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Urban Water Research Association of Australia

Advance Warning of Cyanobacterial Toxicity

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Research Report No 108
November 1996

ISBN 1 876088 20 6

Cover design by Gregory R Smith

Foreword

This report is based on UWRAA Research Project No WR-26: 'Advance Warning of Cyanobacterial Toxicity' which was undertaken during the period January 1993 to September 1996. Organisational responsibility for the project was as follows:

Sponsoring Authority:	Hunter Water Corporation
Project Officer:	Bruce Cole Hunter Water Corporation Newcastle West, NSW
Research Agency:	The University of Newcastle
Principal Researchers:	Dr A T R Sim, Associate Professor J A P Rostas Faculty of Medicine and Health Sciences The University of Newcastle

The project was funded by the Urban Water Research Association of Australia and Hunter Water Corporation.

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1. Project Objectives

(i) To develop a routine assay for the detection of sub-toxic levels of cyanobacterial toxins.
(ii) To identify regions in which algae containing sub-toxic levels of cyanobacterial toxins exist.
(iii) To monitor the temporal variation in levels of these toxins in order that the conditions leading to increased levels might be identified before toxicity arises.

2. Synoposis

- A rapid laboratory assay based on the inhibition of protein phosphatase activity has been developed for the routine monitoring of the microcystin class of cyanobacterial toxins. This test is capable of detecting toxin levels to <0.1ug microcystin-LR per litre of water, without sample concentration. It is therefore most suitable for advance warning of cyanobacterial toxicity.

- The assay has been extensively tested and is now available as a commercial service. Several modifications have been designed and implemented as a result of this testing.

- Using this assay, the existence of previously unreported endogenous cyanobacterial protein phosphatase and proteolytic activities has been demonstrated. These endogenous activities may lead to underestimation of toxin concentration and modifications to the assay have been developed.

- The effectiveness of the assay in detecting unknown toxins or variants which cannot be detected by any other procedure (such as HPLC) has been demonstrated.

- A non-radioactive assay has also been developed, which has the potential for further development as a field based test.

- Irregular occurances of readily accessible cyanobacterial blooms in the Hunter region throughout this project has prevented extensive analysis of algal hot-spots and conditions predisposing to toxicity. Sampling and testing is continuing such that information relating to objectives (ii) and (iii) is likely to be forthcoming in subsequent years.

3. Background

The rise in awareness of toxic cyanobacterial blooms with their associated human health problems and animal deaths in the last few years has highlighted the need for rapid and reliable methods for determining cyanobacterial toxicity. Furthermore, these methods require high sensitivity in order that sub-toxic levels of toxin may be detected. This will allow advance warning of impending toxicity enabling water authorities to effectively reduce the extent and number of cyanobacterial blooms. Furthermore, epidemiological studies now indicate a high correlation between long-term exposure to low levels of the microcystin class of toxin and the development of malignant liver tumours. Water authorities, therefore, should ensure that customers do not have long term exposure to these toxins.

The microcystin class of toxin, of which there are now over 50 variants, was shown in 1990 to be specific for the key cell regulatory enzymes, protein phosphatase types 1 and 2A. This action is identical to the structurally unrelated marine toxin, okadaic acid, often associated with sea-food poisoning. This raised the exciting prospect of developing a test for these toxins based on their ability to inhibit protein phosphatases 1 or 2A. This test would in principle be (i) simple, requiring little instrumentation (ii) sensitive, due to the specificity of action (iii) capable of detecting all variants of the class, thereby being more reliable than instrument based analysis (such as HPLC) which relies on toxin identification (iv) potentially usable in the field for "on-site" toxin detection.

The purpose of this project was to take advantage of the expertise in protein phosphatase research at The University of Newcastle to develop such testing procedures. Dr Sim was integrally associated with identifying the similar mechanism of action of the sea-food toxin okadaic acid and his is the only research group in Australia which focuses on this class of enzyme. Because of the relatively urgent need for these tests, it was decided to take a dual approach to the research :

- The first objective was to take techniques already in use in our laboratory for assaying the activity of protein phosphatases and modify and calibrate these for the routine analysis of cynaobacterial toxins. However, since these tests utilise radiolabelled phosphatase substrates, they are clearly inappropriate for general use by water managers or for the development of a field test.
- The second objective was then to investigate the possibility of developing a non-radioactive form of the test for wider use and development of a field test.

We report here considerable progress and success with both objectives. Furthermore, we make several recommendations for future research and development of commercially available testing procedures.

4. Research Undertaken

4.1 Establishment of a radioactive phosphatase assay for routine toxin quantification.

Protein phosphatase activity is routinely determined by measuring the dephosphorylation of a standard substrate protein (glycogen phosphorylase) labelled with radioactive phosphate. Briefly, the radiolabelled protein is incubated with a source of protein phosphatase and then the reaction is stopped by the addition of trichloroacetic acid (TCA). This precipitates the substrate protein, leaving any radiolabelled phosphate which has been removed by the phosphatase in the supernatant. Measurement of the radioactivity in the supernatant (using a scintillation counter) is then taken as a measure of protein phosphatase activity. The presence of toxin which inhibits protein phosphatases would be detected as a decrease in radioactivity in the supernatant. This assay which was routinely in use in our laboratory, was tested for its ability to detect and quantify microcystin-LR in a range of samples. It was found to be capable of accurately measuring microcystin levels down to 0.1ug toxin per litre water without sample concentration using either purified protein phosphatase 2A or a crude subcellular fraction of chicken brain containing protein phosphatases. It should be noted that, because protein phosphatases are key regulatory enzymes in virtually all cells, including plants, a wide range of sources of enzyme could be used in this assay. Chicken brain was used as the source of protein phosphatase since these animals are inexpensive and readily available and brain is especially rich in protein phosphatases. Furthermore, protein phosphatase activity survives freezing and thawing such that 1-2 months supply of material can be prepared from one chicken, thereby minimising the number of animals used in the test.

Procedures for both fresh and freeze dried water and algal scum sample extraction and preparation were standardised in accordance with those used for other testing procedures. It should be noted that a number of hydrophobic microcystin variants have now been discovered which would not therefore be extracted by standard aqueous methods. The selection of sample preparation procedures is therefore critical for reproducibility and is an area which needs further research before standard techniques can be widely accepted.

Our own standard testing procedures were published (1,2) and made available commercially through our laboratory. Since this test detects any toxin which inhibits protein phosphatases, toxin identification is not required. We consider this a particular advantage over instrument based techniques which rely on the availability of purified standards of all known variants. Our results are reported relative to a standard toxicity of microcystin-LR.

4.2 Evaluation of the Radioactive phosphatase assay.

4.2.1 Detection of algal phosphatase activity.

In the period 1992-94 some 200 samples from around Australia were analysed for toxins which inhibit protein phosphatase activity. During this time a number of important discoveries were made which led to improvements in the test as well as recognition that continued research is necessary in this area. In particular, routine analysis of some algal bloom samples showed the presence of endogenous phosphatase activity which led to both under estimation and in some cases complete masking of toxin levels (1,2) This activity was widely distributed amongst samples. Fortunately we discovered that this endogenous algal phosphatase was insensitive to microcystin and

was not binding toxin. This finding provided a simple way of controlling for this activity, and was published (1, 2) together with the recommendation that future assays take into account this endogenous algal phosphatase by measuring activity both in the absence and presence of an exogenous source of protein phosphatase.

4.2.2 Detection of algal protease activity

A number of additional samples showed the presence of endogenous algal protease activity. This activity was found to digest the substrate protein (glycogen phosphorylase) releasing a peptide fragment containing the radiolabel, which was not precipitated by TCA (results not shown). This lead to quantitation of radioactivity caused by proteolysis and not protein phosphatase activity. Such proteolytic activity has the potential to under estimate the levels of toxin present. In order to prevent false negative results arising from this activity, the TCA supernatant was further processed to convert the free phosphate released by protein phosphatase activity into a phosphomolybdate complex before scintillation counting. This procedure does not trap the peptide fragment released by proteolysis and therefore does not lead to false negative results. While introducing an additional (simple) step into the procedure, this modification is considered essential to achieve accurate toxin quantitation.

4.2.3 Comparison with HPLC Toxin analysis

During this period of investigation our testing procedures were also subject to a number of independent evaluations, including that undertaken for reports published by the Murray Darling Task Force and the Sydney Water Board. These particular studies showed a statistically significant variation between results obtained using the protein phosphatase inhibition assay and those obtained using HPLC. Several laboratories using each test were investigated. While it must be recognised that these studies were not sufficiently calibrated to accurately compare different testing procedures, variation in results reported are likely to be due in part to the above causes of false negatives. Using the modifications described, we now find excellent agreement between results obtained using the phosphatase assay and HPLC (2), although no further independent assessment has been made.

Because this test measures the functional consequences of toxin action, it has the advantage of detecting all toxins which inhibit protein phosphatases. Such an advantage was powerfully demonstrated when analysis of one sample showed the presence of toxin equivalent to 32mg microcystin-LR per litre of water. HPLC analysis failed to identify any peak characteristic of microcystin (2) indicating that the toxicity was due to a previously unidentified toxin. As part of an ARC funded study, the test has also been used to analyse several hundred extracts of marine organisms and we have identified 30 which have previously uncharacterised toxic activity (unpublished observations). One such toxin has been purified and found to be chemically distinct from all other protein phosphatase inhibitors (3), highlighting once again the need for activity based assays rather than those which rely on toxin identification.

4.2.4 Peptide Substrate-based Assay

We also investigated other more readily available substrates for the assay. Glycogen phosphorylase must be phosphorylated by the enzyme glycogen phosphorylase kinase which is expensive and not widely available. As an alternative we investigated the suitability of the peptide GS1-12.This is a synthetic peptide which is phosphorylated by Protein Kinase C. Both are readily available from a number of commercial suppliers. Results published by the Royal Society of Chemistry (2) showed that this peptide was readily dephosphorylated by Protein phosphatase 2A. Therefore we designed assays with the same degree of sensitivity. Furthermore, since we also showed that this peptide substrate was not subject to either endogenous algal phosphatase or protease activity, this substrate provides a simple alternative to glycogen phosphorylase without the loss of sensitivity (2).

4.3 Development of a non-radioactive toxicity test.

The second objective of this project was to investigate the possibility of developing a non-radioactive test which could be used more widely by water management laboratories without access to appropriate radioactivity handling facilities. A number of approaches were considered and investigated :

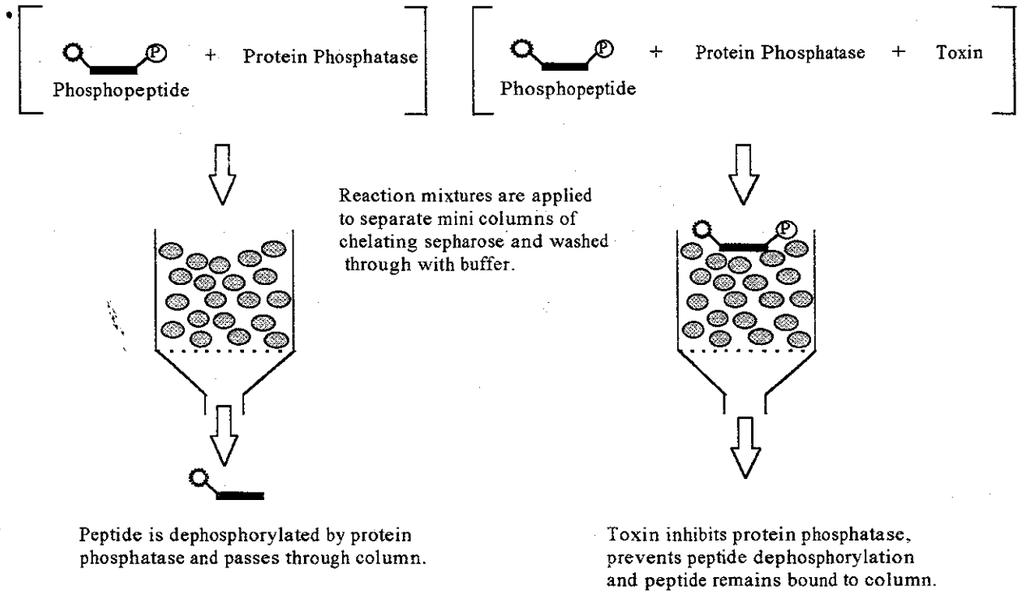
4.3.1 P-nitrophenol phosphate assay

This is a general phosphatase substrate which registers a colorimetric change when dephosphorylated. It is dephosphorylated by most protein phosphatases including alkaline and acid phosphatases. The latter preferentially dephosphorylate this substrate but are not sensitive to microcystin. They are widely distributed and likely to be present in most algal samples. For these reasons while potentially providing the basis for a simple colorimetric test, it was considered not suitable based on the likelihood of high background and significant false negatives. Furthermore, during the term of this project it was shown by my colleagues at the University of Dundee that such an assay was approximately 20-fold less sensitive than current procedures and more prone to non-specific errors.

4.3.2 Malachite Green Assay

This assay formed the basis for another UWRAA funded research project and was therefore not investigated. However, it was also considered unlikely to be satisfactory given that, although this test is identical to the radioactive one but does not use radioactivity, rather it measures the free phosphate released during the assay such that the high and variable background caused by high levels of phosphate in algal samples would render detection of phosphatase mediated changes in phosphate levels difficult. It is our understanding that this has proved to be the case.

4.3.3 Dye-tagged Peptide Substrate Assay



The method shown which was successfully developed and tested for the large part of this project involved the use of a phosphatase substrate which is tagged with a brightly coloured fluorescent marker. This method utilises iron chelate affinity chromatography which specifically binds phosphorylated peptides. The principle behind this assay is that inhibition of protein phosphatase by toxin would lead to higher levels phosphorylated peptide bound to the affinity matrix. The bound peptide can then be eluted from the matrix and the intensity of colour measured colorimetrically as an indication of the amount of toxin present. Furthermore, because the peptide remains bound to the matrix a visual representation of toxin presence was possible, suggesting that this approach may be utilised in the design of a field test.

GS1-12 peptide is commercially supplied in dye-tagged form as part of an assay kit for protein kinase C activity. Since we had shown the untagged GS peptide to be a suitable PP2A substrate (section 4.2.4), this particular dye-tagged substrate was investigated extensively. (The Protein kinase C assay involves the electrophoretic separation of phosphorylated and dephosphorylated forms and was considered inappropriate for development as a routine toxin assay. However, the kit did provide a ready source of peptide and it is now possible to purchase the peptide separately from the assay kit.). Results showed that the tagged peptide was readily dephosphorylated by both crude and purified preparations of protein phosphatase 2A and this could be prevented by microcystin.

While the sensitivity of the assay was similar (<1ug microcystin/l water) to conventional assays a number of problems with reproducibility were encountered. Firstly it was found that the peptide could not be fully phosphorylated by PKC. This led to a high background of

dephosphorylated peptide in all preparations. Secondly, although dephosphorylated by PP2A, the peptide was not an optimal substrate for it and therefore the colorimetric signal obtained was not maximal. In order to address these problems, a different dye-tagged peptide was synthesised. This peptide, which is known to be an optimal substrate for PP2A, was also synthesised as the fully phosphorylated form thereby removing the need for prior phosphorylation and minimising the background due to unphosphorylated peptide. This new peptide proved to be as efficient a substrate as glycogen phosphorylase (see figure 2) but time constraints prevented further investigation. It is therefore considered most appropriate for further development.

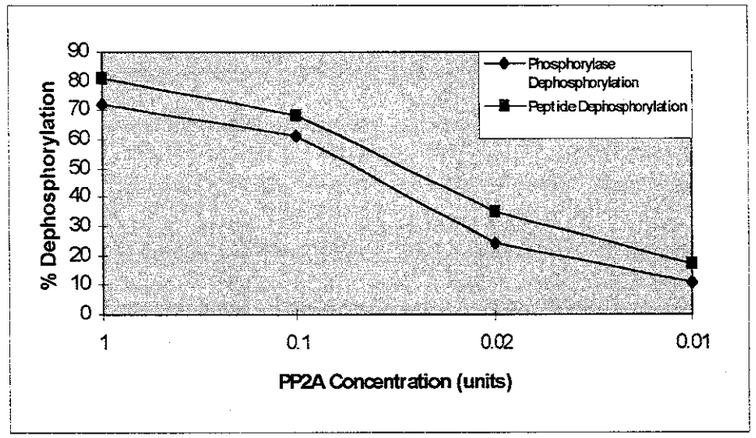


Figure 2 : Comparison of Dephosphorylation by purified PP2A of glycogen phosphorylase and a dye-tagged custom peptide . Glycogen phosphorylase dephosphorylation was measured by the standard radiolabel method and peptide dephosphorylation was measured using the procedure outlined in figure 1.

A second major problem encountered was the discovery that high phosphate concentrations effectively eluted the phosphopeptide from the column leading to false negative results. While this may be controlled in the laboratory, the presence of phosphate in water/algal samples presents a potential hazard for field analysis. This was effectively dealt with by desalting the reaction mixture, prior to application to the column, using C18 reverse phase columns. Subsequently it was found that the peptide could be eluted off the C18 column with methanol and applied directly to the chelating column. This allowed the development of a hybrid column containing both C18 and chelating sepharose facilitating a single step separation. Thus the basic methodology for a non-radioactive laboratory based toxin test has been established. Full development of this assay for routine laboratory quantitation of toxin levels was not an objective of this project and warrants further investigation. In particular, filters based on the chelating sepharose principle are now available commercially which may prove to be simpler to use in these assays.

4.4 Development of a field Test

It is our view that a field test would not serve to replace all other testing procedures but would simply be used to indicate toxin levels within defined ranges. Positive responses would necessitate further analysis in a laboratory. The dye-tagged peptide assay developed above has considerable potential for development into a field test. Investigations made during this project showed that visualisation of toxin presence without instrumentation was possible using the above test but that the sensitivity was necessarily reduced. This is because a higher level of substrate (therefore higher level of phosphatase and higher level of toxin) is needed to gain sufficient signal to be observed with the human eye. Nevertheless, microcystin levels of 10ug/l could be visualised. Furthermore, the use of fluorescent tags suggests that visualisation under UV light may increase the sensitivity. Recently, simple hand held UV illuminators have become available at reasonable cost ($100-$200) such that these may be utilised. Sufficient funds or time was not available to investigate this further.

It is our contention that only tests which can identify all potential protein phosphatase inhibitors will be useful in the long term, therefore assays have to be designed around the binding of toxin to protein phosphatases. Currently the only way this can be measured is by activity based assays, such as those in current use. Transferring laboratory based activity tests to the field raises problems of maintaining sources of active protein phosphatase in these tests. All preparations of protein phosphatase currently require storage at -20 degrees Celsius and only remain active for short periods of time at higher temperatures. Thus strict guidelines relating to storage, shelf life and handling of materials would need to be adhered to if such tests were to be reliable in the field.

Successful design and synthesis of a microcystin binding peptide would facilitate the design of a more robust and therefore reliable field assay. A second approach investigated briefly during this project was to define the microcystin binding site on protein phosphatase 1. This would potentially lead to the development of and assay which did not require the maintenance of phosphatase activity. Colleagues at the University of Dundee provided us with privileged information regarding their own work in this area. This allowed us to synthesise a peptide based on the putative binding site which might then be used to develop a competitive binding assay. Time constraints allowed only the synthesis of one peptide which ultimately proved ineffective at binding microcystin. However, the full microcystin binding site is now known (this site contains the peptide sequenced synthesised by us but is significantly longer) suggesting that design of a synthetic microcystin binding peptide is possible.

5 Conclusions and Recommendations

(a) The investigations carried out and discoveries made during this project illustrate the need for on-going research into the detection and quantitation of toxins which inhibit protein phosphatases. While relatively simple assays have been designed and made commercially available as a service to the industry, the discovery of new toxins and their variants and characterisation of their mechanisms of action will necessitate modification and, potentially, redesign of these tests.

(b) The radioactivity-based protein phosphatase inhibition assay developed during this project remains the most sensitive and simple method for detection of all toxins which inhibit protein phosphatases provided the modifications described here are adhered to.

(c) The non-radioactive protein phosphatase inhibition assay developed during this project provides a viable alternative to the above assay for laboratory based analysis. Further studies are necessary to achieve the same simplicity and sensitivity of the radioactive assay and to develop the procedure for routine use.

(d) The non-radioactive assay provides the basis for a field kit provided strict handling and storage conditions can be maintained. Further studies are necessary.

(e) The discovery of the microcystin binding site on protein phosphatase 1 raises very real possibilities of developing assays which do not require maintenance of enzyme activity. This should be investigated as a priority research area.

6 Publications

1. Sim, A.T.R & Mudge, L-M (1993) Toxicon 31, pages 1179-1186

2. Sim, A.T.R & Mudge, L-M (1994) In "Detection methods for Cyanobacterial Toxins". Royal Society for Chemistry. Eds GA Codd, TM Jeffries, CW Keevil & E Potter. Pages 100-105.

3. Murray, L., Mudge, L-M., Rostas, JAP., Sim, ATR & Capon RJ (1993) Aust. J. Chem 46, pages 1291-1294.

7 Acknowledgements

Excellent technical assistance with this project was provided by Ms Lisa-Maree Mudge and Ms Elizabeth Collins, to whom we are extremely grateful. We would also like to thank Dr Philip Robinson and Dr L Heilmeyer for the generous gifts of Protein Kinase C and phosphorylase kinase used in this project. Lastly, we are most appreciative of the financial and intellectual commitment of the Hunter Water Corporation to this project.

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105	Detection of Cyanobacterial Peptide Toxins by a Non-Radioactive Protein Phosphatase Inhibition Assay	J F Wheldrake A Bliney L Rosenberg
107	Model Guidelines for Domestic Greywater Reuse for Australia	B Jeppesen
109	Guidelines on the Quality of Stormwater and Treated Wastewater for Injection into Aquifers for Storage and Reuse	P Dillon P Pavelic
110	The Destruction of Cyanobacterial Peptide Toxins by Oxidants Used in Water Treatment	B Jeppesen
111	Application of Duckweed in Treating Municipal Wastewater	J Rositano
112	Alternative Overseas Water Treatment and Supply Practices	R A Leng
114	Predicting the Failure Performance of Individual Water Mains	K Mavin
	Environmental Management Guidelines for the Australian Water Industry	S Mills