African Journal of Science and Technology (AJST)
Science and Engineering Series Vol. 6, No. 1, pp. 1 - 10

DETECTION AND QUANTIFICATION OF TOXINS IN CULTURES OF Microcystis aeruginosa (PCC 7820) BY HPLC AND PROTEIN PHOSPHATASE INHIBITION ASSAYFFECT OF BLENDING VARIOUS COLLECTORS AT BULK

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ABSTRACT:- *Increasing anthropogenic eutrophication in lakes, drinking water reservoirs and coastal waters is a world-wide phenomenon leading to the formation of blooms of toxic cyanobacteria. These pose a significant threat to human and animal health hence the need for sensitive methods for their detection, identification and quantification. This report presents two methods: analytical high power liquid chromatography coupled with photo-diode array detection and protein phosphatase inhibition assay for the analysis of the most frequently encountered cyanobacterial hepatotoxins – the microcystins. Four microcystin variants: microcystin - LR, - LY, - LW and - LF were identified and quantified by HPLC in cells and growth media of cultured Microcystis aeruginosa PCC 7820. The protein phosphatase inhibition assay was used to estimate potential toxicity of cyanobacterial extracts and both methods showed good correlation (R2 = 0.91). Although HPLC provides accurate and specific information on the identity and quantity of each microcystin variant, it is quite expensive. The assay method on the other hand is relatively cheaper and can be modified to measure milligramme quantities of sample on a benchtop spectrophotometer but individual microcystin variants cannot be identified*

Keywords: Microcystis aeruginosa, microcystin variants, HPLC-PDA, PPI assay, intra- and extra-cellular toxins.

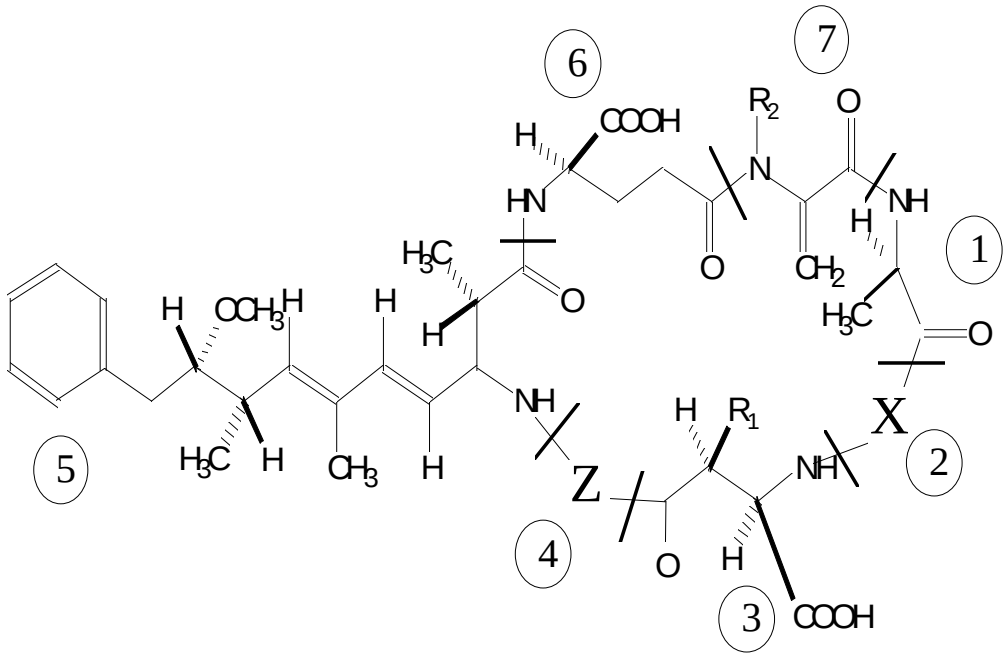
INTRODUCTION

Cyanobacteria (blue-green algae) are photosynthetic prokaryotes usually found in nutrient-enriched or eutrophicated waters world-wide. The seasonal proliferation of cyanobacteria in many aquatic ecosystems including drinking water supplies leads to bloom formation and the production of taste and odour factors and toxins. Many cyanobacterial genera e.g. Microcystis, Oscillatoria, Aphanizomenon, Anabaena have species or strains that produce potent dermato-, hepato- and neuro-toxins implicated in poisoning episodes of wild and domestic animals and human health problems (Belay and Wood 1982, Ochumba 1990; Bell and Codd 1996). Amongst the cyanotoxins, cyclic heptapeptides - microcystins are the most commonly reported in incidents of poisoning and the death of over 50 haemodialysis patients in Caruaru, Brazil, has been attributed to exposure to microcystins in dialysis water (Pouria et al 1998).

Microcystins are composed of seven peptide-linked amino acids (Fig.1) with the general structure:

cyclo-(D-Alanine1 -X2 -D-MeAsp3 -Z4 -Adda5 -D-Glutamate6 -Mdha7).

The amino acid Adda (3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid) is found only in cyanobacteria and crucial to their toxicity. Structural variations have been reported in all seven amino acids but most frequently by substitution of the L-amino acids at positions 2 and 4 and demethylation at positions 3 and /or 7 resulting in about 60 variants. Microcystins are named by the one-letter abbreviation for the 2 variable amino acids e.g. microcystin-LR has leucine and arginine in the variable positions (Sivonen and Jones 1999).



X and Z = the variable L - amino acids.
R_1 and R_2 = H (demethylmicrocystins) or CH_3.
D-MeAsp = D-erythro-?-methylaspartic acid.
Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.
Mdha = N-methyldehydroalanine

Figure 1: General structure of the cyclic heptapeptide hepatotoxin, microcystin showing common variations

The primary effect of microcystins is on liver cells as a result of selective transport mechanisms which concentrate them from the blood into hepatocytes. Their toxicity is linked to inhibition of important cellular enzymes called protein phosphatases types 1 and 2A resulting in hyperphosphorylation of proteins, breakdown of cytoskeletal filaments, liver haemorrhage and death (Yoshizawa et al 1990; Falconer 1994). In addition, low doses of these toxins promote tumour growth and primary liver cancer in humans exposed through drinking water supplies (Yu 1989).

Cyanobacterial toxins represent a global health hazard and several methods have been developed for their detection and quantification. The mouse bioassay was the first method used in toxicity testing of cyanobacteria and although it provides a measure of total toxicity within a few hours, it is not very sensitive or specific (Harada et al 1999). More sophisticated analytical techniques employed include:

a) chromatographic separation followed by detection by UV absorbance, mass spectrometry or fluorometry (Lawton et al 1994, Harada et al 1997, Robillot et al, 2000) b) thin layer chromatography (Pelander et al 1996) c) enzyme-linked immunosorbent assay (ELISA) (Chu et al 1989, Nagata et al 1997) d) protein-phosphatase inhibition assay (An and Carmichael 1994). All these methods are are capable of detecting microcystins within the limit of 1.0 µg l^{-1} of microcystin-LR in drinking water set by the World Health Organisation (WHO 1998). The type of information required and facilities on hand will determine the technique to be used.

This paper describes two methods: high-performance liquid chromatography (HPLC) and protein phosphatase inhibition assay for the detection and quantification of hepatotoxic microcystins in laboratory culture samples of *M. aeruginosa* (PCC 7820).

MATERIALS AND METHODS

Culture of organisms

Microcystis aeruginosa PCC 7820 was obtained from the Institut Pasteur, Paris, France. Stock cultures were grown in sterilized 10 litre flasks with BG-11 medium (Stanier *et al*, 1971) plus nitrate (8.8 mM) for 6 - 8 weeks under continuous illumination (H" 1500 Lux) with cool white fluorescent tubes and sparged with sterile air throughout. Experimental subcultures were prepared by adding 20 ml of BG 11 medium into sterile 50 ml flasks and aseptically inoculating with 1 ml [5 % (v/v)] of *M.aeruginosa* from stock cultures. Four replicate culture flasks prepared and incubated at 25 ^{o}C in a water bath. Flasks were sampled at 3 and 6 weeks interval.

Reagents and Standards

All reagents were of HPLC or analytical grade. Methanol and acetonitrile were purchased from Rathburn Chemicals Ltd (Walkerburn, UK), trifluoroacetic acid (TFA) from Fischer Scientific (Leicestershire, UK), manganese chloride from Fisons (Loughborough, UK). Ultra-pure water was produced with a Milli-Q system (Millipore, Watford, UK). All chemicals used in the PPI assay were obtained from Sigma-Aldrich, Poole, UK).
Microcystin-LR standards were purified from cultures of *M. aeruginosa* by flash chromatography and stored at –20^{o}C (Edwards et al, 1996).

Sample Preparation

Intracellular toxin concentration was determined as follows: One ml of culture sample was dispensed into an eppendorf tube, centrifuged at 14,000 rpm for 5 min and 800 µl of the supernatant was discarded. The pellet containing cyanobacterial cells was then frozen for 24 hours, thawed and extracted in 800 µl of methanol for an hour before analysis by HPLC.
Extracellular toxins were analysed from the culture media as follows: Five ml of culture sample was filtered through a pre-weighed glass fibre filter disc (GF/C Whatman, 55 mm diameter) to remove the cells. Filtered cells were dried at 45^{o}C for 12 hours in an oven and then weighed to determine cell biomass. The supernatant containing only the growth medium was freeze-dried and stored at –20 ^{o}C until required. Prior to HPLC analysis, the freeze-dried supernatant was extracted in 1.5 ml methanol for an hour.

HPLC

Microcystin concentration in extracted samples was determined by HPLC with photo-diode array detection following the method of Lawton et al, 1994. The instrumentation for HPLC consisted of a Waters system with Model 600 solvent pump, Model 717 plus autosampler and a Model 996 photodiode array detector monitoring at 200 – 300 nm with 1.2 nm resolution. The stationary phase was a Symmetry C_{18} column (250 x 4.6 mm I.D., 5 µm particle size, Waters) at a flow rate of 1ml min^{-1} and column temperature 38 ^{o}C. The mobile phase were Milli-Q water (solvent A) and acetonitrile (solvent B) both acidified with 0.05 % v/v TFA. Separation was achieved using the linear gradient outlined in Table 1. Sample injection volume was 50 µl.

Table 1: Linear gradient conditions at 1 ml min^{-1} used in HPLC analysis of microcystins

	Time in minutes						
	0	10	40	42	44	46	55
Solvent A (%)	70	65	30	0	0	70	70
Solvent B (%)	30	35	70	100	100	30	30

Solvent A = water + 0.05 % TFA and Solvent B = acetonitrile + 0.05 % TFA

A methanol blank and microcystin-LR standard (1µg MC-LR / 25 µl injection) was analysed alongside each set of samples. Chromatograms were integrated and analysed at 238 nm with a Millenium^{32} chromatography software (Waters, UK). All microcystins were quantified as microcystin-LR equivalents as follows:

$$\text{Toxin conc. } (\mu g\ ml^{-1}) = \frac{\text{Peak area of sample x 20}}{\text{Peak area of standard}}$$

$$\text{Toxin conc. } (\mu g\ mg) = \frac{\text{toxin conc. } \mu g ml^{-1}}{\text{weight of cells (mg)}}$$

Protein Phosphatase Inhibition (PPI) Assay

The assay was carried out using a modification of colorimetric methods (An and Carmichael 1994, Ward et al 1997 and Liu et al 2002). Protein phosphatase 1 activity was determined by measuring colour production associated with the formation of p-nitrophenol (yellow) from the substrate p-nitrophenyl phosphate (uncoloured) using a microtitre plate reader (Dynex MRX II with Revelation software Version 4.02).

Protein phosphatase-1 (catalytic subunit expressed in *E. coli*, Sigma-Aldrich, Poole, UK) was diluted in 50 mM Tris-HCl pH 7.4 containing 1g l^{-1} bovine serum albumin, 1 mM $MnCl_{2,}$ and 2 mM dithiothreitol. The substrate (5 mM) was prepared in buffer containing 50 mM Tris-HCl pH 8.0, 20 mM $MgCl_{2,}$ 0.2 mM $MnCl_2$ and 0.5 g l^{-1} bovine serum albumin. All buffers were prepared immediately before use. The dose-dependent kinetic activity of PP1 against the substrate (p-nitrophenyl phosphate) was first determined to assess enzyme activity.

Different concentrations of microcystin-LR (0.5 – 500 ng ml^{-1}), test samples and a negative control were prepared with Milli-Q water. The assay was performed by adding 25 µl of PP1 enzyme to 25 µl of test solution in a 96-well polystyrene microtitre plate. The plate was gently shaken and left at room temperature for 4 min followed by the addition of 200 µl of p-nitrophenyl phosphate and incubation at 37 °C. Absorbance readings were taken after 40 min at 405 nm. The standard inhibition curve was plotted using inhibition percentage of enzyme activity against concentrations of microcystin-LR standards.

$$\text{Inhibition percentage} = \frac{A_c - A_s}{A_s} \text{x} 100$$

where A_c is the average absorbance of three negative control samples and A_s is the average absorbance of the test sample or standard. Toxin concentrations in samples were then estimated from the standard curve as microcystin-LR equivalents.

RESULTS AND DISCUSSION

Toxin identification and quantification by HPLC

Reverse-phase HPLC is normally used for the separation of nonpolar or hydrophobic components hence microcystins, a broad group of toxic peptides with differing hydrophobicity are readily separated by this method. The chromatogram revealed four major peaks that can be attributed to microcystins in cultures of *M. aeruginosa* PCC 7820 (Fig 2a). The microcystin peaks were identified by their retention times and absorption spectra. The Adda residue of microcystins includes two conjugated ð-bonds responsible for the characteristic ultraviolet absorbance maximum at 238 nm by most microcystins. However microcystins containing tryptophan (e.g. MC-LW) give an absorption maxima at 222 nm (Fig 2b). The characteristics of the microcystin variants identified MC -LR, -LF, -LW and -LY are presented in Table 2. The variant microcystin-LR with the lowest retention time of 19 min was eluted first being less hydrophobic than the others. Interestingly, the development of sophisticated analytical methods has led to the discovery of other microcystin variants. For example, Lawton et al (1995) identified six microcystins by liquid chromatography - mass spectrometry (LC-MS) while Robillot et al, 2000 reported ten using Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS) in cultures of *M. aeruginosa*. This method provides information on the molecular mass of all peptides in a

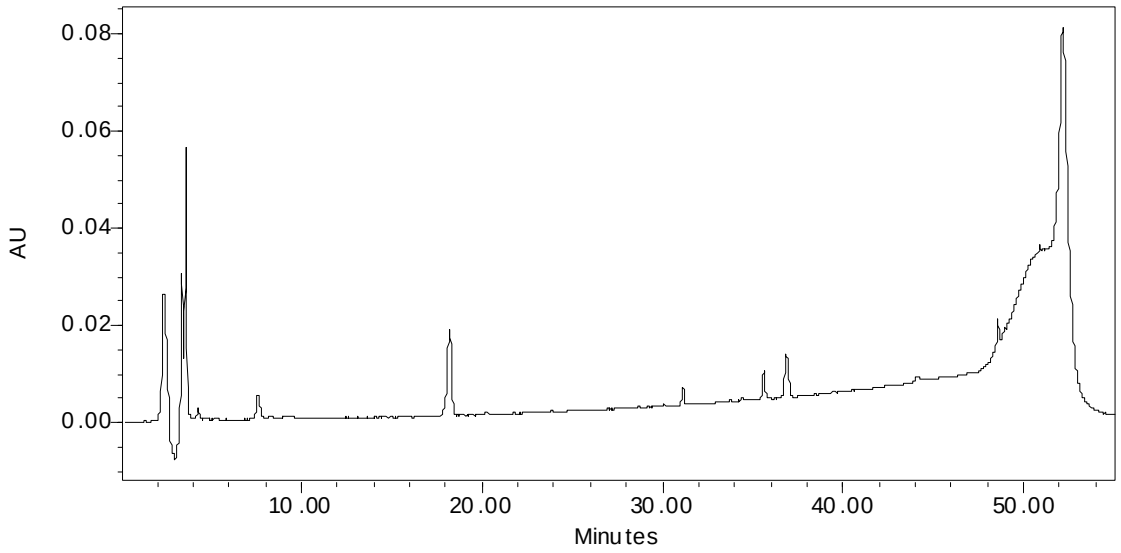


Figure 2(a): Chromatogram of microcystins present in cultures of *M. aeruginosa* PCC 7820: microcystin-LR [1], microcystin-LY [2], microcystin-LW [3] and microcystin-LF [4]

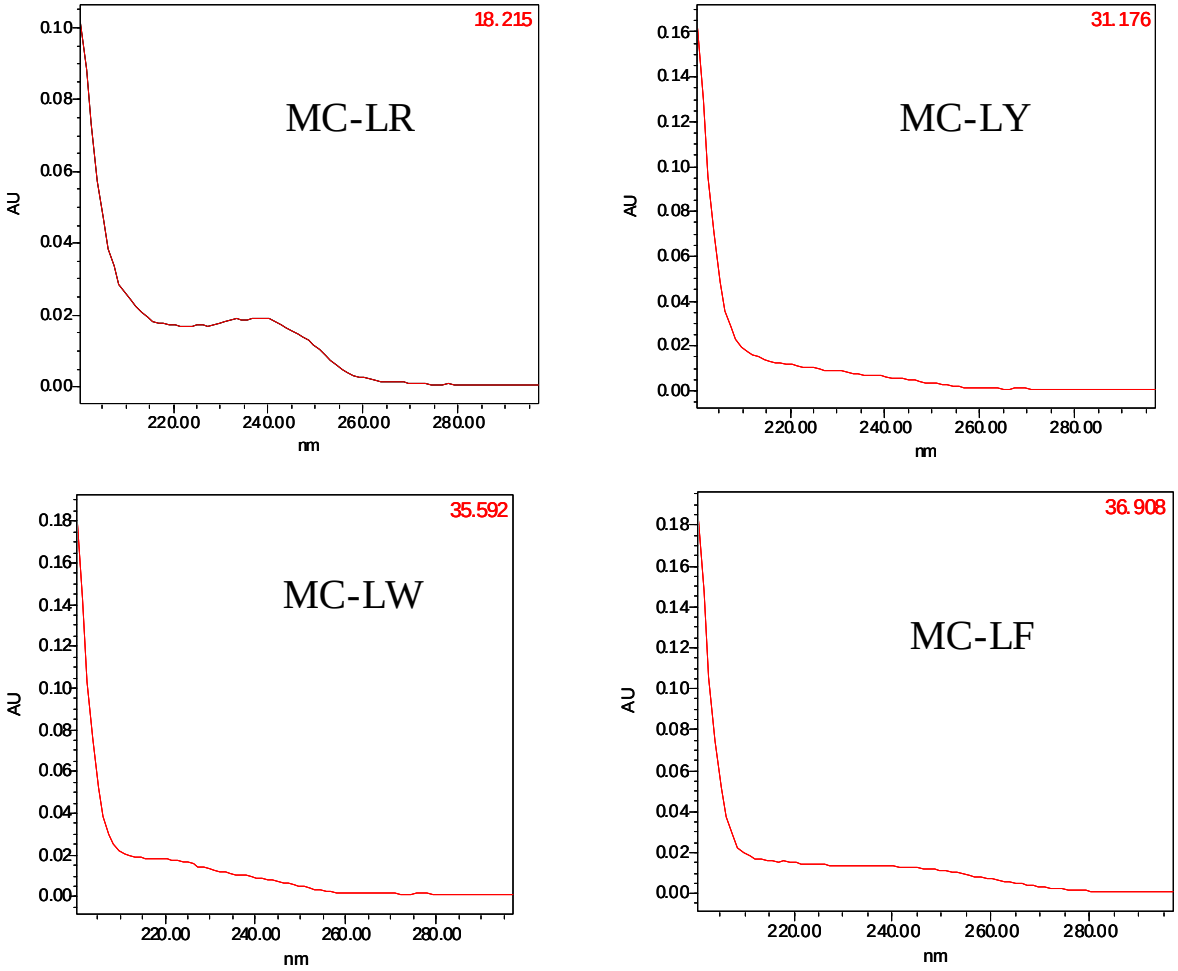


Figure 2(b): Absorption spectra and retention time of each microcystin variant as determined by HPLC-PDA

sample and thus gives strong indications of the microcystin variants present. It is important to know which microcystin variants are present in a sample because their toxicities differ in plants and animals. McElhiney et al 2001 observed that microcystin-LR and -RR had similar inhibitory effect on the growth of mustard seedlings whereas in mice, the toxicity of microcystin-RR was 12 times less than that of microcystin-LR.

All four microcystin variants identified above were present in cell extract (intracellular toxins) and in growth medium (extracellular toxins). The percentage contribution of each variant to total microcystin pool (either intra- or extra-cellular) is relatively constant over time (Table 2) indicating that an increase or decrease in total microcystin concentration results from a corresponding change in the amounts of each variant.

Table 2: Characteristics of microcystin variants identified in this study

Toxin variant (X and Z amino acids)	Retention time (min)	Absorption wavelength (nm)	Percentage contribution to microcystin pool
MC-LR Leucine, Arginine	18	239.1	53 - 62
MC-LF Leucine, Phenylalanine	36.9	239.1	13 - 25
MC-LW Leucine, Tryptophan	35.6	222.7	12 - 16
MC-LY Leucine, Tyrosine	31	239.1	6 - 8

The intra- and extracellular localization of toxins is a vital parameter in the estimation of cell toxic potency because toxins are contained within cyanobacterial cells until decay or death of the bloom. At 3 weeks i.e. during the exponential growth phase, total microcystin concentration was intracellular in all cultures about 5µg ml^{-1} (Figure 3a). But at 6 weeks i.e. the decline phase, intracellular microcystin concentration was reduced in all cultures (< 4µg ml^{-1}) while extracellular microcystin increased concurrently (Fig 3b). This change can be explained by the release of toxins in the medium corresponding with death and lysis of cells when they are out of nutrients. The availability of nutrients has been shown to influence both growth and toxin production in non-nitrogen-fixing cyanobacterial cultures such as *M. aeruginosa* and *O. agardhii* (Codd and Poon 1988; Sivonen 1990).

In Figure 4, toxin amounts expressed as a function of cell biomass (weight) showed that microcystin production at 3 weeks more than doubled that at 6 weeks in all cultures. This conforms with earlier observations that microcystin production in cyanobacterial cells is optimal at the exponential growth phase of the culture / bloom and declines afterwards (Eloff and Westhuizen 1981; Kotak et al 2000). In this study, microcystin concentration in cells seems highest under conditions favouring growth but in contrast Van der Westhuizen and Eloff (1983, 1985) found that the optimum conditions for growth (pH, temperature and light) did not coincide with those for toxin production in their cultures of *M. aeruginosa*.

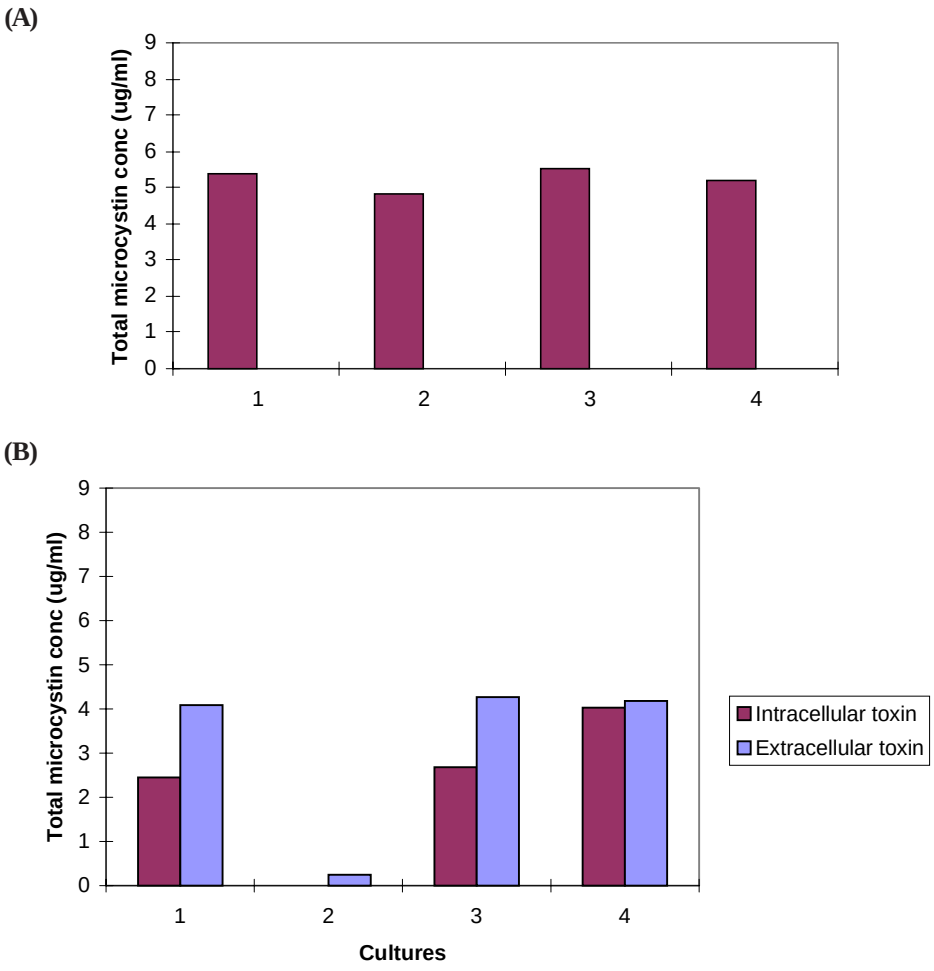


Figure 3: Total microcystin concentration in cultures of *M. aeruginosa* PCC 7820 at 3 weeks (A) and 6 weeks (B)

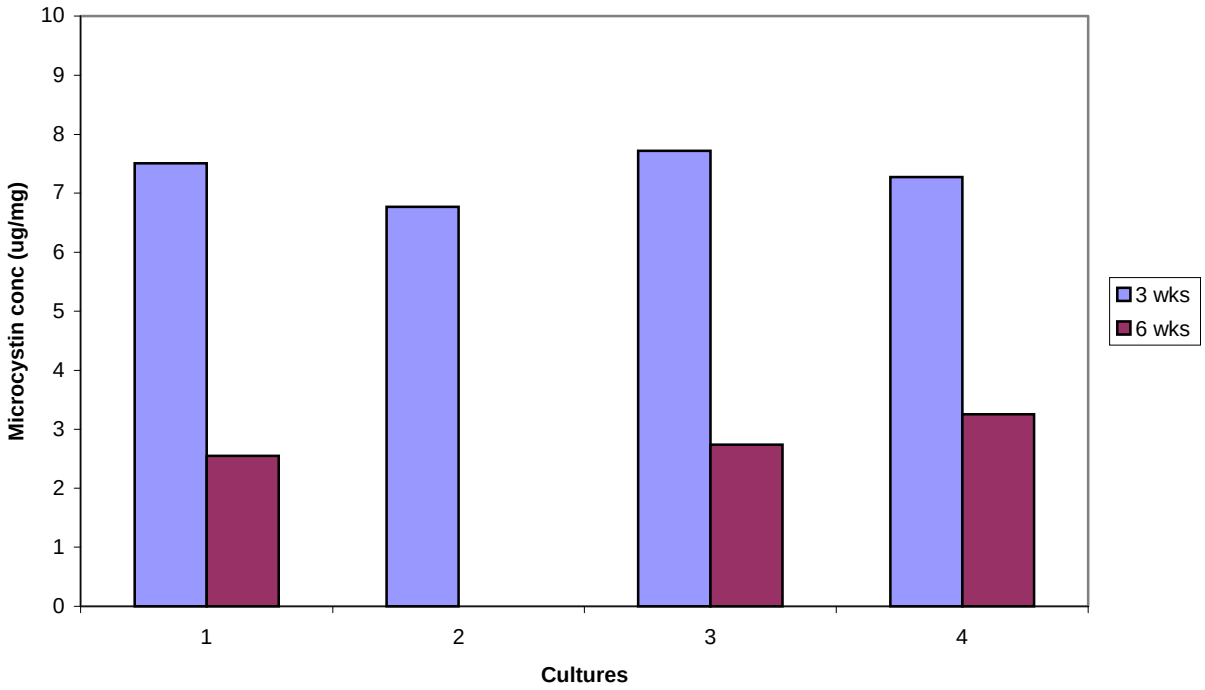


Figure 4: Intracellular microcystin concentration as a function of cell biomass in cultures of *M. aeruginosa* PCC 7820 at 3 and 6 weeks interval

Protein phosphatase inhibition assay

The inhibition of protein phosphatases 1 and 2A by cyanobacterial microcystins is the basis of this simple and quick bioassay. The method is very sensitive and extremely small amounts of microcystin (nanogramme quantities) can be detected in samples. The dose-dependent kinetic activity of protein phosphatase 1 i.e. the lowest concentration of enzyme that gave maximum absorbance with the substrate was 6.25 µg ml^{-1} and this was used in the assays.

The standard curve for the inhibition of PP1 by MC-LR (Fig 5) shows a 100 % inhibition occurring at concentrations greater than 500 ng ml^{-1}. The high reproducibility of the assay is shown by the very small standard error bars. The portion of the curve between 20 and 80 % which is relatively linear and normally used for quantification defines the working range of the assay. The lower limit of detection (20 %) corresponds to about 60 ng ml^{-1} microcystin-LR equivalent hence the assay can detect microcystins at levels eight times lower than the HPLC method using 25 µl of sample in both cases (detection limit for HPLC in this study is approximately 0.5 µg of pure microcystin on the column). The IC_{50} (i.e. concentration of microcystin-LR causing 50 % reduction in PP1 enzyme activity) was about 126 ng ml^{-1}.

Due to insufficient enzyme quantity, only intracellular toxin in 6-week old culture samples of *M. aeruginosa* PCC 7820 were tested. The cell extracts were diluted serially (x 20 or more) to within the detection limits of the assay and incubated with the enzyme. The PP1 assay results (Table 3) are in agreement with that of HPLC analysis ($R^2 = 0.91$). Nevertheless, the assay detected trace amounts of microcystin in sample 2 due to its higher sensitivity. This suggests that for samples containing trace levels of microcystin, analysis by HPLC may lead to the erroneous conclusion that the sample is non-toxic.

Table 3: Results of protein phosphatase inhibition assay for intracellular toxin in 6-week old culture samples of *M. aeruginosa*

	Cultures			
	1	**2**	**3**	**4**
Absorbance	0.31	0.42	0.3	0.33
% inhibition	78.88	71.58	79.9	77.53
MC-LR equivalent (ug ml-1)	7.95	0.28	8.37	18.62

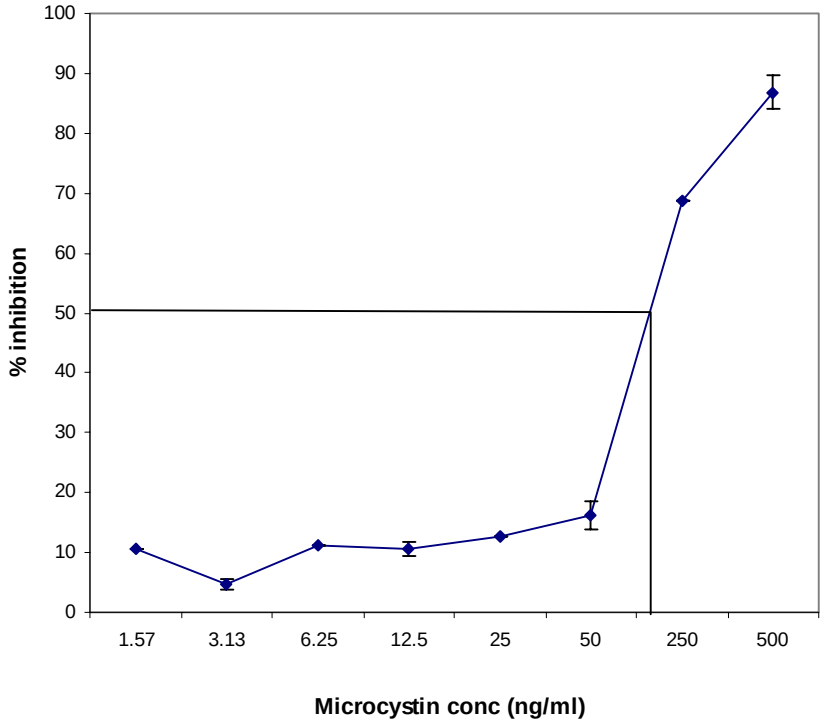


Figure 5: Standard inhibition curve of protein phosphatase 1 by microcystin-LR at 40 min. The solid line shows the estimated IC_{50} (about 126 ng ml^{-1}). Each point is the mean of three observations with the standard error of the mean

Hence it is safer to employ an assay method in combination with HPLC in testing unknown samples. A major drawback of the protein phosphatase inhibition assay is that it is prone to underestimate microcystin content due to intracellular (endogenous) phosphatase activity (Sim and Mudge, 1993).

CONCLUSION

Although analysis of cyanobacterial toxins by HPLC provides accurate and specific information on the identity and quantity of each microcystin variant, it requires special equipment and personnel and has a high sample unit cost. The protein phosphatase inhibition assay on the other hand is relatively cheaper and can be modified to measure milligramme quantities of sample on a benchtop spectrophotometer. It also gives an indication of the potential toxicity of the sample although individual microcystin variants cannot be identified. The assay method would thus be suitable in conditions where it is necessary to determine the overall toxicity of water bloom samples for rapid decision making. In such cases, it is safer to classify a non-toxic sample as toxic than otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank Mr Douglas Graham and Dr Iain Liu for assistance with HPLC analysis and PPI assay respectively. Akin-Oriola was supported by a Royal Society grant (Ref. No: JEB/DEV.SV/12997) and facilities for the research were provided by The Robert Gordon University, School of Life Sciences, Aberdeen, U.K.

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