The Cooperative Research Centre for
Water Quality and Treatment

Evaluation of Potential Matrix Interferences in Phosphatase Inhibition Assays used for Determining Cyanobacterial Hepatotoxins

Research Report 2

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Research Report no 2

April 2000

ISBN 1 876616 03 2

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FOREWORD

Evaluation of Potential Matrix Interferences In Phosphatase Inhibition Assays used for Determining Cyanobacterial Hepatotoxins

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Research Node: Australian Water Quality Centre

CRC for Water Quality and Treatment Project No. 2.3.1.1 - Development of Analytical Methods for Rapid Detection of Toxins in Water

EXECUTIVE SUMMARY

Peptide hepatotoxins such as the microcystins inhibit enzymes (protein phosphatases) responsible for protein dephosphorylation in a quantitative manner. The extent of inhibition of these reactions can therefore be used to determine the concentration of these toxins.

Earlier protein phosphatase inhibition assays relied on measuring radiolabelled phosphate released from the substrate employed. There were several disadvantages associated with these assays, including a requirement for facilities suitable for handling radioactive materials. Approaches using substrates such that a product is formed which can be measured colorimetrically therefore have considerable potential. A procedure using phosvitin as substrate and measuring the phosphate released colorimetrically as its complex with the dye malachite green was developed in an earlier project. While providing high sensitivity, there was some indication that the sample matrix affected the reliability of the results.

Recently a method utilising *p*-nitrophenyl phosphate as substrate, and colorimetrically measuring the *p*-nitrophenol released, was reported (An and Carmichael, 1994). This appeared to be superior to the assay using phosvitin as it was simpler and had equivalent sensitivity. There was some concern that the matrix effects observed with phosvitin might also occur with other substrates such as p-nitrophenyl phosphate. Consequently the assay using *p*-nitrophenyl phosphate as substrate was evaluated in terms of the effects of the sample matrix on its performance. As part of this project, the assay itself was improved to eliminate problems which were encountered, eg, the formation of a precipitate in the assay wells.

The assay as optimised using protein phosphatase 2A was extremely sensitive and reproducible, and adequate for directly determining toxin concentrations in water well below the drinking water guideline level of 1 μg/L without sample preconcentration. It responded equally well to a range of toxins and was not affected by up to 50% methanol in sample extracts. The assay appeared to be without significant matrix interferences, and should be suitable as a routine monitoring tool for water samples. If monitoring water with intact, healthy cyanobacterial cells, some sample pretreatment to release intracellular toxins will be required.

The high sensitivity of the method will result in toxins being found in some cyanobacterial material while less sensitive methods such as HPLC will determine such samples as non-toxic. It is doubtful if any samples of *Microcystis aeruginosa* are truly non-toxic based on the results of this inhibition assay, it is just a matter of degree. This implies that a quantitative definition of “toxic” in terms of cyanobacterial toxicity is required. A figure of 0.1 ppm in an algal extract (corresponding to 25 μg/g freeze-dried material if 10 mg is extracted with 2.5 mL solution) is recommended for consideration. However, the question of the reliability of low level data, ie, < 0.1 ppm, for toxin content of cyanobacterial material still has not been fully resolved.

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1. INTRODUCTION

1.1 Toxic Cyanobacteria

Increasing eutrophication of water bodies leading to increasing frequency of blooms of cyanobacteria (blue-green algae) is placing increasing pressure on these valuable resources, especially in relation to their use as sources of drinking water.

Cyanobacteria can produce toxins belonging to various chemical classes. Over 60 toxins are now recognised comprising neurotoxins, hepatotoxins, cytotoxins and lipopolysaccharide (LPS) endotoxins (Codd *et al.*, 1989; Carmichael, 1997). The hepatotoxins comprise cyclic heptapeptides (microcystins) produced mainly by *Microcystis aeruginosa,* but also by other genera such as *Oscillatoria*, *Anabaena and Nostoc,* and a pentapeptide (nodularin) produced by *N. spumigena* (Carmichael, 1997). Currently there are nearly 50 variants of microcystin which have been characterised (Rinehart *et al.*, 1994) and which may differ in toxicity (Carmichael, 1992.

1.2 Analysis for Cyanobacterial Toxins

Cyanobacteria and their toxins, especially the microcystins, are now a drinking water public health issue with a provisional drinking water guideline of 1 μg/L recently published by World Health Organisation (WHO, 1998) for the most common of the microcystins, microcystin-LR (mLR). Reliable analytical methods capable of determining microcystins in water at concentrations below the guideline level are therefore required to determine compliance with the guideline. Direct methods which require no sample preparation, in particular a concentration step, and which may therefore be used in a field kit, are of particular interest.

Several approaches to determining microcystins are available. Those based on high performance liquid chromatography (HPLC) (Lawton *et al.*, 1994) have been very useful in studying the behaviour of microcystins during water treatment (Nicholson *et al.*, 1994). These chemical methods generally require expensive equipment and highly trained operators, which limits their application in the water industry. A more fundamental disadvantage is the lack of available standards for the various microcystin variants and the subjectivity in identifying responses as microcystins at low concentrations.

Enzyme-linked immunosorbent assays (ELISA) have been used to determine microcystin concentrations in water. This method has the potential for lower cost compared with the chemical methods discussed above but to date success has been variable. As with the chemical methods there are difficulties dealing with the wide range of microcystins which have differing cross-reactivities in the analytical systems employed (Chu *et al.*, 1989; Nagata *et al.*, 1995).

1.3 Phosphatase Inhibition Assays

An alternative approach to measuring the toxins is measuring their associated toxicity. The most common method is the mouse bioassay which involves intraperitoneal (i.p.) injection of extracts. While this method has provided reliable and relevant results regarding the toxicity of concentrated scums, it is not sensitive enough to monitor the toxicity of water samples.

The most promising approach to measuring microcystins utilises recent discoveries of the mode of action of these toxins. MacKintosh *et al.* (1990) demonstrated that microcystins inhibit enzymes responsible for the dephosphorylation of intracellular phosphoproteins, the protein (serine/threonine) phosphatases, in particular PP1 and PP2A. The inhibition of these enzymes appears to be related to the hepatotoxicity of these compounds (Runnegar *et al.*, 1993; Toivola *et al.*, 1994) and is probably also related to their tumour promotion properties.

The basis of this type of procedure is the measurement of phosphate release from a phosphorylated protein or other suitable substrate in the presence of a phosphatase enzyme preparation and an inhibitor such as microcystin. This approach using ^{32}P radiolabelled substrates has been used for the measurement of microcystins in environmental samples (Sim & Mudge, 1993; Craig *et al.*, 1993; Jones & Orr, 1994; Lambert *et al.*, 1994; Lam *et al.*, 1995). The method is sensitive but suffers from a major drawback that the ^{32}P isotope has a short half-life and labelled proteins for the assay have to be prepared on a regular basis using reasonably sophisticated procedures. In addition, many routine laboratories are not set up to carry out radioactive determinations.

Colorimetric assays for the determination of microcystins are therefore attractive, provided sufficient specificity and sensitivity can be achieved. Methods based on using *p*-nitrophenyl phosphate (pNPP) as substrate and measuring the coloured *p*-nitrophenol released have been reported for determining microcystins (An and Carmichael, 1994; Ash *et al.*, 1995; Ward *et al.*, 1997). The commercial availability of recombinant forms of the phosphatase enzymes which obviates the need to isolate natural forms from animal tissues also makes this approach very attractive.

A procedure using PP1 and phosvitin as substrate was recently evaluated (Heresztyn and Nicholson, 1999) and found to be particularly sensitive but did not appear to have much greater sensitivity than the An and Carmichael assay. It also had one extra step, the development of a coloured product from the released phosphate (with malachite green), and the reliability appeared to be influenced by the sample matrix in a way which could not be explained. Some sample matrices appeared to produce erroneously high or false positive results. On this basis the use of pNPP-based assay had a marked advantage over phosvitin. However, it was considered that this matrix effect might be a feature of other phosphatase inhibition assays, whether they be radiolabelled or colorimetrically based. The aim of this study, then, was to further develop this kind of assay for measuring microcystins based on phosphatase inhibition which would be suitable for use in more basic laboratories. In particular, the potential for matrix effects as observed with the phosvitin-based assay which would severely limit the usefulness of the assay, was investigated.

Existing phosphatase inhibition assays with pNPP as substrate both utilise PP1 (Ward *et al.*, 1997; An and Carmichael, 1994). As published, the sensitivity of the two methods varies by a factor of 100 reflecting differences in assay conditions. For example the sensitivity can be greatly influenced by the presence of trace metals such as manganese (Heresztyn and Nicholson, in press). As published, the procedure of Ward *et al.* (1997) was too insensitive (IC_{50} 38 μg/L) to use for monitoring a guideline of 1 μg/L. The An and Carmichael (1994) method appeared satisfactory (IC_{50} 0.3 μg/L). However using it under conditions subsequently recommended by the authors, a much lower sensitivity was obtained. Sensitivities vary depending on whether the toxin concentration reported is that in the solution tested, or in the final assay solution itself, and published IC_{50} values do not always make this clear. For example under the conditions described in Section 1.3, the toxin in the sample aliquot is diluted ten-fold by the enzyme and substrate solutions. Thus there will be a ten-fold difference in the IC_{50}s, depending on whether this applies to the concentration in the original solution or in the final assay solution. A sensitive pNPP-based assay using PP2A had been established by AWT EnSight in Sydney and formed the basis for this study.

There are various publications reporting the inhibition of PP2A compared with PP1. Sensitivities will depend on the enzyme concentration. In order to allow for this variable, true IC_{50}s may be considered those where further reduction in enzyme concentration in the assay causes no reduction in the IC_{50} determined. This is the titration end-point as described by Honkanen *et al.* (1990,1994). The results of Honkanen *et al.* (1990, 1994) which might therefore be considered true indicators of sensitivity indicate that PP2A is approximately 50 times more sensitive than PP1 in its inhibition by microcystin. Consequently PP2A, as used by AWT EnSight, was investigated on the basis that a more sensitive assay for microcystins might be achieved.

2. EXPERIMENTAL

Experimental details are documented in the various sections of “Results and Discussion”. All toxin concentrations refer to concentrations in the solutions tested and therefore can be related to concentrations of interest in water samples. True IC_{50}s, if they represent toxin concentrations in the final assay solution, are approximately an order of magnitude lower. Other experimental details can be found in the phosvitin report (Heresztyn and Nicholson, 1999).

3. RESULTS AND DISCUSSION

3.1 An and Carmichael PP1 Assay Protocol

A workshop held in Brisbane in 1998 (Carmichael and An, 1998) gave a brief background of the nature of protein phosphatase enzymes and their use in the detection and quantification of hepatotoxins such as microcystins and nodularin. The ability of protein phosphatases to dephosphorylate substrates such as *p*-nitrophenyl phosphate, phosvitin, ^{32}P-glycogen phosphorylase a and 4-methylumbelliferyl phosphate can be exploited to measure the presence of hepatotoxins because these enzymes are quantitatively inhibited by these compounds. The mechanism of these assays has been discussed in more detail in the report and paper on the application of the phosvitin assay in the determination of hepatotoxins (Heresztyn and Nicholson, 1999).

Due to a problem with formation of a precipitate in the assay as initially proposed by AWT EnSight, the buffer as detailed at the workshop was also evaluated, and the results are presented here.

The assay presented in the Brisbane workshop had the following protocol which was based on the original publication (An and Carmichael, 1994):

(1) 10 μL sample (mLR) + 40 μL PP1 solution

(2) Incubate 37^{o}C for 5-10 minutes

(3) Add 50 μL pNPP substrate solution E

(4) Read at 405 nm in kinetic mode (5 minute intervals for 40 minutes)

Stock buffer (diluent for Reagents A, B, C)

40 mM Tris HCl

20 mM KCl

30 mM $MgCl_2$

pH 8.6

Reagents A, B, C, D, E

Reagent A 3 mM DTT in stock

Reagent B 1.5 mg/mL BSA in stock buffer

Reagent C 1.5 mM $MnCl_2$ in stock

Reaction solution D 1 part Reagent A + 1 part B + 1 part C

Substrate solution E 40 mM pNPP in Reaction solution D

PP1 solution

PP1 is diluted in Reaction solution D (for PP1 (Boehringer Ingelheim GmbH), 1/800 dilution. - 50mU/50μL)

Unfortunately the assay did not work during the Brisbane workshop, probably due to the enzyme having lost activity and this in itself demonstrates the care needed with these bioassays. Therefore, information on the sort of inhibition curve to be expected from the assay with this enzyme was not available. This assay was tested in these laboratories with PP1 (Calbiochem-Novabiochem Pty Ltd) and PP2A (Promega Corporation). The IC_{50} for the PP1 sigmoidal curve illustrated in Figure 1 is 19.8 ppb mLR with a working range of 4.0 to 97.8 ppb (corresponding to 20% and 80% inhibition). The data for the PP2A curve are as follows: IC_{50} = 14.8 ppb with a working range of 2.9 to 76.4 ppb mLR (PP1 stock soln. = 1 U/mL; PP2A stock soln. = 1.5 U/mL.)

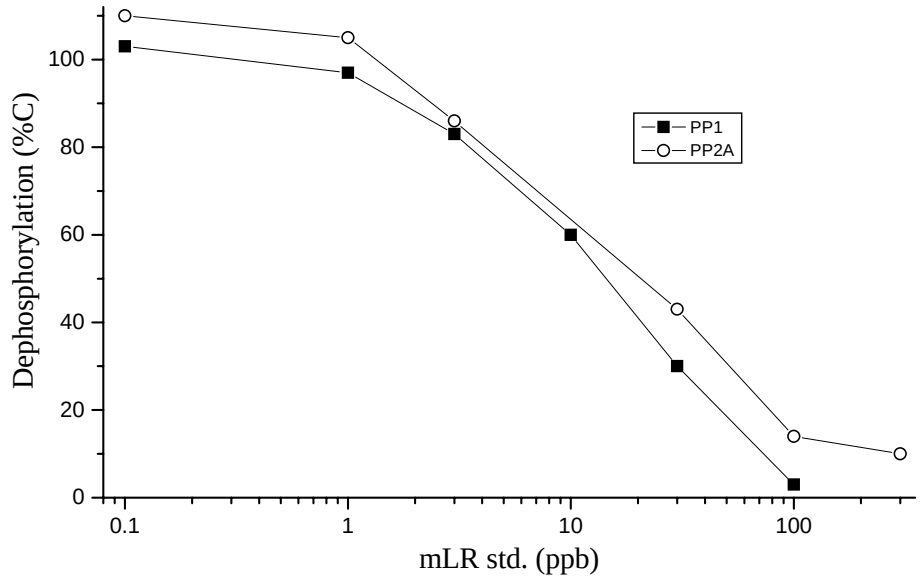

Figure 1. Inhibition curves for Calbiochem PP1 and Promega PP2A, modified An and Carmichael pNPP assay (Brisbane workshop)

Kinetic assay of the enzymic dephosphorylation of pNPP by PP1 and PP2A (Figure 2) showed that PP2A activity starts to level off within about 20 minutes in this buffer system, which accounts for the low absorbances achieved and why larger amounts of this enzyme need to be added to the assay to achieve comparable activity to PP1. Kinetic assays over 4 hours with PP1 indicated the enzyme maintained its activity over this time (data not presented). This buffer, then, does not appear to be entirely suitable for use with PP2A.

Kinetic assays with PP1 and PP2A at different concentrations of mLR are shown in Figure 3. As the mLR concentration increases, the absorbance at any time decreases due to the dephosphorylation of substrate by the enzyme being inhibited by the toxin. The rate of the enzyme reaction (y/x) at each concentration of toxin is also inversely proportional to toxin concentration as expected. Standard curves can be generated either by plotting Vmax versus toxin concentration or absorbance at a particular time versus toxin concentration (endpoint assay). The endpoint assay provided clearer results and it provided more flexibility when making changes to the buffer components of the AWT assay. The incubation time for the assay can be varied depending on time constraints. If older enzyme is used, the incubation time can be lengthened to produce a higher absorbance.

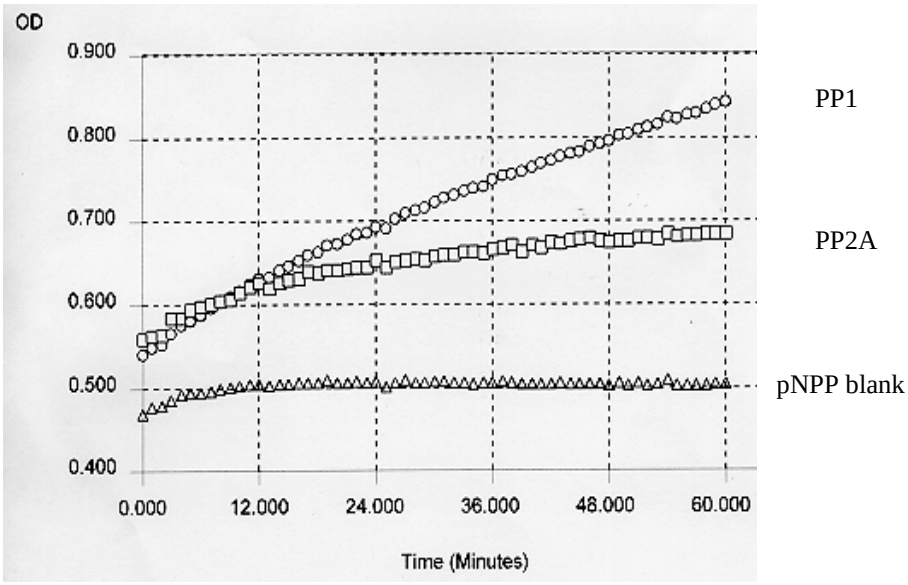

Figure 2. Kinetic assay of dephosphorylation of pNPP by PP1 and PP2A over 1 hour

By graphing the absorbance at 90 minutes (minus blank absorbance) and expressing this value as a percentage of the control, a standard curve for the two enzymes shown in Figure 4, can be used to quantify unknowns.

The IC_{50} for PP1 from Figure 4 is 18.4 ppb mLR. A more concentrated solution of PP2A was used to achieve a ΔOD of 0.3 units in this example. Consequently the sigmoid curve has shifted to the right to give an IC_{50} value of 221 ppb for PP2A. These fluctuations are unexpected, and may be due to older enzyme being used or the fact that the pipettes used were not suitable for the small volumes being dispensed. A new 8-channel Finn pipette was purchased to dispense small volumes such as 40 μL and 50 μL more accurately. The replicate study summarised in Table 1 suggests that the accuracy of the assay is consistent with what is expected from a bioassay (15-20%). However, this can be improved upon as Table 5 in the modified AWT buffer section shows.

3.2 Replicate Assay for PP1 in An and Carmichael Modified Assay

Two enzyme concentrations were used to determine if the assay was more sensitive at lower concentrations of enzyme and also the accuracy of the assay.

The IC_{50} for the sigmoid curves at the 2 enzyme concentrations were: at 0.1 U/mL in the assay IC_{50} = 4.3 ppb; at 0.2 U/mL IC_{50} = 11.2 ppb mLR. These values were an order of magnitude greater than the IC_{50} reported by An and Carmichael (1994) and it was therefore decided this assay was not sensitive enough to replace the phosvitin assay and was abandoned.

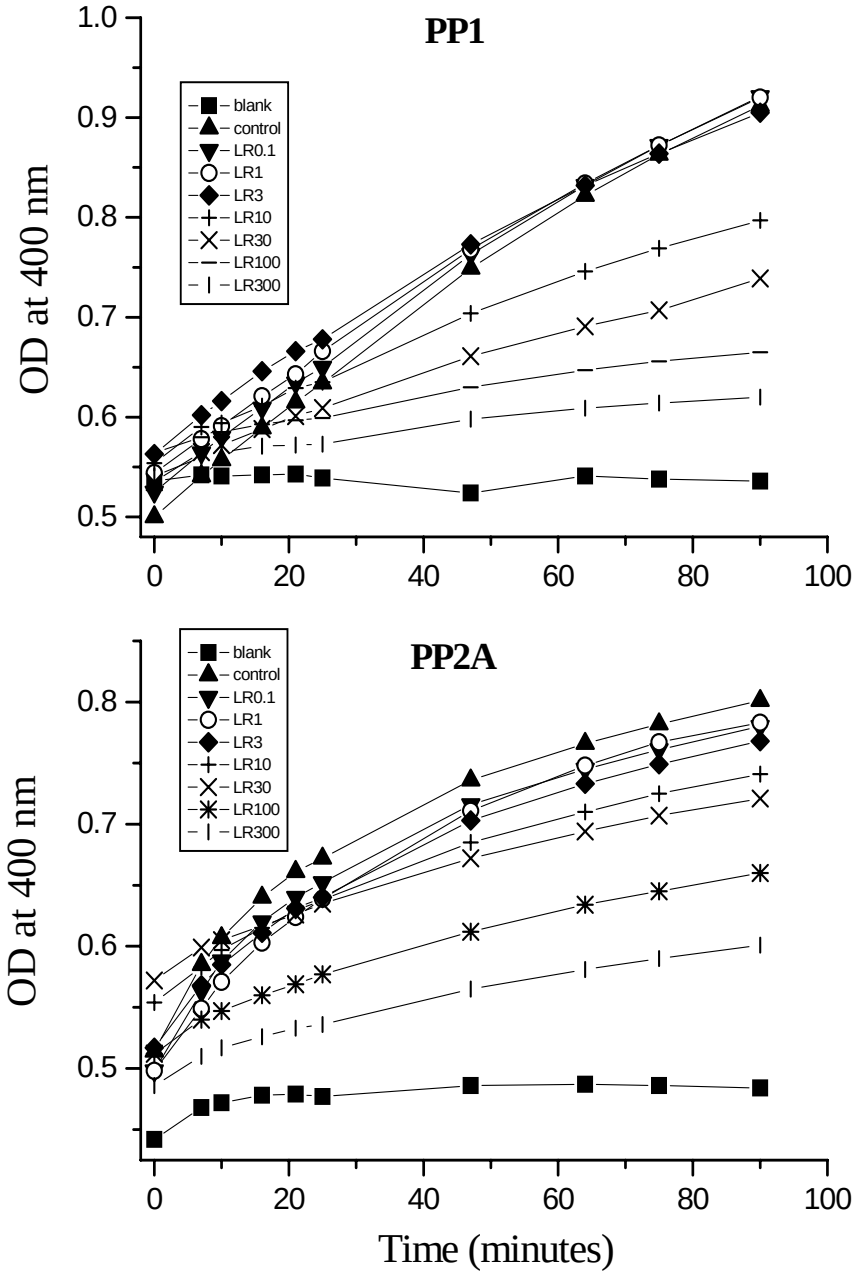

Figure 3. Dephosphorylation of pNPP by Calbiochem recombinant PP1 and Promega PP2A at various concentrations (ppb) of microcystin-LR over 1.5 hours

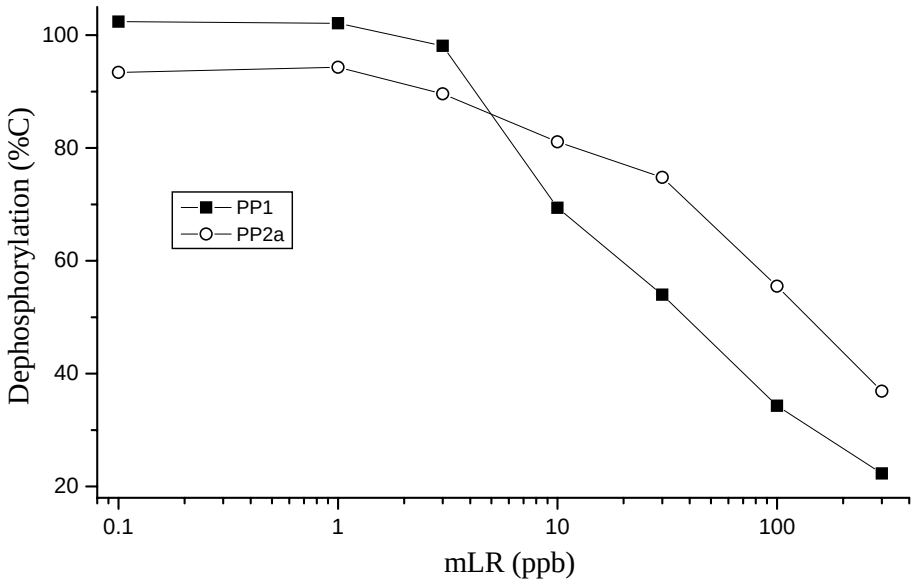

Figure 4. Standard inhibition curves for PP1 and PP2A with modified An and Carmichael assay (Brisbane workshop)

3.3 Phosphatase Inhibition Assay as Provided by AWT

This method was presented to the labs as already optimised. The protocol of the assay is outlined below.

Enzyme diluent

0.1M Tris, pH 7.0	0.5 mL
40 mM DTT	0.05 mL
10 mM $MnCl_2$	0.2 mL
10 mg/mL BSA	0.1 mL
H_2O	0.15 mL

Reaction mix 1

0.4 M Tris, pH 8.1	0.5 mL
10 mM $MnCl_2$	0.04 mL
0.3 M $MgCl_2$	0.28 mL
10 mg/mL BSA	0.2 mL

Reaction mix 2

50 μL reaction mix 1
40 μL pNPP (50 mM)
10 μL 40 mM DTT

Assay: 10 μL sample (mLR) is combined with 10 μL PP2A (0.035-0.04 U) in a 96 well flat-bottomed microtitre plate. The plate is tapped sideways to combine the two solutions and incubated at 37°C for 5 minutes. 100 μL of reaction mix 2 is then added to start the reaction and the plate is again incubated at 37°C for up to 2 hours. The colour is then measured at 405 nm using a microtitre plate reader and the concentration of microcystin calculated from the standard curve.

Table 1. Average of replicate assays (percent of controls) for recombinant PP1 in the An and Carmichael assay

		MLR (ppb)								
	Control	**0.1**	**0.3**	**1**	**3**	**10**	**30**	**100**	**300**	**1000**
PP1 at 0.1 U/mL in assay										
Replicate										
1	0.254	92.8	96	88.1	75.7	46.6	26.4	17.5	11.8	10.5
2	-	105.8	104.3	74.8	69.2	40.5	28.9	21.6	29.9	
3	-	131.3	135	102.3	79	59.2	42.2	40.5	23.2	
4	-	84.8	98.1	78.7	59.4	48.2	27.8	41.6	17	
Average		103.7	108.4	86.0	70.8	48.6	31.3	30.3	20.5	
Std Dev		20.3	18.1	12.2	8.6	7.8	7.3	12.5	7.8	
%		19.6	16.7	14.2	12.1	16.0	23.3	41.3	38.0	
PP1 at 0.2 U/mL in assay										
Replicate										
1	0.492	95.2	85	81.9	79.4	53.8	25.5	19.4	14.5	2.7
2	-	90.5	87.8	85.9	79.4	55.7	34.3	22	13.4	10.4
3	-	92.2	85.1	84.6	83.3	*5.4*	32.8	27.6	29.7	16.2
4	-	106	91	91.9	71.4	57.2	31.6	16.2	20.8	6.4
Average		96.0	87.2	86.1	78.4	55.6	31.1	21.3	19.6	8.9
StdDev		7.0	2.8	4.2	5.0	1.7	3.9	4.8	7.5	5.8
%		7.3	3.2	4.9	6.4	3.1	12.5	22.5	38.3	65.2

Initial assays with this protocol suggested the assay was sensitive, however a white cloudiness was noticed in the microtitre plate wells particularly when the volumes were doubled to increase the absorbance and accuracy of the assay. Kinetic progress of the assay in the presence and absence of microcystin suggested the assay starts to form the precipitate after about 1 hour (Figure 5). Enzymatic dephosphorylation of pNPP accounts for the increase in absorbance between 0 and 0.8 hours. The absorbance then starts to increase significantly which can not all be attributed to enzyme activity and coincides with visual appearance of cloudiness in the microtitre plate wells. As the pNPP blank curve in the An and Carmichael assay in Figure 2 shows, pNPP undergoes some initial hydrolysis over the first 15 to 20 minutes and is reasonably stable after that. The absorbance increase in the assay wells after 1 hour is largely unrelated to enzyme activity. However, the control indicates some enzyme activity otherwise the curve would be parallel to the blank.

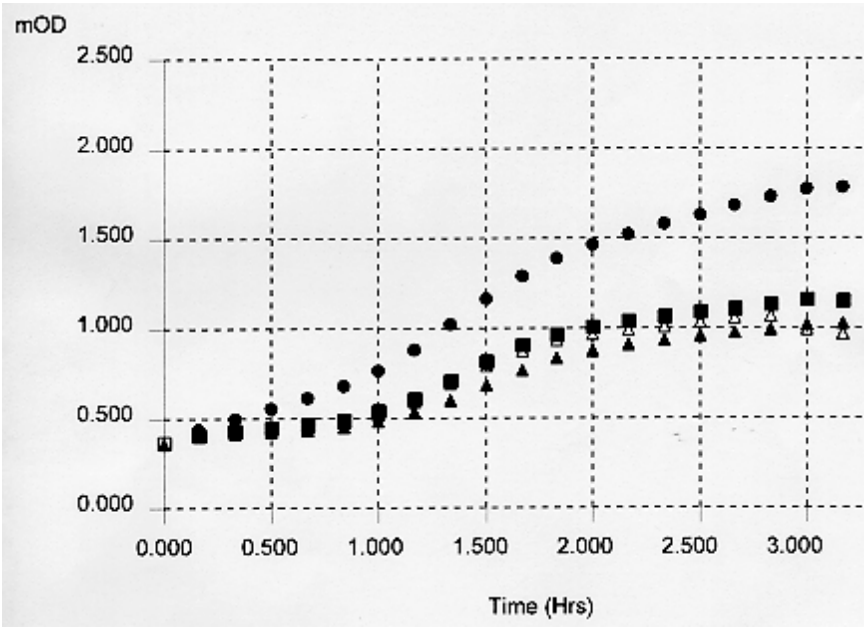

Figure 5. Progress of dephosphorylation of pNPP by PP2A in the presence and absence of mLR over 3 hours

In order to ascertain the cause of the precipitate, the buffer solutions were prepared with one ingredient at a time missing. Scaled up volumes of the assay components were added to test tubes which were incubated in a water bath at 37^{o}C for 1 hour which was adequate for the precipitate to develop.

Table 2. Evaluation of the cause of the precipitate in the assay

Sample (MQ water) 0.05 mL	Enzyme buffer 0.05 mL	Reaction mix2 0.5 mL	Visual Appearance
Present	All components	All components	Ppt. formed
Present	-DTT	-DTT	No ppt.
Present	-Mn	-Mn	Ppt. formed
Present	All components	-Mg	No ppt.

As Table 2 indicates, the only instances in which the precipitate did not form was when Mg or DTT were absent. It therefore appears that the presence of both magnesium and DTT is involved in the formation of the precipitate. After making up fresh solutions and buffers to ascertain the problem was not caused by an incorrect assay component, the assay was also checked at AWT which confirmed the formation of a precipitate.

3.4 Modifying the Buffer to Minimise/Eliminate the Precipitate

Various changes were made to the buffers in four stages and the original and modified buffers were compared at each stage to ensure that enzyme activity was not seriously affected.

Stage 1: The BSA concentration was halved from 10 to 5 mg/mL

DTT was halved from 40 to 20 mM

$MgCl_2$ was decreased from 0.3 to 0.2 M

Figure 6 shows very little difference in PP2A activity at different levels of mLR between the modified and original buffer. By visual observation, the precipitate in the wells for the modified assay appeared to have decreased.

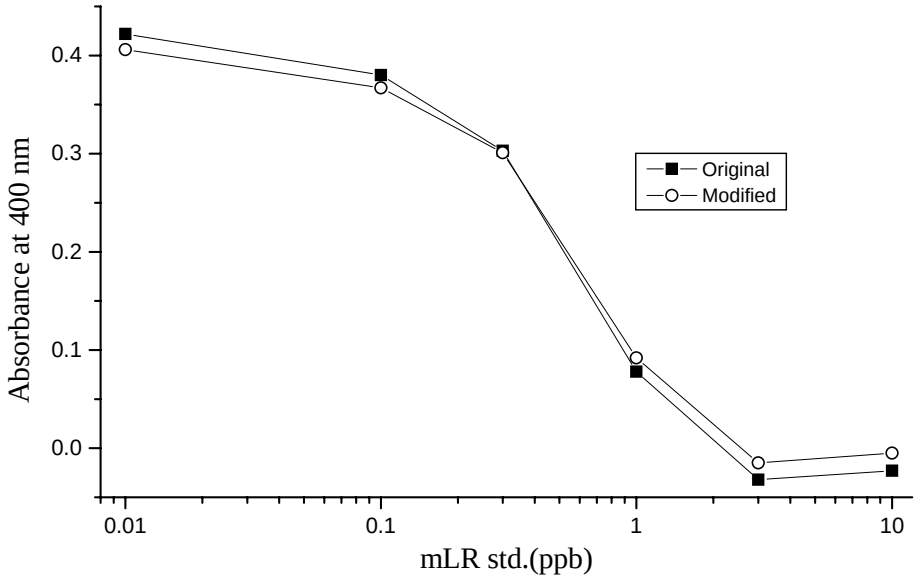

Figure 6. Comparison of the original buffers and those in the modified assay

Stage 2: Tris concentration in reaction mix 1 was changed from 0.4 M, pH 8.1 to

0.25 M Tris, pH 8.5

Mn was omitted from the enzyme diluent and reaction mix

Promega Corporation does not mention PP2A as having a requirement for Mn and does not include this component in their assay protocol (Promega Technical Bulletin 537), so it was decided to try and eliminate this component from the assay altogether. The results are illustrated in Figure 7.

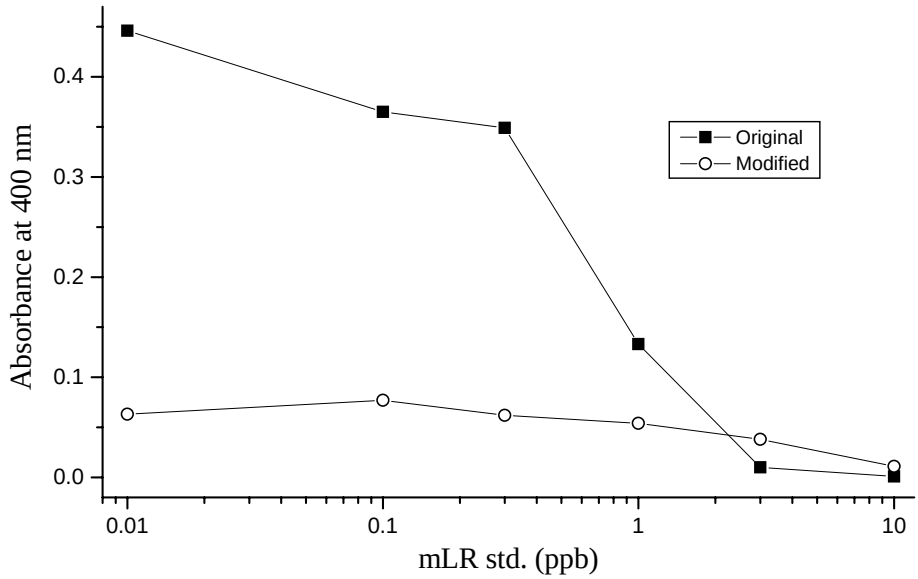

Figure 7. Comparison of original and modified buffer; BSA/DTT halved, Mg 2/3rds, Tris decreased, -Mn ion

The enzyme activity in the modified conditions was clearly affected by the absence of Mn ion. It is unlikely the decreased Tris concentration could have affected the enzyme to such a large degree as the final Tris concentration in the original assay (86 mM) was well in excess of what most assays recommend. Mn ion was therefore incorporated back into the assay. The changes made in stage 1 were still included in all later modifications.

Stage 3: 0.25 M Tris, pH 8.1 was compared with 0.25 M Tris, pH 8.5 in reaction mix 1

pNPP was increased from 50 mM to 60 mM to give a final assay concentration of 20 mM

The effect of decreasing the Tris concentration was again examined. The effect of increasing the pH was also incorporated into this experiment as Promega Corporation use a final buffer pH of about 8.5 and An and Carmichael's (Brisbane workshop) new protocol uses a stock buffer with pH 8.6. The results are shown in Figure 8. Decreasing the Tris concentration, whilst assisting with the precipitate problem, also slightly decreases enzyme activity at pH 8.1. This is not considered significant enough to cause concern. Increasing the pH to 8.5 resulted in an even lower absorbance. This could be a pH effect on the absorbance of p-nitrophenol itself, or enzyme activity is lowered at this pH. It was therefore decided to modify the buffer in reaction mix 1 to 0.25 M Tris, pH 8.1.

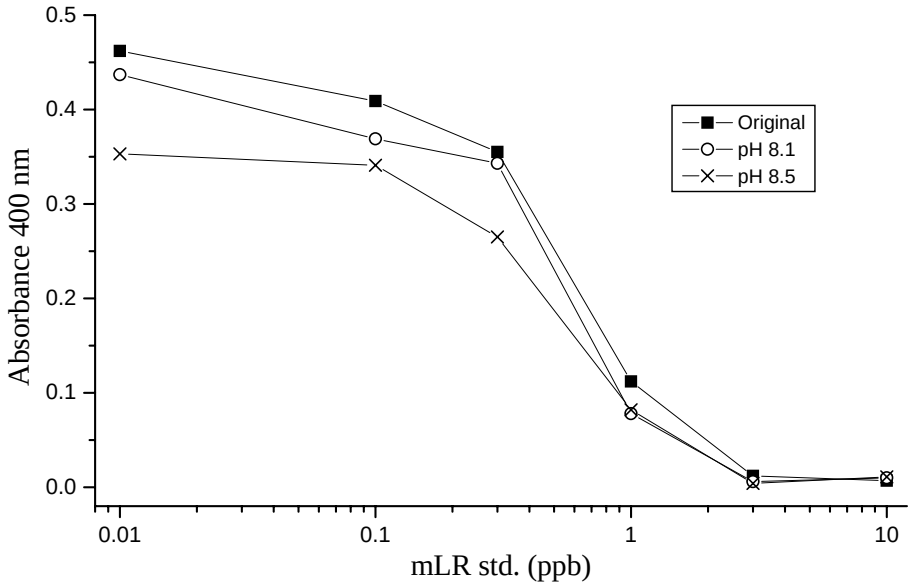

Figure 8. Comparison of original buffer with modified buffers at pH 8.1 and pH 8.5

Scaled up volumes of the buffers were also incubated at 37^{o}C in test tubes. The original buffer went cloudy, the buffer at pH 8.1 had a barely perceptible haze and the buffer at pH 8.5 appeared clear visually.

Stage 4: The last modification tested was the addition of EGTA to assist in keeping the metal ions in solution. EGTA was added to the enzyme diluent to produce a final assay concentration of 0.01 mM. It was combined with the 0.1 M Tris, pH 7.0 and the water addition in the enzyme diluent.

The original buffers ± EGTA were compared with the modified buffers ± EGTA (Figure 9) which by now had the following changes made:

- BSA halved

- DTT halved
- Mg decreased to 2/3 rds
- 0.25 M Tris, pH 8.1 replaced 0.4 M Tris, pH 8.1 in reaction mix 1.

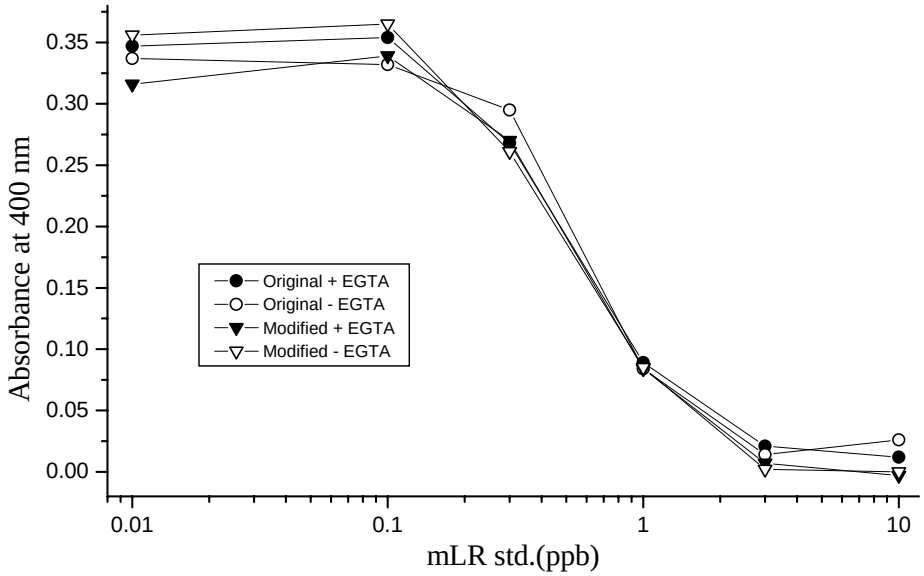

Figure 9. Original and modified buffer +/- EGTA

The absorbance has had the blanks (enzyme solution replaced by diluent) subtracted which negates the effect of the precipitate fairly well. The addition of EGTA does not decrease enzyme activity and it is difficult to see a real difference between the presence or absence of EGTA.

Table 3. Absorbance in the blank wells measured at time intervals

	Original buffers		Modified buffers	
Time (hours)	**-EGTA**	**+EGTA**	**-EGTA**	**+EGTA**
0	0.404	0.423	0.442	0.410
1	0.525	0.541	0.518	0.492
3.5	0.987	1.017	0.569	0.537

These values show that the original buffer produces a precipitate, but it is difficult to conclude whether EGTA helps keep Mg soluble. The modified buffer may still produce a precipitate but the wells looked clear and transparent by visual observation after 3.5 hours.

The formation of the precipitate by kinetic assay was then examined to check whether the unusual curves observed earlier in Figure 5 had been eliminated (see Figure 10).

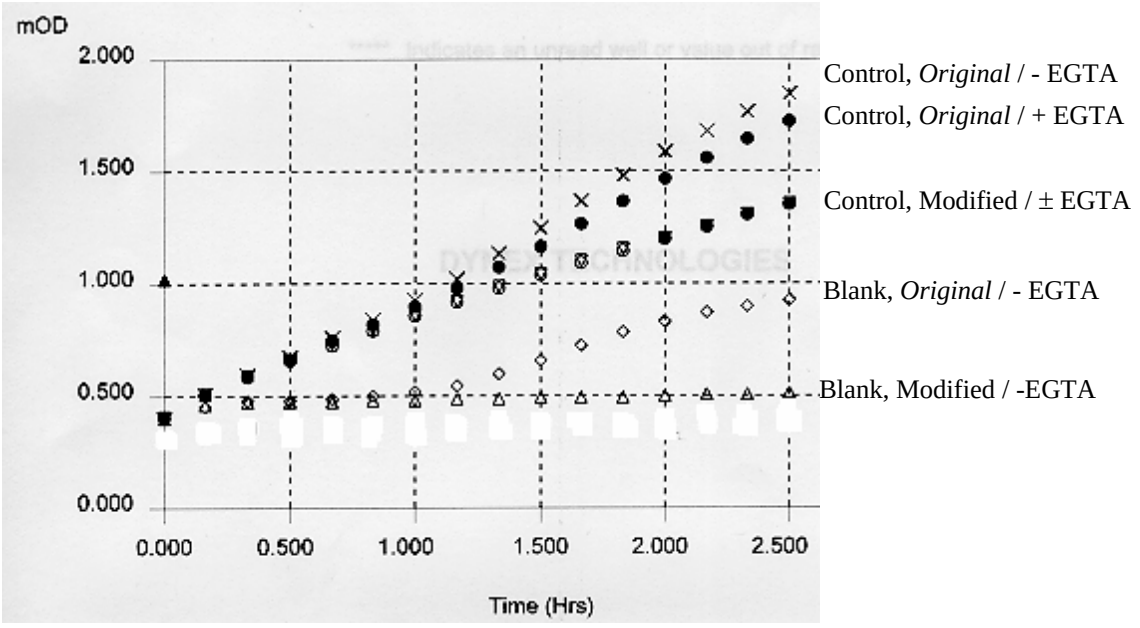

Figure 10. Dephosphorylation of pNPP by PP2A in the absence of microcystin over 2.5 hours

The blank in the modified buffer shows a small increase in absorbance in the first half-hour after which it remains fairly stable. The blank in the original buffers undergoes a significant increase in absorbance after about an hour which corresponds with the visual appearance of the precipitate. Both controls in original buffer, with and without EGTA, show an increase in rate of absorbance at 1-1.5 hours, that can not be attributed to increased enzyme activity. Addition of EGTA may prevent formation of the precipitate to a small degree. The control in the modified buffer exhibits a slowly decreasing rate of reaction over the 2.5 hours. The presence of EGTA has little effect on the enzyme reaction. Since the EGTA does not have an adverse effect, it was decided to keep it in the buffer.

The optimised assay buffers therefore are comprised of the following:

Enzyme diluent

0.08 M Tris, pH 7.0 / 0.19 mM EGTA	0.65 mL
20 mM DTT	0.05 mL
10 mM $MnCl_2$	0.2 mL
5 mg/mL BSA	0.1 mL

Reaction mix 1

0.25 M Tris, pH 8.1	0.5 mL
10 mM $MnCl_2$	0.04 mL
0.2 M $MgCl_2$	0.26 mL
5 mg/mL BSA	0.2 mL

The assay was examined kinetically once more after all the changes had been incorporated into both the enzyme diluent and reaction mix 1. Figure 11 shows that the precipitate problem has been eliminated in the modified buffer. The deviation in the control curve in the original buffer is about 0.5 absorbance units which is the same as that of the blank.

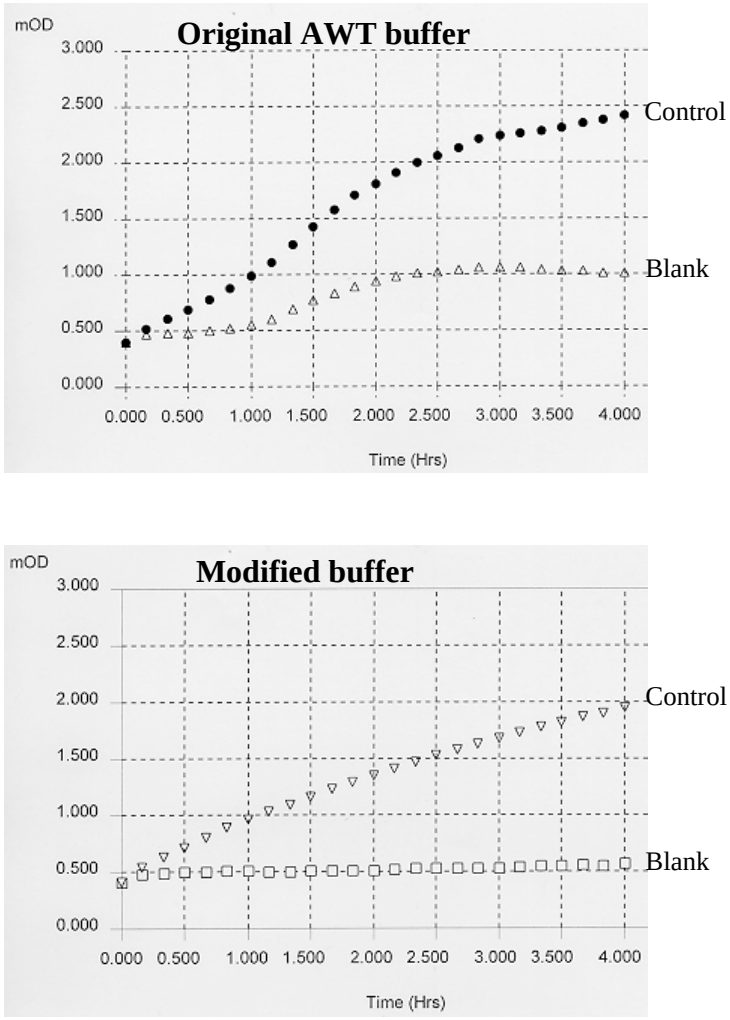

Figure 11. Kinetic assay on the original and modified buffer over 4 hours in the absence of toxin

The accuracy of the modified assay was examined in Corning Costar half area flat bottom microtitre plates where the total assay volume = 120 μL. The depth of the solution in the wells is higher than in standard microtitre plates due to the narrower diameter of the wells. The use of such plates may be advantageous in that a smaller amount of enzyme is required, and costs are therefore reduced. Table 4 displays this data, and should be compared with the data in Table 5 which represents data conducted in standard microtitre plates (well volume = 300 μL) where the volume of the enzyme, sample and substrate was doubled to result in a total final volume of 240 μL.

Table 4. Replicate inhibition assays for microcystin-LR (percent of controls) at two concentrations of PP2A with pNPP as substrate in the half area well plates (total assay vol.=120 μL)

		mLR (ppb								
	Control	**0.01**	**0.03**	**0.1**	**0.3**	**0.6**	**1**	**3**	**10**	**30**
PP2A at 0.125 U/mL										
Replicate										
1	0.130	83.1	94.6	68.5	41.5	13.8	-1.5	7.7	14.6	9.2
2	-	76.9	104.6	57.7	50.8	3.8	-4.6	16.2	15.4	18.5
3	-	88.5	100.0	71.5	33.1	40.8	7.7	14.6	16.2	18.5
4	-	70.0	90.0	57.7	44.6	17.7	10.0	11.5	11.5	11.5
Average		79.6	97.3	63.9	42.5	19.0	2.9	12.5	14.4	14.4
StdDev		6.9	5.5	6.2	6.4	13.6	6.1	3.2	1.8	4.2
%		8.7	5.7	9.7	15.1	71.6	>100	25.6	12.5	29.2
PP2A at 0.25 U/mL										
Replicate										
1	0.240	79.2	88.8	70.4	60.4	51.7	17.1	1.7	-1.7	-6.7
2	-	76.3	87.1	76.7	58.8	53.3	26.3	2.9	0.8	0.4
3	-	74.6	88.8	78.8	72.1	53.8	35.8	11.3	4.6	3.8
4	-	74.6	93.8	63.3		52.1	19.6	-0.4	0.8	-2.5
Average		76.2	89.6	72.3	63.8	52.7	24.7	3.9	1.1	-1.3
StdDev		2.2	2.9	7.0	7.3	1.0	8.4	5.1	2.6	4.5
%		2.9	3.2	9.7	11.4	1.9	34.0	>100	>100	>100

In the early and linear part of the curve the accuracy of the assay is less than or close to 15 % which is the maximum figure acceptable. If this assay volume is to be used, the half area plates are preferred to the standard microtitre plates as the increased depth of assay solution in the well results in a higher absorbance reading as well as reducing enzyme usage. One source of inaccuracy in the assay is the dispensing of 10 μL of enzyme solution with the Finnpipette repeat dispenser. This pipette does not dispense a discrete droplet of solution due to the presence of BSA in the buffer. Therefore 20 μL was the preferred volume in a total assay volume of 240 μL. Graphing the data presented in Table 4 with error bars is shown in Figure 12, and can be compared with Figure 13 where the assay volumes are doubled. The larger volumes obviously result in increased precision.

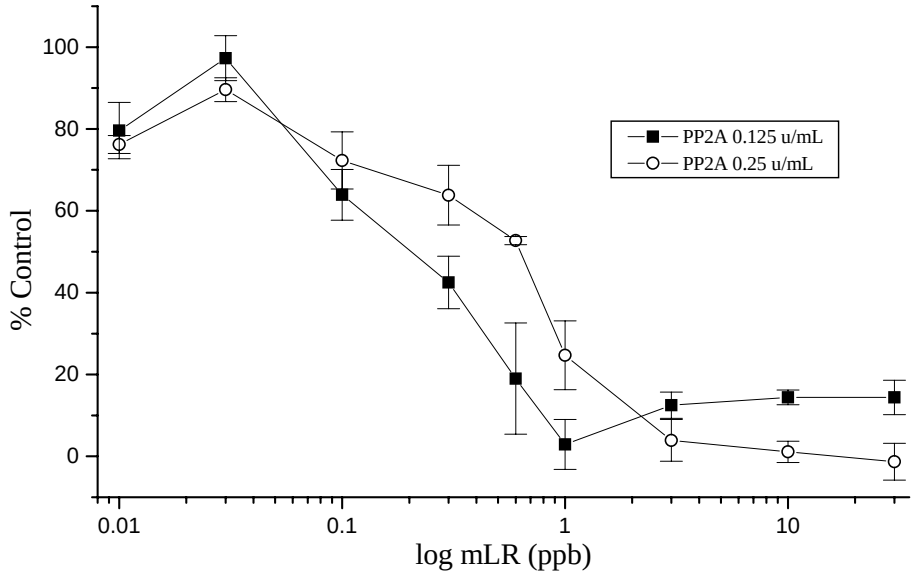

Figure 12. Inhibition curves of replicate study in Corning Costar half area flat bottom plates (data shown in Table 4)

The IC_{50} for the sigmoid curve at 0.125 U/mL PP2A is 0.2 ppb mLR; IC_{50} for the curve at 0.25 U/mL PP2A is 0.7 ppb. The fact that the curve is still shifting to the left with decreasing enzyme concentrations suggests the assay could be further optimised to increase sensitivity. However, the sensitivity is more than adequate for determining toxin to below the drinking water guideline of 1 μg/L.

3.5 Second Replicate Study on Modified Assay

The assay was examined in standard microtitre plates in replicates of four, using 20 μL PP2A, 20 μL mLR standard and 200 μL substrate solution (Table 5). The data are presented as a graph in Figure 13.

3.6 Inhibition Curves for Other Toxin Standards

Standard solutions of nodularin, mRR, mYR, and mLA were compared with the standard curve of mLR (Figure 14). The inhibition curve for each standard has a roughly similar IC_{50}. Given that the purity of purchased standards cannot be guaranteed, it can only be concluded that these five toxins all have similar inhibition constants towards PP2A. Many other research groups (Honkanen et al. 1994, Chaivimol et al. 1993) report similar IC_{50}s for microcystins and this data was summarised in Table 9 of the phosvitin report (Heresztyn and Nicholson, 1999).

Table 5. Replicate study on modified pNPP assay, with double volumes (total vol. = 240 μL)

			mLR (ppb)							
	Absorbance-blank									
Replicate	**Blank**	**Control**	**0.01**	**0.03**	**0.1**	**0.3**	**0.5**	**1**	**3**	**10**
1	0.497	0.520	0.502	0.484	0.456	0.370	0.280	0.121	0.022	0.020
2	0.492	0.496	0.478	0.484	0.425	0.371	0.267	0.090	0.041	0.031
3	0.496	0.497	0.540	0.471	0.446	0.354	0.294	0.082	0.038	0.035
4	0.495	0.475	0.469	0.506	0.461	0.373	0.264	0.136	0.041	0.037
Average	0.495	0.497	0.497	0.486	0.447	0.367	0.276	0.107	0.036	0.031
StdDev	0.002	0.018	0.032	0.015	0.016	0.009	0.014	0.026	0.009	0.008
%	0.4	3.6	6.4	3.0	3.6	2.5	4.9	23.6	26.1	25.2
	Dephosphorylation of pNPP (% Control)									
Replicate										
1		0.497	101.1	97.5	91.9	74.6	56.3	24.2	4.4	4.0
2			96.1	97.5	85.5	74.8	53.8	18.0	8.2	6.3
3			108.7	94.9	89.7	71.2	59.1	16.6	7.7	7.1
4			94.4	101.9	92.8	75.1	53.1	27.3	8.2	7.5
Average			100.1	98.0	90.0	73.9	55.6	21.5	7.1	6.2
StdDev			6.4	2.9	3.3	1.8	2.7	5.1	1.8	1.6
%			6.4	3.0	3.6	2.5	4.9	23.6	26.1	25.2

The % error for this assay is generally less than 5 %.

3.7 Effect of 50% Methanol on the pNPP Inhibition Curve

The C18 cleanup procedure used by the AWQC is a useful means of concentrating hepatotoxins from water or scum material for HPLC analysis. It also eliminates pigments, DOC, salts and other components that might otherwise contribute to the deterioration of the HPLC column as well as interfere with diode array detection of these toxins. The final concentrate from the C18 sep-paks is in 50 % methanol. It may in some instances be useful to analyse this concentrate by phosphatase assay therefore the effect of methanol on the phosphatase inhibition assay was examined using mLR standards made up in 50 % methanol. Figure 15 illustrates a comparison of mLR in 50 % methanol with a standard curve of mLR made up in high purity (MilliQ) water. There is very little difference between the two curves suggesting the presence of methanol in the sample volume of 20 μL does not adversely affect the activity of PP2A or its inhibition by microcystin. It should be noted that the methanol is diluted by a factor of 12 by the enzyme and substrate solutions. Ward et al. (1997) examined the effect of 0 to 100 % methanol in the sample volume on the activity of PP1 to *p*NPP and found that only concentrations of methanol greater than 80 % caused loss of enzyme activity.

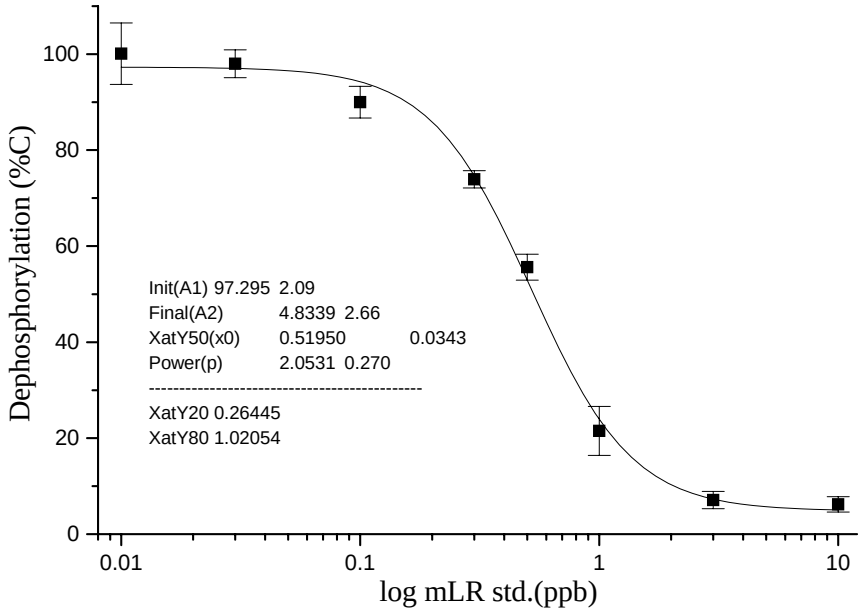

Figure 13. Standard inhibition curve for mLR standards analysed in four replicates with error bars representing standard deviation

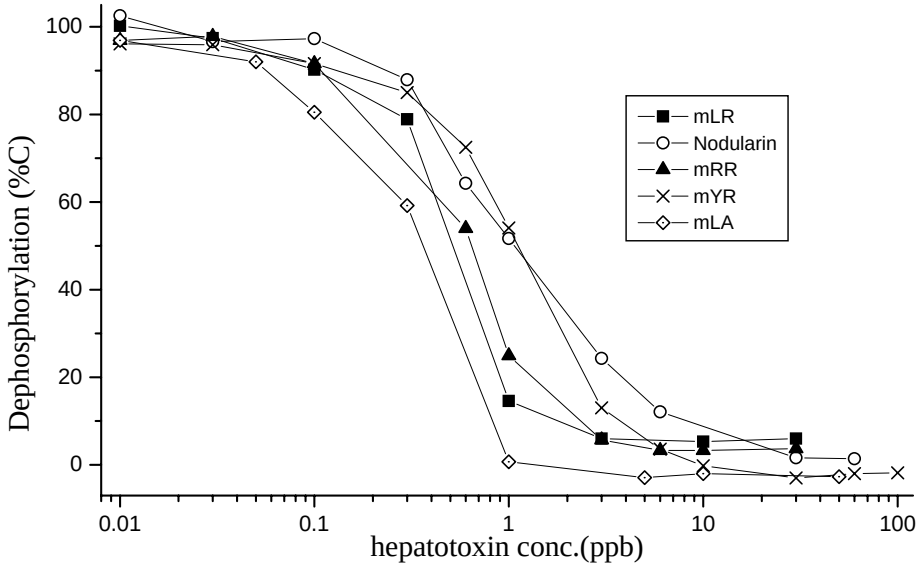

Figure 14. Inhibition of PP2A (pNPP substrate) by mLR, nodularin, mRR, mYR and mLA

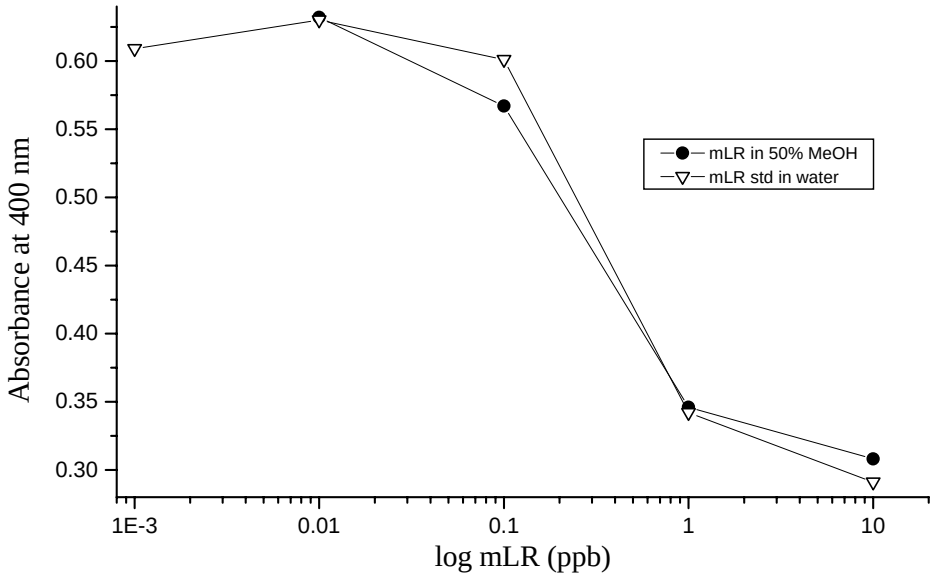

Figure 15. Effect of methanol on PP2A activity in the mLR inhibition assay

3.8 Effect of Matrix on PP2A Inhibition Assay

The five matrices examined in the phosvitin report for their effect on PP1 were again assayed to determine whether they interfered with mLR inhibition of PP2A towards pNPP.

The five matrices were: humic acid solution, 9 mg/mL DOC (*humic acid*)
diluted algal extract, (1/100) (*algal extract*)
a raw water taken from Hindmarsh River (*raw water*)
an ultrafiltered farm dam sample (retentate) (*UF dam*)
MilliQ water as a control (*MQ*)

Microcystin-LR standards in the concentration range of 0.01 to 10 ppb were diluted in these matrices. The control for the assay was MilliQ water which is what is used to quantify unknowns. Graph (a) in Figure 16 suggests that the algal extract may interfere with the assay. However, this sample may in fact contain microcystins at a low concentration which could have contributed to the observed behaviour. The toxin content of around 0.8 ppm determined by this assay is above the detection limit of HPLC (0.06 ppm) but this latter figure only applies if there is one major toxin present. A number of toxins present at concentrations individually below the detection limit and hence not detectable could in total give a concentration much higher such that the detection limit. Time did not permit HPLC analysis of the extract to be repeated using a concentration procedure to resolve this issue. A similar result was found with the phosvitin assay with other matrices (Heresztyn and Nicholson, 1999), and in that case the likelihood of such a result being caused by residual microcystins appeared remote. There is no significant difference between the other matrices investigated here and the standard curve. Graph (b) illustrates a repeat assay with the ultrafiltered farm dam and shows that slight differences between the non-algal matrices in (a) may change in a repeat assay.

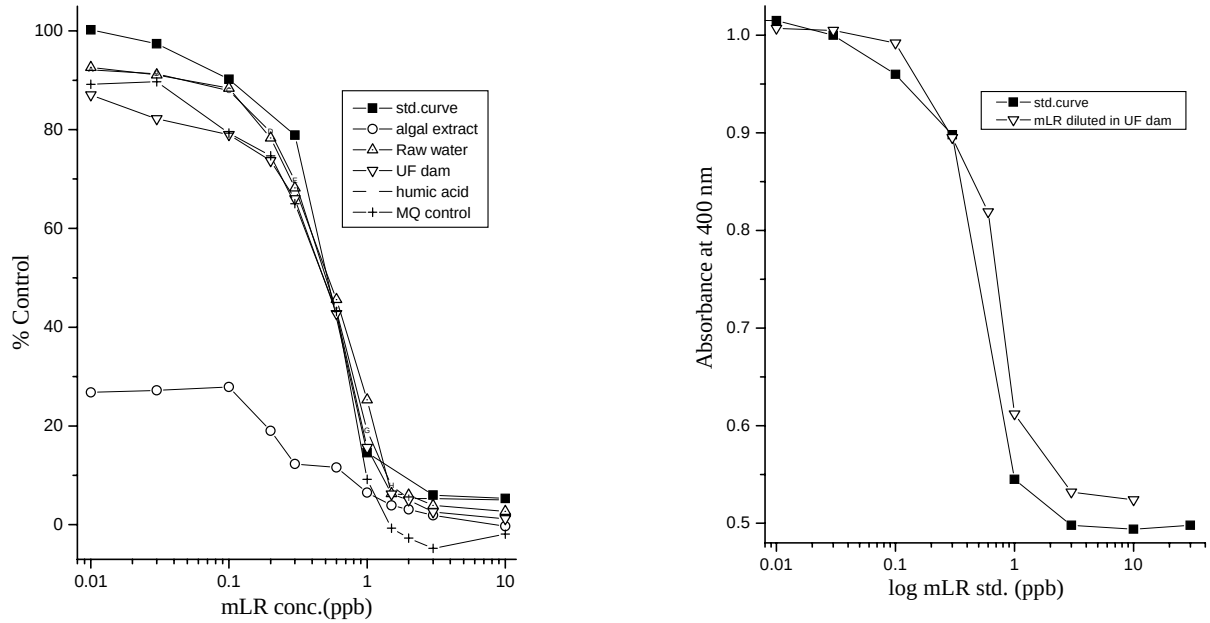

Figure 16. Effect of various matrices on mLR inhibition of PP2A. (a) comparison of 5 matrices with standard curve, (b) repeat assay of UF dam matrix

3.9 Water Spike Experiments

Nodularin and mLR were spiked into water taken from Lake Alexandrina at Milang and Bolivar Lagoon. This would represent a worst case scenario for samples to be analysed from raw waters. The spike concentrations were: 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 100 ppb.

Milang water: pH 7.6

TDS 640 mg/L

DOC 4.7 mg/L

Bolivar WWTP Lagoon: pH 8.05

TDS 1200 mg/L

DOC 17.6 mg/L

Based on the errors that can be expected from this assay (illustrated in Table 5 and Figure 13), the differences between the mLR standard curve and the spiked lagoon wastewater, and the nodularin standard curve and spiked Milang water are within the expected standard deviation. It was therefore concluded that toxins assayed in raw waters are unlikely to exhibit interference from components in these waters.

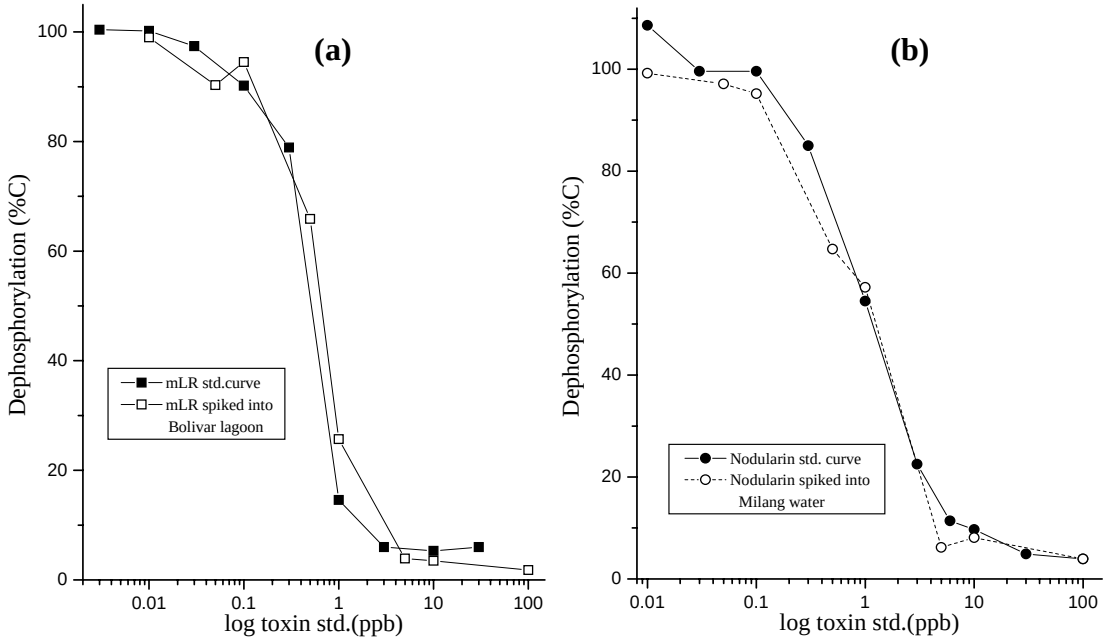

Figure 17. Microcystin and nodularin spiked into two water matrices

3.10 Assay of a Range of Algal Samples by PP2A Inhibition Assay

A number of algal samples were selected for comparison of toxin content by HPLC quantitation with the PP2A inhibition assay (Table 6). Mouse toxicity results are included where the data is available and a number of non-toxic samples were included. Samples with high and low levels of hepatotoxins were also selected to determine the accuracy of the assay across a broad range of toxicity. Within the phosphatase assay data, algal solutions were analysed with and without C18 cleanup. Another cleanup procedure using ultrafiltration units will be discussed with Table 8.

All of the cyanobacterial samples assayed were toxic by phosphatase assay, albeit the toxin contents of some were very low.

Generally, HPLC results corresponded well with phosphatase values. There were some exceptions however, such as Large Torrens and Lake Mokoan (14/3/90) but the differences might not be considered excessive. Repeat analyses were not carried out for confirmation due to time constraints. It is possible that assays such as this detecting such low levels of toxins in matrix of cyanobacterial material might be prone to some interferences and some variation in quantified values is to be expected. For example, the Lake Torrens (Adelaide) samples contained large amounts of *Chlorella* at some stages of the bloom. This study has not been able to resolve this issue. In addition, HPLC results depend on interpretation of spectral information of peaks in the chromatograms as microcystins, and this judgement can affect the results obtained.

Table 6. Protein phosphatase inhibition assay of a range of algal samples analysed by HPLC and mouse bioassay (all values quantified as mLR equivalents except for nodularin)

Sample	Dominant Cyanobacterial species	Mouse Bioassay	HPLC (ppm)[b]	Phosphatase (ppm)[b]	
				Raw	C18 clean
Microcystis blooms, containing microcystins by HPLC					
Mt. Compass farm dam	*M. aeruginosa*	Not tested	72		54
Warren Reservoir	*M. aeruginosa*	Not tested	75.8		68
Pejar Dam, 10/5/95	*M. aeruginosa*		17.5		12.4
Pejar Dam, March 95	*M. aeruginosa*		13.9		14.6
Small Torrens	*M. aeruginosa*	Not tested	6.7	2.8	
Large Torrens	*M. aeruginosa*	Not tested	1.9	8.1	
Mt. Bold Reservoir `88	*M. aeruginosa*	Not tested	35.6	31	35
Lake Wallace 1995	*M. aeruginosa, A. circinalis*	Hepatotoxic	2	2.6	2.7
Lake Mokoan 19/12/89	*M. aeruginosa*		43	32	35
Lake Mokoan 25/4/90	*M. aeruginosa*		53.3	37	39
Lake Mokoan 14/3/90	*M. aeruginosa*		5	1	0.86
AH -(b)	Spiked *Cylindrospermopsis*[c]	Hepatotoxic	50.3	47	
AH -(c)	"	Hepatotoxic	28.7	32	
AH -(e)	"	Hepatotoxic	15.5	14	
Nodularia blooms					
Milang 1991[a]	*N. spumigena*	Hepatotoxic?	33.8	22	28.5
Goolwa 1991[a]	*N. spumigena*	not tested	54.6	51	62
Algal samples either non-toxic by mouse bioassay, or 0 ppm by HPLC					
Bird in Hand 1995	*Microcystis* sp.	not tested	0	0.08	
Pejar Dam, 26/4/95	*M. aeruginosa*	non-toxic	12.7		10.4
Bundaleer Res. 1992	*M. aeruginosa*	non-toxic	0	0.27	0.03
		(ambiguous peaks	8.6)		
Newcastle 1996	*M. aeruginosa*	non-toxic	0	1.5	0.2
		(ambiguous peaks	24.6)		
Pejar Dam 1995	*M. aeruginosa*	non-toxic	4	2.8	4.4
Kennella farm dam	*M. aeruginosa*	not tested	0	0.12	0.012
		(ambiguous peaks	0.3)		
South Gippsland 1999	*M. aeruginosa*	not tested	0	0.22	
Botryococcus scum	(*Botryococcus* sp.)	not tested	-	0.85 ppb	
CC Willowpoint	M. aeruginosa	non-toxic	0	0.009	
CC EPA, NSW	M. wesenbergii	not tested	0	0.0007	
CC Lake Dyer	M. aeruginosa	non-toxic	0	0.4	
CC Pejar dam	M. aeruginosa	non-toxic	0	0.53	
CC Broken Hill	M. aeruginosa	Hepatotoxic	0	0.037	

a – samples analysed for nodularin
b – these quantified values refer to the algal solution resulting from the extraction of a nominal amount of freeze-dried algal material; weight of freeze-dried algae has not been taken into account
c - spiked with microcystin

As all of these samples are blooms, with the exception of the *Cylindrospermopsis* samples which were cultures spiked with microcystin, the presence of other toxic algae also cannot be ruled out. For example, *Botryococcus* is very similar in visual appearance to *Microcystis* and a heavy bloom such as

the one assayed could easily have contained small amounts of *Microcystis* which could have been missed in a microscopic scan. Also, this bloom was very concentrated and *Botryococcus* contains large amounts of lipids so some other inhibitory effect on PP2A cannot be ruled out, however no firm conclusion can be made from this data. The toxicity of the spiked *Cylindrospermopsis* samples assayed also corresponded well with mouse LD_{50} results (A. Humpage, University of Adelaide, personal communication).

Samples cleaned up by C18 appeared to give slightly higher results than the raw or untreated sample which would not be expected as generally the clean up process results in less than quantitative recoveries. However the differences, in most cases, were slight and there was insufficient data to make a statistical assessment.

A significant number of samples found to be non-toxic by mouse bioassay or HPLC were toxic by phosphatase inhibition assay. This is not surprising given that the detection limit by HPLC is around 0.06 ppm mLR which is very much higher than that achieved with this assay. The phosphatase assay is a far more sensitive alternative to the mouse bioassay, and has the added advantage of providing a quantitative value of toxicity of the sample.

Some samples such as Kennella farm dam had very “clean” HPLC traces and surprisingly were toxic, although not highly toxic, using this assay. The presence of one or two late eluting peaks with similar but different UV spectra to mLR raises the question of these so-called ambiguous peaks being toxic microcystins. Again this illustrates the limitation of HPLC in that it relies on spectral interpretation for the identification of microcystins. The results also indicate that nodularin can reliably be determined by this assay.

3.11 Reproducibility of Algal Data Results

A number of algal solutions were analysed on separate days to determine the reproducibility of the phosphatase inhibition assay Table 7). The values quantified on different days showed excellent agreement whether the extracts were cleaned up by C18 or not, and whether they contained high or low levels of microcystins.

3.12 Comparison of Cleanup Procedures

A number of samples were ultrafiltered through Sigma Ultrafree-MC NMWL 5000 filter units prior to quantitation by this phosphatase inhibition assay. Again, the results (Table 8) compare well with values obtained with HPLC and in previous phosphatase assays. The results in the last six samples indicate that microcystin is recovered from these filtration units even when present at low concentration. Thus, if sample cleanup is required, ultrafiltration is a viable alternative to C18 cartridges.

Table 7. Phosphatase inhibition assay of algal solutions on different days

Sample assayed	First phosphatase Result (ppm*)	Second Ppase result (ppm*)
Mt. Compass farm dam	54	49
Warren Reservoir	68	73
Small Torrens	2.8	4
Large Torrens	8.1	8
88-02 Mt. Bold Res. (no C18)	31	38
" C18 cleanup	35	40
Bundaleer Res. (no C18)	0.27	0.31
" C18 cleanup	0.032	0.029
Pejar Dam	2.8	3.5
Lake Wallace	2.6	2.9
Kennella farm dam (no C18)	0.12	0.14
" C18 cleanup	0.012	0.012
L. Mokoan 19/12/89 (no C18)	32	45
" C18 cleanup	35	53
L. Mokoan 25/4 (no C18)	37	43
" C18 cleanup	39	28
95-01	0.079	0.084
L. Mokoan 14/3 (no C18)	1	1.1
" C18 cleanup	0.86	0.9
South Gippsland	0.22	0.24
Botryococcus scum	0.85 ppb	0.95 ppb

* results quantified as mLR equivalents and are in ppm unless indicated otherwise

Table 8. Comparison of phosphatase inhibition assay results of untreated algal solutions with two cleanup procedures

Sample	HPLC (ppm)	Phosphatase (ppm)[a]		
		Raw	C18 clean	Sigma UF
Mt. Bold Reservoir 1988	35.6	31, 38	35, 40	42
Lake Mokoan 1990	53.3	37, 43	39, 28	45
Bird-in-Hand STW 1995	0	0.079, 0.084		0.083
Bundaleer Reservoir 1992	0	0.27, 0.31	0.032, 0.029	0.3
Kennella farm dam	0	0.12, 0.14	0.012, 0.012	0.12
Willowpoint	0	0.0093		0.0087
EPA, NSW	0	0.70 ppb		0.63 ppb
Pejar dam 1995	0	0.53		0.58

a – results in ppm unless stated otherwise, and results of duplicate assays presented

4. SUMMARY AND CONCLUSIONS

The colorimetric protein phosphatase inhibition assay using p-nitrophenyl phosphate as substrate as optimised in this study was extremely sensitive and reproducible. It appeared adequate for directly determining toxin concentrations in water well below the drinking water guideline level of 1 μg/L without sample preconcentration, and should be suitable as a routine monitoring tool. However, some treatment procedure would be required to liberate intracellular toxin from intact, healthy cyanobacterial cells.

Problems such as the appearance of a precipitate during the assay were resolved by altering the concentrations of the buffer components. Precision was best when normal volume microtitre plates were used in the assay. The use of smaller volume plates, while saving on the amount of enzyme required, resulted in poorer precision.

The method responded well to a range of toxic microcystins and was also suitable for determining nodularin. It was not affected by up to 50% methanol in sample extracts.

The assay appeared to be without the matrix interferences which were observed in the assay previously developed where phosvitin was used as substrate. Calibration curves prepared with a number of water matrices were identical to a standard curve prepared with MilliQ water.

The high sensitivity of the method will result in toxins being found in some cyanobacterial material while less sensitive methods such as HPLC will determine such samples as non-toxic. This was demonstrated with a number of samples. It is doubtful if any samples of *Microcystis aeruginosa* are truly non-toxic based on the results of this inhibition assay, it is just a matter of degree. This implies that a quantitative definition of “toxic” in terms of cyanobacterial toxicity is required. A figure of 0.1 ppm in an algal extract (corresponding to 25 μg/g freeze-dried material if 10 mg is extracted with 2.5 mL solution) is recommended for consideration. However, the question of the reliability of low level data, ie, < 0.1 ppm, for toxin content of cyanobacterial material still has not been fully resolved.

It should be mentioned that enzyme inhibition is only a rough approximation of mammalian toxicity. Although the inhibition of phosphatase enzymes is involved in the toxic response, other factors such as intestinal adsorption, excretion rate, etc, will also be important in determining *in vivo* toxicity.

5. RECOMMENDATIONS

It is recommended that the assay be implemented in its optimised form for the determination of peptide hepatotoxins in both cyanobacterial material and water. If used to monitor toxins in cyanobacterial material, concurrent HPLC analyses should be carried out to further determine if the sample matrix affects the assay.

The high sensitivity of the assay will determine bloom samples as toxic when alternative less-sensitive analyses such as HPLC will not detect toxins. Consequently, to describe a bloom as toxic or non-toxic, a more rigorous definition of toxicity is required. Perhaps material with a toxin content below 0.1 ppm in a sample extract could be defined as non-toxic, even though highly sensitive assays will determine the presence of toxins.

Further work on the reliability of the assay for determining toxins at low levels in water should be carried out. This should involve a comparison of the phosphatase inhibition assay results with those obtained by HPLC using pre-concentration.

6. ACKNOWLEDGMENTS

This work was funded by the CRC for Water Quality and Treatment as part of Project No. 2.3.1.1 “Development of Analytical Methods for Rapid Detection of Toxins in Water”. The authors would like to thank Dr Andrew Humpage of the University of Adelaide and colleagues at the Australian Water Quality Centre and AWT EnSight for invaluable advice and discussion. Dr Alistair Sim of the University of Newcastle and Dr Gary Jones of CSIRO Land and Water are also thanked for their helpful comments on phosphatase assays.

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