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Absence of Free-Cylindrospermopsin Bioconcentration in Water Thyme (*Hydrilla verticillata*)

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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; Schembrii 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 $\mu g\ ml^{-1}$. Most recently, Metcalf et al. (2004) demonstrated reduced pollen germination in tobacco plants following CYN exposure between 5 – 1000 $\mu g\ ml^{-1}$.

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

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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 ± 2^0C, continuous light at approximately 6 μEm^{-2}

s^{-1}) or on open laboratory shelves (approximately 4 – 5 $\mu Em^{-2}s^{-1}$).

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 $\mu g\ L^{-1}$. Trials lasted 14 days and were conducted under a 12:12 light:dark photoperiod (illumination, approximately 75 $\mu mols\ m^{-2}s^{-1}$) at 26 ± 4^0C. 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 $\mu g\ L^{-1}$ extracellular CYN, and also after one further week in CYN-free media. The trial was performed under continuous light at 27 ± 2^0C.

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-ionspray interface coupled to a Shimadzu SCL-10A*vp* 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 ng L^{-1} for a 120 μl 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 ±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 μg L^{-1} and 90% at 6.5 μgL^{-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 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 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 1mL of water; 15 ng g^{-1} tissue concentrations equates to only 15 μg 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 μg L^{-1} or above) to record the highest tissue toxins. In contrast, exposure concentrations of 50 μg 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 $\mu S\ cm^{-1}$ 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 > ± 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 ($\mu g\ L^{-1}$ CYN)	Actual Concentration ($\mu g\ L^{-1}$ 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

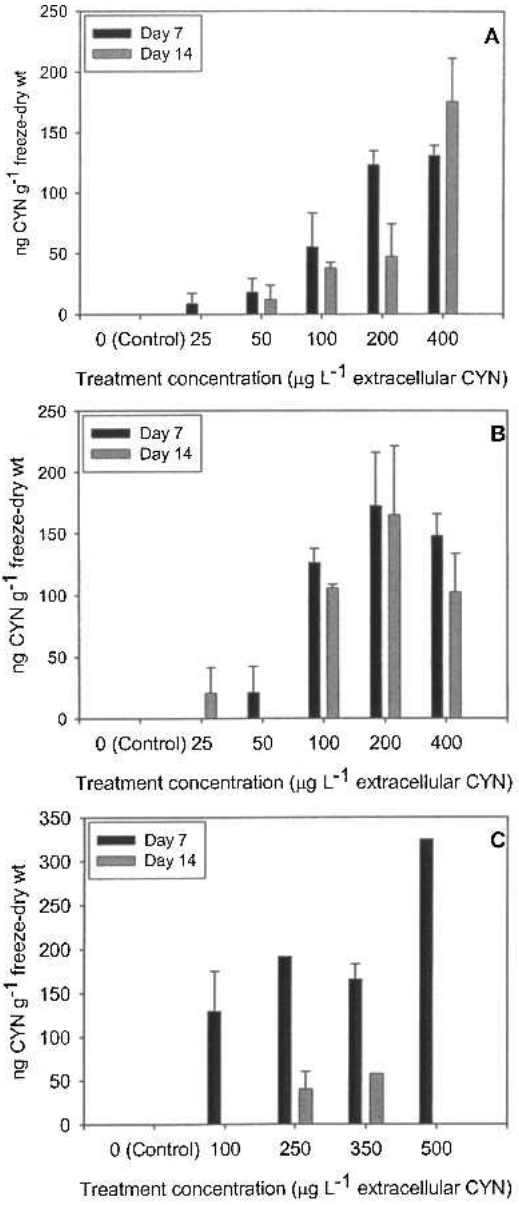

Figure 1. Free-CYN concentration in *H. verticillata* tissues (A) Definitive trial 1. (B) Definitive trial 2. (C) Depuration trial. Bars depict the average of three replicates, except in (C) where only selected samples were tested. Error bars depict standard error.

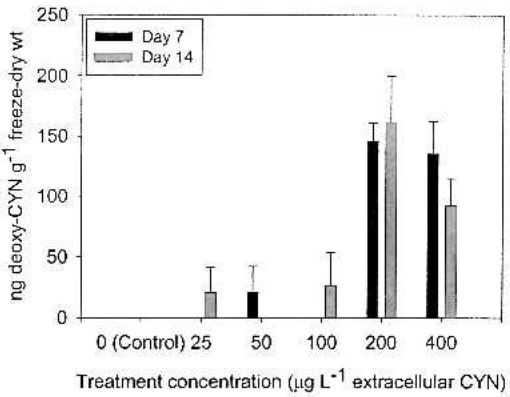

Figure 2. Free deoxy-CYN concentration in *H. verticillata* tissues from definitive trial 2. Bars depict the average of three replicates, error bars depict standard error.

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 biconcentration 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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