Comparison of Methods for Detecting Microcystin and Microcystin-Producing Cyanobacteria

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ABSTRACT

Cyanobacterial samples from Australia, Thailand, Korea, India and the Philippines were analysed at a workshop held in Chiang Mai in April 2004. The workshop participants used the simple but effective analytical screening techniques of Protein Phosphatase inhibition assay (PPi) to measure the microcystin concentrations and used the Polymerase Chain Reaction (PCR) for detecting the presence of the microcystin synthetase gene (mcyA) in fresh and lyophilized samples.

Microscopy showed that 11 of the 20 samples contained toxigenic cyanobacteria (species with the potential to produce microcystin). Eighty-one percents of those samples contained > 1μg/L of microcystin-LR equivalents. The mcyA gene was detected in 64% of the samples.

Key words: Microcystin, Microcystin producing cyanobacteria, Comparative analysis

INTRODUCTION

Contamination of drinking water by toxigenic cyanobacteria is a concern of public health authorities throughout the world. In many countries, public health guidelines for potable and recreational use of water are based on the numerical abundance or biomass of toxigenic cyanobacteria. More recently, public health authorities have introduced guidelines for concentrations of microcystin in drinking water. This hepatotoxin is hazardous to human because low-level, long-term exposure has tumour-promoting effects (Chorus and Bartram, 1999).

A variety of physico-chemical and biochemical methods are currently used to assess water quality in accordance with public health guidelines for microcystin or toxigenic cyanobacteria. These analytical methods differ in sensitivity, cost and technical complexity. Several recent studies have compared the analytical performance of chromatographic methods like HPLC and bioassays like ELISA (Enzyme Linked Immuno-Sorbance Assay) and PPi (Protein Phosphatase inhibition assay) (Fastner et al., 2002; Rapala et al., 2002). Comparative assessments of methods for detection of toxigenic cyanobacteria that have included the detection of microcystin synthetase gene *mcyA* are rare (Hawkins et al., 2004). Researchers seeking to screen algal material for presence of hepatotoxins, or water supply authorities assessing the public health risk from cyanobacterial contamination of water supplies can both benefit from the adoption of new analytical screening methods which are quicker and more sensitive than microscopy and cheaper than HPLC.

A practical workshop was held in Chiang Mai in April 2004, to introduce researchers and water industry professionals to two newly-developed analytical screening methods for detecting microcystin (PPi) and for detecting the *mcyA* gene (PCR). This paper evaluates the performance of the methods in the hands of new users and reports the results of a survey of cyanobacterial bloom samples provided by the workshop participants from their home countries in the Asia Pacific.

MATERIALS AND METHODS

The workshop participants provided twenty freeze-dried or freshly-collected water and scum samples that were expected to contain toxigenic cyanobacteria. The dominant cyanobacterial species in each sample was determined by microscopic examination.

Protein Phosphatase inhibition (PPi)

The colorimetric protein phosphatase inhibition assay was based on the method described by Heresztyn and Nicholson (2001). The protein phosphatase PP2A enzyme (25 units) was purchased from Promega®, U.S.A. (Part# V631). One unit of PP2A enzyme being the amount to hydrolyse one nanomole of pNPP/min at 30°C under the assay conditions specified in Promega Technical Bulletin 537 was used.

The PPi test is quantitative the concentration ranges between 0.2-10 μg/L of microcystin-LR (M-LR). This range is where the change in inhibition is approximately linear with toxin concentration. Outside that range, results are qualitative and are expressed as greater than the quantitative maximum or less than the detection limit.

Other naturally-occurring bioactive compounds can inhibit PP2A enzyme activity, so the PPi test is not specific for microcystin. Furthermore, the degree of inhibition of PP2A varies for different forms of microcystin. The PPi bioassay was calibrated, using the LR form of microcystin (M-LR), therefore, all results are reported as μg/L of M-LR equivalents.

The quantity of microcystin that produced 50% inhibition of the PP2A enzyme activity (IC_{50}) was calculated from the slope of the linear portion of the inhibition curve. The IC_{50} is an indicator of the reproducibility of the method and the sensitivity test for those particular reaction conditions. The absorbance of each sample was corrected, using a reagent blank (PP2A enzyme omitted), which compensated for colour in the sample and for any naturally-occurring phosphatase enzyme in the sample. Each sample was analysed in duplicate and the mean of the two results was reported.

Polymerase chain reaction (PCR)

The samples prepared for PPi were also analysed by PCR for the presence of a component of the microcystin synthetase gene cluster, the *mcyA* gene. Total genomic DNA was extracted from the lyophilised or fresh cyanobacterial material, using a modification of a technique for purification of DNA from gram-negative bacteria (Burns et al., 2004).

Samples were concentrated by centrifugation, then the DNA was extracted and 1% of the extract (1μL) was used for the test. This concentration and dilution process gave a theoretical detection limit of 10 *mcyA* containing cells/mL, assuming no inhibition of the PCR reaction. The PCR method used primers specifically designed to amplify the *mcyA* gene (Table 1). To establish that the PCR was successful, we ran a positive control and an internal standard for each sample. The positive control tested for the presence of 16S rRNA that is specific to cyanobacteria, using primers to amplify the region 27-408 (*E. coli* designation) (Table 1). A negative result for this test would indicate either PCR inhibition or the presence of insufficient cyanobacterial cells in the sample (Burns et al., 2004). An internal standard of cyanobacterial DNA, known to contain the *mcyA* gene was also added as a 'spike' to each sample. This detected any inhibitors to PCR that could produce a false negative result.

Table 1. The PCR primers used in the assay and the corresponding annealing temperatures.

Primer	Target gene	Sequence	Annealing (°C)
MSF	Microcystin	5´ -ATCCAGCAGTTGAGCAAGC	60
MSR		5´ -TGCAGATAACTCCGCAGTTG	
27F	16S rRNA	5´ -AGAGTTTGATCCTGGCTCAG	50
809R		5´ - TTACAA(C/T)CCAA(G/A)(G/A)(G/A)CCTTCCTCCC	

RESULTS

PPi Standard curve

The workshop participants prepared standard curves to measure the inhibition of PP2A enzyme activity by M-LR, for concentrations between 0.2 and 5 μg/L. An example of one of these standard curves is presented in Figure 1. The linear part of the sigmoid inhibition curve, where the concentration of inhibitor can be quantitatively determined from absorbance, was identified by inspection. The quantitative region was from 1-2 μg/L of M-LR and the IC_{50} was calculated from the slope as 1.3 μg/L of M-LR. The standard curves determined by other workshop participants had IC_{50} of 1.9 and 3.6 and all had detection limits of 1μg/L of M-LR.

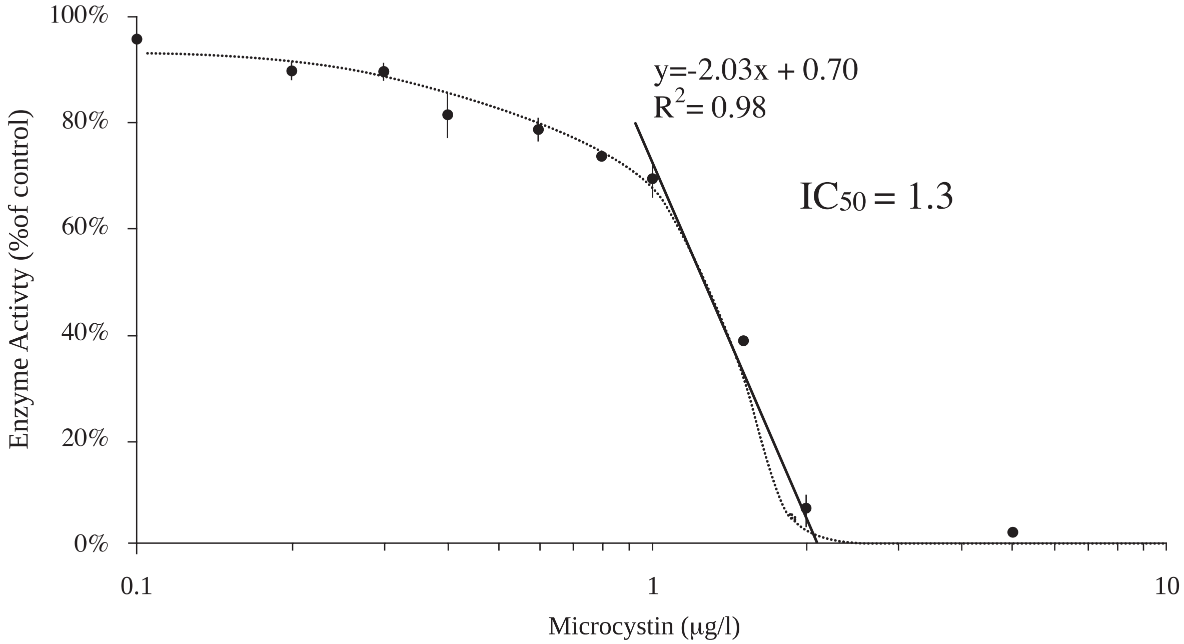

Figure 1. A standard curve showing the inhibition response of the PP2A enzyme under the workshop assay conditions. The error bars around each point show the variation between 2 replicate samples. The IC_{50} value was calculated from the slope of the straight line fitted to the linear portion of the inhibition curve. The R^2 value of 0.98 for the 3 points in the linear section between 1 and 2 µg/L indicates that the analytical precision was satisfactory.

Twenty samples were tested by PPi. The microcystin content of each sample was calculated, using the standard curve and the absorbance of each sample. Nineteen of the samples contained cyanobacteria and eleven samples contained *Microcystis* from microscopic analysis. Nine of the eleven *Microcystis* samples (81%) contained microcystin in excess of 1µg/L by PPi (Table 2). Both strains of *Aphanizomenon* and one of the two *Oscillatoria* strains did contain microcystin. None of the *Anabaena* samples contained microcystin.

Only fifteen of the samples were tested by PCR because of equipment limitations and the results from 14 of these samples could be directly matched with the PPi results (Table 2). The *mcyA* gene was detected in 8 of these samples.

Table 2. A summary of the cyanobacterial species present in each sample with its provenance and a comparison of the results of the PPi and PCR methods. The abundance of each species in a sample was represented by: +++ (dominant); ++ (common); or + (rare). Positive samples by PPi had more than 1μg/L of M-LR equivalents, the detection limit of the assay in our workshop experiments. Positive samples by PCR (+) indicated the *mcyA* gene was present at a concentration of more than 10 cells/L.

Toxigenic species	Source of sample	Sample description (Strain designation)	Microcystin (μg/L)	*mcyA* gene (+/-)	Conclusion
Anabaena only	Korea	Lab strain freeze dried (PP1 04/419)	< 1	not tested	Not comparable as no PCR test
Anabaena only	Korea	Lab strain freeze dried (P10079)	< 1	not tested	Not comparable as no PCR testnot comparable
Anabaena+++	Nong Bua - Thailand	plankton net haul, fresh	< 1	-	PPi and PCR agreed
Aphanizomenon only	Korea	Lab strain freeze dried (P10081)	1.1	not tested	Not comparable as no PCR testnot comparable
Aphanizomenon 2 only	Korea	Lab strain freeze dried (AP -A)	> 1	not tested	Not comparable as no PCR testnot comparable
Microcystis aeruginosa only	Korea	Lab strain freeze dried (KA-3)	1.3	+	PPi and PCR agreed
Microcystis wesenbergi only	Korea	Lab strain freeze dried (CS-MU23)	< 1	-	PPi and PCR agreed
Microcystis+, *Cylindrospermopsis*+++, *Anabaena*+	Nan Province, Thailand	plankton net haul, fresh	< 1	-	PPi and PCR agreed
Microcystis+++	Laguna de Bay, Philippines	plankton net haul, freeze dried	2	+	PPi and PCR agreed
Microcystis+++	Mae Jok, Thailand	plankton net haul, fresh	> 1.5	+	PPi and PCR agreed
Microcystis+++	Phuket, Thailand	plankton net haul, fresh	> 1.5	+	PPi and PCR agreed
Microcystis+++	Mae Kuang Reservoir, Thailand	plankton net haul, fresh	> 1.5	+	PPi and PCR agreed
Microcystis+++	Nong Harn, Thailand	plankton net haul, fresh	> 1.5	+	PPi and PCR agreed
Microcystis+++	SOPA reservoir Sydney, Australia	grab sample of scum	> 1.5	-	PCR false negative too many cells in sample
Microcystis+++	Laguna de Bay, Philippines	plankton net haul, freeze dried	> 2	not tested	Not comparable as no PCR testnot comparable
Microcystis+++, *Cylindrospermopsis*+	Mae Tang, Thailand	plankton net haul, fresh	> 1.5	+	PPi and PCR agreed
No cyanobacteria detected	India	River water sample	< 1	-	PPi and PCR agreed
No cyanobacteria detected	India	River water sample	1.5	not tested	Not comparable as no PCR test
Oscillatoria only	Korea	Lab strain freeze dried (OA-0)	< 1	-	PPi and PCR agreed
Oscillatoria 2 only	Korea	Lab strain freeze dried (CH-0)	> 2	-	PCR false negative too many cells in sample
Synechococcus sp1 and *S. lividus*	Hot Spring at Doi Saket, Thailand	cyanobacterial mat sample from 73°C	not tested	+	not comparable as no PPi test

The results of the two methods agreed in 12 of the comparable 14 samples (7 positives and 5 negatives). However, the results from the two methods did disagree in 2 samples (Table 2). There were high concentrations of toxigenic cyanobacterial cells (*Microcystis or Oscillatoria*) in both these samples and microcystin was detected by PPi, but the PCR test was negative for *mcyA*. We suspected that the polymerase reaction in these two samples was inhibited by other biologically-active compounds associated with the high cyanobacterial cell concentrations. A novel finding was the detection of *mcyA* gene in a sample from a cyanobacterial mat collected from a hot spring at Amphur Doi Saket, Chiang Mai province, Thailand. The mat comprised two *Synechococcus* species, *i.e., Synechococcus spp.* and *S. lividus.*

DISCUSSION

From workshop results, it was evident that the PPi and PCR methods could be quickly and effectively implemented by trained professionals after a brief induction course. The detection limit for the PPi method of 1μg/L of M-LR equivalents which was achieved by each of the workshop participants in their standard curves, was at the level of the World Health Organisation guideline for drinking water. This sensitivity allows analysis of water samples without pre-concentration, reducing cost and the turn-around time for this test.

The proportion of *Microcystis* samples that contained microcystin was greater than the 50% level, typically reported from naturally-occurring *Microcystis* water blooms. This result was influenced by the small sample size but it might reflect a bias in the collection due to participants selecting known or suspected toxic samples for testing.

The close agreement between both sets of results demonstrated the complementary nature of the two assays in providing an enhanced assessment of risk from toxigenic cyanobacteria where the results from only one test might be ambiguous (Hawkins et al., 2004). The PCR test produced two false negative results. These occurred in samples containing high cell concentrations. This limitation of the PCR test has been reported previously (Hawkins et al., 2004). As a false negative result is of greater concern than a false positive in analyses used to make public health decisions, the analyst must dilute samples with high cell concentrations and the method needs to be improved by a cleanup step to prevent this type of PCR inhibition.

Microcystin has been detected in a cyanobacterial mat collected from a Kenyan hot spring, which contained four different mat-forming cyanobacteria; *Phormidium tenebriformis, Oscillatoria willei, Spirulina subsalsa* and *Synechococcus bigranulatus* (Krientiz et al., 2003). However, the microcystin-producing form(s) in the mixed population could not be identified. This is the first report of the presence of the *mcyA* gene in the thermophilic cyanobacteria, *Synechococcus spp.* or *S. lividus.*

CONCLUSION

The workshop format was suitable for training the participants quickly and effectively. Their results demonstrated that the methods were sufficiently robust to deliver useful results even in the hands of relatively inexperienced analysts. The survey found an unusually-high percentage of toxigenic *Microcystis* strains from the Asia Pacific region and the first report of the presence of the *mcyA* gene in the thermophilic *Synechococcus* species, *Synechococcus spp.* or *S. lividus.*

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