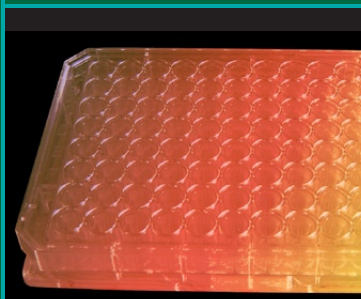




Development of an ELISA for Microcystins



Research Report

27

**COOPERATIVE RESEARCH CENTRE FOR WATER
QUALITY AND TREATMENT**

DEVELOPMENT OF AN ELISA FOR MICROCYSTINS

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FOREWORD

Development of an ELISA method for Microcystins

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CRC for Water Quality and Treatment Project No. 2.3.1.4 – Development of an ELISA for Microcystins

EXECUTIVE SUMMARY

An enzyme linked immunosorbent assay (ELISA) has been developed for the determination of all microcystins and nodularins and their fragments, based on an anti-Adda monoclonal antibody (AD4G2). By virtue of its approximately equal recognition of all variants tested, its monoclonal nature (which instills enhanced reproducibility), and its ability to detect fragments containing Adda, the AD4G2 antibody is currently the best option for estimating the upper limit of sample toxicity.

A robust assay was achieved through the development of a new “inter-washed” format of direct competitive ELISA in order to reduce sample matrix interference. The ELISA is sufficiently robust for analysis of samples over the pH range 3.6-10.2 and samples that contain high humic acid (50 mg/L) content or dissolved organic carbon levels of up to at least 20 mg/L, have salinity levels of up to 5 g/L, or concentrations of up to 0.5 mM copper or 7.5 mM calcium ions. Hence, use of the ELISA is appropriate for all natural waters apart from sea water. It is also able to withstand low solvent concentrations (up to 15% methanol or 5% acetonitrile) in the sample. For calcium ions and acetonitrile, there was no measureable benefit for the inter-washed format over the standard format.

ELISA is predominantly a screening test and can be used to reduce the need for more expensive and time consuming analyses. The results of this study show that the assay is sensitive, having a limit of detection of 0.02 µg/L, and that rough quantification can be made in the microcystin concentration range 0.05 to 5 µg/L. The assay is most accurate around the midpoint of 0.1 µg/L (coefficient of variation of 10 – 14 %, across 10 – 11 plates). The accuracy of the assay between 0.1 and 1 µg/L is ideal for testing samples against guideline upper limits for drinking water (eg. 1 µg/L microcystin-LR).

The performance of the ELISA was evaluated using spiked raw water (dissolved organic carbon content of 20 mg/L). Within the concentration range of 0.01 to 10 µg/L microcystin-LR, the concentration determined by ELISA was between at least 60 and 140% of the actual concentration. Clean-up of samples was shown to be unnecessary for this assay.

The performance of the ELISA was also compared with that of the protein phosphatase inhibition assay (PPIA), high performance liquid chromatography with photo diode array detection (HPLC/PDA), and HPLC with both PDA and mass spectrometric detection (HPLC/PDA/MS). Using spiked raw water samples, while HPLC gave an under-estimation of microcystin at low concentrations (< 0.4 µg/L), both ELISA and PPIA gave an over-estimation of microcystin at low concentrations, and an under-estimation at high concentrations (> 10 µg/L).

Finally, the extracellular and intracellular microcystin concentrations of five cultured *Microcystis* strains were determined and compared between the methods. The ELISA had 2-fold greater sensitivity than the PPIA method used. In general ELISA determined more microcystin than the other methods, as expected. The results suggested that false positives are likely to occur at low concentrations. However, when values of 0.1 µg/L and above are obtained, the likelihood of a false positive is very small and further action should be taken to verify the microcystin variants present using techniques such as HPLC/PDA/MS.

The reproducibility of the assay could be further enhanced, and the probability of obtaining false positives minimised, by implementing an improved data processing method in the future. For sample matrices besides water, a full assessment of the performance of this assay for determining microcystins, nodularins and their fragments is still required.

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ABBREVIATIONS

A ₁	maximum absorbance signal
A ₂	minimum absorbance signal
AD4G2	anti-6 <i>E</i> -Adda monoclonal antibody
Adda	(2 <i>S</i> ,3 <i>S</i> ,8 <i>S</i> ,9 <i>S</i>)-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ALOD	‘absorbance‘ limit of detection
BLOD	‘blank‘ limit of detection
BSA	bovine serum albumin
CaCl ₂	calcium chloride
CuSO ₄	copper sulfate
CV	coefficient of variance (%)
DOC	dissolved organic carbon
EDTA	ethylenediamine tetraacetic acid, disodium salt
ELISA	enzyme linked immunosorbent assay
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
iNOS	inducible nitric oxide synthase
LOD	limit of detection
Mab	monoclonal antibody
MS	mass spectrometry
m/z	mass/charge ratio
NaCl	sodium chloride
NO	nitric oxide
PAC	poly(acrylic) acid
PBS	phosphate buffered saline
PDA	photo-diode array
PPIA	protein phosphatase inhibition assay
PVA	poly(vinyl alcohol)
SPE	solid phase extraction
TMB	3,3',5,5'-tetramethylbenzidine
x ₀	midpoint

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INTRODUCTION

1.1 Microcystins

Cyanotoxins are toxins produced by cyanobacteria, which are found throughout the world, including Australia. Acute exposure to these toxins through ingestion of contaminated water has caused sickness in humans and animals, and in severe cases can lead to death. Concerns over chronic exposure to cyanotoxins are increasing pressure for strict regulatory guidelines.

Microcystins (MCs) are the most commonly found and widely studied group of cyanotoxins. MCs are cyclic heptapeptides (Figure 1) whose structural variations arise from their amino acid content, or group substitution within the amino acids (Sivonen and Jones, 1999). Structural variations include the presence of various L-amino acids at the 2- and 4- positions and the presence or absence of methyl groups at the β -Me-Asp and *N*-methyldehydroalanine (Mdha) residues. Currently over 60 different MC variants have been identified (Sivonen and Jones, 1999; Zeck *et al.*, 2001b). The most commonly found variant is microcystin-LR (MC-LR), which contains L-leucine and L-arginine at the 2- and 4- positions (Figure 1). Blooms tend to comprise a number of cyanobacterial strains, each of which can produce a number of MC variants. Hence, samples collected during a bloom often contain more than one variant.

Another group of cyanotoxins, the nodularins, are pentapeptides that are structurally similar to the MCs and around five variants have been identified (Fischer *et al.*, 2001). Both MCs and nodularins contain the unusual amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda).

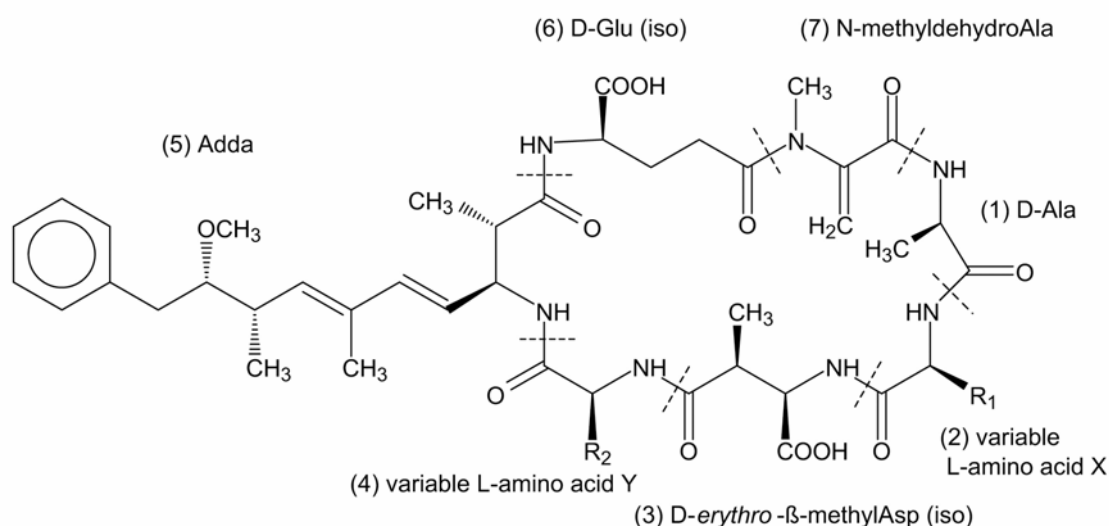


Figure 1: General structure of microcystins

MCs are hepatotoxins that act at very low concentrations (ng/L) especially through repeated exposure. They are potent tumour promoters, that lead to hepatic necrosis and haemorrhage, and cause apoptosis (Baier *et al.*, 2000). On a molecular level MCs inhibit protein phosphatase 1 and 2A, modulate induction of iNOS and therefore NO synthesis, and lead to DNA damage and fragmentation (Baier *et al.*, 2000).

The toxicity of the cyclic peptide toxins has been associated with the 6(*E*)-form of Adda (Harada *et al.*, 1990a,b; Tsuji *et al.*, 1995). Approximately 15% of the known MCs contain Adda moieties that are modified by the presence of a hydroxyl or acetoxy (instead of a methoxy) group at C-9 (Fischer *et al.*, 2001). Although such variants are usually of similar toxicity to the Adda-containing homologues, they are not common constituents of cyanobacteria and usually co-occur with the more common and abundant unmodified Adda-derived variants (Fischer *et al.*, 2001).

1.2 Analysis of microcystins

There are several techniques currently available to quantify MCs in water and cyanobacterial samples, however, they each have their own disadvantages. These disadvantages include the use of expensive equipment, time consuming and labour intensive sample preparations, large sample sizes required, inability to determine individual variants, and the possibility of interference from other compounds in the sample.

Here the problems associated with the three main types of analytical procedure used for MC quantification are described.

a) High Performance Liquid Chromatography (HPLC)

The method used most frequently for MC and nodularin determination is high performance liquid chromatography (HPLC) with photo-diode array (PDA) detection. Although HPLC has the advantage of providing toxin profiles in addition to quantifying toxins, the method involves both skilled labour and expensive equipment - two factors that restrict its use to analytical laboratories. The identification of sample components as toxins involves human interpretation of spectral information. This may lead to mis-identification and erroneous results, especially when toxin concentrations are low and spectral quality is poor.

When compared with the other methods described below, HPLC has the lowest sensitivity with a 1000-fold difference in detection limit. Sample pre-treatment can be performed to extract and concentrate toxins to increase the sensitivity of the procedure. The use of cartridges for sample clean-up prevents interferences from matrix components. However, such sample treatment processes are time consuming and not all toxin variants are necessarily 100% recoverable. In addition, identification of specific MC variants is limited by the small number of analytical standards currently available. Results are usually determined by reference to a MC-LR standard and are expressed as MC-LR equivalents.

In addition, chromatographic methods are not well suited for estimation of toxicity arising from the presence of low levels of several variants because the concentrations of the individual components may be below the limits of detection. Thus toxin content and hence toxicity can be underestimated.

HPLC with mass spectrometric (MS) detection increases the reliability of results since characteristic MC mass fragmentation patterns confirm the presence, and in some cases, the identity of microcystins. This provides for greater confidence in the results obtained.

b) Protein phosphatase inhibition assay (PPIA)

A second analytical method for MC and nodularin determination is a colorimetric protein phosphatase inhibition assay (PPIA). This method is substantially more sensitive than HPLC and samples can be analysed without pre-concentration. The assay is rapid and the equipment required is substantially cheaper than for HPLC. Unlike HPLC, PPIA does not identify the number of individual variants present.

In PPIA inhibition varies with individual variants in a way that is very roughly proportional to their mammalian toxicity. Consequently, the results of this assay are often considered to be an approximation of toxicity, which is very useful information. However, the reports of several workers (An and Carmichael, 1994; Heresztyn and Nicholson, 1999, 2000) indicate that the reactivity of toxin variants only roughly mirrors variant toxicity (An and Carmichael, 1994; Watanabe *et al.*, 1989). Swoboda *et al.* (1994) suggested that the relative differences between toxicity by mouse bioassay and PPIA activity might be due to differences in differential *in vivo* activity and/or cell permeability.

MC-LR is used as a standard and results are expressed in MC-LR equivalents. Other MC variants can be stronger or weaker inhibitors compared with MC-LR and thus an overestimation or underestimation of toxin concentration can result.

Matrix effects may lead to false positive results that require further evaluation. In addition, since inhibition of protein phosphatase is not specific to the MCs and nodularins, other inhibitors in the sample may give false positive results. The presence of such interfering compounds in water samples, however, is unlikely in most circumstances.

c) Enzyme linked immunosorbent assay (ELISA)

A third analytical procedure used is the enzyme linked immunosorbent assay (ELISA). ELISAs are substantially more sensitive than HPLC and may be more sensitive than PPIA. Like PPIA, ELISA is rapid and samples can be analysed without pre-concentration. It also requires similar equipment to PPIA. Unlike HPLC, ELISA does not identify the individual variants present. The specificity of the antibodies used in ELISA eliminates the possibility of other sample components, such as phosphatase inhibitors that interfere with the PPIA, causing false positives. Nevertheless, matrix interference has been reported in the analysis of cellular (Nagata *et al.*, 1997) and water (Rivasseau *et al.*, 1999) samples with ELISA, resulting in low levels of precision at low MC concentrations and in false positives.

Immunoassays, such as ELISAs, overcome the main difficulties of chromatographic methods, such as the need for extensive sample preparation and the high cost of analyses. Another benefit of ELISA is the absence of human interpretation. ELISAs are very sensitive, relatively cheap, do not require a high level of operator expertise, do not require sophisticated instrumentation and many analyses can be run simultaneously.

Many immunoassay for microcystins have been developed using either monoclonal (Mab) or polyclonal (Pab) antibodies (Table 1). More recently recombinant antibody fragments (Scab) have been isolated from phage display libraries. However, the antibodies isolated to date by this technique have not been as sensitive as the polyclonal and monoclonal antibodies already

available. A number of ELISA kits are now commercially available (Abraxis, Beacon Analytical Systems, Elisa Systems, Envirologix, Strategic Diagnostics Inc., Wako Pure Chemical Ind. Ltd).

Direct competitive (Chu *et al.*, 1989; Zeck *et al.*, 2001b), indirect competitive (Baier *et al.*, 2000; Fischer *et al.*, 2001), and sandwich immunoassay (Brooks and Codd, 1988; Nagata *et al.*, 1999) formats have been utilised. The performance of a number of assay formats has been compared using the same antibody (Weller *et al.*, 2001).

ELISA theoretically determines only the toxin molecule against which the antibodies have been generated, however, there is generally some detection of molecules with similar structures. Up until now, the major problem associated with the ELISAs available for MC and nodularin determination has been the unequal cross-reactivities of toxin variants leading to inaccuracies in quantification. This problem has arisen from available systems incorporating antibodies that have been raised against only one of the toxin variants, usually MC-LR (Table 1), with recognition of all the other toxins relying on their similarity in structure to that one variant. Consequently, these antibodies tend to be sensitive to a relatively narrow range of variants and are unlikely to give a true reflection of the total MC concentration present. The difference in cross-reactivities generally results in an underestimation of toxin concentration as some variants have poor cross reactivities compared with MC-LR, the variant against which antibodies have generally been raised. False negatives may also occur in the presence of toxic variants to which the assay is insensitive. However, if the MC-LR equivalent concentrations determined are used as a measure of toxicity, then an overestimation of toxicity may occur if non-toxic variants have significant cross-reactivities with the antibody (An and Carmichael, 1994).

Many studies have been undertaken comparing the performance of the ELISAs developed using these antibodies with PPIA and HPLC (Nagata *et al.*, 1997; Metcalf *et al.*, 2000a; Lawrence *et al.*, 2001; Mikhailov *et al.*, 2001a; Rapala *et al.*, 2002). These antibodies have also been used in a time-resolved fluoro-immunometric assay (Mehto *et al.*, 2001) and in a colorimetric immuno-protein phosphatase inhibition assay (Metcalf *et al.*, 2001). In the most sensitive method reported to date, a detection limit of 2 ng/L MC-LR has been achieved using one particular Mab (M8H5) and a new type sandwich immunoassay (Nagata *et al.*, 1999; Tsutsumi *et al.*, 1998, 2000).

1.3 Generic ELISA for Microcystins

As mentioned above, ELISA is unable to detect individual variants (each with their own toxicity), and hence, an exact measure of toxicity cannot be obtained by ELISA. In order to ensure safety, an upper limit of toxicity could be calculated using the toxicity of the most toxic variant and the total MC concentration, providing all variants are recognised equally. Indeed, the major problem associated with the ELISAs available has been the unequal cross-reactivities of toxin variants leading to inaccuracies in quantification. An excellent summary of the cross-reactivities of antibodies for MC analysis can be found in Metcalf *et al.* (2003).

Table 1. Antibodies raised for the detection of microcystins

MC variant or fragment used ^a	Type of Antibody ^b	Comments on cross-reactivity	Reference
Adda	Mab (AD4G2)	Good for all tested	Zeck <i>et al.</i> , 2001b
Adda	Pab (AB824)	Good for all tested	Fischer <i>et al.</i> , 2001
LR	5 Mab, 1 Pab		Mikhailov <i>et al.</i> , 2001a
LR	Pab		An and Carmichael, 1994
LR	Pab		Brooks and Codd, 1988
LR	Pab	Low for LA and LY	Chu <i>et al.</i> , 1989
LR	Pab		Metcalf <i>et al.</i> , 2000a
LR	Pab	Low for RR, YR and nodularin	Liu <i>et al.</i> , 1996
LR	Scab	Low for RR and LW	McElhiney <i>et al.</i> , 2000
LR	6 Scab	Low for LW and LF	McElhiney <i>et al.</i> , 2002
LR	Scab		Strachan <i>et al.</i> , 2002
LR	Mab		Liu <i>et al.</i> , 2000
LR	Pab		McDermott <i>et al.</i> , 1995
LR	4 Mab		Saito <i>et al.</i> , 1994
LR	6 Mab	Good for all tested (with M8H5)	Nagata <i>et al.</i> , 1995
LR	Pab		Yu <i>et al.</i> , 2002
LR	Mab	Good for 4-arginine MCs	Zeck <i>et al.</i> , 2001a
LR and RR	Pab		Baier <i>et al.</i> , 2000
Nodularin	Mab	Specific to nodularin	Mikhailov <i>et al.</i> , 2001b
LA	Mab		Kfir <i>et al.</i> , 1986a,b

^a Used for conjugating to a protein to form the immunogen. ^b Polyclonal (Pab), monoclonal (Mab), single chain antibody fragment (Scab).

The characteristic feature of both MCs and nodularins is the hydrophobic β -amino acid, Adda (4*E*,6*E*-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). The Adda amino acid is specific to, and present in, almost all of the known toxic MC and nodularin variants. The only exceptions are variants that have minor differences in the groups attached to the double bonds of Adda. These variants occur infrequently and are minor components of

the toxin mixtures. Consequently, an antibody raised against Adda offers potential for equal recognition of most variants. The double bonds of the Adda moiety are also associated with the toxicity of these compounds (Harada *et al.*, 1990a,b). An antibody raised against Adda may also be useful in detecting MC fragments; degradation products and precursors.

Through collaboration with the Technical University of Munich, a monoclonal antibody against Adda (AD4G2) was successfully produced and exhibited good cross-reactivity to a range of MCs (Zeck *et al.*, 2001b). The AD4G2 antibody binds essentially any molecule containing an Adda moiety, including Adda itself.

A similar approach has recently been reported, where a polyclonal antibody (AB824) has been raised against Adda and incorporated into an ELISA (Fischer *et al.*, 2001). However, limited material is available to evaluate the performance of this assay.

Variation in the cross-reactivity pattern is a major drawback of all but three antibodies (AD4G2, AB824 and M8H8) listed in Table 1. Hence, these three antibodies are recommended for use in screening assays in order to detect all MC variants present. The monoclonal antibody raised by Nagata *et al.* (1995) (M8H5) has shown good cross-reactivity to all variants tested (Nagata *et al.*, 1997, Weller *et al.*, 2001), but is unlikely to detect Adda or Adda containing fragments. The monoclonal (AD4G2) and polyclonal (AB824) antibodies raised against Adda, the moiety common to MCs and nodularins (see Section 1.3), have also shown excellent cross-reactivities to a range of MC variants (Zeck *et al.*, 2001b, Fischer *et al.*, 2001). Since AD4G2 and AB824 were raised against Adda they may also recognise MC fragments; degradation products and precursors. It should be noted that in a recent study comparing four ELISAs, detoxified MC-LR conjugation products were shown to have greater reactivity than MC-LR (Metcalf *et al.*, 2002) while they were less toxic than MC-LR.

Considering the cross-reactivities of all antibodies currently available for MC analysis (Metcalf *et al.*, 2003), AD4G2 demonstrates the best performance for the purpose described above. It shows the most equal recognition of the MC variants tested with midpoints of 0.23 - 0.9 $\mu\text{g/L}$, in comparison to 0.37 – 1.22 $\mu\text{g/L}$ for AB824 and 0.12 – 3.16 $\mu\text{g/L}$ for M8H5, and according to these values also has the greatest sensitivity of the three antibodies (which is further enhanced in the study described by this report). In addition, it should be noted that the reproducibility of assays based on polyclonal antibodies will be inherently poorer than the one reported here as only monoclonal antibodies can be produced with constant properties over many years.

The AD4G2 antibody has been incorporated into a generic MC ELISA and optimisation of this assay, for the determination of MCs and nodularins in water samples, is described in this report. Optimisation involved the development of a new “inter-washed” assay format. The robustness of the assay was tested and the assay’s performance was compared with other currently available methods in a validation study with raw water samples, cyanobacterial culture media, and cyanobacterial cells.

EXPERIMENTAL

General

2.1 Materials

High purity UV treated water (Millipore, Milli-Q Water Gradient, Ultrapure Organex cartridge, 18.2 MΩcm) was used in the preparation of all solutions unless otherwise specified. The supplier and purity of all chemicals used is given in Appendix II.

2.2 Preparation of microcystin standard solutions

MC-LR (purity > 98% by HPLC) was purchased from Biomol (Plymouth Meeting, PA, USA). MC-LF and MC-LW were purchased from Alexis (Läufelfingen, Switzerland) as ≥ 95% pure by HPLC (238 nm). MC-RR, MC-YR, and nodularin were purchased from Calbiochem (La Jolla, CA, USA) as ≥ 95% pure by HPLC. Adda was provided by Drs. D. Cundy and T. McCarthy (CSIRO Molecular Science, Clayton South, Victoria, Australia). The synthesis of Adda and Adda-containing dipeptides is described by Cundy *et al.* (1998; 1999). The synthesis of *N*-Ac-Adda is described by Zeck *et al.* (2001a).

Since MCs in aqueous solutions are known to adsorb to plastics (Hyenstrand *et al.*, 2001a,b; Metcalf *et al.*, 2000b), all MC solutions were prepared and stored in glass. Nevertheless, for convenience and consistency, plastic pipette tips were used for transferring all solutions to microtitre plate wells and HPLC vials. This did not lead to underestimation of MC concentrations, since plastic pipette tips were also used for the MC calibration solutions. Brown glass vials were used to minimise light degradation. All glass vials (~ 30 mL), glass pipettes, glass syringes, vial caps and stirrer bars, were thoroughly washed with hypochlorite solution, water, high purity alcohol, and water, prior to preparation of the MC standard solutions.

MC stock solutions were prepared by injection of high purity methanol (to a concentration of 0.1 - 1 g/L) into the supplied vial of toxin and dissolution by sonication in a water bath (FX10, Ultrasonics, Australia) for 2 x 30 min. These solutions were diluted with water to give solutions of approximately 10 mg/L which were subsequently diluted to 1 mg/L. All variants should be soluble in water at 1 mg/L. The concentrations of these aqueous solutions were determined spectrophotometrically at 238 nm using a molar absorptivity value of 39,800 M⁻¹ cm⁻¹ (Harada *et al.*, 1990a). An accurate working standard solution containing 100 µg/L MC was then prepared from the 1 mg/L solution. This was used to prepare a set of aqueous standard working solutions (0, 0.0001 – 100 µg/L) for each variant, by serial dilution, which were stored at 4°C.

2.3 Antibody robustness

The stability of the anti-Adda monoclonal antibody (Mab, AD4G2) was tested using a direct ELISA without competition (*ie.* no MC added).

Plates were pre-coated with anti-mouse IgG (Fc-specific) antibody (affinity purified liquid diluted 1:5 000, 250 µL/well) by incubation (overnight at room temperature with shaking).

Following three washes with washing buffer (see Appendix III), the plates were coated with anti-Adda-Mab (~ 20 mg/L, 1:500 dilution, 200 µL/well) by incubation (> 4h at room temperature with shaking). Following another three washes with washing buffer, 20 µL/well Tris buffer (1M containing 1% EDTA, 1.5 M NaCl and 1% bovine serum albumin (BSA)) was added, followed by 200 µL of the solution to be tested (Table 10), water or PBS (4 replicate wells of each) and incubation (30 min, at room temperature with shaking). After another three washes with washing buffer, tracer (1.7 g/L (Tracer I), diluted 1:100,000 in PBS buffer, 200 µL/well) was added to the aspirated wells and incubated (30 min). Plates were developed and measured as outlined in Figure 12.

Sample preparation

2.4 Sample sources

Water

Pure water was obtained from a Milli-Q water system (Millipore, Milli-Q Water Gradient, Ultrapure Organex cartridge, 18.2 MΩcm). Tap water was used as the treated water sample. Raw water was obtained from the Warren Reservoir (Barossa Valley, South Australia). These water samples were filtered through glass microfibre (GF/C, 90 mm) filter papers under vacuum to remove particulates. A set of eight MC-LR standards (0, 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µg/L) were then prepared in each of the three waters (using the method in Section 2.2). The dissolved organic carbon (DOC) concentration of the pure, treated and raw water samples were determined using a Portable Total Organic Carbon analyser (Sievers 820, Sievers Instruments Inc., USA).

In addition, 2.69 L of filtered raw water was spiked with the 10 mg/L aqueous solution of MC-LR to give a final concentration of 0.4 µg/L. Serial dilutions of this solution were made to yield solutions containing 0.1 (2.64 L) and then 0.03 µg/L (2.02 L). Un-spiked raw water (2.02 L) was also retained as a 'blank' for analysis. Two 1 L samples were taken from each solution for concentration (see Section 2.5) and the remainder was retained for analysis as the 'original' solution.

Cyanobacteria

Five *Microcystis aeruginosa* strains were selected from the Australian Water Quality Centre's culture collection to provide samples containing a range of microcystin variants. MIC040C was isolated from an Eden Valley, South Australia, farm dam, MIC050D and MIC050G were isolated from the Torrens Lake, Adelaide, MIC309A was isolated from the Mount Bold Reservoir, South Australia, and MIC325B (CCAP 1450/1) was from the Culture Collection of Algae and Protozoa (Cumbria, England).

These strains were cultured in ASM-1 media (Appendix I) in an incubator (Contherm, Digital Series, Biocell Incubator) on a 12 hour day/night cycle at 25°C and a light intensity of 50 µmol photons m⁻² s⁻¹. A 1 mL aliquot in 20mL ASM-1 was initially used, followed by scale-up to 200 mL (after 1 week) and then to 2L once a green colour developed. Cultures were harvested approximately 1 month after the initial culture was prepared.

Once each 2 L culture had grown sufficiently, three 200 mL and three 400 mL aliquots were filtered through microfibre GF/C (90 mm) filter papers and each of the six filtrates was made up to 1 L with water and retained as media samples (containing extracellular toxin). Exact volumes after filtration were noted. Those samples arising from the initial 200 mL were found to contain sufficient toxin for measurement, so the 400 mL samples were not required. A cellular sample (containing intracellular toxin) was retained on filter papers for each strain cultured. The filter papers were stored in plastic vials at 4°C.

2.5 Concentration of spiked raw water and cyanobacterial culture media samples

Sample clean-up and MC concentration was performed by the following method for both the spiked raw water samples, and the cyanobacterial culture media samples containing extracellular toxin.

Solid phase extraction C₁₈ cartridges (Waters, Sep-Pak®, Vac 3cc) were conditioned with 2 cartridge volumes of methanol, followed by 5 cartridge volumes of water. Samples (1 L) were then passed through the cartridge at a flow rate of approximately 5 mL/min. The cartridge was not allowed to dry out before the entire sample had been passed through. Cartridges were then washed with 6 cartridge volumes of water, 5 cartridge volumes of 10% methanol in water, and 4 cartridge volumes of 20% methanol in water. Each cartridge was air-dried for 5 min under vacuum. MCs were eluted dropwise with 3 cartridge volumes of 90% methanol in water. The eluate was collected in a 12 mL glass sample tube, and evaporated to dryness under nitrogen on a heating block at 45°C. The inside of the tube was washed down with approximately 2 mL of methanol during the evaporation process, to minimise toxin losses and improve the yield. The residue was reconstituted in 150 µL of methanol, vortexed for 1 min, and then 100 µL of water was added and vortexed for a further minute. Each final solution was placed in a disposable syringe and filtered through a 0.45 µm PVDF membrane filter (Gelman Laboratory, Acrodisc LC 13 mm syringe filter) into a 2 mL solid capped brown glass vial for storage at 4°C.

2.6 Preparation of cyanobacterial cellular extracts

Intracellular toxins were extracted from each cellular sample retained on the filter papers (see Section 2.4) by the following method. The filter papers were cut up into approximately 5 mm x 5 mm squares, placed in plastic screw top vials, to which 5 mL methanol was added, and left in the freezer overnight at -18°C. The methanol was then decanted into a 10 mL glass tube, and the paper pieces were re-extracted with an additional 5 mL of methanol and vortexed for 1 min. The two extracts were combined and evaporated to dryness under nitrogen at 45°C. The inside of the tube was washed down with approximately 2 mL of methanol during the evaporation process, to minimise toxin losses and improve the yield. The residue was reconstituted in 500 µL of methanol, vortexed for 30 sec, and then 500 µL of water was added and vortexed for a further 30 sec. Each final solution was placed in a disposable syringe and filtered through a 0.45 µm PVDF membrane filter (Gelman Laboratory, Acrodisc LC 13 mm syringe filter) into a 2 mL solid capped brown glass vial for storage at 4°C.

2.7 General

All vials and caps used in the preparation of samples were soaked in methanol and washed several times with deionised water prior to use.

The concentrated spiked raw water and culture media samples (Section 2.5) and cellular extracts (Section 2.6) were placed in plastic HPLC vial inserts and used directly for HPLC/PDA and HPLC/PDA/MS analysis. Three HPLC/PDA replicate injections were made for the cellular extract (intracellular) samples, while one injection of each of triplicate culture media (extracellular) samples and duplicate spiked raw water samples were made.

Based on the HPLC results, these samples were diluted with pure water to a total MC concentration that was within the working range of both PPIA and ELISA. The precision of these methods is greatest close to their midpoints, hence, the optimal dilution concentrations for ELISA and PPIA were approximately 0.12 and 0.28 µg/L, respectively. For convenience, and based on the observation (Hyenstrand *et al.*, 2001b) that smaller percentage losses occur with higher concentration solutions (< 1% loss with MC concentrations > 0.15 µg/L), dilution steps were performed using plastic pipette tips. Solutions were stored in solid capped brown glass vials at 4°C. All samples (spiked raw water, and extracellular and intracellular cyanobacterial samples) were analysed in five replicate wells for both ELISA and PPIA.

Optimisation of ELISA

2.8 Zeck's ELISA procedure

Microtitre plates (96-well, Greiner bio-one, Microton, flat-bottom, high-binding) were coated overnight at room temperature with 250 µL anti-mouse IgG (Fc-specific) serum diluted 1:3000 in carbonate buffer (40 mM, pH 9.6). After washing (3 x) with PBS (pH 7.6, 0.007 M phosphate, containing 0.015 M NaCl and 0.05% Tween 20), the wells were incubated with the diluted cell culture supernatant of monoclonal antibody AD4G2 (200 µL, *ca.* 12 µg/L Mab in PBS (pH 7.6, 0.08 M sodium phosphate, 0.15 M NaCl)) for 3 h at room temperature. After a further wash (3 x), 20 µL per well PBS buffer (pH 7.6, 0.8 M phosphate, containing 1.5 M NaCl) was added and subsequently MC standards in the concentration range from 0.0001 to 100 µg/L or samples (200 µL per well) were added to the plate. After incubation of the plate for 1 h, peroxidase tracer (*N*-Ac-Adda-HRP conjugate, 50 µL per well, 0.2 mg/L in PBS buffer (pH 7.6, 0.08 M sodium phosphate, 0.15 M NaCl)) was added and the plate incubated for 15 min in the dark. The plate was subsequently washed (3 x) and substrate solution (0.15 g/L tetramethyl benzidine and 0.004% hydrogen peroxide in citrate buffer (pH 3.8, 200 mM), 200 µL per well) was added. After a suitable development time (up to 30 min in the dark) the reaction was stopped with dilute sulfuric acid (5%, 100 µL per well) and the absorbance was measured at 450 nm (reference wavelength 620 nm) with a microplate reader (Model MRX, Dynex Technologies Ltd). Since the development reaction is temperature dependent, some adjustment in development time between 20 and 30 min is required.

This ELISA protocol gave a midpoint of 0.33 µg/L and a limit of detection of 0.07 µg/L (Zeck *et al.*, 2001b) and was used as a starting point for optimisation of the generic (Adda) MC ELISA. The ELISA was optimised for sensitivity and robustness in order to lower the

assay's detection limit and range of quantification, and to minimise interferences from the sample matrix.

2.9 Optimised standard direct competitive ELISA procedure

Microtitre plates (96-well, Greiner bio-one, Microlon, flat-bottom, high-binding) were coated overnight at room temperature with 300 μL anti-mouse IgG (Fc-specific) serum diluted 1:3000 in carbonate buffer (40 mM, pH 9.6). After washing (3 x) with PBS (pH 7.6, 0.008 M potassium phosphate, containing 0.015 M NaCl and 0.05% Tween 20), the wells were incubated with the diluted cell culture supernatant of monoclonal antibody AD4G2 (200 μL , *ca.* 25 $\mu\text{g/L}$ Mab in PBS (pH 7.6, 0.08 M phosphate, 0.15 M NaCl)) for 4 h at room temperature. After a further wash (3 x), 20 μL per well Tris buffer (pH 7.4, 1 M containing 4% EDTA, 1.5 M NaCl, and 1% BSA) was added, and subsequently MC standards in the concentration range from 0.0001 to 100 $\mu\text{g/L}$ or samples (200 μL per well) were added to the plate. After incubation of the plate for 2 h, peroxidase tracer (microcystin-LR-HRP conjugate, 50 μL per well, 55 $\mu\text{g/L}$ in Tris buffer (pH 7.4, 0.1 M, containing 0.4% EDTA, 0.15 M NaCl and 0.5% BSA)) was added and the plate incubated for 15 min in the dark. The plate was subsequently washed (3 x) and substrate solution (0.15 g/L tetramethyl benzidine and 0.004% hydrogen peroxide in citrate buffer (pH 3.8, 200 mM), 200 μL per well) was added. After a suitable development time (up to 30 min in the dark) the reaction was stopped with dilute sulfuric acid (5%, 100 μL per well) and the absorbance was measured at 450 nm (reference wavelength 620 nm) with a microplate reader (Model MRX, Dynex Technologies Ltd).

2.10 Optimised inter-washed direct competitive ELISA procedure

Microtitre plates (96-well, Greiner bio-one, Microlon, flat-bottom, high-binding) were coated overnight at room temperature with 300 μL anti-mouse IgG (Fc-specific) serum diluted 1:3000 in carbonate buffer (40 mM, pH 9.6). After washing (3 x) with PBS (pH 7.6, 0.008 M potassium phosphate, containing 0.015 M NaCl and 0.05% Tween 20), the wells were incubated with the diluted cell culture supernatant of monoclonal antibody AD4G2 (50 μL , *ca.* 25 $\mu\text{g/L}$ Mab in PBS (pH 7.6, 0.08 M phosphate, 0.15 M NaCl)) for 4 h at room temperature. After a further wash (3 x), 50 μL per well Tris buffer (pH 7.4, 0.5 M containing 2% EDTA, 0.75 M NaCl, and 0.5% BSA) was added, and subsequently MC standards in the concentration range from 0.0001 to 100 $\mu\text{g/L}$ or samples (200 μL per well) were added to the plate. After incubation of the plate for 2 h at room temperature the plate was washed (2 x). Peroxidase tracer (microcystin-LR-HRP conjugate, 50 μL per well, 100 $\mu\text{g/L}$ in Tris buffer (pH 7.4, 0.1 M containing 0.4% EDTA, 0.15 M NaCl and 0.5% BSA)) was added and the plate incubated for 5 min in the dark. The plate was subsequently washed (3 x) and substrate solution (0.15 g/L tetramethyl benzidine and 0.004% hydrogen peroxide in citrate buffer (pH 3.8, 200 mM), 200 μL per well) was added. After a suitable development time (up to 30 min in the dark) the reaction was stopped with dilute sulfuric acid (5%, 100 μL per well) and the absorbance was measured at 450 nm (reference wavelength 620 nm) with a microplate reader (Model MRX, Dynex Technologies Ltd).

2.11 Optional plate-protection procedure

Initially a range of poly(vinyl alcohol)s (PVAs, with average M_w of 9,000 – 10,000, 22,000 and 49,000) and bovine serum albumin (BSA) (1% solutions) were tested in both Tris (1 M containing 1% EDTA and 0.05% Tween 20) and PBS (0.8 M containing 2% EDTA and 0.05% Tween 20) buffers. Combinations of 0.5% PVA and 0.5% BSA were also tested in each buffer.

Tris buffer (1 M containing 1% EDTA and 0.05% Tween 20) was then used for testing the effectiveness of nine protection solutions after microtitre plate storage at 44.5°C for 6 days. The tested solutions were: (i) 5% poly(vinyl alcohol) (average M_w 9,000 – 10,000, 80% hydrolysed), (ii) 5% poly(ethylene glycol) (average M_n ca. 8,000), (iii) 5% sucrose, (iv) 5% mannitol, (v) 5% bovine serum albumin, (vi) 5% casein, (vii) 1% poly(vinyl alcohol), (viii) 1% bovine serum albumin, and (ix) 0.5% poly(vinyl alcohol) plus 0.5% bovine serum albumin.

Between the antibody coating and sample incubation steps plates may be protected and stored for at least 3 months at 4°C by the following procedure.

After washing (3 x) a solution containing 0.5% polyvinyl alcohol (average M_w 9,000 – 10,000, 80% hydrolysed) and 0.5% BSA in Tris buffer (0.1 M containing 0.4% EDTA and 0.15 M NaCl) was added (300 μ L per well). The solution was immediately tipped out, the plate tapped to remove any excess liquid from the wells, and left to dry at room temperature overnight in the dark. Protected plates were then stored at 4°C with desiccant (silica gel).

It was also noted that plates coated with IgG solution may be stored at 4 °C for at least a week before use.

Testing of ELISA

2.12 ELISA robustness

For testing the ELISA's robustness the sample incubation step of the ELISA procedure was modified slightly. Rather than addition of 200 μ L of standard solution, 100 μ L of standard was added and then 100 μ L of the solution to be tested. Duplicate measurements were made for each solution.

The buffers used for testing the pH dependence of the assay were prepared as outlined in Table 2. A glycine/HCl buffer containing 0.01 M NaCl was used for pH 1.4, 2.2, 2.8, and 3.6. Phosphate buffer containing 0.01 M NaCl was used for pH 6.2 and 7.1. A glycine/NaOH buffer containing 0.01 M NaCl was used for pH 8.4, 10.2 and 11.7.

To the plate wells containing 50 μ L (inter-washed) or 20 μ L (standard) sample buffer was added 100 μ L standard solution and 100 μ L of the buffer solution to be tested, resulting in a final tested buffer concentration of 0.004 M in the wells. The equivalent sample pH was determined by measuring the pH of a solution containing equal volumes of pure water and the buffer solution to be tested.

Table 2. Preparation of buffers used for testing pH stability

pH/buffer	Glycine/NaCl (0.1 M)	HCL (0.1 M)	Water (total volume)
pH 1.4 Glycine/HCl, 0.01 M NaCl	1.11 mL	8.89 mL	1.1 mL (filled to 11.1 mL)
pH 2.2 Glycine/HCl, 0.01 M NaCl	1.014 mL	0.986 mL	8.14 mL (filled to 10.14 mL)
pH 2.8 Glycine/HCl, 0.01 M NaCl	810 µL	190 µL	7.1 mL (filled to 8.1 mL)
pH 3.6 Glycine/HCl, 0.01 M NaCl	903 µL	97 µL	8.03 mL (filled to 9.03 mL)
	KH ₂ PO ₄ (1 M)	K ₂ HPO ₄ (1 M)	NaCl (0.1 M)
pH 6.2 Phosphate buffered saline (0.01 M)	88.9 µL	11.1 µL	100 µL (filled to 10 mL with water)
pH 7.1 Phosphate buffered saline (0.01 M)	41.3 µL	58.7 µL	100 µL (filled to 10 mL with water)
	Glycine/NaCl (0.1 M)	NaOH (0.1 M)	Water (total volume)
pH 8.4 Glycine/NaOH, 0.01 M NaCl	947 µL	53 µL	8.47 mL (filled to 9.47 mL)
pH 10.2 Glycine/NaOH, 0.01 M NaCl	1072 µL	928 µL	8.72 mL (filled to 10.72 mL)
pH 11.7 Glycine/NaOH, 0.01 M NaCl	1000 µL	3000 µL	6 mL (filled to 10 mL)

2.13 Cross-Reactivities

Standard solutions were prepared for each MC variant as described in Section 2.2. The cross-reactivity of each variant was calculated as follows:

$$\text{Cross-reactivity (\%)} = \frac{\text{Concentration of microcystin - LR at 50 \% inhibition (mol/L)}}{\text{Concentration of microcystin analogue at 50 \% inhibition (mol/L)}} \times 100 \quad (2.0)$$

Errors were propagated according to Gauß's law on error extrapolation (Weltner, 1981).

2.14 Pure, treated and raw waters

The MC-LR standard solutions prepared in pure, treated and raw waters were used to obtain fitted calibration curves in each water on multiple microtitre plates. Each plate contained standards for four calibration curves (run in columns 1-3, 4-6, 7-9, 10-12). For each water three replicate wells of each standard concentration were used as the data for fitting to the calibration curve. Ten calibration curves were obtained for pure water (over 9 plates), and eleven for treated water and for raw water (over 8 and 8 plates, respectively). This data was collected on different plates on different days. Sets of standards for the three different waters were rotated across the four plate positions (columns 1-3, 4-6, 7-9, or 10-12).

Validation against existing methods

2.15 High performance liquid chromatography (HPLC/PDA)

Samples were analysed for microcystin using a reverse-phase HPLC method. The WatersTM HPLC system comprised a Model 600 controller, a Model 600 pump, a Model 717 plus autosampler, and a Model 996 photodiode array detector, which were operated using Millenium³²® software (1997, Waters Corporation, MA, USA). A reverse-phase C₁₈ column (Phenomenex, Desc. Luna, 5 µm pore, 150 x 4.6 mm) was washed and stored in methanol. The mobile phase gradient used is outlined in Table 3. All solvents were of high analytical purity and were vacuum filtered through a 0.45 µm membrane filter paper (Gelman Laboratory, Supor^R, 450 Grid, 47 mm), before use. A flow rate of 1 mL/min and an injection volume of 50 µL were used. The limit of detection of this HPLC/PDA method was 0.06 µg/L with samples concentrated 1000-fold.

Table 3. HPLC gradient for microcystin analysis^a

Time (min)	% Solvent A	% Solvent B
0	100	0
12.5	50	50
15	0	100
21	0	100
23	100	0
32	100	0

^a Where solvent A consisted of 30% acetonitrile with 0.05% trifluoroacetic acid, and solvent B was comprised of 55% acetonitrile with 0.05% trifluoroacetic acid.

The retention times and absorbance maxima of some common MCs are listed in Table 4. Individual peaks in the chromatograms were identified as MC peaks by comparison of spectral shape, absorbance maxima, and retention time, with those in Table 4. Individual MC concentrations were quantified by comparison of peak area with that of MC-LR standards, and were expressed as MC-LR equivalents.

Table 4. Retention times and absorbance maxima of some common microcystin variants obtained using the described HPLC method (AWQC, unpub. data)

Variant	Retention time (min)	Absorbance maxima (nm)
RR	7.4	238.2
YR	14.8	231.2
LR	15.6	238.2
LA	23	238.2
LW	27.7	221.8
LF	28.8	238.2

2.16 HPLC/mass spectroscopy (HPLC/PDA/MS)

An Agilent Zorbax LC column (2.1 x 150 mm, 5 μ m) coupled to a Hewlett Packard series 1100 HPLC system equipped with mass spectrometric (electrospray) and photo-diode array detection, was used for MC quantification. Data analysis was performed using Agilent software. Microcystin-LR was detected using ions of 995 and 996 m/z. Microcystin-RR and -YR were detected using ions of 520 and 1038 m/z, and 1045 and 1046 m/z, respectively. The limit of detection of this method was 0.05 μ g/L with samples concentrated 4000-fold (ie. 200 μ g/L without concentration) (AWQC, unpub. data).

Both photo-diode array and mass spectrometric detection were used to quantify the amount of MC present and the average of these results was used as the concentration of MC in the sample.

2.17 Protein phosphatase inhibition assay (PPIA)

The protein phosphatase inhibition assay was carried out according to Heresztyn and Nicholson (2001). Briefly, enzyme diluent, reaction buffer, and substrate solutions (see Appendix IV) were prepared in brown glass vials (~ 30 mL) and stored on ice. Samples/standards (20 μ L/well) and the enzyme solution (20 μ L/well) were applied to each well and incubated (37°C, 5 min, with shaking). After addition of substrate solution (200 μ L/well) the plate was incubated (37°C, 1.5 – 2 hrs, with shaking) prior to measurement of the absorbance at 450 nm with a microplate reader (Model MRX, Dynex Technologies Ltd).

Data processing

2.18 Microtitre plate data processing

The results obtained from ELISA and PPIA were analysed using Origin 6.1 (OriginLab Corporation, Northampton, MA, USA) as described here.

The calibration curve was fitted to the following equation:

$$\text{Concentration } (\mu\text{g/L}) = x_0 [(A_1 - \text{abs})/(\text{abs} - A_2)]^{1/p} \quad (2.1)$$

The limit of detection was calculated using two methods. Firstly, the mean (χ) and standard deviation (σ) was calculated for the absorbances of all (3-5) blank (containing no microcystin) samples on the microtitre plate. The 'blank' limit of detection (BLOD) was then taken as the concentration corresponding to the following absorbance:

$$\text{abs} = \chi - 3 \sigma \quad (2.2)$$

Secondly, the absorbance corresponding to 85% of the absorbance change was calculated as follows:

$$\text{abs} = [0.85 \times (A_1 - A_2)] + A_2 \quad (2.3)$$

The 'absorbance' limit of detection (ALOD) was then taken as the concentration corresponding to this absorbance.

Values were only reported as zero if they were less than both the BLOD and the ALOD.

For the protein phosphatase inhibition assay, using solutions of known MC concentration, the BLOD appeared to be a more appropriate method of calculating the limit of detection, as the ALOD appeared to give high values. Whereas, using solutions of known MC concentration for ELISA, the method of calculating the more appropriate limit of detection varied randomly.

Five replicates were run for each sample solution. The absorbance values were sorted into increasing order and the lowest and highest were discarded. Of the three remaining values both the lowest and highest were repeated to give five values, and the trimmed mean (χ) and standard deviation (σ) were calculated from these. The mean absorbance was converted to concentration using equation 2.1. The MC concentration range of the sample was calculated as follows:

$$\text{min abs} = \chi - 3 \sigma \quad (2.4)$$

$$\text{max abs} = \chi + 3 \sigma \quad (2.5)$$

These absorbance values were then converted into the maximum and minimum concentrations, respectively, using equation 2.1, and expressed as the concentration range of the sample. This range is an indication of the variation within one plate.

Where samples were prepared in triplicate one standard deviation of the final mean concentration is also reported. The lowest of the minimum concentrations and the highest of the maximum concentrations are reported as the range. This range takes into account all variations arising from sample preparation, plate-to-plate, and day-to-day variations.

In all figures containing calibration curves the errors represent the range, whereas in the plots with fitted parameters the errors represent the standard error.

2.19 Chromatography data processing

The results obtained from HPLC/PDA and HPLC/MS/PDA were analysed using Excel 97 SR-2 as described here.

Concentrations were calculated by comparison of peak areas with that of a microcystin-LR standard as follows:

$$\text{Concentration of sample} = \frac{[\text{Peak area of sample}]}{[\text{Peak area of standard}]} \times \text{Concentration of standard} \quad (2.6)$$

RESULTS AND DISCUSSION

3.1 Overview of Optimisation

In the optimisation experiments the amount of anti-Adda antibody was varied first. Reduced amounts showed that lower midpoints and a decreased limit of detection could be achieved. Consequently, a new assay was designed where the coating volume was reduced from 200 μL to 50 μL . Together with a decreased tracer incubation time this led to an improved midpoint. In order to protect the antibody throughout the assay identical volumes of sample buffer and antibody solution were used. While optimising the assay a different formula of the sample buffer was found to improve the reproducibility of the assay.

Advantages of an inter-washed direct competitive ELISA format

In addition to these changes, the binding between the anti-Adda Mab and the Adda moiety of MCs was found to be sufficiently strong that the standard format of a direct competitive ELISA could be modified by the introduction of an additional washing step. The principle of this new 'inter-washed' ELISA format is shown in Figure 2. The washing step introduced between the sample and tracer incubations (Figure 2) protects the least stable component of the assay – the enzyme of the tracer conjugate – from adverse effects from components of the sample matrix. In the standard format components of the sample matrix remain in the wells when the tracer molecule is added and, as a result, these components can interfere with the assay. The major benefit of the inter-washed format is that such components are washed out of the well by the additional washing step, and hence, the stability and reliability of the assay should be improved.

Storage

In order to improve the shelf life of antibody coated plates a protection buffer was applied and aspirated, the plates left to dry in the dark and then stored with desiccant in a sealed container at 4°C. Plates protected in this manner were stable for at least 3 months.

3.2 ELISA optimisation

In optimising the ELISA the range of parameters varied included buffer components, reagent concentrations, incubation times, solution volumes and amount of washing. The goals of the optimisation were to improve the assay's sensitivity and stability, whilst not compromising the absorbance signal obtained. During the optimisation phase both the standard and inter-washed formats were used and compared. When fitted values were more different than the sum of their standard errors, this was considered to be a significant effect.

3.2.1 IgG pre-coating

Plates were pre-coated with 0, 50, 250 and 300 μL anti-mouse IgG F_c antibody to determine the optimal volume. The results revealed that 50 μL was sufficient volume for the assay to be performed. The absorbance signal obtained with the use of either 250 or 300 μL was almost the same, and was three-fold higher than that obtained with 50 μL . These larger volumes prevent adsorption of protein/matrix to the polystyrene surface of the microtitre plates, and hence, 300 μL was selected for the optimised assay.

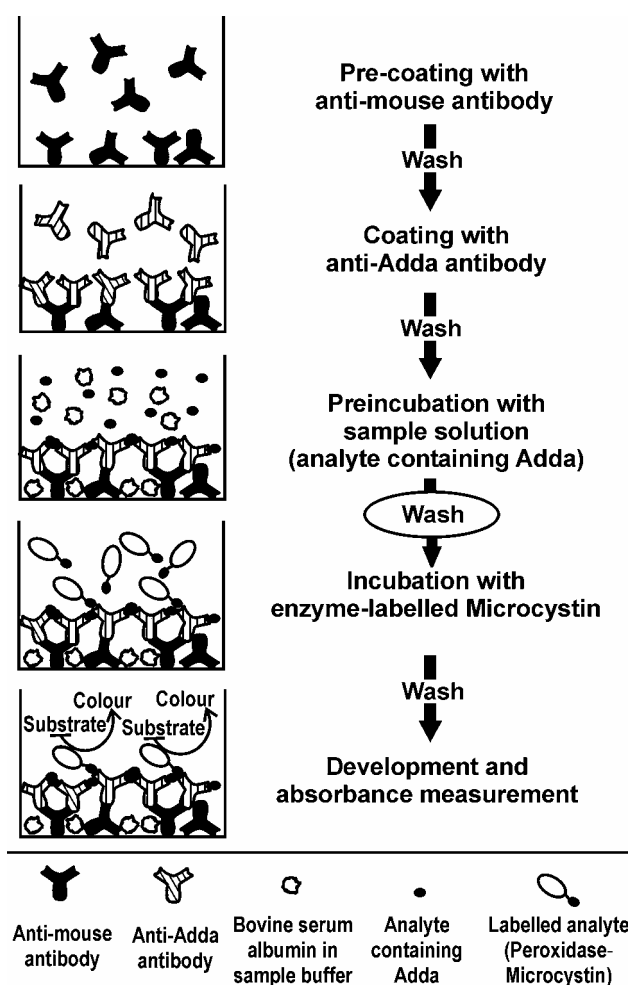


Figure 2: Principle of the inter-washed format of the direct competitive ELISA

3.2.2 Anti-Adda Mab coating

A cell culture supernatant containing anti-Adda Mab was used and the antibody concentration of the coating solution was optimised. The concentration of anti Adda Mab solution added to the plate in the final procedure (Figure 12) was approximately 25 µg/L.

3.2.3 Sample incubation

Sample waters may range considerably in pH. The sample buffer should have sufficient buffering capacity to maintain a constant pH in the wells, despite the pH of the sample added. Two buffers, phosphate buffered saline (PBS) and tris(hydroxymethyl)aminomethane/HCl (Tris) buffer, were selected for maintaining a neutral pH and studied.

In the standard format 20 μL of 'sample buffer' solution was added to the plate and diluted (11 x) to a total volume of 220 μL with sample, whereas, for the inter-washed format 50 μL of solution was added to the plate and diluted (5 x) to a total volume of 250 μL (see Figures 11 and 12). In initial investigations the concentrations of PBS and Tris were varied between 0.08 and 0.8 M, and 0.5 and 1.0 M, respectively, in both formats. This resulted in concentrations of 0.007 - 0.16 M PBS and 0.045 - 0.2 M Tris in the wells.

As a result of the difference in dilution factor for each format, the buffer concentration used in the standard format was then changed to twice that used in the inter-washed format, leading to approximately the same concentration in the wells of both formats. This two-fold difference in 'sample buffer' concentrations applies unless otherwise specified.

Additional components may be added to the sample buffer to avoid the effects of sample constituents (such as humic acids or metal ions) that may interfere with the assay. Such additives include bovine serum albumin (BSA) for reducing non-specific binding, ethylenediamine tetraacetic acid disodium salt (EDTA) for binding metal ions, and sodium chloride (NaCl) for adjusting ionic strength. In order to optimise the sample buffer composition, the effects of a number of possible sample components (ie. humic acid and calcium) were studied using potential sample buffer solutions.

Buffer components in the presence of humic acid

In this section identical buffers were used for both the standard and inter-washed formats. Initially the performance of Tris buffer (1 M), PBS buffer (0.08 or 0.8 M potassium phosphate buffer containing 0.15 or 1.5 M NaCl, respectively), water, BSA (1% in water), EDTA (1% in 0.08 M PBS containing 0.15 M NaCl), NaCl (1.5 M in 0.08 M PBS containing 0.15 M NaCl) and Tris buffer (1 M Tris buffer containing 1.5 M NaCl, 1% EDTA, and 1% BSA (pH 7.2)) were compared in the absence of MC and presence of increasing humic acid concentrations (0 to 300 mg/L).

The drift in absorbance signal across the microtitre plate (A0 - H0, Figure 3) was small in comparison to the changes in signal caused by high humic acid concentrations. In the presence of PBS alone or containing EDTA or NaCl a decrease in absorbance signal was observed with increasing humic acid concentration. Water alone or containing BSA (1%) did not offer satisfactory elimination of matrix effects. Although Tris buffer (1 M) alone showed an increase in absorbance signal at high humic acid concentrations (≥ 100 mg/L), in the presence of 1.5 M NaCl, 1% EDTA and 1% BSA, it was the most effective at stabilising the assay (Figure 3).

In the standard format a Tris buffer containing 1.5 M NaCl, 1% EDTA and 1% BSA (pH 7.2) offered the best stabilisation of both the antibody and the enzyme in the presence of humic acid. In the inter-washed format 1% BSA in water showed slightly better stabilisation, than Tris buffer containing 1.5 M NaCl, 1% EDTA and 1% BSA, only at very high humic acid levels (> 100 mg/L). The absorbance signals obtained for a sample solution containing no MC but varying concentrations of humic acid (0 to 1 g/L), in both the standard and inter-washed formats, with either water or Tris buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA) as the 'sample buffer' solution, are compared in Figure 4. The additional washing step in the inter-washed format improved the assay's performance in the presence of humic acid.

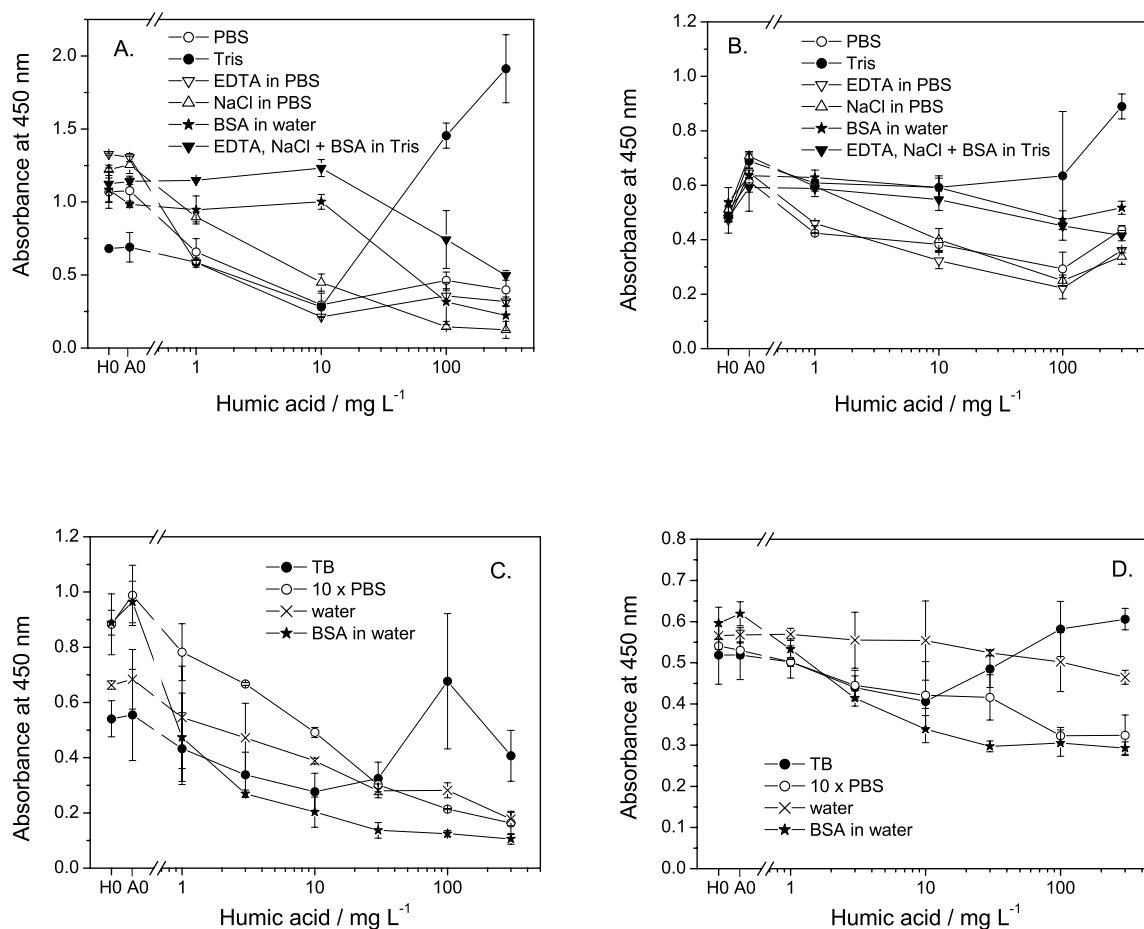


Figure 3. Effect of various sample buffer solutions on the absorbance of a standard solution containing no microcystin (blank), in the presence of increasing humic acid concentration using both standard (A, C) and inter-washed (B, D) formats. PBS, 0.08 M phosphate buffer containing 0.15 M NaCl (pH 7.4); Tris, 1 M Tris (pH 8.0); EDTA in PBS, 1% EDTA in PBS (pH 6.9); NaCl in PBS, 1.5 M NaCl in PBS (pH 6.8); BSA in water, 1% w/v BSA in water (pH 7.7); EDTA, NaCl + BSA in Tris, 1 M Tris containing 1% EDTA, 1.5 M NaCl and 1% BSA (pH 7.2); TB, 1 M Tris (pH 7.4)); 10 x PBS, 0.8 M phosphate buffer containing 1.5 M NaCl (pH 7.4). Median values \pm range are shown. A0 and H0 are the absorbances of the first and last pipetted blank when no humic acid was present. The difference between A0 and H0 is an indication of the signal drift across the microtitre plate.

The effect of humic acid on the MC-LR calibration curve was compared using 1 M Tris buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA, pH 7.2) as the sample buffer (Figure 5). Only the absorbance signals of the low MC concentration standards were affected by humic acid. The standard format showed a substantial decrease in A_1 between 10 and 100 mg/L humic acid. The inter-washed format showed a slight decrease in A_1 at 100 mg/L. Thus the inter-washed format would produce more accurate results in the presence of high concentrations of humic acids.

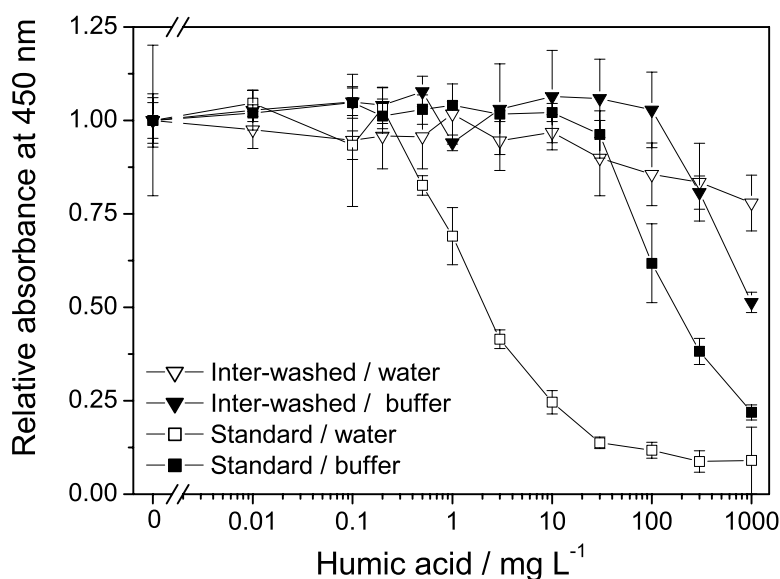


Figure 4. Absorbance signal (normalised to 1.0) obtained in the absence of microcystin for water solutions containing increasing humic acid concentrations, using both water and Tris buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA) as sample buffers, and using both standard and inter-washed formats. Median values \pm range are shown

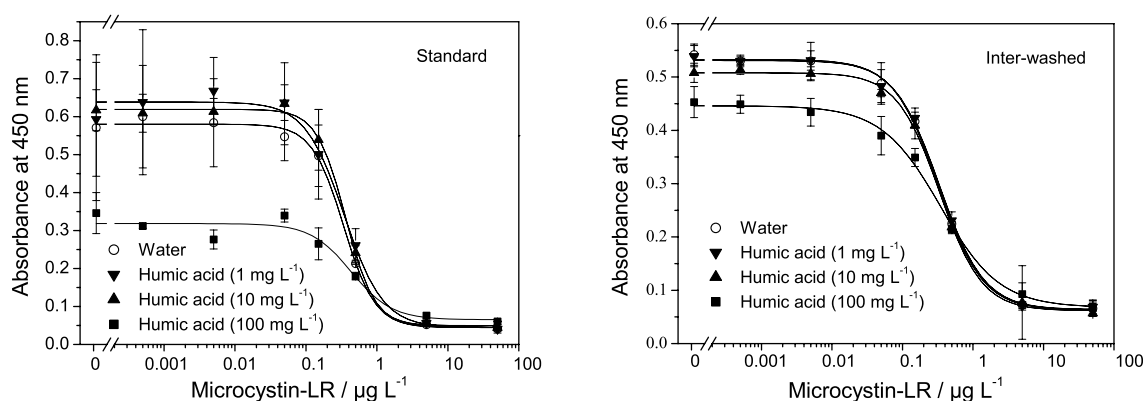


Figure 5. Effect of humic acid on the microcystin-LR calibration curve using both standard and inter-washed formats with a Tris (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA, pH 7.2) sample buffer

Buffer components in the presence of calcium

In this section identical buffers were used for both the standard and inter-washed formats.

a) PBS

In natural waters calcium is present in concentrations of up to 5 mM (200 mg/L calcium ions). In this study samples containing concentrations of up to 9 mM calcium (1 g/L calcium chloride) were used. With PBS buffer a calcium phosphate precipitate may form. In the inter-washed format, for the amount of calcium used, 2% EDTA in 0.8 M PBS sample buffer (diluted 1:5 to give 0.4% EDTA in the wells) sufficiently complexed the calcium ions and no precipitation was observed photometrically at 450 nm (tested in plates without antibody-coating). Although the same sample buffer (2% EDTA in 0.8 M PBS) was used in the standard format, calcium precipitation could still be observed with a sample containing 9 mM calcium. This was probably due to the lower EDTA concentration (0.18%) in the wells, resulting from the 1:11 dilution in this format.

Using standard MC-LR solutions the calibration curve obtained with 0.5 g/L calcium chloride in PBS (0.8 M containing 2% EDTA) was compared with that in the absence of calcium (Table 5). In PBS (0.8 M containing 2% EDTA) the presence of 0.5 g/L calcium chloride an increased maximum absorbance signal (A_1) and decreased midpoint (x_0) was seen in the standard format, while no effect was observed in the inter-washed format. In addition, the standard curve in 0.8 M PBS was compared with that in 0.8 M PBS containing 2% EDTA. The presence of 2% EDTA resulted in an increase in A_1 in the inter-washed format. In the presence of calcium and absence of EDTA, precipitation occurred. When compared with 0.8 M PBS alone, the presence of calcium and 2% EDTA resulted in an increase in A_1 and a decrease in midpoint in both formats.

The effect of increasing calcium concentration (0 to 7.5 mM) on the MC-LR calibration curve was studied using a 0.8 M PBS buffer containing 2% EDTA, 1.5 M NaCl and 1% BSA (pH 7.6). Calcium had no significant effect on the midpoints of either the standard or the inter-washed format. An increase in A_1 was observed in the standard format with increasing calcium concentration, however, in the inter-washed format there was an initial decrease and then increase in signal with increasing calcium concentration. These results agree well with those observed in the absence of 1% BSA (Table 5). Hence, BSA does not appear to influence calcium complexation.

b) Tris

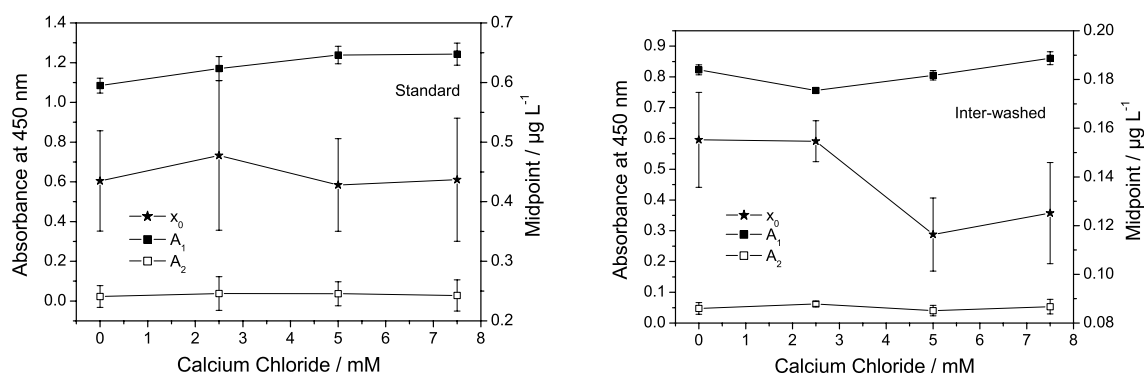
Using a Tris buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA, pH 7.4) the effect of increasing calcium concentration (0 to 7.5 mM) on the MC-LR calibration curve was studied using sample incubation times of 30 min and 3 h. No significant difference was observed between the 30 min and the 3 h incubation time. With sufficient sample incubation time a slight increase in A_1 was observed in the standard format and the midpoint was unaffected in either format, over this range of calcium concentrations (Figure 6).

Addition of either 20 or 50 μ L of either 1% EDTA in 1 M Tris buffer (containing 1.5 M NaCl) or 2% EDTA in 0.8 M PBS buffer to the well prior to the addition of a solution containing calcium (0 to 1 g/L CaCl_2) were compared in terms of preventing precipitation. Using 1% EDTA in 1 M Tris buffer, 20 μ L is sufficient for complexing 1 g/L calcium chloride (9 mM), however, when using 2% EDTA in 0.8 M PBS, 50 μ L is required. This difference may arise from the fact that besides EDTA, tris(hydroxymethyl)aminomethane (Tris) is able to complex calcium ions.

Table 5. Variation of fitted parameters in the presence of calcium chloride (0.5 g/L) and/or with the addition of 2% EDTA to a PBS sample buffer^a

Format	Chemicals added	A_1^b	A_2^c	x_0^d
Standard	2% EDTA	+ or –	=	= or –
Inter-washed	2% EDTA	+	+ or =	– or =
Standard	CaCl ₂ (0.5 g/L) + 2% EDTA	+	=	–
Inter-washed	CaCl ₂ (0.5 g/L) + 2% EDTA	+	=	–
Standard	CaCl ₂ (0.5 g/L) + 2% EDTA Compared with 2% EDTA	+	=	–
Inter-washed	CaCl ₂ (0.5 g/L) + 2% EDTA Compared with 2% EDTA	=	=	=

^a A + or – indicates a substantial increase or decrease in fitted parameter value (exceeding the variation incorporated within the standard errors of the values compared) when compared with the fitted values obtained with PBS (0.8 M containing 1.5 M NaCl) alone (unless otherwise specified). + + or – – indicates > 50% change, = indicates no detectable change. ^b Maximum absorbance (absorbance of blank). ^c Minimum absorbance. ^d Midpoint (total microcystin concentration at which the amounts of antibody-bound and free microcystin are equal).

**Figure 6. Effect of calcium chloride concentration on the parameters fitted to the MC-LR standard curves after 3 h incubation. A_1 and A_2 are the maximum and minimum absorbances, respectively, and x_0 is the midpoint**

In order to complex all calcium ions plus any other metal ions present in the sample, 4% EDTA in 1 M Tris buffer should be adequate in the standard format, whereas, 2% EDTA in 0.5 M Tris buffer (50 μ L) should be sufficient for the inter-washed format. The effects of buffer concentration in the presence of metal ions and humic acid are discussed below.

Buffer components in the presence of copper

The influence of copper ions on the ELISA was also investigated in the inter-washed format. Copper is an algicide that may be added to water in the presence of cyanobacteria. The highest concentration of copper ions expected in waters is 1.6 mM (100 mg/L copper). Most treatments aim for a copper concentration of around 2 mg/L, however, localised concentrations may be much higher. In this study either 8 mM copper (CuSO_4) was added in a 50 μL aliquot and diluted 5 times in the wells to give 1.6 mM, or 3.2 mM copper was added in a 100 μL aliquot and diluted 2.5 times in the wells to give 1.28 mM copper (equivalent to adding a 200 μL sample containing 1.6 mM copper into a well containing 50 μL sample buffer, as in the inter-washed format).

When 0.1 M Tris buffer (containing 0.1% EDTA, 0.15 M (8.76 g/L) NaCl and 0.1% BSA) was used as the sample buffer it did not have sufficient buffering capacity to maintain a constant pH of 7.4 in the presence of 8 mM (500 mg/L) copper alone or with the addition of 0.3% EDTA. When 8 mM copper was added the pH dropped to 6.0, and when an additional 0.3% EDTA was added to this solution the pH dropped to 5.1. With this buffer (diluted to 0.02 M Tris in the wells) a decrease in midpoint was observed in the presence of 1.6 mM (100 mg/L) copper with either a total of 0.02% (0.5 mM) or 0.08% (2 mM) EDTA as the final concentrations in the wells.

At a slightly alkaline pH a $\text{Cu}(\text{OH})_2$ precipitate will form. This was observed with the 0.1 M Tris sample buffer, however, by using a more concentrated sample buffer (0.5 M Tris) which buffered more effectively, the precipitate was no longer observed.

When 0.5 M Tris buffer (containing 0.75 M NaCl and 0.5% BSA) was used as the sample buffer the final concentration in the wells was 0.1 M Tris buffer. No effects on A_1 or midpoint were observed in the presence of 1.28 mM copper (equivalent to 1.6 mM copper in a sample) and 0.4% (10 mM) EDTA. This was equivalent to incorporating 2% EDTA in the Tris buffer.

When sufficient EDTA (2% in sample buffer, inter-washed format) was present no metal ion effect was observed in the presence of samples containing either 1.6 mM copper sulfate alone, or in combination with 5 mM calcium chloride.

Assuming calcium and copper ions are present at the highest expected concentrations and taking a two-fold excess of the molar equivalent of EDTA for these two ions, suggests that 2% EDTA should be sufficient for complexation of metal ions in water samples. Addition of either 2 or 5% EDTA in the absence of calcium or copper to a 0.5 M Tris sample buffer did not affect A_1 or the midpoint. Consequently, 2% EDTA was incorporated in a 0.5 M Tris sample buffer (containing 0.75 M NaCl and 0.5% BSA) in the final procedure (Figure 12).

Buffer concentration in the presence of humic acid or calcium

MC-LR calibration curves obtained in the presence of water, humic acid (50 mg/L) or calcium (5 mM) solution using undiluted Tris buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA) and half-strength buffer (0.5 M) were compared.

In the standard format the results obtained for 0.5 M Tris buffer tended to have a lower A_1 than those obtained with 1 M buffer, and in the presence of either humic acid or calcium a slightly lower midpoint was also observed. In this format a higher A_1 was obtained in the

presence of calcium using both 0.5 and 1 M Tris buffer, and a lower A_1 was only obtained in the presence of humic acid using 0.5 M buffer (Table 6). Hence, 1 M Tris buffer with an increased level of EDTA, to complex calcium ions, was expected to be suitable.

In the inter-washed format the results obtained for 0.5 M Tris buffer tended to have a slightly higher A_1 than those obtained with 1 M buffer. In this format a slightly higher A_1 (and A_2) and a lower midpoint were obtained with the 0.5 M Tris buffer in the presence of humic acid (Table 6). Hence, 0.5 M Tris buffer gave similar or maybe slightly improved results when compared to 1 M Tris buffer.

Table 6. Variation of fitted parameters with addition of humic acid (50 mg/L) or calcium (5 mM) in the presence of full or half-strength Tris buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA, pH 7.4)^a

Format	Chemicals added	A_1^b	A_2^c	x_0^d
Standard	Humic acid in 0.5 M Tris buffer	–	=	–
Inter-washed	Humic acid in 0.5 M Tris buffer	+	+	–
Standard	Humic acid in 1 M Tris buffer	=	=	=
Inter-washed	Humic acid in 1 M Tris buffer	=	=	=
Standard	Calcium in 0.5 M Tris buffer	+	=	–
Inter-washed	Calcium in 0.5 M Tris buffer	=	+	–
Standard	Calcium in 1 M Tris buffer	+	=	=
Inter-washed	Calcium in 1 M Tris buffer	=	=	=

^a A + or – indicates a marked increase or decrease in fitted parameter value (exceeding the variation incorporated within the standard errors of the values compared) when compared with the fitted values obtained with pure water in either 0.5 or 1 M Tris buffer alone. ++ or – – indicates > 50% change, = indicates no detectable change. ^b Maximum absorbance (absorbance of blank). ^c Minimum absorbance. ^d Midpoint (total microcystin concentration at which the amounts of antibody bound and free microcystin are equal).

Tween 20 in the presence of humic acid or calcium

Tween 20 is often used to keep hydrophobic substances in solution and it aids wetting of the polystyrene plate surface. It has been used for stabilising enzymes and preventing adsorption to plastics (Sundberg *et al.*, 1995).

Firstly, the effects of adding Tween to either or both sample buffer (0.5 or 1% v/v) and tracer buffer (0.1% v/v) were investigated (Table 7). Sample buffer was comprised of Tris buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA, half-strength for the inter-washed format) and tracer buffer consisted of dilute Tris buffer (0.1 M containing 0.1% EDTA, 0.15 M NaCl and 0.5% BSA).

Addition of Tween 20 to either or both sample buffer (1% v/v for standard, 0.5% v/v for inter-washed) and tracer buffer (0.1% v/v) resulted in a decrease in A_1 in both formats, with the greatest change (> 2 -fold) observed when Tween was added to both buffers in the standard format.

No change in midpoint was observed in either format when Tween (0.1%) was added to only tracer buffer, however, when Tween ($\geq 0.5\%$) was added to only sample buffer or Tween was present in both buffers in either format, an increase in midpoint resulted. The increase was greatest (2-fold) when Tween was added to both buffers in the inter-washed format.

Table 7. Variation in fitted parameters when Tween 20 was added to sample and/or tracer buffers^a

Format	Chemicals added	A_1^b	A_2^c	x_0^d
Standard	Tween (1%) in sample buffer	–	=	=
Inter-washed	Tween (0.5%) in sample buffer	–	=	+
Standard	Tween (0.1%) in tracer buffer	–	=	=
Inter-washed	Tween (0.1%) in tracer buffer	–	–	=
Standard	Tween in both buffers	– –	=	+
Inter-washed	Tween in both buffers	–	=	++

^a A + or – indicates a marked increase or decrease in fitted parameter value (exceeding the variation incorporated within the standard errors of the values compared) when compared with the fitted values obtained in the absence of Tween. ++ or – – indicates $> 50\%$ change, = indicates no detectable change. ^b Maximum absorbance (absorbance of blank). ^c Minimum absorbance. ^d Midpoint (total microcystin concentration at which the amounts of antibody bound and free microcystin are equal).

The effect of adding 0.025% v/v Tween to the Tris sample buffer (1 M containing 1% EDTA and 1% BSA, half-strength for inter-washed) in the presence of water, humic acid (50 mg/L) or calcium (5 mM) solution was then investigated (Table 8). The slight increase in A_1 with 5 mM calcium verified that metal ion effects are not completely removed by 1% and 0.5% EDTA in the sample buffer for the standard and inter-washed formats, respectively.

In the standard format the addition of Tween resulted in an increase in absorbance, especially in the presence of calcium. The midpoints obtained in the presence of humic acid or calcium were lowered by the addition of Tween and there was greater variation between midpoints (for water, humic acid, and calcium) in the presence of Tween.

In the inter-washed format the addition of Tween resulted in an increase in A_1 and a decrease in midpoint. There was greater variation between midpoints (for water, humic acid, and calcium) in the absence of Tween.

Table 8. Variation in fitted parameters with the addition of humic acid (50 mg/L) or calcium chloride (5 mM) in the presence or absence of Tween 20 (0.025%) in the sample buffer (1 M Tris containing 1% EDTA, 1.5 M NaCl and 1% BSA, pH 7.4, half strength for inter-washed)^a

Format	Chemicals added	A ₁ ^b	A ₂ ^c	x ₀ ^d
Standard	Humic acid	=	=	=
Inter-washed	Humic acid	+	=	+
Standard	CaCl ₂	+	=	=
Inter-washed	CaCl ₂	+	=	–
Standard	Tween 20	+	=	=
Inter-washed	Tween 20	+	=	–
Standard	Humic acid + Tween 20	+	+	–
Inter-washed	Humic acid + Tween 20	+	=	=
Standard	CaCl ₂ + Tween 20	+	=	–
Inter-washed	CaCl ₂ + Tween 20	+	=	–

^a A + or – indicates a marked increase or decrease in fitted parameter value (exceeding the variation incorporated within the standard errors of the values compared) when compared with the fitted values obtained in the absence of Tween 20, humic acid and calcium chloride. + + or – – indicates > 50% change, = indicates no detectable change. ^b Maximum absorbance (absorbance of blank). ^c Minimum absorbance. ^d Midpoint (total microcystin concentration at which the amounts of antibody bound and free microcystin are equal).

The addition of Tween 20 (0.025%) to the sample buffer gave similar, maybe slightly improved, results. Since the presence of Tween 20 in the sample buffer keeps hydrophobic substances in solution, this may affect the cross reactivities of various microcystin variants in the assay. Tween 20, however, was omitted from the final sample buffer for reasons outlined in Section 3.2.5.

Final sample buffer constituents

The final sample buffer selected for the standard format was 1 M Tris buffer containing 4% EDTA, 1.5 M NaCl and 1% BSA (pH 7.4) and for the inter-washed format the sample buffer comprised 0.5 M Tris buffer containing 2% EDTA, 0.75 M NaCl and 0.5% BSA (pH 7.4). The buffering capacity of this solution was sufficient to maintain the pH of the solution in the plate well at neutral pH under the anticipated conditions of use. The concentration of EDTA was sufficient to eliminate metal ion effects at realistic concentrations. The concentration of the sample buffer needed to be five times greater than that of the protection or tracer buffers for the inter-washed format, since it was diluted 5-fold in the plate wells upon addition of the sample.

Sample incubation time

Sample incubation time was varied between 2 h and overnight. No significant difference was found between incubation for 2 ¼ h and 3 ½ h or between incubation for 4 h or 8 h. Overnight incubation appeared to give significantly better results, probably due to equilibrium being attained. With overnight incubation the assay appeared to be more sensitive, however, MCs may have adsorbed to the polymer surface with the additional incubation time, leading to a decreased MC content in the sample and a reduced midpoint. Since overnight incubation was not practical for routine testing and an incubation time of 2 h gave acceptable results this length of incubation time was considered sufficient.

3.2.4 Washing

The washing steps were only studied for the inter-washed format as they were already well defined for the standard format.

Washing between sample and tracer incubation

The number of washes with washing buffer (0.008 M phosphate buffer containing 0.015 M NaCl and 0.05% Tween 20) undertaken between the sample incubation and tracer incubation steps in the inter-washed format was varied between one and four. The effect of the number of washes on the fitted minimum absorbance, A_2 , and the midpoint, x_0 , are shown in Figure 7. The timing of the washes is also shown, since this can affect the kinetics of the binding interactions. The value of A_2 was notably lower with 1 x wash than with 2, 3, or 4 x wash. This can be explained by the extra washes possibly dissociating bound MCs and vacating antibody sites that are then available for the binding of additional tracer molecules. Washing 3x rather than 2x before tracer addition decreased A_1 by about 0.1 while A_2 remained unchanged, hence the magnitude of the absorbance signal decreased. No obvious difference in midpoint was observed between one and four washes. Since no marked change in A_2 was observed after 2 x wash, and a slightly lower midpoint was achieved, this was used in the final protocol.

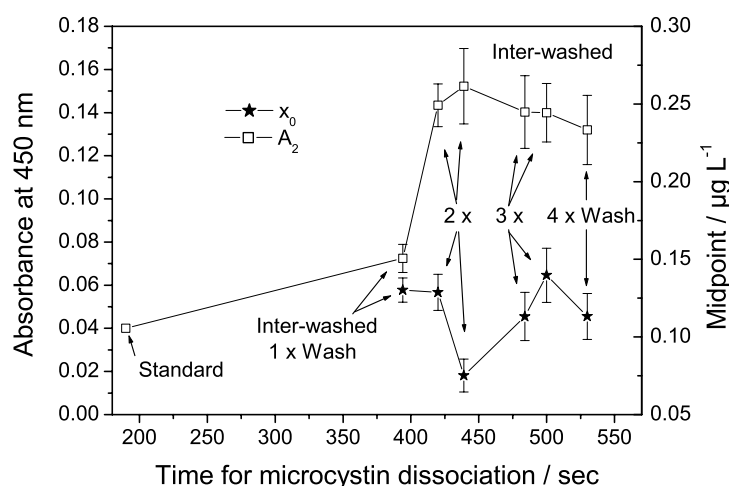


Figure 7. Variation in the fitted minimum absorbance, A_2 , and the midpoint, x_0 , with number of washes between sample and tracer incubations. A_2 for the standard format with no wash is included, however, the standard and inter-washed procedures do differ and a direct comparison is not possible

Washing between tracer incubation and development

The number of washes undertaken following tracer incubation and prior to colour development was varied between one and four in the inter-washed format. After 2 x wash no further reduction in A_1 and A_2 was observed (Figure 8). The markedly higher absorbances obtained after only 1 x wash may result from weak non-specific binding of tracer in the wells, which appears to be removed by subsequent washes.

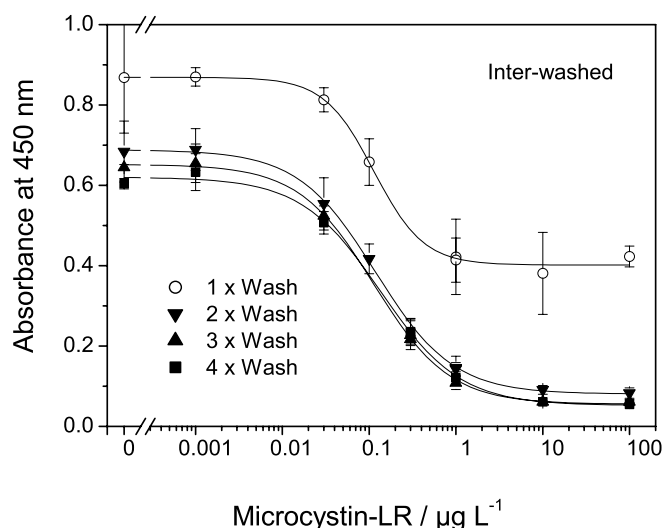


Figure 8. Variation in the microcystin-LR calibration curve with number of washes between tracer incubation and colour development

Since no difference was found between washing with 2 x washing buffer / 1 x water and 3 x washing buffer, 3 x washing buffer was used for washing after tracer incubation in the final protocol.

3.2.5 Tracer incubation and development

Addition of BSA to tracer buffer

The effect of adding BSA (0, 0.1, 0.5, 1%) to a PBS (0.08 M containing 0.15 M NaCl) 'tracer buffer' for preparation of the tracer solution was investigated in the presence of humic acid (10 mg/L) which was added to all standard solutions (Figure 9). In both the standard and the inter-washed format A_1 increased in the order: $0 < 1 < 0.1 < 0.5\%$ BSA, and A_2 increased in the order: $0 \leq 1 \leq 0.5 \leq 0.1\%$ BSA. Hence, the absorbance signal was maximised with 0.5% BSA.

In the standard format, a lower midpoint was obtained with the addition of 0.1% BSA to the tracer solution. In the inter-washed format no significant difference in midpoint was observed for 0 – 1% BSA. In the standard format the difference in A_1 obtained with 0 – 1% BSA in the tracer buffer was 0.61, whereas in the inter-washed format it was only 0.45. In the inter-washed format most humic acid will be washed away with the additional washing step prior to addition of tracer, hence, even without BSA the assay should be less susceptible to interference from humic acids and a smaller change in A_1 with addition of BSA was expected.

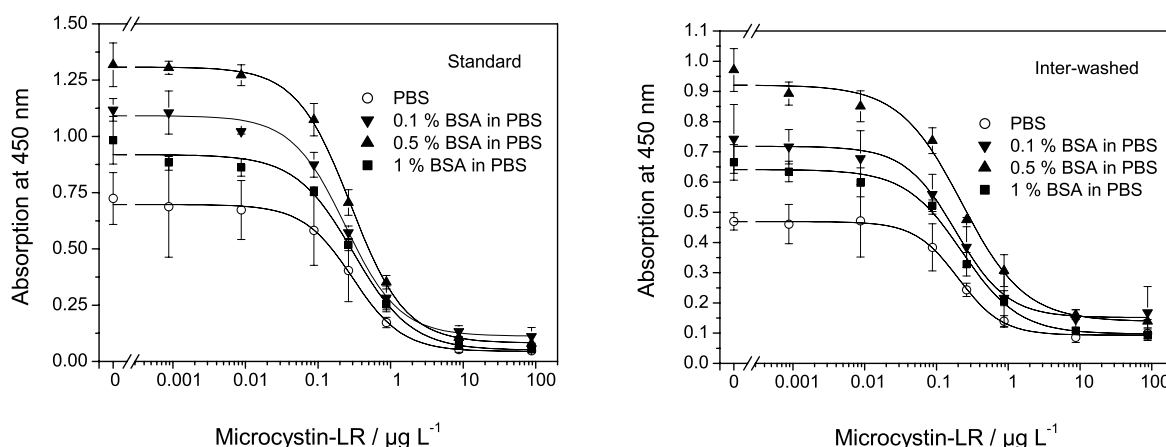


Figure 9. Influence of bovine serum albumin (BSA) in the tracer buffer (0.08 M PBS containing 0.15 M NaCl) on the microcystin-LR calibration curve in the presence of humic acid (10 mg/L added to the MC standard solutions) using both standard and inter-washed formats

It appears that the presence of 0.5% BSA in the tracer buffer is optimal for improving A_1 in both formats. While lower BSA concentrations may not be adequate to minimise interference from humic acids and stabilise the enzyme, higher BSA concentrations may block antibody binding sites leading to a lower signal.

Addition of Tween and/or polyacrylic acid to tracer buffer

The addition of Tween 20 to sample and/or tracer buffers is discussed in Section 3.3.3. The addition of polyacrylic acid (PAC) was expected to stabilise the tracer and prevent non-specific binding of humic acid.

The effects of adding Tween 20 (0.01% or 0.1%) and/or polyacrylic acid (PAC, 0.01 or 0.1%) to the tracer buffer (0.1 M Tris containing 0.1% EDTA, 0.15 M NaCl and 0.5% BSA) were studied (Table 9).

In both the standard and inter-washed formats Tween (0.01%) decreases A_1 slightly, while A_2 and the midpoints are unaffected. With Tween (0.1%) a slight decrease in A_2 was observed in the inter-washed format. At a concentration of 0.1%, PAC markedly increases A_1 and leaves the midpoint unchanged in both formats. When both Tween (0.01%) and PAC (0.01 or 0.1%) were present, no effect was observed on the midpoint or A_2 in both formats. However, the increase in A_1 observed in the inter-washed format was not observed in the standard format. There was no evidence of PAC stabilising the tracer molecule. Neither PAC nor Tween 20 appeared to reduce the high A_2 that was thought to arise from non-specific binding of the tracer. As a detergent, Tween 20 may enhance dissociation of the antibody-MC complex, leading to an increased A_2 .

Table 9. Variation of fitted parameters with addition of Tween 20 and/or polyacrylic acid (PAC) to the tracer buffer (0.1 M Tris containing 0.1% EDTA, 0.15 M NaCl and 0.5% BSA)^a

Format	Chemicals added	A ₁ ^b	A ₂ ^c	x ₀ ^d
Standard	Tween (0.01%)	–	=	=
Inter-washed	Tween (0.01, 0.1%)	–	= / –	=
Standard	PAC (0.01 / 0.1%)	= / +	= / =	= / =
Inter-washed	PAC (0.01 / 0.1%)	– / +	= / +	+ / =
Standard	Tween (0.01%) and PAC (0.01 / 0.1%)	= / –	= / =	= / =
Inter-washed	Tween (0.01%) and PAC (0.01 / 0.1%)	+ / +	= / =	= / =

^a A + or – indicates a marked increase or decrease in fitted parameter value (exceeding the variation incorporated within the standard errors of the values compared) when compared with the fitted values obtained with tracer buffer alone. ++ or -- indicates > 50% change, = indicates no detectable change. ^b Maximum absorbance (absorbance of blank). ^c Minimum absorbance. ^d Midpoint (total microcystin concentration at which the amounts of antibody bound and free microcystin are equal).

Although Tween appears to support the washing process by eliminating non-specific binding of the tracer (Section 3.2.4), the results in Tables 7 and 9 suggest that it inhibits the tracer enzyme reaction leading to a decreased A₁. Consequently, Tween was omitted from the final sample and tracer buffers.

Concentration and incubation time

The concentration of the tracer and antibody were optimised together with the tracer incubation time and development time. The incubation time was varied between 4 and 12.5 minutes and the development time was varied between 12 and 31 min. A tracer incubation time of 5 min was selected as optimal, with a development time of 20 to 30 min. The concentration of tracer molecule added to the plate in the final inter-washed format procedure (Figure 12) was 0.1 µg/mL microcystin-LR-horse-radish-peroxidase. The concentration of tracer molecule added to the plate in the final standard format procedure (Figure 11) was approximately 0.075 µg/mL microcystin-LR-horse-radish-peroxidase.

3.3 Optimisation of storage procedure

In order to reduce the time between receiving samples and obtaining results, microtitre plates may be coated with antibody and stored ready for application of samples and standard solutions. If the antibody is not stable under the storage conditions the sensitivity and absorbance signal of the assay will decrease and, if the antibody is sufficiently degraded, the assay may not function at all. In this study a number of solutions were tested for their ability to stabilise antibody bound to microtitre plates.

Poly(vinyl) alcohols (PVAs) have previously been shown to effectively stabilise peroxidase-enzyme conjugates, peroxidases, and other glycoprotein enzymes, at concentrations as low as 0.5% PVA, allowing their transport and storage without need of refrigeration. These stabilisation effects are dependent on the molecular weight and degree of hydrolysis of the PVA used (Boyd *et al.*, 1994, 1996; Dankwardt *et al.*, 1998). It has been suggested that a higher carbohydrate content of the molecule to be stabilised results in more effective stabilisation as a result of hydrogen bonding between the alcohol groups of PVA and the carbohydrate groups of the molecule inhibiting non-functional conformations (Boyd *et al.*, 1996). Hence, PVAs were studied for their ability to stabilise the glycoprotein antibody coating on the microtitre plates.

Poly(vinyl) alcohol and BSA

Firstly, two buffers were selected for consideration; (i) Tris buffer (1 M containing 1% EDTA and 0.05% Tween, pH 7.4), and (ii) PBS buffer (0.8 M containing 2% EDTA and 0.05% Tween, pH 7.6). Poly(vinyl) alcohols (average M_w 22 000, 98% hydrolysed (PVA-22) or 49 000, 98% hydrolysed (PVA-49)) and BSA, individually (1% solutions) or in combination (0.5 + 0.5%), were tested for their protective effects.

In all cases protective solutions prepared in Tris buffer were much more effective than solutions prepared in PBS buffer, however, the difference was more marked in the standard format with up to an 11-fold difference in A_1 . The ability of the PVA solutions tested to protect the antibody coated plate for storage decreased as follows: 1% PVA-49 > 1% PVA-22 > Tris buffer alone. The ability of the PVA/BSA solutions tested to protect the antibody coated plate for storage decreased as follows: 0.5% BSA + 0.5% PVA-22 \geq 0.5% BSA + 0.5% PVA-49 > 1% BSA.

Since a combination of BSA and PVA appeared to give the best protection this was investigated further. The protective effects of the following solutions were compared; (i) water, (ii) Tris buffer, (iii) Tris buffer with 1.5 M NaCl added, (iv) Tris buffer with 1% PVA-22, (v) Tris buffer with 0.5% PVA-22 and 0.5% BSA, (vi) Tris buffer with 1% BSA. The combination of 0.5% PVA and 0.5% BSA gave the best results for A_1 and the midpoint. The presence of NaCl in the Tris buffer did not appear to have any adverse effects.

In Tris buffer (0.1 M containing 0.1% EDTA, 0.15 M NaCl and 0.05% Tween, pH 7.4), the protective effects of solutions containing 0.5% BSA and 0.5% of each of three poly(vinyl) alcohols; average M_w 9 000 - 10 000 (80% hydrolysed) (PVA-9), 22 000 (PVA-22) and 49 000 (PVA-49), were compared using an incubation time of 5.5 h (Figure 10). Comparison with Tris buffer alone (and Tris buffer without Tween) showed that protection of the coated plate with BSA/PVA yields a substantially (> 3-fold) higher A_1 . PVA-9 and PVA-49 showed the highest A_1 and appeared to be more effective than PVA-22. No effect on midpoint was observed. PVA-9 was selected for use in the final procedure. Immediate emptying of solution from the wells without incubation also yielded sufficient protection, hence no incubation was used in the final protocol.

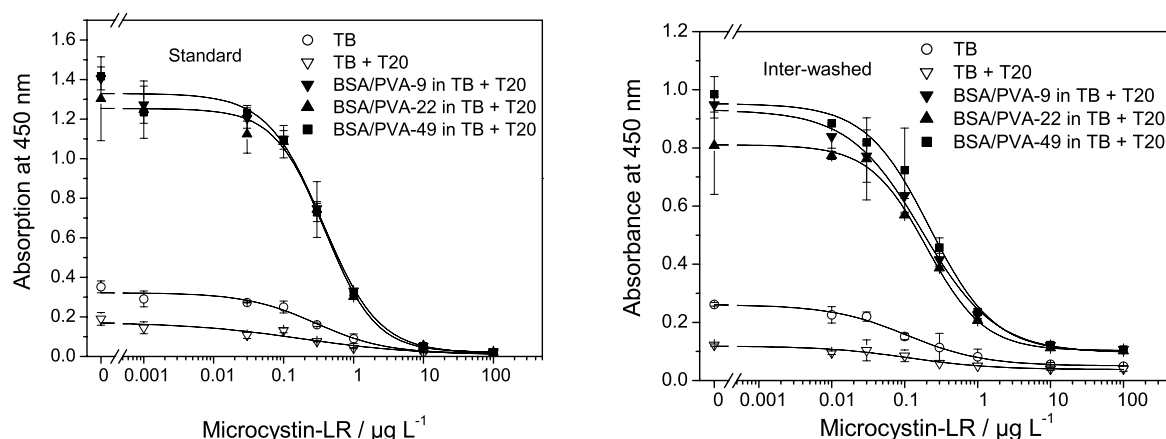


Figure 10. The effect of poly(vinyl) alcohol average M_w used in protecting antibody coated plates on the performance of the assay. TB, 0.1 M Tris buffer containing 0.1% EDTA and 0.15 M NaCl; T20, 0.05% Tween 20; BSA/PVA, 0.5% BSA + 0.5% PVA

Protection buffer concentrations

In optimising the plate protection buffer, the Tris buffer (1 M containing 1% EDTA and 1.5 M NaCl, pH 7.4) was diluted to give 0.1, 0.5 and 1 M Tris solutions which contained 0.3%, 0.25% and 0.5% BSA/PVA-9, respectively, to study the effect of buffer and BSA/PVA-9 concentration. All solutions were suitable. The most dilute solution (0.1 M with 0.3% BSA/PVA) gave the lowest midpoint and a higher A_1 than that obtained with the 0.5 M Tris buffer containing 0.25% BSA/PVA in the inter-washed format, whereas almost no difference was observed between the solutions in the standard format. On drying, crystals were only observed in the standard format for the two higher concentration solutions. Hence, 0.1 M Tris was selected for the protection buffer. A concentration of 0.3 - 0.5% PVA/BSA was considered to be optimal.

Plate protection in Tris buffer was also effective when Tween 20 was omitted. Other results obtained in the presence and absence of Tween 20 suggested that Tween 20 may decrease absorbance. Nevertheless, Tween 20 was retained in the final buffer solution in order to keep all corners of the wells covered with solution.

Temperature stability

In another experiment, protected microtitre plates were stored at 44.5°C for 6 days. Over this period a large drop in A_1 (to $\leq 50\%$ of the value obtained when the assay was run directly after coating with antibody, without protection, and without exposure to heat) was observed and the midpoints also varied considerably. The A_1 value obtained decreased for protectant solutions in the order: 0.5% poly(vinyl alcohol) + 0.5% bovine serum albumin > poly(vinyl alcohol) (5 or 1%), bovine serum albumin (5 or 1%), casein (5%) > ethylene glycol (5%), sucrose (5%), mannitol (5%), and tris buffer (1 M containing 1% EDTA and 0.05% Tween 20). The midpoints obtained for sucrose (5%) and mannitol (5%) were substantially greater than for the other protectant solutions, indicating the lack of suitability of these solutions.

Final protection solution constituents

A solution containing 0.5% BSA and 0.5% PVA (M_w 9 000 – 10 000) in Tris buffer (0.1 M containing 0.4% Na_2EDTA , 0.15 M NaCl and 0.05% Tween 20, pH 7.4, for both formats) appeared to provide good stabilisation of the antibody adsorbed on the plates and enabled storage of antibody coated plates for at least 3 months at 4°C. The procedure established for protecting antibody coated plates for storage, with minimum loss of signal and sensitivity, is outlined in the assay procedure (Figure 12) and in Section 2.11.

3.4 Optimised inter-washed and standard ELISA protocols

The optimised procedures for the standard and inter-washed formats are described in Sections 2.9 and 2.10, and in the practical layouts in Figures 11 and 12, respectively. The reagents and solutions used are detailed in Appendix II.

The inter-washed assay (Section 2.10) offers improved sensitivity in comparison to the original protocol (Section 2.8), with a lower midpoint of 0.1 µg/L consistently obtained.

3.5 Antibody robustness

The robustness of the anti-Adda monoclonal antibody (AD4G2) under various conditions (see Section 2.3) is given in Table 10. Concentrations of additives and pH in the sample are referred to in this section.

Detergents

Detergents are often used in immunoassays in order to improve microtitre plate surface wetting and to keep hydrophobic molecules in solution. Here three detergents were tested. The antibody was stable in the presence of at least 15% of Tween[®]20 or Brij[®]78, however, in the presence of Triton[®]N-101 it was only stable up to 0.125% (w/v).

Solvents

When immunoassays are used to analyse MCs in water samples that have been concentrated by solid phase extraction, or eluates from HPLC analysis (when the two techniques are coupled together), solvents will usually be present in the samples applied to the microtitre plate. The two solvents most commonly used in sample preparation and in HPLC mobile phases, methanol and acetonitrile, were tested.

Solvents had the most severe effects on the antibody's stability of any conditions tested. Methanol could only be tolerated up to 40%, and acetonitrile up to 25%.

Salts

Sodium chloride will be present in brackish water samples tested. Salts are also often added to solutions for varying ionic strength. Here the effects of three salts were studied. Sodium chloride and ammonium sulfate were tested up to concentrations that exceeded their solubility limits (359 g/L NaCl and 760 g/L $(\text{NH}_4)\text{SO}_4$), whereas, sodium sulfate was still soluble up to the maximum concentration tested (298 g/L). The antibody appeared to be very stable in high concentrations of a range of salts.

Test Number:
S-Adda-ELISA

Date:

Stage	Procedure	Solution Preparation Details	Time (min)
IgG Pre-coating	Goat anti-mouse IgG (1:3 000) in carbonate buffer, 300 µL/well Overnight, RTS	10 µL IgG in 30 mL	
Wash	3 x washing buffer		
α-Adda Mab Coating	Adda Mab cell supernatant (1:800)* in PBS, 200 µL/well 4 h – Overnight, RTS	25 µL Mab [§] in 20 mL	
Wash	3 x washing buffer		
Protection	Protection buffer + 0.5% BSA, 300 µL/well Solution aspirated / tapped out, plate dried overnight at room temperature in the dark, before storage at 4°C with desiccant	0.15 g BSA in 30 mL	
Wash	3 x washing buffer		
Sample Incubation	Tris buffer (1 M, 4% Na ₂ EDTA, 1.5 M NaCl) + 1% BSA, 20 µL/well MC-LR standards or samples, 200 µL/well 2 h (can leave longer), RTS	0.022 g BSA in 2.2 mL	
Tracer Incubation	Tracer* in Tris buffer (diluted 1:10) + 0.5% BSA, 50 µL/well 15 min, RTS, *time is critical* Cover with aluminium foil to keep in the dark	Tracer III (1:12 000), Orig Tracer (1:17 000) (Tracer II 1:30 000) [#]	
Wash	3 x washing buffer		
Development	Substrate solution (25 mL substrate buffer + 300 µL TMB solution + 100 µL H ₂ O ₂ solution), 200 µL/well 15-30 min*, RTS, cover with aluminium foil to keep in the dark		
Stop	5% Sulphuric acid, 100 µL/well		
Read	Measure absorbance at 450 nm in a microplate reader Then do: File/SaveAs		

RTS = incubation at room temperature (20-25°C), with shaking; PBS = phosphate buffered saline; BSA = bovine serum albumin added fresh before use.

* Adjust dilution or time to get a blank absorbance (A_1) of 0.8-1.0 and a midpoint (x_0) of 0.15-0.3 µg/L in the results. [§] 25 µg/L. [#] 0.05-0.1 µg/mL.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 11: Optimised Standard ELISA protocol

Test Number:
W-Adda-ELISA

Date:

Stage	Procedure	Solution Preparation Details	Time (min)
IgG Pre-coating	Goat anti-mouse IgG (1:3 000) in carbonate buffer, 300 µL/well Overnight, RTS	10 µL IgG in 30 mL	
Wash	3 x washing buffer		
α-Adda Mab Coating	Adda Mab cell supernatant (1:800)* in PBS, 50 µL/well 4 h – Overnight, RTS	7 µL Mab [§] in 5.6 mL	
Wash	3 x washing buffer		
Protection	Protection buffer + 0.5% BSA, 300 µL/well Solution aspirated / tapped out, plate dried overnight at room temperature in the dark, before storage at 4°C with desiccant	0.15 g BSA in 30 mL	
Wash	3 x washing buffer		
Sample Incubation	Tris buffer (0.5 M, 2% Na ₂ EDTA, 0.75 M NaCl) + 0.5% BSA, 50 µL/well MC-LR standards or samples, 200 µL/well 2 h (can leave longer), RTS	0.026 g BSA in 5.2 mL	
Wash	2 x washing buffer		
Tracer Incubation	Tracer* in Tris buffer (diluted 1:5) + 0.5% BSA, 50 µL/well 5 min, RTS, *time is critical* Cover with aluminium foil to keep in the dark	Tracer III (1:9 000), Orig Tracer (1:17 000) [#] 0.31 µL Orig Tracer in 5.27 mL 0.026 g BSA in 5.27 mL	
Wash	3 x washing buffer		
Development	Substrate solution (25 mL substrate buffer + 300 µL TMB solution + 100 µL H ₂ O ₂ solution), 200 µL/well 20-30 min*, RTS, cover with aluminium foil to keep in the dark		
Stop	5% Sulphuric acid, 100 µL/well		
Read	Measure absorbance at 450 nm in a microplate reader Then do: File/SaveAs		

RTS = incubation at room temperature (20-25°C), with shaking; PBS = phosphate buffered saline; BSA = bovine serum albumin added fresh before use.

* Adjust dilution or time to get a blank absorbance (A_1) of 0.8-1.0 and a midpoint (x_0) of 0.05-0.15 µg/L in the results. [§] 25 µg/L. [#] 0.1 µg/mL.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
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Figure 12: Optimised Inter-washed ELISA protocol

Table 10. Robustness of the anti-Adda monoclonal antibody under various conditions^a

Type of additive	Component tested	Concentration range tested in sample	Maximum concentration at which AD4G2 is stable ^a
Detergents	Tween [®] 20 in PBS ^b	0 – 15% (v/v)	At least 15% (v/v)
	Triton [®] N-101 in PBS ^b	0 – 20% (w/v)	0.125% (w/v)
	Brij [®] 78 in PBS ^b	0 – 20% (w/v)	At least 20% (w/v)
Solvents	Methanol in PBS ^b	0 – 80% (v/v)	40% (v/v)
	Acetonitrile in PBS ^b	0 – 60% (v/v)	25% (v/v)
Salts	NaCl in PBS ^b	0 – 398 g/L	At least 398 g/L
	Na ₂ SO ₄ in water	0 – 210 g/L	At least 210 g/L
	(NH ₄) ₂ SO ₄ in water	0 – 763 g/L	At least 763 g/L
Acids	Trifluoroacetic acid in PBS ^b	0 - 0.1% (v/v)	0.005% (v/v)
	Formic acid in PBS ^b	0 – 1% (v/v)	0.005% (v/v)
	Acetic acid in PBS ^b	0 – 1% (v/v)	0.05% (v/v)
pH level	pH	1.4, ^c 2, ^c 3, ^c 5, ^d 6, ^d 8.8, ^e 10, ^f 11, ^f 12, ^f 12.8 ^f	5 – 11
Other	Humic acid in water	0 – 10 g/L	100 mg/L
	Glycine in water	0 – 10 g/L	At least 10 g/L
	H ₂ O ₂ in water	0 – 35% (v/v)	10% (v/v)
	EDTA in water	0 – 10 g/L	At least 10 g/L

^a The antibody was defined as stable providing its activity after incubation with the solution tested was $\geq 90\%$ of that after incubation in water. ^b Phosphate buffered saline (PBS, pH 7.6) was comprised of potassium dihydrogen phosphate (10 mmol/L), dipotassium hydrogen phosphate (70 mmol/L), and sodium chloride (145 mmol/L). ^c Glycine (0.1 mol/L) was mixed with HCl (0.1 mol/L) to give pH 1.4, 2, and 3. ^d Potassium dihydrogen phosphate (0.1 mol/L) and dipotassium hydrogen phosphate (0.1 mol/L) were mixed to give pH 5 and 6. ^e Tris(hydroxymethyl)aminomethane (0.1 mol/L) and HCl (0.1 mol/L) were mixed to give pH 8.8. ^f Glycine (0.1 mol/L) was mixed with NaOH (0.1 mol/L) to give pH 10, 11, 12, and 12.8.

Acids and pH

Acids are often used in sample preparation techniques and in methods such as HPLC, which may be coupled with immunoassays for analysis of the eluates. Here the three most commonly used acids were tested. Trifluoroacetic, formic and acetic acids had severe effects on the antibody's stability, with stability only being retained up to 0.005% or 0.05% (v/v) acid.

Besides such acids affecting the pH of samples, tested waters themselves will range in pH. These tests demonstrate that the antibody is stable when exposed to samples buffered over the pH range of 5 to 11, providing 1 M Tris sample buffer (20 µL) is added to the well prior to 200 µL of sample solution.

Other

- Humic acid

Humic acids are often present in natural waters. The concentration of humic acid tested here exceeded its solubility limit, as a suspension was observed at 10 g/L humic acid. The antibody was stable in solutions containing up to 100 mg/L humic acid. It is expected that most waters tested will have humic acid contents much less than this.

- Glycine

Glycine is a commonly used buffer that could be useful, for example, in antibody purification or immunoaffinity applications. The antibody was stable over the full range of glycine concentrations tested (up to 10 g/L).

- Hydrogen peroxide

Hydrogen peroxide is often used for disinfection purposes and in the ELISA procedure itself. Since enzymes are known to be very sensitive to hydrogen peroxide, the stability of the antibody towards hydrogen peroxide was considered useful information. The antibody was stable at up to 10% hydrogen peroxide. Another anti-MC antibody, MC10E7, has been reported to be stable with 1% hydrogen peroxide (Zeck *et al.*, 2001a), however, it is unknown whether or not a higher concentration of hydrogen peroxide was tested.

- EDTA

In order to remove interference from metal ions in samples, EDTA will often be added to immunoassays. The antibody appears to be very stable in EDTA (up to at least 1%).

3.6 ELISA robustness

In this section the performance of the inter-washed ELISA format (Figure 12) was compared with the standard ELISA format (Figure 11) in the presence of a number of reagents that have the potential to interfere with the assay.

The standard ELISA referred to here is that reported by Zeck *et al.* (2001b) with slight modifications as described in Section 2.9 and Figure 11. The coating volume was maintained at 200 µL but the concentration of antibody was decreased, the sample buffer constituents were changed to be the same as those used in the inter-washed format assay (slightly different final concentration in the well), and the plates were protected.

The main differences between the standard and inter-washed assay formats compared here are: (i) the additional washing step between sample incubation and tracer incubation, and consequent changes in tracer incubation time, in the inter-washed format, and (ii) a 50 μL volume was used for both antibody coating and addition of sample buffer prior to sample application (rather than 200 μL for antibody coating and 20 μL of sample buffer prior to sample application) in order to keep the entire surface coated with antibody moist during sample application in the inter-washed format.

The effects of a range of salts, solvents and pH on these two assay formats are now described. When values differed more than the sum of 2 x the standard error on the fitted value obtained with pure water (or at pH 7) plus the standard error on the value being compared, this was considered to be a marked effect.

3.6.1 Salts

Sodium chloride

The influence of sodium chloride (NaCl) on the assay is important, since MCs can be present in brackish waters. In sea water, the concentration of total dissolved salts is 35 g/L and for sodium plus chloride ions is about 30 g/L. The effect of NaCl on the standard and inter-washed assay formats is shown in Figure 13. Concentrations of NaCl in excess of 50 g/L are extreme and not representative of real samples.

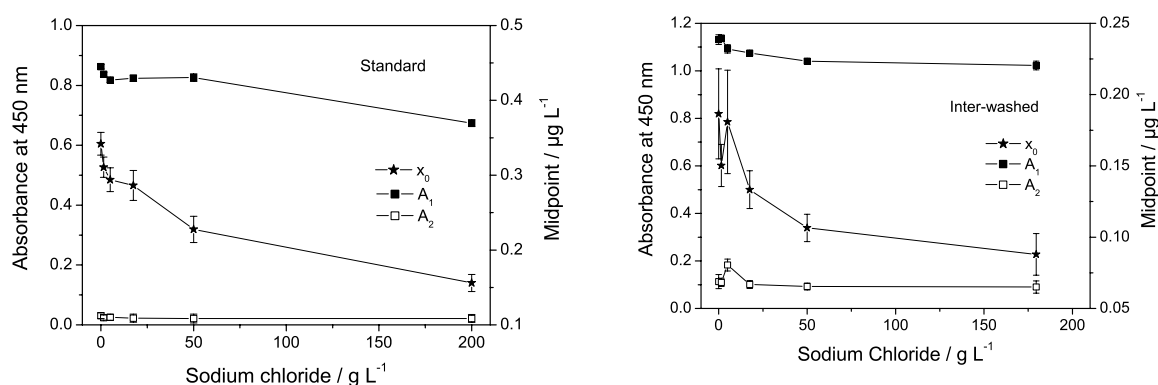


Figure 13. Influence of sodium chloride concentration in the sample on parameters (x_0 , A_1 , A_2) fitted to the microcystin-LR standard curves in both the inter-washed and standard formats

Addition of the sample buffer leads to a final concentration of 0.15 M (8.85 g/L) NaCl in the wells, when no NaCl is present in the sample. Nevertheless, the results shown in Figure 13 indicate that there is still a marked salt effect. Since the antibody itself is not affected by NaCl (Section 3.5), the high salt concentration must affect either the conformation of MCs and their binding to the antibody, or the enzymatic activity of the tracer, or both. The decrease in absorbance signal is < 10% in the inter-washed format, which is not considered to be a marked change, whereas in the standard format a larger decrease is observed. Since the salt is removed prior to addition of the tracer in the inter-washed format, these results imply that salt does affect the tracer, which is directly exposed to high salt concentrations in the standard format.

In the inter-washed format, the midpoint is unaffected by NaCl in the samples up to a concentration of 5 g/L, whereas, in the standard format, the midpoint is not stable in the presence of NaCl in samples. Two commercially available MC ELISA kits have been shown to be influenced by full strength sea water (Metcalf *et al.*, 2000b). Salinity has also been found to affect MC-LR quantification by ELISA using antibodies raised by Metcalf (1999), resulting in an apparent increase in MC-LR equivalents with increasing salt concentrations.

Copper sulfate

Copper sulfate may be added to waters containing cyanobacteria as an algicide. The effect of CuSO_4 on the standard and inter-washed assay formats is shown in Figure 14.

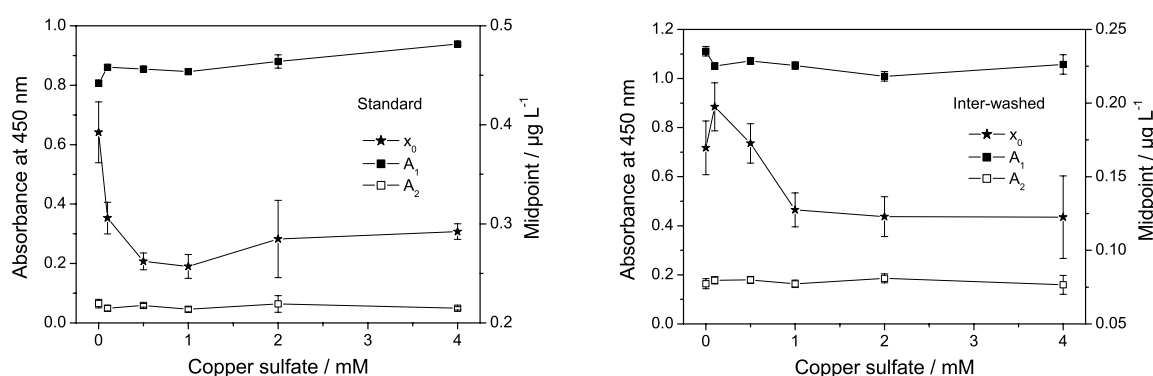


Figure 14. Influence of copper sulfate concentration on the parameters fitted to microcystin-LR standard curves in both the inter-washed and standard formats

The MC-LR standard curves showed no obvious changes up to 4 mM copper sulfate. Nevertheless, plots of the fitted parameters (Figure 14) indicate there are some marked changes. The change in absorbance signal is < 10% in the inter-washed format, which is not considered to be a large change, whereas in the standard format a slight, but noticeable, increase is observed.

In the standard format a marked decrease in the midpoint is observed between 0 and 0.5 mM copper (160 mg/L CuSO_4), while between 1 and 4 mM copper little change in the midpoint is seen. Although the midpoint is stable up to 0.5 mM copper in the inter-washed format, it appears to decrease between 0.5 and 1 mM copper. Hence, the inter-washed format can be defined as stable up to 0.5 mM copper.

Copper ions have been reported to bind MCs (Humble *et al.*, 1997). While the ion is proposed to locate itself within the MC cavity, binding to the oxygen in Adda is a possibility that would form a higher energy complex (Yan *et al.*, 2000). Such interactions are likely to result in electrostatic and conformational changes, and concomitant changes in the interaction between MC, or the MC-enzyme tracer (in the standard format), and the antibody, leading to changes in the equilibrium and thus the midpoint. No effect of copper on horseradish peroxidase has been reported.

3.6.2 Humic acid

Humic acids are found in natural waters and are known to interfere with immunoassays. The effect of humic acid on the standard and inter-washed assay formats is shown in Figure 15.

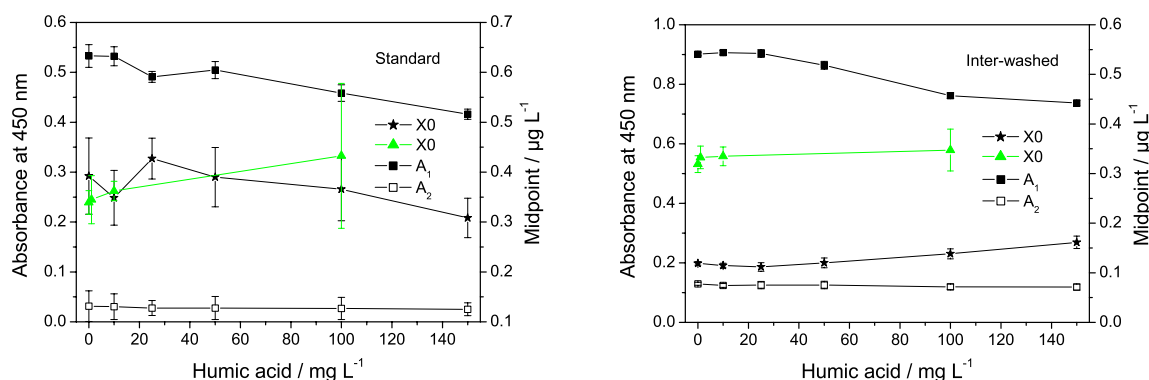


Figure 15. Influence of humic acid concentration on the parameters fitted to microcystin-LR standard curves in both the inter-washed and standard formats. Midpoint data from two experiments are shown. The additional x_0 shown in grey is from the experimental data of Section 3.2.3

Although the antibody is unaffected by up to 100 mg/L humic acid (Section 3.5), at concentrations of greater than 50 mg/L humic acids appear to influence both assay formats. This is seen by similar decreases in the maximum absorbance signal in both formats. In the experiments of Section 3.2.3 it was shown that the absorbance of the blank halved between 10 and 100 mg/L humic acid in the standard format, a decrease much greater than that seen in the inter-washed format (Figure 5).

While a slight increase in midpoint is evident in the inter-washed format, no significant change is observed in the standard format. In the experiments of Section 3.2.3 no significant difference in midpoint was observed in both formats up to 100 mg/L humic acid.

The difference between the experiments described in Section 3.2.3 and those reported here was a shorter sample incubation time (30 min rather than 2 h) and the same Tris sample buffer (1 M containing 1% EDTA, 1.5 M NaCl and 1% BSA) was used for both formats. While humic acids may not affect the stability (conformation or activity) of the antibody, they may bind non-specifically to the antibody's Adda binding site and may also affect the activity of the enzyme tracer. With humic acid, the final optimised inter-washed assay format did not appear to offer significant advantages over the final optimised standard assay format.

3.6.3 Sample pH

The pH of natural waters can vary considerably, from approximately 6.0 to 8.5. More extreme changes in pH may affect hydrogen bonding and electrostatic interactions in the immunoassay. Buffers (0.01 M solutions, diluted to the equivalent of 0.005 M solutions in the plate) comprising either glycine/NaCl/NaOH, glycine/NaCl/HCl or $\text{KH}_2\text{PO}_4/\text{NaCl}/\text{K}_2\text{HPO}_4$ were used to maintain individual pH values in the range 3.6 to 11.7. The effect of pH on the standard and inter-washed assay formats is shown in Figure 16.

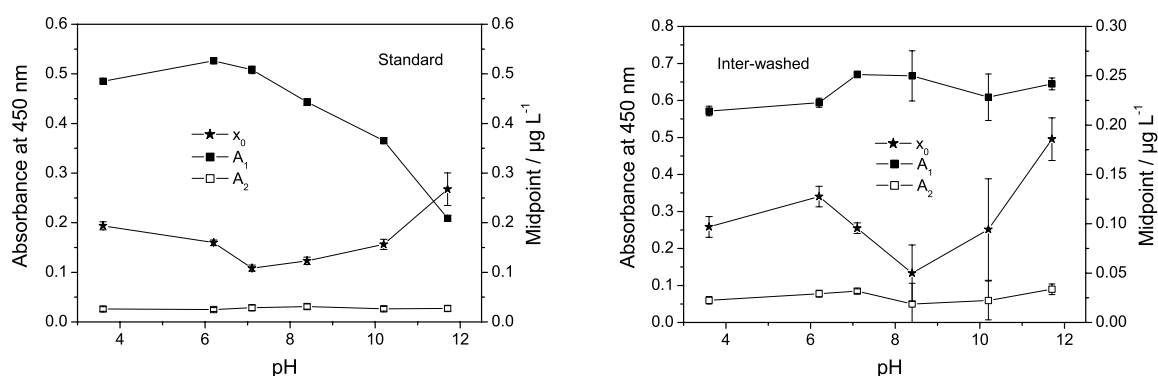


Figure 16. Influence of sample pH on the parameters fitted to microcystin-LR standard curves in both the inter-washed and standard formats

The results in Figure 16 show that pH has a considerable effect on the standard format assay, as evidenced by the dramatic decrease in maximum absorbance at $\text{pH} \geq 8$. The additional washing step of the inter-washed format appears to increase the reliability of the assay at high pH, with only 15% variation in absorbance signal observed over the pH range 3.6 to 11.7.

There appears to be a trend towards an increase in midpoint with pH between 8 and 12 in both formats. In the standard format a decrease in midpoint is observed between pH 3.6 and 7.1, while no marked change is observed in this region in the inter-washed format. In a separate inter-washed format experiment, while no absorbance signal was obtained at pH 1.4, within the pH range of 2.8 to 7.1 no pH effect on absorbance signal or midpoint was detected.

The AD4G2 antibody is stable between pH 5 and 11 (Section 3.5). Due to the presence of sample buffer in the well, the antibody will not be exposed to the extreme pH of the sample. However, the sample buffer was not able to completely control pH effects. For example, when a pH 11.7 sample was added to the plate, the pH of the solution in the well was 0.7 or 0.4 pH units above that obtained when a pH 7.1 sample was added, for the standard or inter-washed formats, respectively. Such shifts in pH can have severe effects on ELISA results. In the standard format, the increase in pH in the well when alkaline samples are used ($\text{pH} \geq 8$) is sufficient to reduce the activity of the enzyme tracer (horse-radish peroxidase has optimal activity at pH 6.5), leading to a decrease in maximum absorbance. In the inter-washed assay, because high and low pH samples solutions are removed prior to addition of the enzyme tracer (at pH 7.4), sample pH has less effect than in the standard format.

Although MCs are rather hydrophobic, they also possess polar functions; carboxylic acids, amino and amido groups. The hydrophobicity of MC-LR has been shown to decrease between pH 1 and 10 (De Maagd *et al.*, 1999). The pKa values of the two ionizable carboxyl groups and one ionizable amino group of MC-LR are reported to be 2.09, 2.19 and 12.48, respectively, although these were measured in free amino acids and can be slightly different for amino acids incorporated in a peptide (De Maagd *et al.*, 1999) and can also vary depending on the environment of the peptide (Rivasseau *et al.*, 1998). According to these pKas, below pH 2.09 MC-LR will have a single positive charge, between pH 2.09 and 2.19 it will be uncharged, between 2.19 and 12.48 it will have a single negative charge, and above pH 12.48 it will have a double negative charge. However, according to Rivasseau *et al.* (1998), ionization of MC carboxylic groups occurs at pH 3.3 - 3.4. Of course pKas and ionisable groups will vary with each MC variant, however, above pH 3.5 MCs become either amphoteric or negatively charged (Rivasseau *et al.*, 1998). These changes in the MC molecule with varying pH may contribute to the variation in binding parameters.

In the analysis of commercially available MC ELISA kits, a decrease in MC concentration determined was observed with increasing pH between 6.25 and 10 in one kit, but not in the other (Metcalf *et al.*, 2000b). An increase in midpoint would result in such an effect. The reason for the lack of an effect in the second kit tested may be the extra dilution step involved in the procedure.

When pure water alone was used instead of the sample buffer solution, the effect of pH on the fitted parameters in the inter-washed format is seen in Figure 17. From the midpoints in Figure 17, the actual pH in the well for Figure 16 can be estimated to be between pH 5.5 and 7 in the inter-washed assay when samples of pH 3.6 to 11.7 were analysed.

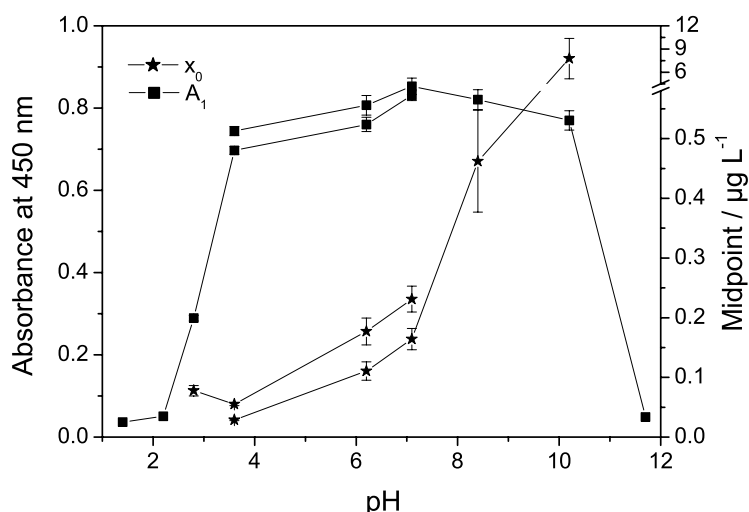


Figure 17. Influence of sample pH on the parameters fitted to microcystin-LR standard curves in the inter-washed format when pure water was used instead of sample buffer. Data from two experiments are shown. The data obtained at pH 1.4, 2.2 and 11.7 could not be fitted to equation 2.1, hence no errors are shown on the A_1 values

The A_2 values were constant at around 0.1 for all pH levels. The results shown in Figure 17 suggest an optimal pH of 3.6. With reduced pH during sample incubation a reduced midpoint of down to 0.03 $\mu\text{g/L}$ can be achieved while maintaining a strong absorbance signal. Although for standard analysis such conditions at the edge of the pH stability of the antibody (Section 3.5) are not recommended, this sensitivity improvement may be useful for special applications. A similar effect, of a decreasing midpoint, was observed with the decrease in pH that occurred upon addition of EDTA and copper during optimisation experiments (Section 3.2.3).

These results indicate the importance of pH control in the assay, which necessitates that the sample buffer is highly effective in countering the buffering capacity of water samples in order to maintain the pH selected. In the current study (with the exception of Figure 17) the optimised sample buffer at pH 7.4 was used. As can be seen from Figure 17, the greatest change in midpoint occurs above pH 4, in the region between the pKas of the carboxyl and amino groups. Hence, a sample buffer at pH 3.6 would appear to give optimal sensitivity and reproducibility. These results demonstrate the importance of ionic interactions in antibody-MC binding.

3.6.4 Solvents

Solvents have effects on the solubility of MCs and the viscosity of solutions. They can also affect the binding region of the antibody and possibly destroy the activity of the antibody or the enzyme.

Acetonitrile

The effect of acetonitrile on the standard and inter-washed assay formats is shown in Figure 18.

In the standard format, without a washing step, acetonitrile has a greater influence on the maximum absorbance signal, whereas, its influence on the midpoint is comparable in the two formats. This implies that acetonitrile is affecting both the antibody-MC binding equilibrium and the activity of the enzyme tracer. Acetonitrile may also remove the antibody coating from the microplate wells. Both formats can be defined as unaffected by up to 5% acetonitrile.

Methanol

The effect of methanol on the standard and inter-washed assay formats is shown in Figure 19.

In the standard format, without a washing step, methanol has a greater influence on the maximum absorbance signal, similar to that seen with acetonitrile.

Interestingly, methanol increases the midpoint less in the standard format than it does in the inter-washed format. The viscosity of methanol:water solutions increases up to 40% methanol and then decreases. Hence, changes in viscosity do not appear to be correlated with the observed change in midpoint. Alternatively methanol may remove different amounts of antibody coating from the microtitre plate wells in the standard and inter-washed formats.

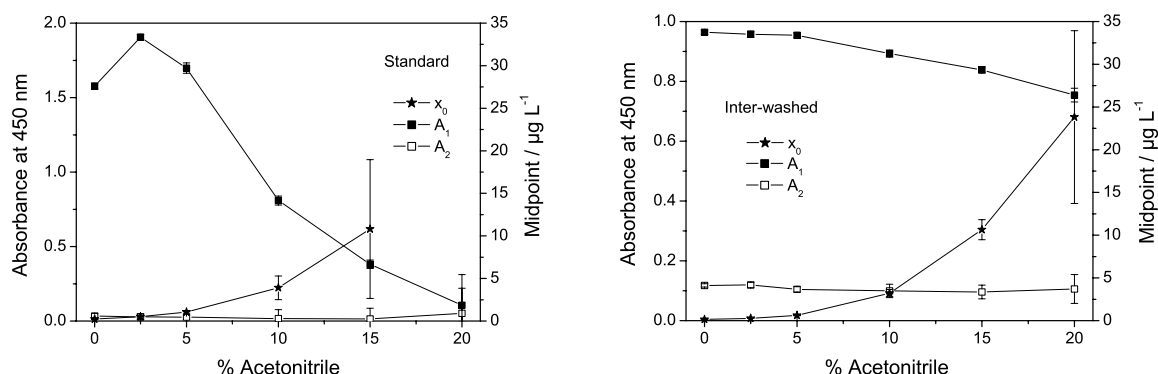


Figure 18. Influence of the acetonitrile content (% v/v) of the sample on the fitted parameters in the standard and inter-washed formats

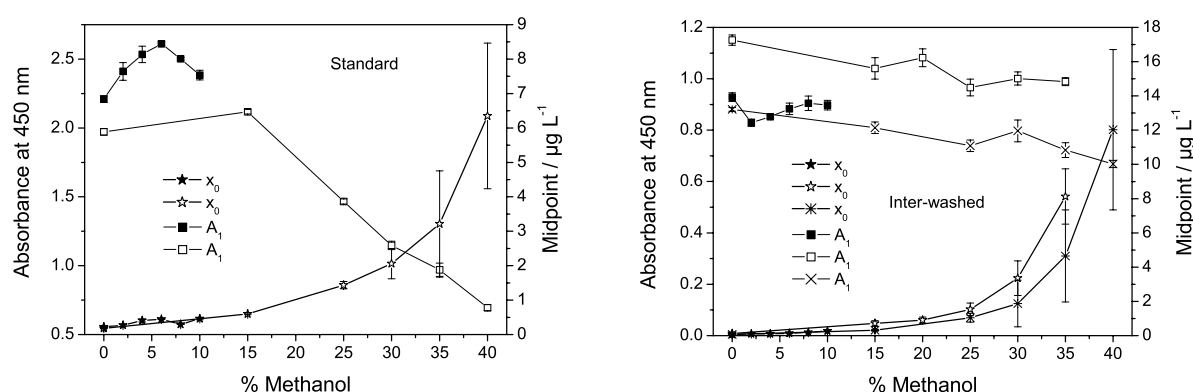


Figure 19. Influence of the methanol content (% v/v) of the sample on the midpoint and maximum absorbance signal obtained in the standard and inter-washed formats. Data from two experiments are shown

The standard and inter-washed formats can be defined as unaffected by up to $\leq 10\%$ and 15% methanol, respectively. Methanol (up to 30% v/v) has previously been reported to dramatically affect two commercially available MC ELISA kits leading to false positive results with one kit and an underestimation or decrease in MC-LR concentration determined using both kits (Metcalf *et al.*, 2000b). The absence of false positives with the second kit was thought to result from the additional dilution step used in the procedure for this kit. Other MC ELISAs have also been reported to be affected by methanol (Metcalf, 1999; Metcalf *et al.*, 2000a).

As seen above, the washing step before the tracer addition in the inter-washed format leads to more consistent blank absorbance signals in the presence of both acetonitrile and methanol, but does not prevent the increase in midpoint observed with increasing solvent content in the standard format. Both assay formats are less affected by methanol than by acetonitrile, and show considerably less stability towards these solvents than does the antibody alone (25% acetonitrile, 40% methanol (Section 3.5)).

The inter-washed format could be used up to the solvent concentration limit set by the antibody stability (Section 3.5) providing the calibration is done in the presence of solvent. However, most samples would not contain such high solvent concentrations, and the methanol content of those samples obtained as solid phase extraction (SPE) extracts could be reduced by dilution or evaporation to dryness and reconstitution in a solvent mixture containing less methanol.

3.7 Cross-reactivity of microcystin variants

The anti-6E-Adda monoclonal antibody (AD4G2) was expected to recognise all MC and nodularin variants with approximately equal sensitivity, regardless of their specific amino acid composition.

Cross-reactivities were calculated from the molar concentration of the variant giving 50% inhibition (IC_{50}) of the binding of tracer (MC-HRP) to the antibody coated solid phase (ie. the MC concentration that results in free MC binding to 50% of the antibody binding sites available) and are expressed relative to the IC_{50} for MC-LR. The IC_{50} is also referred to as the midpoint or x_0 (equation 2.1).

The cross-reactivities are given in Table 11.

A lower limit of detection and lower midpoint (in mol/L) will be achieved when the molar cross-reactivity is high.

No marked differences were found between the cross-reactivities reported prior to optimisation (Zeck *et al.*, 2001b) and those obtained in either the optimised standard or inter-washed formats. Unlike previously generated antibodies, AD4G2 recognises the Adda moiety of all MC variants tested to date and shows similar cross-reactivity to each variant.

The cross-reactivity of the range of variants tested was excellent despite the absence of certified reference standards and the uncertainties in the degree of purity of the variants. Some of the deviation from 100% cross-reactivity can be explained by the fact that all commercially available variant standards contained impurities (guaranteed purity $\geq 95\%$ by HPLC, but such materials may contain inorganic impurities that are not detectable by HPLC) and others have found that the actual quantities of the variants supplied ranged between $\pm 20\%$ of the quantity stated by the supplier (Rapala *et al.*, 2002; Fischer *et al.*, 2001). Since cross-reactivities were calculated based on the UV-absorbance at 238 nm (molar absorbance coefficient for MC-LR at 238 nm = $39,800 \text{ M}^{-1} \text{ cm}^{-1}$ (Harada *et al.*, 1990a)) rather than the toxin mass specified by the supplier (weighing the small quantities supplied is not feasible), cross-reactivities between 80 and 120% are most likely not differentiable from 100%.

Table 11: Cross-reactivities of microcystin variants tested, compared with microcystin-LR

Variant	Molar Cross Reactivity in the Standard Format (%) (Zeck <i>et al.</i> , 2001b)	Molar Cross Reactivity in the Standard Format (%)	Molar Cross Reactivity in the Inter-washed Format (%)
Nodularin-R	163 ± 41	171 ± 54	187 ± 75
Microcystin-YR	120 ± 19		90 ± 9
[D-Asp ³]Microcystin-RR	109 ± 31		
Microcystin-LY	103 ± 15		
Microcystin-LR	100 ^a	100 ^a	100 ^a
N-Ac-Adda-methylamide	99 ± 54		
[D-Asp ³]Microcystin-LR	97 ± 10		
Microcystin-LW	84 ± 32		97 ± 16
Microcystin-WR	76 ± 10		
Microcystin-RR	70 ± 16	87 ± 27	79 ± 17
Microcystin-LF	69 ± 19		90 ± 9
Microcystin-LA	66 ± 24		
[D-Asp ³]Microcystin-HtyrR	62 ± 8		
[D-Asp ³]Dhb ⁷ -Microcystin-RR	51 ± 5		
Adda	27 ± 12	28 ± 9	17 ± 54 ^b
N-Ac-Adda	25 ± 4		

^a By definition. ^b Could be detected in the inter-washed format only because of insufficient washing between the sample and tracer incubation steps. This washing step washes away the small Adda molecule.

The low cross-reactivities found for Adda and N-Ac-Adda are proposed to result from the presence of a negative charge on the carboxylic acid that was not present in the immunogen (Zeck *et al.*, 2001b). This was supported by the conversion of the carboxyl group to a methylamide group resulting in 99% cross-reactivity for N-Ac Adda-methylamide (Zeck *et al.*, 2001b). Nevertheless, the midpoints of Adda and N-Ac-Adda are < 1 µg/L, and hence, the assay even permits measurement of microcystin fragments that contain Adda.

The hydrophobicity of variants increases in the order: YR < LR < RR (Rivasseau *et al.*, 1998), however, hydrophobicity does not appear to have any affect on the cross-reactivities of these variants.

The high cross-reactivity of nodularin probably results from conformational effects. The smaller ring size of nodularin may cause less steric hindrance than MCs in the binding of the Adda moiety to the antibody and result in a better fit in the binding site.

In determining the cross-reactivity of Adda very high minimum absorbance signals (A_2) were obtained in the inter-washed format. The additional 2 x wash after sample incubation in this format seems to release bound Adda, thus allowing more tracer molecules to bind to the antibody. The fact that the washing procedure seems to affect small weakly bound molecules may be advantageous, since microcystin fragments are likely to be less toxic (Bourne *et al.*, 1996). Although the aim of this study was to produce an assay having equal cross-reactivities with all MCs, in practice it is preferable that the assay has greater affinity for, and more strongly binds, the more toxic variants, in order to give the best indication of the sample's potential toxicity. If detection of the smaller weakly bound fragments is not required, a more rigorous washing step may be incorporated to ensure all weakly bound molecules are washed away.

3.8 Comparison between the standard and inter-washed ELISAs

The stabilities of the standard and inter-washed ELISA formats towards a range of chemicals are compared in Table 12. Marked improvements in the maximum concentration tolerable are seen with the inter-washed format in the presence of NaCl, CuSO₄, high pH while slight improvements were observed in the presence of methanol. Use of the inter-washed format did not provide any marked advantage over the standard format in the presence of humic acid or acetonitrile. This suggests that these chemicals exert effects on the components of both assays that are the same, such as the binding between the antibody and MC, and it is unlikely they are affecting the activity of the enzyme tracer.

No marked differences in cross-reactivities between the standard and inter-washed formats were observed, with the exception of Adda being released more readily by the additional wash in the inter-washed format.

In a comparison between the standard and inter-washed formats, the same amount (concentration and volume (either 200 or 50 μ L)) of antibody was applied to each plate. Although the same amount (concentration and volume) of tracer was added to each plate, in the standard format the tracer concentration in the well was 5.4- to 6-fold lower (as the sample dilutes the tracer concentration in the well), and the tracer incubation time was 3-fold longer than in the inter-washed format. In addition, when an antibody coating volume of 200 μ L was used, only 25% of the antibody coated surface area was covered by tracer in the inter-washed format. Under these conditions both formats had similar midpoints, however, the maximum absorbance varied. For example, in the inter-washed format a 50% lower A_1 than that of the standard format was obtained, due to the tracer being washed off with the inter-washing step. To keep the absorbance signals comparable between the two formats a greater amount of tracer could be added in the inter-washed format. However, taking the factors mentioned above into account in kinetic calculations, the absorbance signals obtained in both

formats were comparable. Protection of antibody coated plates seems to result in higher non-specific tracer binding and hence a higher A_2 .

Table 12. Optimal pH range and maximum tested concentrations of various chemicals in the sample that are tolerated by the standard and inter-washed ELISA formats without affecting the fitting parameters

Chemical/Conditions	Standard format	Inter-washed format
pH	6.2-8.4	3.6-10.2
NaCl	0 g/L	5 g/L
CuSO ₄	0 mM	0.5 mM
CaCl ₂ ^a	7.5 mM	7.5 mM
Humic acid ^b	50 mg/L	50 mg/L
Acetonitrile	5% (v/v)	5% (v/v)
Methanol	≤ 10% (v/v)	15% (v/v)

^a Slightly different experimental conditions were used (see Figure 6, Section 3.2.3)). ^b However, Figures 4 and 5 (slightly different experimental conditions were used (see Sections 3.2.3 and 3.6.2)) imply that the inter-washed format is more stable toward humic acid than the standard format.

Using the procedures outlined in Section 2.9 and 2.10 and Figures 11 and 12, the midpoints and A_1 obtained in the inter-washed format were generally lower than in the standard format. This probably arises from the lower amount of antibody coated on the plate in the inter-washed format. Increasing the volume of antibody in the inter-washed format gave a slight increase in A_1 , but a higher midpoint. A higher A_2 value was consistently obtained with the inter-washed format, when compared with the standard format. This results from the much higher tracer concentration in the wells and also from the additional wash causing some dissociation of the MC-antibody complex.

In the case of the AD4G2 antibody the inter-washed assay format is more robust than the standard assay format. These benefits may also be achieved with other immunoassays, however, they can only be realised when the interaction between the antibody and the analyte is sufficiently strong.

3.9 Pure, treated and raw waters

Calibration curves for raw, treated and pure water spiked with microcystin-LR are shown in Figure 20. No significant difference was found between the calibration curves in these three waters, with dissolved organic carbon levels between 1.5 and 20 mg/L.

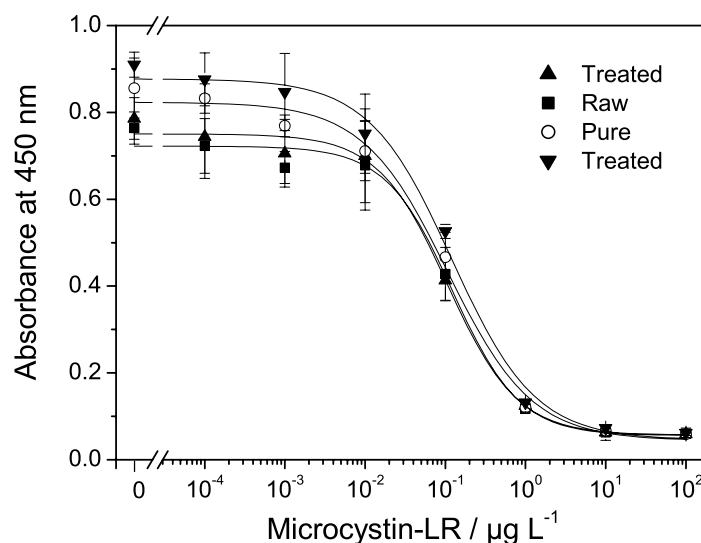


Figure 20. Calibration curves for raw, treated and pure waters spiked with microcystin-LR. A replicate of the treated water standards (run in columns 1-3 and 10-12) shows the extent of variation within in a single microtitre plate

It should be noted that a small variation in absorbance around the midpoint translates to a considerable variation in the corresponding MC concentration as a result of the log-scale of the x-axis.

Effect of plate position

Due to the time taken for application of solutions to plate wells, differences in results may arise with differences in plate position. While some variation across the plate was seen in individual experiments (Figure 20), averaged values determined in position 1 (columns 1-3), position 2 (columns 4-6), position 3 (columns 7-9) and position 4 (columns 10-12) show that no significant differences arise from the position on the plate (Table 13). Considering each of the four plate positions ($n = 8$), the coefficient of variance (CV) obtained for the midpoint was $< 24\%$ and the greatest CV (of up to 34%) was obtained for the A_2 values. These values are an indication of the variability within a microtitre plate position with three different waters over 8 microtitre plates.

Effect of dissolved organic carbon

Nine experiments were carried out in which calibration curves for raw, treated and pure water were rotated through the microtitre plate positions (columns 1-3, 4-6, 7-9, 10-12). Fitted parameters for each water, from at least ten calibration curves (each with 3 replicate wells) obtained across the range of plate positions, are compared in Table 14. No significant difference was found between the fitted parameters in the three waters (Table 14) suggesting that dissolved organic carbon (DOC) contents of up to 20 mg/L in natural raw waters do not affect the assay. For each water the CV of the midpoint was $< 19\%$ and the greatest CV (of up to 28%), was obtained for the A_2 values. These values are an indication of repeatability; plate-to-plate and day-to-day variability.

Another ELISA recently developed (Fischer *et al.*, 2001), which employs a polyclonal anti-Adda antibody (AB824), reported negligible matrix effects over a concentration range spanning four orders of magnitude. Standard MC-YR curves were determined in spiked PBS, tap water, and river water. Nevertheless, no indication was given of the DOC content or the potential for components present in the tested waters to interfere with the assay. In contrast, the raw water selected for this study has a high dissolved organic carbon content and has caused matrix effects in other assays.

It is rare that waters with a DOC or humic acid level of > 30 mg/L need to be analysed for MCs. These results and those of Sections 3.2.3 and 3.6 (humic acid) suggest that sample preparation (solid phase extraction) may be omitted when using this assay for the determination of MCs in water samples.

The results of Table 14 suggest that the sample matrix of tested waters will not affect the accuracy or precision of the ELISA. Due to slight variations in cross-reactivities (see Section 3.7) there will be some minor variations in the LOD and limit of quantification for different variants, however, these will vary much less than those of previously developed ELISAs.

Limit of detection

Comparison of the results obtained using two methods of determining the limit of detection (LOD) (Table 14) suggests that the LOD (abs) method (Section 2.18) is most appropriate, at least when only three replicate wells are used for the LOD (blank) calculation. This is demonstrated by the high CV and inability to determine values in all cases (lower n) seen in Table 14. The LOD (blank) calculation may be improved by using five or more blank wells.

There was no significant difference in LOD for the three waters tested. The results of Table 14 suggest a LOD of $0.022 \mu\text{g/L}$ for the ELISA, however, a LOD should be determined for each plate from the standard curve.

Range of quantification

The three waters were spiked with known amounts of MC-LR to give eight standard MC-LR solutions for each water, which were used to obtain calibration curves and fitted parameters. Using these fitted parameters and the average absorbance of each solution, the concentration of each solution measured by the ELISA was then back-calculated and compared with the known concentration (Table 14). For solutions of MC-LR concentration 0.0001 to $0.01 \mu\text{g/L}$, the concentrations determined were less than the limit of detection. Very high CVs were obtained at the low and high MC-LR concentrations. At the low levels the concentration of MC-LR appeared to be overestimated, whereas, at high levels the concentration was under estimated (also see Table 16). Comparison between the actual and the calculated concentrations revealed that between 0.01 and $10 \mu\text{g/L}$ the calculated value was between 60 and 140% of the actual value.

ELISAs are predominantly screening tests, and these results suggest that quantification can be made in the concentration range 0.022 to 10 µg/L within $\pm 40\%$ accuracy. However, a quantification range of 0.05 – 5 µg/L was considered to be more reasonable, with a CV of $< 40\%$ (across 10-11 plates) estimated from Table 14. The accuracy of the assay between 0.1 and 1 µg/L is ideal for testing samples against current guideline upper limits in drinking water, such as the 1 µg/L MC-LR upper limit proposed by the World Health Organisation (Gupta, 1998).

Table 13. Average parameter values obtained in plate positions 1 to 4^a

Parameter	Position 1		Position 2		Position 3		Position 4	
	Mean	SD (CV)	Mean	SD (CV)	Mean	SD (CV)	Mean	SD (CV)
A ₁ ^b	0.8	0.1 (14.9)	0.8	0.1 (13.2)	0.88	0.06 (6.9)	0.88	0.09 (10.0)
A ₂ ^c	0.06	0.01 (15.2)	0.06	0.01 (19.9)	0.06	0.02 (33.5)	0.07	0.01 (21.5)
x ₀ ^d (µg/L)	0.12	0.02 (18.6)	0.13	0.01 (8.5)	0.12	0.02 (13.4)	0.13	0.03 (23.6)
p ^e (L/µg)	1.0	0.1 (12.5)	1.0	0.2 (15.9)	0.9	0.2 (21.9)	0.9	0.1 (15.9)

^a Each water spiked with microcystin-LR at 0 (blank), 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µg/L concentrations. Three replicate wells of each standard were run on each plate. SD is standard deviation, CV is the coefficient of variance (%). Position 1 (columns 1-3; 2 pure, 3 treated and 3 raw water standard curves), position 2 (columns 4-6; 3 pure, 2 treated and 3 raw water standard curves), position 3 (columns 7-9; 3 pure, 3 treated and 2 raw water standard curves), position 4 (columns 10-12; 2 pure, 3 treated and 3 raw water curves). ^b Maximum absorbance of fitted curve. ^c Minimum absorbance of fitted curve. ^d Midpoint (IC₅₀) of fitted curve. ^e Slope of fitted curve at the midpoint.

Table 14. Fitted parameters, limit of detection, and accuracy of quantification, determined from 10-11 replicate measurements of spiked pure, treated and raw waters^a

	Pure ^b			Treated ^c			Raw ^d		
DOC ^e (mg/L)	1.5 ± 1			7 ± 1			20 ± 1		
Parameters	<i>n</i> ^m	Mean	SD (CV)	<i>n</i> ⁿ	Mean	SD (CV)	<i>n</i> ⁿ	Mean	SD (CV)
A ₁ ^f	10	0.86	0.12 (13.4)	11	0.89	0.10 (10.8)	11	0.80	0.06 (7.7)
A ₂ ^g	10	0.06	0.01 (22.7)	11	0.06	0.02 (28.1)	11	0.07	0.01 (17.2)
x ₀ ^h (µg/L)	10	0.13	0.02 (17.2)	11	0.13	0.02 (14.1)	11	0.12	0.02 (18.4)
p ⁱ (L/µg)	10	1.00	0.17 (17.3)	11	0.96	0.17 (17.9)	11	0.95	0.17 (17.7)
LOD _{av} (blank) ^j (µg/L)	10	0.029	0.037 (127.2)	9	0.017	0.014 (85.2)	10	0.017	0.014 (85.8)
LOD _{av} (abs) ^k (µg/L)	10	0.023	0.009 (39.8)	11	0.022	0.009 (41.0)	11	0.020	0.009 (44.0)
Microcystin-LR standard concentration ^l (µg/L)									
0.0001	3	0.003	0.004 (124.4)	7	0.004	0.005 (127.4)	5	0.003	0.003 (104.4)
0.001	9	0.008	0.009 (111.4)	10	0.004	0.003 (60.0)	10	0.005	0.002 (55.3)
0.01	10	0.011	0.003 (30.4)	11	0.014	0.005 (33.5)	11	0.014	0.004 (33.2)
0.1	10	0.09	0.01 (14.1)	11	0.09	0.01 (13.0)	11	0.10	0.01 (10.2)
1	10	1.2	0.3 (24.5)	11	1.4	0.4 (32.6)	11	1.2	0.3 (26.4)
10	10	6	3 (43.8)	11	11	15 (131.7)	11	7	4 (54.3)
100	7	8	5 (69.2)	9	16	11 (72.7)	8	14	7 (52.2)

^a Each water spiked with microcystin-LR at 0 (blank), 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µg/L concentrations. Three replicate wells of each standard were run on each plate. *n* is the number of replicate measurements used to determine the mean, SD is standard deviation, CV is coefficient of variance (%). ^b Milli-Q water (18.2 MΩcm). Results from ten plates. ^c Treated water. Results from eleven plates. ^d Raw water from the Warren Reservoir (SA, April 2002). Results from eleven plates. ^e Dissolved organic carbon content. ^f Maximum absorbance of fitted curve. ^g Minimum absorbance of fitted curve. ^h Midpoint (IC₅₀) of fitted curve. ⁱ Slope of fitted curve at the midpoint. ^j LOD_{av} (blank) is the average of the limits of detection calculated from three blank replicates for each water on each plate, where the limit of detection is the concentration corresponding to the absorbance = (mean of three blanks) – 3 x (standard deviation of three blanks). ^k LOD_{av} (abs) is the average of the limits of detection calculated from 85% of the absorbance range (A₁ - A₂) for each water on each plate, where the limit of detection is the concentration corresponding to the absorbance = (0.85 x (A₁ - A₂)) + A₂. ^l The concentration of each standard MC-LR solution was back-calculated for each water on each plate from the mean absorbance of three replicate wells and the fitted parameters. The average, standard deviation, and coefficient of variance of these concentrations in each water are reported. ^m Where *n* < 10, this indicates that the concentration of standard could not be back calculated from the parameters since the absorbance was either > A₁ or < A₂. ⁿ Where *n* < 11, this indicates that the concentration of standard could not be back calculated from the parameters since the absorbance was either > A₁ or < A₂.

3.10 Validation with spiked high DOC water

The ELISA was validated against PPIA, HPLC/PDA and HPLC/PDA/MS using raw water (DOC = 20 mg/L) spiked with MC-LR. For ELISA and PPIA, individual results (obtained from five replicate wells) were only classified as zero if they were below the LOD, calculated by both methods (see Section 2.18). Comparisons between the actual spiked concentration and the concentrations determined using each method can be made from Table 15.

False positives were seen with ELISA, however, in one case the error shows that no significant MC was present. A false negative was seen with PPIA. False negatives have previously been reported with PPIA (Sim and Mudge, 1994) due to endogenous phosphatase activity. A reported approach to avoid this is to heat samples prior to PPIA to destroy endogenous phosphatases.

With both ELISA and PPIA an over-estimation was found at low MC concentrations ($< 0.4 \mu\text{g/L}$) as mentioned in Section 3.9, whereas, an under-estimation was apparent with HPLC/PDA, especially at these low MC concentrations (Table 15). Under-estimation using HPLC may result from low intensity peaks not being quantified or from inaccuracies in picking the start and end of a peak for integration as occurs with very low concentrations. The over-estimation using HPLC/PDA/MS cannot be explained, especially as good agreement was found between the PDA and MS detection.

This ELISA has 2-fold greater sensitivity than the PPIA method used. As can be seen in Figure 21, in general ELISA determines more MC than do PPIA or HPLC/PDA. Similar observations have been made in other comparison studies (Fischer *et al.*, 2001, Chu *et al.*, 1990). Cyanobacterial samples may contain protein phosphatase activity (Sim and Mudge, 1993, 1994) that will mask the presence of toxin in PPIA. This can be compensated for by including a sample blank without addition of enzyme in the assay. In any case, this is unlikely to be the reason for the lower results seen here for PPIA, especially following solid phase extraction (Section 2.5). No losses from the use of plastic pipette tips were seen in the dilution of the HPLC samples for ELISA and PPIA analysis.

Solid phase extraction (SPE) was used for sample clean up and concentration prior to quantification with HPLC/PDA and HPLC/PDA/MS. These extracts were diluted with pure water to within the range of quantification of ELISA and PPIA. In addition, aliquots of the spiked solutions, taken prior to SPE (original solutions), were analysed by ELISA and PPIA. Between 72 and 113% of the MC in the original solutions was determined in the diluted post-SPE solutions by ELISA or PPIA (Table 16), suggesting that the sample matrix was not causing interference.

Table 15. Comparison of methods for determination of microcystin concentrations in raw water^a samples spiked with Microcystin-LR

MC-LR conc. spiked (µg/L)	ELISA ^b original solution ^c		PPIA ^b original solution ^c		ELISA ^{bd}		PPIA ^{bd}		HPLC/PDA ^e	HPLC/PDA/MS ^e
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Mean ± SD
0.00	0.05 ± 0.01	0.02 – 0.07	0.000 ± 0.000	0.000 – 0.000	0.01 ± 0.02	0.00 – 0.06	0.000 ± 0.000	0.000 – 0.065	0.000 ± 0.000	
0.03	0.08 ± 0.02	0.05 – 0.13	0.000 ± 0.000	0.000 – 0.051	0.068 ± 0.009	0.051 – 0.095	0.079 ± 0.003	0.000 – 0.122	0.012 ± 0.002	
0.10	0.16 ± 0.02	0.12 – 0.21	0.118 ± 0.004	0.099 – 0.134	0.18 ± 0.04	0.15 – 0.21	0.13 ± 0.04	0.08 – 0.17	0.064 ± 0.007	0.147 ± 0.008
0.40	0.57 ± 0.08	0.48 – 0.67	0.378 ± 0.008	0.340 – 0.411	0.41 ± 0.06	0.30 – 0.53	0.30 ± 0.04	0.27 – 0.33	0.370 ± 0.002	0.816 ± 0.053

^a Obtained from the Warren Reservoir, South Australia, in April 2002, having a dissolved organic carbon level of 20 mg/L after filtration through a GF/C filter. ^b Values are the mean of the trimmed means of duplicate samples, measured in 5 replicate wells, ± 1 standard deviation (SD). The range in brackets gives the minimum and maximum of the duplicate concentration ranges obtained using the trimmed mean ± 3 SD (of the trimmed data from 5 replicate wells) for each sample. ^c Spiked solution prior to concentration and clean up procedure used for all other samples tested. ^d The same samples that were concentrated by solid phase extraction for HPLC/PDA/MS and HPLC/PDA detection were diluted for analysis by ELISA and PPIA. ^e Values are the mean of duplicate samples ± 1 standard deviation. For HPLC/PDA/MS values obtained from PDA and MS detection were averaged for each of the duplicate samples.

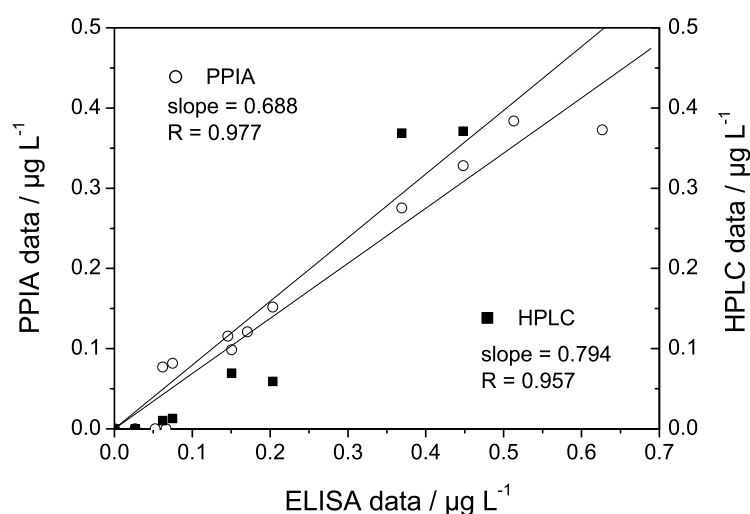


Figure 21. Correlation between the microcystin-LR concentrations determined by ELISA, PPIA and HPLC for spiked raw water (dissolved organic carbon content of 20 mg/L). Measurements by ELISA and PPIA both pre- and post-solid phase extraction are included, since sample matrix effects were considered to be minimal and recovery was between 72 and 113% (Table 16)

Table 16. Recovery of microcystin from raw water following solid phase extraction (SPE) and analytical recovery of spiked microcystin-LR by ELISA, PPIA, HPLC/PDA and HPLC/PDA/MS

MC-LR spiked (µg/L)	SPE Recovery (%) by ELISA ^a	SPE Recovery (%) by PPIA ^b	Recovery (%) by ELISA (pre/post SPE)	Recovery (%) by PPIA (pre/post SPE)	Recovery (%) by HPLC/PDA	Recovery (%) by HPLC/PDA/MS
0.03	85.0		266.7 / 226.7	0.0 / 263.3	40.0	
0.10	112.5	110.2	160.0 / 180.0	118.0 / 130.0	64.0	147.0
0.40	71.9	79.4	142.5 / 102.5	94.5 / 75.0	92.5	204.0

^a From Recovery (%) by ELISA (pre/post SPE) data. ^b From Recovery (%) by PPIA (pre/post SPE) data.

3.11 Validation with cyanobacterial culture media

The ELISA was validated against PPIA, HPLC/PDA and HPLC/PDA/MS using extracellular solutions from cyanobacterial cultures. For ELISA and PPIA, individual results (obtained from five replicate wells) were only classified as zero if they were below the LOD, calculated by both methods (see Section 2.18). The results obtained are summarised in Table 17.

In the pure water control MCs appear to have been determined by ELISA and HPLC, however, the large errors on these values indicate no significant MC is present.

Table 17. Comparison of methods for the determination of extracellular and intracellular microcystin concentrations ($\mu\text{g/L}$) in *Microcystis* cultures

Extracellular^a										
<i>Microcystis</i> strain	ELISA Mean	SD (CV)	Range	PPIA Mean	SD (CV)	Range	HPLC/PDA Mean	SD (CV)	HPLC/PDA/MS Mean \pm SD (CV)	Microcystin variants present ^g
Control ^b	0.9	1.2 (132.7)	0.0 – 3.2	0.0	0.0	0.0 – 2.6	0.06	0.06 (111.7)		
MIC040C ^c	1.0	0.2 (19.9)	0.0 – 1.9	0.0	0.0	0.0 – 6.2	0.00	0.00		
MIC050D ^d	3.2	0.4 (12.0)	2.4 – 5.2	5.1	1.8 (34.8)	2.9 – 7.9	2.4	0.4 (17.9)		YR, LR
MIC050G ^d	1.7	0.4 (24.0)	0.0 – 4.1	0.0	0.0	0.0 – 2.0	0.00	0.00	0.00 \pm 0.00	
MIC309A ^e	39	7 (17.2)	28 – 54	39	5 (13.4)	0 – 69	26	2 (7.9)		YR, LR, LA
MIC325B ^f	49	25 (50.0)	17 – 82	37	17 (47.0)	17 – 58	49	4 (9.2)	70 \pm 20 (31.6)	RR, YR, W ^h , 4 others
Intracellularⁱ										
<i>Microcystis</i> strain	ELISA ^j Mean		Range	PPIA ^j Mean		Range	HPLC/PDA ^k Mean	SD (CV)	HPLC/PDA/MS ^l Mean	Microcystin variants present ^g
MIC040C ^c	0.000		0.000 – 0.047	0.000		0.000 – 0.075	0.00	0.00		
MIC050D ^d	66		52 – 82	47		46 – 48	55	4 (6.4)		YR, LR, LA, 2 others
MIC050G ^d	2.6		2.3 – 2.9	1.8		1.7 – 1.8	2.2	0.3 (13.0)	2.8	LR, up to 3 others
MIC309A ^e	61		60 – 63	37		33 – 42	51	2 (3.3)		YR, LR, LA, 2 others
MIC325B ^f	140		120 – 160	71		65 – 76	112	3 (2.3)	114	RR, YR, W ^h , up to 4 others

^a Culture media were concentrated and cleaned up (see Section 2.5). Samples were diluted appropriately for ELISA and PPIA detection. Values are the mean of three independent samples for HPLC/PDA and HPLC/PDA/MS, and the mean of the trimmed means of the three independent samples for ELISA and PPIA, \pm one standard deviation. For the triplicate samples, trimmed means were determined from five replicate wells. The range in brackets gives the minimum and maximum of the triplicate concentration ranges obtained using the trimmed mean \pm 3 SD (of the trimmed data from 5 replicate wells) for each sample. For HPLC/PDA/MS values obtained from PDA and MS detection were averaged for each of the triplicate samples. ^b Milli-Q water was used as the control. ^c Isolated from an Eden Valley farm dam. ^d Isolated from the Torrens Lake, Adelaide, South Australia. ^e Isolated from the Mount Bold Reservoir. ^f CCAP 1450/1. ^g Determined from HPLC/PDA and HPLC/PDA/MS. ^h Variant containing tryptophan (identified by characteristic absorbance peak at 222 nm). ⁱ Cellular material was extracted as in Section 2.6. Samples were diluted appropriately for ELISA and PPIA detection. ^j Values are the trimmed mean of a single sample, measured in five replicate wells. The concentration range in brackets is given by the trimmed mean \pm 3 SD (of the trimmed data from 5 replicate wells). ^k Values are the mean of three replicate measurements of the one sample for HPLC/PDA \pm one standard deviation.

^l The value quoted is the average of the concentrations determined from PDA and MS detection for the one sample.

The *Microcystis* strains selected produce a range of MC variants. HPLC/PDA/MS revealed up to eight different microcystin variants (with retention times of 15.75, 16.0, 16.9, 17.0 (MC-RR), 18.3, 19.4 (MC-YR), 20.6 (absorbance maximum at 222 nm indicates this is a tryptophan variant), and 23.4 min) in the culture media of MIC325B. Microcystin-RR comprised *ca.* 50% of the microcystin-LR equivalents determined and a variant containing tryptophan was the second most abundant (*ca.* 37%). Microcystin-YR was also present at *ca.* 3%, while microcystin-LR was not detected. No microcystin variants were detected in the culture media of MIC040C and MIC050G by the HPLC methods, despite the 4000-fold concentration step.

In two cases, for strains MIC040C and MIC050G, the ELISA determined MC although no MC was detected by PPIA or HPLC/PDA. While it is possible that the ELISA determines MC fragments, degradation products or MC precursors, which are unlikely to be detected at least by HPLC, analysis of other dilutions of these samples suggests that these results were, in fact, false positives. The results for the other three strains are in good agreement for all methods tested. The CVs obtained with HPLC/PDA are slightly better than those obtained with the other methods. For ELISA and PPIA, large errors result at higher MC concentrations, within the region from the curvature of the inhibition curve to higher concentrations, as minor differences in absorbance translate to large differences in MC concentration. For this reason, samples should be diluted to concentrations within the range of quantification for analysis.

In general ELISA determines more MC than does PPIA or HPLC/PDA (Figure 22, and Section 3.10). The slope on this plot is slightly higher than those of Figure 21 (in raw water) for PPIA, but similar to those of Figure 21 for HPLC/PDA.

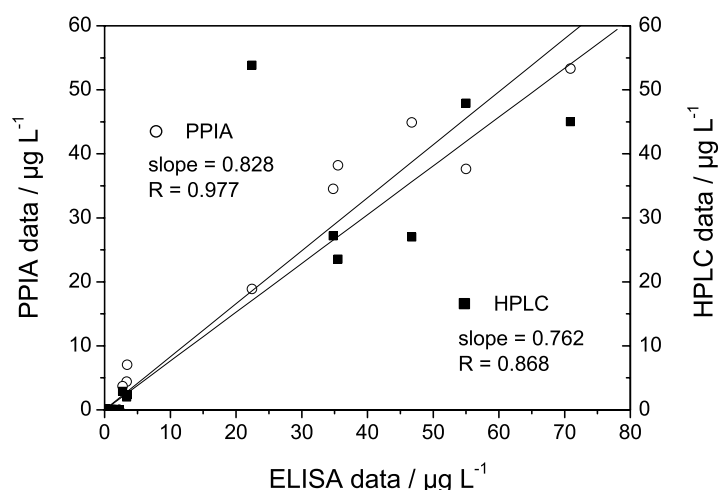


Figure 22. Correlation between the microcystin-LR equivalent concentrations determined by ELISA, PPIA and HPLC for five cyanobacterial culture media samples

3.12 Validation with cyanobacterial extracts

The ELISA was validated against PPIA, HPLC/PDA and HPLC/PDA/MS using extracts from cultured cyanobacterial cells. For ELISA and PPIA, individual results (obtained from five replicate wells) were only classified as zero if they were below the LOD, calculated by both methods (see Section 2.18). The results obtained are also summarised in Table 17.

The lower CV obtained for the HPLC/PDA method in this section results from the fact that a single sample was measured in triplicate, rather than three separate samples being measured as in the previous section.

HPLC/PDA/MS revealed at least five microcystin variants (with retention times of 16.8, 17.0 (MC-RR), 18.3, 19.4 (MC-YR), and 20.6 (absorbance maximum at 222 nm indicates this is a tryptophan variant)) in the MIC325B cells. Microcystin-RR comprised *ca.* 60% of the microcystin-LR equivalents determined and a variant containing tryptophan was the second most abundant (*ca.* 27%). Microcystin-YR was also present at *ca.* 4%, while microcystin-LR was not detected. This composition is very similar to that of the culture media. MIC050G cells were found to contain four microcystin variants (with retention times of 19.9 (MC-LR), 24.1, 24.9, and 26.1 min), although no MCs were detected in the culture media by HPLC or PPIA. Microcystin-LR comprised *ca.* 28% of the total microcystin-LR equivalents determined. Overall, the same variants were found in the cellular extracts as in the culture media samples, although in general, additional variants were seen in the cellular extracts. In healthy cultures most MC is retained intracellularly, and hence, it is expected that MC variants would be more readily detected in cellular extracts rather than in culture media. Indeed, the total MC-equivalent concentrations for the cellular extracts were markedly higher than those for the culture media.

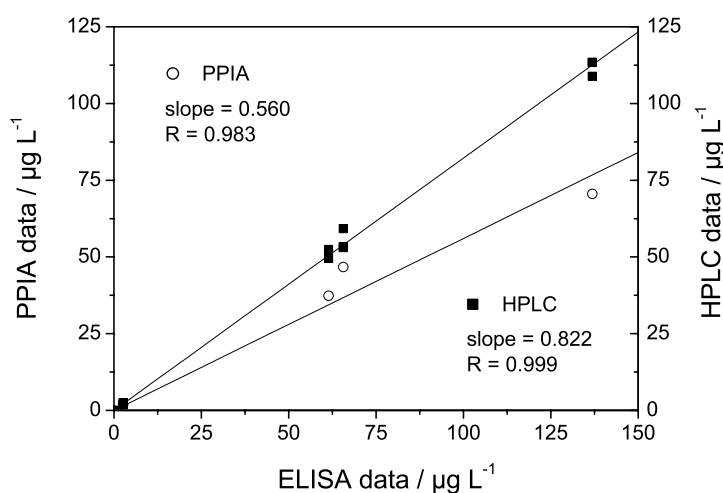


Figure 23. Correlation between the microcystin-LR equivalent concentrations determined by ELISA, PPIA and HPLC for five cultured cyanobacterial cell samples

Once again ELISA was found to determine more MC than either PPIA or HPLC/PDA (Figure 23, and Sections 3.10 and 3.11). The slope on this plot is slightly lower than those of Figure 21 (in raw water) and Figure 22 (culture media) for PPIA, but similar to those of Figures 21 and 22 for HPLC/PDA. Concentrations determined by HPLC/PDA appear to consistently be 80% of those determined by ELISA. In general ELISA data have been slightly higher than HPLC data with detection by absorbance at 254 nm (Chu *et al.*, 1990). Concentrations determined by PPIA were less than those of either HPLC/PDA or ELISA, for the raw water and cellular extract samples, but were similar to those of HPLC/PDA for the culture media samples. This may suggest that PPIA is more susceptible to negative interference from dissolved organic carbon or cellular material. ELISAs have been reported to generally give higher values than PPIA (Fischer *et al.*, 2001). The ELISA used here recognises all MC variants approximately equally, whereas, PPIA detects some variants with less sensitivity than MC-LR. Both this ELISA and PPIA may detect MC fragments.

3.13 Validation Summary

The results obtained with the generic MC ELISA, based on the anti-Adda monoclonal antibody AD4G2, correlated reasonably well with PPIA and HPLC results ($R \geq 0.87$). As anticipated, the ELISA was able to determine more MC than either PPIA or HPLC. This is probably due to the approximately equal recognition of all variants, in contrast to PPIA, the ability to detect MC fragments, and the fact that ELISA does not suffer from the drawback of the difficulty in quantifying low concentrations of variants by HPLC.

The drawbacks of the generic MC ELISA are the false positives obtained, the relatively wide sample concentration ranges obtained, and the relatively low reproducibility (high CVs). Through improvements in data analysis techniques these drawbacks may be addressed. For example, by running serial dilutions (10 x) and using two of these (within the range of quantification) to determine the sample concentration false positives will be determined and more accurate sample concentrations will be determined (due to the combination of over-estimation at low concentrations and under-estimation at high concentrations).

SUMMARY AND CONCLUSIONS

In view of the wide range of cyanobacterial cyclic peptide toxins that exert serious detrimental human health effects, and the fact that new variants continue to be found, many of which are as toxic as MC-LR, sensitive determination of the whole spectrum of cyclic peptide toxin variants is required in order to effectively manage risk. Ideally the determined response would be in proportion to the toxicity of each analyte, however, this is difficult to achieve. In order to best approximate the upper limit of toxicity, methods that determine all variants are required. Effective methods of analysis must have similar sensitivities to all variants and be able to determine concentrations well below the guideline limits, since the toxic effects of the various MCs are expected to be additive in the worst case scenario.

The common structural feature specific to the cyanobacterial cyclic peptide toxins, 6E-Adda, has been associated with their toxicity. A monoclonal antibody (Mab) raised against 6E-Adda was used to develop a direct competitive ELISA for the determination of all microcystin and nodularin variants that was ideal for application as a screening assay to estimate the upper limit of sample toxicity.

In the generic (Adda) microcystin ELISA developed in this study, the anti-Adda Mab (AD4G2) exhibited good cross reactivity with a wide range of cyclic peptide toxin variants tested and also probably detected MC fragments containing the Adda moiety. This assay was expected to recognise MC and nodularin fragments containing Adda as well as linear MCs and nodularins that can result from microbial degradation (hydrolysis) of these cyclic peptides by microcystinases (Bourne *et al.*, 1996). This is important since at least some linear peptides arising from the degradation of MC-LR are potent inhibitors of protein phosphatases (Bourne *et al.*, 1996) and hence have the potential to induce acute or chronic toxicity effects. Although these degradation products are of importance for public health risk assessment, they are unlikely to be recognised by the majority of antibodies described in Section 1.2. The generic (Adda) microcystin ELISA may also be useful in detecting toxin precursors.

By developing a new “inter-washed” direct competitive ELISA format, the robustness of the generic (Adda) microcystin ELISA was improved. The ELISA is unaffected by samples having a pH within the range of 3.6 to 10.2, which far exceeds the pH range expected for water samples. It is unaffected by sodium chloride at concentrations of up to 5 g/L and hence can be used for determining toxins in both fresh or brackish water samples. The assay is also stable in the presence of up to 0.5 mM copper sulfate and up to 7.5 mM calcium chloride, both of which exceed the concentrations expected in water samples. The ELISA is unaffected by humic acid at concentrations of up to 50 mg/L, which is around the maximum concentration expected in water samples. Nevertheless, the inter-washed assay format does not completely stabilise the assay under all conditions anticipated. For example, the interaction between Adda and the anti-Adda antibody can still be disturbed by the presence of methanol (at greater than 15% v/v) or acetone (at greater than 5% v/v) in the sample matrix. Outside the optimal conditions the inter-washed format is generally less affected than the standard format. The cross-reactivity pattern and midpoint are essentially the same for both the standard and inter-washed formats.

The reproducibility of the generic (Adda) microcystin ELISA was determined from at least ten calibration curves in each of three waters (pure, treated, raw) spiked with MC-LR, which were run on different microtitre plates on different days. The greatest CV for the midpoint was 18% (raw water) and for A₁ was 13% (pure water).

Advantages of this generic ELISA over currently available microcystin analysis tools include:

- Approximately equal cross-reactivity of all variants tested to date (a good comparison is available in Metcalf *et al.*, 2003)
- Sensitivity (limit of detection = 0.02 µg/L)
- Concentration and clean-up steps are not required for the analysis of water samples
- Robustness (stable over a relatively wide range of conditions)
- Antibody coated plates can be prepared in advance and protected to enable storage at 4°C for at least 3 months
- Time required to obtain results (< 3 h)
- Small sample volumes required (200 µL)
- Simplicity
- Cost-effectiveness

For obtaining a total peptide toxin concentration the assay described in this report is superior to the ELISAs previously developed, by virtue of the similarity in cross reactivities obtained with the anti-Adda antibody (AD4G2). Only one other ELISA has recently been developed with similar ability. Fischer *et al.* (2001) raised a polyclonal anti-Adda antibody (AB824) and developed an indirect competitive Adda-ELISA. Fischer's assay exhibits similar sensitivity to the direct competitive Adda ELISA reported here. Nevertheless, the assay described here should be more reproducible, since monoclonal antibodies can be produced with constant properties over many years. Data on the robustness of Fischer's assay has not been available, however, the robustness of the assay reported here is expected to be greater given the format used. Both assays appear to have good cross-reactivity patterns, however, Fisher's assay has not been tested with as many variants.

The integrative nature of ELISA sums the total Adda content of the sample, presenting the analyst with a value representing the total concentration of MCs, nodularins, and their fragments. This overcomes a problem of chromatographic methods, which are known to underestimate the total toxicity of samples due to the difficulty of quantifying very low levels of multiple variants which are detected individually. The other type of assay often considered as a screening test is PPIA. PPIA results are considered to more closely reflect the total toxicity of the sample. However, differences in hydrophobicity of the variants affect their ability to cross the cell membrane, and hence, their ability to inhibit protein phosphatase does not necessarily reflect their actual toxicity. Although the toxicity of MCs has been associated with the Adda moiety, some of the molecules detected with the generic (Adda) microcystin ELISA will have low or negligible toxicity, and hence, the results of this ELISA will provide an upper limit of toxicity.

ELISAs are generally more sensitive than PPIA and have been reported to have much better repeatability than PPIA (Fischer *et al.*, 2001). While PPIA can suffer from the occurrence of false positive results, caused by protein phosphatase inhibitors other than MCs or nodularins in the sample, and false negative results, caused by the presence of endogenous phosphatases in the sample, this ELISA also shows false positives. Nevertheless, for a screening method it is preferable that false positives, rather than false negatives, are obtained.

The generic (Adda) microcystin ELISA was validated against PPIA, HPLC/PDA and HPLC/PDA/MS, using raw water (DOC = 20 mg/L) spiked with MC-LR, *Microcystis* culture media (extracellular toxins) and cellular extracts (intracellular toxins). A dissolved organic carbon content of up to 20 mg/L in natural raw water did not affect the ELISA. The position of sample solutions on the microtitre plate did not significantly affect the results obtained. Results obtained with ELISA correlated well ($R \geq 0.87$) with those obtained by PPIA and the chromatographic methods, however the ELISA gave consistently higher results. The results obtained by PPIA for raw water, culture media and cellular extract samples were on average only 69, 83, and 56% of those determined by ELISA. The results obtained by HPLC for raw water, culture media and cellular extract samples were on average only 79, 76, and 82% of those determined by ELISA. Comparison of analyses prior to and post sample clean-up and pre-concentration suggested that these steps may be omitted when using the ELISA for determining MC and nodularin concentrations in water samples. A full assessment of sample matrix effects in the analysis of cellular samples is still required.

The generic (Adda) microcystin ELISA based on the monoclonal anti-Adda antibody (AD4G2), in the inter-washed format, is ideal for sample screening – it is robust, offers high sensitivity, and broad specificity to cyanobacterial cyclic peptide toxin variants. It will aid in the monitoring and toxicological assessment of waters in the presence of cyanobacterial blooms. It will reduce the total cost and workload of toxin monitoring associated with safety testing, since only positive samples will require a full analysis. It will also minimise the risk of water containing novel but toxic MC or nodularin variants being declared “toxin-free”. Its sensitivity allows detection of microcystin levels at least 20 times lower than the WHO guideline, and hence, may be useful as an early warning system prior to a bloom. The rapid results it provides will be useful to managers of water supplies in making decisions such as temporary withdrawal of raw water sources if the MC concentration is above guideline values for drinking water (World Health Organisation; National Health and Medical Research Council). Hence, the assay will assist in selecting safer water supplies, thus improving water quality and human and animal health.

RECOMMENDATIONS

In order to further enhance reproducibility and minimise the chance of obtaining false positives, improved methods of data collection and analysis should be considered as outlined in the text. A more comprehensive assessment of the performance of the generic (Adda) microcystin ELISA for the analysis of water samples is recommended with the new data processing method. The ELISA's performance in the analysis of algal scum samples, algal dietary supplements, fish and shellfish tissue, and blood serum, is yet to be evaluated. A full assessment of matrix effects should also be undertaken, analysing both culture media and cellular samples both with and without clean-up, concentration, and dilution. Although protected plates have been found to be stable for up to 3 months at 4°C, a more thorough evaluation of their stability with time and temperature is suggested.

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APPENDIX I

ASM-1 media

Compound	Final Concentration (mg/L)
NaNO ₃	170
K ₂ HPO ₄	17.4
Na ₂ HPO ₄	14.2
MgCl ₂ .6H ₂ O	40.62
MgSO ₄ .7H ₂ O	49.33
CaCl ₂ .2H ₂ O	29.4
FeCl ₃ .6H ₂ O	1.0835
H ₃ BO ₃	2.47
MnCl ₂ .4H ₂ O	1.3683
ZnCl ₂	0.44
Na ₂ EDTA	6.64
CoSO ₄ .7H ₂ O	0.0216
CuCl ₂ .2H ₂ O	0.00013

After autoclaving the pH of the media was adjusted to 7.6 – 7.65 using 1 M sodium hydroxide under sterile conditions in a laminar flow cabinet (Gelman Sciences, Clean Bench, HLF series, Laminar Flow Work Station) to minimise contamination from airborne microbes. The ASM-1 media was usually prepared in 10 L batches in large spring water containers and stored at 4°C in a refrigerator until required.

APPENDIX II

Chemicals used

acetic acid Merck (Darmstadt, Germany), p.a., 100%
acetonitrile Merck (Darmstadt, Germany) and Riedel-de Haën, HPLC grade Sigma-Aldrich (Seelze, Germany) for HPLC grade
Adda Adda was provided by Drs. D. Cundy and T. McCarthy (CSIRO Molecular Science, Clayton South, Victoria, Australia)
ammonium sulfate Merck (Darmstadt, Germany) for biochemistry
bovine serum albumin (BSA) Fraction V, A3059, Sigma, (Steinheim, Germany) 99% A-3803, Sigma
Brij®78 Fluka (Buchs, Switzerland)
casein from bovine milk C-5890, Sigma
dimethylsulfoxide Merck, > 99.5% D-8418, Sigma
di-potassium hydrogen orthophosphate anhydrous, 1.05104, Merck, (Darmstadt, Germany), p.a., 99% 10436 3A, BDH, 99.0% (MW=174.18)
ethylenediamine tetraacetic acid, disodium salt (EDTA) dihydrate, Fluka (Buchs, Schweiz), micro select > 99% 180-500G, Ajax, 99.0-101.0% (MW=372.2)
formic acid Fluka (Neu-Ulm, Germany), puriss. p.a., > 98%
glycine Merck (Darmstadt, Germany), for molecular biology
horseradish peroxidase (POD or HRP) 814393, Roche Diagnostics, Boehringer Mannheim (Germany), EIAgrade
humic acid, sodium salt 7824, Roth (Karlsruhe, Germany)
hydrochloric acid Merck (Darmstadt, Germany), p.a. 10125 6J, BDH, 35.4% (MW=36.46)
hydrogen peroxide 35%, 1.08600, Merck (Darmstadt, Germany), medical extra pure 30%, 18312, Riedel-de Haën, stabilised, extra pure
mannitol Ajax analytical reagent
methanol Merck (Darmstadt, Germany) HPLC grade

microcystin-LR from <i>Microcystis aeruginosa</i> , 350-012, Alexis (Lausen, Switzerland), ≥ 98% 101043-37-2, Biomol, >98% (MW=995.2)
microcystin-LF 350-081-C025, Alexis Biochemicals, ≥ 95%
microcystin-LW 350-080-C025, Alexis Biochemicals, ≥ 95%
microcystin-YR 475819, Calbiochem, ≥ 95%
microcystin-RR 475816, Calbiochem (La Jolla, CA, USA), ≥ 95% 350-043-C100, Alexis Biochemicals, ≥ 97%
nodularin 488002, Calbiochem (La Jolla, CA, USA), ≥ 95%
polyacrylic acid 8417, middle viscosity, Roth (Karlsruhe, Germany)
poly(ethylene glycol), Average M_n ca. 8 000 20, 245-2, Aldrich
poly(vinyl alcohol), 80% hydrolysed, Average M_w=9 000 – 10 000 36,062-7, Aldrich
poly(vinyl alcohol), 98% hydrolysed, Average M_w=22 000 81382, Fluka, (Neu-Ulm, Germany)
poly(vinyl alcohol), 98% hydrolysed, Average M_w=49 000 81383, Fluka (Buchs, Switzerland)
potassium dihydrogen citrate 60214, Fluka (Neu-Ulm, Germany) >99% (MW=230.22)
potassium dihydrogen orthophosphate 60219, Fluka, Neu-Ulm, > 99.5% Merck (Darmstadt, Germany), p.a. 10203.4B, BDH, 99.5% (MW=136.09)
protein phosphatase-2A catalytic subunit, 25 Units, V631A, 5258914, Promega
sodium azide S-2002, Sigma-Aldrich (Steinheim, Germany) (MW=65.01)
sodium carbonate (Na_2CO_3) dry, 1.06395, Merck (Darmstadt, Germany) 99.5% D3247, Ajax, 99.9-100.1% (MW=105.99)
sodium chloride 1.01540, Merck (Darmstadt, Germany), for molecular biology, >99.5% 10241.4J, BDH, > 99.5% (MW=58.44)
sodium hydrogen carbonate 1.06329, Merck (Darmstadt, Germany) > 99.5% Merck, 99.7-100.3% (MW=84.01)
sodium hydroxide Merck (Darmstadt, Germany), p.a.
sodium sulfate 1.06649, Merck, (Darmstadt, Germany), p.a., 99% 23,859-7, Aldrich, > 99% (MW=142.04)

sorbic acid, potassium salt (potassium sorbate) S-1751, Sigma (Deisenhofen, Germany) > 99% (MW=150.2)
sucrose 24,761-8, Aldrich, 99+%
sulfuric acid 100731, Merck, Darmstadt, 95-97% 10276 7Y, BDH, > 98% (MW=98.07)
3,3',5,5'-tetramethylbenzidine (TMB) 3.2.1.1 Note: this is the basic form 1.08622, Merck, (Darmstadt, Germany), > 99% T-2885, Sigma (MW=240.3)
trifluoroacetic acid Fluka (Neu-Ulm, Germany), ≥ 99% GC, < 0.05% H ₂ O
tris(hydroxymethyl)amino methane (TRIS) 93362, Fluka, (Neu-Ulm, Germany), for molecular biology grade, > 99.8% Sigma-Aldrich (Steinheim, Germany) ultrapure grade T-1503, Sigma, Reagent Grade, > 99.9% (MW=121.1)
Triton[®] N-101 37220, Serva, (Heidelberg, Germany)
Tween[®] 20 (polyoxyethylene (20) sorbitan monolaurate) 655204 Calbiochem molecular biology grade Merck (Darmstadt, Germany) for molecular biology 2509-500mL, Asia Pacific Specialty Chemicals
water, pure UV-treated pure water (Millipore, Milli-Q Water Gradient, Ultrapure Organex cartridge, 18.2 MΩcm)

Antibodies used

Antibody	"Antigen"	Species/kind	Supplier	Literature
AD4G2 cell supernatant (~ 20 mg antibody / L)	N-Acetyl-Adda	mouse, monoclonal antibody, IgG ₁	M.Weller, Technical University of Munich	Zeck <i>et al.</i> , (2001b)
anti-mouse-IgG, (affinity purified liquid – 2 mg)	mouse-IgG, F _c -fragment	goat, polyclonal antibody	670291, ICN Biochemicals (Costa Mesa, CA, USA) ICN Pharmaceuticals (Eschwege, Germany)	

Enzyme-MC tracer used

Microcystin-LR-horseradish peroxidase was prepared as outlined in Zeck *et al.* (2001a).

Batch No.	Enzyme concentration (g/L)	Dilution used	Enzyme concentration ^a used (µg/mL)
I	1.7	1 : 17 000	0.1
II	0.9	< 1: 6000 – 12 000	
III	0.912	1:9 000	0.1

^a Concentration (g HRP/mL rather than activity) was determined by UV measurement.

APPENDIX III

ELISA solutions

The following solutions should be stable for at least one month at 20°C. Fresh buffer should be prepared each month or if unusual results are obtained. Washing buffer concentrate should be kept at 4°C to permit longer storage. TMB and hydrogen peroxide solutions may also be stored at 4°C, and were prepared in capped brown glass vials (~ 30 mL) to stop degradation by light. Volumes listed below are sufficient for 32 plates, with the exception of the TMB solution (16 plates).

Carbonate buffer – 1 L

For IgG-coating (pH 9.6)

- 1.59 g (15 mM) Na_2CO_3
- 2.94 g (35 mM) NaHCO_3
- 0.195 g (3 mM) NaN_3
- add 1000 mL pure water

6 x Phosphate-buffered saline (PBS) – 300 mL

For preparing concentrated washing buffer and PBS (pH 7.6)

- 2.45 g (60 mM) KH_2PO_4
- 21.95 g (420 mM) K_2HPO_4
- 15.25 g (870 mM) NaCl
- add 300 mL pure water

Phosphate-buffered saline (PBS) – 240 mL

For anti-Adda-Mab-coating (pH 7.6). Dilute 6 x as required
(contains 0.01 M KH_2PO_4 , 0.07 M K_2HPO_4 , 0.145 M NaCl)

- 40 mL of 6x PBS
- add 200 mL pure water

60x Concentrated Washing-buffer – 250 mL

(pH 7.6, stable for 3 months at 4°C)
(contains 6x PBS buffer, 3% Tween 20)

- 7.5 g (3% w/v) Tween[®] 20
- add 250 mL of 6x PBS

Washing-buffer – 1.8 L

(pH 7.6, dilute 60 x as required)
(contains 0.001 M KH_2PO_4 , 0.007 M K_2HPO_4 , 0.015 M NaCl , 0.05% Tween 20)

- 30 mL of 60x concentrated washing buffer
- add 1800 mL pure water

Trihydroxy amino methane (Tris) buffer – 500 mL

For sample-incubation, and for dilution to 1/5 Tris buffer (pH 7.4)

(contains 0.5 M Tris, 2% Na₂EDTA, 0.75 M NaCl)

30.28 g (0.5 M) Tris(hydroxymethyl)aminomethane

200 mL HCl (1 M) (adjust to pH 7.4)

10 g (2% w/v) Na₂EDTA

21.92 g (0.75 M) NaCl

add 300 mL water

This buffer is used as the base for three steps in the ELISA:

For protection:

use 1/5 dilution + 0.5% (w/v) poly vinyl alcohol + 0.05% (w/v) Tween 20, add bovine serum albumin (0.5% w/v) fresh before use

For sample-incubation:

add bovine serum albumin (0.5% w/v) fresh before use

For tracer-incubation:

use 1/5 dilution and add bovine serum albumin (0.5% w/v) fresh before use

1/5 Tris buffer – 1.25 L

(pH 7.4, dilute 5 x as required)

For tracer-incubation and preparation of protection buffer.

(contains 0.1 M Tris, 0.4% Na₂EDTA, 0.15 M NaCl)

250 mL Tris buffer

add 1000 mL pure water

add bovine serum albumin (0.5% w/v) fresh before use

Protection buffer – 1 L

(pH 7.4)

(contains 0.1 M Tris, 0.4% Na₂EDTA, 0.15 M NaCl, 0.05% Tween 20, 0.5% poly(vinyl alcohol))

0.5 g (0.05% w/v) Tween 20

5 g (0.5% w/v) poly(vinyl alcohol)

add 1000 mL 1/5 Tris buffer

add bovine serum albumin (0.5% w/v) fresh before use

Substrate buffer – 1 L

(pH 3.8, may be stable for more than a month)

49.04 g (200 mM) KH₂-citrate

0.15 g (1 mM) sorbic acid, potassium salt

add 1000 mL water

TMB solution – 5 mL

0.0625 g 3,3',5,5'-tetramethylbenzidine (TMB) base
5 mL dimethylsulfoxide (DMSO)

1% Hydrogen peroxide solution – 5 mL

0.15 mL 30% hydrogenperoxide
add 5 mL water

5% Sulfuric acid – 500 mL

27.5 ml (95-97% sulphuric acid) **OR** 25.5 mL (98%)
add 500 mL water

APPENDIX IV

PPIA solutions

The following solutions should be stable for at least one month at 4°C. The pH of each solution should be adjusted before making up to volume with pure water.

0.19 mM EGTA in 0.08 M Tris buffer (pH 7.0) – 250 mL

2.42 g Tris(hydroxymethyl)aminomethane
0.019 g ethylene glycol – bis[β -aminoethyl ether]*N,N,N',N'*-tetraacetic acid) (EGTA)
0.1M hydrochloric acid (pH adjusted to 7.0)

0.25 M Tris buffer (pH 8.1) – 250 mL

7.57 g Tris(hydroxymethyl)aminomethane [Trizma^R Base]
0.1 M hydrochloric acid to adjust pH

10 mM Manganese chloride – 250 mL

0.50 g manganese chloride tetrahydrate

0.2 M Magnesium chloride – 250 mL

10.17 g magnesium chloride hexahydrate

The following solutions were prepared fresh before use. Volumes vary depending on the number of microtitre plate wells to be used.

20 mM Dithiothreitol (DTT) in 0.01 M Sodium acetate (pH 5.2)

0.205 g sodium acetate
0.01 M acetic acid (to adjust pH)
made up to the calibration mark with high purity(Milli-Q) water.

The final solution was made up fresh in a 30 mL brown glass vial and stored on ice before applying to microplate as follows:

0.0071 g Dithiothreitol (D.T.T)
Prepared 0.01 M Sodium acetate solution (pH 5.2) was then added to the DTT, dissolved and stored in an ice bath until applying to the plate.

5 mg/mL Bovine serum albumin

This solution was prepared fresh for each plate.

0.20 g bovine serum albumin
4 mL high purity(Milli-Q) water

60 mM *p*-Nitrophenylphosphate

This solution was prepared fresh for each PPIA plate in a 30 mL brown glass vial.

0.20 g *p*-nitrophenylphosphate disodium hexahydrate(*p*-NPP)
high purity (Milli-Q) water (9 mL) was added to the *p*-NPP, dissolved and stored on ice before use.

The solutions listed above are mixed as follows to prepare the final solutions fresh before use.

Reaction buffer

Solutions of 0.25 M Tris buffer (7.5 mL), 10 mM manganese chloride (600 µL), 0.2 M magnesium chloride (3.9 mL) and 5 mg/mL bovine serum albumin (3 mL) were combined in a 30 mL brown glass vial, stirred and stored on ice.

Substrate solution

Solutions of 60 mM *p*-nitrophenylphosphate (8 mL), 20 mM dithiothreitol in 0.01 M sodium acetate (2 mL) and reaction buffer (10 mL) were combined in a 30 mL brown glass vial, stirred and stored on ice.

Enzyme diluent

Solutions of 0.019 mM EGTA in 0.08 M Tris buffer (1.625 mL), 20 mM dithiothreitol in 0.01 M sodium acetate (125 µL), 10 mM manganese chloride (500 µL) and 5 mg/mL bovine serum albumin (250 µL) were combined in a 30 mL brown glass vial, stirred and stored on ice.

Enzyme solution

In a 30 mL brown glass vial immediately before starting the PPIA reaction, 12 µL of PPTase-2A enzyme (Promega, PPTase-2A, catalytic subunit, 25 u, 500u/mL) was added to the enzyme diluent (2 mL) and stirred to obtain an enzyme concentration of 3 enzyme units/mL. The dilution was then stored on ice until applied to the plate.

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