Determination of Microcystins in Waters and Extracts of Cyanobacteria by a Protein Phosphatase Inhibition Assay

Report by Carol MacKintosh, University of Dundee,
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Methods for the Examination of Waters and Associated Materials

Contents
0 Introduction
1 Performance characteristics of the method
2 Principle
3 Hazards
4 Reagents
5 Apparatus
6 Sample storage
7 Analytical procedure
8 Calculation
9 References

0 Introduction

Microcystins are cyclic heptapeptide toxins produced by various species of cyanobacteria (blue-green algae). Microcystin-LR (Fig. 1) is the best studied of approximately 50 structural variants of microcystins that have been identified (for example, Ref. 1). Nodularins are cyclic pentapeptides that are similar in chemical structure to the microcystins, and are produced by species of cyanobacteria that are usually found in esturine and brackish waters.

In 1990, it was discovered that microcystins and nodularins exert their toxic effects because they are extremely potent and specific inhibitors of protein phosphatases 1 and 2A (PP1 and PP2A), two classes of enzymes that regulate many processes in eukaryotic cells (Ref. 2).

It is important that any technique used for screening these toxins is sensitive and able to detect all toxic chemical variants. The high affinity binding of microcystins and nodularins to PP1 and PP2A (for example, Ref. 2) underlies the exquisite sensitivity by which these toxins can be detected and quantified in the assay described here. Quantitative data is given here for microcystin-LR, microcystin-RR and microcystin-YR (Fig. 1) because only these variants are commercially available. However, the 3-dimensional structure of microcystin-LR bound to PP1 (Ref. 3) shows that positions X and Y in the microcystin ring (Fig. 1) do not interact directly with the enzyme. Therefore, microcystins that vary in positions X and Y in the ring (Fig. 1) would have identical inhibitory properties to microcystin-LR in the assay described here. Modification of the methyl-dehydroalanine residue does not affect inhibition of PP1 and PP2A in the assay described here (Ref. 4). Moreover, all of the toxic variants of microcystin that have been tested inhibit PP1 and PP2A (C.M. unpublished) and Appendix II shows that all of the microcystins in a complex mixture inhibited PP1 and PP2A.
1. Performance characteristics of the method

1.1 Substances determined

Potent inhibitors of PP1 and PP2A. Compounds of this type that are relevant to freshwater analysis are the microcystins and nodularins. and these toxins are the subject of the method described here.

1.2 Types of samples

Raw and drinking waters. Soluble extracts of cyanobacterial blooms. Fractions collected from HPLC separations or other fractionation techniques. Extracellular body fluids. The assay is NOT suitable for assaying the microcystins and nodularins that have bound covalently to protein phosphatases in the liver or other eukaryotic tissues (for example, in some postmortem samples) (Ref. 4).

1.3 Basis of the method

Portions of raw samples are filtered, diluted in Buffer B and can be tested directly for inhibition of PP1 and/or PP2A in a batchwise manner as part of a rapid screening programme. After primary screening, inhibitory samples can be fractionated using HPLC or other chromatographic methods. Following chromatographic separation, portions of fractions are diluted in Buffer B (at least a 30-fold dilution) and assayed for inhibition of PP1 and PP2A. The profile of inhibitory activity observed after HPLC indicates how many toxins are present in the sample (for example, Appendix II). If desired, the chemical identity of peaks of inhibitory activity can be determined by mass spectrometry and other physiochemical techniques.

1.4 Range of application

Unrestricted. Samples giving greater than 50% inhibition in the standard assay should be reported as ‘off-scale’ and can be retested after dilution.

1.5 Limit of detection and sensitivity

For the standard assay described here, from >1pg to 5pg (equivalent to 10 µL of a 100 to 500 ng/L solution) added to a 30 µL assay (Fig. 2).

1.6 Calibration curves

The PP1γ (human gene expressed in E. coli) and PP2A (cardiac muscle) (from Life Technologies) used in performance tests at 0.2 mU/mL (see Section 4.4) gave the detection curves for MC-LR, -RR and -YR that are shown in Fig. 2.
The calibration curves depend on concentration of enzyme used because the toxin-enzyme interactions are stoichiometric (Ref. 2). PP1 and PP2A from different sources may have different sensitivities to microcystin inhibition (Refs. 2 and 5).

1.7 Standard deviation

Duplicate analyses that vary by more than 5% indicate pipetting error and should be discounted.

1.8 Bias
Apart from poor sample storage, there is no obvious reason why false negatives would be reported. There may be other inhibitors of protein phosphatases (see interferences). However, microcystins give the distinctive ratio of inhibition of PP1 and PP2A shown in Fig. 2 and testing both enzymes in parallel should allow microcystins to be distinguished from other inhibitors. All positives in initial screening should be examined further as described in 1.3 Basis of the Method.

1.9 Interferences

Fluoride and phosphate ions inhibit protein phosphatases with IC$_{50}$ values (concentrations giving 50% inhibition in the assay) of 133mg/L and 142 mg/L respectively for PP1, and 361 mg/L and 285 mg/L respectively for PP2A.
Organophosphorus compounds do not contain phosphate groups and, therefore, there is no reason to believe that these compounds would be inhibitors of PP1 or PP2A. Organophosphorus compounds tested (Dichlorvos, Diazinon) did not inhibit PP1 or PP2A when tested at concentrations of up to 100 µg/L.

1.10 Sources of error
(a) Sample collection and storage. Variability in cyanobacterial content of raw waters, degradation of microcystin during storage are likely to be major sources of error for any method used to assay microcystins (Ref. 6).
(b) Calibration of microcystin standards. Microcystin stocks of 500 µg sold by Calbiochem were found to be accurate within 5% as judged by HPLC and amino acid analysis (not shown). Furthermore, protein phosphatase inhibition curves for each microcystin variant coincided (Fig. 2).
(c) Assay procedure. Correct storage and thawing of enzymes is critical to maintain constant activities from day to day.

1.11 Time required for analysis (primary screening)

Provided that enzymes, substrates and solutions have been prepared, one operator can perform a maximum of 40 single protein phosphatase assays in approximately 2 hours, equivalent to 10 samples assayed in duplicate with each of PP1 and PP2A.
2 Principle

2.1.1 Standard assay
PP1 and PP2A catalyse the hydrolysis of $^{32}$P-labelled glycogen phosphorolase $a$ to give glycogen phosphorolase $b$ and inorganic phosphate ($^{32}$P$_i$). The reaction is stopped with trichloroacetic acid (TCA), protein is removed by centrifugation and $^{32}$P-inorganic phosphate ($^{32}$P$_i$) released into the supernatant is counted. Samples to be tested for inhibition of PP1 or PP2A are added to parallel assays. Controls 'blanks' contain samples but no PP1 or PP2A and 'totals' contains $^{32}$P-labelled glycogen phosphorolase $a$ but no PP1, PP2A or TCA.

2.1.2 Standard assay with molybdate extraction modification
Very rarely, there may be a release of TCA-soluble $^{32}$P caused by the sample alone (a high blank) which is due to proteases in the sample that digest the phosphorolase substrate and release small TCA-soluble $^{32}$P-labelled peptides. If 'blanks' are greater than 5% of 'total' counts assays are repeated with the modification that the TCA-supernatant is extracted with acid-molybdate before counting. Acid-molybdate forms a complex with $^{32}$P$_i$ but not with peptides, therefore the molybdate extraction modification eliminates interference by proteases.

3 Hazards

Microcystins have been shown to promote tumours in laboratory animals. Skin contact, ingestion and inhalation must be avoided. Acids are corrosive. While working with $^{32}$p, minimise handling time, maximise distance and use shielding (1 cm-thick Plexiglas body shield, tube rack and storage box). Consult local health and safety regulations about safety training, licensing of premises and disposal of $^{32}$p (low level) waste.

4 Reagents

4.1 Solutions for dilutions and standard assay

Use double-distilled deionised water and analytical quality grade reagents.

4.1.1. 5M Sodium hydroxide. Dissolve 20g of sodium hydroxide in 80 mL of water. Cool and dilute to 100 mL with water. Mix well.

4.1.2. 5M Hydrochloric acid. Dilute 36 mL of hydrochloric acid ($d_20$ 1.18) with water. Cool and make up to 100 mL with water. Mix well.

4.1.3. Tris-HCl (121g/L, pH7.5 (20°C)). Dilute 12.1g of tris(hydroxymethyl)aminomethane (Tris) in 80 mL of water. Add 5M hydrochloric acid to pH 7.5 at 20°C and make up to 100 mL with water. Mix well.

4.1.4. EGTA (46 g/L, pH 7). Add 70 mL water to 4.6 g ethylene glycol-bis(β-aminoethyl-ether) N, N', N'-tetraacetic acid (EGTA). Add 5M sodium hydroxide to pH 7 and make up to 100 mL with water. Mix well.
4.1.5. **BSA (100 mg/mL).** Dissolve 1g of enzyme grade bovine serum albumin (BSA) in 10 mL of water. Mix well. Store at -20°C in 1mL aliquots.

4.1.6. **Caffeine (14.6 g/L, pH 7.0).** Add 50 mL of water to 1.46 g of caffecine. Add 5M sodium hydroxide to pH 7 and make up to 100 mL with water. Store in a dark bottle at room temperature.

4.1.7. **Buffer A.** Mix 5 mL Tris-HCl (121g/L, pH7.5 (20°C), 0.1 mL EGTA (46 g/L, pH 7), 0.1 mL 2-mercaptoethanol and 1 mL BSA (100 mg/mL). Make up to 100 mL with water. Mix well. Refrigerate and discard after 2 weeks.

4.1.8. **Buffer B.** Mix 5 mL Tris-HCl (121g/L, pH7.5 (20°C), 0.1 mL EGTA (46 g/L, pH 7), 0.1 mL 2-mercaptoethanol and 0.1 mL of a 30% (w/v) solution of Brij-35*. Make up to 100 mL with water. 0.03% (v/v). Mix well. Refrigerate and discard after 2 weeks.

4.1.9. **Buffer B X 10.** Mix 50 mL Tris-HCl (121g/L, pH7.5 (20°C), 1 mL EGTA (46 g/L, pH 7), 1 mL 2-mercaptoethanol and 1 mL of a 30% (w/v) solution of Brij-35*. Make up to 100 mL with water. Mix well. Refrigerate and discard after 2 weeks.

4.1.10. **Buffer C.** Mix 5 mL Tris-HCl (121g/L, pH7.5 (20°C), 0.1 mL EGTA (46 g/L, pH 7), 0.1 mL 2-mercaptoethanol and make up to 100 mL with water. Mix well. Refrigerate and discard after 2 weeks.

4.1.11. **TCA (20% w/v).** Dissolve 20g trichloroacetic acid (TCA) in 80 mL water and make up to 100 mL with water. Store in a stoppered glass bottle.

4.1.12. **Scintillation fluid.** Scintillation fluid must be compatible with both aqueous and organic solvents, for example Optiphase Hi-Safe 3 (Wallac Prod. no. 1200-437).

4.2 **Additional solutions for molybdate extraction modification of assay**

4.2.1. **Acid phosphate.** Gradually add 1.45 mL of sulphuric acid (d<sub>20</sub> 1.84) to 80 mL of water. Cool and dilute to 100 mL with water. Add 17 mg of potassium phosphate, monobasic (KH<sub>2</sub>PO<sub>4</sub>). Mix well.

4.2.2. **Butan-1-ol/heptane (1:1 (w/v)).** Mix 50 mL butan-1-ol with 50 mL heptane.

4.2.3. **Ammonium molybdate (5% (w/v)).** Dissolve 5g ammonium molybdate in 80 mL of water. Make up to 100 mL with water. Mix well.
4.3 Standard solutions of toxins

Microcystin-LR, for example Calbiochem (Cat. no. 475815), Gibco BRL (Cat. no. 610-3130SA) or Bio-Mol Research Laboratories (Cat. no. E1-193).
Microcystin-YR, for example Calbiochem (Cat. no. 475819).
Microcystin-RR, for example Calbiochem (Cat. no. 475816).

4.3.1. Stock solutions of microcysts (1 mg/mL)
Add 100 µL methanol, followed by 400 µL of water to a septum-sealed vial containing 500 µg of microcystin. Mix well. Store at -20°C.

4.3.2. Solutions for preparation of calibration curves
Dilute stock solutions of microcystin in Buffer B to give concentrations of 0.3, 1, 3, 10, 30, 100, 300, 1000 and 3000 ng/L. Store aliquots at -20°C and test calibration curves periodically. (Solutions of pure microcystins stored in Buffer B at -20°C have been found to give constant results when tested periodically over a period of 2 years. (CM, not shown))

4.4 Protein phosphatases 1 and 2A (PP1 and PP2A)

Protein phosphatase 1γ, for example Life Technologies (Cat. no. 10191-013).
Protein phosphatase 2A, for example Life Technologies (Cat. no. 10192-011).
Store at -20°C.

Dilute enzymes to 0.6 milliunits/mL in Buffer A, freeze aliquots in liquid nitrogen and store at -80°C. Thaw by brief incubation at 30°C and store on ice on day of use. A milliunit (mU) is defined as the amount of enzyme which releases 1 nmole (equivalent to 19 ng/mL) of 32P-inorganic phosphate from 32P-labelled phosphorylase in 1 minute in the standard assay described here.

(N.B. I have retained the convention of defining enzyme activity in terms of nmols/mL/min to maintain consistency with all previous publications and because enzymes are sold in Unit defined in nmols/mL/min). One nmole/mL of inorganic phosphate is equivalent to 19 ng/mL.

4.5. Assay substrate (32P-labelled glycogen phosphorylase a)

N.B. The present method assumes that the substrate, 32P-labelled glycogen phosphorylase a, will be provided to the analytical laboratory. For background information, protocols for the preparation and aliquoting of 32P-phosphorylase a are given in Ref. 7 and in the instructions provided with ‘The Protein Phosphatase Assay System’ kit that is sold by Life Technologies, Gibco BRL (Cat. No. 13188-016).

32P-labelled glycogen phosphorylase a is prepared every 4-6 weeks to a specific radioactivity of 10⁹ counts per minute/nmol (cpm/nmol). Store refrigerated. DO NOT FREEZE. On the day of use, add 0.1 mL Caffeine (14.6 g/L, pH 7.0) and 0.4 mL Buffer C to an aliquot of substrate. The protein concentration will be 3 mg/mL and one aliquot is sufficient for 50 assays. Store on ice and discard after 1-2 days or when ‘Blank’ counts rise to >5% of the ‘Total.’
4.6 Sample dilution

(a) Water samples
Mix 90 µL of filtered sample with 10 µL of Buffer B X 10.

(b) Methanolic extracts of cyanobacteria.
Extract (disrupted by sonication or other suitable method) in a ratio of 1 g dry weight of cyanobacteria to 2 mL methanol. Filter or centrifuge to clarify the extract. Dilute clarified extract in Buffer B. (Dilutions of between 1000-fold and 1 000 000-fold of the extract are suggested).

5. Apparatus
Gilson pipettes P20, P200 and P1000 (or equivalent) and disposable tips
Microfuge tubes with attached caps
Water bath set to 30°C fitted with racks to hold microfuge tubes
Microcentrifuge (to hold microfuge tubes and capable of 14 000 x g)
Vortex mixer
Timer
Scintillation counter capable of counting $^{32}$P
Plexiglass racks, safety glasses, lined working area, waste $^{32}$P storage area
Ice bucket

6. Sample collection and storage

The procedure for collection and storage of cyanobacterial samples that is outlined in the attached document (Appendix 1) is being considered by the DOE Toxins Panel.

7. Analytical procedures

7.1 Standard assay
7.1.1. Add 10 µL (PP1 or PP2A or Buffer A) plus 10 µL (diluted Sample or Buffer B or Microcystin control) to a set of microfuge tubes as follows (each combination in duplicate):

<table>
<thead>
<tr>
<th>10 µL</th>
<th>10 µL</th>
<th>Tube label</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1 or PP2A (0.6 mU/mL)</td>
<td>Buffer B</td>
<td>Control</td>
</tr>
<tr>
<td>PP1 or PP2A (0.6 mU/mL)</td>
<td>Sample</td>
<td>Sample</td>
</tr>
<tr>
<td>Buffer A</td>
<td>Buffer B</td>
<td>Control blank</td>
</tr>
<tr>
<td>Buffer A</td>
<td>Sample</td>
<td>Sample blank</td>
</tr>
<tr>
<td>PP1 or PP2A (0.6 mU/mL)</td>
<td>1000ng/L MC-LR</td>
<td>MC-LR control</td>
</tr>
</tbody>
</table>

7.1.2 Start the reaction by adding diluted 10 µL phosphorylase a to every tube (at 10-15 sec intervals) and incubate for 10 min at 30°C.

7.1.3 Stop the reaction by adding 0.1 ml of 20% TCA to each tube and vortex mix.
7.1.4 Centrifuge tubes at 12 000g for 2 min.
7.1.5 Transfer 0.1 ml of each supernatant to a second set of microfuge tubes.
7.1.6 Prepare a tube labelled ‘Total’ containing 10μL phosphorylase a and 0.1 mL of 20% TCA.
7.1.7 Add 1 mL of scintillation fluid to each tube and count using a 32P-program in a scintillation counter.

7.2 Molybdate extraction modification of standard assay
7.2.1 Perform the standard assay up to the end of step 7.1.5.
7.2.2 Add 0.2 mL of acid-phosphate, 0.5 mL of butan-1-ol/heptane and 0.1 mL of 5% ammonium molybdate to every tube, except the ‘Total’ and vortex vigorously.
7.2.3 Stand for 10 min to allow aqueous and organic phases to separate.
7.2.4 Transfer 0.3 mL of the organic (top) phase into fresh tubes.
7.2.5 Prepare a tube labelled ‘Total’ containing 10μL phosphorylase a and 0.3 mL butan-1-ol/heptane.
7.2.6 Add 1 mL scintillation fluid to each tube and count using a 32P-program in a scintillation counter.

8. Calculation Procedure
8.1.1 Protein phosphatase activity in mU/mL in the standard assay is calculated from the control (100% activity) as follows (worked example in Table 1):
\[ \frac{\text{cpm in Control tube} - \text{cpm in Control blank}}{\text{cpm in Total}} \times 1.3 \]
(N.B. 100μl out of 130 μl transferred)

The 100% activity should be 0.2 mU/ mL.

8.1.2 Protein phosphatase activity in mU/mL in the modified assay is calculated from the control (100% activity) as follows:
\[ \frac{\text{cpm in Control tube} - \text{cpm in Control blank}}{\text{cpm in Total}} \times 1.3 \times 1.6 \]
(N.B. 0.3 ml out of 0.5 ml counted). The 100% activity should be 0.2 mU/mL.

8.1.3 Activity in sample tubes is calculated as a percentage of the 100% activity control (worked example in Table 1). Inhibitory activity in ‘microcystin equivalents’ is read from the calibration curves shown in Fig. 2.
9. References


Additional references for background reading


Table 1A - Raw data and calculations for inhibition of PP1 by microcystins

The calculated activities (%) in the presence of the stated concentrations of microcystins are plotted in Fig. 2A.

Control assays

<table>
<thead>
<tr>
<th></th>
<th>cpm (duplicate)</th>
<th>average</th>
<th>minus blank</th>
<th>Activity (%)</th>
<th>mU/mL*</th>
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<td>MC-LR blank</td>
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<td>Total</td>
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* PP1 activity in mU/mL is calculated from the control assay as described in section 8.1.

Microcystin-LR

Concentration of MC-LR (ng/L)

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Microcystin-YR

Concentration of MC-YR (ng/L)

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Microcystin-RR

Concentration of MC-RR (ng/L)

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<td>30000</td>
<td>196, 243</td>
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Table 1B - Raw data and calculations for inhibition of PP2A by microcystins

The calculated activities (%) in the presence of the stated concentrations of microcystins are plotted in Fig. 2B.

Control assays

<table>
<thead>
<tr>
<th></th>
<th>cpm (duplicate)</th>
<th>average</th>
<th>minus blank</th>
<th>Activity (%)</th>
<th>mU/mL*</th>
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<td></td>
<td>171</td>
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<td>MC-LR blank</td>
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<td></td>
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* PP2A activity in mU/mL is calculated from the control assay as described in section 8.1.

Microcystin-LR

Concentration of MC-LR (ng/L)

<table>
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<tr>
<th>Preassay</th>
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<th>cpm (duplicate)</th>
<th>average</th>
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<th>Activity (%)</th>
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Microcystin-YR

Concentration of MC-YR (ng/L)

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<th>Activity (%)</th>
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Microcystin-RR

Concentration of MC-RR (ng/L)

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<th>cpm (duplicate)</th>
<th>average</th>
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<th>Activity (%)</th>
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Appendix II - An example to show use of the protein phosphatase assay to aid the identification of microcystins separated by HPLC.

Sample preparation and chromatography
A sample of freeze-dried cyanobacteria known to contain a mixture of different microcystins was obtained from Dr. Peter Thiel, Medical Research Council, South Africa. An extract was prepared by sonicating 0.5g cyanobacteria in 1 mL methanol. The extract was filtered through a GF/C filter, diluted 10-fold in water and applied to an equilibrated Waters Sep-pak C18 cartridge. The cartridge was washed in 20% methanol and microcystins were eluted in 0.1% trifluoroacetic acid (TFA) in acetonitrile. The sample was taken to near dryness by rotary evaporation, diluted in 500 µL 0.1% TFA in water and 50 µL was chromatographed on a C18 (Vydac) column equilibrated with 0.1% TFA in water and developed with a gradient of acetonitrile in 0.1% TFA using a flow rate of 1 mL/min. Absorbance was monitored at 238 nm and fractions of 0.5 ml were collected. The principles and practice of this separation method are similar to the method described in Lawton, L.A., Edwards, C. and Codd, G.A. 'Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters', Analyst 1994 119, 1525-1530.

Protein phosphatase assays
Preliminary assays of selected fractions for inhibition of PP1 were carried out at a range of different dilutions in Buffer B (not shown). A relatively low dilution (30, 000-fold preassay dilution) of each fraction was chosen in order to show the inhibition of PP1 by the microcystins-YA, -LA and -L-Aba (the three microcystins which barely register towards the end of the trace of A268 absorbance (see HPLC profile on next page). The detection of these microcystins as clear peaks of inhibition of PP1 demonstrate the high degree of sensitivity of this assay. For this reason, the PP1 inhibition assays for most of the microcystin peaks are 'off-scale.' The assignments of microcystins are from information provided by Dr Thiel's laboratory.
Figure 1. Structure of microcystin.

X and Y represent the most variable residues in the ring. For example, in microcystin-LR, residue X is leucine and Y is arginine. In microcystin-YR, residue X is tyrosine and Y is arginine.
Figure 2A. Inhibition of 0.2 mU/mL of the purified PP1γ catalytic subunit by microcystin-LR (squares), microcystin-YR (circles) and microcystin-RR (diamonds).
Figure 2B. Inhibition of 0.2 mU/mL of the purified cardiac muscle PP2A catalytic subunit by microcystin-LR (squares), microcystin-YR (circles) and microcystin-RR (diamonds).

Activity (%) vs. Microcystin in assay (ng/L)
Sampling Strategy for Blue-Green Algae.

The following are guidelines for taking water samples for Cyanobacterial toxin analysis.

HANDLING OF SAMPLE.

1.1 Due to the harmful and potent nature of the toxins, samplers are advised to wear gloves. If conditions are such that waves are breaking on the shoreline or spray is evident, suitable respiratory apparatus may be considered necessary. Samples should be marked as toxic and be handled by glove between sampler and analyst.

1.2 Prior to collection, it should be established whether the samples are to be analysed for EXTRACELLULAR, INTRACELLULAR or TOTAL toxin content, as this would have implications on the care needed when handling and transporting the sample. TOTAL toxin determination (where toxin partitioning in the cells/water column is not required) requires no special fragile handling procedure. For EXTRACELLULAR toxin analysis, the cells must be separated from the water sample prior to transport. Details are given in the appropriate methods. For INTRACELLULAR analysis, the sample must be handled such as to minimise agitation which could lead to the rupture of cells prior to analysis.

1.3 Sample bottles should be filled completely with no air space, in order to reduce the incidence of cell lysis.

LOCATION OF SAMPLE COLLECTION.

2.1 Where a single sample point is used to represent the water body, the sample should be collected to represent the maximum possible toxin available. A sample point must include the most downwind point of the water body at the time of sampling. A sample of any scum or bloom should be taken. A sample should also be taken at the upwind point to establish the minimum toxin concentration.

2.2 Samples should generally be taken at the water surface, although benthic samples may be required where benthic algal cells (i.e. Oscillatoria sp.) are suspected of causing a toxic problem.

SAMPLING EQUIPMENT.

3.1 Samples should only be collected in the appropriate glass bottles and stored at a designated temperature, as specified in the analytical method. The method also specifies the volume of water required.
TIME INTERVALS FOR ANALYSIS & SAMPLE STORAGE.

4.1 Samples should be analysed within 24 hours after collection, where possible. Storage conditions should be adhered to, as specified in the method. Care should be taken when storing samples containing cells at low temperatures, so as to prevent cell lysis through temperature shock. Sample cooling to storage temperature should be gradual.

SAMPLE RECORDING SHEET.

5.1 Samplers should give information on; weather conditions, wind direction, water temperature, time and date of sample and location of sample, on a data sheet to be submitted with the water sample. The sample bottle should be clearly marked to identify the sample and cross-reference to the datasheet.

Elizabeth Potter  NRA 210294