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The Detection and Quantification of Cyanobacterial Toxins in Water Using the Brine Shrimp (*Artemia salina)* Assay

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Abstract

The cyanobacteria (blue-green algae) are an ancient and ubiquitous group of prokaryotes that form dense growths or blooms in eutrophic water bodies. The blooms are of interest to aquaculturists and water management authorities due to their production of taste and odour compounds and/or potent natural toxins. The study focused on method development for toxin extraction and detection in cyanobacterial blooms based on its lethal effect on the brine shrimp, *Artemia salina*. The standard, pure microcystin-LR and extracts of cyanobacterial cells were assessed by an immersion assay and the results were analysed by the probits method using the software Ldp Line. The LC_{50} for pure microcystin-LR was 6.80 µg ml^{-1} while that of samples ranged from 0.80 - 33.58 mg ml^{-1}. The cultured bloom, *M. aeruginosa* PCC 7813 with 3.68 µg mg^{-1} MC-LR equivalents of toxin was the most toxic sample while the natural bloom from loch Carlisle was the least toxic with 0.18 µg mg^{-1} MC-LR equivalents of toxin. The brine shrimp assay provides a simple and sensitive method for routine monitoring of blooms, particularly in developing countries where sophisticated equipment are not available.

Introduction

Cyanobacteria are one of the oldest dated life-forms on earth. Their mass occurrence in lakes, rivers, water reservoirs and coastal waters due to eutrophication is of major environmental, economic and scientific importance. This is because bloom-forming cyanobacteria such as *Microcystis*, *Anabaena* and *Oscillatoria* produce a wide range of toxic secondary metabolites. These toxins predominantly affect the nervous, hepatic and dermatologic systems of wild and domestic animals, fishes, birds and man, causing poisoning and death (Carmichael & Falconer, 1993; Pouria *et al*., 1998; Falconer, 1999).

Microcystin, the most common cyanobacterial toxin was first extracted from *M. aeruginosa*, a unicellular cyanobacterium that forms spongy masses in a gelatinous sheath. The microcystins are a group of over 60 cyclic heptapeptide hepatotoxins made up of three D-amino acids (1, 3, 6) one dehydroaminoacid (7), two strain-dependent variable L-amino acids (2 and 4) and a unique β-amino acid (5) found only in cyanobacteria and crucial to their toxicity (Fig. 1). The toxins are named by the one-letter abbreviation for the two variable amino acids and other minor structural modifications (e.g. microcystin-LR has leucine and arginine in the variable positions).

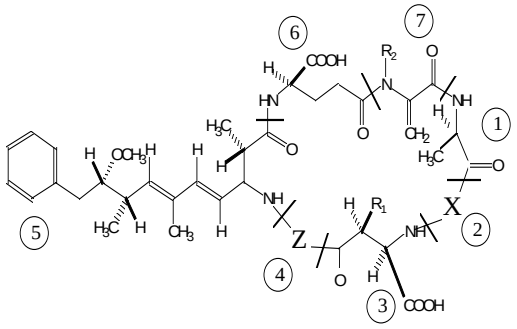

Fig. 1. Structure of microcystins (Sivonen & Jones, 1999).

Water-borne microcystins have been implicated in the promotion of tumours (Nishiwaki-Matsushima *et al*., 1992) and liver cancer (Ueno *et al*., 1996; Fleming *et al*., 2002). The exposure of crop plants to microcystins through contaminated irrigation water inhibits photosynthesis and the growth of root hairs (McElhiney *et al*., 2001). Outbreaks

of poisoning by toxic cyano-bacteria also occur in chlorinated tap water supplies (Falconer, 1999) because conventional water treatment techniques such as coagulation, sedimentation and filtration have been shown to be only partly effective in removing microcystins.

The monitoring of blooms in water resources is dependent upon adequate methods of detection and quantification. Mouse bioassay was the first method used in toxicity testing of cyanobacterial blooms (Eloff, 1982) but current methods include high-performance liquid chromatography (HPLC), mass spectrometry (Lawton *et al*., 1995) and enzyme-linked immunosorbent assays (ELISA) (Metcalf *et al*., 2000). In Africa, information on the occurrence or effects of cyanobacterial blooms is scanty. With the exception of South Africa, few countries in the continent have research programmes or knowledge in this field (Scott, 1991; Codd, 2000).

The paper reports the findings of the study on method development for the detection and quantification of microcystins in laboratory cultures and natural blooms of cyanobacteria using the brine shrimp (*Artemia salina*) assay.

Materials and methods

Source of standard and cyanobacterial material

The standard toxin used, microcystin-LR, was purified from laboratory cultures by flash chromatography (Lawton & Edwards, 2001). Laboratory cultures of *M. aeruginosa* PCC 7813 (Institut Pasteur, Paris) and *M. aeruginosa* Sciento (Sciento, Manchester, UK) and natural samples collected during bloom episodes from Loch Balgavies (15th August 1994) and Carlisle (21st June 1995) in Scotland were freeze-dried and stored at –20 °C.

Study area

Loch Balgavies is a freshwater, dimictic eutrophic lake near Forfar, Scotland, and the axenic clone referred to as *M. aeruginosa* PCC 7820 was isolated from algal blooms collected from this lake (Codd & Carmichael, 1982). Loch Carlisle is a freshwater lake near Carlisle, Scotland, which experiences blooms of algae in the summer.

Preparation of standard and samples

The standard was prepared by adding 250 µg of 80% aqueous methanol to 250 µg of microcystin-LR. Serial dilution of the standard stock was carried out with brine shrimp media to give final concentrations of 40, 20, 10, 5, 2.5, 1.25 µg ml^{-1} microcystin-LR.

To determine the toxicity of cyano-bacterial cells, samples must be extracted and this was carried out for 1 h in 80% aqueous methanol at a concentration of 50 mg dry weight of cells per ml. Extracts were diluted with brine shrimp media to give concentrations ranging from 50–0.78 mg ml^{-1}.

Hatching of cysts and bioassay

Brine shrimp cysts purchased from Sciento, Manchester, UK were stored at –20 °C before use. Brine shrimp media was prepared by diluting the stock solution with distilled water in the ratio 1:7 (Harada *et al*., 1999). A 100 mg of cysts was added and incubated at 25 °C for 48 h. The hatched larvae were pipetted into fresh media and 100 µl of the suspension (containing about 25 active larvae) were transferred into 96-well polystyrene microtitre plates. Cyanobacterial extract (100 µl) was added to test wells while brine shrimp media (100 µl) was added to control wells. All treatments were carried out in triplicate and plates were covered and incubated at 25 °C for 18 h. Different concentrations of the standard microcystin-LR were incubated along with samples. After incubation, dead larvae in each well (or those swimming in a fixed position) were counted under the microscope and percentage mortality was calculated using the mean of three replicates.

The concentration of standard micro-cystin-LR or extract required to kill 50% of the larval population (LC_{50}) was determined by probit analysis (Finney 1971) using the software Ldp Line (*http://embakr.tripod. com/ldpline*).

Results and discussion

A sigmoid dose-response curve was obtained for all samples with mortality increasing with dose of toxin or extract. Pure microcystin-LR caused 100% mortality of shrimps at 20 µg ml^{-1} (Fig. 2a) while for cultures – PCC 7813 and Sciento, 100% mortality was achieved at 25 and 50 mg ml^{-1} dry weight of cells respectively (Fig. 2b). These results indicate that microcystin-LR and extracts of cyanobacterial blooms were toxic to *A. salina* in a dose-dependent manner. However for natural samples, total mortality was not recorded at the highest concentration (Fig. 2b). This

suggests that the natural samples are less toxic than laboratory cultures, but it is possible for such samples to contain other cyanotoxins which this assay cannot detect since it has been fully evaluated for microcystins only (Codd, 2000). A probit transformation of the dose-response curve for pure microcystin-LR (Fig. 3) using the software Ldpline gave an LC_{5O} of 6.80 µg ml^{-1}. This is comparable with previous studies in which the LC_{50} for MC-LR were 3.75 and 4.58 µg ml^{-1} (Delaney & Wilkins, 1995; Metcalf *et al.*, 2002), respectively, while that for demethyl MC-RR was 5.0 µg ml^{-1} (Kiviranta *et al.*, 1991). In addition, the analyses of 18 hepatotoxic natural bloom samples by the brine shrimp assay gave LC_{50} values from 1.0–40.0 mg ml^{-1}, and this compared favourably with mouse bioassay and HPLC results (Lawton *et al.*, 1994).

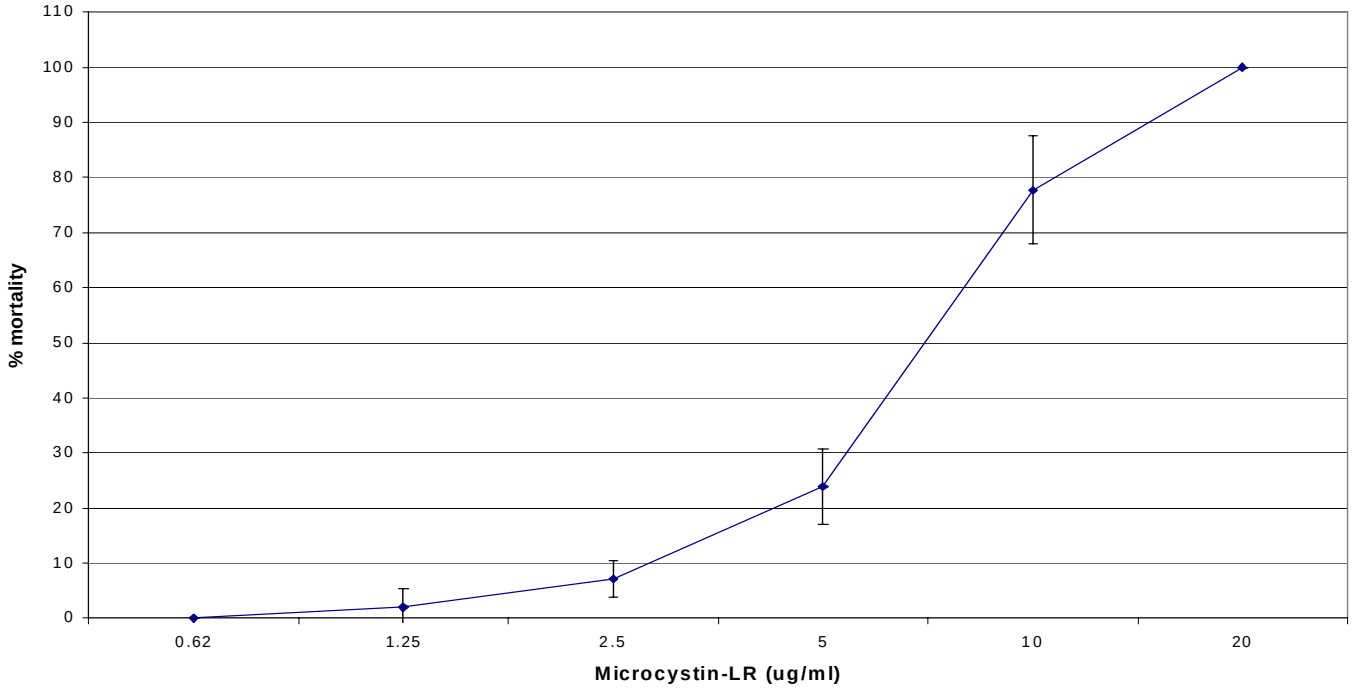

Fig. 2a. Brine shrimp assay for microcystin-LR

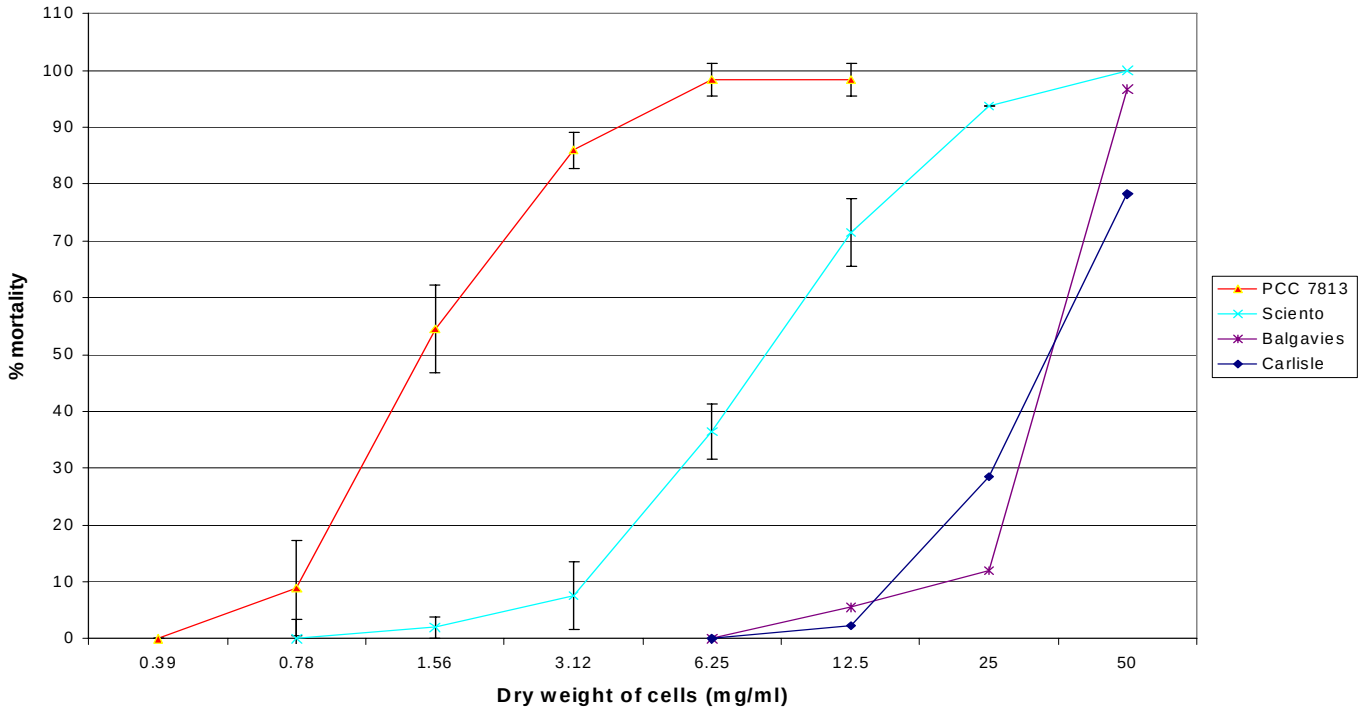

Fig. 2b. Brine shrimp assay for cultured and natural cyanobacterial blooms

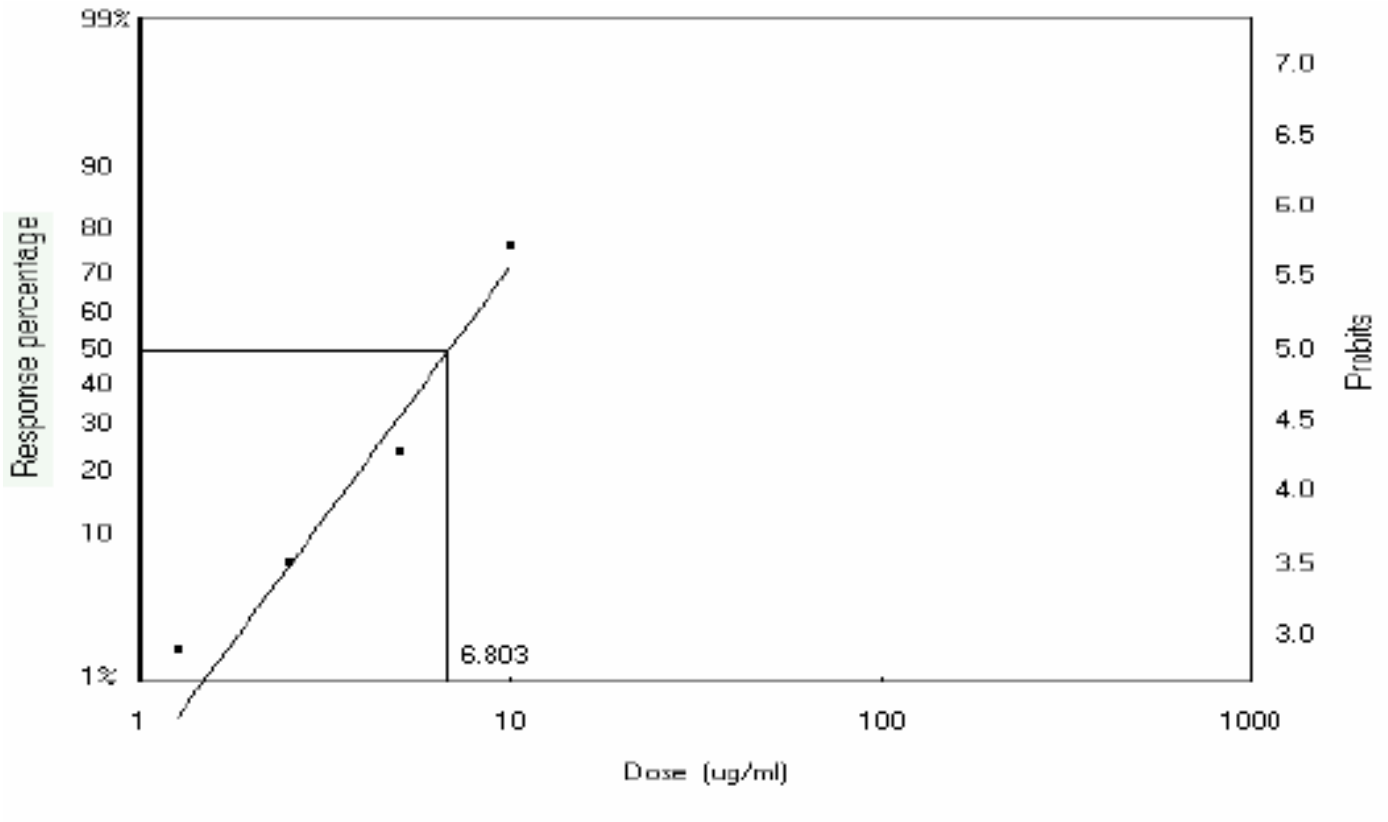

Fig. 3. Dose-response transformation for the standard, pure microcystin-LR

Toxin concentration in each sample was estimated as MC-LR equivalents from the standard dose-response graph and the results are presented in Table 1. The culture PCC 7813 was the most toxic sample with LC_{50} 0.80 mg ml^{-1} and 3.68 μg mg^{-1} MC-LR equivalents of toxin while the natural sample from loch Carlisle was the least toxic with LC_{50} 33.58 mg ml^{-1} and 0.18 μg mg^{-1} MC-LR equivalents of toxin. Thus, on a dry weight basis, *M. aeruginosa* PCC 7813 was still the most toxic sample while the bloom sample from Carlisle was the least toxic.

TABLE 1

Brine shrimp LC_{50} values for microcystin-LR and cyanobacterial blooms, and toxin concentration in blooms expressed as MC-LR equivalents

Sample	*LC_{50}*	*Toxin concentration (MC-LR equivalents μg mg^{-1})*
Standard toxin (¼g ml^{-1})Microcystin-LR	6.80	
Cultured Blooms (mg ml^{-1}) *M. aeruginosa* PCC 7813*M. aeruginosa* Sciento	0.808.38	3.680.73
Natural Blooms (mg ml^{-1}) Loch Balgavies Loch Carlisle	24.9733.58	0.260.18

Values shown for toxin concentration are means, n = 3

It was noted that for the same dry weight of cells, blooms recorded different LC_{50} values and toxin concentrations probably indicating that the species and/or strain of cyanobacteria present in a bloom is very important in determining its toxicity. Some strains of the same cyanobacterial species vary in toxicity by as much as three orders of magnitude hence the occurrence in minor amounts of a highly toxic strain amongst larger numbers of non-toxic strains, may render a bloom sample toxic (Sivonen & Jones, 1999).

The paucity of information about the occurrence and impacts of algal blooms in Africa is more probably due to a lack of detection methods that are adaptable for use in developing country laboratories. Most analytical methods require expensive equipment or kits and lengthy clean-up/concentration procedures which hinder their use in laboratories with modest resources. Much of the research on algal blooms in West Africa depends on microscopic identification of species, estimation of nutrient and chlorophyll *a* concentrations in water (Nwankwo, 1993; Akin-Oriola, 2003; Addico & Frempong, 2004; Oben & Oben, 2004). However, this approach is inadequate as it gives no indication of the toxicity or actual toxin concentration in cells bearing in mind that the WHO safety guideline for cyanotoxins is 1.0 mg l^{-1} of water.

The long-established and extensively-used mouse bioassay method relies on an in-bred strain of laboratory animals and specially trained operators which increases its cost implications. This study is therefore signifi-cant in demonstrating the use of the brine shrimp immersion assay as a sensitive alternative method for initial toxicity screen-ing of blooms for microcystin hepatotoxins. In addition, the brine shrimp assay is simpler, faster, more humane and cheaper as the costs of keeping animals is excluded. It is therefore a more acceptable method for laboratories with limited financial and technical resources.

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