Protective efficacy of the antioxidants vitamin E and Trolox against *Microcystis aeruginosa*, microcystin-LR and menadione toxicity in *Artemia franciscana* nauplii

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Running head: Vitamin E and Trolox protect *Artemia* nauplii against MC-LR toxicity

ABSTRACT: This study was undertaken to evaluate the protective efficacy of the antioxidants vitamin E and Trolox against the toxicity of microcystin-LR (MC-LR), *M. aeruginosa* aqueous extract (CE) and a reference toxin menadione sodium bisulfite (MSB) in *Artemia franciscana* nauplii. This was achieved by using the well established brine shrimp bioassay. The experiment was conducted in two stages, with 1) 12 h mortality time course and 2) LC₅₀ determination for 12 and 24 h exposures. Treatments consisted of MC-LR, CE and MSB alone and with 4 h pre-treatments of either vitamin E or Trolox. Sensitivity of *A. franciscana* nauplii with 24 h LC₅₀ values of 11.0 (10.1-12.1) µg/mL for MSB and 9.5 (8.8-10.4) µg/mL for MC-LR were in general agreement with values reported for *Artemia* sp. Both antioxidant pre-treatments resulted in significant reductions in mortality of approximately 50% at 9 h post-exposure when challenged by either 40 µg/mL MC-LR or 20 µg/mL MSB. In contrast, the antioxidant pre-treatments offered little to no protection from CE suggesting that other uncharacterised bioactive compounds contributed to overall toxicity. The described bioassay is easily accessible, inexpensive, rapid, complies with animal ethics guidelines of many countries and thus provides a potential alternative to the mouse bioassay for the initial screening for chemo-protectants of MC-LR toxicity.

INTRODUCTION

Anthropogenic eutrophication appears to be the direct cause of many cyanobacterial blooms in inland water systems and reservoirs. This presents considerable risk to public and ecological health, as cyanobacteria can produce toxic secondary metabolites. These toxins can result in morbidity and mortality in humans (Azevedo et al. 2002; Texeira et al. 1993), death in domestic animals and wildlife (Duy et al. 2000; Stewart et al. 2007), concentration in plant tissue and inhibition of growth (McElhiney et al. 2001; Mitrovic et al. 2005) and can potentially transfer into humans and animals via food chains (Crush et al. 2008). Based on mammalian models, cyanobacterial toxins are grouped according to the physiological systems, organs, tissues or cells which they mainly affect and are categorised as: neurotoxins, hepatotoxins, cytotoxins and irritants/gastrointestinal toxins (Codd et al. 2005). Of the hepatotoxins, the microcystins (MCs) are the most prevalent worldwide and are produced by several bloom forming species, with *Microcystis aeruginosa* (Kützing) Kützing (taxonomic authority as per Guiry and Guiry 2008) being the most common (Sivonen and Jones 1999).

MCs are a heterogeneous group, with more than 60 isoforms described (Sivonen and Jones 1999), that share a common chemical structure containing three D-amino acids, two variable

L-amino acids and the two unusual non-proteinogenic amino acids, N-methyldehydroalanine and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Botes et al. 1984; Botes et al. 1985). Primarily, it is the two variable L-amino acids in positions 2 and 4 which account for the different microcystin isoforms, with a two letter suffix used in naming the various amino acids that are present in the toxin. For example MC-LR contains leucine (L) and arginine (R) (Figure 1).

Fig. 1. The cyclic heptapeptide structure of microcystin-LR (MC-LR). Common structural features of microcystins are positions 1 (D-Alanine), 3 (Methylaspartic acid), 5 (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda)), 6 (D-Glutamic acid) and 7 (N-Methyldehydroalanine). The variable amino acids in positions 2 and 4 are leucine (L) and arginine (R) respectively.

As MC-LR is one of the most commonly occurring isoforms in the environment (e.g. Anjos et al. 2006; Kemp and John 2006; Vasconcelos et al. 1996), it is also one of the most studied (de Figueiredo et al. 2004). The toxicity of MCs has resulted in the World Health Organisation (WHO) establishing a guideline for MC-LR of not more than 1µg/L in drinking water (WHO 1998). However, due to limited commercial supply of calibrated standards and access to high performance liquid chromatography (HPLC) mass spectral (MS) analysis, MC concentrations are commonly reported as MC-LR equivalence regardless of which isoform/s is present (McElhiney and Lawton 2005).

One of the best understood mechanisms of MC-LR toxicity is its ability to act as potent inhibitor of protein phosphatases types 1 and 2A (Honkanen et al. 1990; MacKintosh et al. 1990). MC-LR is preferentially taken up by hepatocytes, where the increased phosphorylation of intracellular proteins results in cell death (Eriksson et al. 1990; Hooser 2000). Haemorrhagic shock rapidly leads to death (Falconer 2005), with post mortem pathology revealing extensive haemorrhagic necrosis and disruption of the liver sinusoids (Kaya 1995).

Epidemiological studies from China suggest a possible relationship between hepatic and colorectal cancer in humans and chronic exposure to MC in drinking water (Yu 1995; Zhou et al. 2002). This data is supported by experimental findings which demonstrate tumour promotion by MC in the livers and colons of rodents (Falconer 2005). The potential for MC exposure via potable water is not likely to be confined only to developing nations. For instance, in the USA, samples taken from several water supply plants after regular treatment, were found to contain MCs at concentrations exceeding the WHO guideline [reported in (Fleming et al. 2002)].

MC-LR is also likely to be involved in other pathways of toxicity, such as oxidative stress (Ding and Ong 2003; Monserrat et al. 2003; Wiegand and Pflugmacher 2005). In tilapia fish intraperitoneally administered MC-LR results in the induction of adaptive responses, such as the increase in several antioxidant enzymes, including glutathione, that are biomarkers of the main symptoms of oxidative stress; lipid peroxidation (Prieto et al. 2006). *In vitro* cultured rat hepatocytes also show a corresponding increase in glutathione levels in response to MC-LR treatment, with this toxin initiating reactive oxygen species formation (Bouaicha and Maatouk 2004; Ding et al. 2001). Furthermore, these cell line studies provide evidence that MC-LR induced oxidative stress could be involved in hepatotoxicity and carcinogenicity.

Antioxidant defence of the cell is mediated by enzymatic and non-enzymatic mechanisms. While it is possible that chronic exposure to low levels of MC-LR may stress or overwhelm enzymatic defences, there remains the possibility that prophylactic treatment with non-enzymatic defences may afford protection. Indeed, this approach has been a topic of recent research seeking protective agents, such as green tea, flavonoids, vitamin E and Trolox, against MC-LR toxicity in mice (Gehring et al. 2003; Jayaraj et al. 2007; Lakshmana Rao et al. 2004; Xu et al. 2007). The use of the mouse bioassay, for screening potential chemo-protectants, however, is faced with several constraints. Typically, these include animal ethics

concerns, expense, specialised animal husbandry resources, small number of test subjects/replicates per treatment and time required to complete the assay (days to weeks). Thus, an alternative bioassay, without these limitations, could prove useful.

The *Artemia* nauplii bioassay has been employed successfully for the detection of MC-LR toxicity (Akin-Oriola and Lawton 2006; Lee et al. 1999; Metcalf et al. 2002) and a variety of compounds involved in superoxide-mediated toxicity (Matthews 1995). It appears, however, that use of this bioassay as recommended by Matthews (1995) for the investigation of compounds that afford protection against damage by superoxide, or other active oxygen species, has not been trialled with MC-LR. In view of this, the present study was undertaken to evaluate the protective efficacy of the antioxidants vitamin E and Trolox against MC-LR, *M. aeruginosa* aqueous extract and the reference toxin menadione sodium bisulfite in *Artemia* nauplii.

MATERIALS AND METHODS

MC-LR extraction and quantification

MC-LR was isolated from an environmental bloom sample dominated by *Microcystis aeruginosa* (identified by Queensland Health Scientific Services, Coopers Plains, Queensland (QHSS)) collected from a farm dam in the vicinity of Warwick (28° 13' 0" S, 152° 1' 0" E) southeast Queensland, Australia in 2002. Initial HPLC-MS/MS analysis (QHSS) determined that the sample contained several MCs, with MC-LR and MC-LA being the dominant isoforms. The sample was freeze-dried and stored in the dark at -20°C.

A Waters Prep HPLC System (Model 600E) was used to purify MC-LR using reverse phase C₁₈ chromatography. All reagents were of HPLC or analytical grade and the water was Milli-Q[®] 18 MOHM. The general method described by Lawton et al. (1994) was employed, with appropriate modifications to suit our laboratory conditions. To extract the intracellular toxin, the lyophilised sample (2 g) was transferred to a plastic tube containing water (100 mL) and the cells were disrupted using an ultrasonic pulsing probe (Sonifier[®] S-450, Branson Ultrasonics Corporation, USA) using 20 second cycles of alternate sonication/ice bath immersion for a total sonication treatment of 10 min. Cell debris was separated using centrifugation (95 000 G; 30 min) with the supernatant filtered (0.7 µm glass fibre filter) and passed through an Alltech high capacity solid phase C₁₈ extraction cartridge (10 g; 25× 30

mm; 50 μ m) which had previously been washed with methanol (Mallinckrodt Inc, USA) (50 mL) followed by water (50 mL). After all the solution had been passed through, the cartridge was washed with water (50 mL) followed by 20% methanol (50 mL). The MC containing fraction was eluted from the column with 75% methanol (2×50 mL). The solvent was removed at 40°C under vacuum (Rotavapor[®], Büchi Labortechnik AG, Switzerland). The dried fraction was dissolved in methanol (10 mL) and made up to 20 mL with water. To separate the MCs, the sample was filtered (0.7 μ m glass fibre filter) and a sub-sample (2 mL) injected into a Waters Bondapak[®] radially compressed C₁₈ column (100 $\times$ 25 mm; 10 μ m, 125 Å) and eluted with an acetonitrile (Labscan Asia Co Ltd, Thailand)/water gradient containing 0.05% v/v trifluoroacetic acid (Fisons, FSE, (NSW) Australia) at a speed of 10 mL/min. The toxin fractions were monitored using Waters 486 tuneable absorbance detector at an absorbance of 238 nm. Identical fractions, from multiple runs, were collected and pooled. The solvent was removed by rotary vacuum evaporation, diluted with water (1:1) and freeze-dried overnight. Each fraction was re-dissolved in 50% acetonitrile and re-chromatographed twice, initially with a Waters Prep Nova-Pak[®] radially compressed C₁₈ column (100 $\times$ 25 mm; 6 μ m, 60 Å) and finally with an Alltech, Apollo C₁₈ (150 $\times$ 22 mm, 5 μ m) column. The acetonitrile was evaporated under rotary vacuum and the sample was diluted with water and freeze-dried overnight to yield an amorphous white powder.

Purity and quantification of MC-LR sample (dissolved in tetradeuteromethanol (Aldrich Chemical Co Inc, USA)/deuterium oxide (Cambridge Isotope Laboratory Inc, USA) (CD₃OD/D₂O, 1:1) was analysed by Proton Nuclear Magnetic Resonance (¹H NMR) Spectroscopy, HPLC-MS/MS, HPLC photo-diode array detection and amino acid analysis and found to be > 95% purity. The MC-LR (500 μ g) was stored in sealed glass ampoules and stored in the dark at -20°C until use.

For testing, pure MC-LR (500 μ g) was dissolved in 200 μ L of methanol after which 800 μ L water was added, bringing the stock solution to 500 μ g/mL in 80% aqueous methanol.

***Microcystis aeruginosa* aqueous extract (CE)**

An environmental bloom sample dominated by *Microcystis aeruginosa* (identified as per Baker and Fabbro (1999)) was collected from Lake Samsonvale (North Pine Dam) (27° 16' 18.84" S, 152° 55' 8.76"), southeast Queensland in 2005. The cells were concentrated by standing the sample overnight, causing the buoyant cells to accumulate at the top of the water

column as a dense scum. The scum was harvested and freeze-dried. The stock solution was prepared by milling (TEMA Engineering Ltd, UK) the lyophilised material to fine powder and suspending 400 mg of this material in 10 mL of synthetic seawater (SW) (34 g/L distilled water; Reef Salt, AZOO Co, USA) to produce a 0.40% w/v suspension. This was vortexed, freeze-thawed (4 cycles), steeped overnight at 4°C, centrifuged (5 500 G; 15 min) and the supernatant syringe filtered (0.45 and 0.22 µm). This aqueous cell-free cyanobacterial extract (CE) was prepared fresh before use. This same method was used to prepare a sample for quantitative HPLC-MS/MS analysis for cyanotoxins performed by QHSS.

Reference toxin for oxidative stress (MSB)

Menadione sodium bisulfite (MSB) (2-methyl-1,4-naphthoquinone sodium bisulfite) (purity $\geq 95\%$, Sigma-Aldrich Co, USA), a stable water-soluble derivative of Vitamin K₃, was selected as a reference toxin to induce oxidative stress as per Matthews (1995). It was dissolved in SW to give a 100 mg/mL stock that was made fresh before use.

Antioxidants

Vitamin E acetate (α -tocopherol) (purity > 96%) and the water soluble vitamin E derivative 6-hydroxy-2,5,7,8-tetramethyl-chromon-2-carboxylic acid (purity > 97%), commonly known by the trade name Trolox, were both obtained from Sigma-Aldrich Co, USA. Vitamin E was dissolved in 60% methanol to give a 10 mg/mL stock and Trolox was prepared as a 1.5 mg/mL stock in 60% methanol. Both antioxidant stocks were refrigerated overnight. These stocks were diluted with SW to a concentration of 400 µg/mL and then immediately used for bioassay.

***Artemia* incubation, hatching and collection**

The species identity, *Artemia franciscana* Kellogg, and harvest location, Great Salt Lake, Utah, USA were verified by the supplier, North American Brine Shrimp (NABS), LLC, USA. This is consistent with *Artemia* zoogeography (Van Stappen 2002).

Toxicity was tested using a modified form of the *Artemia* nauplii lethality assay developed by Meyer (1982). *A. franciscana* cysts (0.5 g) were incubated for 24 h in 500 mL of vigorously aerated SW at of 25°C $\pm$ 2°C with continuous artificial light 2000 Lux (1 $\times$ 11 Watt reflector

lamp). After 24 h of incubation, the newly hatched nauplii and SW were poured into a sorting tray and held for a further 24 h under the same temperature but with continuous ambient light of 500 Lux (2×36 Watt cool white fluorescent tubes). The phototactic nauplii were concentrated to a suitable density, by using a point light source, and 400 μ L of SW containing approximately 27 (mean 26.6, $n = 275$, SE 0.53) nauplii were transferred by pipette to the each well of the 48 well tissue culture plates for immediate use in bioassay.

***Artemia* nauplii bioassay**

To investigate the ability of antioxidants vitamin E and Trolox to block the toxic effect of MSB, MC-LR and CE, the bioassay was conducted in two stages, with 1) 12 h time course with mortality monitored hourly and 2) LC₅₀ determination for 12 and 24 h exposures. For the LC₅₀ determination, a six-step 50% serial dilution series (using SW), was prepared from each stock giving the starting concentrations of 160 μ g/mL for MSB and MC-LR and 40 mg dry weight (dw) of lyophilised material per mL for CE. Anti-oxidant pre-treatment involved adding 200 μ L of either vitamin E or Trolox to the wells of a 48 well plate containing 400 μ L of SW and *A. franciscana* nauplii. After 4 h, 200 μ L of each of the appropriate toxin dilutions were added to the wells bringing the total volume to 800 μ L. Thus, the final concentrations in the bioassay were 100 μ g/mL for the antioxidants with the maximum concentration of 40 μ g/mL for MSB and MC-LR and 10 mg dw/mL for CE. Toxin only (control), treatments consisted of the addition of 200 μ L of SW instead of antioxidant pre-treatment, followed 4h later by the addition of 200 μ L of the toxin dilutions. All tests were performed in at least triplicate. At least one negative control consisting of 200 μ L SW, SW plus vitamin E, and SW plus Trolox, followed by the addition of 200 μ L after 4 h, was also run in at least triplicate for each plate. Methodology was identical for the 12 h time course study with the exception that only one toxin concentration was used. The single concentrations in the bioassay were MSB 20 μ g/mL, MC-LR 40 μ g/mL and 10 mg dw/mL CE. Conditions during the treatment period were $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with continuous artificial light 500 Lux (2×36 Watt cool white fluorescent tubes).

For the LC₅₀ determination, the wells were examined at 12 and 24 h post treatment. For the 12 h time course, wells were examined hourly. The number of dead and moribund (no movement detected over 10 second period) nauplii were counted using an Olympus SZ40 stereo zoom microscope (Olympus Co. Ltd., Japan). After the final exposure period, all

nauplii were sacrificed using 100 μL of 10% glacial acetic acid solution (v/v in distilled water) and counted to determine the total number per well.

Statistics

LC_{50} values, with $\pm 95\%$ confidence limits, were calculated by Probit analysis (Finney 1971) using the software package XLSTAT Version 7.5.2 (Addinsoft, USA). The Paired T -Test was used to calculate statistical significance between control and treated groups with a P value < 0.05 considered to be statistically significant.

RESULTS AND DISCUSSION

All treatments resulted in sigmoidal dose-response curves with the 12 and 24 h LC_{50} values obtained by Probit analysis (Table 1). Sensitivity (24 h) to the reference toxin MSB of LC_{50} 11.0 (10.1-12.1) $\mu\text{g/mL}$ compared well with that reported by Matthews (1995) of IC_{50} 7.2 (5.8-9.1) $\mu\text{g/mL}$. The 24 h LC_{50} of 9.5 (8.8-10.4) $\mu\text{g/mL}$ for MC-LR was also in general agreement with previously reported values, over a similar exposure period, with 6.8 (Akin-Oriola and Lawton 2006) and 5.7 $\mu\text{g/mL}$ (Chen et al. 2006). Comparison of these values indicated that the *Artemia* nauplii bioassay is quite reproducible despite variations in methods, laboratories and analysts. Spontaneous mortality (mean %) at 24 h in the negative controls of synthetic seawater ($n = 24$) and synthetic seawater plus vitamin E ($n = 12$) and synthetic seawater plus Trolox ($n = 12$) was $\leq 0.5\%$, which is well within the $< 3\%$ recommended by Meriluoto et al. (2000). Regarding the source of *Artemia*; the studies cited state their test species as *Artemia salina*. However, there is a common taxonomic discrepancy in citing the identity of brine shrimp nauplii used for bioassay as *A. salina*, when the material of North American origin is invariably *A. franciscana* (Ruebhart et al. 2008).

Table 1: LC_{50} values for *A. franciscana* nauplii treated with MSB, MC-LR and CE individually and with 100 $\mu\text{g/mL}$ antioxidant pre-treatment (4 h exposure). All bioassays were performed in at least triplicate.

Treatment	LC_{50} ($\pm 95\%$ confidence intervals) value at time (h)	
	12	24
MSB ($\mu\text{g/mL}$)	16.1 (14.9-17.5)	11.0 (10.1-12.1)
Vitamin E + MSB ($\mu\text{g/mL}$)	21.2 (20.0-22.7)	12.7 (11.9-13.6)

Trolox + MSB ($\mu\text{g/mL}$)	17.8 (17.0-18.7)	10.5 (9.9-11.2)
MC-LR ($\mu\text{g/mL}$)	30.3 (28.0-33.0)	9.5 (8.8-10.4)
Vitamin E + MC-LR ($\mu\text{g/mL}$)	33.7 (30.7-37.6)	9.0 (8.2-9.9)
Trolox + MC-LR ($\mu\text{g/mL}$)	32.5 (29.4-36.4)	7.8 (7.1-8.6)
CE (dw mg/mL)	2.6 (2.4-2.7)	0.95 (0.89-1.0)
Vitamin E + CE (dw mg/mL)	3.2 (3.0-3.5)	0.99 (0.91-1.1)
Trolox + CE (dw mg/mL)	4.0 (3.7-4.4)	1.0 (0.92-1.1)

Time course studies (Figure 2) for *A. franciscana* exposed to 20 $\mu\text{g/mL}$ MSB, 40 $\mu\text{g/mL}$ MC-LR and 10 mg dw/mL CE produced > 70% mortality for all treatments at 12 h. Hourly monitoring was conducted as the effects of antioxidants can be overcome by pro-oxidants over relatively short time frames. Indeed, 12 and 24 h LC_{50} values (Table 1) indicate that pre-treatment plus toxin values were similar to toxin only values. Nevertheless, statistically significant ($P < 0.05$) reductions in mortality due to antioxidant pre-treatment were obtained during the time course for MSB with vitamin E at 7-12 h and with Trolox at 8-11 h, MC-LR with vitamin E at 7-9 h and with Trolox at 9 h, and CE with vitamin E at 9 h. For comparison across treatments, the 9 h exposure time was chosen (Figure 3). At this exposure time, the protective efficacy of 100 $\mu\text{g/mL}$ vitamin E pre-treatment resulted in a statistically significant ($P < 0.05$) reduction in mortality of *A. franciscana* nauplii of approximately 50% when challenged by either 20 $\mu\text{g/mL}$ MSB or 40 $\mu\text{g/mL}$ MC-LR (Figure 3 (A) and (B)). Similarly, pre-treatment with 100 $\mu\text{g/mL}$ Trolox against MC-LR exposure also resulted in a statistically significant ($P < 0.05$) reduction in mortality of *A. franciscana* nauplii by approximately 50% (Figure 3 (B)).

Toxicity of the reference toxin, the quinone-containing compound MSB, is attributed to the formation of several reactive oxygen species (Boelsterli 2003; Chiou et al. 2003). These are superoxide anions, hydrogen peroxide and hydroxyl radicals; hence, the value of this toxin in the screening of chemo-protectants of oxidative stress (Abe and Saito 1996; Chen and Cederbaum 1997). Interestingly, the 24 h LC_{50} values for MSB 13.5 (12.7-14.3) and MC-LR 9.5 (8.8-10.4) (Table 1) were similar, with the protective efficacy of vitamin E and Trolox against both these challenges, significant and of a comparable magnitude (Figure 3 (A) and (B)). Thus, this raises the prospect of using MSB as a surrogate for MC-LR in the

preliminary testing of potential chemo-protectants although the mechanisms of action of both toxins require further study. This is an attractive option because there is a limited commercial supply of MC standards, with their availability complicated by strict national and international controls governing production, through to transportation of cyanotoxins (Metcalf et al. 2006; Phelan and Downing 2007). In contrast, MSB is readily available and relatively inexpensive.

FIG 2. Time course of mean ($n \geq 3$, $\pm$ SE) mortality for *A. franciscana* nauplii exposed to (A) MSB 20 µg/mL, (B) MC-LR 40 µg/mL and (C) CE 10 dw mg/mL over 12 h. Toxin (control) (▲), toxin with vitamin E 100 µg/mL 4 h pre-treatment (■) and toxin with Trolox 100 µg/mL 4 h pre-treatment (□).

Figure 3: Protective efficacy of 4 h pre-treatment with antioxidants on mean ($n \geq 3$, $\pm$ SE) mortality after 9 h exposure to (A) MSB 20 µg/mL, (B) MC-LR 40 µg/mL and (C) CE 10 dw mg/mL. *A. franciscana* nauplii were (1) treated with toxin (control), (2) toxin with Vitamin E 100 µg/mL pre-treatment and (3) toxin with Trolox 100 µg/mL pre-treatment. Paired *T*-Tests were carried out on antioxidant pre-treatment groups (2) and (3) versus toxin only group (1) with significant differences shown (** $P < 0.05$).

The mouse bioassay has also been used to test the efficacy of Vitamin E and Trolox pre-treatment against MC-LR toxicity. For example, pre-treatment with vitamin E 48 h prior to an intraperitoneally injected lethal dose of MC-LR resulted in 50% survival (Hermansky et al. 1991). Similarly, 24 h Trolox pre-treatment to a lethal MC-LR dose was found to protect 25% of the test subjects (Lakshmana Rao et al. 2004). Thus, the protective efficacy of both vitamin E and Trolox against MC-LR toxicity is common to both mice and *A. franciscana* nauplii. Investigation into the role of vitamin E in protecting against MC-LR toxicity in mice by Gehringer et al. (2003) demonstrated a significant reduction in lipid peroxidation, presumably by its ability to quench free radicals. The aforementioned study also revealed that vitamin E supplementation significantly reduced the MC-LR induced decrease in glutathione S-transferase (sGST) levels when compared to MC-LR only groups. This suggests that cellular enzymatic defence mechanisms were supported by the antioxidant action of vitamin E pre-treatment.

The degree of toxicity for the cell-free aqueous *M. aeruginosa* extract (CE) of 24 h LC₅₀ of 0.95 (0.89-1.0) mg dw/mL (Table 1) was high as per the guidelines (< 2 mg/mL) by Meriluoto et al. (2000) for the interpretation of sample toxicity using the *Artemia* nauplii bioassay. The HPLC-MS/MS analysis determined the CE contained a total of 30.67 µg/mL MCs, with MC-LR 20 µg/mL, MC-LA 8.4 µg/mL and other minor MCs 2.27 µg/mL. Thus, it contained approximately half as much MC-LR and three quarters the total MCs as the pure cyanotoxin treatment of 40 µg/mL. As for the toxicity of MC-LA, it appears that there is no data on *Artemia* sensitivity to this isoform, however, in mice the LD₅₀ for MC-LR is similar to that of MC-LA (Kuiper-Goodman et al. 1999). The CE treatment of 10 mg dw/mL resulted in resulted > 80% mortality 9 h post-exposure to all of the three treatments (Figure 3 (C)). Although statistically significant ($P < 0.05$), protection against CE by vitamin E was marginal while there was no significant ($P < 0.05$) protection by Trolox.

The relatively greater toxicity of CE compared to pure MC-LR in this study is consistent with previous studies. For example, environmental bloom material dominated by *M. aeruginosa*, containing microcystins, has been found to exert greater toxicity than pure cyanobacterial toxins at equivalent concentrations across a variety of organisms (Pietsch et al. 2001). This is likely due to *Microcystis* cell extracts containing other, as yet uncharacterised, bioactive compounds that contribute to overall toxicity (Falconer 2007); possibly acting either independently, additively or synergistically with MCs. To explain the comparatively strong

toxicity of *Microcystis* cell extract, Pietsch et al. (2001) postulated that cyanobacterial lipopolysaccharides (LPS), present in extracts, may be involved in inhibiting soluble sGST activity, thereby, blocking the detoxification of MCs. Since then, it has been demonstrated that cyanobacterial LPS is of low toxicity ($LC_{50} > 1000 \mu\text{g/mL}$) to *Artemia* nauplii and, when used at a sub-lethal dose in a pre-treatment, it actually confers protection against MC-LR toxicity as evidenced by significantly increased LC_{50} values (Lindsay et al. 2006).

Cyanobacterial extracts may also contain other toxins that are not commonly characterised in toxicity studies. For instance, new antibacterial metabolites have been isolated from *Nostoc commune* including an anthraquinone (1,8-dihydroxy-4-methylanthraquinone) (Jaki et al. 2000). Work done in our laboratory (unpublished results) shows that a closely related compound, aloe emodin (1,8-dihydroxy-3-methylanthraquinone) is toxic to *A. franciscana* (72 h LC_{50} $62 \mu\text{g/mL}$). With synthesis of 1,8-dihydroxy-4-methylanthraquinone now possible (Wang et al. 2002), future studies are likely to involve screening studies for the occurrence of this compound in other species of cyanobacteria as well as invertebrate toxicity studies. Another novel toxin, isolated from cyanobacteria that has also been shown toxic to *Artemia* sp., includes malynamide-type secondary metabolites (hermitamides) from *Lyngbya majuscula* (Tan et al. 2000). Thus, bioassay guided search for new compounds in cyanobacteria is likely to reveal a host of new toxins that are not currently tested for using present HPLC-MS/MS methods.

In addition to possibly containing uncharacterised toxins, it is likely that the *Microcystis* cell extract also contained antioxidants. While it appears that a profile of antioxidants is unavailable for *M. aeruginosa*, it is known that the cyanobacterial dietary supplement Spirulina contains several antioxidant phenolic compounds, β -carotene and α -tocopherol (Miranda et al. 1998). Thus, it is possible that other cyanobacteria, including the genus *Microcystis* may also contain these antioxidants. If so, the antioxidants present in the *Microcystis* cell extract, in combination with the antioxidant pre-treatments of vitamin E or Trolox may have acted additively to produce a pro-oxidant effect. Indeed, evidence has been produced suggesting that α -tocopherol does not function as a classic antioxidant but may develop into a pro-oxidant when co-antioxidants are exhausted (Bowry et al. 1992; Bowry and Stocker 1993; Kontush et al. 1996).

In conclusion, this study demonstrated that the antioxidants vitamin E and Trolox provided protection against MSB, a commonly used compound that causes oxidative stress. These

antioxidants also protected against MC-LR toxicity in a similar manner, providing further evidence that this cyanotoxin induces oxidative stress. The described bioassay is easily accessible, inexpensive, rapid and complies with animal ethics guidelines (e.g. NHMRC 2004). It is envisaged that its use may provide an alternative to the mouse bioassay for the initial screening for chemo-protectants of MC-LR toxicity.

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REFERENCES

- Abe K, Saito H. 1996. Menadione toxicity in cultured rat cortical atocytes. *The Japanese Journal of Pharmacology* 72:299-306.
- Akin-Oriola GA, Lawton LA. 2006. The detection and quantification of cyanobacterial toxins in water using the brine shrimp (*Artemia salina*) assay. *West Africa Journal of Applied Ecology* 9(Jan -Jun 2006): Paper 16 of 18.
- Anjos FM, Bittencourt-Oliveira MC, Zajac MP, Hiller S, Christian B, Erler K, Luckas B, Pinto E. 2006. Detection of harmful cyanobacteria and their toxins by both PCR amplification and LC-MS during a bloom event. *Toxicon* 48(3):239-245.
- Azevedo SMFO, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK. 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru - Brazil. *Toxicology* 181-182:441-446.
- Baker PD, Fabbro LD. 1999. *A Guide to the Identification of Common Blue-Green Algae (Cyanoprokaryotes) in Australian Freshwaters*. Albury, Australia.
- Boelsterli UA. 2003. *Mechanistic Toxicology: The Molecular Basis of How Chemicals Disrupt Biological Targets*. London, United Kingdom: Taylor & Francis. p 166.
- Botes DP, Tuinman AA, Wessels PL, Viljoen CC, Kruger H, Williams DH, Santikarn S, Smith RJ, Hammond SJ. 1984. The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*. *Journal of the Chemical Society, Perkin Transactions* 1:2311-2318.
- Botes DP, Wessels PL, Kruger H, Runnegar MTC, Santikarn S, Smith RJ, Barna JCJ, Williams DH. 1985. Structural studies on cyanoginosins-LR, -YR, -YA, and -YM, peptide toxins from *Microcystis aeruginosa*. *Journal of the Chemical Society, Perkin Transactions* 1:2747-2748.
- Bouaicha N, Maatouk I. 2004. Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation in primary cultured rat hepatocytes. *Toxicology Letters* 148(1-2):53-63.
- Bowry VW, Ingold KV, Stocker R. 1992. Vitamin E in human low-density lipoprotein: When and how this antioxidant becomes a pro-oxidant. *Biochemistry Journal* 288:341-344.
- Bowry VW, Stocker R. 1993. Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *Journal of the American Chemical Society* 115(14):6029-6044.

- Chen Q, Cederbaum AI. 1997. Menadione cytotoxicity to Hep G2 cells and protection by activation of nuclear factor-kB. *Molecular Pharmacology* 52:648-657.
- Chen Y-M, Lee T-H, Lee S-J, Lin J-Z, Huang R, Chou H-N. 2006. Potential of a simple solid-phase extraction method coupled to analytical and bioanalytical methods for an improved determination of microcystins in algal samples. *Journal of Chromatography B* 844(1):134-141.
- Chiou T-J, Chu S-T, Tzeng W-F. 2003. Protection of cells from menadione-induced apoptosis by inhibition of lipid peroxidation. *Toxicology* 191(2-3):77-88.
- Codd GA, Morrison LF, Metcalf JS. 2005. Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology* 203(3):264-272.
- Crush JR, Briggs LR, Sprosen JM, Nichols DE. 2008. Effect of irrigation with lake water containing microcystins on microcystin content and growth of ryegrass, clover, rape, and lettuce. *Environmental Toxicology* 23(2):246-252.
- de Figueiredo DR, Azeiteiro UM, Esteves SM, Goncalves FJM, Pereira MJ. 2004. Microcystin-producing blooms - a serious global public health issue. *Ecotoxicology and Environmental Safety* 59(2):151-163.
- Ding W-X, Ong CN. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiology Letters* 220(1):1-7.
- Ding W-X, Shen M-H, Ong C-M. 2001. Critical role of reactive oxygen species formation in microcystin-induced cytoskeleton disruption in primary cultured hepatocytes. *Journal of Toxicology and Environmental Health, Part A* 64(6):507-519.
- Duy TN, Lam PK, Shaw GR, Connell DW. 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. *Reviews of Environmental Contamination and Toxicology* 163:113-85.
- Eriksson JE, Gronberg L, Nygard S, Slotte JP, Meriluoto JAO. 1990. Hepatocellular uptake of 3H-dihydromicrocystin-LR, a cyclic peptide toxin. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1025(1):60-66.
- Falconer IR. 2005. Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins. Boca Raton: CRC Press. p 109-139.
- Falconer IR. 2007. Cyanobacterial toxins present in *Microcystis aeruginosa* extracts--More than microcystins! *Toxicon* 50(4):585-588.
- Finney DJ. 1971. Probit Analysis. 3rd ed. Cambridge: Cambridge University Press.
- Fleming LE, Rivero C, Burns J, Williams C, Bean JA, Shea KA, Stinn J. 2002. Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. *Harmful Algae* 1(2):157-168.
- Gehring MM, Govender S, Shah M, Downing TG. 2003. An investigation of the role of vitamin E in the protection of mice against microcystin toxicity. *Environmental Toxicology* 18(2):142-148.
- Guiry MD, Guiry GM. 2008. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. (cited 5 March 2008). Available from: <http://www.algaebase.org>
- Hermansky SJ, Stohs SJ, Eldeen ZM, Roche VF, Mereish KA. 1991. Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. *Journal of Applied Toxicology* 11(1):65-73.
- Honkanen RE, Zwiller J, Moore RE, Daily SL, Khatra BS, Dukelow M, Boynton AL. 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *The Journal of Biological Chemistry* 265(32):19401-19404.
- Hooser SB. 2000. Fulminant hepatocyte apoptosis in vivo following microcystin-LR administration in rats. *Toxicologic Pathology* 28(5):726-733.
- Jaki B, Heilmann J, Sticher O. 2000. New Antibacterial Metabolites from the Cyanobacterium *Nostoc commune* (EAWAG 122b). *Journal of Natural Products* 63(9):1283-1285.

- Jayaraj R, Deb U, Bhaskar ASB, Prasad GBKS, Lakshmana Rao PV. 2007. Hepatoprotective efficacy of certain flavonoids against microcystin induced toxicity in mice. *Environmental Toxicology* 22:472-479.
- Kaya K. 1995. Toxicology of microcystins. In: Watanabe MF, Harada K-i, Carmichael WW, Fujiki H, editors. *Toxic Microcystis*. Boca Raton: CRC Press. p 175-202.
- Kemp A, John J. 2006. Microcystins associated with *Microcystis* dominated blooms in the Southwest wetlands, Western Australia. *Environmental Toxicology* 21(2):125-130.
- Kontush A, Finckh B, Karten B, Kohlschutter A, Beisiegel U. 1996. Antioxidant and prooxidant activity of alpha-tocopherol in human plasma and low density lipoprotein. *Journal of Lipid Research* 37(7):1436-1448.
- Kuiper-Goodman T, Falconer I, Fitzgerald J. 1999. Human health aspects. In: Chorus I, Bartram J, editors. *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Managment*. London, United Kingdom: E & FN Spon. p 114-153.
- Lakshmana Rao PV, Gupta N, Jayaraj R. 2004. Screening of certain chemoprotectants against cyclic peptide toxin microcystin-LR. *Indian Journal of Pharmacology* 36(2):87-92.
- Lawton LA, Edwards C, Codd GA. 1994. Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst* 119:1525-1530.
- Lee TH, Chen YM, Chou HN. 1999. Toxicity assay of cyanobacterial strains using *Artemia salina* in comparison with the mouse bioassay. *Acta Zoologica Taiwanica* 10(1):1-9.
- Lindsay J, Metcalf JS, Codd GA. 2006. Protection against the toxicity of microcystin-LR and cylindrospermopsin in *Artemia salina* and *Daphnia* spp. by pre-treatment with cyanobacterial lipopolysaccharide (LPS). *Toxicon* 48(8):995-1001.
- MacKintosh C, Beattie KA, Klumpp S, Cohen P, Codd GA. 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(2):187-192.
- Matthews RS. 1995. *Artemia salina* as a test organism for measuring superoxide-mediated toxicity. *Free Radical Biology and Medicine* 18(5):919-922.
- McElhiney J, Lawton L, Leifert C. 2001. Investigations into the inhibitory effects of microcystins on plant growth, and the toxicity of plant tissues following exposure. *Toxicon* 39(9):1411-1420.
- McElhiney J, Lawton LA. 2005. Detection of the cyanobacterial hepatotoxins microcystins. *Toxicology and Applied Pharmacology* 203(3):219-230.
- Meriluoto J, Lawton L, Harada K-i. 2000. Isolation and detection of microcystins and nodularins, cyanobacterial peptide hepatotoxins. In: Holst O, editor. *Methods in Molecular Biology*, Vol. 145: Bacterial Toxins - Methods and Protocols Totowa, USA: Humana Press. p 65-87.
- Metcalf JS, Lindsay J, Beattie KA, Birmingham S, Saker ML, Torokne AK, Codd GA. 2002. Toxicity of cylindrospermopsin to the brine shrimp *Artemia salina*: comparisons with protein synthesis inhibitors and microcystins. *Toxicon* 40(8):1115-1120.
- Metcalf JS, Meriluoto JAO, Codd GA. 2006. Legal and security requirements for the air transportation of cyanotoxins and toxigenic cyanobacterial cells for legitimate research and analytical purposes. *Toxicology Letters* 163(2):85-90.
- Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica* 45:31-34.
- Miranda MS, Cintra RG, Barros SBM, Mancini-Filho J. 1998. Antioxidant activity of the microalga *Spirulina maxima*. *Brazilian Journal of Medical and Biological Research* 31(8):1075-1079
- Mitrovic SM, Allis O, Furey A, James KJ. 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(3):345-352.
- Monserat JM, Pinho GLL, Yunes JS. 2003. Toxicological effects of hepatotoxins (microcystins) on aquatic organisms. *Comments on Toxicology* 9(2):89-101.
- NHMRC. 2004. Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Canberra: National Health and Medical Research Council. 3, 5, 7 & 28 p.
- Phelan RR, Downing TG. 2007. Optimization of laboratory scale production and purification of microcystin-LR from pure cultures of *Microcystins aeruginosa*. *African Journal of Biotechnology* 6(21):2451-2457.
- Pietsch C, Wiegand C, Valeria A, M. , Nicklisch A, Wunderlin D, Pflugmacher S. 2001. The effect of a cyanobacterial crude extract on different aquatic organisms: evidence for cyanobacterial toxin modulating factors. *Environmental Toxicology* 16:535-542.
- Prieto AI, Jos A, Pichardo S, Moreno I, Camean AM. 2006. Differential oxidative stress responses to microcystins LR and RR in intraperitoneally exposed tilapia fish (*Oreochromis sp.*). *Aquatic Toxicology* 77(3):314-321.
- Ruebhart DR, Cock IE, Shaw GR. 2008. Brine shrimp bioassay: Importance of correct taxonomic identification of *Artemia* (Anostraca) species. *Environmental Toxicology* 9999(9999):NA.
- Sivonen K, Jones G. 1999. Cyanobacterial toxins. In: Chorus I, Bartram I, editors. *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring, and Management*. London: E & FN Spon p41-111.
- Stewart I, Seawright AA, Shaw GR. 2007. Cyanobacterial poisoning in livestock, wild mammals and birds - and overview. In: Hudnell HK, editor. *Proceedings of the Interagency, International Symposium on Cyanobacterial Harmful Algae Blooms*. *Advances in Experimental Medicine & Biology* p599-623.
- Tan LT, Okino T, Gerwick WH. 2000. Hermitamides A and B, toxic malyngamide-type natural products from the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products* 63(7):952-955.
- Texeira MGL, Costa MCN, de Carvalho VLP, Pereira MS, Hage E. 1993. Gastroenteritis epidemic in the area of the Itaparica Dam, Bahia, Brazil. *Bulletin of the Pan American Health Organization* 27(3):244-253.
- Van Stappen G. 2002. Zoogeography. In: Abatzopoulos TJ, Beardmore JA, Clegg JS, Sorgeloos P, editors. *Artemia: Basic and Applied Biology*. Dordrecht, The Netherlands: Kluwer Academic Publishers. p 171-224.
- Vasconcelos VM, Sivonen K, Evans WR, Carmichael WW, Namikoshi M. 1996. Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Research* 30(10):2377-2384.
- Wang A, Wu C-H, Biehl E. 2002. Unambiguous synthesis and spectral characterization of 1,8-dihydroxy-4-methylanthraquinone. *ARKIVOC Part (i)*:80-84.
- Wiegand C, Pflugmacher S. 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicology and Applied Pharmacology* 203(3):201-218.
- Xu C, Shu WQ, Cao J, Qiu ZQ, Zhao Q, Chen JA, Zeng H, Fu WJ. 2007. Antagonism effects of green tea against microcystin induced oxidant damage on liver and kidney (in Chinese, Abstract in English). *Zhonghua Yu Fang Yi Xue Za Zhi* [Chinese journal of preventive medicine] 41(1):8-12.
- Yu SZ. 1995. Primary prevention of hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology* 10(6):674-682.
- Zhou L, Yu H, Chen K. 2002. Relationship between microcystin in drinking water and colorectal cancer. *Biomedical and Environmental Science* 15(2):166-171.