- Quinn G.P., Keough M.J., 2002. Experimental design and data analysis for biologists, Cambridge University Press, Cambridge.

- Rader R.B., Belk M.C., Keleher M.J., 2003. The introduction of an invasive snail (*Melanoides tuberculata*) to spring ecosystems of the Bonneville Basin, Utah. *Journal of Freshwater Ecology* 18, 647-657.
- Rasmussen J.P., Campbell R., Monis P.T., Saint C.P., 2005. The genetic determinants of cylindrospermopsin production and their detection using real-time PCR. Abstracts of the 5th Workshop of the Australian Research Network on Algal Toxins, Moreton Bay Research Station, Queensland, Australia, 9 - 11 July 2005.
- Reinikainen M., Ketola M., Walls M., 1994. Effects of the concentrations of toxic *Microcystis aeruginosa* and an alternative food on the survival of *Daphnia pulex*. *Limnology and Oceanography* 39, 424-432.
- Reisner M., Carmeli S., Werman M., Sukenik A., 2004. The cyanobacterial toxin cylindrospermopsin inhibits pyrimidine nucleotide synthesis and alters cholesterol distribution in mice. *Toxicological Sciences* 82, 620-627.
- Ressom R., San Soong F., Fitzgerald J., Turczynowicz L., El Saadi O., Roder D., Maynard T., Falconer I., 1994. Health effects of toxic cyanobacteria (blue green algae), National Health and Medical Resources Council, Canberra.
- Richey L.J., Carbonneau D.A., Schoeb T.R., Taylor S.K., Woodward A.R., Clemmons R., 2001. Potential toxicity of cyanobacteria to American alligators (*Alligator mississippiensis*). Florida Fish and Wildlife Conservation Commission, Florida.
- Riethmuller N., Camilleri C., Franklin N., Hogan A., King A., Koch A., Markich S.J., Turley C., van Dam R., 2003. Ecotoxicological testing protocols for Australian tropical freshwater ecosystems. Environmental Research Institute of the Supervising Scientist, Environment Australia, Darwin, Australia.
- Rinehart K.L., Namikoshi M., Choi B.W., 1994. Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). *Journal of Applied Phycology* 6, 159-176.
- Romanowska-Duda Z., Tarczyska M., 2002. The influence of microcystin-LR and hepatotoxic cyanobacterial extract on the water plant *Spirodela oligorrhiza*. *Environmental Toxicology* 17, 434-440.
- Romanowska-Duda Z., Mankiewicz J., Tarczyska M., Walter Z., Zalewski M., 2002. The effect of toxic cyanobacteria (blue-green algae) on water plants and animal cells. *Polish Journal of Environmental Studies* 11, 561-566.
- Runnegar M.T., Kong S., Zhong Y., Lu S.C., 1995. Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochemical Pharmacology* 49, 219-225.
- Runnegar M.T., Kong S.M., Zhong Y.Z., Ge J.L., Lu S.C., 1994. The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochemical and Biophysical Research Communications* 201, 235-241.

- Runnegar M.T., Xie C., Snider B.B., Wallace G.A., Weinreb S.M., Kuhlenkamp J., 2002. *In vitro* hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. *Toxicological Sciences* 67, 81-87.
- Sainty G.R., Jacobs S.W.L., 1994. Waterplants in Australia, Sainty and Associates, Darlinghurst.
- Saker M., 2000. Cyanobacterial blooms in tropical north Queensland water bodies. Ph D thesis. School of Tropical Biology, James Cook University, Townsville, Australia.
- Saker M.L., Eaglesham G.K., 1999. The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the redclaw crayfish *Cherax quadricarinatus*. *Toxicon* 37, 1065-1077.
- Saker M.L., Griffiths D.J., 2000. The effect of temperature on growth and cylindrospermopsin content of seven isolates of the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju from water bodies in northern Australia. *Phycologia* 39, 349-354.
- Saker M.L., Griffiths D.J., 2001. Occurrence of blooms of the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju in a north Queensland domestic water supply. *Marine and Freshwater Research* 52, 907-915.
- Saker M.L., Neilan B.A., 2001. Varied diazotrophies, morphologies and toxicities of genetically similar isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from Northern Australia. *Applied and Environmental Microbiology* 67, 1839-1845.
- Saker M., Thomas A.D., Norton J.H., 1999a. Cattle mortality attributed to the toxic cyanobacterium *Cylindrospermopsis raciborskii* in an outback region of North Queensland. *Environmental Toxicology* 14, 179-182.
- Saker M.L., Neilan B.A., Griffiths D.J., 1999b. Two morphological forms of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolated from Solomon Dam, Palm Island, Queensland. *Journal of Phycology* 35, 599-606.
- Saker M.L., Nogueira I.C.G., Vasconcelos V.M., Neilan B.A., Eaglesham G.K., Pereira P., 2003. First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. *Ecotoxicology and Environmental Safety* 55, 243-250.
- Saker M.L., Metcalf J.S., Codd G.A., Vasconcelos V.M., 2004. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. *Toxicon* 43, 185-194.

- Samecka-Cymerman A., Kempers A.J., 1996. Bioaccumulation of heavy metals by aquatic macrophytes around Wroclaw, Poland. *Ecotoxicology and Environmental Safety* 35, 242-247.
- Schembri M.A., Neilan B.A., Saint C.P., 2001. Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 16, 413-421.
- Schuytema G.S., Nebeker A.V., 1998. Comparative Ttxicity of diuron on survival and growth of Pacific treefrog, Bullfrog, Red-legged frog, and African clawed frog embryos and tadpoles. *Archives of Environmental Contamination and Toxicology* 34, 370-376.
- Schwimmer M., Schwimmer D., 1968. 'Medial aspects of phycology' in D. F. Jackson, (ed.). *Algae, Man and the Environment*. New York, Syracuse University Press, pp. 279-358.
- Scott G.R., Sloman K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology* 68, 369-392.
- Seawright A.A., Nolan C.C., Shaw G.R., Chiswell R.K., Norris R.L., Moore M.R., Smith M.J., 1999. The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 14, 135-142.
- Senogles P.-J., Scott J.A., Stratton H., 2001. Photocatalytic degradation of the cyanotoxin cylindrospermopsin, using titanium dioxide and UV irradiation. *Water Research* 35, 1245-1255.
- Shaw G., Sufenik A., Livne A., Chiswell R.K., Smith M.J., Seawright A.A., Norris K.R., Eaglesham G., Moore M.R., 1999. Blooms of the cylindrospermopsin containing cyanobacterium, *Aphanizomenon ovalisporum* (Forti) in newly constructed lakes, Queensland, Australia. *Environmental Toxicology* 14, 167-177.
- Shaw G., Seawright A.A., Moore M.R., Lam P.K.S., 2000. Cylindrospermopsin, a cyanobacterial alkaloid: evaluation of its toxicologic activity. *Therapeutic Drug Monitoring* 22, 89-92.
- Shaw G., McKenzie R.A., Wickramasinghe W.A., Seawright A.A., K. E.G., Fabbro L.D., 2002. Comparative toxicity of the cyanobacterial toxin, Cylindrospermopsin, between mice and cattle: human implications. Abstracts of the 4th Workshop of the Australian Research Network for Algal Toxins, Australian Institute of Marine Science, Townsville, 14th July 2002.
- Shen X., Lam P.K.S., Shaw G.R., Wickramasinghe W., 2002. Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. *Toxicon* 40, 1499-1501.

- Siegl G., MacIntosh C., Stitt M., 1990. Sucrose-phosphate synthase is dephosphorylated by protein phosphatase 2A in spinach leaves. *FEBS Letters* 270, 198-202.
- Sijm D.T.H., Hermens J.L., 2000. 'Internal effect concentration: link between bioaccumulation and ecotoxicity for organic chemicals' in B. Beek, (ed.). *Bioaccumulation New Aspects and Developments*. Springer, Berlin, pp. 167-233.
- Singh D.K., Agarwal R.A., 1986. Toxicity of pesticides to fecundity, hatchability and survival of young snails of *Lymnaea acuminata*. *Acta hydrochimica et hydrobiologia*. 14, 191-194.
- Singh A., Tyagi M.B., Kumar A., Thakur J.K., Kumar A., 2001. Antialgal activity of a hepatotoxin-producing cyanobacterium, *Microcystis aeruginosa*. *World Journal of Microbiology and Biotechnology* 17, 15-22.
- Sipiä V., 2001. Accumulation of cyanobacterial hepatotoxins and okadaic acid in mussel and fish tissues from the Baltic Sea. *Finnish Institute of Marine Research - Contributions* 3.
- Sipiä V., Kankaanpää H., Lahti K., Carmichael W.W., Meriluoto J., 2001a. Detection of Nodularin in Flounders and Cod from the Baltic Sea. *Environmental Toxicology* 16, 121-126.
- Sipiä V., Kankaanpää H., Flinkman J., Lahti K., Meriluoto J., 2001b. Time-dependent accumulation of cyanobacterial hepatotoxins in Flounders (*Platichthys flesus*) and Mussels (*Mytilus edulis*) from the Northern Baltic Sea. *Environmental Toxicology* 16, 330-336.
- Sivonen K., Jones G. 1999. 'Cyanobacterial toxins' in I. Chorus, J. Bartram, (eds). *Toxic cyanobacteria in water*. E & FN Spoon, London.
- Smith M.J., Kay W.R., Edward D.H., Papas P.J., Richardson J., Simpson J.C., Pinder A.M., Cale D.J., Horwitz P.H., Davis J.A., Yung F.H., Norris R.H., Halse S.A., 1999. AusRivAS: using macroinvertebrates to assess ecological condition of rivers in Western Australia. *Freshwater Biology* 41, 269-282.
- Smith S., Kwan M.K.H., 1989. Use of aquatic macrophytes as a bioassay method to assess relative toxicity, uptake kinetics and accumulated forms of trace metals. *Hydrobiologia* 188/189, 345-351.
- Soares R., Magalhães V.F., Azevedo L.O., 2004. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (Cichlidae) under laboratory conditions. *Aquatic Toxicology* 70, 1-10.
- Stephan C.E., Mount D.I., Hansen D.J., Gentile J.H., Chapman G.A., Brungs W.A., 1985. Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. United States Environmental Protection Agency, Washington, DC.

- Stillman M.J., Shaw C.F., Suzuki K., 1992. Metallothioneins synthesis, structure and properties of metallothioneins, phytochelatins and metal-thiolate complexes. VCH Publishers, Inc., New York.
- Stirling D.J., Quilliam M.A., 2001. First report of the cyanobacterial toxin cylindrospermopsin in New Zealand. *Toxicon* 39, 1219-1222.
- Streit B., 1998. 'Bioaccumulation of contaminants in fish' in T. Braunbeck, D. E. Hinton, B. Streit, (eds). Fish Ecotoxicology. Birkhäuser Verlag, Basel, Switzerland, pp. 353-387.
- Sutton D.L., Portier K.M., 1989. Influence of allelochemicals and aqueous plant extracts on the growth of duckweed. *Journal of Aquatic Plant Management* 27, 90-97.
- Takahashi E.M., Shaw G.R., Yu Q., Eaglesham G., Connell D.W., 2005. Occurrence of DSP, ASP and gymnodimine around North Stradbroke Island, Australia. Abstracts of the 5th Workshop of the Australian Research Network for Algal Toxins, Moreton Bay Research Station, Queensland, Australia, 9-11th July 2005.
- Takeda S., Mano S., Ohto M., Nakamura K., 1994. Inhibitors of protein phosphatases 1 and 2A block the sugar-inducible gene expression in plants. *Plant Physiology* 106, 567-574.
- Tarczyska M., Nalecz-Jawecki G., Romanowska-Duda Z., Sawicki J., Beattie K.A., Codd G.A., Zalewski M., 2001. Tests for the toxicity assessment of cyanobacterial bloom samples. *Environmental Toxicology* 16, 383-390.
- Tencalla F., Dietrich D., 1997. Biochemical characterization of microcystin toxicity in rainbow trout (*Oncorhynchus mykiss*). *Toxicon* 35, 583-595.
- Terao K., Ohmori S., Igarshi K., Ohtani I., Watanabe M.F., Harada K.I., Ito E., Watanabe M., 1994. Electron microscope studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. *Toxicon* 32, 833-843.
- Törökné A.K., Asztalos M., Bánkiné M., Bickel H., Borbély G., Carmeli S., Codd G.A., Fastner J., Huang Q., Humpage A.R., Metcalf J.S., Rábai E., Sukenik A., Surányi G., Vasas G., Weiszfeiler V., 2004. Interlaboratory comparison trial on cylindrospermopsin measurement. *Analytical Biochemistry* 332, 280-284.
- Tripathi P.K., Singh A., 2002. Toxic effects of Dimethoate and Carbaryl pesticides on carbohydrate metabolism of freshwater snail *Lymnaea acuminata*. *Bulletin of Environmental Contamination and Toxicology* 68, 606-611.
- Tripathi R.D., Rai U.N., Gupta M., Chandra P., 1996. Induction of phytochelatins in *Hydrilla verticillata* (l. f.) Royle under cadmium stress. *Bulletin of Environmental Contamination and Toxicology* 56, 505-512.

- Truscott R., McCrohan C.R., Bailey S.E.R., White K.N., 1995. Effect of aluminium and lead on activity in the freshwater pond snail *Lymnaea stagnalis*. *Canadian Journal of Fisheries and Aquatic Sciences* **52**, 1623-1629.
- Tyler M.J., 1999. Australian frogs a natural history, Reed New Holland, Sydney.
- Underwood A.J., 1981. Techniques of analysis of variance in experimental marine biology and ecology. *Oceanography and Marine Biology. An Annual Review*, 19, 513-605.
- Underwood A.J., 1997. Experiments in ecology - Their logical design and interpretation using analysis of variance, Cambridge University Press, Melbourne.
- USEPA (United States Environmental Protection Agency), 1994. Chlorophyll determination (Standard Operating Procedure #2030). United States Environmental Protection Agency, Washington, D. C.
- USEPA (United States Environmental Protection Agency), (1996). OPPTS 850.4400 Aquatic Plant Toxicity Test Using *Lemna* Spp., Tiers I and II, United States Environmental Protection Agency, Washington, D. C.
- Utkilen H., Gjølme N., 1995. Iron-stimulated toxin production in *Microcystis aeruginosa*. *Applied and Environmental Microbiology* **61**, 797-800.
- van der Oost R., Beyer J., Vermeulen N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* **13**, 57-149.
- Vanderploeg H.A., Liebig J.R., Carmichael W.W., Agy M.A., Johengen T.H., Fahnenstiel G.L., Nalepa T.F., 2001. Zebra mussel (*Dreissena polymorpha*) selective filtration promoted toxic *Microcystis* blooms in Saginaw Bay (Lake Huron) and Lake Erie. *Canadian Journal of Fisheries and Aquatic Sciences* **58**, 1208-1221.
- Vasas G., Gáspár A., Surányi G., Batta G., Gyémánt G., M-Hamvas M., Máthé C., I. G., Molnár E., Borbély G., 2002. Capillary electrophoretic assay and purification of cylindrospermopsin, a cyanobacterial toxin from *Aphanizomenon ovalisporum*, by plant test (Blue-Green *Sinapis* Test). *Analytical Biochemistry* **302**, 95-103.
- Vasconcelos V.M., 1995. Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*. *Aquatic Toxicology* **32**, 227-237.
- Vasconcelos V.M., Oliveira S., Teles F.O., 2001. Impact of a toxic and a non-toxic strain of *Microcystis aeruginosa* on the crayfish *Procambarus clarkii*. *Toxicon* **39**, 1461-1470.
- Vehovszky Á., Kiss T., Kovács A., Hiripi L., Vörös L., 1997. P-41 Membrane effects of algal toxins on identified snail neurons. Abstracts of posters presented at the

- 6th meeting of the International Neurotoxicology Association. Available from http://www.neurotoxicology.org/ina6_posters.htm, accessed 27th September 2005.
- Vesterkvist P.S.M., Meriluoto J.A.O., 2003. Interaction between microcystins of different hydrophobicities and monolayers. *Toxicon* 41, 349-355.
- Vinagre T.M., Alciati J.C., Yunes J.S., Richards J., Bianchini A., Monserrat J.M., 2002. Effects of extracts from the cyanobacterium *Microcystis aeruginosa* on Ion regulation and Gill Na⁺, K⁺ - ATPase and K⁺ -dependent phosphatase activities of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). *Physiological and Biochemical Zoology* 75, 600-608.
- Voutsas E., Magoulas K., Tassios D., 2002. Prediction of the bioaccumulation of persistent organic pollutants in aquatic food webs. *Chemosphere* 48, 645-651.
- Walker C.H., Hopkin S.P., Sibly R.M., Peakall D.B., 2001. Principles of ecotoxicology, Taylor & Francis, London.
- Wang W., 1990. Literature review on duckweed toxicity testing. *Environmental Research* 52, 7-22.
- Ward C.J., Codd G.A., 1999. Comparative toxicity of four microcystins of different hydrophobicities to the protozoan, *Tetrahymena pyriformis*. *Journal of Applied Microbiology* 86, 874-882.
- Watanabe M.F., Tsuji K., Watanabe Y., Harada K.I., Suzuki M., 1992. Release of a heptapeptide toxin (microcystin) during the decomposition process of *Microcystis aeruginosa*. *Natural toxins* 1, 48-53.
- Watanabe M.F., Park H.-D., Kondo F., Harada K.I., Hayashi H., Okino T., 1997. Identification and estimation of microcystins in freshwater mussels. *Natural toxins* 5, 31-35.
- Wei J., Liebert H.P., Braune W., 2000. Influence of microcystin-RR on growth and photosynthetic capacity of the duckweed *Lemna minor* L. *Journal of Applied Botany* 74, 100-105.
- Welker M., Bickel H., Fastner J., 2002. HPLC-PDA detection of cylindrospermopsin - opportunities and limits. *Water Research* 36, 4659-4663.
- Whitaker B.R., 2001. 'Water Quality' in K. M. Wright, B. R. Whitaker, (eds). Amphibian medicine and captive husbandry. Krieger Publishing, Malabar, Florida, pp. 147-157.
- White S.H., Duivenvoorden L.J., Fabbro L. 2004. *Effects of Cyanotoxins on Aquatic Snails and Shrimp*. Proceedings of the AWA (Qld) Regional Conference, 11-14th November, 2004, Kingfisher Bay, Fraser Island, Australian Water Association, Artarmon, NSW.

- White S.H., Duivenvoorden L.J., Fabbro L.D., 2005. Impacts of a toxic *Microcystis* bloom on the macroinvertebrate fauna of Lake Elphinstone, Central Queensland, Australia. *Hydrobiologia* 548, 117-126.
- Whitton B.A., Potts M., (eds). 2000. The ecology of cyanobacteria their diversity in time and space. Kluwer Academic Publishers, Dordrecht.
- WHO (World Health Organisation), 2004. Guidelines for drinking-water quality. Volume I: recommendations, World Health Organisation, Geneva.
- Wiedner C., Visser P.M., Fastner J., Metcalf J.S., Codd G.A., Mur L.R., 2003. Effects of light on the microcystin content of *Microcystis* strain PCC 7806. *Applied and Environmental Microbiology* 69, 1475-1481.
- Wiegand C., Pflugmacher S. 2001. 'Effects of microcystin-LR on detoxication enzymes' in I. Chorus, (ed.) Cyanotoxins occurrence, causes, consequences. Springer-Verlag, Berlin, pp. 253-257.
- Wiegand C., Pflugmacher S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicology and Applied Pharmacology* 203, 201-218.
- Wiegand C., Peuthert A., Pflugmacher S., Carmeli S., 2002. Effects of microcin SF608 and microcystin-LR, two cyanobacterial compounds produced by *Microcystis* sp., on aquatic organisms. *Environmental Toxicology* 17, 400-406.
- Wilbur H.M., 1997. Experimental Ecology of Food Webs: Complex systems in temporary ponds. *Ecology* 78, 2279-2302.
- Williams D.E., Dawe S.C., Kent M.L., Andersen R.J., Craig M., Holmes C.F., 1997. Bioaccumulation and clearance of microcystins from salt water mussels, *Mytilus edulis*, and *in vivo* evidence for covalently bound microcystins in mussel tissues. *Toxicon* 35, 1617-1625.
- Wood S.A., Holland P.T., 2005. Preferred and alternative methods for analysis and characterisation of cyanotoxins in drinking-water. Cawthron Report No. 1044. Prepared for the Ministry of Health, Cawthron, Nelson, New Zealand.
- Yamasaki S., 1993. Probable effects of algal bloom on the growth of *Phragmites australis* (Cav.) Trin. ex Steud. *Journal of Plant Research* 106, 113-120.
- Yin L., Huang J., Li D., Liu Y., 2005. Microcystin-RR uptake and its effects on the growth of submerged macrophyte *Vallisneria spiralis* (Lour.) Hara. *Environmental Toxicology* 20, 308-313.
- Yokoyama A., Park H., 2002. Mechanism and prediction for contamination of freshwater bivalves (Unionidae) with the cyanobacterial toxin microcystin in hypereutrophic Lake Suwa, Japan. *Environmental Toxicology* 17, 424-433.

- Yokoyama A., Park H., 2003. Depuration kinetics and persistence of the cyanobacterial toxin microcystin-LR in the freshwater bivalves *Unio douglasiae*. *Environmental Toxicology* 18, 61-67.
- Young F.M., Thomson C., Metcalf J.S., Lucocq J.M., Codd G.A., 2005. Immunogold localisation of microcystins in cryosectioned cells of *Microcystis*. *Journal of Structural Biology* 151, 208-214.
- Zurawell R., 2001. Occurrence and toxicity of microcystins in the freshwater pulmonate snail *Lymnaea stagnalis*. Ph D thesis. Department of Biological Sciences, University of Alberta, Edmonton, Alberta.
- Zurawell R., Kotak B., Prepas E., 1999. Influence of lake trophic status on the occurrence of microcystin-LR in the tissue of pulmonate snails. *Freshwater Biology* 42, 707-718.
- Zylstra U., 1972. Uptake of particulate matter by the epidermis of the freshwater snail *Lymnaea stagnalis*. *Netherlands Journal of Zoology* 22, 299-306.

Appendix A: recipe for ASM1 algal growth media

ASM1 growth media was prepared from an adaptation of Gorham *et al.* (1964) (Table A). Following preparation of media, the pH was brought to 7.7 – 7.9 by addition of sodium hydroxide (NaOH; 1 molar) or hydrochloric acid (HCl; 1 molar). Media was buffered using HEPES (N-2'-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and autoclaved (120°C, 15 minutes).

Table A. Ingredients for ASM1 algal growth media

Stock Solution Preparation	Working Solution Preparation
85 g L ⁻¹ NaNO ₃	2 mL
17.4 g L ⁻¹ K ₂ HPO ₄	1 mL
14.2 g L ⁻¹ Na ₂ HPO ₄	1 mL
40.62 g L ⁻¹ MgCl ₂ ·6H ₂ O	1 mL
49.33 g L ⁻¹ MgSO ₄ ·7H ₂ O	1 mL
29.4 g L ⁻¹ CaCl ₂ ·2H ₂ O	1 mL
1.0835 g L ⁻¹ FeCl ₃ ·6H ₂ O	1 mL
2.47 g L ⁻¹ H ₃ BO ₃	1 mL
1.3683 g L ⁻¹ MnCl ₂ ·4H ₂ O	1 mL
0.44 g L ⁻¹ ZCl ₂	1 mL
6.64 g L ⁻¹ Na ₂ EDTA	1 mL
0.216 g L ⁻¹ CoSO ₄ ·7H ₂ O	0.1 mL
0.013 g L ⁻¹ CuCl ₂ ·2H ₂ O	0.01 mL
Reverse osmosis water	To final volume of 1L

REFERENCE

Gorham, P. R., Mc Lachlan, J., Hammer U. T., Kim, W. K., 1964. Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Breb. *Verhandlungen Internationale Vereinigung für Theoretische und Angewandte Limnologie* 15: 796-804.

Appendix B: determination of cylindrospermopsin and deoxy-cylindrospermopsin in water samples

All toxin analyses were conducted at Queensland Health Scientific Services, Coopers Plains, Queensland (Australia); Geoff Eaglesham (QHSS) kindly provided the following method.

Cylindrospermopsin (CYN) and deoxy-cylindrospermopsin (deoxy-CYN) were determined by high performance liquid chromatography – tandem mass spectrometry using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) equipped with a turbo-ion spray interface coupled to a Perkin Elmer (Perkin Elmer, Norwalk, Connecticut) series 200 HPLC system (Eaglesham *et al.* 1999; Norris *et al.* 1999).

Separation was achieved using a 5 micron 150 X 4.6 mm Alltima C₁₈ column (Alltech, Australia) run at 35°C, and a flow rate of 0.8 ml min⁻¹ with a linear gradient starting at 100% A for 0.1 minutes, ramped to 100% B in 5 minutes, held for 1 minute and then to 100% A in 2 minutes and equilibrated for 7 minutes. The dead volume in the system modifies the actual gradient at the column and equates to approximately 3 minutes at 100% A before the start of the gradient (A = 1% methanol/deionised water, B = 60% methanol/deionised water, both 5mM in ammonium acetate). The column effluent was split to achieve a flow rate of 0.25 ml min⁻¹ to the mass spectrometer. The mass spectrometer was operated in the positive ion, multiple reaction-monitoring mode using nitrogen as the collision gas under the conditions listed in Table B.

Table B. Operation of mass spectrometer during determination of toxins in water and culture samples. CYN = cylindrospermopsin.

Analyte	Quantitation transition	Confirmation transition (% of Quant. Trans.)	Declustering potential, Focusing potential	Collision Energy (Quant., Conf.)	Retention time (mins)
CYN	416.3->194.1	416.3->176.1 (36%)	19, 125	47, 52	6.33
Deoxy-CYN	400.3->194.1	400.3 -> 176.1 (36%)	19, 125	47, 52	6.89

Positive samples were confirmed by retention time and by comparing transition intensity ratios between the sample and an appropriate concentration standard from the same run. Samples were only reported as positive if the two transitions were present, retention time was within 0.15 minutes of the standard and the relative intensity of the confirmation transition was within 20% of the expected value. The value reported was that for the quantitation transition.

Using a 150 μL injection volume the limit of detection for this method is typically less than $0.2 \mu\text{g L}^{-1}$ and response is linear to at least $500 \mu\text{g L}^{-1}$. For each batch of samples, one sample was run in duplicate, in addition to a blank and control sample. Over a six-month period the average concentration of the control sample was calculated as $23 \mu\text{g L}^{-1}$ with a standard deviation of 6.4%. This method generally gives 95% confidence limits of $\pm 13\%$ as determined from controls run with each sample batch (Geoff Eaglesham, personal communication).

Full HPLC and MS conditions and linearity data are stored in a method validation folder. Method 15506 (quantitative analysis using the PE/SCIEX API 300 mass spectrometer) should be consulted for the operating instructions and mobile phase preparation for the HPLC and Mass Spectrometer.

REFERENCES

- Eaglesham, G. K., Norris, K. R., Shaw, G. R., Smith, M. J., Chiswell, R. K., Davis, B. C., Neville, G. R., Seawright A. A., Moore, B. S., 1999. Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. *Environmental Toxicology* 14: 151-154.
- Norris, K. R., Eaglesham, G. K., Pierens, G., Shaw, G. R., Smith, M. J., Chiswell, R. K., Seawright, A. A., Moore, M. R., 1999. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 14: 163-165.

Appendix C: cylindrospermopsin and deoxy-cylindrospermopsin extraction and detection from plant and animal tissues

All plant extraction and analyses were conducted at Queensland Health Scientific Services (QHSS). Animal tissues were partially prepared at Central Queensland University, before being sent to QHSS for toxin determinations. Note that methods for the detection of deoxy-CYN became available only partway through the project, hence these analyses were not done for some plant tissues.

To extract plant (*Spirodela*, *Hydrilla*) material, 5 mL of HPLC grade water was added to the freeze-dried tissue, and homogenised using an Ultra Turrax homogeniser (IKA Works, Asia) at 24,000 rpm for approximately 1 minute. Water was considered a suitable extractant due to the hydrophilic nature of CYN, and according to the success of other authors (Saker & Eaglesham 1999). The homogenate was allowed to settle and 1.5 mL of the supernatant was filtered through a 0.45 micron filter (Millex-HV, Millipore Corporation, Bedford, Massachusetts) into a sample vial for analysis by high performance liquid chromatography – tandem mass spectrometry.

For extraction of animal (*Melanoides*, *Bufo*) tissues, the freeze-dried material was resuspended in 12 – 15mL of Milli-Q water (quality, 18.2 MΩ cm⁻¹ or better) and homogenised using UltraTurrax (24,000 rpm for approximately one minute each). Samples were immediately frozen to limit enzymatic breakdown of CYN and transported (frozen) to QHSS. Frozen samples were thawed, centrifuged at 3000 rpm and the supernatant filtered using 0.45 micron syringe filters (Millex HV, Millipore Corporation, Bedford, MA).

Cylindrospermopsin (CYN) and deoxy-cylindrospermopsin (deoxy-CYN) were determined by HPLC-MS/MS using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) equipped with a turbo-ionspray interface coupled to a Shimadzu SCL-10Avp HPLC system (Shimadzu Corporation, Kyoto, Japan) (Eaglesham *et al.* 1999; Norris *et al.* 1999).

Separation was achieved using a 5 micron 150 X 4.6 mm Alltima C₁₈ column (Alltech Associates (Australia) Baulkham Hills, NSW) run at 35°C, and a flow rate of 0.8 ml min⁻¹ with a linear gradient starting at 100% A for 2.0 minutes, ramped to 100% B in 5 minutes, held for 1 minute and then to 100% A in 0.2 minutes and equilibrated for 5 minutes. The dead volume in the system modifies the actual gradient at the column and equates to approximately 3 minutes at 100% A before the start of the gradient (A = 1% methanol/deionised water, B = 60% methanol/deionised water, both 5 mM in ammonium acetate).

The column effluent is split to achieve a flow rate of 0.25 ml min⁻¹ to the mass spectrometer. The mass spectrometer is operated in the positive ion, multiple reaction-monitoring mode using nitrogen as the collision gas with conditions as listed in Table C.

Table C. Operation of mass spectrometer during determination of tissue toxins. CYN = cylindrospermopsin.

Analyte	Quantitation transition	Confirmation transition (% of Quant. Trans.)	Declustering potential, Focusing potential	Collision Energy (Quant., Conf.)	Retention time (mins)
CYN	416.3->194.1	416.3->176.1 (36%)	19, 125	47, 52	6.33
Deoxy-CYN	400.3->194.1	400.3 -> 176.1 (36%)	19, 125	47, 52	6.89

Positive samples are confirmed by retention time and by comparing transition intensity ratios between the sample and an appropriate concentration standard from the same run. Most positive samples will also contain deoxy-CYN. Samples were only reported as positive if the two transitions are present, retention time is within 0.15 minutes of the standard and the relative intensity of the confirmation transition is within 20% of the expected value. The value reported is that for the quantitation transition.

Using a 120 μL injection volume, the limit of detection for *Spirodela* and *Hydrilla* tissues was less than $0.2 \mu\text{g L}^{-1}$ (equivalent to 1.0 nanogram in 5 ml) and response was linear to at least $500 \mu\text{g L}^{-1}$. For *Melanoides* and *Bufo* tissues, a slightly higher limit of detection was achieved at $0.5 \mu\text{g L}^{-1}$ (approximately equivalent to 3.0 nanograms per 5 mL sample) due to increased interference(s). For example, in the *Melanoides* samples, the signal to noise ratio was approximately 3:1.

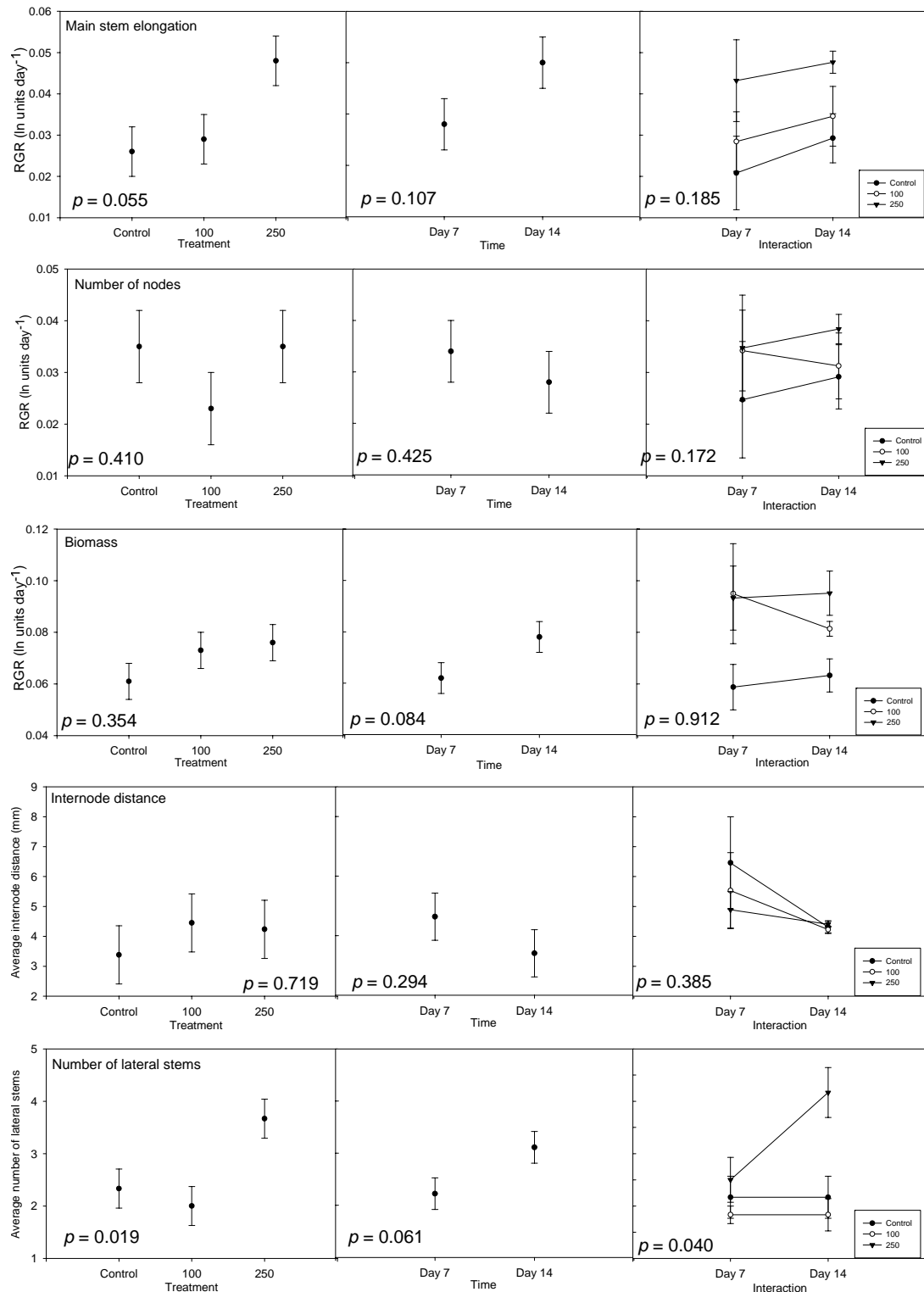
REFERENCES

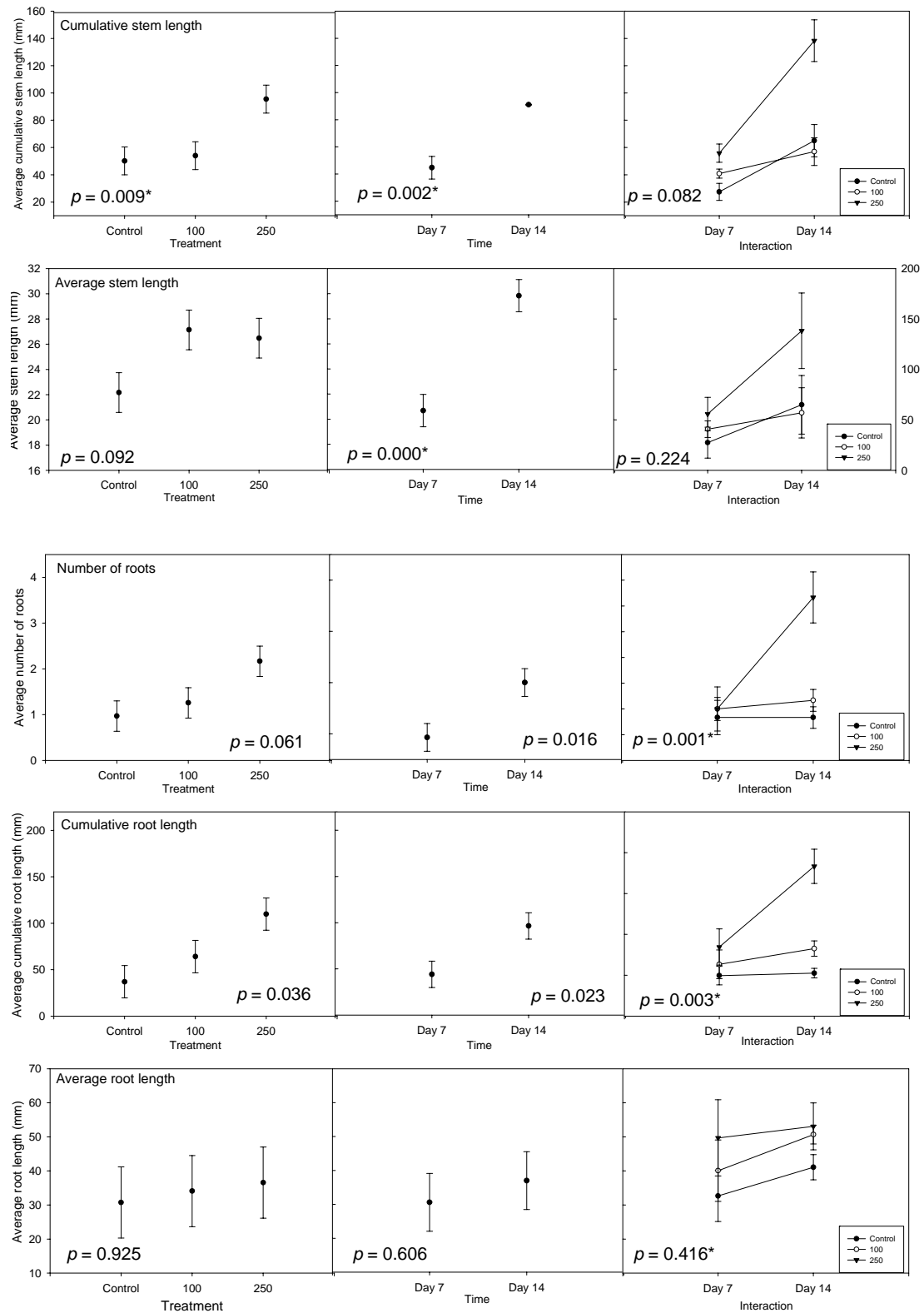
- Eaglesham, G. K., Norris, K. R., Shaw, G. R., Smith, M. J., Chiswell, R. K., Davis, B. C., Neville, G. R., Seawright A. A., Moore, B. S., 1999. Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. *Environmental Toxicology* 14: 151-154.
- Norris, K. R., Eaglesham, G. K., Pierens, G., Shaw, G. R., Smith, M. J., Chiswell, R. K., Seawright, A. A., Moore, M. R., 1999. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 14: 163-165.
- Saker, M. L., Eaglesham, G. K., 1999. The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus*. *Toxicon* 37: 1065-1077.

Appendix D: *Hydrilla verticillata* growth graphs

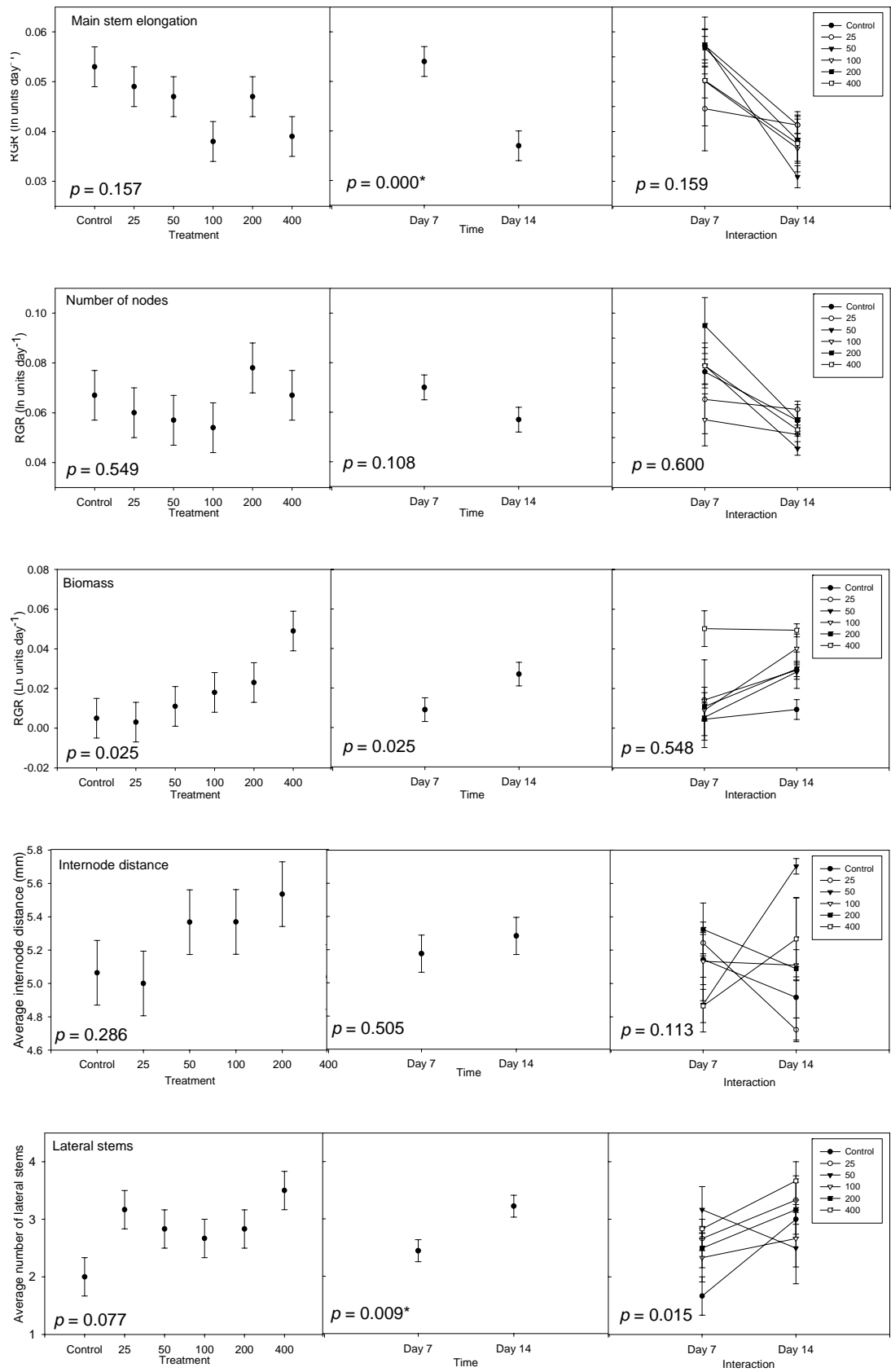
Growth of *Hydrilla verticillata* following exposure to *Cylindrospermopsis raciborskii* whole cell extracts containing cylindrospermopsin. Graphs show means (pooled for treatment and time) \pm standard error; ' p ' values are univariate results; '*' indicates significance at $\alpha = 0.010$ (heterogeneous datasets).

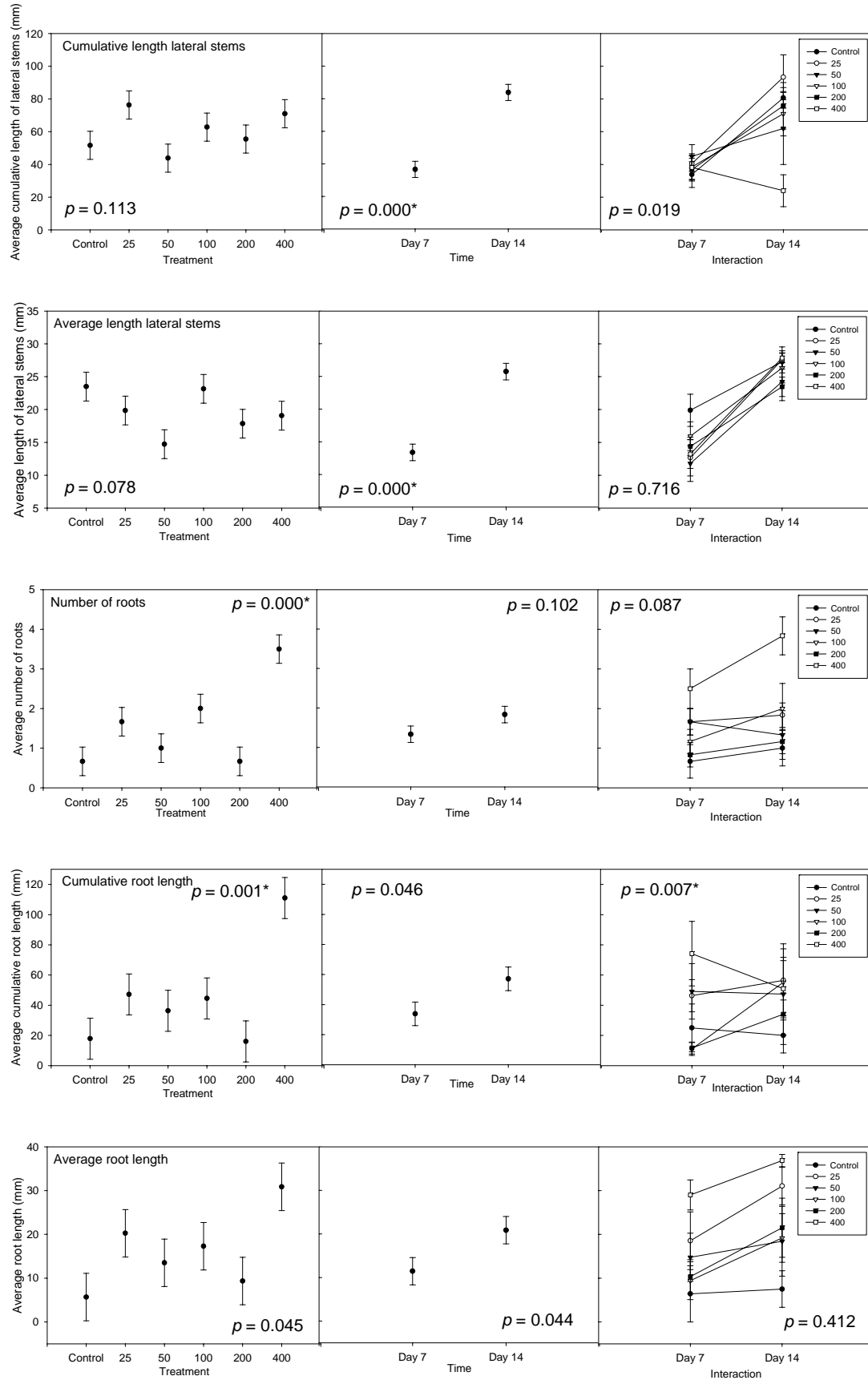
TRIAL ONE

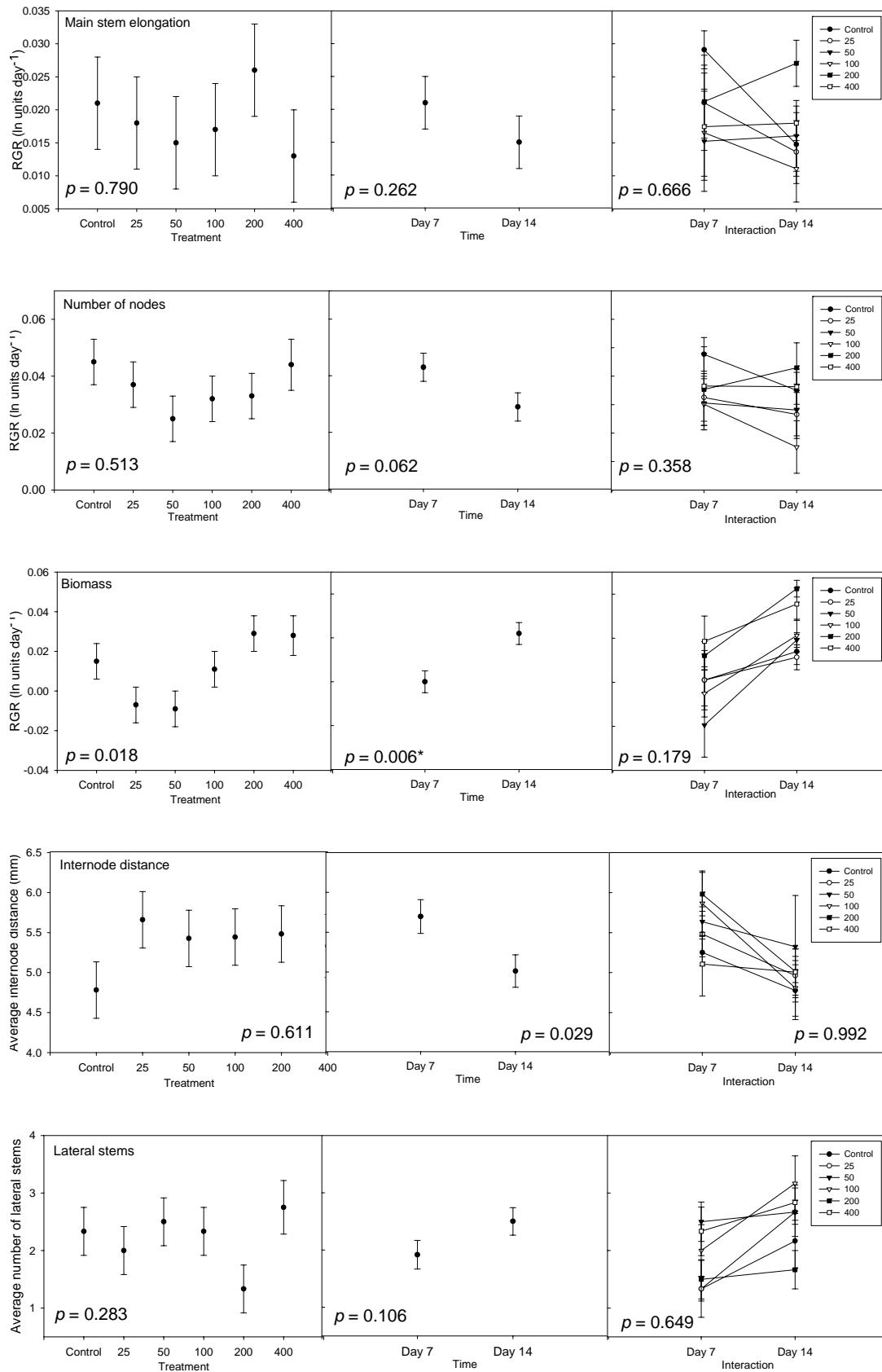


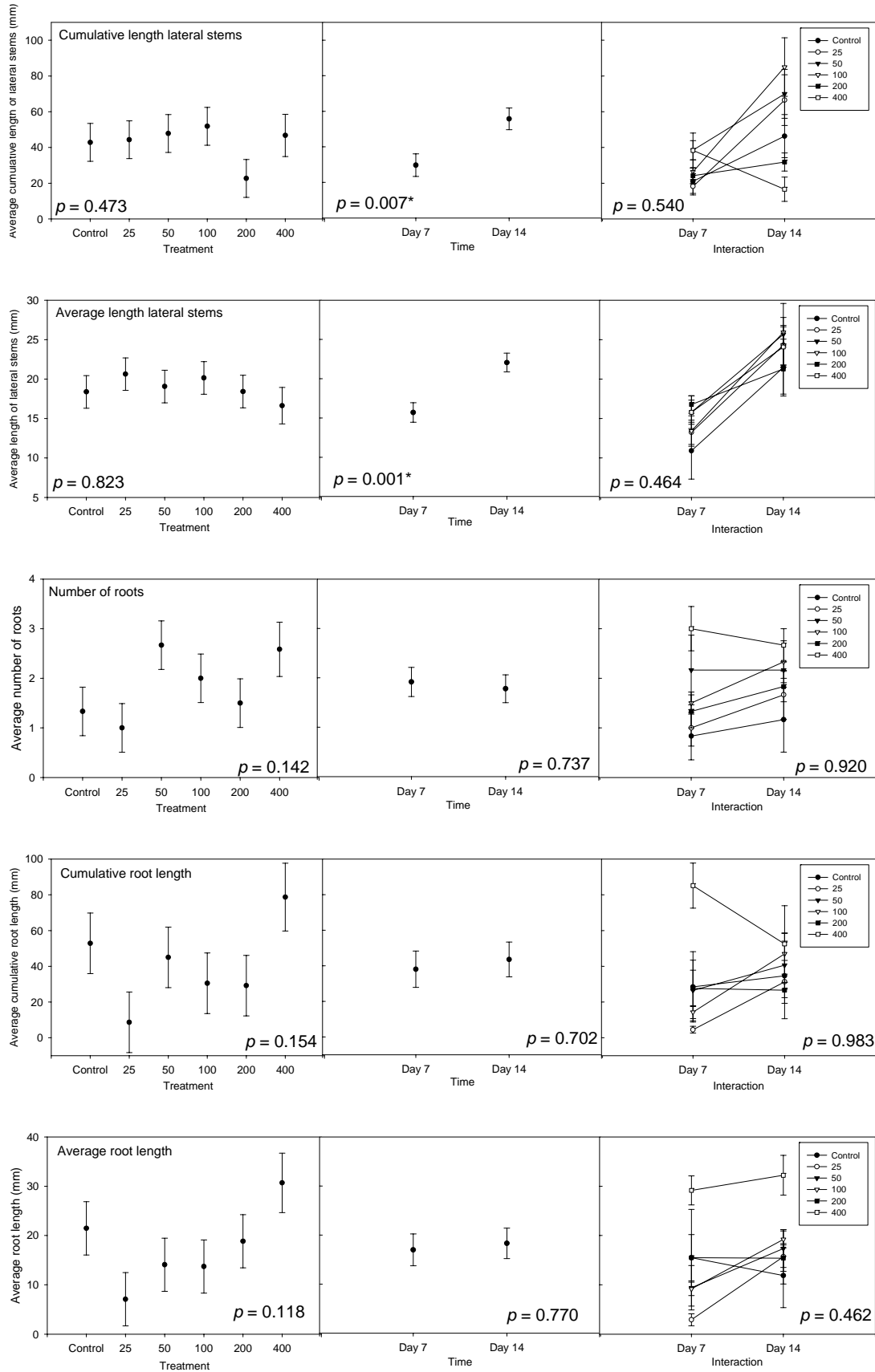


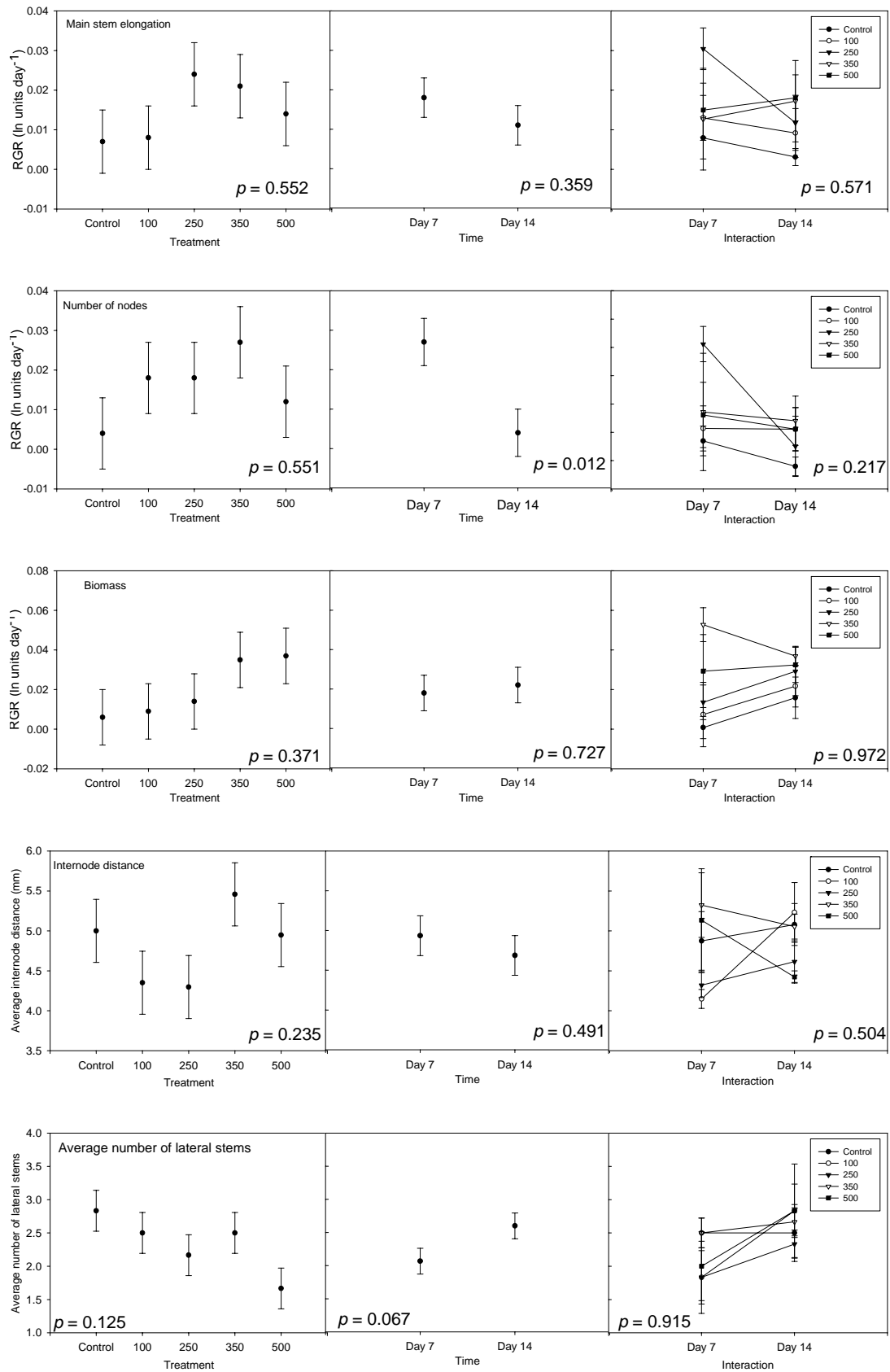
TRIAL TWO

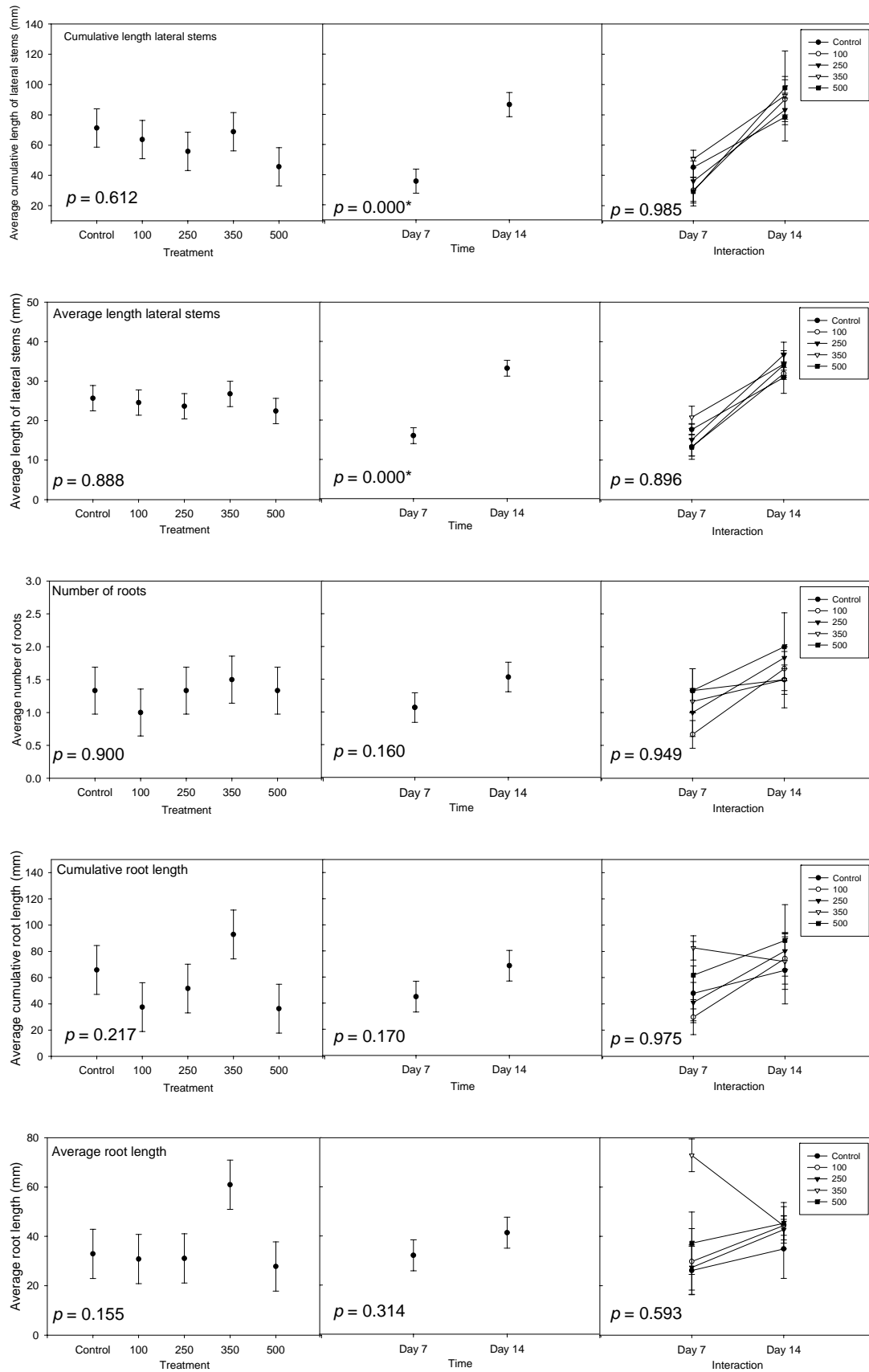




TRIAL THREE



TRIAL FOUR (DEPURATION TRIAL)



Appendix E: protocols for the preparation of tadpole histology sections

Bufo marinus tadpoles were fixed whole for two to three months in 10% neutral buffered formalin (prepared using 10% formalin (1000mL); 4 g of sodium acid phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$); and 6.5 g of anhydrous disodium phosphate (Na_2HPO_4)) (Drury & Wallington 1980, p. 49).

The following protocol was adopted from a modification of Berrill (2002) and Walsh & CQU biology staff (2000). Tadpoles were placed into labelled cassettes and rinsed in running tap water for one hour; and subjected to the following series:

Dehydration:

1. 30% EtOH	1 hour
2. 50% EtOH	1 hour
3. 70% EtOH	1 hour
4. 100% EtOH	1 hour
5. 100% EtOH	1 hour

Clearing

6. 50% EtOH / 50% Histolene	1 hour
7. 100% Histolene	1 hour

Wax infiltration

8. Paraffin (56°C)	1 hour
9. Paraffin (56°C)	1 hour

Specimens were embedded whole in Paraplast®+ (Medos, Queensland) at 59°C, with the body oriented vertically and the nose facing down (Shandon Histocentre embedding station). Sections were cut at 4 – 10 µm (Spencer 820 microtome), using serial sectioning, with tissues generally comprising the nose tip, mouth (pharyngeal) area, eyes, heart (pericardial) region, foregut, midgut, hindgut and tail. Ribbons were placed into a flotation bath at 52 – 53°C with slide adhesive added at the ratio of 2 mL adhesive to 1L tap water. Slide adhesive was prepared using 2 g gelatin, 2 g potassium dichromate, and 100mL distilled water. Sections were floated onto frosted-end slides and incubated overnight (minimum) at 53°C.

Slides were stained using Harris' Modified Haematoxylin (SigmaAldrich; HHS-32) and Eosin Y solution, alcoholic, with phloxine (SigmaAldrich; HT110-3-32), using the following protocol:

Deparaffinise:

Clearing agent (SigmaAldrich Histochoice; H2779)	3 minutes
Clearing agent	3 minutes

Rehydrate:

Isopropanol absolute (SigmaAldrich)	3 minutes
Isopropanol 90%	3 minutes
Isopropanol 70%	3 minutes
Deionised water rinse	4 minutes

Stain:

Harris' modified haematoxylin	1 minute
Reverse osmosis water	4 minutes
Tap water*	3 minutes

Counterstain:

Deionised water	4 minutes
Eosin Y solution	15 seconds

Dehydration:

Isopropanol 70%	6 dips
Isopropanol 90%	6 dips
Isopropanol absolute	6 dips

Clearing:

Clearing agent	1 minute
Clearing agent	2 – 3 minutes

Mounting:

Slides were then permanently mounted using 'DPX'.

* Rockhampton municipal water supply is generally alkaline and was used in placement of Scott's blueing solution.

REFERENCES

Berrill M., 2002. Histology protocols. Available from www.trentu.ca/biology/berrill/histology_protocols.htm , accessed 31 May 2005.

Drury RAB & Wallington EA, 1980. Carleton's Histological Technique, Oxford University Press, Oxford.

Walsh, K., Central Queensland University (CQU) biology staff, 2000. Histotechniques Laboratory Manual, Central Queensland University Division of Teaching and Learning Services, Rockhampton.

Appendix F: Copies of published papers arising from the thesis

In order of acceptance:

1. **White, S.H.**, Duivenvoorden, L. J. & Fabbro, L. D., 2005. A Decision-making Framework for Ecological Impacts Associated with the Accumulation of Cyanotoxins (Cylindrospermopsin and Microcystin). *Lakes and Reservoirs: Research and Management* 10: 25-37.
2. **White, S.H.**, Duivenvoorden, L. J. & Fabbro, L. D., 2005. Absence of free-cylindrospermopsin bioconcentration in water thyme (*Hydrilla verticillata*). *Bulletin of Environmental Contamination and Toxicology* 75 (3): 574-583.
3. **White, S.H.**, Duivenvoorden, L. J., Fabbro, L. D. & Eaglesham, G. K., 2006. Influence of intracellular toxin concentrations on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata*). *Toxicon* 47(5): 497-509.

A decision-making framework for ecological impacts associated with the accumulation of cyanotoxins (cylindrospermopsin and microcystin)

Susan H. White,* Leo. J. Duivenvoorden and Larelle D. Fabbro

Freshwater Ecology Group, Centre for Environmental Management, Central Queensland University, Rockhampton, Queensland, Australia

Abstract

Toxins produced by cyanoprokaryotes are a key issue in aquatic management because of their potential to exert adverse effects on humans and aquatic biota. The information gap regarding bioaccumulation and biomagnification processes associated with cyanotoxins, however, has resulted in inadequacies in the management and maintenance of biological diversity in lakes and reservoirs affected by toxic cyanoprokaryote blooms. This paper examines the potential for, and effects of, bioaccumulation of two common cyanotoxins, microcystin and cylindrospermopsin, in aquatic organisms. The factors influencing cyanotoxin bioavailability are discussed in the context of the challenges associated with understanding and managing toxin accumulation. Based on the characteristics of cyanotoxin bioavailability, exposure and uptake routes, a theoretical, predictive model for cyanotoxin bioaccumulation is proposed. Key concepts include monitoring changes in toxin availability throughout the progression of a toxic bloom and the prediction of ecological effects based on internal tissue concentrations. The model explores the minimum requirements that managers must undertake in order to properly assess bioaccumulation risk in terms of frequency of toxin testing, toxin fraction determination and assessment of aquatic organisms.

Key words

bioaccumulation, blue-green algae, cyanoprokaryotes, cylindrospermopsin, ecological risk assessment, microcystin, modelling.

INTRODUCTION

Reports of lakes and reservoirs affected by toxic cyanoprokaryote (blue-green algal) blooms are increasing around the world (e.g. Padisák 1997; Chorus & Bartram 1999; Bouvy *et al.* 2000; Frank 2002). Such blooms demand attention because of their potential toxicity to terrestrial and aquatic animals and the possible health risks posed for humans in recreational and drinking-water storage bodies (Carmichael *et al.* 2001; Carmichael & Falconer 1993; Falconer 2001).

Two globally common freshwater toxins are cylindrospermopsin (CYN) and microcystin (MC). The former are produced by strains of *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*, *Anabaena bergii* var. *limnetica* and *Raphidiopsis curvata* (Li *et al.* 2001; Shaw *et al.* 1999; Schembri *et al.* 2001), while the latter are

produced by *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc* (Rinehart *et al.* 1994; Botes *et al.* 1984). These toxins exert acute and chronic lethal and sublethal effects on a range of organisms. Historically, Schwimmer and Scwhimmer (1968) reported accounts of deaths linked to blue-green algal intoxication in cattle, sheep, horses, dogs, cats, domestic fowl (geese, chickens, ducks), birds, squirrels, mice, turkeys and extensive fish kills. More recent work also has demonstrated a range of sublethal effects to other aquatic flora and fauna, including growth inhibition and reproductive effects in zooplankton (Ferrão-Filho *et al.* 2002a; Ferrão-Filho & Azevedo 2003), macroinvertebrates and aquatic insects (Beattie *et al.* 2003; Delaney & Wilkins 1995; Hiripi *et al.* 1998), accumulation in crayfish (Liràs *et al.* 1998; Saker & Eaglesham 1999) and reduced growth in aquatic plants (Kirpenko 1986; Pflugmacher 2002; Romanowska-Duda & Tarczyska 2002).

Adverse human health effects associated with MC and CYN include gastrointestinal complaints, skin allergies

*Corresponding author. Email: s.white@cqu.edu.au

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and, possibly, tumour promotion and increased risks of primary liver cancer (Ueno *et al.* 1999; Xu *et al.* 2000; Falconer 2001). Microcystin was found to be responsible for the deaths of dialysis patients treated with water from the MC-containing Tabocas Reservoir (Komarek *et al.* 2001), while CYN was implicated in the so-called Palm Island mystery disease, which resulted in the hospitalization of > 100 children suffering acute gastroenteritis (Byth 1980; Hawkins *et al.* 1985).

This paper examines the potential for increased toxicity of MC and CYN resulting from bioaccumulation and biomagnification processes. Currently, lack of data and scientific understanding regarding bioaccumulation has contributed to inadequacies in management procedures addressing the ecological and human health hazards associated with exposure to these toxins. A new management approach also is suggested, whereby a predictive model is used to enable managers of affected lakes and reservoirs to gauge periods of high bioaccumulation risk based on toxin availability and to estimate adverse effects to aquatic biota based on tissue toxin concentrations.

BIOACCUMULATION OF CYANOTOXINS: EVIDENCE AND EFFECTS

The extensive range of effects associated with direct cyanotoxin exposure in aquatic animals, and evidence of their dose-dependency (Carmichael 1996; Chong *et al.* 2000; Zimba *et al.* 2001; Vasas *et al.* 2002), provide a compelling argument to study the bioaccumulation and biomagnification potential of cyanotoxins in aquatic organisms.

Several factors indicate that cyanotoxins might bioaccumulate and biomagnify similarly to other toxicants, such as heavy metals and pesticides (Sivonen & Jones 1999). Cylindrospermopsin and MC are both stable (Ohtani *et al.* 1992; Harada 1996), which is considered necessary for accumulation to occur (van der Oost *et al.* 2003). Both also have structural features conducive to accumulation processes. Microcystin is generally hydrophobic (Vestervik & Meriluoto 2003), thereby increasing the likelihood of its partitioning into lipid tissues of aquatic organisms and, although being a large compound (800–1100 Da; Sivonen & Jones 1999), is possibly cell-permeant (Duy *et al.* 2000). Though CYN is hydrophilic, it is a relatively small compound (Sivonen & Jones 1999; Shaw *et al.* 2000), usually represented by extracellular toxin fractions (Griffiths & Saker 2003; Hawkins *et al.* 2001). Thus, CYN accumulation might result from contact via gills and dermal surfaces, or from accidental ingestion by drinking.

The octanol–water partitioning coefficient (K_{ow}), a value often used to indicate bioaccumulation potential, also suggests likely accumulation of these cyanotoxins. Values

of $\log K_{ow} > 3$ are considered to have high potential (Franke *et al.* 1994). Estimated values for MC-LR, MC-LY, MC-LW and MC-LF, using high-performance liquid chromatography techniques, were 2.16, 2.92, 3.46 and 3.56, respectively (Ward & Codd 1999).

Finally, the evolution of aquatic organisms alongside cyanoprokaryotes might have allowed the development of mechanisms to counter cyanotoxicity (Lirås *et al.* 1998), enabling organisms to continue to accumulate large amounts of toxins without side-effects. Such organisms represent excellent vectors through which toxins can transfer up the food chain (Christoffersen 1996), thereby enabling biomagnification.

Only two published works to date have examined the bioaccumulation of CYN. Saker and Eaglesham (1999) detected CYN in the muscle, hepatopancreas and abdominal tissues of the crayfish *Cherax quadricarinatus*, at a maximum concentration of $4.3 \mu\text{g g}^{-1}$ freeze dried tissue. Toxin also was reported ($1.2 \mu\text{g g}^{-1}$ freeze dried tissue) in the visceral tissue of rainbow fish (*Melanotaenia eachamensis*). Most recently, Saker *et al.* (2004) reported the accumulation of CYN in the swan mussel (*Anodonta cygnea*), with maximum levels of $61.5 \mu\text{g g}^{-1}$ (haemolymph), $5.9 \mu\text{g g}^{-1}$ (viscera) and $2.52 \mu\text{g g}^{-1}$ (whole body). In contrast, MC accumulation has been reported at $\text{ng} - \mu\text{g g}^{-1}$ levels in organisms representing all components of aquatic systems, including aquatic plants (Wiegand & Pflugmacher 2001), zooplankton (Ferrão-Filho *et al.* 2002b), snails (Kotak *et al.* 1996; Zurawell 2001; Ozawa *et al.* 2003), mussels and clams (Yokoyama & Park 2003, 2002; Eriksson *et al.* 1989; Prepas *et al.* 1997; Williams *et al.* 1997), and higher animals such as crayfish (Vasconcelos 1995; Lirås *et al.* 1998) and fish (Xie *et al.* 2004; Magalhães *et al.* 2001; Mohamed 2001).

A key issue surrounding the bioaccumulation and biomagnification of cyanotoxins is the possibility for toxic blooms to exert greater toxicity due to higher concentrations of internalized toxins being accrued in bodily tissues. There currently is a dearth of information regarding the sublethal effects of cyanotoxins, especially in aquatic organisms. Most studies have focused on effects at the lowest levels of biological organization. As a result, ecosystem-level effects remain the least well understood of all possible impacts, especially the long-term implications for higher-level consumers (Landsberg 2002). Therefore, a relevant question is what new effects might be possible due to increased tissue toxin levels and exposure?

At the organ level, the effects of MC exposure include necrosis and haemorrhaging of liver tissue, hepatic malfunction and heart stress in laboratory rats and fish (Falconer *et al.* 1981; Dawson 1998; Heinze 1999; Fischer *et al.* 2000; Best *et al.* 2001). In aquatic plants, MC

exposure reduces growth rates and seed germination, and alters photosynthetic pigmentation (Pflugmacher 2002; Casanova *et al.* 1999; McElhiney *et al.* 2001; Romanowska-Duda & Tarczynska 2002). Reduced embryo survival, developmental abnormalities and population changes have also been reported in fish (Oberemm *et al.* 1999; Oberemm 2001; Liu *et al.* 2002). These developmental effects might have significant implications as prolonged or recurrent toxic blooms, which cover entire or successive breeding seasons (Baker & Humpage 1994), might prevent the development of larval and young stages of organisms. Other effects include reduced species diversity (MacKintosh *et al.* 1990), altered predator–prey interactions and other behavioural changes (Oberemm 2001).

Little is known regarding MC transfer in food webs. The sensitivity of aquatic organisms to MC exposure is known to vary with trophic level. Fish, crustaceans and rotifers appear to be most tolerant ($0.5\text{--}20\ \mu\text{g L}^{-1}$), followed by phytoplankton ($0.05\text{--}1\ \mu\text{g mL}^{-1}$) and macrophytes ($0.001\text{--}0.05\ \mu\text{g mL}^{-1}$) (Christoffersen 1996). Kotak *et al.* (1996) examined MC–LR bioaccumulation in different trophic levels and found detectable MC only in grazers, with a strong possibility of MC–LR transfer to terrestrial food webs. Ferrão-Filho *et al.* (2002b) concluded that zooplankton were more efficient at transferring MC to higher trophic organisms than were other organisms (e.g. molluscs). Such food chain transfers represent potential human risks from consuming organisms, such as crayfish, mussels and fish (Saker & Eaglesham 1999). Moreover, they have implications for biomanipulation management approaches as consumers might remove toxic phytoplankton from the water (Boon *et al.* 1994; Matveev *et al.* 1994), only to accumulate it into other food web compartments.

The effects of CYN exposure are critically understudied. Cylindrospermopsin is associated with severe hepatic necrosis (Terao *et al.* 1994), which results from the inhibition of protein synthesis (Shaw *et al.* 2000) and, possibly, DNA disruption (Shen *et al.* 2002). Cylindrospermopsin toxicity in mammals is associated with hepatic and kidney malfunctions (Falconer *et al.* 1999; Duy *et al.* 2000). It also has been found to exert effects on molluscan neurones and neurotransmitter receptors (Kiss *et al.* 2002) and causes mortality in insects and cattle (Hiripi *et al.* 1998; Saker *et al.* 1999). Studies have not been conducted on chronic CYN exposure in any species (Carson 2000).

CURRENT ALGAL MANAGEMENT APPROACHES

In 2001, the World Health Organization released an internationally accepted guide to the monitoring, assessment and management of toxic algal blooms (Chorus

2001). However, these guidelines focus more on human health consequences than on environmental risks. The recommended safe levels for drinking water are generally aimed at protecting human users; although toxin accumulation into aquatic biota is mentioned as potentially hazardous for consumers, no strategies for minimizing ecological risks are proposed. In Australia, a global hotspot for toxic blooms, monitoring and management of toxic blooms occurs at both national and state levels (Jones *et al.* 2002; Queensland Department of Natural Resources and Mines 2003). Risk levels are assigned on the basis of cell and toxin concentration data; again, these data are not interpreted with respect to bioaccumulation and/or biomagnification.

The effects exerted by MC and CYN clearly indicate that consideration of ecological risk is required for a total approach to managing toxic blooms. Guidelines that rely on cell concentration data for calculating human risks are not adequate for this purpose. Similarly, while critical for management of drinking water bodies, reactive management strategies, such as flocculation, sedimentation and chlorination (Hoeger *et al.* 2004), are inadequate for ecological risk management as they focus on removing algal cells and toxins already present in the system. Predictive modelling approaches, from which likely periods of bioaccumulation and biomagnification can be calculated, are best equipped to minimize the toxic effects of blooms in relation to both human and ecological risks.

CHALLENGES IN MODELLING CYANOTOXIN ACCUMULATION

Modelling the bioaccumulation processes of cyanotoxins is likely to be highly complex, as cyanotoxins, unlike most other toxicants, are produced within living cells. For most (synthetic) toxicants, the likelihood of bioaccumulation depends on bioavailability, as well as other species-specific factors, including feeding, metabolism, growth dilution and uptake efficiencies (McCarty & Mackay 1993). With cyanotoxins, however, bioavailability itself is governed by further factors, including the type and concentration of available toxins, their spatial and temporal variability and their uptake routes. Understanding and inclusion of these factors is critical for effective bioaccumulation modelling.

Many factors influence toxin production by cyanoprokaryote cells, though the significance of each remains poorly understood. The toxin content of blooms can vary, depending on the number and physiological status of algal cells (Orr & Jones 1998), and on various environmental conditions (Utkilen & Gjelme 1995; Saker & Griffiths 2000). Although a single species (or strain) of cyanoprokaryotes can be identified as being toxigenic, this does not necessarily mean that toxin production occurs (Chorus *et al.* 2001).

Moreover, increased cyanoprokaryote cell densities do not necessarily correspond to increased toxicity (Bickel *et al.* 2000; Jacoby *et al.* 2000). Many studies have examined likely triggers for toxin production and the conditions under which maximal toxin production might occur, including relationships between cell division rates and the toxin content of cells (e.g. Orr & Jones 1998; Bickel *et al.* 2000), and environmental parameters such as nutrient, temperature and light conditions (Long *et al.* 2001; Saker & Griffiths 2000; Saker & Neilan 2001). Toxin decomposition processes, which also play a role in toxin content, depend on factors such as time, pH, temperature, light, water quality and microbial activity (Lahti *et al.* 1997b; Chiswell *et al.* 1999).

Variability in toxin production also is related to the toxin composition of a given bloom, even for monospecific occurrences. For example, > 65 variants of MC have been identified (Carmichael 2001), although a natural bloom rarely contains > 10 (Eaglesham, pers. comm. 2002). Similarly, *C. raciborskii* can produce more than one variant of CYN (Hawkins *et al.* 1997; Falconer 1999; Norris *et al.* 2002). Additive or synergistic effects also might result from the presence of more than one toxin or toxin variant. The implication for bioaccumulation modelling here is that predictions of toxicity based on identification and cell counts of toxigenic species are not sufficient as toxin production is highly variable within these parameters.

Furthermore, cyanotoxins might be represented by three different toxin fractions. First, intracellular toxins are those held within algal cells. Second, extracellular toxins are those lysed from cells into the surrounding water. Third, incorporated toxins are intracellular or extracellular toxins which, as a result of uptake and/or adsorption processes, become part of or are attached to, the cells of aquatic organisms. Incorporated toxins might be reversibly bound to protein phosphatases or other cellular enzymes, but do not include toxins existing in the alimentary canal of algal grazers, which might later pass out of the organism. Blooms of different cyanotoxins are comprised of different toxin fractions. For example, MC is usually intracellular (Long *et al.* 2001; Berg *et al.* 1987; Watanabe *et al.* 1992; Lahti *et al.* 1997b), although Wiedner *et al.* (2003) found extracellular concentrations to be 20% higher in cultures with higher light irradiance. In contrast, CYN is mostly extracellular (Chiswell *et al.* 1999; Carson 2000). Some evidence suggests variations in toxin fractions might be controlled, even at the species level, based on environmental conditions (Hawkins *et al.* 2001).

As a result of their influence on the uptake route, the availability of the different toxin fractions is critical to modelling its bioaccumulation. Intracellular toxins might pose significant risks to organisms which ingest toxin-

laden cells, either through grazing or accidental ingestion. In contrast, aquatic plants might be at greater risk from extracellular toxins if transdermal uptake is possible. Incorporated toxins might represent risks for consumers not exposed to toxins directly (e.g. terrestrial birds). Consequently, the availability of particular toxin fractions will be significant in influencing bioaccumulation in a given set of organisms (e.g. intracellular toxins for grazers).

The temporal and spatial variability of toxins also impacts the availability of toxins to aquatic biota. Temporal variation in each of the toxin fractions occurs as a result of production, decomposition and uptake processes during the progression of toxic blooms, which results in some fractions being more abundant at certain times. These dynamics can be mapped, for example, for a *Microcystis* bloom (Fig. 1). The early stages of a toxic bloom are associated mostly with intracellular toxin, consistent with production and storage within cyanoprokaryote cells. As the bloom ages, however, some cells die and lyse, releasing small amounts of extracellular toxins into the water. Meanwhile, newly made cells continue to add to intracellular toxin levels. In the latter stages of the bloom, the situation becomes reversed; as more cells lyse, intracellular toxin production is slowed and extracellular toxin becomes more abundant. Furthermore, incorporated toxins might now be present in aquatic organisms capable of accumulation. Finally, during and after the bloom collapse, extracellular toxins might either persist or be rapidly degraded (Lahti *et al.* 1997a), while incorporated toxins remain until depuration and/or clearance occurs (Falconer 1993; Zurawell 2001; Beattie *et al.* 2003; Yokoyama & Park 2003).

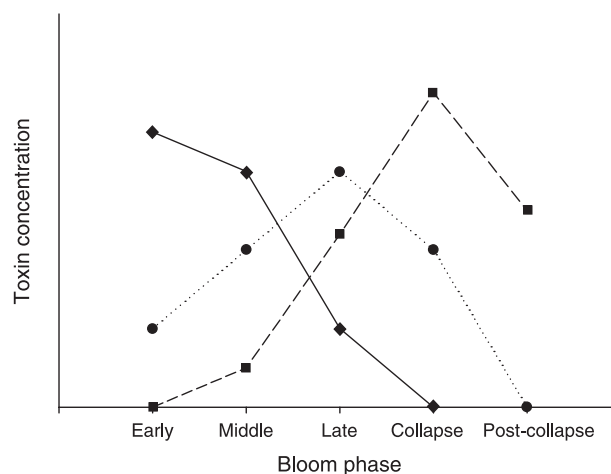


Fig. 1. Speculated change in microcystin toxin fractions with *Microcystis* bloom progression. ◆, intracellular; ●, extracellular; ■, incorporated.

Although Christoffersen (1996) suggested peak toxicity occurred in the later stages of toxic blooms, when cells lyse and release large amounts of toxins into the water, this might not necessarily be true for bioaccumulation effects (Fig. 1). Rather, as each toxin fraction becomes dominant, certain exposure routes vary in importance, and organisms with those uptake routes become susceptible to toxin bioaccumulation and its effects. For example, plants cannot ingest algal cells and, therefore, are not at risk from intracellular toxins. Thus, plants might not be predisposed to bioaccumulation early in bloom formation. Conversely, algal grazers could be considered to be at higher risk during this time. Thus, the dynamics of toxin fraction availability is a key issue to identifying peak bioaccumulation risk times in different aquatic organisms.

Spatial change in toxin fractions also might impact on bioavailability to aquatic organisms. Although intracellular toxins are held within living cells, for example, the distribution of the cells is not homogenous in a system. As a result, concentrations of intracellular toxin might occur at certain depths, related to gas-vacuolate cells regulating buoyancy according to light preferences. Wind-induced cell (thus, intracellular toxin) accumulations might occur in surface species, such as *Microcystis* (Webster 1990), while other subsurface species (e.g. *C. raciborskii*) might impact pelagic fauna. Furthermore, sinking, senescing cells contribute to an abundance of extracellular toxins in benthic habitats. Accordingly, the habitats of aquatic animals might predispose them to exposure to certain toxin fractions, an example being benthic animals and sinking, senescing cells releasing extracellular toxin.

A final factor influencing the likelihood of bioaccumulation is the individual capability of each aquatic animal to respond to cyanotoxins. Some organisms might be able to minimize or avoid exposure to cyanotoxins, thereby decreasing the likelihood of its bioaccumulation. Zurawell (2001) surmised, for example, that sessile filter feeders might experience the greatest exposure because they are unable to readily move to another location and have limited alternatives for food. However, coexistence of such animals with toxic algae over evolutionary time scales also might have given them the opportunity of developing tolerance to the toxins. Internal physiology also could be significant; interactions between toxins and internal proteins might occur in some organisms (Kankaanpää *et al.* 2002), while others might convert toxins to a different form or compound, particularly for CYN (Runnegar *et al.* 1994). These kinds of processes might limit or prevent bioaccumulation of cyanotoxins and their effects.

The range of factors influencing cyanotoxin bioavailability and, thus, bioaccumulation potential, clearly makes con-

structing a predictive model for bioaccumulation difficult to accomplish. However, the ecological effects associated with cyanotoxin bioaccumulation makes a clear case for developing such a model.

A MODEL FOR PREDICTING TOXIN BIOACCUMULATION AND ITS RISKS

Based on current knowledge of general bioaccumulation processes and of the specific nature of cyanotoxins, three factors are critical in enabling the bioaccumulation of cyanotoxins: (i) the toxin is in a form readily available for uptake by a given organism (i.e. fraction availability); (ii) the toxin is available in sufficiently high concentrations for bioaccumulation to occur (i.e. toxin threshold); and (iii) the toxin is present for a sufficient period of time to allow its accumulation into organismal tissues (i.e. exposure). The first of these factors, fraction availability, already has been discussed in the context of influencing bioaccumulation through associations with different uptake routes.

The second requirement for bioaccumulation is a suitable toxin concentration. A threshold concentration presumably exists, below which toxin accumulation does not occur, with the metabolic capabilities of aquatic organisms regulating or preventing its accumulation in tissues. Moreover, the concentration is likely to differ between species, based on the metabolic capabilities of each species (Fig. 2). The first animal ('non-regulator') does not have the metabolic capability to process (depurate, detoxify or metabolize) toxin, so that once exposure to the toxin occurs, it accumulates in the tissues. The second animal ('regulator'), although exposed to the same toxin concentration, is capable of processing small amounts of it ($< 4 \mu\text{g L}^{-1}$), thereby preventing bioaccumulation from low-level exposure. This capability fails at higher toxin levels, however, and a threshold is eventually reached ($12 \mu\text{g L}^{-1}$),

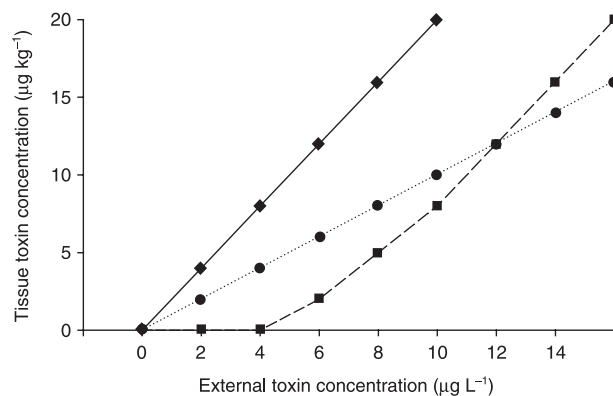


Fig. 2. Bioaccumulation thresholds in animals of different metabolic capabilities. ◆, level above which bioaccumulation occurs; ●, non-regulator organism; ■, regulator organism.

at which toxins accumulate in tissues. Although it is difficult to estimate likely thresholds for MC and CYN accumulation from current literature, this knowledge is critical to managing toxin bioaccumulation.

The third requirement for toxin accumulation, minimum exposure time, can vary, depending on the uptake route(s). Organisms accumulating toxins from dermal exposure, for example, might need only short periods of time, while bioaccumulation from orally ingested cells might depend on feeding activity and metabolism, particularly as the latter changes on the basis of reproductive status and season (Vasconcelos *et al.* 2001; Yokoyama & Park 2002).

Once toxin bioaccumulation risk has been predicted on the basis of fraction availability, concentration and exposure time, determining the risk of ecological effects will depend on internal tissue concentrations. Basing ecological risk assessment and management strategies on internal concentrations is considered more reliable than doing so on the basis of water concentrations, primarily because the issues of uptake and metabolism have already been resolved (Sijm & Hermens 2000). In fact, the use of

internal tissue concentrations is now highly recommended over other methods (Connell *et al.* 1999). However, for this approach to be successful, it is crucial that problems, such as the preferential storage of toxins into some organs, have been identified in order to avoid underestimation of toxin levels. A strong knowledge base is required to predict likely sublethal or lethal effects corresponding to internal toxin levels. Unfortunately, however, there is virtually no data currently available in regard to tissue toxin levels and sublethal or lethal toxicities for cyanotoxins.

A useful, accurate management model clearly will need to address the following four principal elements regarding the prediction of cyanotoxin bioaccumulation and its ecological effects in aquatic organisms:

1. Knowledge of toxin concentrations in the water, including the concentrations of each toxin fraction.
2. Understanding of the likely toxin uptake routes for principal aquatic organisms of concern.
3. Knowledge of the minimum concentration thresholds and exposure times required for bioaccumulation to occur.
4. Data linking internal tissue toxin concentrations with the expected sublethal and lethal effects.

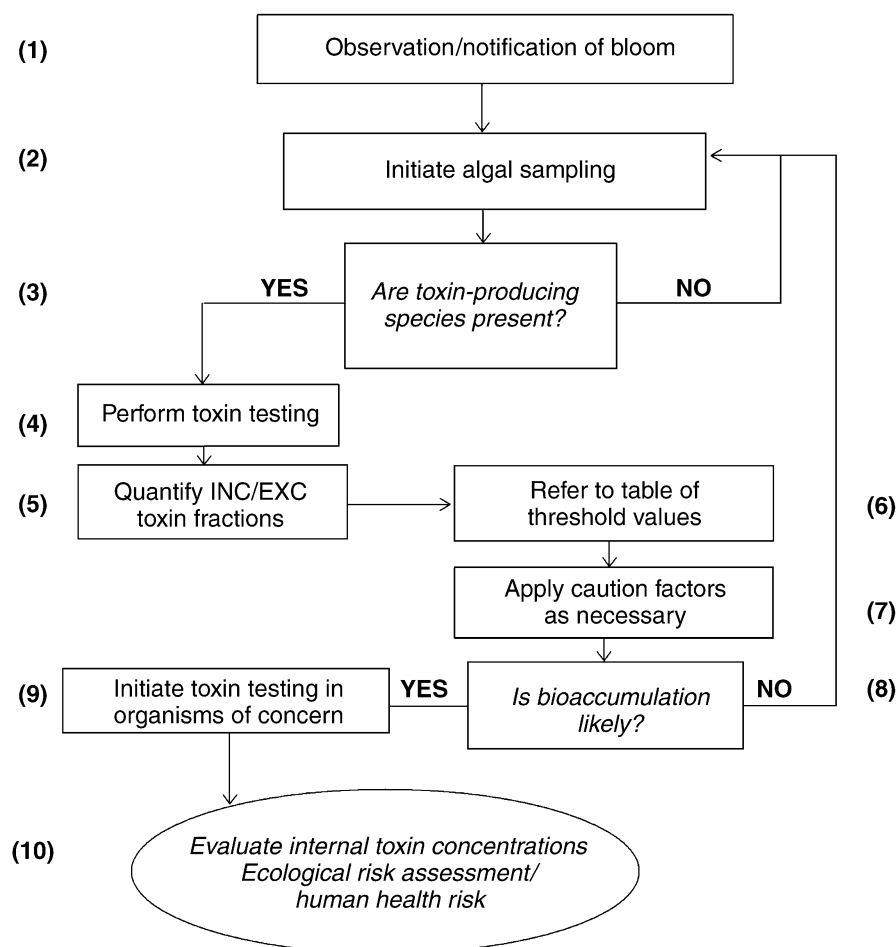


Fig. 3. Proposed step-wise model for monitoring and management of cyanotoxin bioaccumulation and its ecological effects. INC, intracellular; EXC, extracellular.

The model proposed below (Fig. 3) combines these elements within a decision framework, utilizing a predictive modelling approach to toxic algal bloom management, specifically regarding toxin bioaccumulation. A worked example is provided at the end of this discussion.

The step-wise process (Fig. 3) begins with monitoring of water bodies likely to be affected by toxic cyanoprokaryote blooms, based on prior history and/or current conditions (step 1). Algal sampling is conducted (step 2) and samples are examined for potentially toxigenic species (step 3). Where these are present at levels of concern (Chorus & Bartram 1999; Chorus 2001), toxin testing is initiated (step 4). At this stage in the process, all the procedures are already accepted algal monitoring protocols. However, this model requires further, separate quantification of the intracellular and extracellular toxin fractions (step 5), as these are critical in determining bioaccumulation potential.

The sampling results are then evaluated by referring to a table of threshold values (step 6) for aquatic organisms, calculated from data of laboratory-executed and field-validated ecotoxicity experiments. As no such data are yet available, an exemplar table for MC, using arbitrary values, is provided in Table 1. The table identifies a species of concern and its likely toxin uptake route(s), and then provides the specific threshold value(s) above which toxin bioaccumulation is deemed likely.

Species with multiple uptake routes can still be evaluated by this method as it does not matter how many routes are available, as long as each threshold is not exceeded. These require the use of caution factors (step 7); examples are included in Table 1. Caution values have

been used in other ecotoxicological applications, often to account for the differences between laboratory-measured lethal effects and the expected sublethal effects occurring in the field, or short versus long-term experimental data (Markich & Camilleri 1996). Here, however, their role is to ensure that two subthreshold fraction values are not interpreted as being risk-free. The caution values act to decrease the singular thresholds, after which the risk from each fraction should be re-evaluated, using the new (reduced) threshold value. Using such factors provides 'an extra margin of safety beyond the known or estimated sensitivities of aquatic organisms' (Stephan *et al.* 1985).

Returning to the model, the likelihood of toxin bioaccumulation is determined (step 8) by calculating if any thresholds have been exceeded, before or after application of caution factors. If so, tissue testing is initiated for those organisms considered at risk of toxin bioaccumulation (step 9). The results of these tests can then be compared against known sublethal and lethal toxicities for similar internal concentrations in order to assess the likely ecological and human health effects of toxin bioaccumulation (step 10).

A WORKED EXAMPLE

The following example demonstrates the decision pathway for managers of a reservoir stocked with *Cherax* crayfish. Early in the bloom, algal sampling and enumeration identifies the predominance of potentially toxic *Microcystis*, prompting the need for toxin analysis. The first toxin analysis is performed, with the obtained results being $3 \mu\text{g L}^{-1}$ intracellular toxin and $2 \mu\text{g L}^{-1}$ extracellular

Table 1. Bioaccumulation risk threshold values for microcystin (for illustrative purposes only; values not verified)

Species	Primary exposure route(s)	Intracellular threshold	Extracellular threshold	Caution factor(s)
Aquatic plant (<i>Lemna</i> sp.)	Dermal	NA	$1 \mu\text{g L}^{-1}$	NR
Macroinvertebrates	Dermal, oral	$2 \mu\text{g g}^{-1}$	$1 \mu\text{g L}^{-1\dagger}$	NR
<i>Tubifex</i> sp. (tube worm)				
<i>Thiara</i> sp. (herbivorous snail)	Oral	$0.5 \mu\text{g g}^{-1}$	$6 \mu\text{g L}^{-1\dagger}$	NR
<i>Eusthenia</i> sp. (carnivorous stonefly)	Incorporated (in prey)	NA (do not graze on algae)	$4 \mu\text{g L}^{-1\dagger}$	NR
Higher animals				
<i>Cherax</i> sp. (omnivorous crayfish)	Oral, dermal	$4 \mu\text{g g}^{-1}$	$3 \mu\text{g L}^{-1}$	Reduce threshold values by 1/2 in presence of 2 subthreshold values
<i>Bidyanus</i> sp. (silver perch)	Oral, incorporated, dermal, drinking	$10 \mu\text{g g}^{-1}$	$4 \mu\text{g L}^{-1}$	Reduce threshold values by 1/2 in presence of 2 subthreshold values

[†]Possible route, unlikely to be a major contributor (hence, caution factors are not required).

NA, not applicable; NR, not required (only one uptake route is considered to be of significance).

toxin. According to Table 1, the risk evaluation values for *Cherax* are $4 \mu\text{g L}^{-1}$ for the intracellular fraction and $3 \mu\text{g L}^{-1}$ for the extracellular fraction. Based on individual toxin fractions, no risk thresholds have been exceeded for *Cherax*. However, as *Cherax* has multiple uptake routes, caution factors must be introduced. As more than two subthreshold values are present, each individual threshold value must be reduced by half. As a result, both thresholds are exceeded, requiring tissue toxin testing to be initiated, with the results being used to determine the possible ecological effects of bioaccumulation.

MODEL EVALUATION

This model is unique in addressing the challenges posed by predictive modelling of cyanotoxin bioaccumulation values. Interspecific variability in toxin production is addressed by regular toxin testing, based on the species composition of the algal bloom. Temporal and spatial change in toxin fraction variability is accounted for because the toxin tests separately quantify intracellular and extracellular fractions, and each organism has likely uptake routes nominated (hence, likely exposure risk). Finally, the responses of aquatic organisms to cyanotoxin exposure (uptake, metabolism and adverse effects) are each considered by dividing the risk evaluation table according to species.

The decision-making process followed in this model might need to be repeated several times in the event of a particularly long toxic bloom, especially where early toxin sampling indicates toxin levels are below threshold values. In this event, monitoring should be continued in order to ensure levels do not rise above risk concentrations at a later date. Incorporating exposure times into this model is difficult because of the need to introduce almost continuous toxin testing to document fluctuations in toxin concentrations between tests. Thus, it might be useful to initiate toxin testing at critical stages of a bloom, examples being the 'middle', 'late' and 'collapse' stages shown in Fig. 1, possibly using predictions from cell count data.

The model does not attempt to model the processes of toxin uptake and depuration rates, nor the possibility of metabolic transformation or conjugation of toxins, although these have been studied in some organisms (Zurawell 2001; Pflugmacher 2002; Yokoyama & Park 2003). The model also does not include the possibility of incorporated toxins as a further uptake route (toxin source) for some animals (i.e. biomagnification). This is because incorporating the complexities of these elements might risk underestimation of the bioaccumulation hazard, especially if all components are not properly taken into account (Franke *et al.* 1994).

This model could easily be integrated into current algal management approaches, especially because field officers would already be familiar with required sampling methods. Furthermore, there are no requirements for extra testing or data collection methods other than those used at present – except for performing toxin analyses on intracellular and extracellular, rather than total, fractions. The model also simplifies the rather complex concepts regarding algal bloom periodicity and toxicity.

Drawbacks to this model include potential doubling of toxin analyses costs by requiring the cell-bound and non-cell bound fractions to be determined separately, and for toxin analyses to be repeated in the event of long bloom periods, or in blooms where bioaccumulation effects persist past the bloom collapse. One downfall of using the risk threshold approach (Table 1) is that it presumes uptake always will occur in the presence of toxin fractions. This might be likely for intracellular toxin; oral consumption of MC appears to correspond well with toxin uptake in gastropods (Kotak *et al.* 1996; Yokoyama & Park 2002). This situation, however, is uncertain for incorporated and extracellular toxins. The model also has a certain degree of error, in that the results of toxin analysis might not reflect exactly the ratios of toxin fractions in the sampled water body due to variability in sampling (e.g. sample collection, transport and storage) and toxin detection methods. In particular, lysing of cells and/or degradation of dissolved fractions in storage might underestimate actual toxin concentrations (Nicholson & Burch 2001). Although evaluating these levels will give only a gross indicator of the bioaccumulation risk, the model nevertheless will still act as a valuable screening tool to minimize costly tissue testing.

Future improvements to this model can be the inclusion of toxin exposure thresholds for time and the impact of environmental parameters (pH, temperature) in affecting uptake and bioaccumulation processes. As research data become available, the model can be expanded to include more species. This could possibly include species not specified in the risk evaluation table by examining their trophic level and food preferences and, hence, likely modes of exposure (e.g. oral ingestion). The model also could be used with respect to human health risk assessment, an example being its use for decision-making regarding the suitability of aquatic organisms for consumption.

A number of challenges still exist with respect to determining the ecological effects (and human health risks) of cyanotoxin bioaccumulation. As a result of insufficient relevant studies, the range of sublethal effects associated with direct toxicity for many cyanotoxins remains unknown, much less their bioaccumulation

effects. The inability to accurately detect incorporated MC and CYN also represents a considerable barrier to linking internal tissue concentrations with toxicity. This model represents an important step in toxic algae management, by considering the ecological effects of bioaccumulation and biomagnification of cyanotoxins in aquatic organisms.

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REFERENCES

- Baker P. D. & Humpage A. R. (1994) Toxicity associated with commonly occurring cyanobacteria in surface waters of the Murray–Darling basin, Australia. *Aust. J. Mar. Freshwat. Res.* **45**, 773–86.
- Beattie K. A., Ressler J., Wiegand C. *et al.* (2003) Comparative effects and metabolism of two microcystins and nodularin in the brine shrimp *Artemia salina*. *Aquat. Toxicol.* **62**, 219–26.
- Berg K., Skulberg O. M. & Skulberg R. (1987) Effects of decaying toxic blue-green algae on water quality – a laboratory study. *Arch. Hydrobiol.* **108**, 549–63.
- Best J. H., Eddy F. B. & Codd G. A. (2001) Effects of purified microcystin-LR and cell extracts of *Microcystis* strains PCC 7813 and CYA 43 on cardiac function in brown trout (*Salmo trutta*) alevins. *Fish Physiol. Biochem.* **24**, 171–8.
- Bickel H., Lyck S. & Utkilen H. (2000) Energy state and toxin content – experiments on *Microcystis aeruginosa* (Chroococcales, Cyanophyta). *Phycologia* **39**, 212–8.
- Boon P. I., Bunn S. E., Green J. D. & Shiel R. J. (1994) Consumption of cyanobacteria by freshwater zooplankton: Implications for the success of ‘top-down’ control of cyanobacterial blooms in Australia. *Aust. J. Mar. Freshwat. Res.* **45**, 875–87.
- Botes D. P., Tuiman A. A., Wessels P. L. *et al.* (1984) The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*. *J. Chem. Soc. Perkin Trans.* **1**, 2311–8.
- Bouvy M., Falcão D., Marinho M., Pagano M. & Moura A. (2000) Occurrence of *Cylindrospermopsis* (Cyanobacteria) in 39 Brazilian tropical reservoirs during the 1998 drought. *Aquat. Microbiol. Ecol.* **23**, 13–27.
- Byth S. (1980) Palm Island mystery disease. *Med. J. Aust.* **2**, 40–2.
- Carmichael W. W. (1996) Toxic *Microcystis* and the environment. In: *Toxic Microcystis* (eds M. Watanabe, K. I. Harada, W. W. Carmichael & H. Fujiki) pp. 1–12. CRC Press, Boca Raton.
- Carmichael W. W. (2001) Health effects of toxin-producing Cyanobacteria: ‘The CyanoHABs’. *Human Ecol. Risk Assess.* **7**, 1393–407.
- Carmichael W. W., Azevedo S. M., Molica R. *et al.* (2001) Human fatalities from Cyanobacteria: chemical and biological evidence for cyanotoxins. *Environ. Health Perspect.* **109**, 663–8.
- Carmichael W. W. & Falconer I. R. (1993) Diseases related to freshwater blue-green algal toxins, and control measures. In: *Algal Toxins in Seafood and Drinking Water* (ed. I. R. Falconer) pp. 187–209. Academic Press, Sydney.
- Carson B. (2000) Cylindrospermopsin – a review of toxicological literature. National Institute of Environmental Health Services, Research Triangle Park, North Carolina, USA.
- Casanova M. T., Burch M. D., Brock M. A. & Bond P. M. (1999) Does toxic *Microcystis aeruginosa* affect aquatic plant establishment? *Environ. Toxicol.* **14**, 97–109.
- Chiswell R. K., Shaw G. R., Eaglesham G. *et al.* (1999) Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: Effect of pH, temperature and sunlight on decomposition. *Environ. Toxicol.* **14**, 155–61.
- Chong M. W. K., Gu K. D., Lam P. K. S., Yang M. & Fong W. F. (2000) Study on the cytotoxicity of microcystin-LR on cultured cells. *Chemosphere* **41**, 143–7.
- Chorus I. (ed.). (2001) *Cyanotoxins Occurrence, Causes, Consequences*. Springer-Verlag, Berlin.
- Chorus I. & Bartram J. (eds). (1999) *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management*. E & FN Spon, London.
- Chorus I. V., Neisel J., Fastner C. *et al.* (2001) Environmental factors and microcystin levels in waterbodies. In: *Cyanotoxins Occurrence, Causes, Consequences* (ed. I. Chorus) pp. 159–77. Springer-Verlag, Berlin.
- Christoffersen K. (1996) Ecological implications of cyanobacterial toxins in aquatic food webs. *Phycologia* **35** (Suppl.), 42–50.
- Connell D. W., Chaisuksant Y. & Yu J. (1999) Importance of internal biotic concentrations in risk evaluations with aquatic systems. *Mar. Pollut. Bull.* **39**, 54–61.
- Dawson R. M. (1998) The toxicology of microcystins. *Toxicon* **37**, 953–62.
- Delaney J. M. & Wilkins R. M. (1995) Toxicity of Microcystin-LR, isolated from *Microcystis aeruginosa*, against various insect species. *Toxicon* **33**, 771–8.
- Duy T. N., Lam P. K. S., Shaw G. R. & Connell D. W. (2000) Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. *Rev. Environ. Contam. Toxicol.* **163**, 113–86.

- Eriksson J. E., Meriluoto J. & Lindholm T. (1989) Accumulation of a peptide toxin from the cyanobacterium *Oscillatoria agardhii* in the freshwater mussel *Anodonta cygnea*. *Hydrobiologia* **183**, 211–6.
- Falconer I. R. (1993) Mechanism of toxicity of cyclic peptide toxins from blue-green algae. In: *Algal Toxins in Seafood and Drinking Water* (ed. I. R. Falconer) pp. 177–86. Academic Press, Sydney.
- Falconer I. R. (1999) An overview of problems caused by toxic blue-green algae (Cyanobacteria) in drinking and recreational water. *Environ. Toxicol.* **14**, 5–11.
- Falconer I. R. (2001) Toxic cyanobacterial bloom problems in Australian waters: Risks and impacts on human health. *Phycologia* **40**, 228–33.
- Falconer I. R., Hardy S. J., Humpage A. R., Frosio S. M., Tozer G. J. & Hawkins P. R. (1999) Hepatic and renal toxicity of the blue-green alga (Cyanobacterium) *Cylindrospermopsis raciborskii*. Male Swiss albino mice. *Environ. Toxicol.* **14**, 143–50.
- Falconer I. C., Jackson A. R., Langley J. & Runnegar M. (1981) Liver pathology in mice in poisoning by the blue-green algae *Microcystis aeruginosa*. *Aust. J. Biol. Sci.* **34**, 179–87.
- Ferrão-Filho A. & Azevedo S. M. F. O. (2003) Effects of unicellular and colonial forms of toxic *Microcystis aeruginosa* from laboratory cultures and natural populations on tropical cladocerans. *Aquat. Ecol.* **37**, 23–35.
- Ferrão-Filho A., Domingos P. & Azevedo M. T. P. (2002a) Influences of a *Microcystis aeruginosa* Kützinger bloom on zooplankton populations in Jacarepaguá Lagoon (Rio de Janeiro, Brazil). *Limnologia* **32**, 295–308.
- Ferrão-Filho A., Kozłowsky-Suzuki B. & Azevedo S. M. F. O. (2002b) Accumulation of microcystins by a tropical zooplankton community. *Aquat. Toxicol.* **59**, 201–8.
- Fischer W. J., Hitzfield B. C., Tencalla F., Eriksson J. E., Mihailov A. & Dietrich D. R. (2000) Microcystin-LR toxicodynamics, induced pathology, and immunohistochemical localisation in livers of blue-green algae exposed rainbow trout (*Onorhynchus mykiss*). *Toxicol. Sci.* **54**, 365–73.
- Frank C. (2002) Microcystin-producing cyanobacteria in recreational waters in southwestern Germany. *Environ. Toxicol.* **17**, 361–6.
- Franke C., Studinger G., Berger G. *et al.* (1994) The assessment of bioaccumulation. *Chemosphere* **29**, 1501–14.
- Griffiths D. J. & Saker M. (2003) The Palm Island mystery disease 20 years on: a review of research on the cyanotoxin cylindrospermopsin. *Environ. Toxicol.* **18**, 78–93.
- Harada K. I. (1996) Chemistry and detection of microcystins. In: *Toxic Microcystis* (eds M. Watanabe, K. I. Harada, W. W. Carmichael & H. Fujiki) pp. 103–48. CRC Press, Boca Raton.
- Hawkins P. R., Chandrasena N. R., Jones G. J., Humpage A. R. & Falconer I. R. (1997) Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. *Toxicon* **35**, 341–6.
- Hawkins P. R., Putt E., Falconer I. R. & Humpage A. R. (2001) Phenotypical variation in a toxic strain of the phytoplankter, *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) during batch culture. *Environ. Toxicol.* **16**, 460–7.
- Hawkins P. R., Runnegar M. T. C., Jackson A. R. B. & Falconer I. R. (1985) Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated from a domestic water supply reservoir. *Appl. Environ. Microbiol.* **50**, 1292–5.
- Heinze R. (1999) Toxicity of the cyanobacterial toxin microcystin-LR to rats after 28 days intake with drinking water. *Environ. Toxicol.* **14**, 57–60.
- Hiripi L., Nagy L., Kalmar T., Kovacs A. & Voros L. (1998) Insect (*Locusta Migratoria Migratorioides*) test monitoring the toxicity of cyanobacteria. *Neurotoxicology* **19**, 605–8.
- Hoeger S. J., Shaw G. R., Hitzfield B. C. & Dietrich D. R. (2004) Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants. *Toxicon* **43**, 639–49.
- Jacoby J. M., Collier D. C., Welch E. B., Hardy F. J. & Crayton M. (2000) Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. *Can. J. Fish Aquat. Sci.* **57**, 231–40.
- Jones G., Baker P. D., Burch M. D. & Harvey F. L. (2002) National protocol for the monitoring of cyanobacteria and their toxins in surface waters. Agriculture and Resource Management Council of Australia and New Zealand (National Algal Management), Canberra, ACT, Australia. Draft V5.0.
- Kankaanpää H. P., Vuorinen J., Sipilä V. & Keinänen M. (2002) Acute effects and bioaccumulation of nodularin in sea trout (*Salmo trutta* M. *trutta* L.) exposed orally to *Nodularia spumigena* under laboratory conditions. *Aquat. Toxicol.* **61**, 155–68.
- Kirpenko N. I. (1986) Phytopathic properties of blue-green algae toxin. *Hydrobiol. J.* **22**, 44–7.
- Kiss T., Vehovsky Á., Hiripi L., Kovács M. & Vörös L. (2002) Membrane effects of toxins isolated from a cyanobacterium, *Cylindrospermopsis raciborskii*, on identified molluscan neurones. *Comp. Biochem. Physiol., C* **131**, 167–76.

- Komarek J., Azevedo S., Domingos P., Komarkova J. & Tichý M. (2001) Background of the Caruaru tragedy: a case taxonomic study of toxic cyanobacteria. *Algol. Stud.* **103**, 9–29.
- Kotak B. G., Zurawell R., Prepas E. & Holmes C. F. (1996) Microcystin-LR concentration in aquatic food web compartments from lakes of varying trophic status. *Can. J. Fish Aquat. Sci.* **53**, 1974–85.
- Lahti K. M., Niemi J., Rapala K. S. & Sivonen K. (1997a) Biodegradation of cyanobacterial hepatotoxins – characterization of toxin degrading bacteria. In: *Harmful Algae* (eds B. Reguera, J. Blanco, M. Fernandez & T. Watt) pp. 363–5. Proceedings of the VIII International Conference on Harmful Algae; 25–29 June 1997, Vigo, Spain. Intergovernmental Oceanographic Commission of UNESCO, Santiago de Compostela, Spain.
- Lahti K., Rapala J., Färdig M., Niemelä M. & Sivonen K. (1997b) Persistence of cyanobacterial hepatotoxin, Microcystin-LR, in particulate material and dissolved in lake water. *Water Res.* **31**, 1005–12.
- Landsberg J. H. (2002) The effects of harmful algal blooms on aquatic organisms. *Rev. Fish Sci.* **10**, 113–390.
- Li R., Carmichael W., Brittain S. *et al.* (2001) First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata* (Cyanobacteria). *J. Phycol.* **37**, 1121–6.
- Lirás V., Lindberg M., Nystrom P., Annadotter H., Lawton L. & Graf B. (1998) Can ingested cyanobacteria be harmful to the signal crayfish (*Pacifastacus leniusculus*)? *Freshwat. Biol.* **39**, 233–42.
- Liu Y., Song L., Li X. & Liu T. (2002) The toxic effects of microcystin-LR on embryo-larval and juvenile development of loach, *Misgurnus mizolepis* Gunthe. *Toxicon* **40**, 395–9.
- Long B. M., Jones G. J. & Orr P. T. (2001) Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. *Appl. Environ. Microbiol.* **67**, 278–83.
- MacKintosh C., Beattie K. A., Klumpp S., Cohen P. & Codd G. A. (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **264**, 187–92.
- Magalhães V. F., Soares R. & Azevedo S. (2001) Microcystin contamination in fish from the Jacarepaguá Lagoon (Rio de Janeiro, Brazil): Ecological implication and human health risk. *Toxicon* **39**, 1077–85.
- Markich S. J. & Camilleri C. (1996) Investigation of metal toxicity to tropical biota: Recommendations for revision of the Australian water quality guidelines. Office of the Supervising Scientist of the Alligator Rivers Region, Jabiru, NT, Australia. Internal Report No. 223.
- Matveev V., Matveeva L. & Jones G. J. (1994) Study of the ability of *Daphnia carinata* King to control phytoplankton and resist cyanobacterial toxicity: Implications for biomanipulation in Australia. *Aust. J. Mar. Freshwat. Res.* **45**, 899–904.
- McCarty L. S. & Mackay D. (1993) Enhancing ecotoxicological modelling and assessment. *Environ. Sci. Technol.* **27**, 1719–28.
- McElhiney J., Lawton L. & Leifert C. (2001) Investigations into the inhibitory effects of microcystins in plant growth, and the toxicity of plant tissues following exposure. *Toxicon* **39**, 1411–20.
- Mohamed Z. A. (2001) Accumulation of cyanobacterial hepatotoxins by *Daphnia* in some Egyptian irrigation channels. *Ecotoxicol. Environ. Safety* **50**, 4–8.
- Nicholson B. C. & Burch M. D. (2001) *Evaluation of Analytical Methods for Detection and Quantification of Cyanotoxins in Relation to Australian Drinking Water Guidelines*. Cooperative Research Centre for Water Quality and Treatment, Canberra.
- Norris K. R., Seawright A. A., Shaw G. *et al.* (2002) Hepatic xenobiotic metabolism of cylindrospermopsin *in vivo* in the mouse. *Toxicon* **40**, 471–6.
- Oberemm A. (2001) Effects of cyanotoxins on early life stages of fish and amphibians. In: *Cyanotoxins Occurrence, Causes, Consequences* (eds I. Chorus) pp. 240–8. Springer-Verlag, Berlin.
- Oberemm A., Becker J., Codd G. A. & Steinberg C. (1999) Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. *Environ. Toxicol.* **14**, 77–88.
- Ohtani I., Moore R. E. & Runnegar M. T. C. (1992) Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. *J. Am. Chem. Soc.* **114**, 7941–2.
- van der Oost R., Beyer J. & Vermeulen N. P. E. (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environ. Toxicol. Pharmacol.* **13**, 57–149.
- Orr P. & Jones G. (1998) Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. *Limnol. Oceanogr.* **43**, 1604–14.
- Ozawa K., Yokoyama A., Ishikawa K., Kumagi M., Watanabe M. & Park H.-D. (2003) Accumulation and depuration of microcystin produced by the cyanobacterium *Microcystis* in a freshwater snail. *Limnology* **4**, 131–8.
- Padisák J. (1997) *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding highly adaptive

- cyanobacterium: worldwide distribution and review of its ecology. *Archiv. Hydrobiol. Suppl. 107 (Monogr. Stud.)* **4**, 563–93.
- Pflugmacher S. (2002) Possible allelopathic effects of cyanotoxins, with reference to Microcystin-LR, in aquatic ecosystems. *Environ. Toxicol.* **17**, 407–13.
- Prepas E. E., Kotak B. G., Campbell L. M., Evans J. C., Hrudey S. E. & Holmes C. F. (1997) Accumulation and elimination of cyanobacterial heptatotoxins by the freshwater clam, *Anodonta grandis simpsonia*. *Can. J. Fish Aquat. Sci.* **54**, 41–6.
- Queensland Department of Natural Resources and Mines (2003) *Queensland Harmful Algal Bloom Operational Procedures*. Harmful Algal Blooms Steering Committee, Queensland Department of Natural Resources and Mines, Rocklea, Qld, Australia.
- Rinehart K. L., Namikoshi M. & Choi B. W. (1994) Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). *J. Appl. Phycol.* **6**, 159–76.
- Romanowska-Duda Z. & Tarczyska M. (2002) The influences of microcystin-LR and hepatotoxic cyanobacterial extract on the water plant *Spirodela oligorrhiza*. *Environ. Toxicol.* **17**, 434–40.
- Runnegar M. T., Kong S. M., Zhong Y. Z., Ge J. L. & Lu S. C. (1994) The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochem. Biophys. Res. Commun.* **201**, 235–41.
- Saker M. L. & Eaglesham G. K. (1999) The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus*. *Toxicon* **37**, 1065–77.
- Saker M. L. & Griffiths D. J. (2000) The effect of temperature on growth and cylindrospermopsin content of seven isolates of the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju from water bodies in northern Australia. *Phycologia* **39**, 349–54.
- Saker M. L., Metcalf J. S., Codd G. A. & Vasconcelos V. M. (2004) Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. *Toxicon* **43**, 185–94.
- Saker M. & Neilan B. A. (2001) Varied diazotrophies, morphologies and toxicities of genetically similar isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from Northern Australia. *Appl. Environ. Microbiol.* **67**, 1839–45.
- Saker M., Thomas A. D. & Norton J. H. (1999) Cattle mortality attributed to the toxic cyanobacterium *Cylindrospermopsis raciborskii* in an outback region of North Queensland. *Environ. Toxicol.* **14**, 179–82.
- Schembri M. A., Neilan B. A. & Saint C. P. (2001) Identification of genes implicates in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. *Environ. Toxicol.* **16**, 413–21.
- Schwimmer M. & Schwimmer D. (1968) Medical aspects of phycology. In: *Algae, Man and the Environment* (ed. D. F. Jackson) pp. 279–358. Syracuse University Press, New York.
- Shaw G., Seawright A. A., Moore M. R. & Lam P. K. S. (2000) Cylindrospermopsin, a cyanobacterial alkaloid: Evaluation of its toxicologic activity. *Ther. Drug Monitor.* **22**, 89–92.
- Shaw G., Sufenik A., Livne A. *et al.* (1999) Blooms of the cylindrospermopsin containing cyanobacterium, *Aphanizomenon ovalisporum* (Forti) in newly constructed lakes, Queensland, Australia. *Environ. Toxicol.* **14**, 167–77.
- Shen X., Lam P. K. S., Shaw G. R. & Wiickramasinghe W. (2002) Gentoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. *Toxicon* **40**, 1499–501.
- Sijm D. T. H. & Hermens J. L. (2000) Internal effect concentration: link between bioaccumulation and ecotoxicity for organic chemicals. In: *Bioaccumulation: New Aspects and Developments* (ed. B. Beek) pp. 167–233. Springer, Berlin.
- Sivonen K. & Jones G. (1999) Cyanobacterial toxins. In: *Toxic Cyanobacteria in Water* (eds I. Chorus & J. Bartram) pp. 41–111. E & FN Spon, London.
- Stephan C. E., Mount D. I., Hansen D. J., Gentile J. H., Chapman G. A. & Brungs W. A. (1985) *Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and their Uses*. US Environmental Protection Agency, Washington DC.
- Terao K. S., Ohmori K., Igarshi I. *et al.* (1994) Electron microscope studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. *Toxicon* **32**, 833–43.
- Ueno Y., Makita Y., Nagata S. *et al.* (1999) No chronic oral toxicity of a low dose of microcystin-LR, a cyanobacterial hepatotoxin, in female BALB/c mice. *Environ. Toxicol.* **14**, 45–55.
- Utkilen H. & Gjelme N. (1995) Iron-stimulated toxin production in *Microcystis aeruginosa*. *Appl. Environ. Microbiol.* **61**, 797–800.
- Vasas G., Gáspár A., Surányi G. *et al.* (2002) Capillary electrophoretic assay and purification of cylindrospermopsin, a cyanobacterial toxin from *Aphanizomenon ovalisporum*, by plant test (blue-green *Sinapis* test). *Anal. Biochem.* **302**, 95–103.
- Vasconcelos V. M. (1995) Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*. *Aquat. Toxicol.* **32**, 227–37.

- Vasconcelos V. M., Oliveira S. & Teles F. O. (2001) Impact of a toxic and a non-toxic strain of *Microcystis aeruginosa* on the crayfish *Procambarus clarkii*. *Toxicon* **39**, 1461–70.
- Vesterkvist P. S. M. & Meriluoto J. A. O. (2003) Interaction between microcystins of different hydrophobicities and monolayers. *Toxicon* **41**, 349–55.
- Ward C. J. & Codd G. A. (1999) Comparative toxicity of four microcystins of different hydrophobicities to the protozoan, *Tetrahymena pyriformis*. *J. Appl. Microbiol.* **86**, 874–82.
- Watanabe M. F., Tsuji K., Watanabe Y., Harada K. I. & Suzuki M. (1992) Release of a heptapeptide toxin (microcystin) during the decomposition process of *Microcystis aeruginosa*. *Nat. Toxins* **1**, 48–53.
- Webster I. (1990) Effect of wind on the distribution of phytoplankton cells in lakes. *Limnol. Oceanogr.* **35**, 989–1001.
- Wiedner C., Visser P. M., Fastner J., Metcalf J. S., Codd G. A. & Mur L. R. (2003) Effects of light on the microcystin content of *Microcystis* strain PCC 7806. *Appl. Environ. Microbiol.* **69**, 1475–81.
- Wiegand C. & Pflugmachers. (2001) Uptake of Microcystin-LR in aquatic organisms. In: *Cyanotoxins Occurrence, Causes, Consequences* (ed. I. Chorus) pp. 249–52. Springer-Verlag, Berlin.
- Williams D. E., Dawe S. C., Kent M. L., Andersen R. J., Craig M. & Holmes C. F. (1997) Bioaccumulation and clearance of microcystins from salt water mussels, *Mytilus edulis*, and *in vivo* evidence for covalently bound microcystins in mussel tissues. *Toxicon* **35**, 1617–25.
- Xie L., Xie P., Ozawa K., Honma T., Yokoyama A. & Park H.-D. (2004) Dynamics of microcystins-LR and -RR in the phytoplanktivorous silver carp in a sub-chronic toxicity experiment. *Environ. Pollut.* **127**, 431–9.
- Xu L. H., Lam P. K. S., Chen J. P. *et al.* (2000) Use of protein phosphatase inhibition assay to detect microcystins in Donghu Lake and a fish pond in China. *Chemosphere* **41**, 53–8.
- Yokoyama A. & Park H. (2002) Mechanism and prediction for contamination of freshwater bivalves (Unionidae) with the cyanobacterial toxin microcystin in hypereutrophic Lake Suwa, Japan. *Environ. Toxicol.* **17**, 424–33.
- Yokoyama A. & Park H. (2003) Depuration kinetics and persistence of the cyanobacterial toxin microcystin-LR in the freshwater bivalves *Unio douglasiae*. *Environ. Toxicol.* **18**, 61–7.
- Zimba P. V., Gaunt P. S., Brittain S. & Carmichael W. (2001) Confirmation of catfish, *Ictalurus punctatus* (Rafinesque) mortality from *Microcystis* toxins. *J. Fish Dis.* **24**, 41–7.
- Zurawell R. (2001) Occurrence and toxicity of microcystins in the freshwater pulmonate snail *Lymnaea stagnalis*. PhD thesis. University of Alberta, Alberta, Canada.

Absence of Free-Cylindrospermopsin Bioconcentration in Water Thyme (*Hydrilla verticillata*)

S. H. White,¹ L. J. Duivenvoorden,¹ L. D. Fabbro¹

¹ Freshwater Ecology Group, Center for Environmental Management, Central Queensland University, Bruce Highway, Rockhampton, Q4701, Australia

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Cylindrospermopsin (CYN) is a blue-green algal toxin first isolated and identified in 1992 (Ohtani et al. 1992). CYN is produced by several blue-green algae including *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*, *Anabaena bergii* var *limnetica* and *Raphidiopsis curvata* (Li et al. 2001; Schembri et al. 2001; Shaw et al. 1999; Stirling and Quilliam 2001). CYN studies are becoming increasingly important: this algal toxin remains one of the least well understood, despite compelling evidence for widespread environmental and human health risk. Furthermore, *C. raciborskii*, the most common producer of CYN, is a highly adaptive and invasive species being commonly reported from blooms worldwide (Briand et al. 2004; Padisák 1997).

CYN exerts a wide range of toxic effects on plants and animals. In the well-publicized 'Palm Island Mystery Disease' of 1979, 138 children and 10 adults were hospitalised suffering acute gastroenteritis following CYN ingestion (Byth 1980; Griffiths and Saker 2003). General modes of action for CYN include cytotoxicity and hepatotoxicity, however inhibition of protein synthesis is the primary mechanism (Chong et al. 2002; Hawkins et al. 1985). Inhibition of glutathione synthesis has also been closely studied (Runnegar et al. 1995; 1994). Other lesser-known mechanisms include implications for the inhibition of molluscan neurones (Kiss et al. 2002), and strong evidence for cardiotoxicity, genotoxicity, tumour promotion and carcinogenicity (Bernard et al. 2003; Falconer and Humpage 2001; Ohtani et al. 1992; Shen et al. 2002).

Despite the likelihood for CYN's protein synthesis inhibition to be as equally applicable to primary producers as to animals, to date, only two studies have examined CYN toxicity on plant species. Neither study examined aquatic macrophytes. Vasas et al. (2002) demonstrated the inhibitory effects of CYN on the metabolism of *Sinapis* mustard seedlings, with 50% growth reduction apparent at $18.2 \mu\text{g ml}^{-1}$. Most recently, Metcalf et al. (2004) demonstrated reduced pollen germination in tobacco plants following CYN exposure between $5 - 1000 \mu\text{g ml}^{-1}$.

Only two studies have investigated the potential for CYN to bioaccumulate; again, no studies have involved plants. However, the potential for bioaccumulation of algal toxins is particularly significant: the occurrence of direct lethal and sublethal toxicities associated with exposure may increase in the presence of high toxin

Correspondence to: S. H. White

concentrations which result from bioaccumulation. Furthermore, there are implications for transfer of toxins throughout aquatic food chains, and possibly, contamination of human food sources.

Toxin uptake sources in affected aquatic ecosystems include both intracellular (cell-bound) and extracellular (lysed, dissolved) toxins, while potential uptake routes are represented by dermal exposure, drinking of aqueous concentrations and oral consumption of suspended particles. Toxin accumulation risk in aquatic plants is solely represented by uptake of the extracellular fraction via the plant cell walls, as no consumption of algal cells takes place. Hence, in this paper, the process of toxin accumulation into plant tissues will be referred to as bioconcentration. This is consistent with the definition that bioconcentration is a special case of bioaccumulation that refers to uptake from toxins available from the water column, usually via epithelial tissues or in drinking water (Hall 2003).

Despite plants being at risk only from extracellular toxin, CYN uptake potential remains high, as most available surface area is in direct contact with toxins in the water column, and because CYN in both natural blooms and culture environments may be predominated by the extracellular, rather than intracellular, toxin fraction, depending on bloom age (Griffiths and Saker 2003; Hawkins et al. 2001).

Detection of CYN using liquid chromatography and tandem mass spectrometry (LC/MS/MS) is currently the method of choice (Nicholson and Burch 2001). However, an important limitation of current CYN studies, particularly for bioaccumulation work, is the inability of current analytical techniques to detect enzymatically-bound CYN within the cells of animals and plants. CYN may bind to such tissues because of its structural features (Duy et al. 2000), and because similar problems have been experienced with detection of another cyanotoxin, microcystin (Kankaanpää et al. 2002; Williams et al. 1997). This is problematic as the concentrations of CYN within aquatic organisms may be underestimated, as chemical detection is suitable only for unbound (free) CYN. Hence, this paper examines only free-CYN bioconcentration in the rooted, submersed aquatic macrophyte *Hydrilla verticillata*, using environmentally realistic CYN test concentrations.

MATERIALS AND METHODS

Hydrilla verticillata (l.f. Royle) was obtained from a private dam in Cawarral, Central Queensland and from an ornamental pond on the CQU campus. Neither of these collection points are known to experience blooms of *Cylindrospermopsis raciborskii*. Plants were washed thoroughly in tap water to remove epiphytic growth and other aquatic biota, and cultured under experimental conditions for a least one week prior to commencement of tests.

Cylindrospermopsin (CYN) was obtained from cultures of *Cylindrospermopsis raciborskii* (strains CQU-FR001 and -FR002) grown in ASM-1 media buffered to pH 7.8 (Gorham et al. 1964). Culturing took place in glass Schott bottles in either a controlled-temperature room ($25 \pm 2^{\circ}\text{C}$, continuous light at approximately $6 \mu\text{Em}^{-2}$

s⁻¹) or on open laboratory shelves (approximately 4 – 5 $\mu\text{Em}^{-2}\text{s}^{-1}$).

Following initial range-finding tests, two definitive bioconcentration trials were conducted, along with one further trial to examine toxin depuration in *H. verticillata*. All trials took the form of semi-static renewal tests. Test sections of *H. verticillata* were obtained by separating lateral stems of generally uniform size from mother plants: each fragment was comprised of an apical tip with no lateral stems or roots.

Definitive trials examined exposure to extracellular CYN at six treatment concentrations, namely 0, 25, 50, 100, 200 and 400 $\mu\text{g L}^{-1}$. Trials lasted 14 days and were conducted under a 12:12 light:dark photoperiod (illumination, approximately 75 $\mu\text{mol m}^{-2}\text{s}^{-1}$) at $26 \pm 4^{\circ}\text{C}$. Plants were harvested at days 7 and 14. In the depuration trial, the CYN and deoxy-CYN (an analog of CYN) concentrations of tissues were studied after seven days exposure to 0, 100, 250, 350 and 500 $\mu\text{g L}^{-1}$ extracellular CYN, and also after one further week in CYN-free media. The trial was performed under continuous light at $27 \pm 2^{\circ}\text{C}$.

All extracellular CYN test concentrations (150ml volume) were prepared by freeze-thawing pooled *C. raciborskii* cultures of known CYN toxicity (based on a single subsampling event), and diluting to the desired concentration using ASM-1 media. Controls used ASM-1 algal culturing media only. Test chambers were glass crystallisation dishes (300ml capacity); these were covered with soda-glass watch glasses to minimise evaporation. Dishes were always randomly placed on the trial bench, and repositioned regularly, following renewal of test solutions. All treatments were prepared using six replicates ($n = 3$ for each of two exposure intervals).

To minimise the effects of toxin degradation over the trial period, test volumes (including controls) were renewed by half at three-day intervals, using 75ml of freshly prepared extracellular CYN solution. The displaced solutions were reserved, pooled within treatments and two samples randomly selected from each trial for analysis of CYN content (see below). Test chambers were measured for pH (TPS LC80A) and conductivity (TPS LC84) prior to solution renewal throughout all trials.

All plant samples from the definitive trials and selected samples from the depuration trial were analysed for free-CYN concentration. Following harvest, plants were rinsed with approximately 200mL distilled water and blotted dry using paper towels. Samples were weighed (fresh weight, to 0.01g), frozen and freeze-dried (approximately 48hr, Virtis Sentry freeze-drier with Alcatel vacuum pump). Following re-weighing, samples were sent to the Queensland Health Scientific Service (QHSS, Brisbane) for analysis. Here, five ml of HPLC grade water was added to each sample, which was then homogenised at 24,000 rpm for approximately 1 min (Ultra Turrax homogeniser, IKA Works, Asia). The homogenate was allowed to settle before 1.5 ml of supernatant was filtered through a 0.45 micron filter (Millex-HV, Millipore Corp., Bedford, MA) into a sample vial.

Non-enzymatically bound cylindrospermopsin (free-CYN) was determined from both plant and water samples (e.g., treatment concentrations and original cultures) using HPLC/Electrospray/MS/MS [AB/Sciex API 300 mass spectrometer, Applied Biosystems, Concord, On. Canada, equipped with a turbo-ion spray interface coupled to a Shimadzu SCL-10Avp HPLC system, Shimadzu Corp., Kyoto, Japan] (Eaglesham et al. 1999). Positive samples were confirmed by both retention time (6.13 minutes) and by comparing transition intensity ratios between the sample and an appropriate concentration standard from the same run. The limit of detection for this method is $< 200 \text{ ng L}^{-1}$ for a $120 \mu\text{L}$ injection volume (equivalent to 1.0 nanograms in 5 ml). One sample per batch was run in duplicate, in addition to a blank and control sample. This method generally gives 95% confidence limits of $\pm 13\%$ as determined from controls run with each sample batch (G. K. Eaglesham, *personal communication*). Spike recoveries for 5 ml homogenate spiked with CYN averaged 97.5% at $3.2 \mu\text{g L}^{-1}$ and 90% at $6.5 \mu\text{g L}^{-1}$. Samples from the second definitive trial were also analysed for deoxy-CYN content (retention time, 6.63 minutes).

As the standard deviation of some replicates was zero, preferred statistical analyses (analysis of variance) could not be conducted on the collected data.

RESULTS AND DISCUSSION

A maximum of $176 \text{ ng free-CYN g}^{-1}$ freeze-dried tissue was recorded from *H. verticillata* in the definitive trials (Figure 1 a, b). Based on a regression equation calculated from the fresh and freeze-dried data from both definitive trials, this was roughly equivalent to $15 \text{ ng free-CYN g}^{-1}$ fresh weight ($y = 0.3823 + (0.0825x)$; $r^2 = 0.9140$; SigmaStat 3.0). There was no evidence to indicate free-CYN bioaccumulation had occurred: maximum tissue concentrations did not exceed exposure concentrations. That is, assuming 1 gram of plant tissue is equivalent to 1 mL of water; 15 ng g^{-1} tissue concentrations equates to only $15 \mu\text{g L}^{-1}$ media concentration. The average bioconcentration factor (tissue concentration divided by exposure concentration) for both definitive trials was only 0.045. In contrast, bioaccumulation is indicated by bioconcentration factors > 1 .

Deoxy-CYN concentrations were similarly low, peaking at approximately 160 ng g^{-1} freeze-dried tissue (Figure 2), roughly equivalent to 15 ng g^{-1} fresh weight ($y = 0.1208 + (0.0908x)$; $r^2 = 0.9517$). The bioconcentration factor could not be calculated as deoxy-CYN exposure concentrations were unknown.

Despite the lack of bioaccumulation, there was a trend for plants in high-level CYN exposure concentrations ($100 \mu\text{g L}^{-1}$ or above) to record the highest tissue toxins. In contrast, exposure concentrations of $50 \mu\text{g L}^{-1}$ or below resulted in little or no free-CYN in the tissues. This also occurred during the exposure phase of the depuration trial, although little or no free-CYN was present by the end of the depuration phase (Figure 1c). Other experimental work with CYN and the floating duckweed *S. oligorrhiza* has indicated that any detected free-CYN is likely to represent toxin adsorbed to the cell wall of *H. verticillata*, rather than toxin that has been taken up to become truly intracellular (White et al. *unpublished data*). In particular, CYN

values recorded from the depuration trial are consistent with adsorption and 'rinsing' of toxins from the plant surface area.

Test solution conductivity values ranged from 210 – 770 $\mu\text{S cm}^{-1}$ over the trial periods. Higher values recorded towards the end of trial periods probably resulted from evaporative concentration. High test solution pH values (>9.0 units) were also recorded during trials, particularly in the higher CYN-concentration treatments and towards the end of the exposure periods. High pH values may result from the original culture banks used to prepare treatment solutions: *C. raciborskii* cultures of similar age to those used in the trials have since had pH values measured between 9.3 – 9.7. Increases in pH may have also resulted from photosynthetic activity during exposure. Since elevated pH values are common in naturally occurring blooms no attempt was made to buffer pH during trials.

Declines in CYN concentration were experienced during trials (Table 1). Current OECD ecotoxicity guidelines stipulate that test concentrations should not vary by $>\pm 20\%$ (OECD 2000) although such guidelines typically relate to inert toxicants (e.g., pesticides, heavy metals). Subsequent laboratory experimentation has indicated that preparing treatments based on a single CYN value for culture strength (as done for these trials) may inaccurately represent true CYN content. Increased accuracy may be achieved by increasing the number of determinations of original culture strength. CYN decomposition may also have occurred during the trial: although previous work indicates a long half-life for CYN (Chiswell et al. 1999), the effect of the elevated pH values in accelerating decomposition is unknown. Detection of trace CYN concentrations after the depuration phase may represent CYN introduced by association with *H. verticillata*, as plant material was not rinsed before transfer into CYN-free media.

Table 1. Extracellular CYN concentrations of two randomly chosen treatment solutions from each trial. Values indicate the CYN concentration in three pooled replicates of test solution.

Trial Type (Number)	Days since half-solution replacement	Prepared Concentration ($\mu\text{g L}^{-1}$ CYN)	Actual Concentration ($\mu\text{g L}^{-1}$ CYN)	Percent (%) Remaining
Definitive 1	1	50	41.2	82
Definitive 1	2	50	43.3	87
Definitive 2	2	50	32	64
Definitive 2	5	400 ^a	138 ^a	35 ^a
Depuration	1	250 ^b	229 ^b	92 ^b
Depuration	2	0 ^c	5 ^c	NA ^c

^a - Last solution renewal missed due to lack of available toxin

^b - Exposure phase

^c - Depuration phase

NA - Not applicable

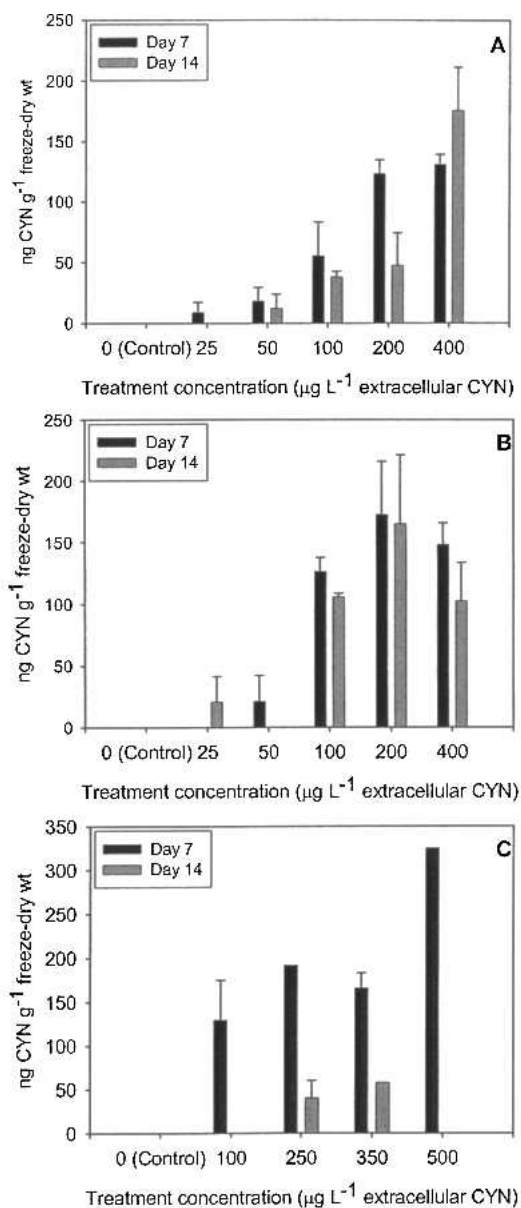


Figure 1. Free-CYN concentration in *H. verticillata* tissues (A) Definitive trial 1. (B) Definitive trial 2. (C) Depuration trial. Bars depict the average of three replicates, except in (C) where only selected samples were tested. Error bars depict standard error.

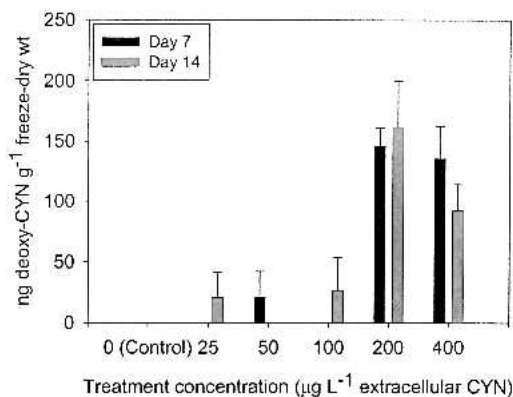


Figure 2. Free deoxy-CYN concentration in *H. verticillata* tissues from definitive trial 2. Bars depict the average of three replicates, error bars depict standard error.

The lack of free-CYN bioconcentration in *H. verticillata* indicates one of three possibilities. Firstly, *H. verticillata* may be incapable of extracellular-CYN uptake, and is thus not able to receive a 'bioavailable' CYN dose. Secondly, toxin uptake may be possible, however intracellular toxin could then be transported out of the cell at the same rate at which it enters, resulting in no net accumulation. Lastly, intracellular CYN may become enzymatically bound, modified or metabolised within plant tissues and hence is not able to be detected via HPLC. The latter problem has been demonstrated to occur with another algal toxin, microcystin, making extraction and detection difficult (Kankaanpää et al. 2002; Williams et al. 1997). High levels of bioaccumulated free-CYN have been recorded from aquatic organisms other than plants (Saker and Eaglesham 1999; Saker et al. 2004). Pflugmacher (2002) theorised that microcystin, at least, could be taken up by plant chloroplasts, to later become non-enzymatically bound or enzymatically conjugated to GSH. This may also be true of CYN, thus explaining the apparent lack of bioconcentration in plants, despite high levels being recorded from animals. The capability for plants to transport CYN out of cells is unknown.

This is the first study to demonstrate a lack of free-CYN bioconcentration in an aquatic macrophyte. In the absence of an improved analytical method for detection of bound or modified CYN, radiolabelling or enzyme-marking studies may provide further information regarding the uptake pathway(s) and possible bioaccumulation of total CYN and its metabolites in aquatic macrophytes.

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REFERENCES

- Bernard C, Harvey M, Biré R, Krysz S, Fontaine JJ (2003) Toxicological comparison of diverse *Cylindrospermopsis raciborskii* strains: Evidence of liver damage caused by a French *C. raciborskii* strain. *Environ Toxicol* 18:176-186
- Briand JF, Leboulanger C, Humbert J-F, Bernard C, Dufour P (2004) *Cylindrospermopsis raciborskii* (Cyanobacteria) invasion at mid-latitudes: selection; wide physiological tolerance, or global warming? *J Phycol* 40:231-238
- Byth S (1980) Palm Island mystery disease. *Med J Australia* 2:40-42
- Chiswell RK, Shaw GR, Eaglesham G, Smith MJ, Norris KR, Seawright AA, Moore MR (1999) Stability of cylindrospermopsin, the toxin from the cyanobacterium, *Cylindrospermopsis raciborskii*: Effect of pH, temperature and sunlight on decomposition. *Environ Toxicol* 14:155-161
- Chong MWK, Wong BSF, Lam PKS, Shaw GR, Seawright AA (2002) Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. *Toxicon* 40:205-211
- Duy TN, Lam PKS, Shaw GR, Connell DW (2000) Toxicology and risk assessment of freshwater cyanobacterial (Blue-Green Algal) toxins in water. *Rev Environ Contam Toxicol* 163:113-186
- Eaglesham G, Norris KR, Shaw GR, Smith MJ, Chiswell RK, Davis BC, Neville GR, Seawright AA, Moore BS (1999) Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. *Environ Toxicol* 14:151-154
- Falconer IR, Humpage AR (2001) Preliminary evidence for *in vivo* tumour initiation by oral administration of extracts of the blue green alga *Cylindrospermopsis raciborskii* containing the toxin cylindrospermopsin. *Environ Toxicol* 16:192-195
- Gorham PR, Mc Lachlan J, Hammer UT, Kim WK (1964) Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Breb. *Verhandlungen. Int Vereinigung Theoret Angewandte Limnol* 15:796-804
- Griffiths DJ, Saker ML (2003) The Palm Island mystery disease 20 years on: A review of research on the cyanotoxin cylindrospermopsin. *Environ Toxicol* 18:78-93
- Hall JE (2003) Bioconcentration, bioaccumulation, and biomagnification in Puget Sound biota: Assessing the ecological risk of chemical contaminants in Puget Sound. *J Environ* 1:1-19.
- Hawkins PR, Putt E, Falconer IR, Humpage AR (2001) Phenotypical variation in a toxic strain of the phytoplankter, *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) during batch culture. *Environ Toxicol* 16:460-467
- Hawkins PR, Runnegar MTC, Jackson ARB, Falconer IR (1985) Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated from a domestic water supply reservoir. *Appl Environ Microbiol* 50:1292-1295
- Kankaanpää H, Vuorinen PJ, Sipilä V, Keinänen M (2002) Acute effects and bioaccumulation of nodularin in sea trout (*Salmo trutta* m. *trutta* L.) exposed

- orally to *Nodularia spumigena* under laboratory conditions. *Aquat Toxicol* 61:155-168
- Kiss T, Vehovsky Á, Hiripi L, Kovács M, Vörös L (2002) Membrane effects of toxins isolated from a cyanobacterium, *Cylindrospermopsis raciborskii*, on identified molluscan neurones. *Comp Biochem Physiol C* 131:167-176
- Li R, Carmichael WW, Brittain S, Eaglesham G, Shaw G, Liu Y, Watanabe M (2001) First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata* (Cyanobacteria). *J Phycol* 37: 1121-1126
- Metcalf JS, Barakate A, Codd GA (2004) Inhibition of plant protein synthesis by the cyanobacterial hepatotoxin, cylindrospermopsin. *FEMS Microbiol Lett* 235:125-129
- Nicholson BC, Burch MD (2001) Evaluation of analytical methods for detection and quantification of cyanotoxins in relation to Australian drinking water guidelines. National Health and Medical Research Council of Australia/WSAA/CRC for Water Quality and Treatment, Canberra.
- Ohtani I, Moore RE, Runnegar MTC (1992) Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. *J Amer Chem Soc* 114:7941-7942
- OECD (Organisation for Economic Cooperation and Development) (2000). Guidance document on aquatic toxicity testing of difficult substances and mixtures. Organisation for Economic Cooperation and Development, Environmental Directorate, Paris
- Padisák J (1997) *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding highly adaptive cyanobacterium: worldwide distribution and review of its ecology. *Archiv fuer Hydrobiol. Suppl* 107 (Monographic Studies) 4:563-593
- Pflugmacher S (2002) Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR, in aquatic ecosystems. *Environ Toxicol* 17:407-413
- Runnegar MT, Kong S, Zhong Y, Lu SC (1995) Inhibition of reduced glutathione synthesis by cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochem Pharmacol* 49:219-225
- Runnegar MT, Kong SM, Zhong YZ, Ge JL, Lu SC (1994) The role of glutathione in the toxicity of a novel cyanobacterial alkaloid cylindrospermopsin in cultured rat hepatocytes. *Biochem Biophys Res Comm* 201:235-241
- Saker ML, Eaglesham GK (1999) The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cherax quadricarinatus*. *Toxicon* 37:1065-1077
- Saker ML, Metcalf JS, Codd GA, Vasconcelos VM (2004) Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. *Toxicon* 43:185-194
- Schembri MA, Neilan BA, Saint CP (2001) Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. *Environ Toxicol* 16:413-421
- Shaw G, Sufenik A, Livne A, Chiswell RK, Smith MJ, Seawright AA, Norris KR, Eaglesham G, Moore MR (1999) Blooms of the cylindrospermopsin containing cyanobacterium, *Aphanizomenon ovalisporum* (Forti) in newly constructed lakes, Queensland, Australia. *Environ Toxicol* 14:167-177

- Shen X, Lam PKS, Shaw GR, Wickramasinghe W (2002) Genotoxicity investigation of a cyanobacterial toxin, cylindrospermopsin. *Toxicon* 40:1499-1501
- Stirling DJ, Quilliam MA (2001) First report of the cyanobacterial toxin cylindrospermopsin in New Zealand. *Toxicon* 39:1219-1222
- Vasas GA, Gáspár G, Surányi G, Batta G, Gyémánt M, M-Hamvas C, Máthé GI, Molnár E, Borbély G (2002). Capillary electrophoretic assay and purification of cylindrospermopsin, A cyanobacterial toxin from *Aphanizomenon ovalisporum*, by plant test (Blue-Green Sinapis Test). *Anal Biochem* 302: 95-103
- Williams DE, Dawe SC, Kent ML, Andersen RJ, Craig M, Holmes CF (1997) Bioaccumulation and clearance of microcystins from salt water mussels, *Mytilus edulis*, and *in vivo* evidence for covalently bound microcystins in mussel tissues. *Toxicon* 35:1617-1625

Influence of intracellular toxin concentrations on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata*)

S.H. White^{a,1,*}, L.J. Duivenvoorden^{a,1}, L.D. Fabbro^{a,2}, G.K. Eaglesham^b

^a Freshwater Ecology Group, Centre for Environmental Management, Central Queensland University, Rockhampton, Qld 4702, Australia

^b Queensland Health Scientific Services, 39 Kessels Road, Coopers Plains, Qld 4109, Australia

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Abstract

Scant information is available regarding the bioaccumulation of cylindrospermopsin (CYN) in aquatic organisms, particularly in invertebrates. This study examined toxin bioconcentration and bioaccumulation in the aquatic snail, *Melanoides tuberculata*, following exposure to freeze-thawed whole cell extracts and a live *Cylindrospermopsis raciborskii* culture containing CYN. Both bioconcentration and bioaccumulation were evident, but exposure to toxin in the freeze-thawed solutions resulted in minor tissue contamination compared with that resulting from live *C. raciborskii* exposure. Thus, whilst CYN uptake resulted from both extracellular and intracellular exposures, the availability of intracellular toxin was critical in affecting tissue CYN values. *M. tuberculata* did not bioconcentrate CYN into the shell. Bioaccumulation of the analog deoxy-CYN was also recorded. Knowledge of intracellular toxin concentrations may be critical in evaluating the bioaccumulation, ecological and human health risks associated with contaminated systems.

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1. Introduction

Cylindrospermopsin (CYN) is a protein-synthesis inhibiting algal toxin (Terao et al., 1994) produced by several cyanoprokaryote species including *Cylindrospermopsis raciborskii* (in Australia, Thailand, Hungary, New Zealand and the United States), *Umezakia natans* (Japan),

Aphanizomenon ovalisporum (Israel and Australia), *Anabaena bergii* var *limnetica* and *Raphidiopsis curvata* (Ohtani et al., 1992; Harada et al., 1994; Shaw et al., 1999; Schembri et al., 2001; Stirling and Quilliam, 2001; Li et al., 2001a). Highly toxic CYN-producing blooms appear to be increasing globally (Padisák, 1997; Briand et al., 2004). Hence, understanding the range of effects associated with CYN exposure, including the potential for tissue contamination, is becoming increasingly important.

Bioaccumulation, simply defined, is a process whereby uptake of a toxicant results in tissue concentrations exceeding those present in the surrounding environment (Burkhard et al., 2003). In the case of algal toxins, uptake may occur from extracellular, intracellular or tissue-bound toxin fractions. Thus, accumulation can be further separated into bioconcentration, where uptake results exclusively from dissolved toxins; and bioaccumulation, where uptake may

* Corresponding author. Tel.: +61 7 4930 9647; fax: +61 7 4930 9209.

E-mail address: s.white@cqu.edu.au (S.H. White).

¹ Present address: Biological and Environmental Sciences, Building 6, Central Queensland University, Bruce Highway, Rockhampton, Qld 4702, Australia.

² Present address: Centre for Environmental Management, Building 9, Central Queensland University, Bruce Highway, Rockhampton, Qld 4702, Australia.

result from both dissolved and cellular toxins (Mackay and Fraser, 2000; Voutsas et al., 2002; van der Oost et al., 2003).

The primary modes of CYN uptake are unknown. The relative abundance of intracellular and extracellular toxin fractions, including spatial and temporal variation, depends largely on bloom speciation and the age of a toxic bloom. This may be critical, since fraction bioavailability is inextricably linked to available toxin uptake routes. Recently, this concept has been investigated as a possible means of predicting and managing bioaccumulation risk for aquatic organisms living in contaminated waters (White et al., 2005). However, field and experimental research data are needed to further develop this predictive management framework. To date, only two studies have examined bioaccumulation of CYN in aquatic organisms (Saker and Eaglesham, 1999; Saker et al., 2004). *Cylindrospermopsis* has also been recorded from the cladoceran *Daphnia magna* (Nogueira et al., 2004a). In the latter, however, bioaccumulation was not present, since reported bioaccumulation factors (BAFs; tissue toxin concentrations divided by available toxin) were <1 .

This study examines accumulation of CYN and its analog, deoxy-CYN, in the freshwater gastropod, *Melanoidea tuberculata*. Since gastropods are grazer species, multiple methods of CYN uptake are possible. Herbivorous species may ingest cellular toxins by grazing (intentionally or accidentally) on toxic blue-green algae. Snails may also be vulnerable to dissolved transdermal uptake as a result of their submerged habit, and consequent prolonged contact with dissolved toxins. Several gastropod species are already known to bioaccumulate microcystin (Kotak et al., 1996; Zurawell, 2001; Yokoyama and Park, 2002, 2003; Ozawa et al., 2003), but studies have not examined CYN bioaccumulation.

2. Materials and methods

M. tuberculata (Müller, 1774) is an introduced Asian prosobranch snail typically found in stagnant or slow-flowing waters (Dillon, 2000). The species is relatively common in central Queensland waterways. *M. tuberculata* were collected from Moores Ck (Rockhampton, Qld). A subsample of Moores Ck water collected at the site was subsequently shown to have no CYN present. Specimens were cultured in a large plastic tub filled with Moores Ck water and housed in a controlled climate room ($24 \pm 2^\circ\text{C}$) under 12:12 light:dark photoperiod. Snails were provided mixed green algae obtained from the collection site. Snail species confirmation was provided by Dr Winston Ponder (Australian Museum, Sydney).

2.1. Bioconcentration trials

Bioconcentration was defined as the uptake of toxins from an exclusively extracellular source. Two trials

examined CYN bioconcentration by exposing *M. tuberculata* to double freeze-thawed *C. raciborskii* whole cell extracts containing extracellular CYN. Treatments included controls plus five CYN concentrations at 25, 50, 100, 200 and $400\ \mu\text{g L}^{-1}$. Test solutions (200 mL volume) were prepared by diluting cultures of *C. raciborskii* (strain CQU FR001) of known toxicity to the desired test concentrations. In the first trial, the control and dilution waters used were filtered, aged, non-sterile tap water. Control and dilution waters in the second definitive trial were filtered (Whatman GF/F glass microfibre) creek water obtained from the specimen collection site. All solutions, including controls, were replenished at 2-day intervals throughout both trials to ensure constant CYN concentrations.

Trials were conducted under 12:12 L:D photoperiod regime ($80\ \mu\text{mol photon m}^{-2}\text{ s}^{-1}$). Test vessels were rectangular glass dishes with glass lids. Stainless steel mesh (Termi-mesh commercial-grade termite barrier) rectangles were cut and positioned at the surface to prevent snails escaping exposure to the test solutions. Small holes were punched through the mesh to accommodate aerators. Test chambers were randomly arranged and half-submersed in a water bath at $26.5 \pm 1.0^\circ\text{C}$. Water was kept well circulated by the use of Thermoline temperature regulation and water circulation devices. Water temperatures were recorded half-hourly using StowAway TidBit DataLoggers. Chamber aeration was provided by a HiBlow electric air compressor (Sakuragawa Pty Ltd, Japan).

Water quality data were collected at 48 h intervals, including measurements of conductivity (TPS LC84), pH (TPS 80A), dissolved oxygen (TPS WP-82Y) and total ammonia (Aquasonic or Aquarium Pharmaceuticals Inc., freshwater total ammonia salicylate test kits). Water hardness and alkalinity of the control/dilution water was measured from 150 mL filtered subsamples ($0.45\ \mu\text{m}$ Millipore nylon filter) and determined using atomic absorption spectroscopy and alkalinity titrations.

Snails were not fed during the experimental period since toxin adsorption to food sources may have influenced the bioavailability of extracellular CYN. For example, snails may have been able to graze on adsorbed toxin, even though it was extracellular. Minimizing food provisions, sources also reduced faecal matter, which may otherwise have decreased dissolved oxygen concentrations and hence the biological activity of some materials (ASTM, 2003). Exposure periods of similar length, including without food provisions, have been used successfully by other authors (Lajtner et al., 1996; Klobucar et al., 1997).

Trials commenced by randomly assigning three snails per flask. Snails were harvested ($n=$ three flasks per treatment) on days 7 and 14, euthanized by freezing and stored frozen until CYN analyses were carried out. Since trial 1 differed from all other experiments by the use of tap water in the controls, tissues from selected snails only were analysed for CYN in this trial. All snail specimens were analysed in the second (and third) trials.

When snails were harvested on day 7 of the first definitive trial, shells were reserved during dissection to determine the concentration of CYN associated with the shell component. Shells were pooled within treatment groups ($n=9$ shells per treatment), weighed (fresh weight) and frozen (unrinsed), before thawing and macerating into fine slurry using a glass mortar and pestle with 2 mL of Milli-Q water. The slurry was washed into centrifuge tubes and stored frozen until toxin analyses were carried out.

2.2. Bioaccumulation trial

Bioaccumulation was defined as toxin uptake resulting from both intracellular and extracellular sources. One trial examined the bioaccumulation of toxin during exposure to a live *C. raciborskii* culture. Test conditions were as described in the extracellular trials, with the exception of the treatment solutions. These were prepared by pooling several 1 L *C. raciborskii* cultures into a fish tank on the day prior to trial commencement. Treatments were prepared by thoroughly mixing the tank culture with a metal spoon, subsampling and diluting to test concentrations of 10, 20, 30, 40 and 50%, again using filtered creek water. Controls were filtered creek water only.

Total (CYN_{TOT}), intracellular (CYN_{INC}) and extracellular (CYN_{EXC}) toxin fractions were monitored closely throughout the trial. On day 0, and every 48 h thereafter, two 50 mL subsamples were collected from the source culture (tank), and two subsamples per treatment concentration (pooled sample of all replicates within a given treatment). For each pair, one sample was filtered (Whatman GF/F glass microfibre) and frozen for determination of CYN_{EXC} ; the second was frozen without filtering for determination of CYN_{TOT} (intracellular plus extracellular fractions). Total CYN only was determined from controls since no CYN was expected to be present. Variability within treatment replicates was also tested on random samples.

C. raciborskii cell concentrations were monitored in selected treatments and the source culture by periodically collecting 10 mL aliquots and preserving with Lugol's iodine. Later, duplicate trichome counts were performed using the Sedgewick-Rafter counting chamber method: each chamber was counted for a total of 25 squares or 100 trichomes. Trichome counts were converted to approximate cell counts by dividing average trichome length ($n=200$ trichomes; from control and various experimental treatments) by the known cell length of *C. raciborskii* strain FR001 in culture (Fabbro et al., 2001). Duplicate counts were averaged and CYN cell quotas calculated from known CYN_{TOT} and CYN_{INC} concentrations.

2.3. Toxin analyses

Specimens were thawed and the shell manually dissected from the soft tissues. Tissues were rinsed in distilled water and placed in a centrifuge tube, freeze-dried (approximately

24–48 h, Virtis Sentry freeze-drier with Alcatel vacuum pump) and brought to room temperature before being reweighed. Tissues were then homogenized in Milli-Q water (Ultraturrax; 24,000 rpm for approximately 1 min) and immediately frozen. Samples were sent to Queensland Health Scientific Services for analysis of CYN and deoxy-CYN concentration. Frozen samples were thawed, centrifuged at 3000 rpm and the supernatant filtered using 0.45 μm syringe filters (Millex HV, Millipore Corp., Bedford, MA). Detection of free (non-bound) toxins was achieved via high performance liquid chromatography/tandem mass spectrometry, using an AB/Sciex API 300 mass spectrometer (Applied Biosystems, Concord, Ont., Canada) equipped with a turbo-ion-spray interface coupled to a Shimadzu SCL-10Avp HPLC system (Kyoto, Japan) (Eaglesham et al., 1999; Norris et al., 1999). This technique has limit of detection ≤ 3.0 ng per 5 mL sample (approximately equivalent to 0.5 $\mu\text{g L}^{-1}$) for both CYN and deoxy-CYN. Spike recoveries averaged 87.5% at concentrations between 1.8 and 5.7 $\mu\text{g L}^{-1}$ ($n=4$).

3. Results

3.1. Water quality and toxin concentrations

Oxygen saturation of the test solutions generally remained $\geq 80\%$ saturation; no gross changes in pH or conductivity occurred either within or between treatments (Table 1). Total ammonia concentrations also remained ≤ 1.0 ppm, excepting some 400 $\mu\text{g L}^{-1}$ treatments in the extracellular trials. Measured CYN concentrations were generally at or above nominal test concentrations in extracellular trials (Table 2). In the live exposure trial, the average toxin concentration in the source culture was 659 $\mu\text{g L}^{-1}$, whilst concentrations for the control and culture treatments were 1, 91, 167, 223, 294 and 406 $\mu\text{g L}^{-1}$, respectively (Table 3). Maximum and minimum CYN exposure concentrations were therefore easily comparable with the nominal test values used in the extracellular trials (0–400 $\mu\text{g L}^{-1}$). Toxin was detected in one control sample only (Table 3). Extracellular toxin represented between 72 and 81% of total CYN (Fig. 1B, Table 3). Average deoxy-CYN concentrations ranged between 3 and 12 $\mu\text{g L}^{-1}$ (controls excluded), or 1.8–3.3% deoxy-CYN relative to total CYN (Table 3).

Approximate toxin quotas (Q_{CYN}) for *C. raciborskii* cells were calculated by dividing CYN_{INC} concentrations by cell count data. In the source culture, average Q_{CYN} was 0.09 pg cell $^{-1}$, whilst experimental treatments recorded an average Q_{CYN} of 0.21 pg cell $^{-1}$ (Table 4). However, if calculated from CYN_{TOT} concentrations (intracellular plus extracellular toxin), Q_{CYN} values were approximately four- to fivefold higher (Table 4). Too few samples were taken to identify trends in Q_{CYN} 's over the experimental period.

Table 1
Ranges for water quality parameters measured throughout trials

	Trial 1 (extracellular)	Trial 2 (extracellular)	Trial 3 (live exposure)
Illumination ($\mu\text{mol photon m}^{-2} \text{s}^{-1}$)	60–75	60–75	60–75
Temperature ($^{\circ}\text{C}$)	25.5 ± 1	24.5 ± 1.5	25.0 ± 1.5
Alkalinity (mg L^{-1} of CaCO_3) ^a	140	119	110
Hardness (mg L^{-1} of CaCO_3) ^a	312	357	195
pH	7.6–8.8	8.0–8.8	7.9–8.9
Conductivity ($\mu\text{S cm}^{-1}$)	279–465	85–139	527–873
Dissolved oxygen (% saturation)	≥ 73	≥ 80	$\geq 62^b$
Ammonia (ppm)	0–5	0–2.0	0–1.0

^a Control/dilution water, prior to trial.

^b Outliers, values typically $\geq 80\%$.

3.2. Toxin in the shell

Toxin deposition into the shell appeared to be generally dose-dependent (Fig. 2). However, the bioconcentration factor (BCF; calculated as tissue toxin ($\mu\text{g kg}^{-1}$ wet weight) divided by exposure concentrations ($\mu\text{g L}^{-1}$)) averaged 0.12 (controls excluded), indicating no bioconcentration.

3.3. CYN bioconcentration and bioaccumulation

CYN concentrations in *M. tuberculata* varied greatly (Fig. 3A–C). Maximum tissue concentrations corresponded with highest exposure concentrations ($400 \mu\text{g L}^{-1}$) and longest exposure periods (14 days) (Fig. 3A, B). Considerable differences in toxin concentrations were recorded from *M. tuberculata* the first and second trials, despite both trials using the same exposure regime (Fig. 3 A, B).

Tissue toxin concentrations were dramatically increased in the live culture trial compared with the whole-cell extract trials. Whilst the effect of exposure concentrations was relatively weak, increasing the exposure time from 7 to 14 days typically resulted in tissue CYN values being almost doubled (Fig. 3C). Toxin concentrations were also more variable than the second definitive trial (Table 5).

A strong, positive correlation was evident between tissue CYN concentrations and CYN exposure concentrations (total or extracellular) in all trials (Table 6). In the live trial, tissue toxin concentrations were significantly positively correlated with total CYN_{TOT}, CYN_{INC} and CYN_{EXC}, and the ratio of intracellular to extracellular toxin. Exposure time was significantly positively correlated with

absolute tissue toxin values in the live exposure trial only (Table 6).

Extracellular CYN exposure resulted in bioconcentration only in definitive trial 1, when exposures were $\geq 200 \mu\text{g L}^{-1}$ (Table 7). The maximum BCF was 1.48, recorded after 14 days exposure to $400 \mu\text{g L}^{-1}$. Generally, increased exposure times and exposure concentrations resulted in higher BCFs (Fig. 4A). Bioaccumulation values were strikingly different in the live exposure trial. Every treatment (excluding controls) recorded bioaccumulation, with BAFs being >100 , especially in the second week of exposure (Table 7). Again, high BAFs corresponded with increased exposure concentration and exposure time (Fig. 4B). However, CYN treatment concentration significantly influenced the BCF of *M. tuberculata* only in the first extracellular trial (Table 8). Increasing exposure time significantly increased BAF values in the live exposure trial (Table 8).

3.4. Deoxy-CYN bioconcentration and bioaccumulation

Deoxy-CYN concentrations were analysed in trials 2 (extracellular exposure) and 3 (live exposure) only. Overall, average tissue deoxy-CYN concentrations were far lower than CYN concentrations, peaking at $7113 \mu\text{g kg}^{-1}$ (dry weight) during the live culture trial (Fig. 5A, B). BCFs could not be calculated in the extracellular trial since deoxy-CYN exposure concentrations were unknown. In the live trial, BAFs ranged from 20.4 to 249.3 (Table 9). Increased BAFs corresponded with increased exposure times (Fig. 6), in similarity to the CYN bioaccumulation results. However,

Table 2
CYN concentrations of selected treatment solutions from extracellular trials

Trial	Day(s) since solution renewal	Test concentration ($\mu\text{g L}^{-1}$ CYN)	Actual concentration ($\mu\text{g L}^{-1}$ CYN)	Percent (%) remaining	Actual concentration ($\mu\text{g L}^{-1}$ deoxy-CYN)	Deoxy-CYN (% of CYN)
Definitive 1	1	100	100	100	nt	na
Definitive 1	1	200	250	125	nt	na
Definitive 2	2	50	31	62	9.1	29
Definitive 2	1	400	336	84	95.1	28

Values indicate the CYN concentration of three pooled replicates. nt, not tested; na, not applicable.

Table 3

Values for CYN toxin fractions in original culture, control and experimental treatments during trial 3

Day of trial	Toxin concentrations ($\mu\text{g L}^{-1}$ or %EXC) in experimental treatments						
	Source	Control	10%	20%	30%	40%	50%
CYN_{TOT}							
0	438	nt	nt	Nt	nt	nt	nt
2	542	nd	57	5.5 ^a	184	255	320
4	420	nd	71	132	210	299	357
6	714	nd	81	155	213	287	370
8	631	nd	73	150	209	396	491
10	985	nd	130	191	227	336	460
12	879	5.8	117	182	264	264	449
14	nt	nd	110	191	258	223	394
Average \pm standard error	659 \pm 81	na	91 \pm 10	167 \pm 9 ^a	223 \pm 11	294 \pm 22	406 \pm 24
CYN_{EXC}							
0	370	nt	nt	nt	nt	nt	nt
2	369	nt	46	75	132	181	246
4	382	nt	61	115	174	232	284
6	501	nt	67	130	186	236	303
8	372	nt	64	122	171	293	362
10	563	nt	84	131	178	nt	331
12	628	nt	81	133	201	355	354
14	nt	nt	102	156	230	287	nt
Average \pm standard error		na	72 \pm 7	123 \pm 9	182 \pm 11	264 \pm 23	313 \pm 17
% EXC							
0	84	nt	nt	nt	nt	nt	nt
2	68	nt	78	na ^a	72	71	77
4	91	nt	85	87	83	78	80
6	70	nt	83	84	88	82	82
8	59	nt	87	81	82	74	74
10	57	nt	65	68	78	na	72
12	71	nt	69	73	76	134	79
14	nt	nt	93	82	89	129	na
Average \pm standard error	72 \pm 5	na	80 \pm 4	79 \pm 3	81 \pm 2	nc ^b	77 \pm 1
INC/EXC ratio (average)	nc	0	0.28	0.27	0.23	0.30	0.30
Deoxy-CYN_{TOT}							
0	5.6	nt	nt	nt	nt	nt	nt
2	6.2	nd	0.9	0.6	5.4	6.3	8.9
4	11.4	nd	2.3	3.4	5.4	9.8	11.3
6	19.3	nd	2.6	3.8	5.0	9.8	10.7
8	7.5	nd	1.2	1.8	2.4	9.8	12.9
10	26.2	nd	4.2	5.3	7.0	3.5	13.9
12	9.2	nd	3.4	5.7	9.2	8.1	14.4
14	nt	nd	3.3	7.2	7.8	5.3	12.1
Average \pm standard error	12 \pm 2.9	na	3 \pm 0.5	4 \pm 0.8	6 \pm 0.8	7 \pm 1.0	12 \pm 0.7
Average deoxy-CYN (%average total CYN)	1.8	na	3.3	2.4	2.7	2.4	3.0

nt, not tested; na, not applicable; nc, not calculated; nd, not detected ($<0.5 \mu\text{g L}^{-1}$).^a Day 2 result discarded, given the outlier nature and the result recorded for the day 2 extracellular component.^b Some (anomalous) results exceeded 100%.

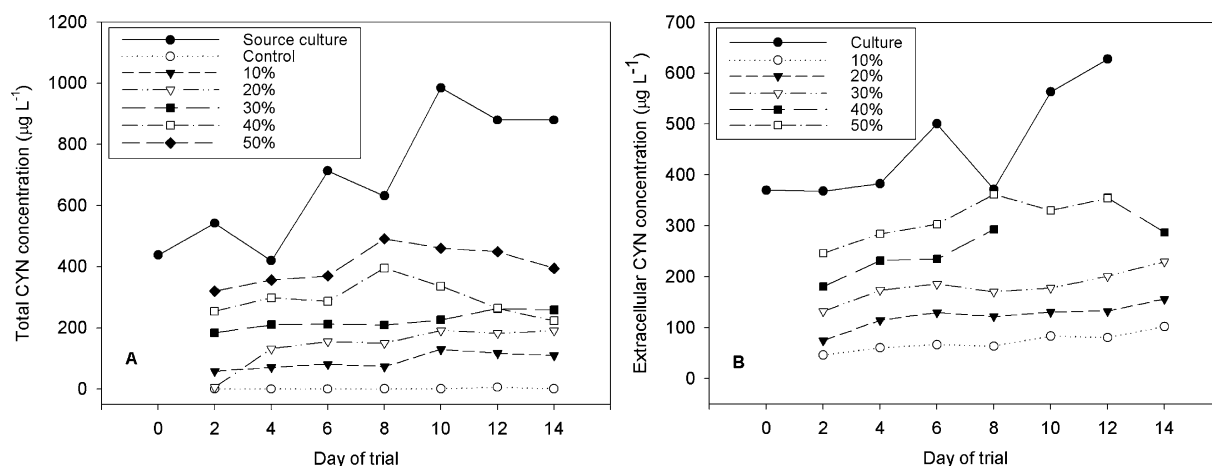


Fig. 1. CYN concentrations during trial 3: (A) total CYN concentrations; (B) extracellular CYN concentrations (controls not tested).

the BAFs of CYN and deoxy-CYN were significantly different in the live trial ($p > 0.050$; one-way ANOVA), with average total deoxy-CYN BAFs being higher than those for CYN (85.4 and 72.3, respectively). Both CYN treatment concentration and exposure time were significantly correlated with deoxy-CYN concentrations in the tissues (Table 10). However, only exposure time was significantly correlated with deoxy-CYN BAFs.

4. Discussion

4.1. Water quality

Differences in the conductivity, alkalinity and hardness of dilution waters reflected rain periods that occurred between specimen (and hence control water) collections. Since *M. tuberculata* thrives in these conditions naturally, such variations are considered to have had minimal

influence on the snails. Elevated total ammonia concentrations may be associated with large quantities of faecal matter.

4.2. CYN, deoxy-CYN and cell concentrations

Deoxy-CYN concentrations relative to CYN concentrations ranged widely, averaging 29% (second trial) to just 2.5% (third trial) (Tables 2 and 3). The production of deoxy-CYN may depend on at least three factors: the species and/or strain responsible for production; culture age; and the makeup of culturing media, particularly regarding to nitrogen availability. Deoxy-CYN production has been reported at 27–200% the quantity of CYN for an Australian *C. raciborskii* grown in Jaworski's media (Norris et al., 2001) but <10% for a Thailand strain grown in CT media containing nitrogen (Li et al., 2001b). *R. curvata* produces the analog at 2000 times the

Table 4
Cell quotas of *C. raciborskii* based on total and intracellular-only CYN values

Treatment	Cells (mL ⁻¹): average ± standard error	CYN _{TOT} (ng mL ⁻¹)	Cell quota (pg cell ⁻¹)	CYN _{INC} (ng mL ⁻¹)	Cell quota (pg cell ⁻¹)
Source day 0	1,288,950 ± 365,203	438	0.34	68	0.05
Source day 4	2,032,575 ± 26,440	420	0.21	37	0.02
Source day 10	2,118,505 ± 16,525	985	0.46	421	0.20
Source day 12	1983 ± 661	879	nc ^a	251	nc ^a
Average			0.34		0.09
40% day 2	853,351 ± 36,355	255	0.30	73	0.09
30% day 4	38,149 ± 7271	210	1.52	36	0.26
40% day 4	521,529 ± 7271	299	0.57	67	0.13
10% day 6	31,992 ± 529	81	2.53	14	0.44
30% day 8	409,159 ± 661	209	0.51	38	0.09
50% day 8	1,052,642 ± 47,923	491	0.47	129	0.12
20% day 10	295,467 ± 6610	191	0.65	61	0.21
30% day 14	120,302 ± 200	258	2.14	42	0.35
Average			1.09		0.21

^a Not calculated due to unexpectedly low cell concentrations.

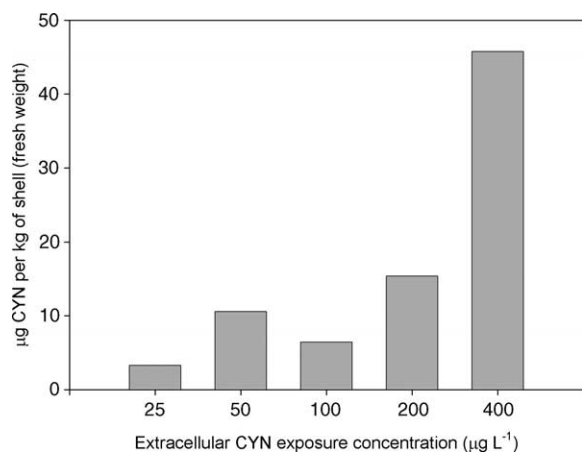


Fig. 2. CYN recorded from the shells of *M. tuberculata* following 7 days exposure to extracellular toxin.

concentration of CYN (Li et al., 2001a). In the current work, cultures for the extracellular and live trials were cultured in ASM1 algal media (Gorham et al., 1964) and were approximately 5 and 6 months old, respectively.

Q_{CYN} values compared with other values reported for cultured *C. raciborskii* (Fabbro et al., 2001), but were much higher than those recorded from natural settings. For example, Saker et al. (2004) reported a Q_{CYN} value of $0.03 \text{ pg cell}^{-1}$ for a Townsville strain of *C. raciborskii*. Average Q_{CYN} 's in experimental treatments (with snails added) were nearly triple those of the source culture (containing no snails). This could indicate a competitive response by *C. raciborskii* in relation to snail grazing. For example, increased Q_{CYN} 's may lower the palatability of *C. raciborskii* or increase toxicity: both could be effective in deterring potential grazers. High toxin quotas may also be linked with feeding inhibition: Pereira et al. (2004) demonstrated reduced cell clearance of mussels fed *Aphanizomenon issatschenkoi* when those cells had a high cell toxin content.

4.3. CYN associated with shell

M. tuberculata did not bioconcentrate CYN into shell material ($\text{BCF} < 1$), though small quantities of toxin suggest toxin adsorption to the mantle or shell wall. Possible interaction(s) between CYN and the proteinaceous and calcareous components of gastropods shells are not known. Thus, bound CYN may have been deposited into the shell, but was unable to be extracted via HPLC/MS-MS. Bioconcentration could take place as the shell layer is secreted from the mantle; however, far longer periods of CYN exposure may be required for this to occur.

4.4. CYN bioconcentration and bioaccumulation

CYN accumulation has only been studied in one other freshwater mollusc: *Anodonta cygnea* mussels recorded

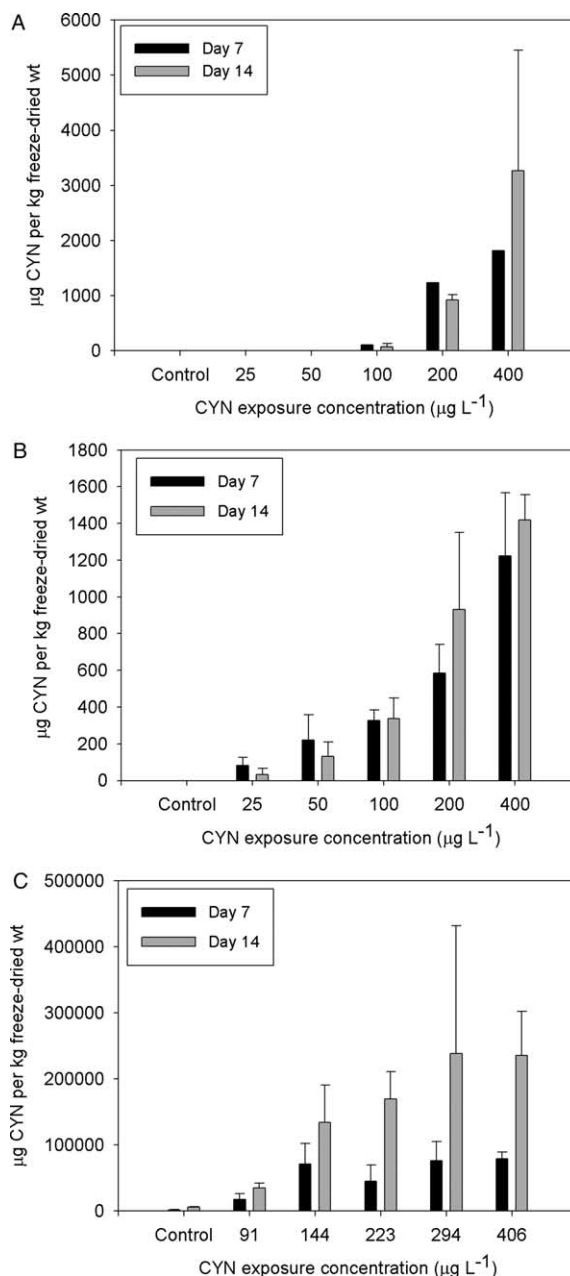


Fig. 3. CYN (expressed as dry-weight equivalent) in *M. tuberculata*: (A) definitive trial 1; (B) definitive trial 2; (C) definitive trial 3. Graphs show average ($n=3$, except in trial 1 where only selected samples were tested), bars show standard error.

maximum CYN values in the haemolymph of 408 µg L^{-1} after 14 days exposure (Saker et al., 2004). When compared with the total CYN of the surrounding media (90 µg L^{-1}), this results in a BAF of 4.53. *M. tuberculata* recorded an average BAF value of 49.57 from the whole tissues in similar exposure conditions (Table 7). The higher BAF in *M. tuberculata* could be attributed to many factors: the

Table 5

Variability in data recorded from trial 2 (extracellular) and trial 3 (live exposure)

	Definitive trial 2		Definitive trial 3	
	Week 1	Week 2	Week 1	Week 2
Average CYN ($\mu\text{g kg}^{-1}$) ^a	406.1	475.6	48,445	136,446
Average standard deviation	213.5	225.0	29,981.9	105,586.1
Average standard error	123.2	129.9	17,310	60,960
% Error (standard error/average)	30.3	27.3	35.7	44.7

Values calculated from group averages per treatment ($n=6$).

^a Freeze-dry weight.

studies differed in terms of temperature, *C. raciborskii* strain, size of test species and toxin extraction methods. Studies of microcystin have demonstrated that molluscs are capable of accumulating this toxin at concentrations from 0.136–630 $\mu\text{g kg}^{-1}$ dry weight (Zurawell, 2001; Yokoyama and Park, 2003), which compares with the range of CYN accumulation values reported here (up to 250 $\mu\text{g kg}^{-1}$, Fig. 3).

Bioconcentration and bioaccumulation of CYN in *M. tuberculata* may have been underestimated since any toxin bound to tissue or metabolized was not measured. Spike recoveries indicating an average recovery of 87.5% of CYN are acceptable. Equally, bioaccumulation may have been overestimated, since toxins derived from the stomach content of *M. tuberculata* would be included in the total toxin result. Large proportions of toxins have been reported from the digestive glands of molluscs (Eriksson et al., 1989; Vasconcelos, 1995; Saker et al., 2004). Dissection of the alimentary tract prior to analyses could have reduced this problem; however, this was not done since the study was designed to emphasize environmental relevance. That is, making the distinction between ‘alimentary tract’ and ‘rest

Table 6

Pearson product moment correlations between tissue CYN concentrations and exposure regime

Trial	Exposure concentration	Exposure time
Definitive 1	$p=0.014; 0.685; 12$	ns
Definitive 2	$p=0.000; 0.865; 36$	ns
Definitive 3		$p=0.026; 0.363; 36$
CYN _{TOT}	$p=0.005; 0.457; 36$	
CYN _{EXC}	$p=0.004; 0.466; 36$	
CYN _{INC}	$p=0.005; 0.460; 36$	
CYN _{INC} /CYN _{EXC} ratio	$p=0.035; 0.352; 36$	

Cells show p -value, correlation coefficient and sample size. ns, not significant; $p>0.050$.

Table 7

Bioconcentration and bioaccumulation factors for CYN-exposed *M. tuberculata*; nd, no data available due to snail deaths; EXC, extracellular only; total, extracellular and intracellular.

Trial	Exposure regime			BCF or BAF ^a (average \pm standard error)
	Concentration ($\mu\text{g L}^{-1}$)	Toxin type	Period (days)	
Trial 1	25	EXC	7	0 ^b
	50	EXC	7	0 ^b
	100	EXC	7	0.13 ^b
	200	EXC	7	1.10 ^b
	400	EXC	7	1.00 ^b
	25	EXC	14	nd
	50	EXC	14	nd
	100	EXC	14	0.10 \pm 0.10
	200	EXC	14	0.97 \pm 0.11
	400	EXC	14	1.48 \pm 0.41
Trial 2	25	EXC	7	0.43 \pm 0.23
	50	EXC	7	0.62 \pm 0.16
	100	EXC	7	0.51 \pm 0.08
	200	EXC	7	0.48 \pm 0.08
	400	EXC	7	0.60 \pm 0.15
	25	EXC	14	0.26 \pm 0.26
	50	EXC	14	0.64 \pm 0.35
	100	EXC	14	0.56 \pm 0.14
	200	EXC	14	0.56 \pm 0.13
	400	EXC	14	0.66 \pm 0.11
Trial 3	91	Total	7	39.52 \pm 18.36
	167	Total	7	49.13 \pm 12.45
	223	Total	7	33.33 \pm 16.67
	294	Total	7	54.10 \pm 20.31
	406	Total	7	43.42 \pm 6.53
	91	Total	14	49.57 \pm 11.05
	167	Total	14	120.97 \pm 27.09
	223	Total	14	124.42 \pm 5.34
	294	Total	14	98.99 \pm 53.29
	406	Total	14	109.73 \pm 30.94

^a Bioconcentration factor (extracellular trials) or bioaccumulation factor (live trials).

^b No standard error since only selected samples analysed.

of the snail’ is meaningless, since predators typically consume the entire soft tissues of snails.

Considerable variability in tissue toxin concentrations was recorded between the extracellular trials, despite both having identical exposure regimes. The variability of the data could indicate the impact of stress on toxin uptake, metabolism and depuration. For example, additional or synergistic effects (such as poor water quality combined with CYN toxicity) may have resulted in a reduced ability to metabolize toxin. However, only selected samples were analysed for CYN in trial 1. The peak toxin value (3267 $\mu\text{g g}^{-1}$ freeze dried weight) was the average of two samples that had vastly different toxin concentrations (5951 and 583 $\mu\text{g g}^{-1}$). If these data are discounted, values for trial 1 compare favourably with those of trial 2.

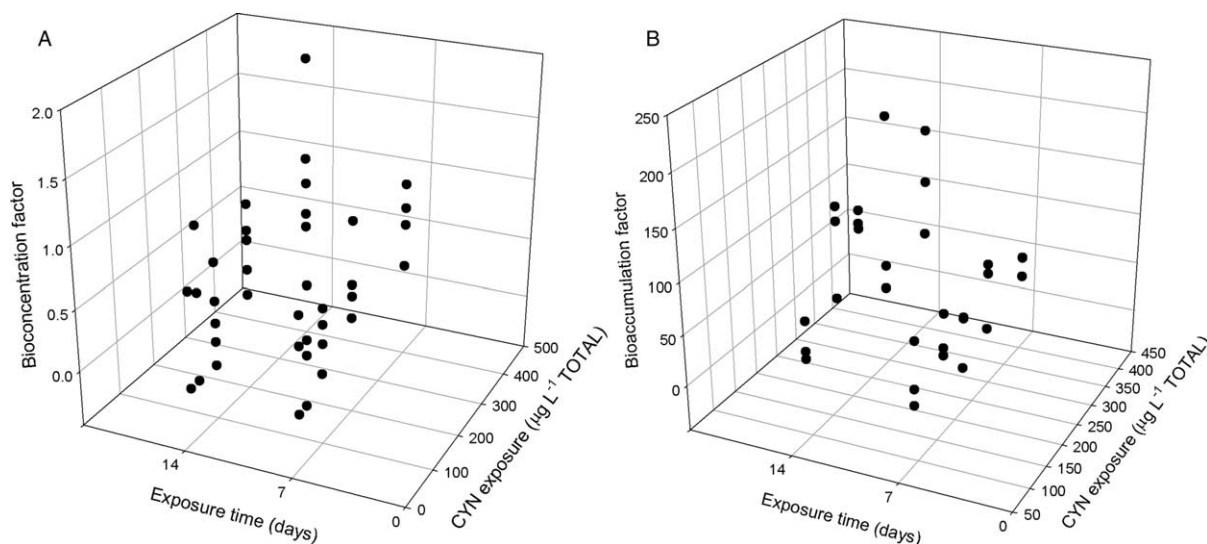


Fig. 4. Three-dimensional scatterplot comparing exposure concentration, exposure time, and bioconcentration factors (A) or bioaccumulation factors (B) for *M. tuberculata*.

The high variability in tissue toxin concentrations recorded during the live trial (Table 7) probably results from the multiple influences on uptake rates. This element of CYN bioaccumulation is also shared with microcystin: previous exposure, differential grazing rates, different snail habitats and the spatial distribution of toxin-laden cells within the water column all contributed to variability in microcystin concentrations of freshwater snails (Prepas et al., 1997; Zurawell et al., 1999). However, in the present work, most of these were controlled by the laboratory setting: grazing is thus likely to be the primary cause of variation.

4.4.1. Effects of toxin exposure concentrations and exposure time

High CYN_{TOT} concentrations are likely to result in snails having higher levels of tissue contamination than those in lower-CYN concentration environments. However, overall BAF or BCF values are likely to be similar in both. In contrast, longer exposure times will result in both higher absolute tissue toxin values and significantly increased BAFs, at least where intracellular toxin is present. A significant effect of exposure time was present only during live trial, possibly because tissue harvests were only conducted on days 7 and 14. Other authors have examined tissue toxin concentrations at 2-day or even hourly intervals, and found accumulation and depuration to be highly variable over these timescales (Zurawell, 2001; Saker et al., 2004).

4.4.2. Effects of toxin fraction availability

The difference between bioconcentration and bioaccumulation in *M. tuberculata* was considerable: exposure to

live *C. raciborskii* culture resulted in far higher toxin loads in *M. tuberculata* despite CYN_{TOT} concentrations being comparable to those used in the extracellular trials. Moreover, intracellular toxin accounted for less than one-quarter of total toxin, but led to over 100% increases in BAFs compared to BCFs. Intracellular toxin therefore appears to be critical in elevating CYN bioaccumulation values in *M. tuberculata*. Again, this is similar to microcystin accumulation: Prepas et al. (1997) reported that 50 µg L⁻¹ dissolved MC exposure over 3 days did not result in toxin bioaccumulation in the freshwater clam, *Anodonta*. In contrast, just 8 µg L⁻¹ total MC exposure (both cellular and dissolved fractions present) resulted in tissue concentrations of 776 ± 569 µg kg⁻¹ dry weight (Prepas et al., 1997).

Table 8

Summary results for Pearson product moment correlations between bioconcentration or bioaccumulation values for *M. tuberculata* and experimental conditions

Trial	Exposure concentration	Exposure time
Definitive 1 ^a	$p=0.001$; 0.861; 11	ns
Definitive 2 ^b	ns	ns
Definitive 3 ^b		$p=0.001$; 0.582; 30
CYN _{TOT}	ns	
CYN _{EXC}	ns	
CYN _{INC}	ns	
CYN _{INC} /CYN _{EXC} ratio	ns	

ns, not significant ($p>0.050$). Cells contain p -value, correlation coefficient and sample size.

^a Selected values only.

^b Data for controls omitted.

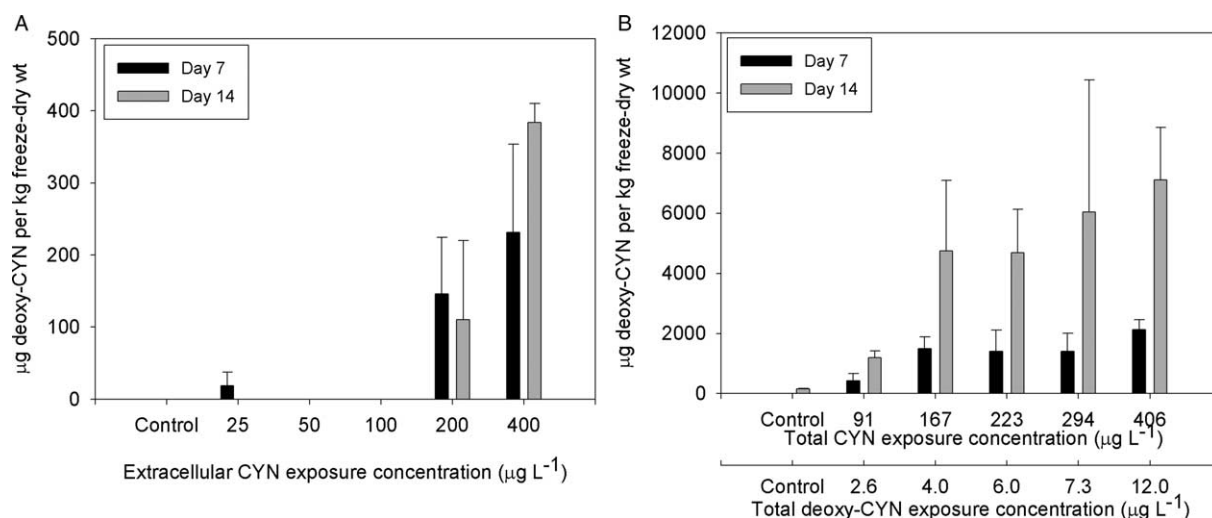


Fig. 5. Deoxy-CYN in the soft tissues of *M. tuberculosis* in (A) definitive trial 2 (extracellular toxin) and (B) definitive trial 3 (live culture exposure). Note: treatment deoxy-CYN values not known in definitive trial 2.

CYN exposure concentration strongly impacts the potential for tissue contamination (absolute toxin values, $\mu\text{g kg}^{-1}$) in *M. tuberculosis*. Toxin contamination cannot be accurately predicted from exposure concentration alone: length of exposure time and toxin fraction availability must also be considered. Exposure concentrations are also inadequate in predicting the extent of tissue contamination relative to toxins available in the surrounding environment (BCF and BAF values). Rather, these depend more closely on exposure time and, most importantly, the presence of intracellular toxin. In natural blooms, the proportions of dissolved and cell-bound toxins may be highly variable due to algal growth phase, degradation and dilution (Zurawell et al., 1999). Thus, it seems that accurate predictions of bioaccumulation risk cannot be made unless such ratios are properly and

regularly quantified, such as in the predictive management approach suggested by White et al. (2005).

4.5. Deoxy-CYN bioconcentration and bioaccumulation

Patterns of deoxy-CYN bioconcentration and bioaccumulation were similar to those of CYN, albeit with much lower final tissue concentrations. Deoxy-CYN BAFs were also significantly less than those for CYN. This reflects the fact that deoxy-CYN exposure concentrations were also far lower ($0\text{--}12 \mu\text{g L}^{-1}$) than those for CYN ($0\text{--}406 \mu\text{g L}^{-1}$). Consequently, exposure to, and accumulation

Table 9
Deoxy-CYN bioaccumulation factors for *M. tuberculosis* exposed live *C. raciborskii* treatments (controls not included)

Exposure regime		BAF (average \pm standard error)
Concentration ($\mu\text{g L}^{-1}$)	Period (days)	
3	7	29.4 ± 15.85
4	7	38.4 ± 1.45
6	7	31.3 ± 10.92
7	7	99.1 ± 30.94
12	7	20.4 ± 9.15
3	14	249.3 ± 8.22
4	14	73.6 ± 27.64
6	14	134.2 ± 27.51
7	14	66.7 ± 52.97
12	14	112.0 ± 27.69

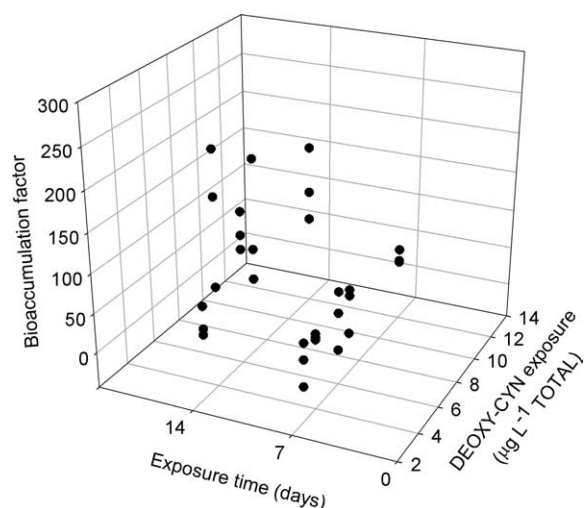


Fig. 6. Three-dimensional scatterplot comparing exposure concentration, exposure time, and bioaccumulation factors for *M. tuberculosis* exposure to deoxy-CYN. Controls not included.

Table 10

Pearson product moment correlations between tissue deoxy-CYN concentrations and bioaccumulation factor with exposure regime

	Exposure concentration	Exposure time
Tissue toxin concentration (μg deoxy-CYN kg^{-1} fresh weight)	$p=0.040$; 0.377; 30	$p=0.004$; 0.513; 30
Bioaccumulation factor	ns	$p=0.001$; 0.556; 30

Cells contain p -value, correlation coefficient and sample size. ns, not significant; $p > 0.050$. Controls not included. ns, not significant ($p > 0.010$).

of, deoxy-CYN may be considered less critical to freshwater aquatic organism in comparison to CYN.

4.6. Possible methods of uptake

During the extracellular trial, the only possible methods of toxin uptake were transdermal uptake (active or passive transport) or accidental drinking of aqueous toxin. The relative contribution of each of these pathways with respect to final tissue toxin values can only be speculated. Transdermal uptake could result from simple diffusion, where CYN may pass through the cell membranes of the dermis or gill epithelia. Possibly, the protective shell layer may limit transdermal uptake by shielding the fleshy tissues from toxin contact.

The live exposure trial introduced an entirely new route of uptake, since grazing represents an obvious opportunity to ingest large volumes of toxin-laden cells. *M. tuberculata* might graze on *C. raciborskii*, however, gastropods usually graze on attached periphyton. In the natural environment, planktonic *C. raciborskii* trichomes are uncommon in the benthic habitat of *M. tuberculata*. On the other hand, snails could consume the filaments that settle out of the water or accumulate in littoral areas during dense blooms. This has already been suggested to occur with *Microcystis* and *Nodularia* (Zurawell et al., 1999; Sipilä et al., 2001). Faecal strings of *M. tuberculata* were not examined for trichomes.

Digestion in freshwater gastropods requires particles to enter via the scraping radula, followed by grinding of material in the gizzard (Dillon, 2000). Only particles $\leq 0.4 \mu\text{m}$ are able to pass into the digestive diverticulae, ready for phagocytosis and/or extracellular digestion (Dillon, 2000). Based on this evidence, *C. raciborskii* cells could not leave the alimentary canal without having been lysed, and any toxin present in the cells would become liberated in the alimentary tract. However, intact *Microcystis* colonies have been reported from the faecal strings of *Lymnaea stagnalis*, indicating that cyanoprokaryote cells could escape the mechanical breakdown performed by the gizzard (Zurawell et al., 1999). Unlike *Cylindrospermopsis*, however, *Microcystis* colonies are typically encapsulated in mucilaginous sheaths: these could be crucial in preventing cell (and colony) lysis in the gut (Zurawell, 2001).

Given the dramatic increase in bioaccumulation values in conjunction with the introduction of (comparatively) small quantities of cell-bound toxin, grazing is considered to

represent the major uptake route. Conversely, extracellular toxin is likely to contribute to only minor tissue contamination. This conclusion is supported by the results of several studies of algal toxin accumulation (Kotak et al., 1996; Prepas et al., 1997; Zurawell et al., 1999; Ozawa et al., 2003; Saker et al., 2004).

4.7. Implications for management

Gastropods may form a substantial dietary component of many freshwater and terrestrial species (Dillon, 2000). High CYN concentrations in *M. tuberculata* may indicate the potential for biomagnification of CYN into higher trophic levels, potentially representing a critical issue for human consumption if, for example, commercially important fish predate on CYN-laden snails. This is especially important since CYN and other toxins may persist in the tissues for lengthy time periods. For example, toxins have been detected from organisms some 30–65 days after transfer into toxin-free water (Eriksson et al., 1989; Saker et al., 2004).

Ultimately, target setting for protection of aquatic systems will focus primarily on whether or not tissue contamination results in CYN imposing greater ecological risks for aquatic species, or if it causes tissue toxin concentrations to exceed trigger values for human consumption. This study has shown that changes in the relative abundance of toxin fractions, rather than overall CYN concentrations, show the most promise in predicting likely CYN bioaccumulation values in *M. tuberculata*.

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References

- ASTM. 2003. E 1192-97 Standard Guide for Conducting Tests on Aqueous Effluents with Fishes, Macroinvertebrates, and Amphibians¹. American Society for Testing and Materials International, West Conshohocken, PA, 14 pp.

- Briand, J.F., Le Boulanger, C., Humbert, J.-F., Bernard, C., Dufour, P., 2004. *Cylindrospermopsis raciborskii* (Cyanobacteria) invasion at mid-latitudes: selection; wide physiological tolerance, or global warming? *Journal of Phycology* 40, 231–238.
- Burkhard, L.P., Cook, P.M., Mount, D.R., 2003. The relationship of bioaccumulative chemicals in water and sediment to residues in fish: a visualisation approach. *Environmental Toxicology and Chemistry* 22, 2822–2830.
- Dillon, R.T., 2000. *The Ecology of Freshwater Molluscs*. Cambridge University Press, Cambridge.
- Eaglesham, G., Norris, K.R., Shaw, G.R., Smith, M.J., Chiswell, R.K., Davis, B.C., Neville, G.R., Seawright, A.A., Moore, B.S., 1999. Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. *Environmental Toxicology* 14, 151–154.
- Eriksson, J.E., Meriluoto, J., Lindholm, T., 1989. Accumulation of a peptide toxin from the cyanobacterium *Oscillatoria agardhii* in the freshwater mussel *Anadonta cygnea*. *Hydrobiologia* 183, 211–216.
- Fabbro, L.D., Baker, M., Duivenvoorden, L.J., Pegg, G., Shiel, R., 2001. The effects of the ciliate *Paramecium* cf. *caudatum* Ehrenberg on toxin producing cylindrospermopsis isolated from the Fitzroy river, Australia. *Environmental Toxicology* 16, 489–497.
- Gorham, P.R., McLachlan, J., Hammer, U.T., Kim, W.K., 1964. Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Breb. *Verhandlungen Internationale Vereinigung für Theoretische und Angewandte Limnologie* 15, 796–804.
- Harada, K.I., Ohtani, I., Iwamoto, K., Suzuki, M., Watanabe, M.F., Watanabe, M., Terao, K., 1994. Isolation of cylindrospermopsin from a cyanobacterium *Umezakia natans* and its screening method. *Toxicon* 32, 73–84.
- Klobucar, G.I.V., Lajtner, J., Erben, R., 1997. Lipid peroxidation and histopathological changes in the digestive gland of a freshwater snail *Planorbis cornutus* L. (Gastropoda, Pulmonata) exposed to chronic and sub-chronic concentrations of PCP. *Bulletin of Environmental Contamination and Toxicology* 58, 128–134.
- Kotak, B.G., Zurawell, R., Prepas, E., Holmes, C.F., 1996. Microcystin-LR concentration in aquatic food web compartments from lakes of varying trophic status. *Canadian Journal of Fisheries and Aquatic Sciences* 53, 1974–1985.
- Lajtner, J., Erben, R., Klobucar, G.I.V., 1996. Histopathological effects of phenol on the digestive gland of *Amphimelania holandri* Fér. (Gastropoda, Prosobranchia). *Bulletin of Environmental Contamination and Toxicology* 57, 458–464.
- Li, R., Carmichael, W.W., Brittain, S., Eaglesham, G., Shaw, G., Liu, Y., Watanabe, M., 2001a. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata* (Cyanobacteria). *Journal of Phycology* 37, 1121–1126.
- Li, R., Carmichael, W.W., Brittain, J.E., Eaglesham, G.K., Shaw, G.R., Mahakhan, A., Noparatnaraporn, N., Yongmanitchai, W., Kaya, K., Watanabe, M.M., 2001b. Isolation and identification of the cyanotoxin cylindrospermopsin and deoxycylindrospermopsin from a Thailand strain of *Cylindrospermopsis raciborskii* (Cyanobacteria). *Toxicon* 39, 973–980.
- Mackay, D., Fraser, A., 2000. Bioaccumulation of persistent organic chemicals: mechanisms and models. *Environmental Pollution* 110, 375–391.
- Nogueira, I.C.G., Saker, M.L., Pflugmacher, S., Wiegand, C., Vasconcelos, V.M., 2004. Toxicity of the Cyanobacterium *Cylindrospermopsis raciborskii* to *Daphnia magna*. *Environmental Toxicology* 19, 453–459.
- Norris, K.R., Eaglesham, G., Pierens, G., Shaw, G., Smith, M.J., Chiswell, R.K., Seawright, A.A., Moore, M.R., 1999. Deoxycylindrospermopsin, an analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 14, 163–165.
- Norris, R.L., Eaglesham, G.K., Shaw, G.R., Senogles, P., Chiswell, R.K., Smith, M.J., Davis, J.A., Seawright, A.A., Moore, M.R., 2001. Extraction and purification of the zwitterions cylindrospermopsin and deoxycylindrospermopsin from *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 16, 394–396.
- Ohtani, I., Moore, R.E., Runnegar, M.T.C., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. *Journal of the American Chemical Society* 114, 7941–7942.
- Ozawa, K., Yokoyama, A., Ishikawa, K., Kumagi, M., Watanabe, M., Park, H.-D., 2003. Accumulation and depuration of microcystin produced by the cyanobacterium *Microcystis* in a freshwater snail. *Limnology* 4, 131–138.
- Padisák, J., 1997. *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding highly adaptive cyanobacterium: worldwide distribution and review of its ecology. *Archiv für Hydrobiologie. Supplement* 107 (Monographic Studies) 4, 563–593.
- Pereira, P., Dias, E., Franca, S., Pereira, E., Carolino, M., Vasconcelos, V.M., 2004. Accumulation and depuration of cyanobacterial paralytic shellfish toxins by the freshwater mussel *Anodonta cygnea*. *Aquatic Toxicology* 68, 339–350.
- Prepas, E.E., Kotak, B.G., Campbell, L.M., Evans, J.C., Hrudey, S.E., Holmes, C.F., 1997. Accumulation and elimination of cyanobacterial hepatotoxins by the freshwater clam *Anodonta grandis simpsonia*. *Canadian Journal of Fisheries and Aquatic Sciences* 54, 41–46.
- Saker, M.L., Eaglesham, G.K., 1999. The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the redclaw crayfish *Cherax quadricarinatus*. *Toxicon* 37, 1065–1077.
- Saker, M.L., Metcalf, J.S., Codd, G.A., Vasconcelos, V.M., 2004. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. *Toxicon* 43, 185–194.
- Schembri, M.A., Neilan, B.A., Saint, C.P., 2001. Identification of genes implicates in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. *Environmental Toxicology* 16, 413–421.
- Shaw, G., Sufenik, A., Livne, A., Chiswell, R.K., Smith, M.J., Seawright, A.A., Norris, K.R., Eaglesham, G., Moore, M.R., 1999. Blooms of the cylindrospermopsin containing cyanobacterium, *Aphanizomenon ovalisporum* (Forti) in newly constructed lakes, Queensland, Australia. *Environmental Toxicology* 14, 167–177.
- Sipiä, V., Kankaanpää, H., Flinkman, J., Lahti, K., Meriluoto, J., 2001. Time-dependent accumulation of Cyanobacterial Hepatotoxins in Flounders (*Platichthys flesus*) and Mussels (*Mytilus edulis*) from the Northern Baltic Sea. *Environmental Toxicology* 16, 330–336.

- Stirling, D.J., Quilliam, M.A., 2001. First report of the cyanobacterial toxin cylindrospermopsin in New Zealand. *Toxicon* 39, 1219–1222.
- Terao, K., Ohmori, S., Igarshi, K., Ohtani, I., Watanabe, M.F., Harada, K.I., Ito, E., Watanabe, M., 1994. Electron microscope studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. *Toxicon* 32, 833–843.
- van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13, 57–149.
- Vasconcelos, V.M., 1995. Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*. *Aquatic Toxicology* 32, 227–237.
- Voutsas, E., Magoulas, K., Tassios, D., 2002. Prediction of the bioaccumulation of persistent organic pollutants in aquatic food webs. *Chemosphere* 48, 645–651.
- White, S.H., Duivenvoorden, L.J., Fabbro, L.D., 2005. A decision-making framework for ecological impacts associated with the accumulation of cyanotoxins (cylindrospermopsin and microcystin). *Lakes & Reservoirs: Research and Management* 10, 25–37.
- Yokoyama, A., Park, H., 2002. Mechanism and prediction for contamination of freshwater bivalves (Unionidae) with the cyanobacterial toxin microcystin in hypereutrophic Lake Suwa, Japan. *Environmental Toxicology* 17, 424–433.
- Yokoyama, A., Park, H., 2003. Depuration kinetics and persistence of the cyanobacterial toxin microcystin-LR in the freshwater bivalves *Unio douglasiae*. *Environmental Toxicology* 18, 61–67.
- Zurawell, R., 2001. Occurrence and Toxicity of Microcystins in the Freshwater Pulmonate Snail *Lymnaea stagnalis*. PhD Thesis, Department of Biological Sciences, University of Alberta, Edmonton, Alberta.
- Zurawell, R., Kotak, B., Prepas, E., 1999. Influence of lake trophic status on the occurrence of microcystin-LR in the tissue of pulmonate snails. *Freshwater Biology* 42, 707–718.