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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) LC$_{50}$ 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 LC$_{50}$ 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 (Gehringer 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 chemoprotectants, 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 C\textsubscript{18} 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 C\textsubscript{18} extraction cartridge (10 g; 25× 30
mm; 50 µm) 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 µm glass fibre filter) and a sub-sample (2 mL) injected into a Waters Bondapak® radially compressed C\textsubscript{18} column (100 × 25 mm; 10 µm, 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 C\textsubscript{18} column (100 × 25 mm; 6 µm, 60 Å) and finally with an Alltech, Apollo C\textsubscript{18} (150 × 22 mm, 5 µm) 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) (CD\textsubscript{3}OD/D\textsubscript{2}O, 1:1) was analysed by Proton Nuclear Magnetic Resonance (\textsuperscript{1}H NMR) Spectroscopy, HPLC-MS/MS, HPLC photo-diode array detection and amino acid analysis and found to be > 95% purity. The MC-LR (500 µg) was stored in sealed glass ampoules and stored in the dark at -20°C until use.

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

\textit{Microcystis aeruginosa} aqueous extract (CE)

An environmental bloom sample dominated by \textit{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 ≥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 ± 2°C with continuous artificial light 2000 Lux (1 × 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 µL 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 µg/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 µL of either vitamin E or Trolox to the wells of a 48 well plate containing 400 µL of SW and *A. franciscana* nauplii. After 4 h, 200 µL of each of the appropriate toxin dilutions were added to the wells bringing the total volume to 800 µL. Thus, the final concentrations in the bioassay were 100 µg/mL for the antioxidants with the maximum concentration of 40 µg/mL for MSB and MC-LR and 10 mg dw/mL for CE. Toxin only (control), treatments consisted of the addition of 200 µL of SW instead of antioxidant pre-treatment, followed 4h later by the addition of 200 µL of the toxin dilutions. All tests were performed in at least triplicate. At least one negative control consisting of 200 µL SW, SW plus vitamin E, and SW plus Trolox, followed by the addition of 200 µL 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 µg/mL, MC-LR 40 µg/mL and 10 mg dw/mL CE. Conditions during the treatment period were 25°C ± 2°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

LC₅₀ values, with ± 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 LC₅₀ values obtained by Probit analysis (Table 1). Sensitivity (24 h) to the reference toxin MSB of LC₅₀ 11.0 (10.1-12.1) µg/mL compared well with that reported by Matthews (1995) of IC₅₀ 7.2 (5.8-9.1) µg/mL. The 24 h LC₅₀ of 9.5 (8.8-10.4) µg/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 µg/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 ≤ 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: LC₅₀ values for A. franciscana nauplii treated with MSB, MC-LR and CE individually and with 100 µg/mL antioxidant pre-treatment (4 h exposure). All bioassays were performed in at least triplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LC₅₀ (+ 95% confidence intervals) value at time (h)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>MSB (µg/mL)</td>
<td>16.1 (14.9-17.5)</td>
</tr>
<tr>
<td>Vitamin E + MSB (µg/mL)</td>
<td>21.2 (20.0-22.7)</td>
</tr>
</tbody>
</table>
Trolox + MSB (µg/mL) | 17.8 (17.0-18.7) | 10.5 (9.9-11.2)
MC-LR (µg/mL) | 30.3 (28.0-33.0) | 9.5 (8.8-10.4)
Vitamin E + MC-LR (µg/mL) | 33.7 (30.7-37.6) | 9.0 (8.2-9.9)
Trolox + MC-LR (µg/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 µg/mL MSB, 40 µg/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 LC50 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 µg/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 µg/mL MSB or 40 µg/mL MC-LR (Figure 3 (A) and (B)). Similarly, pre-treatment with 100 µg/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 LC50 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 ($n \geq 3$, ± 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 ($n \geq 3$, ± 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 LC$_{50}$ 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 LD$_{50}$ 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 > 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\(_{50} > 1000 \mu g/mL\)) 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 µg/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 malyngamide-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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