

## A review of analytical methods for assessing the public health risk from microcystin in the aquatic environment

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### ABSTRACT

We surveyed the occurrence of toxigenic cyanobacteria, the *mcyA* component of the microcystin synthetase gene and microcystin in aquatic systems in temperate Australia and tropical Thailand. The survey methods, microscopy, protein phosphatase inhibition assay, enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), were evaluated for screening raw water for public health risk from microcystin producing toxigenic cyanobacteria.

Three tests, ELISA, PCR and protein phosphatase inhibition (PPI), were judged very useful because of their sensitivity and speed of analysis. ELISA ranked slightly higher because of its superior specificity for microcystin. The PCR was highly sensitive, but there were three false negative results, whilst the PPI was cheap but less specific than ELISA. Some of the microcystin quantifications results were validated by high performance liquid chromatography (HPLC).

The combination of a gene probe for the *mcy* gene complex with an ELISA or PPI assay for microcystin is proposed as a powerful screening technique for raw waters that can provide multi-level risk assessment including 'incipient risk', when microcystin producing cyanobacterial strains are rare and microcystin concentrations are below the detection limit for biochemical or chromatographic analyses.

**Key words** | microcystin, public health risk assessment, toxigenic cyanobacteria

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### INTRODUCTION

Contamination of drinking water by cyanobacterial toxins is a concern for public health and water authorities throughout the world. Microcystin is an especially hazardous cyanotoxin in freshwaters, because of its tumour promoting effects (Chorus and Bartram 1999).

Early guidelines for use of potable or recreational waters contaminated by cyanobacteria recognised the danger of microcystin, but the public health risk assessment was usually based on the concentration of toxigenic cyanobacterial cells (e.g. NSW BGATF 1992). As analytical techniques for cyanotoxins have improved, guidelines have expanded to include microcystin concentration (Chorus & Bartram 1999; NHMRC 2001).

The recent description of the complete pathway for microcystin synthesis by Tillett *et al.* (2001) offers another way to assess the likelihood of microcystin contamination of environmental waters, through detection of the microcystin synthetase gene complex (*mcy*). The veracity of polymerase chain reaction (PCR) based gene probe analysis has been demonstrated for laboratory strains of cyanobacteria (Neilan *et al.* 1999).

A variety of physico-chemical and biochemical methods to measure microcystin or toxigenic cyanobacterial concentrations are now available. These methods differ in detection limit, cost and technical complexity. Several recent studies have compared the accuracy of biochemical

assays such as enzyme linked immunosorbent assay (ELISA) and protein phosphatase inhibition (PPI) with high performance liquid chromatography (HPLC) for detection of microcystin (Fastner *et al.* 2002; Rapala *et al.* 2002). The use of PCR-based genetic methods for screening environmental water samples has been very limited to date (Baker *et al.* 2001; Bittencourt-Oliveira 2003).

This paper assesses the performance of emerging analytical methods for the measurement of the public health risk from microcystin that are available to water and public health authorities. The methods were ranked on cost, reliability and simplicity in a routine laboratory setting to provide a pragmatic assessment, relevant to the water industry.

## METHODS

Twenty water bodies in Australia and five water bodies in Thailand that were expected to contain toxigenic cyanobacteria were sampled by local volunteers during the summer of 2003 (see Figure 1). The bloom samples were stored on ice in the dark and freighted to a central laboratory (Sydney Water) where the sample was split into three sub-samples then shipped on to the other participating laboratories.

At the Sydney Water laboratory all samples were tested for chlorophyll-a according to *Standard Methods* (1998),

examined by microscopy to determine the concentration of toxigenic cyanobacteria and analysed for the concentration of microcystin-LR equivalents by PPI.

The National Research Centre for Environmental Toxicology (EnTox) initially screened the samples for microcystins by ELISA using the Abraxis<sup>™</sup> antibody, and Queensland Health Scientific Services quantified the microcystin in some samples by HPLC/Diode array (QHSS Method # 15605), while the Microbiology Department of the University of New South Wales tested each sample for the presence of a component of the microcystin synthetase gene cluster *mcyA* by PCR. The specific methods used in the survey are described briefly here; a more detailed review is available in Chorus & Bartram (1999).

As a test of the reproducibility of the biochemical analyses, sub-samples of a microcystin producing strain of *Microcystis* (PCC7806) were prepared and stored frozen, then submitted with different sample batches to the respective analytical laboratories.

## Microscopy

The method consisted of conventional light microscopy with 10 times sample concentration, mild sonication to disrupt colonies, Lugols fixation and 20h sedimentation. The method detection limit was 10 cells ml<sup>-1</sup> for each cyanobacterial species after sonication. Microscopic examination cannot detect microcystin but can identify the presence of toxigenic cyanobacteria (i.e. species with the potential to produce cyanotoxins). The estimated cost for each test (excluding labour) was US\$1.50 and the labour time was 50 minutes per test.

## Protein phosphatase inhibition (PPI)

The colorimetric protein phosphatase inhibition assay was based on the method described by Heresztyn & Nicholson (2001). Protein phosphatase PP2A was purchased from Promega Corporation, Australia. It was supplied in lots of 25 units, one unit being defined as the amount of enzyme required to hydrolyse one nanomole of pNPP min<sup>-1</sup> at 30°C under the specified assay conditions (Promega Technical Bulletin 537). The IC<sub>50</sub> was between 0.4 and 0.8 µg l<sup>-1</sup>

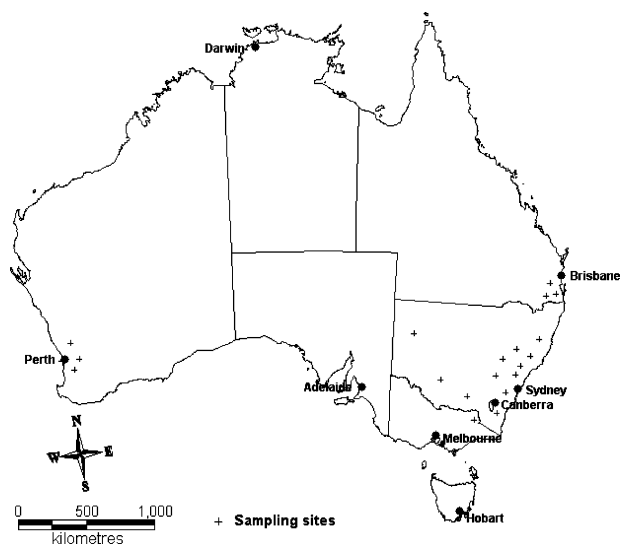


Figure 1 | Sites sampled in Australia in summer 2003 for toxigenic cyanobacteria.

(microcystin –LR equivalents) and the response range was  $0.3\text{--}1\text{ }\mu\text{g l}^{-1}$ . Each sample was always tested in duplicate and the mean of the two results was reported. The PPI method is not specific for microcystin, as the phosphatase enzyme can be inhibited by other compounds that occur in environmental samples. All reagents are commercially available and the estimated cost of each test (excluding labour) was US\$15. The labour time for an analytical run was 4–6 person hours depending on the number of tests. The laboratory's usual practice was to analyse a maximum of 12 samples in a single run, which equates to 4 hours for one test or 30 minutes per test if 12 samples are analysed.

#### Enzyme-linked immunosorbent assay (ELISA)

The method was an indirect ELISA, sensitive to the ADDA moiety of microcystin as described in Fischer *et al.* (2001). We used Abraxis antibody to screen for microcystins. All samples were run (in triplicate) on plates coated with antibody in-house. Normal variability between replicates was no more than 5%. These were then averaged. The commercially available ELISA (Abraxis LLC, Product No. 520011) costs US\$400 per 96 well plate. A single use plate can accommodate 15 samples, plus standards and blanks. The ELISA was reported specific for all variants of microcystin and nodularin. The estimated cost of each test (excluding labour) was US\$30 and the labour time was similar to PPI, 30 minutes per test.

Both the ELISA and the PPI methods have low limits of detection, so sample pre-concentration is not required. These tests have a limited linear response to microcystins, within a narrow range ( $0.2\text{--}10\text{ }\mu\text{g l}^{-1}$ ). Sample concentrations above the linear range were diluted to give semi-quantitative results.

The inhibitory power of different analogues of microcystin to the phosphatase enzyme PP2A used in the PPI assay reflects how tight the binding forces are between the enzyme and its inhibitor. This to some extent reflects the toxicity. The only known crystal structure for a phosphatase enzyme with bound inhibitor, PP1 and Mic-LR, was described by Goldberg *et al.* (1995). That structure suggests that the different structural analogues of microcystin will have different interactions between the inhibitor and the

phosphatase enzyme, leading to different inhibitory power, which in turn should be a measure of toxicity.

The ELISA ideally should show no cross reactivity. However, the EnTox laboratory has observed favourable interactions between microcystin and the antibody, when the arginine position is occupied by a relatively small side chain (as in Mic-LA and Mic-L-Aba). This difference in reactivity caused these microcystin analogues to be over-estimated compared with Mic-LR. Both the PPI and the ELISA assays were calibrated using microcystin-LR and all results were expressed as microcystin-LR equivalents.

#### Polymerase chain reaction (PCR)

The PCR test for the *mcyA* gene only determines presence or absence of this DNA fragment. In this study, the PCR test amplified *mcyA* DNA when 10 or more cells were present and there was no inhibition. Water samples (100 ml) were concentrated by centrifugation, then the DNA was extracted and 1% of the extract ( $1\text{ }\mu\text{l}$ ) was used for the test. This concentration and dilution process gave a theoretical detection limit of 10 *mcyA* containing cells per ml. The PCR method was highly specific for the presence of the *mcyA* component of the microcystin synthetase gene cluster. All PCR samples were run in at least duplicate. A PCR test for the presence of cyanobacterial 16S rRNA using the 27/809 primers was used as a control to establish that reactions were successful on a given sample. Negative results could indicate reaction inhibition or insufficient cyanobacterial cells (Burns *et al.* 2004), and when no amplification was evident samples were spiked with known cyanobacterial DNA to confirm the sample DNA status. The estimated cost to test each sample (excluding labour) was US\$8, but the primers are not yet commercially available. The time for the PCR analysis was approximately 8 hours for 12 samples. As this can be run as a batched test where all the samples are analysed together, the time required is similar for 1 or 12 samples.

#### HPLC

The HPLC method was a standard protocol of the Queensland Health Scientific Services (QLD Health Method

# 15605). Fifty ml of sample was sonicated to lyse cells using a Branson 450 watt ultrasonic probe. The solution was then filtered using a 0.45 micron disk filter. Nodularin was added to all samples as a check on recovery. Microcystins are concentrated onto a C18 cartridge (Alltima C18 7.5 mm × 4.6 mm, guard column) by pumping 25 ml of the filtered solution through the cartridge. The cartridge was then switched into the analytical HPLC system (Shimadzu, LC10A) and the eluted microcystins were separated on an Alltima C18 column (150 mm × 4.6 mm, Alltech, Australia, kept at 40°C) using an 8 mM ammonium acetate/acetonitrile gradient (flow 1 ml min<sup>-1</sup>, 15% acetonitrile to 35% acetonitrile in 25 min). Microcystins were identified from their characteristic UV spectra using a Shimadzu diode array detector. Quantitation was performed by comparing the areas of the microcystin peaks identified in the sample to the area of a standard microcystin LR of known concentration and reported as total microcystins (μg l<sup>-1</sup>). A detection limit of 0.2 μg l<sup>-1</sup> for individual microcystins is achievable by this method. If more than one microcystin is present the reporting limit for the method is 0.5 μg l<sup>-1</sup> total microcystins. The HPLC method takes about 3.5 hours for each analysis because concentration and chromatography cannot be run in parallel, although automation can speed up the processing. Analyses by HPLC were not replicated. The estimated cost for each sample (excluding labour) was US\$50.

### Recovery

The recovery of microcystin in all tests was assessed by inclusion of internal standards. The results of the PPI and ELISA tests were rejected if the IC<sub>50</sub> for the internal Mic-LR standard did not fall within the acceptable IC<sub>50</sub> range previously defined for that specific batch of ELISA or protein phosphatase reagents. The PCR is a non-quantitative, presence/absence method. Two internal controls were used, firstly DNA extracted from a cyanobacterium and secondly DNA from a known *mcyA* producing cyanobacterium. The PCR results were rejected on the basis of low recovery if either of these internal controls was not positive. This occurred on several occasions for the PCR analyses, particularly with samples with high concentrations of cells

of *mcyA* producing cyanobacteria. The HPLC used a known concentration of nodularin as the internal standard.

### Method performance

We ranked the performance of each method into five categories: microcystin detection limit, cost, level of analyst training required, **selectivity** for microcystin, and turnaround time (TAT). The adequacy of each method was first determined by whether the detection limit reported by the analytical laboratories was below the WHO guideline for microcystin (or toxigenic cell equivalents). The microscopy and PCR methods report concentration of toxigenic cells and so to compare these methods with the WHO guideline, we inter-converted between *Microcystis* cells and microcystin per cell by assuming each *Microcystis* cell contained 10<sup>-15</sup> g microcystin (after Orr & Jones 1998).

We assigned a quantitative performance characteristic to each method in each category from our laboratory experience (i.e. cost, turnaround time). The cost estimate was restricted to consumables and equipment depreciation, because of the wide variation in labour cost internationally. The experience required to train an analyst in the method and the selectivity of each method were assigned subjectively, based on the experience of the lab managers involved in the study.

We normalised the performance of each method within a category against the least desirable method (i.e. slowest or most costly). Therefore the lowest percentage score corresponds to the highest ranking. We then ranked those methods with adequate detection limits, by taking the mean of the normalised result for each category.

## RESULTS AND DISCUSSION

The survey analysed 53 grab samples from suspected cyanobacterial blooms in 25 different water bodies. These samples were from 20 waterbodies in temperate zone Australia and five waterbodies in tropical Thailand. All samples were not analysed by all methods, because of logistical difficulties transporting them between laboratories. The breakdown of number analysed, positive results and the detection limit for each method is given in Table 1.

**Table 1** | Presence of toxigenic cyanobacteria, microcystin and *mcyA* gene in cyanobacterial bloom samples

	Toxic cyanobacteria by microscopy	± <i>mcyA</i> by PCR <sup>A</sup>	Mic-LR eq. <sup>B</sup> by PPI	Mic-LR eq. <sup>B</sup> by ELISA	Mic-LR eq. <sup>B</sup> by HPLC
Samples tested	53	51	53	35	7
Positive samples	85%	43%	44%	26%	100% <sup>C</sup>
Detection limit <sup>D</sup> (ug l <sup>-1</sup> )	≈ 0.001	≈ 0.001	0.3	0.2	0.5 <sup>E</sup>
Detection limit <sup>F</sup> (cells ml <sup>-1</sup> )	10	10	≈ 3,000	≈ 2,000	≈ 5,000

<sup>A</sup>Reported as presence or absence of the microcystin synthetase gene (*mcyA*).

<sup>B</sup>The result as Mic-LR represents the effect of all microcystin analogues expressed in terms of the standard, Mic-LR.

<sup>C</sup>Only samples positive by ELISA were tested.

<sup>D</sup>Detection limit in ug l<sup>-1</sup> for the cell detection tests (PCR and microscopy) assumes 100 fg of microcystin per *Microcystis* cell.

<sup>E</sup>Detection limit (DL) for all microcystin analogues combined. If present only as Mic-LR, DL is 0.2 ug l<sup>-1</sup>.

<sup>F</sup>Detection limit for the microcystin tests in cells ml<sup>-1</sup> assumes one *Microcystis* cell per 100 fg of microcystin.

All 53 samples were examined microscopically and 45 samples contained toxigenic cyanobacteria, either *Microcystis* or *Anabaena circinalis*. The PPI and PCR tests indicated microcystin (or equivalent) and *mcyA* gene was present in just under half the samples tested.

The ELISA method was under-represented as only 35 of the 53 samples were tested. In this subset, microcystin was detected in 26% of the samples (Table 1). When the ELISA results were compared with matched PPI tests, microcystin (as m-LR equivalents) was not detectable in 21 samples and was found in 9 samples by both methods. Five sample results were ambiguous, as the PPI test detected microcystin equivalents while the ELISA did not. All analytical results from samples containing microcystin by any test are presented in Table 2. Microcystin concentrations ranged from the detection limit to more than 100 ug l<sup>-1</sup>. Seven of the positive tests by ELISA or PPI were confirmed by HPLC for quality assurance.

### Comparison of *mcyA* and microcystin assays

We supplemented the microcystin assay data from the 35 ELISA results with results from 13 additional samples tested only by PPI and compared the combined bioassay data with the detections of *mcyA* gene by PCR. These results are presented in Table 3, displayed in quadrants representing varying levels of 'risk' of presence of microcystin.

The risks are defined by the presence (+) or absence (-) of either the *mcyA* gene or the toxin, microcystin. This analysis recognises two categories when the risk to public health was high (++): first, when the *mcyA* gene is present AND microcystin is present at concentrations above the WHO guideline of 1 ug l<sup>-1</sup> (13 samples). The second 'High risk' category (- +), was when the *mcyA* gene was not detected, but microcystin exceeded the WHO guideline. This result would occur when microcystin was present in water but cells and DNA were absent because of loss by filtration, cell lysis or sedimentation.

There were eight samples in the 'Incipient risk' category (+ -) that contained *mcyA* but where microcystin was below the biochemical assay detection limits. We are confident these are not PCR false positives, as toxigenic cyanobacteria were observed microscopically and the PCR test was several hundred times more sensitive than the biochemical assays. A gene probe survey of *Microcystis* populations collected from Brazilian reservoirs for the *mcyB* gene found that toxic and non-toxic genotypes coexisted (Bittencourt-Oliveira 2003). In such situations the public health risk is incipient, because the genetic capacity for microcystin production can exist, even though the toxic strain may be rare.

There were 24 samples in the 'No risk' category (- -) that contained no detectable *mcyA* or microcystin and these samples represented about half of all the blooms. This is consistent with other reports from Australian surveys (see WHO 2003).

**Table 2** | Comparison of the quantification of microcystin by three detection methods

Chlorophyll ( $\mu\text{g l}^{-1}$ )	<i>Microcystis</i> (cells $\text{ml}^{-1}$ )	<i>Anabaena</i> (cells $\text{ml}^{-1}$ )	Microcystin by ELISA ( $\mu\text{g l}^{-1}$ )	Microcystin by PPI ( $\mu\text{g l}^{-1}$ )	Microcystin by HPLC ( $\mu\text{g l}^{-1}$ )	ELISA and PPI agreement
131	100	21,600	<0.2	0.3	nt	No
17	8,700	<10	<0.2	0.5	nt	No
26	<10	15,000	<0.2	0.3	nt	No
50	1,600	9,000	<0.2	4	nt	No
22	3,000	<10	<0.2	1	nt	No
1,254	433,000	513,000	>40	47	80	Yes
440	5,000,000	1,000,000	>40	>100	60	Yes
1,400	>50,000,000	<10	>40	>100	>100	Yes
725	5,000,000	<10	10–40	14	10	Yes
320	2,000,000	<10	10–40	15	15	Yes
nt	PCC7806 * *	0	>40	65	55	Yes
nt	PCC7806 * *	0	>40	61	18	Yes
210	990,000	<10	6	22	nt	Yes *
273	70,500	1,500	>40	33	nt	Yes *
836	<10	2,300	8–40	6	nt	Yes *
100	30,000	3,000	8–40	41	nt	Yes *

nt—not tested.

\*results of the two microcystin assay methods agree within the range of error of these semi-quantitative tests.

\*\*Monospecific culture of *Microcystis* PCC 7806 used as internal reference standard.

### Quality assurance

For quality assurance and to test the quantitation of the ELISA and PPI tests, seven samples containing toxigenic cells in which microcystin was detected by ELISA were also analysed by HPLC (Table 2). The quality assurance samples included duplicates of a reference material, a pure culture of a toxic *Microcystis* strain (PCC7806). The results of all the methods were consistent within the respective ranges of each test, although the PPI and HPLC result did differ by an order of magnitude in one case at the extreme of quantitation (800 vs. 60  $\mu\text{g l}^{-1}$ ).

### Additional information from HPLC

HPLC is recommended as the standard method for the determination and quantitation of microcystins (Chorus & Bartram 1999). In this study we have compared the usefulness of microscopy, PCR and two semi-quantitative microcystin assays for screening water for the risk to public health from microcystin toxicity. HPLC was used to quantify the microcystin in a limited number of samples to check on the accuracy of quantitation of the PPI and ELISA.

The results of the screening tests were unanimous in 43 of the 53 samples (Table 4). We reviewed the 10 results

**Table 3** | Risk assessment matrix and comparison of microcystin assays with PCR

	PPI or ELISA results	
	microcystin present (+)	microcystin not detected (-)
PCR	13	8
<i>mcyA</i> present (+)	High risk (++)	Incipient risk (- +)
PCR	3 <sup>A</sup>	24
<i>mcyA</i> absent (-)	High risk (+ -)	Low risk (- -)

<sup>A</sup>false negative by PCR: toxigenic cells and microcystin present from both PPI and ELISA but *mcyA* gene was not detected by PCR.

where there was disagreement between one or more tests to determine whether an unequivocal method of toxin identification (HPLC) could have improved our understanding of the potential risk and allowed us to better reconcile apparent differences between the tests results. In the first three samples in Table 4 (samples 1–3), the PPI and the ELISA results disagreed. In these samples, further quantitation by HPLC could have resolved this problem and confirmed the presence or absence of microcystin. In these samples, even though toxigenic cyanobacteria were identified microscopically, the absence of *mcyA* suggests the PPI result may have been a false positive, as these cells should not have been able to synthesise microcystin. Inhibition of phosphatase enzyme activity by compounds other than microcystin has been described before (e.g. Robillot & Hennion 2004) and a variation to the PPI method, to deactivate non-microcystin inhibitors of the protein phosphatase enzyme and improve the specificity of the test has been reported recently (Rapala *et al.* 2002).

There were two other examples (samples 4 and 5) where the PPI and ELISA results disagreed. In these cases quantitation by HPLC was unlikely to have resolved this disagreement because the conflicting results were at or below the detection limit (DL) of the HPLC analysis of 0.5 ug l<sup>-1</sup>. These two samples both contained toxigenic cells and *mcyA* gene, suggesting the ELISA result may have been a false negative.

There were two samples (samples 6 and 7) in which only the PCR was positive. These are samples where PCR

identified an incipient risk. Although microcystin was not detectable, the DL for PCR is 10 *mcyA* containing cells per ml whereas the DL for ELISA, PPI and HPLC was 2,000, 3,000 and 5,000 highly toxic *Microcystis* cells, respectively. In these cases HPLC cannot add more information.

The difference in result between PCR and the other three tests could not be explained satisfactorily for the results of samples 8–10. We propose the PCR non-detection of *mcyA* in these samples was a false negative, because the other three tests detected high concentrations of either toxic cyanobacteria or microcystin.

The PCR protocol does include a check for inhibition of DNA amplification. Inhibition of amplification was reported in some high algal biomass samples. The chlorophyll concentration of samples 8–10 was more than 100 ug l<sup>-1</sup>, but there was no evidence of PCR inhibition. Therefore these three results cast a doubt over the reliability of the PCR test at *high cell concentrations*. This could be addressed by standard dilution of concentrated samples. The quantitation of microcystin in these three samples by HPLC would neither prove nor disprove the presence of the *mcyA* gene.

### Performance ranking all methods

We ranked the overall performance of each method for detection limit and selectivity for microcystin, analytical cost, analytical skill needed and turnaround time. These rankings are offered as a qualitative measure of the relative usefulness of each method for assessing public health risk from cyanobacterial contamination of recreational or potable water supplies.

The mouse assay was excluded because it was not sufficiently sensitive to detect microcystin (or equivalent cell concentrations) below the WHO guideline level of 1 ug l<sup>-1</sup>. PCR and microscopy were much more sensitive than the other techniques (Table 5).

The rank order of the methods was usually different between categories. The chromatographic methods (LC/MS and HPLC) were the most selective (PCR although highly selective for *mcyA* does not detect microcystin); microscopy was the cheapest method; and PPI and ELISA had the quickest turnaround time (TAT) because these methods do

**Table 4** | Samples where results from all tests were not in unanimous agreement

Sample number	Chlorophyll ( $\mu\text{g l}^{-1}$ )	Microcystis (cells $\text{ml}^{-1}$ )	Anabaena (cells $\text{ml}^{-1}$ )	ELISA	PPi	<i>mcyA</i> PCR	Comments	Added information from HPLC
1	50	1,600	9,000	<0.2	4	-ve	Absence of <i>mcyA</i> suggests false negative by PPi	Result may be resolved by HPLC
2	22	3,000	<10	<0.2	1.2	-ve	Absence of <i>mcyA</i> suggests false negative by PPi	Result may be resolved by HPLC
3	26	<10	15,000	<0.2	0.3	-ve	Absence of <i>mcyA</i> suggests false negative by PPi	Result may be resolved by HPLC
4	17	8,700	<10	<0.2	0.5	+ve	Presence of toxigenic cells and <i>mcyA</i> suggests ELISA false negative	Result not resolved by HPLC (at DL)
5	131	70	22,000	<0.2	0.3	+ve	Presence of toxigenic cells and <i>mcyA</i> suggests ELISA false negative	Result not resolved by HPLC (< DL)
6	13	<10	<10	<0.2	<0.3	+ve	Incipient risk (see Table 3)	Result not resolved by HPLC as PCR is a genetic test
7	60	<10	<10	nt	<0.3	+ve	Incipient risk (see Table 3)	Result not resolved by HPLC as PCR is a genetic test
8	210	990,000	<10	6	22	-ve	PCR false negative	Result not resolved by HPLC as no conflict in PPi and ELISA results
9	100	30,000	3,000	8-40	41	-ve	PCR false negative	Result not resolved by HPLC as no conflict in PPi and ELISA results
10	1,400	50,000,000	<10	>40	1,000	-ve	PCR false negative	Result not resolved by HPLC as no conflict in PPi and ELISA results

nt-not tested.



**Table 5** | Methods ranked on cost, selectivity, turnaround time and training needs

Method	DL <sup>A</sup> (micn-ug l <sup>-1</sup> )	DL (cells ml <sup>-1</sup> )	Selective <sup>B</sup> (microscopy)	Cost <sup>C</sup> (LCMS)	Time <sup>D</sup> (mouse)	Training (LCMS)	Rank score <sup>E</sup>
PPi	0.3	3,000	63%	19%	25%	20%	0.32
ELISA	0.2	2,000	50%	42%	25%	20%	0.34
PCR	0.001	10	56%	9%	50%	29%	0.36
Microscopy	0.001	10	100%	3%	50%	42%	0.49
HPLC	0.5	5,000	38%	63%	50%	74%	0.56
LC/MS	0.5	5,000	13%	100%	75%	100%	0.72
Mouse	8	80,000	75%	13%	100%	42%	

<sup>A</sup>DL - detection limit.

<sup>B</sup>All methods within each category were normalised against the 'worst' method in the category (identified in brackets).

<sup>C</sup>US\$ cost of consumables and equipment (excludes labour).

<sup>D</sup>Time - Turnaround time for analysis.

<sup>E</sup>The lowest score is the most preferred method.

not require concentration. These two methods also required the least analyst training.

The average ranking for each test (across all criteria) identified PPi, ELISA and PCR as most useful, because of ease of use, low cost and rapid turnaround time. The PCR method was disadvantaged by the occurrence of three false negatives. A drawback of PPi was the occurrence of the suspected false positives.

The ELISA method was the best technique for screening water samples for public health risk from toxigenic cyanobacteria because of its reliability and its high specificity for microcystin. The detection limit and specificity of PCR and the low cost and the potential for improvement in the PPi technique encourages their continued use in a combined assay format.

The traditional methods of microscopy and HPLC ranked in the mid-range, but these had quite different performance attributes. Microscopy is cheap and sensitive but will always produce false positives. Half the toxigenic blooms in this study were non-toxic. HPLC is a highly selective test, but it is a relatively expensive method with a long turnaround time. Both microscopy and HPLC required greater operator competence than the biochemical tests.

## CONCLUSIONS

The ELISA test was judged best for screening environmental water samples for microcystin-like compounds because of its reliability, specificity to microcystin, low cost, high sensitivity and ease of analysis.

The PCR test was highly sensitive and the *mcyA* gene was detected in significantly more samples than microcystin indicating a capacity to detect situations in which public health risk was incipient. The test was also relatively cheap, but it did produce false negatives at high cell concentrations. We expect that simple improvements to the PCR and PPi protocols will significantly increase the usefulness of both these tests for environmental analysis in future.

ELISA and PPi have been recommended as useful screening techniques, when combined with more specific assays based on structural recognition of microcystin, such as HPLC or LC/MS (Robillot & Hennion 2004). We propose a combined *mcyA* gene/microcystin assay protocol for screening raw water samples. The *mcyA* PCR complements the microcystin assay methods by enhancing the specificity of those tests and provides an additional level of public health risk assessment, by detecting an 'incipient risk'

when microcystin producing cyanobacteria are present but microcystin is not detectable.

In summary, undertaking an HPLC analysis of each of the 53 samples could have improved our understanding of three ambiguous results where the ELISA and the PPI results did not agree. However, all methods employed singly have limitations and in a routine operational setting, PCR plus either ELISA or PPI is a very rapid and effective way to screen batches of water samples for risk from microcystin, reserving HPLC for final confirmation and quantitation.

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## REFERENCES

- Baker, J. A., Neilan, B. A., Entsch, B. & McKay, D. B. 2001 Identification of cyanobacteria and their toxigenicity in environmental samples by rapid molecular analysis. *Environ. Toxicol.* **16**, 472–482.
- Bittencourt-Oliveira, M. C. 2003 Detection of potential microcystin-producing cyanobacteria in Brazilian reservoirs with a *mcyB* molecular marker. *Harmful Algae* **2**, 51–60.
- Burns, B. P., Saker, M. L., Moffit, M. C. & Neilan, B. A. 2004 Molecular detection of the genes responsible for cyanobacterial toxin production in the genera *Microcystis*, *Nodularia* and *Cylindrospermopsis*. *Meth. Mol. Biol.* **268**, 213–222.
- Chorus, I. & Bartram, J. 1999 *Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management*. E&F Spon, London.
- Fastner, J., Codd, G. A. & Metcalf, J. S. 2002 An international intercomparison exercise for the determination of purified microcystin LR and microcystin in cyanobacterial field material. *J. Analyt. Bioanalyt. Chem.* **374**(3), 437–444.
- Fischer, W. J., Garthwaite, I., Miles, C. O., Ross, K. M., Aggen, J. B., Chamberlin, A. R., Towers, N. A. & Dietrich, D. R. 2001 Congener-independent immunoassay for microcystins and nodularins. *Environ. Sci. Technol.* **35**, 4849–4858.
- Goldberg, J., Huang, H-B., Kwon, Y-G., Greengard, P., Nairn, A. C. & Kuriyan, J. 1995 Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **376**, 745–753.
- Heresztyn, T. & Nicholson, B. C. 2001 Determination of cyanobacterial toxins directly in water using a protein phosphatase inhibition assay. *Wat. Res.* **35**(13), 3049–3056.
- Neilan, B. A., Dittmann, E., Rouhiainen, L., Bass, A., Schaub, V., Sivonen, K. & Börner, T. 1999 Non-ribosomal peptide synthesis and toxigenicity of cyanobacteria. *J. Bacteriol.* **181**, 4089–4097.
- NHMRC 2001 *Australian Drinking Water Guidelines and Framework for Management of Drinking Water Quality*. National Health and Medical Research Council and Agriculture and Resource Management Council of Australia and New Zealand, Commonwealth of Australia, Canberra, [www.nhmrc.gov.au/publications](http://www.nhmrc.gov.au/publications).
- NSW BGATF 1992 *Final Report of the New South Wales Blue-green Algal Task Force*. NSW Department of Water Resources, Parramatta, NSW.
- Orr, P. T. & Jones, G. J. 1998 Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. *Limnol. & Oceanogr.* **43**(7), 1604–1614.
- Rapala, J., Erkoma, K., Kukkonen, J., Sivonen, K. & Lahti, K. 2002 Detection of microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography-UV detection and enzyme-linked immunosorbent assay: Comparison of methods. *Analytica Chimica Acta* **466**(2), 213–231.
- Robillot, C. & Hennion, M-C. 2004 Issues arising when interpreting the results of the protein phosphatase 2A inhibition assay for the monitoring of microcystins. *Analytica Chimica Acta* **512**, 339–346.
- Standard Methods for the Examination of Water and Wastewater* 1998 20th edition, American Public Health Association/American Water Works Association/Water Environment Federation: Washington, DC.
- Tillett, D., Parker, D. L. & Neilan, B. A. 2001 Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcyA*) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. *Appl. Environ. Microbiol.* **67**, 2810–2818.
- WHO (World Health Organization) 2003 *Guidelines for Safe Recreational Water Environments. Volume 1: Coastal and Fresh Waters*. World Health Organization, Geneva.

## Author Queries

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