Influence of intracellular toxin concentrations on cylindrospermopsin bioaccumulation in a freshwater gastropod (*Melanoides tuberculata)*

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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 in comparison 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.

Keywords: *Cylindrospermopsis raciborskii*, bioaccumulation, bioconcentration, blue-green algae, cyanobacteria, deoxy-cylindrospermopsin.

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; Schembrii *et al.* 2001; Stirling & 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 result from both dissolved and cellular toxins (Mackay & 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 is needed to further develop this predictive management framework. To date, only two studies have examined bioaccumulation of CYN in aquatic organisms (Saker & Eaglesham 1999; Saker *et al.* 2004). Cylindrospermopsin 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 (tissue toxin concentrations divided by available toxin) were < 1.

This study examines accumulation of CYN and its analog, deoxy-CYN, in the freshwater gastropod, *Melanoides 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 & Park 2002; Ozawa *et al.* 2003; Yokoyama & Park 2003), but studies have not examined CYN bioaccumulation.

2. Materials and Methods

Melanoides 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, Queensland). 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 ± 2^0C) 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 μg L^{-1}. Test solutions (200mL 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 two-day intervals throughout both trials to ensure constant CYN concentrations.

Trials were conducted under 12:12 L:D photoperiod regime (80 μmol photon $m^{-2}s^{-1}$). 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 ± 1.0 ^{0}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 was collected at 48h 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 150mL filtered subsamples (0.45 μm 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 sources also reduced faecal matter, which may otherwise have decreased dissolved oxygen concentrations and hence the biological activity of some materials (ASTM 2003). Similarly long exposure periods without food 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 seven and fourteen, euthanized by freezing and stored frozen until CYN analyses were carried out. Since trial one 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 seven 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 2mL 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 one-litre *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 zero, and every 48h thereafter, two 50mL 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 10mL 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 – 48h, Virtis Sentry freeze-drier with Alcatel vacuum pump) and brought to room temperature before being reweighed. Tissues were then homogenised

in Milli-Q water (Ultraturrax; 24,000 rpm for approximately one minute) 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 micron 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, Ontario, Canada) equipped with a turbo-ionspray 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 5mL sample (approximately equivalent to 0.5 μg L^{-1}) for both CYN and deoxy-CYN. Spike recoveries averaged 87.5% at concentrations between 1.8 and 5.7 μg L^{-1} (n = 4).

3. Results

3.1 Water quality and toxin concentrations

Oxygen saturation of the test solutions generally remained ≥ 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 μg 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 μg L^{-1}, whilst concentrations for the control and culture treatments were 1, 91, 167, 223, 294 and 406 μg 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 μg L^{-1}). Toxin was detected in one control sample only

(Table 3). Extracellular toxin represented between 72 – 81 % of total CYN (Figure 1B, Table 3). Average deoxy-CYN concentrations ranged between 3 – 12 μg 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 five-fold higher (Table 4). Too few samples were taken to identify trends in Q_{CYN}'s over the experimental period.

3.2 Toxin in the shell

Toxin deposition into the shell appeared to be generally dose-dependant (Figure 2). However, the bioconcentration factor (BCF; calculated as tissue toxin (μg kg^{-1} wet weight) divided by exposure concentrations (μg L^{-1})) averaged 0.12 (controls excluded), indicating no bioconcentration.

3.3 CYN bioconcentration and bioaccumulation

CYN concentrations in *M. tuberculata* varied greatly (Figure 3A-C). Maximum tissue concentrations corresponded with highest exposure concentrations (400 μg L^{-1}) and longest exposure periods (fourteen days) (Figure 3A, B). Considerable differences in toxin concentrations were recorded from *M. tuberculata* the first and second definitive trials, despite both trials using the same exposure regime (Figure 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 seven to fourteen days typically resulted in tissue CYN values being almost doubled (Figure 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 one, when exposures were ≥ 200 μg L^{-1} (Table 7). The maximum BCF was 1.48, recorded after fourteen days exposure to 400 μg L^{-1}. Generally, increased exposure times and exposure concentrations resulted in higher BCFs (Figure 4A). Bioaccumulation values were strikingly different in the live exposure trial. Every treatment (excluding controls) recorded bioaccumulation, with bioaccumulation factors (BAFs) being >100, especially in the second week of exposure (Table 7). Again, high BAFs corresponded with increased exposure concentration and exposure time (Figure 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 two (extracellular exposure) and three (live exposure) only. Overall, average tissue deoxy-CYN concentrations were far lower than CYN concentrations, peaking at 7,113 μg kg^{-1} (dry weight) during the live culture trial (Figure 5A, B). Bioconcentration factors 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 (Figure 6), in similarity to the CYN bioaccumulation results. However, 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 bioaccumulation factors.

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, 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). *Raphidiopsis curvata* produces the analog at 2000 times the 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 five and six 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 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 high cell toxin content.

4.3 CYN associated with shell

M. tuberculata did not bioconcentrate CYN into shell material (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 mollusk: *Anodonta cygnea* mussels recorded maximum CYN values in the haemolymph of 408 µg L^{-1} after fourteen days exposure (Saker *et al.* 2004). When compared with the total CYN of the surrounding media (90 µg L^{-1}), this results in a bioaccumulation factor 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 studies differed in terms of temperature, *C. raciborskii* strain, size of test species and toxin extraction methods. Studies of microcystin have demonstrated that mollusks are capable of accumulating this toxin at concentrations from 0.136 – 630 µg kg^{-1} dry weight (Zurawell 2001; Yokoyama & Park 2003), which compares with the range of CYN accumulation values reported here (up to 250 µg kg-1, Figure 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 of the snail' is meaningless, since predators typically consume the entire soft tissues of snails.

10 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 one. The peak toxin value (3267 μg g^{-1} freeze dried weight) was the average of two samples that had vastly different toxin concentrations (5951 and 583 μg g^{-1}). If these data are discounted, values for trial one compare favourably with those of trial two.

20 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.

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 seven and fourteen. Other authors have examined tissue toxin concentrations at two-day or even hourly intervals, and found accumulation and depuration to be highly variable over these timescales (Zurawell 2001; Saker *et al.* 2004).

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 three 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).

CYN exposure concentration strongly impacts the potential for tissue contamination (absolute toxin values, μg kg^{-1}) in *M. tuberculata.* 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 – 12 μg L^{-1}) than those for CYN (0 – 406 μg L^{-1}). Consequently, exposure to, and accumulation 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 dermisor 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; Sipiä *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 ≤ 0.4 μm 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 to 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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Figure captions

Figure 1. CYN concentrations during trial three (A) total CYN concentrations; (B) extracellular CYN concentrations (controls not tested).

Figure 2. CYN recorded from the shells of *M. tuberculata* following seven days exposure to extracellular toxin.

Figure 3. CYN (expressed as dry-weight equivalent) in *M. tuberculata*: (A) definitive trial one, (B) definitive trial two, (C) definitive trial three. Graphs show average (n = 3, except in trial one where only selected samples were tested), bars show standard error.

Figure 4. 3-D scatterplot comparing exposure concentration, exposure time, and bioconcentration factors (figure A) or bioaccumulation factors (figure B) for *M. tuberculata.*

Figure 5. Deoxy-CYN in the soft tissues of *M. tuberculata* in (A) definitive trial two (extracellular toxin); and (B) definitive trial three (live culture exposure). Note: treatment deoxy-CYN values not known in definitive trial two.

Figure 6. 3-D scatterplot comparing exposure concentration, exposure time, and bioaccumulation factors for *M. tuberculata* exposure to deoxy-CYN. Controls not included.

Table 1. Ranges for water quality parameters measured throughout trials. nt = not tested.

	Trial one (extracellular)	**Trial two (extracellular)**	**Trial three (live exposure)**
Illumination (μmol photon $m^{-2}s^{-1}$)	60 – 75	60 – 75	60 – 75
Temperature (^{0}C)	25.5 ± 1 ^{0}C	24.5 ± 1.5 ^{0}C	25.0 ± 1.5 ^{0}C
Alkalinity (mg/L of CaCo3) [a]	140	119	110
Hardness (mg/L of CaCo3)[a]	312	357	195
pH	7.6 – 8.8	8.0 – 8.8	7.9 – 8.9
Conductivity (μS cm^{-1})	279 – 465	85 – 139	527 – 873
DO (% Saturation)	≥ 73	≥ 80	≥ 62[b]
Ammonia (ppm)	0 – 5	0 – 2.0	0 – 1.0

[a] Control/dilution water, prior to trial; [b]outliers, values typically ≥ 80%.

Table 2. CYN concentrations of selected treatment solutions from extracellular trials. Values indicate the CYN concentration of three pooled replicates. nt=not tested; na=not applicable.

Trial	Day(s) since solution renewal	Test concentration ($\mu g\ L^{-1}$ CYN)	Actual concentration ($\mu g\ L^{-1}$ CYN)	Percent (%) remaining	Actual concentration ($\mu g\ L^{-1}$ 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

Table 3. Values for CYN toxin fractions in original culture, control and experimental treatments during trial three. nt=not tested; na=not applicable; nc=not calculated; and nd=not detected (<0.5 μg L^{-1}).

Day of Trial	Toxin concentrations (μg 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
Avg ± std. error	*659±81*	*na*	*91±10*	*167±9*[a]	*223±11*	*294±22*	*406±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
Avg ± std. error		*na*	*72±7*	*123±9*	*182±11*	*264±23*	*313±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
Avg ± std. error	*72±5*	*na*	*80±4*	*79±3*	*81±2*	*nc*[b]	*77±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
Avg ± std. error	*12±2.9*	*na*	*3±0.5*	*4±0.8*	*6±0.8*	*7±1.0*	*12±0.7*
Average deoxy-CYN (%average total CYN)	*1.8*	*na*	*3.3*	*2.4*	*2.7*	*2.4*	*3.0*

[a] Day two result discarded, given the outlier nature and the result recorded for the day two extracellular component; [b] some (anomalous) results exceeded 100%.

Table 4. Cell quotas of *C. raciborskii* based on total and intracellular-only CYN values. nc=not calculated.

Treatment	**Cells mL^{-1} Average ± std error**	**CYN_{TOT} (ng mL^{-1})**	**Cell quota (pg cell^{-1})**	**CYN_{INC} (ng mL^{-1})**	**Cell quota (pg cell^{-1})**
Source day 0	1288950 ± 365203	438	0.34	68	0.05
Source day 4	2032575 ± 26440	420	0.21	37	0.02
Source day 10	2118505 ± 16525	985	.046	421	0.20
Source day 12	1983 ± 661	879	nc[a]	251	nc[a]
Average			*0.34*		*0.09*
40% day 2	853351 ± 36355	255	0.30	73	.009
30% day 4	138149 ± 7271	210	1.52	36	0.26
40% day 4	521529 ± 7271	299	0.57	67	0.13
10% day 6	31992 ± 529	81	2.53	14	0.44
30% day 8	409159 ±661	209	0.51	38	0.09
50% day 8	1052642 ± 47923	491	0.47	129	0.12
20% day 10	295467 ± 6610	191	0.65	61	0.21
30% day 14	120302 ± 200	258	2.14	42	0.35
Average			*1.09*		*0.21*

[a] not calculated due to unexpectedly low cell concentrations.

Table 5 Variability in data recorded from trial two (extracellular) and trial three (live exposure).Values calculated from group averages per treatment (*n* = 6).

	Definitive trial two		Definitive trial three	
	Week one	**Week two**	**Week one**	**Week two**
Average CYN (μg 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

[a] Freeze-dry weight

Table 6 Pearson Product Moment Correlations between tissue CYN concentrations and exposure regime. Cells show p value; correlation coefficient; sample size. ns=not significant; p > 0.050.

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	

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]
	Concentration (µg L^{-1})	Toxin type	Period	(average ± standard error)
Trial one	25	EXC	7 days	0[b]
	50	EXC	7 days	0[b]
	100	EXC	7 days	0.13[b]
	200	EXC	7 days	1.10[b]
	400	EXC	7 days	1.00[b]
	25	EXC	14 days	nd
	50	EXC	14 days	nd
	100	EXC	14 days	0.10 ± 0.10
	200	EXC	14 days	0.97 ± 0.11
	400	EXC	14 days	1.48 ± 0.41
Trial two	25	EXC	7 days	0.43 ± 0.23
	50	EXC	7 days	0.62 ± 0.16
	100	EXC	7 days	0.51 ± 0.08
	200	EXC	7 days	0.48 ± 0.08
	400	EXC	7 days	0.60 ± 0.15
	25	EXC	14 days	0.26 ± 0.26
	50	EXC	14 days	0.64 ± 0.35
	100	EXC	14 days	0.56 ± 0.14
	200	EXC	14 days	0.56 ± 0.13
	400	EXC	14 days	0.66 ± 0.11
Trial three	91	Total	7 days	39.52 ± 18.36
	167	Total	7 days	49.13 ± 12.45
	223	Total	7 days	33.33 ± 16.67
	294	Total	7 days	54.10 ± 20.31
	406	Total	7 days	43.42 ± 6.53
	91	Total	14 days	49.57 ± 11.05
	167	Total	14 days	120.97 ± 27.09
	223	Total	14 days	124.42 ± 5.34
	294	Total	14 days	98.99 ± 53.29
	406	Total	14 days	109.73 ± 30.94

[a] Bioconcentration factor (extracellular trials) or bioaccumulation factor (live trials); [b] no standard error since only selected samples analysed.

Table 8 Summary results for Pearson Product Moment Correlations between bioconcentration or bioaccumulation values for *M. tuberculata* and experimental conditions. ns=not significant (p > 0.050). Cells contain p value, correlation coefficient and sample size.

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	

[a] Selected values only; [b] data for controls omitted.

Table 9. Deoxy-CYN bioaccumulation factors for *M. tuberculata* exposed live *C. raciborskii* treatments (controls not included).

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

Table 10. Pearson Product Moment Correlations between tissue deoxy-CYN concentrations and bioaccumulation factor with exposure regime. Cells contain p value, correlation coefficient and sample size. ns=not significant; p > 0.050. Controls not included. ns=not significant (p > 0.010).

	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

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Figure 1

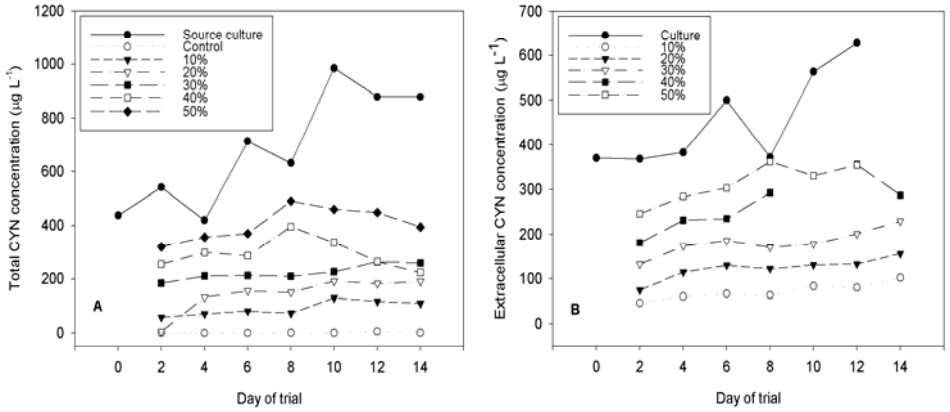
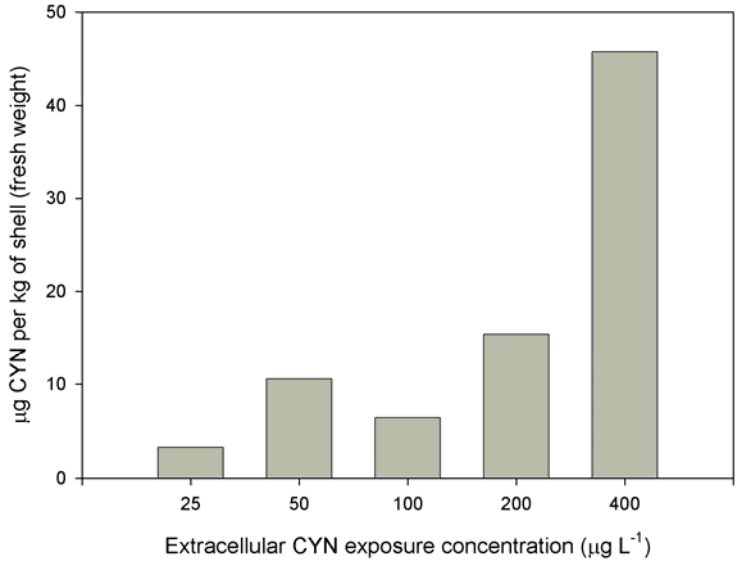


Figure 2



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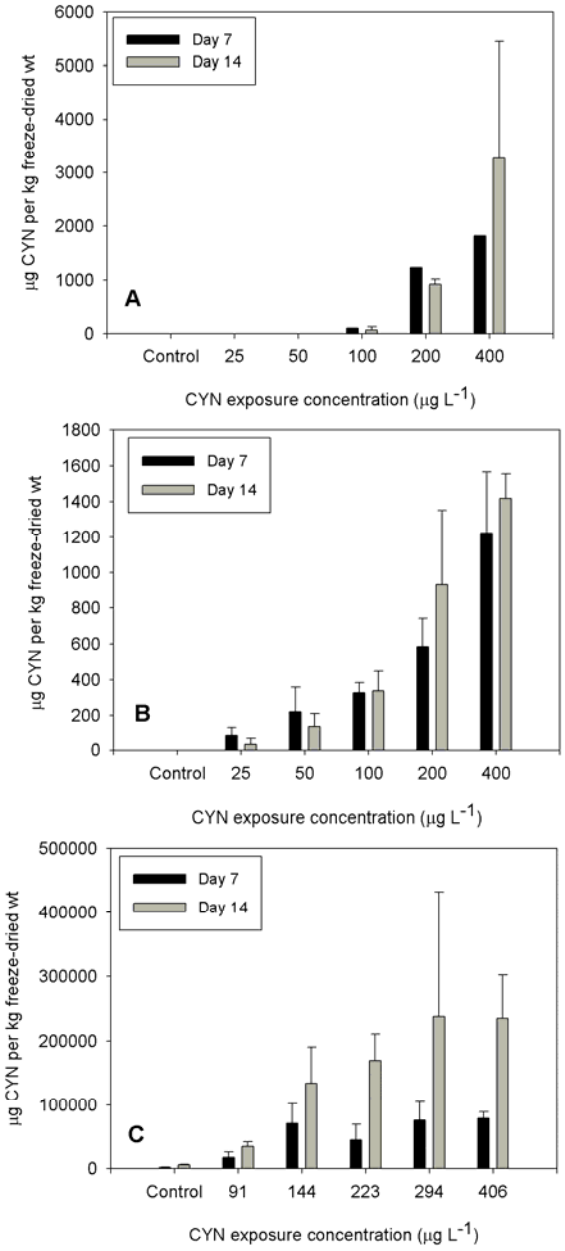
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Day 14
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4000
3000
2000
1000
0
A
Control
25
50
100
200
400
CYN exposure concentration (µg L-1)
1800
1600
1400
1200
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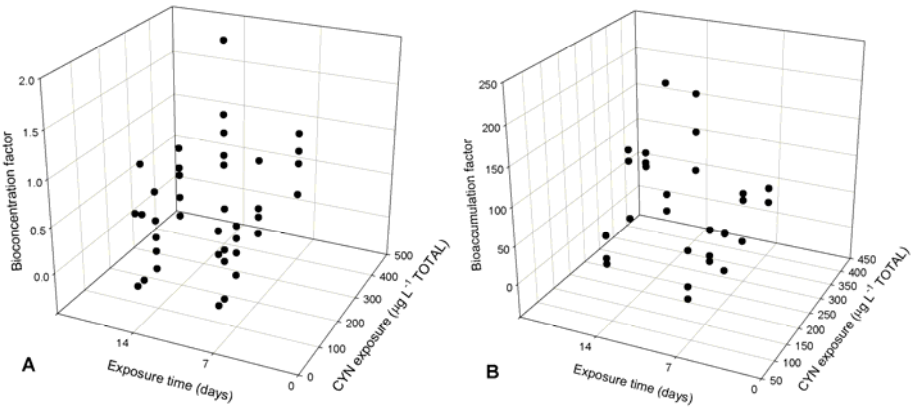
Figure 4



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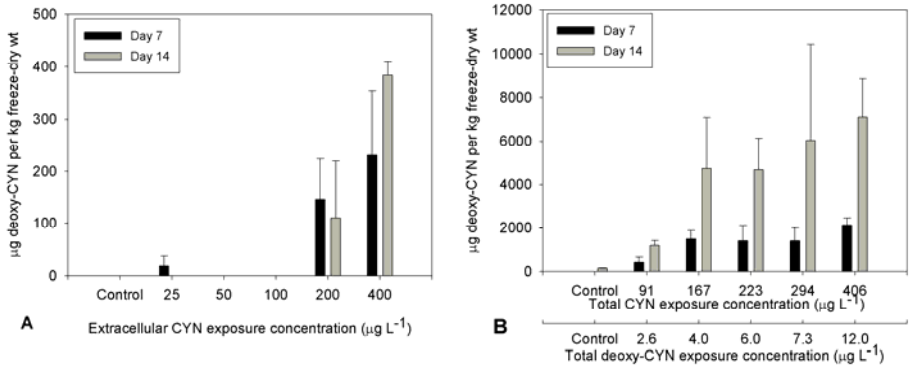

Figure 6