# An Assessment of a Re-usable Protein Phosphatase Biosensor for Microcystin and Okadaic Acid Detection

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## An Assessment of a Re-usable Protein Phosphatase Biosensor for Microcystin and Okadaic Acid Detection

**By Laura L. Allum**

The fluorescent protein phosphatase inhibition assay (PPINA), modified into a pre-biosensor design that utilises a micro-filter plate, was assessed on its performance to detect okadaic acid (OA), the major diarrhetic shellfish poisoning (DSP) toxin, and microcystins (MC), using two enzymes, protein phosphatase type 2A (PP-2A) and a recombinant protein phosphatase type 1 (R.PP-1). The pre-biosensor design consisted of immobilising the enzyme prior to conducting the assay, so that the enzyme could be retained for re-use. Modifications were made to the immobilised PP-2A and R.PP-1 method to maximise the activity and stability of the enzyme. These modifications involved adjusting cofactors in the assay, altering pH and adjusting components in the buffers during the immobilisation procedure. Both of these enzymes showed high activity in the optimised, immobilised system and were stable over a long period of time (600 hours).

The feasibility of the immobilised PPINA for use as a biosensor, for MC and OA detection, was determined by constructing dose-response curves for each toxin with PP-2A and R.PP-1. Under the optimised, immobilised assay conditions, OA and MC-LR inhibited PP-2A dose-dependently, with $IC_{50}$ values of 55 and 80 nM, respectively. Similarly, immobilised R.PP-1 was inhibited by MC dose-dependently, with an $IC_{50}$ value of 150 nM, under optimised assay conditions. This compares with $IC_{50}$ values of 5.5 and 3.2 nM for PP-2A against OA and MC-LR respectively, and 0.9 nM for R.PP1 against MC-LR, in the standard, unimmobilised PPINA format. Thus, the sensitivities of the immobilised enzymes were at least 10-fold less for MC-LR and OA detection, compared to the unimmobilised PPINA format. These detection limits for the immobilised PP-2A and R.PP-1 enzymes were outside the proposed guidelines of 1 μg of MC-LR/L for drinking water set by the WHO, and 160 μg of OA equivalents/kg in mussel meat set by the European Commission on Standards. Furthermore, R.PP-1 was not suitable for detecting OA, as immobilised and unimmobilised

enzyme activity was induced (rather than inhibited) between 10-200 nM and 1-100 nM of OA, respectively. PP-2A is the preferred enzyme over R.PP-1 for OA and MC detection, because of the greater sensitivity of this enzyme to both toxins.

The immobilised enzyme assay system (using PP-2A and R.PP-1) was tested for re-usability, by determining if the toxin inhibitors (i.e. OA and MC-LR) can be removed from the system by washing with Tris buffer (pH 7.0). Removal of the toxins should result in enzyme activity being restored in the assay to pre-toxin levels, which would render the assay suitable for repeated use. The immobilised PP-2A enzyme was not suitable for re-use, as permanent binding occurred between the enzyme and both toxins. Consequently, this prevents the use of non-replaceable PP-2A enzyme in a biosensor format. In contrast, R.PP-1 showed some potential suitability in this format for MC detection, as enzyme activity was restored to pre-toxin levels after 20 washes.

The immobilised R.PP-1 and PP-2A enzyme assays were applied to the analysis of algal and mussel samples naturally contaminated with MCs and DSP toxins, respectively. This was carried out to compare the sensitivity of the immobilised PPINA with LC-MS and other PPINA methods. The mussel and algal tests gave an insight into the applicability of the pre-biosensor method. Firstly, matrix effects, caused by confounding substances in sample extracts, were shown to interfere with the enzyme-toxin binding. Secondly, sample dilution was found to be important to maximise toxin detection. Further studies to address these issues are discussed.

This study is the first to assess the suitability of the immobilised PPINA method for translation into a prototype biosensor format. The results indicate that while PP-2A is the preferred enzyme because of its enhanced sensitivity over R.PP-1, it is not amenable for re-use in a biosensor format. Nevertheless, the enzyme could be used in a biosensor as a disposable component. However, further improvements to enhance sensitivity to the toxins are necessary before translation to a biosensor format is considered. This study is also the first to demonstrate the use of the fluorimetric substrate, MUMP for the R.PP-1 enzyme assay.

Keywords: protein phosphatase inhibition assay; protein phosphatase type 2A; recombinant protein phosphatase type 1; okadaic acid; microcystin; diarrhetic shellfish toxins; biosensor

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# Terms and Abbreviations

| | |
|---|---|
| AP | acid phosphatase |
| ASP | amnesic shellfish poisoning |
| BSA | bovine serum albumin |
| CFP | ciguatera fish poisoning |
| DDAO | dimethylacridinone phosphate |
| DiFMUP | difluoromethylumbelliferyl phosphate |
| DSP | diarrhetic shellfish poisoning |
| DTT | dithiothreitol |
| DTX | dinophysistoxins |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene-bis[oxyethylenenitrilo]tetraacetic acid |
| ELISA | enzyme linked immunosorbent assay |
| FDP | fluorescein diphosphate |
| GOD | glucose oxidase |
| HAB | harmful algal bloom |
| HEPES | 4-(2-Hydroxyethyl) piperazine-1-ethansulfonic acid |
| ip | intraperitoneal |
| $IC_{50}$ | inhibiting concentration 50% |
| LC-MS | liquid chromatography with mass spectrometry |
| $LC_{50}$ | lethal concentration 50% |
| $LD_{50}$ | lethal dose 50% |
| MC | microcystin |
| MC-LR | microcystin-LR |
| $MgCl_2$ | magnesium chloride |
| MPL | maximum permissible level |
| MOPS | 3-Morpholinopropanesulfonic acid |
| MU | mouse unit |
| MUMP | methylumbelliferyl phosphate |
| MUM | methylumbelliferone |
| NODLN | nodularin |

| | |
|---|---|
| NSP | neurotoxic shellfish poisoning |
| OA | okadaic acid |
| PCB | polychlorinated biphenyls |
| *p*NPP | *para*-nitrophenyl phosphate |
| PPINA | protein phosphatase inhibition assay |
| PP-1 | protein phosphatase 1 |
| PP-2A | protein phosphatase type 2A |
| PSP | paralytic shellfish poisoning |
| PTX | pectenotoxin |
| R.PP-1 | recombinant protein phosphatase type 1 |
| TDI | total daily intake |
| WRT | with reference to |
| YTX | yessotoxin |

# Chapter 1 Introduction

## 1.1 Microalgae and Cyanoprokaryotes

Microalgae play an important role in biological ecosystems as they are a crucial food source in the aquatic food chain. Of the several thousand known species of microalgae, a small number can proliferate to form algal blooms. Blooms may contain between 20,000-50,000 algae in 1ml of water (Klassen and Watkins, 1999) and usually last for short periods, at particular times of the year. Only a few bloom forming algae are capable of producing potent toxins and are responsible for the phenomenon known as 'harmful algal blooms' (HABs).

Cyanobacteria, formally known as blue-green algae, are cyanoprokaryotes that can also form water blooms and can produce potent toxins (cyanotoxins). Cyanobacteria constitute a major source of natural toxin input into the environment, particularly in freshwater systems (Carmichael, 1997). In New Zealand freshwaters, blooms that contain 15,000-20,000 cells/ml of cyanobacteria, is generally used as the cut-off level for recreational contact with the water body (K. Smith, personal communication, 14th November 2005).

Both microalgal toxins and cyanotoxins are found within cells, biological tissue of consumers (due to bioaccumulation) and in watersheds when they are either released by the cell or loosely bound, so that changes in cell permeability or age allow their release into the environment (Carmichael, 2001). Primary exposure routes to these toxins are through drinking contaminated water (gastrointestinal exposure), contact with contaminated recreational waters (dermal exposure), or consuming contaminated seafood. Algal and cyanobacterial blooms can be triggered during appropriate conditions of temperature, light, pH, calm conditions, carbon availability, turbidity, nutrients and/or water salinity (Pitois *et al.*, 2001).

Although algal and cyanobacterial blooms have occurred throughout history, public health and economic impacts of these events have increased in frequency, intensity and geographic distribution in recent years (Daranas *et al.*, 2001). A number of explanations have been proposed for the apparent increase in blooms (Hallegraeff, 1995):

- increased scientific awareness of toxic species (through increased technologies to detect them)

- increased utilisation of coastal water for aquaculture
- stimulation of plankton blooms by eutrophication and/or unusual climatic conditions or global climate change
- transport of dinoflagellates as resting cysts, either in ships ballast water or associated with translocation of shellfish stocks, from one area to another.

## 1.2 Algal and Cyanobacterial Toxins

Marine algal toxins are estimated to be responsible for greater than sixty-thousand human toxicity incidents per year, with an overall mortality rate of 1.5% (van Dolah, 2000). These incidents have occurred through consuming seafood contaminated with toxic algae, and through breathing aerosolised (e.g. brevetoxins) or volatilised (e.g. *Pfiesteria* toxin) toxins. Many marine algal toxins act on similar mechanisms within the nervous system. However, the resultant biological effect varies for each toxin group, from minor short-term effects to fatality. These effects have been classified into the following syndromes: diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), ciguatera fish poisoning (CFP), amnesic shellfish poisoning (ASP) and estuary syndrome (*Pfiesteria* spp.). Toxic marine algae of primary concern are the unicellular phytoplankton species, of which dinoflagellates comprise the greatest number of members, however diatoms also account for a small proportion. Bloom forming cyanobacteria are predominantly fresh- and brackish water residing, although some studies have highlighted there presence in the coastal environment (Chen *et al.*, 1993; Williams *et al.*, 1997).

Two of the toxins mentioned here, are relevant to my research and will be further discussed. Microcystins (MCs) are toxins produced from several cyanobacterial species from the following taxa, *Microcystis, Anabaena, Nostoc, Oscillatoria* and *Planktothrix* (Carmichael, 2001). Microcystins are capable of inflicting serious gastrointestinal illness in humans, livestock and wildlife, through drinking contaminated water (Carmichael, 1997). More than sixty-seven of these hepatotoxins have been identified in which most have been isolated from laboratory strains of *Microcystis* (greater than 70%) (Codd *et al.*, 2001). Microcystin-LR (MC-LR) is considered the most toxic of all MCs (Carmichael, 1997). Therefore, analytical methods generally use MC-LR as the standard in which to measure MC toxicity against.

Okadaic acid (OA) is a marine toxin produced by toxic dinoflagellates and is associated with the gastrointestinal syndrome, DSP. The DSP toxin family also consist of analogues of OA, dinophysistoxins-1, -2, and -3 (Mountfort *et al.*, 2001). Poisoning occurs from ingesting shellfish contaminated with high levels of these toxins in their tissues (Quilliam, 1995; Mountfort *et al.*, 1999). Globally, the most common DSP producing dinoflagellates are *Dinophysis* spp. and *Prorocentrum* spp. (Yasumoto *et al.*, 1995; Vieytes *et al.*, 1997; Cordova *et al.*, 2001).

Microcystins and OA have a similar mechanism of action in biological systems as both toxin families share the same pharmacologic receptors, protein phosphatase enzymes, which are the primary sites of toxin action. The toxins are potent and specific inhibitors of protein phosphatase type 2A (PP-2A) and protein phosphatase type 1 (PP-1), which are two of four major phosphatases in mammalian cells responsible for dephosphorylating serine and threonine residues (Vieytes *et al.*, 1997). Consequently, diarrhetic effects are produced when these toxins are orally ingested. Symptoms usually begin within a couple of hours and resolve within 2-3 days (van Dolah, 2000). Okadaic acid and MCs have also been implicated in long-term health effects, including severe damage to intestinal mucosa (Hungerford and Wekell, 1992b) and tumour promotion (Carmichael, 1997; Pitois *et al.*, 2001). These toxins have a worldwide-occurrence and pose a potential threat to the public and animal health, and also to the shellfish industry.

## *1.3 Microcystin and Okadaic Acid Detection*

Several countries have imposed limits on the level of acceptable MCs and DSP toxins, in potable water and shellfish, respectively, in order to protect the consumers (Shumway, 1990; Chorus and Bartram, 1999). The overseas market represent a significant export trade for New Zealand shellfish products (>75%) (P. McNabb, personal communication, 14 November 2005). Thus, there is a strong incentive to comply with international regulatory levels. The principal quality control method used to measure MC and DSP toxins in the environment, is the mouse bioassay (Mountfort *et al.*, 1999). However, there are many disadvantages in using this method, such as low sensitivity and the propensity to give false positives (Ramstad, 2001b). Consequently, considerable effort has been channelled towards developing more suitable and sensitive analytical methods for routine toxin detection. Chromatographic methods and chemical

assays have been tailored to detect all major marine and freshwater algal toxins. These methods differ largely in their approach and method of analysis. The protein phosphate inhibition assay (PPINA) is an example of an enzyme assay that utilises the inhibitory effect of the toxin on protein phosphatase enzymes. The PPINA has been developed for OA and MC detection with several protein phosphatase enzymes, the most common include PP-2A (Bialojan and Takai, 1988; Cohen *et al.*, 1989; Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990; Takai and Mieskes, 1991; Takai *et al.*, 1992a; Takai *et al.*, 1992b; Simon and Vernoux, 1994; Ash *et al.*, 1995; Takai *et al.*, 1995; Tubaro *et al.*, 1996; Vieytes *et al.*, 1997; Shimizu *et al.*, 1998; Hummert, 2000; Leira *et al.*, 2000), PP-1 (Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990; Takai and Mieskes, 1991; Takai *et al.*, 1992a; Ash *et al.*, 1995; Takai *et al.*, 1995; Shimizu *et al.*, 1998; Hummert, 2000; Holmes *et al.*, 2002) and recombinant forms of PP-1 (Zhang *et al.*, 1992; An and Carmichael, 1994; Zhang, 1994; Shimizu *et al.*, 1998; Heresztyn and Nicholson, 2001; Rapala *et al.*, 2002). The PPINA has the advantage over chromatographic methods in being able to detect the total potential toxicity, rather than identifying specific toxin congeners (Rapala *et al.*, 2002). The PPINA has been designed into a biosensor format by Mountfort *et al.,* (manuscript in preparation-a), whereby the enzyme is immobilised onto microbeads before incorporation into the assay. The immobilised PPINA is essentially carried out in a microfilter plate, rather than the standard multiwell plate, so that enzyme is retained in each well after an assay. The assay modification affords a means by which the conditions for operating a fill-flow type biosensor (Gooding and Hall, 1998) can be reproduced in rapid format, i.e. multiple environmental samples can potentially be analysed for OA and MC in the field. Performance evaluation of the immobilised enzyme (i.e. pre-biosensor format) would need to be carried out prior to design of the prototype device.

## *1.4 Research Aims and Objectives*

### 1.4.1 Research aim

To optimise conditions for an immobilised PPINA, which utilises a microfilter plate, using two enzymes, PP-2A and a recombinant PP-1 (R.PP-1), and to evaluate the feasibility of this method for use in a prototype biosensor, for MC and OA detection.

### 1.4.2 Specific objectives

1. To optimise conditions for the immobilised PPINA using PP-2A and a R.PP-1 enzyme.
2. To determine the feasibility of using the immobilised PPINA in a pre-biosensor format by constructing dose-response curves, and determining the $IC_{50}$ and assay detection limit for MC-LR and OA, with PP-2A and R.PP-1.
3. To determine if the immobilised assay system is reusable with PP-2A and R.PP-1, after treatment with toxin inhibitors.
4. To test the robustness of the method by analysing environmental samples that are naturally contaminated with MCs and DSP toxins.

## *1.5 Hypotheses*

1. The immobilised protein phosphatase enzymes can be optimised by varying components during the immobilisation process (coupling buffer and pH), and in the assay (cofactors and pH).
2. Dose-response studies can be conducted for each enzyme against OA and MC-LR, to determine the sensitivity, and detection range of the assay.
3. Inhibited PP-2A and R.PP-1 enzyme activity can recover when washed with Tris buffer.
4. Environmental samples can be tested using the pre-biosensor format to determine the concentration of DSP (OA and DTXs) and MC toxins, in each sample.

# Chapter 2 Literature Review

## 2.1 Introduction

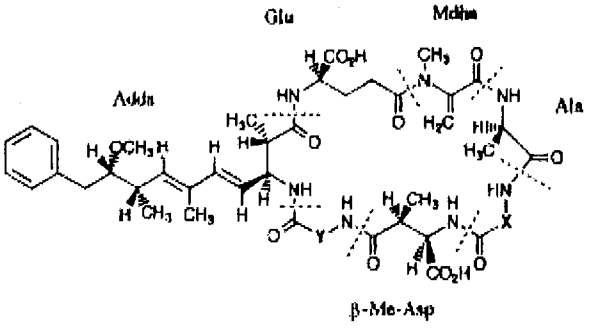
This literature review has been set out in three main sections. The first section reviews MCs and DSP toxins in detail and discusses their structure, mechanism of action, health effects, global increase, functional roles and depuration. The second section discusses current toxic microalgal and cyanobacterial problems in New Zealand and reviews past and present methods of detection, including an in depth look at the PPINA. The third section reviews biosensors, as on-site detection tools for microalgal and cyanobacterial toxins, and discusses the application of the PPINA for use as a biosensor, in place of current methods for MC and DSP detection in New Zealand.

## 2.2 Algal and Cyanobacterial Toxins

### 2.2.1 Microcystins

The general structure of MCs is cyclo [-D-Ala-"X"-D-MeAsp-"Y"-ADDA-D-Glu-Mdha] where X and Y are variable L-amino acids, X equals leucine (L), arginine (R) and tyrosine (Y) and Y equals arginine (R), alanine (A) and methionine (M) (Figure 2-1) (Carmichael, 1997). The D-MeAsp is D-erythro-β-methylaspartic acid, Mdha is N-methyldehydroalanine, and ADDA is a hydrophobic β-amino acid (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid (Carmichael, 1997). The 6*E* geometry in the ADDA moiety is considered a prerequisite for toxicity in microcystin congeners (Fischer *et al.*, 2001). Structural and compositional variations of MCs differ in the two amino acids and additional methylation/demethylation of the other amino acids, particularly methylaspartic acid and/or methyldehydroalanine (Rinehart *et al.*, 1994). Other MC variants have modified ADDA units and include acetyl-demethyl (ADMADDA) and demethyl (DMADDA) variants. The X and Y combinations for the heptapeptide toxins typically include LR, LA, YA, YM, RR, YR, WR, AR and FR. Microcystins are typically hydrophilic, with the exception of hydrophobic MC-LL, -LV and –LM identified from *Microcystis* spp. in Canada (Craig *et al.*, 1993). Table 2-1 lists some of

the common cyanobacterial hepatotoxins (refer to appendix 7.1 for a more comprehensive list). Microcystin-LR is considered the most toxic of all MCs considering its low intraperitoneal $LD_{50}$ in rodents (50 μg/kg) (refer to appendix 7.2 for a list of toxicity values for common MCs) and comparatively high number of conjugates identified (Rinehart *et al.*, 1994). Microcystin-LR is also the most commonly isolated MC. Thus, MC-LR is used as the standard in most analytical methods to measure MC toxicity against.




| | X | Y |
|---|---|---|
| Microcystin-LA | Leu | Ala |
| Microcystin-LR | Leu | Arg |
| Microcystin-YR | Tyr | Arg |
| Microcystin-YA | Tyr | Ala |
| Microcystin-YM | Tyr | Met |

**Figure 2-1.** Structures of five common microcystins (Botes *et al.*, 1985)

Closely related to MCs is the low molecular weight, monocyclic pentapeptide, nodularin (NODLN), which is produced by cyanobacteria. Nodularins also contain ADDA but lack one of the L- and D-amino acids found in the MCs. Nodularin structure is cyclo [-D-MeAsp-L-Arg-ADDA-D-Glu-2-(methylamino)-2-dehydrobutyric acid (known as Mdhb)] (Figure 2-2) (Sivonen, 1996). Approximately, five congeners of NODLNs have been identified (Fischer *et al.*, 2001). *Nodularia* spp. are the primary producers of NODLN and have been reported typically in brackish waters worldwide, with *N. spumigena* being the first cyanobacterium reported in the

literature in 1878 (Carmichael *et al.*, 1988). *Nodularia spumigena* was first characterised in New Zealand and like all *Nodularia* spp. was identified as being able to produce potent, hepatotoxic peptides of similar composition but lower molecular weight than the heptapeptide MCs (Carmichael *et al.*, 1988).

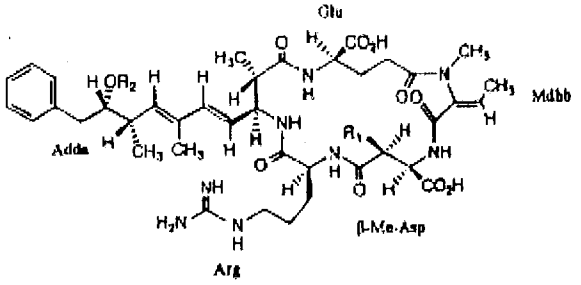



**Figure 2-2.** Structure of nodularin (R1, R2 = CH3) (Harada, 1996).

## 2.2.2 Diarrhetic shellfish poisoning toxins

Diarrhetic shellfish poisoning toxins are structurally unrelated to MCs (Figure 2-3). They consist of a class of acidic polyether toxins with OA and dinophysistoxin-1 (DTX-1) being the primary congeners. Structural derivatives of DTXs include DTX-2, -3, -4, -5 and -6. Dinophysistoxin-2 has only been discovered and identified in Irish mussels (Carmody *et al.*, 1995), where it continues to be the predominant toxin in the form of DTX-2B and -2C (Draisci *et al.*, 1998). Dinophysistoxin-3 has a fatty acyl group attached through the 7-OH group of OA, hence it has been named acyl OA (Figure 2-3). Ester derivatives of OA, referred to as the diol esters, have been isolated and identified from differing strains of *Prorocentrum lima* (Norte *et al.*, 1994). Dinophysistoxin-4 is not an end-product of toxin synthesis but is enzymatically transformed to OA-diol ester 8 and then to OA, thus it acts as a precursor for lipophilic toxins such as OA (Pan *et al.*, 1999). Structural studies by Holmes *et al.* (1990) have suggested that the free carboxyl group, the hydroxyl group at the 24$^{th}$ carbon chain, and the spatial conformation are all essential for toxin activity. Table 2-2 lists the common DSP toxins and the species associated with their production.

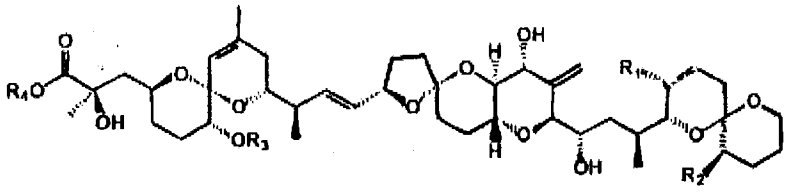
**Table 2-1: Common microcystins and nodularins identified from species and strains of *Anabaena, Microcystis,* and *Nodularia* (Rinehart *et al.*, 1994; Carmichael, 1997).**

| Toxin Strain | MW* | Molecular formula | Toxin Producing Species | Reference |
|---|---|---|---|---|
| MC-LR | 994 | $C_{49}H_{74}N_{10}O_{12}$ | *A. flos-aquae* | (Carmichael *et al.*, 1988) |
| | | | *M. aeruginosa* | (Watanabe *et al.*, 1988) |
| | | | *M. viridis* | |
| MC-LA | 909 | $C_{46}H_{67}N_7O_{12}$ | *M. aeruginosa* | (Botes *et al.*, 1985) |
| MC-AR | 952 | $C_{49}H_{68}N_{10}O_{12}$ | *M. aeruginosa* | (Namikoshi *et al.*, 1992) |
| MC-YA | 959 | $C_{49}H_{65}N_7O_{13}$ | *M. aeruginosa* | (Botes *et al.*, 1985) |
| MC-LF | 985 | $C_{52}H_{71}N_7O_{12}$ | *M. aeruginosa* | (Azevedo *et al.*, 1994) |
| MC-LY | 1001 | $C_{52}H_{71}N_7O_{13}$ | *M. aeruginosa* | (Stoner *et al.*, 1989) |
| MC-FR | 1028 | $C_{52}H_{72}N_{10}O_{12}$ | *M. aeruginosa* | (Namikoshi *et al.*, 1992) |
| MC-YM | 1035 | $C_{51}H_{69}N_7O_{14}S$ | *M. aeruginosa* | (Botes *et al.*, 1985) |
| MC-RR | 1037 | $C_{49}H_{75}N_{13}O_{12}$ | *A. flos-aquae* | (Sivonen *et al.*, 1992) |
| | | | *M. aeruginosa* | (Zhang *et al.*, 1991) |
| | | | *M. viridis* | (Kusumi *et al.*, 1987) |
| MC-YR | 1044 | $C_{52}H_{72}N_{10}O_{13}$ | *M. aeruginosa* | (Botes *et al.*, 1985) |
| | | | *M. viridis* | (Watanabe *et al.*, 1988) |
| MC-WR | 1067 | $C_{54}H_{73}N_{11}O_{12}$ | *M. aeruginosa* | (Namikoshi *et al.*, 1992) |
| NODLN | 824 | $C_{41}H_{60}N_8O_{10}$ | *N. spumigena* Strain L575 | (Laub *et al.*, 2002) |

* MW- molecular weight

Pectenotoxins (PTX) are a family of cyclic polyether macrolide toxins that have been isolated from shellfish involved in DSP (Daranas *et al.*, 2001). Yessotoxin (YTX), a desulfated polyether toxin, has also co-occurred with DTX-1 and DTX-3 from scallops collected in Mutsu Bay, Japan (Murata *et al.*, 1987). Pectenotoxin has been shown to be hepatotoxic and diarrheagenic in high concentrations only (Zhou *et al.*, 1994). Yessotoxin, when given orally (i.e. consumed), doesn't get absorbed and is mainly excreted in the faeces. Studies have shown YTX to be only mildly diarrheagenic with pathogenic effects evident through intraperitoneal (i.p.) injection only (Ogino *et al.*, 1998). Although YTX and PTX have tentatively been included in the greater DSP toxin family, they are not protein phosphatase inhibitors (Food Standards Agency, 2003).

Prorocentrolide and prorocentrolide B, nitrogenous polyether lactones, have additionally been isolated from DSP producing species, *P. lima* and *P. maculosum,* respectively (Daranas *et al.*, 2001). Although the mechanism of action of these fast acting toxins is not yet understood, they are not considered protein phosphatase inhibitors (Daranas *et al.*, 2001).



Okadaic acid: R1=$CH_3$; $R_2$,$R_3$,$R_4$=H

DTX1: $R_1$,$R_2$=$CH_3$; $R_3$,$R_4$=H

DTX2: $R_1$=H; $R_2$=$CH_3$; $R_3$,$R_4$=H

DTX3: $R_1$,$R_2$=$CH_3$; $R_3$=acyl; $R_4$=H

**Figure 2-3.** Structure of common DSP toxins (Daranas *et al.*, 2001).

### 2.2.3 Mechanism of Action

Microcystins and DSP toxins have a high affinity for the liver and utilise the bile acid transport system to enter the hepatocytes (Dawson and Holmes, 1999). In the liver, MCs and DSP toxins bind to and inhibit serine/threonine protein phosphatases for selective classes, protein phosphatase type 1 (PP-1) and protein phosphatase type 2A (PP-2A). Microcystins inhibit both of these enzymes equivalently ($IC_{50}$~0.1 nM (MacKintosh *et al.*, 1990)), whilst DSP toxins inhibit PP-2A at an equivalent sensitivity ($IC_{50}$ 0.1 nM) but inhibit PP-1 at a reduced sensitivity ($IC_{50}$ 10 nM) under similar conditions (Cohen *et al.*, 1989).

Protein phosphatase type 1 and PP-2A are two of four major classes of protein phosphatases that have been identified in eukaryotic cells (Vieytes *et al.*, 1997). Protein phosphatase type 1 dephosphorylates the β-subunit of phosphorylase kinase C specifically, whereas PP-2A dephosphorylate the α-subunit of phosphorylase kinase C preferentially (Cohen and Cohen, 1989). Protein phosphatase type 2 enzymes also comprise PP-2B and PP-2C. Protein phosphatase type 2B is 1000 fold less sensitive than PP-2A to MC-LR, and PP-2C is not inhibited by MC-LR (Honkanen *et al.*, 1990).

**Table 2-2: Common diarrhetic shellfish toxins identified from *Dinophysis*, *Prorocentrum* spp. and *Lingulodium* sp.** (Yasumoto *et al.*, 1995)

| Toxin | Mw | Molecular formula | Toxicity[a] μg/kg | Toxic Species | Reference |
|---|---|---|---|---|---|
| OA | 804 | $C_{44}H_{68}O_{13}$ | 200 | *D.fortii* *D.acuta,* *D.acuminata* *P.lima* | (MacKenzie *et al.*, 2002) |
| DTX-1 | 818 | $C_{45}H_{70}O_{13}$ | 160 | *D.fortii,* *D.mitra,* *D.rotundata,* *D.tripos* *D.acuta,* *D.norvegica* *P.lima* | (Imai *et al.*, 2003) (Ramstad *et al.*, 2001) |
| DTX-2 | 804 | $C_{44}H_{68}O_{13}$ | | *D.acuta,* *D.acuminata* | (Carmody *et al.*, 1995) |
| DTX-3[b] | 1056 | $C_{61}H_{100}O_{14}$ | 250 | *D.acuta* | (Rhodes *et al.*, 2001) |
| PTX-1 | 874 | $C_{47}H_{70}O_{15}$ | 250 | *D.acuta* | (MacKenzie *et al.*, 2002) |
| PTX-2 | 858 | $C_{47}H_{70}O_{14}$ | 230 | *D.acuta* *D.fortii* D.caudata | (Draisci *et al.*, 1996) (MacKenzie 2002) |
| YTX | 1186 | $C_{55}H_{80}O_{21}S_2Na_2$ | 100 | *P. reticulatum* *Lingulodium polyedra* | (Mackenzie *et al.*, 1998; Rhodes *et al.*, 2001) |

[a] intraperitoneal injection to mice

[b] 7-O-palmitoyl DTX-1, the most abundant component

Serine/threonine protein phosphatases are essential in the signalling cascades in eukaryotic cells, which regulate important cellular processes such as ion balance, neurotransmission and metabolism (Anderson, 1994). Signalling is achieved through regulating the number of phosphate groups on protein chains. Inhibition of these enzymes therefore leads to hyperphosphorylation of cellular proteins such as cytokeratin 8 and 18, and vimentin, causing dramatic cell deformation through disaggregation of the intermediate filaments (Chou *et al.*,

1990; Eriksson *et al.*, 1990; Eriksson *et al.*, 1992). An early symptom of exposure is diarrhea caused by the continual secretion of sodium from intestinal cells (Eriksson *et al.*, 1992). The structural changes in the liver of animals from an acute lethal dose of MC or NODLN, are a breakdown in liver tissue architecture and blood accumulation in the liver, with blood loss to the circulatory system and subsequent death (Falconer, 2001). Increased phosphorylation has also been associated with tumour promotion in various organs. Protein phosphatase type 1 and PP-2A are believed to function, in part, as tumour suppressors in normal cells as they are the chief enzymes that reverse the action of protein kinase C (Cohen and Cohen, 1989). Thus, MC and DSP toxins act as tumour promoters by inhibiting these enzymes.

### 2.2.4 Health Implications

#### 2.2.4.1 Acute health incidents

Exposure to cyanobacterial blooms has been linked to many cases of illness, since the first publication on cyanobacteria toxicity in 1878 (Flint, 1966). The most clearly demonstrated effects have been due to the ingestion of MCs (Falconer, 2001). Incidents have been documented in North and South America, Europe, Australia, Canada, Asia, South Africa, New Zealand, Israel, China and the Soviet Union (Flint, 1966; Craig *et al.*, 1993; Azevedo *et al.*, 1994; Chorus and Bartram, 1999; Domingos *et al.*, 1999; Fastner *et al.*, 1999; Giovannardi *et al.*, 1999; de Magalhaes *et al.*, 2001). A review of poisoning events in toxic cyanobacterial research can be found in Carmichael (2001). Most toxicity cases have only produced sub-lethal health effects, such as the suspected involvement of cyanobacteria in the Half disease, which historically affected fish-eating people along the Baltic coast in the 1920's and 1930's (Pitois *et al.*, 2001). In Australia, a sickness known as Barcoo fever was believed to be caused by the ingestion of cyanobacteria which produced diarrhetic effects (Dawson and Holmes, 1999). There have also been numerous reports of gastro- and hepatoenteritis (inflammation of the stomach and intestines, and liver, respectively), and contact irritation following exposure to MC in drinking and recreational water, respectively (Chorus and Bartram, 1999; Falconer, 2001). The only confirmed human deaths (55 fatalities) from MC have been limited to exposure through renal dialysis at a haemodialysis centre in Caruaru, Brazil in 1996 (Domingos *et al.*, 1999; Carmichael, 2001).

Unlike incidents involving MCs, DSP has only been a documented problem for a relatively short period. The first incidence of human shellfish related illness from DSP toxins occurred in Japan in the late 1970's, in which *Dinophysis fortii* was identified as the causative organism (van Dolah, 2000). Since this time, seasonal occurrences of DSP have been reported in a number of countries, including Japan, Europe, North and South America, Australia, India, Thailand, Indonesia and New Zealand (Draisci *et al.*, 1996; Mackenzie *et al.*, 1998; van Dolah, 2000; Ramstad, 2001a; Uribe *et al.*, 2001; Imai *et al.*, 2003). In some countries, DSP blooms have been a more significant problem than other algal blooms, due to the characteristically longer bloom period (Sivonen, 1996). In humans, most DSP toxicity cases have produced diarrhetic effects and symptoms are self-limiting. Routine shellfish monitoring in developed countries can prevent human poisonings, as contaminated shellfish beds are identified and closed to the public. However, in developing countries which have not adopted monitoring programmes, particularly those areas that rely heavily on the sea for food, have a higher incidence of illness as a result of toxic algal blooms (Carmichael, 1997). There have been no confirmed human deaths as a result of DSP to date.

### 2.2.4.2 Symptoms of acute toxin exposure

Symptoms of dermal exposure to cyanotoxins include allergic reactions, skin irritation, blisters and itching, shortly followed by dermatitis (Carmichael, 2001). Ingestion related illnesses include gastroenteritis and hepatoenteritis, with symptoms including vomiting, diarrhea, nausea, throat irritations, headache, abdominal pain, respiratory difficulty, and other pneumonia-related symptoms (Carmichael, 2001).

Symptoms of DSP are similar to MC toxicity, as they both share the same pharmacologic receptors. However, there is potential for exposure to higher oral concentrations of DSP toxins as exposure is to seafood bioaccumulated with DSP toxins, rather than in the water. Hence, symptoms of gastrointestinal exposure to seafood containing DSP toxins can be more severe. Severe cases of DSP can induce symptoms within 30 minutes, whereas mild cases may take several hours to develop after ingestion (van Dolah, 2000). Recovery of DSP takes approximately 2-3 days regardless of treatment. Diarrhetic shellfish toxins have varying toxicities (cell densities of *D.fortii* as low as 200 cells/L have resulted in DSP) and only OA, DTX-1 and DTX-3, are thought to be responsible for diarrhea (Hungerford and Wekell, 1992a).

### 2.2.4.3 Chronic health incidents

Initial epidemiological evidence from chronic, long-term exposure to MCs was obtained from Dr Shun Zhang at the Shanghai Medical University, China and Wayne Carmichael at Wright State University in the United States (Carmichael, 1997). They revealed a positive correlation between drinking surface water (containing MC) and high rates of primary liver cancer in certain areas of China. Another study of chronic exposure, was undertaken on human lymphocytes *in vitro* (Pitois *et al.*, 2001). The study concluded that MCs produced more chromosomal damage than known carcinogens, benzene and sodium arsenite, and produce similar damage to polychlorinated biphenyls (PCBs). Their results suggest that toxic cyanobacteria are a more serious environmental hazard than previously thought. A report by Ito *et al.* (1997), confirmed the carcinogenic activity of MCs in mice, as they developed large tumour nodules in the livers after one-hundred i.p. injections of the toxin.

Considering that DSP is a relatively newly identified toxin, cases of chronic human exposure have yet to be identified. However, the tumour promoting activity of MC and DSP toxins has been well described in laboratory studies as discussed in several publications (Haystead *et al.*, 1989; Humpage and Falconer, 1999; Falconer, 2001; Pitois *et al.*, 2001).

### 2.2.4.4 Effects on terrestrial and aquatic animals

A large number of livestock poisonings have been reported for MC, some have been reported in detail, while others are anecdotal. There have been a number of reported livestock (sheep, cattle, waterfowl, lambs and turkeys) and domestic animal (horses and dogs) deaths in several countries (including NZ) as a result of MC exposure. A review of these animal poisoning events can be found in Chorus and Bartram (1999), Carmichael (2001) and Falconer (2001). Chronic exposure of livestock to cyanobacteria (Carbis *et al.*, 1994) has revealed a substantial reduction in bile acid synthesis in sheep due to the inhibition of PP-1 and PP-2A in the liver by cyanotoxins. A decrease in the bile acid pool results in a cascade of digestion related problems in sheep, which is of obvious concern to the livestock industry.

A number of studies have demonstrated that MCs and DSP toxins can negatively impact on aquatic organisms, such as amphibians, fish and zooplankton (Chen *et al.*, 1993; Williams *et al.*, 1997; Amorim and Vasconcelos, 1999; Holland *et al.*, 2005). For example, glycogen synthase activity (regulated by PP-1 and PP-2A) was shown to be effected in rainbow trout,

*Oncorhynchus mykiss*, following exposure to OA (Svensson and Forlin, 1998). Acute effects on heart rate and development were shown in zebrafish following MC exposure (Oberemm *et al.*, 1999). The inhibitory effects of MC on zooplankton protein phosphatases have also been reported (DeMott and Dhawale, 1995). In a comparative study between fish, crustaceans and phytoplankton, deposit feeding crustaceans were found to have the highest MC concentration with a maximum value of 103.3 μg/kg (Total Daily Intake (TDI) 0.52 μg/kg/day), even during periods of low ambient concentration (water concentration <0.02 μg/l) (Magalhaes *et al.*, 2003). Considering that MC are degraded by absorption to clay material and bacterial degradation (Magalhaes *et al.*, 2003), benthic sediment feeders, such as crustaceans, would be expected to be most at risk of exposure.

Microcystins have been a problem for cultivated species. For example, MCs were believed to cause disease in some freshwater farm-reared fish and maricultured shrimp (Carmichael, 2001). Microcystins were also believed to have been responsible for the net-pen liver disease of farm-reared Atlantic salmon in the Pacific Northwest (Andersen *et al.*, 1993). This disease had devastating effects on farmed salmon and caused the loss of millions of dollars to the fish farming industry in British Columbia and Washington State (Dawson and Holmes, 1999).

Microcystins have also been identified in estuaries and coastal waters (Vasconcelos, 1995; Codd, 1998). For example, the presence of MC and NODLN in oceanic and coastal shellfish has been reported at concentrations three-fold higher than acceptable quarantine levels for OA (Chen *et al.*, 1993). A recent report to the NZ Food Safety Authority measured the levels on MC in the Pacific Oyster using ELISA, LC-MS/MS and the PP2A assay, and revealed that guideline value (1 μg/l) had been exceeded in some cases (Holland *et al.*, 2005).

### 2.2.5 Toxin Tolerance levels

The World Health Organisation (WHO) has set a provisional guideline value for MC-LR in drinking water of 1 μg/L (1 nM) (World Health Organization, 2003). The WHO recommends a maximum total daily intake for consumption of cyanobacterial products (MCs) as 0.04 μg/kg as a safe dose. International guidelines for the protection of bathers in recreational waters have been discussed by Chorus and Bartram (1999). These include the following guidance levels

which have been taken from human epidemiological studies, WHO drinking water guidelines and case histories of animal and human poisonings:

1. To post on-site advisory signs and inform relevant authorities when cyanobacterial cell counts exceed 20 000 per ml or 10 μg/L of Chlorophyll *a* (Chl *a*) with dominance of cyanobacteria.
2. To restrict bathing and investigate further when cyanobacterial cell counts exceed 100 000 per ml or 50 μg/L of Chl *a* with dominance of cyanobacteria.
3. To take immediate action to prevent contact with scum and possibly prohibit bathing when cyanobacterial scum formation occurs in bathing areas.

In New Zealand, warning signs are typically put out when cell concentrations of cyanobacteria are above 20 000 cells/ml (this is a guideline value only) (K. Smith, personal communication, 22 November, 2005). The Ministry of Health (2005) has now included cyanotoxins (including MC and NODLN) in the new Drinking-water Standards for New Zealand 2005, which has set a guideline value consistent with that for MCs in drinking water set by the WHO (1 μg/L).

Most countries with established regulations for DSP toxins have set tolerance levels at the limit of detection for the mouse bioassay. One mouse unit (MU) corresponds with 4 μg OA in 100 g mussel meat, which is the minimum quantity of toxin capable of killing a 20 g mouse within 24 hours after an i.p. injection (Ramstad, 2001b). An acceptable level of 4-5 MU per 100 g (Luckas, 1992) is usually applied or 16-20 μg of OA in 100 g mussel meat (equivalent to 0.8 μg/g hepatopancreas) (Carmody *et al.*, 1995; Ramstad, 2001b). The acceptable guidelines are now expressed as 160 μg/kg (European Commission, 2002). The minimum dose of OA and DTX-1 necessary to induce diarrhea in adults is estimated to be 40 and 36μg, respectively (Ramstad, 2001b, 2001a). European guidelines also have a limit for YTX which is 1 μg/g (European Commission, 2002). The New Zealand mussel industry must comply with these guidelines for export trade to Europe, as it accounts for approximately 15% of shellfish export in New Zealand (P. McNabb, personal communication, 14 November, 2005).

### 2.2.6 Global Increase of Algal Toxins

The eutrophication of lakes, reservoirs and low tidal flushing watersheds has without doubt been the most consequential factor leading to increased cyanobacterial blooms worldwide (Pitois *et al.*, 2001). Conditions that facilitate cyanobacterial blooms depend on nutrient

concentration (namely P and $NH_3$), light intensity, temperature, pH, population stability, degree of mixing and mass of water body (Herath, 1997; Chorus and Bartram, 1999; Pitois *et al.*, 2001). Other biological factors such as presence of herbivores (i.e. zooplankton that directly feed on algae) can also play a role in decreasing bloom abundance (Herath, 1997). Although eutrophication is a natural process, anthropogenic eutrophication is believed to be largely responsible for increased blooms (Pitois *et al.*, 2001), as a result of intensive agricultural and industrial activities. Due to the wide-spread distribution of cyanobacteria (particularly as cysts) in lakes and rivers (Chorus and Bartram, 1999), any inland and low tidal flushing watershed that exists within agricultural, industrial or metropolitan areas is potentially at risk of a cyanobacterial bloom.

Figure 2-4, shows the global increase of reported marine HABs as reported in 1970 and 1999. There have been a number of hypotheses to explain the global increase of algal blooms as discussed in section 1.1. The global transport of algal cysts and motile cells in ships ballast water is potentially one of the most significant factors leading to the global distribution of toxic algae in coastal waters. Ballast water has been responsible for the transfer of pathogenic bacteria, protists, algae, zooplankton, benthic invertebrates and fish (Ruiz *et al.*, 1997). For example, potentially harmful and non-indigenous dinoflagellates were detected in over 50% of ballast water samples taken from one hundred and twenty-seven boats in a Scottish port (Macdonald and Davidson, 1998). Algal cysts or cells in receiving waters may bloom if the environmental conditions are favourable, which can be facilitated during periods of anomalous weather events, such as El Nin□ and/or global climate change. For example, the first documented marine HAB in New Zealand was caused by *Gymnodinium mikimotoi*, a neurotoxin producer now known as *Karenia c.f. mikimotoi*, which was bought in by an oceanic Japanese vessel during an El Ni□o event (Rhodes *et al.*, 2001).

Recently, there has been some discussion as to the correlation between increased marine farming practises and marine algal blooms. In New Zealand, aquaculture is mostly based on filter feeding molluscs that probably produce a net loss of nutrients from the ecosystem (Rhodes *et al.*, 2001), in comparison to sea-cage finfish farms and alike, that add nutrients to the ecosystem (of which there are few, in New Zealand). Rhodes *et al.* (2001), suggest there has been no evidence that the expansion of mussel farms in the Marlborough Sounds (major shellfish growing area in New Zealand) has resulted in the increased incidence of HABs.


**Figure 2-4.** The global increase of reported marine algal toxins. Encircled areas indicate where outbreaks have occurred or toxin has been detected at levels sufficient to cause harm to humans or the environment (van Dolah, 2000). PSP = paralytic shellfish poisoning, NSP = neurotoxic shellfish poisoning, DSP = diarrhetic shellfish poisoning, ASP = amnesic shellfish poisoning, Ciguatera = ciguatera fish poisoning, and *Pfiesteria* = estuary syndrome (*Pfiesteria sp.*).

### 2.2.7 Toxin synthesis

Not all toxigenic species of cyanobacteria will produce toxic blooms at all times, as toxicity can be variable within, and between blooms of the same species (Falconer, 2001). The toxin content of cyanobacteria is thought to be influenced by similar physiochemical parameters that influence algal blooms (this is in addition to species and strain of toxin, bloom

heterogeneity, culture age, and other factors that contribute to toxin degradation) (Falconer, 2001). For example, Orr and Jones (1998) found that environmental parameters ultimately influenced toxin production by influencing cell division rates of cyanobacteria rather than by directly affecting the metabolic pathways of toxin production. White *et al.* (2003) supported this theory by showing that in tropical environments, light and nutrient availability played a key role in allowing high toxin production by cyanobacteria, by influencing cell division rates and degradation of the toxin in the water column.

As for DSP toxins, geographic and temporal variation seems to affect toxin synthesis. For example, DTX-1 is a common toxin produced by *D .fortii* in Japan, whilst OA is the predominating toxin produced by *D. fortii* in Europe (Hungerford and Wekell, 1992a). Intraspecies toxin synthesis has also been shown to vary within a given area, from season to season and with depth. For example, Edebo *et al.,* (1988) revealed differences in the total DSP concentration at variable depths within the same sampling site.

Factors affecting toxin synthesis in marine algae have been studied by several authors (Quilliam *et al.*, 1996; Pan *et al.*, 1999; Imai *et al.*, 2003). Growth rate (Pan *et al.*, 1999) and age-dependant (Quilliam *et al.*, 1996) toxicity has been demonstrated in OA producing dinoflagellates. Recent research by Imai *et al.* (2003), supported the hypothesis that particular toxic phytoplankton species such as *Dinophysis* spp., may originally be non-toxic and may only become toxic secondarily, through the ingestion of toxic small-sized phytoplankton (nano-and/or picophytoplankton) as a result of mixotrophy (i.e. autotrophic and heterotrophic).

### 2.2.8 Functional roles of toxins

The physiological and ecological roles of MCs remain largely unclear. Possible explanations for the functional roles of MCs have been summarised by Hitzfield *et al.* (2000). These include: provide protection from grazers; provide an ecological advantage over algae; regulate protein phosphatase; or simply act as storage substances. Keating (1978) provided some evidence to suggest that MCs inhibit the growth of diatoms. However, this was not supported by the recent finding by Shi *et al.* (1999) that cyanobacterial phosphatases are insensitive to MCs. The functional roles of DSP toxins are less clear and very limited information exists in the literature.

### 2.2.9 Depuration

Depuration is the natural or unnatural process of removing toxins from shellfish, algae or water. Natural detoxification is an effective way to reduce toxicity, whereby shellfish are maintained in the sea for several weeks once the outbreak has subsided (Gonzalez *et al.*, 2002). Natural depuration however is slow and depends on the metabolic rate of the shellfish, which is subsequently affected by varying environmental conditions, such as low temperature and salinity (Gonzalez *et al.*, 2002). Depuration rate also differs between shellfish species. For example, higher concentrations of OA have been found in blue mussels (*Mytilus galloprovincialis)* than in Greenshell™ mussels (*Perna canaliculus*) in NZ, with the conversion product DTX-3 (7-O-acyl OA) also being found in Greenshell™ mussels (Rhodes *et al.*, 2001). This latter finding suggested that the diol ester derivative acyl OA is a more important contaminant in New Zealand shellfish than previously thought (Mountfort *et al.*, 2001). Croci *et al.* (1994) reviewed the literature on the depuration of OA in shellfish and revealed that some studies showed 30 days were required before all traces of OA in toxic mussels in aquaculture ponds were eliminated. This review also highlighted the significant variability in toxin concentration reported within a matter of days and suggested that weekly sampling of shellfish beds may not be sufficient for maximum protection of public health.

Due to the extreme thermo-resistance of DSP toxins, thermal treatment has not been an effective solution to reduce toxicity (Gonzalez *et al.*, 2002). Only two other studies have experimented with increasing depuration efficiency. The first reported study used ozonised water to reduce toxicity whereby a considerable reduction in toxicity occurred within 3 days for some cases (variable results were obtained however) (Croci *et al.*, 1994). Further studies by Gonzalez *et al.*, (2002) exposed OA to a supercritical mixture of carbon dioxide with acetic acid. This non-toxic and food compatible treatment was highly effective with 90% of the toxin eliminated in 30 minutes for some samples. Croci *et al.* (1994) believes that not all components of the DSP toxins are capable of being removed once they have accumulated in shellfish, and future studies are needed to determine this.

Microcystins are extremely stable and resistant to many chemical processes at neutral pH, such as boiling, chemical hydrolysis or oxidation. Natural detoxification processes are only effective at high temperatures, and at elevated or low pH. For example, MC undergo slow photochemical breakdown in full sunlight which can be accelerated with humic substances which

act as photosensitises (Chorus and Bartram, 1999). A number of treatment techniques are currently used in the treatment of watersheds contaminated with MC. These typically include aeration, treatment with algicides (copper sulphate has proved most effective), biological agents (e.g. bacteria) and activated carbon (Carmichael, 1997).

The depuration of MC from shellfish has been studied by several authors. Williams *et al.* (1997) revealed that the mussel, *M. edulis*, can rapidly clear MC from their tissues (both unbound and covalently bound to protein phosphatase enzymes) in a short period (4 days) when they were regularly flushed with untreated seawater. Amorin and Vasconcelos (1999) revealed that depuration of MCs by the mussel, *M. galloprovincialis*, was a slow process when mussels were not exposed to a flow-through water system, as the mussels were being re-contaminated by their faeces containing MCs. It is likely that the former study performed tests under more natural conditions.

## *2.3 Microalgae and Cyanobacteria in New Zealand and Methods of Detection*

### 2.3.1 Microalgae and Cyanobacteria in New Zealand

#### 2.3.1.1 Marine Microalgae

New Zealand has a substantial Greenshell™ mussel (*P. canaliculus*) industry with a fifty-five country export trade and earnings of approximately US$62 million (Mussel Industry Council Records, Rhodes *et al.*, 2001). Since the first outbreak of shellfish poisoning in New Zealand during summer 1992/1993, (DSP was responsible, in part) (Epstein and Jenkinson, 1993), New Zealand's aquaculture industry has been acutely aware of the potential of algal toxins to risk consumer safety and inhibit international trade in shellfish products. In New Zealand, the shellfish industry and the government are actively involved in routine monitoring of shellfish beds. The shellfish industry funds, by levies of marine farm license holders, weekly phytoplankton monitoring at 30 commercial harvesting sites (Rhodes *et al.*, 2001). The central government is responsible for biotoxin monitoring at approximately 80 public sites throughout the country (Rhodes *et al.*, 2001). These figures have likely increased since 2001. Monitoring has avoided the harvesting of contaminated shellfish. For example, in 2001 the north-west coast of

the South Island was issued public health warnings against consuming local shellfish, as mussels were found to be contaminated, in part, with DSP toxins (MacKenzie *et al.*, 2002).

New Zealand tests for all major toxin classes, whilst most other countries only test for 1 or 2 toxin groups (Garthwaite, 2000). All major classes of shellfish toxins have now been detected in NZ with levels above the maximum permissible level (MPL) for all classes at some time in the last 5 years (Garthwaite, 2000). The analyses of seawater samples for microalgae are carried out at the Cawthron Institute, Nelson. This laboratory has been awarded International Accreditation New Zealand (IANZ), which is covered under the ISO-IES Guide 25 as the recognised international laboratory standard (Rhodes *et al.,* 2001).

Seasonal marine HABs in New Zealand commonly start with diatoms in early spring, followed by dinoflagellates in summer and further diatom blooms in autumn (Rhodes *et al.,* 2001). *Dinophysis acuta*, *D. acuminata* and *Prorocentrum lima* are common bloom forming, DSP producing phytoplankton in New Zealand. Although *D. acuta* and *D. acuminata* are producers of DTX-2 in Europe, OA is the main toxin related to these two phytoplankton species in New Zealand. These species are thought to co-occur possibly as a result of favouring the same environmental conditions (a high water column with a high nutrient status and a shallow strongly defined pycnocline) (MacKenzie *et al.*, 2002).

### 2.3.1.2 Cyanobacteria

The first published record of the suspected involvement of toxic cyanobacteria in livestock deaths in New Zealand, occurred in a farm in lake Waipukurau, in which a bloom of *M. aeruginosa* was accountable (Flint, 1966). Current cyanobacterial blooms in New Zealand are common in the Rotorua Lakes, Lake Ellesmere and Lake Forsyth in Canterbury, Lake Waihola in Dunedin and a number of lakes in Waikato, Northland, Wellington and Horowhenua (A. Crowe, personal communication, 9 December 2004). Many unrecorded accounts of poisoning by cyanobacteria in New Zealand were also believed to have occurred throughout the last century (Flint, 1966). Recorded cyanobacterial blooms have been increasing in frequency in recent decades, particularly in Australia (Carmichael, 2001). Thus, it is possible that a similar pattern may occur within New Zealand. For example, large scale agricultural farming in New Zealand, particularly in the livestock industry, can facilitate the inland eutrophication of watersheds, and

give rise to favourable conditions for cyanobacteria to multiply. Thus, many fresh- and brackish watersheds are potential candidates for cyanobacterial blooms in New Zealand.

The analyses of freshwater samples for cyanobacteria are carried out in the Phytoplankton Laboratory at the Cawthron Institute. These analyses typically include State of the Environment periphyton monitoring, drinking water supply monitoring, oxidation pond monitoring and bloom monitoring and are mainly for regional, district and city councils (K. Smith, personal communication, 22 November, 2005).

### 2.3.2 Current Methods of Toxin Detection

The main regulatory method to date for microalgal toxin detection involves the mouse bioassay and it remains the primary detection method in New Zealand and throughout the rest of the world. Confirmation of toxin identification in the mouse bioassay is usually followed by an enzyme linked immunosorbent assay (ELISA) or LC-MS. In 2001, liquid chromatography with mass spectrometry (LC-MS) was accredited in New Zealand for routine detection at the Cawthron Institute, Nelson, which detects domoic acid (DA) (responsible for ASP) and DSP toxins.

There are many disadvantages of the mouse bioassay and other alternative methods appear to be more sensitive for screening toxins (Table 2-3). All methods available have advantages and disadvantages (Table 2-4). The mouse bioassay has low sensitivity and specificity, is a lengthy procedure and relies on live animals for testing. The PPINA is rapid, cheap and sensitive, and has a high throughput format, however it lacks specificity. Chromatographic methods are very expensive and require highly skilled personnel to operate the equipment. Stoichiometric methods, such as LC-MS and ELISA are also constrained by the availability of standards for the full range of DSP toxins, MC and NODLN variants in a sample. An exception, is the development of a new indirect ELISA developed by Fischer *et al.* (2001) for MC detection, which uses antibodies to the ADDA moiety. Most microcystins have an ADDA moiety and the 6*E* geometry moiety is responsible for toxicity in microcystin congeners. Thus, the new ELISA can recognise many toxic congeners. However, stoichiometric methods do not provide any indication of toxicity of a sample and assign equal weight to toxin congeners that differ in their toxic potential.

The major advantage of the PPINA over the former methods is its ability to detect total bioactivity of toxins as detection is based on functional activity rather than on specific structural components (Carmichael and An, 1999). This is useful as toxicity is directly proportional to inhibitory activity on the enzyme, thus indicating unsafe toxin levels. The PPINA is the method that has been further developed for the purpose of this thesis.

**Table 2-3: Comparison of Methods**

| Method | Sensitivity | Specificity | References |
|---|---|---|---|
| *Mouse Bioassay* | Low (ppm range) | Low | (Carmichael and An 1999) |
| *LC-MS* | High[a] (ppb-ppt range) | High | (Lawrence *et al.*, 2001) |
| *ELISA* | High[a] (ppb-ppt range) | High/ Low[b] | (Carmichael and An 1999) |
| *PPINA* | High[a] (ppb-ppt range) | Low | (Carmichael and An 1999) |

[a] detection limit <1 μg/100 g mussel tissue

[b] Only low specificity for MC detection with Fischer *et al.* (2001) method.

### 2.3.3 Protein Phosphatase Inhibition Assay

Purified catalytic subunits of PP-2A and PP-1 have been used to develop a PPINA, for the qualitative measurement of DSP toxins and MCs in the aquatic environment. Protein phosphatase type 1 and PP-2A are the most abundant phosphatases in mammalian tissues with catalytic subunits of 38 and 36 kDa, respectively (Toivola *et al.*, 1994). Protein phosphatase type 2A is found mainly in the cytosolic fraction, whereas PP-1 is found mainly in the mitochondrial and post-mitochondrial particulate fractions (Toivola *et al.*, 1994). These phosphatases dephosphorylate serine/threonine residues on a variety of regulatory and structural proteins (Cohen, 1989). Microcystins and DSP toxins specifically bind to and inhibit the activity of these phosphatases (Takai and Mieskes, 1991; Takai *et al.*, 1992a; Honkanen *et al.*, 1994). As a result, the PPINA was developed based on the ability of these toxins to prevent protein phosphatase enzymes dephosphorylating colorimetric or fluorimetric substrates. The assay is carried out in 96-multiwell plates and the hydrolysis of the substrate is qualitatively measured on a spectrometer or fluorimeter. The decrease in hydrolysis of the substrate is inversely proportional to the concentration of toxin.

**Table 2-4: Advantages and disadvantages of available methods for microcystin and diarrhetic shellfish poisoning detection.**

| Method | Advantages | Disadvantages | References |
|---|---|---|---|
| *Mouse Bioassay* | Detects toxicity in biological tissue, hence the bioactive component of the sample. | Low specificity, sensitivity and long procedure. Relies on large samples of live animals for testing. Only suitable for detection of acute levels. Expensive. Tendency to produce false positives. I.p. administration may not parallel natural exposures. | (Carmichael and An 1999) |
| *LC* | High sensitivity. Good correlation with other methods. | Expensive and required highly skilled personnel. Constrained by the availability of standards for the full range of toxin variants found in samples. Provides no indication of toxicity. | (Laub et al. 2002) (Lawrence et al. 2001) (Fischer et al. 2001) |
| *ELISA* | Broad specificity for cyclic peptide toxin congeners. Based on the structural components of the sample. Cheap and easy to perform. Good correlation with other methods. | Provides no indication of toxicity. Assigns equal weight to toxin congeners, even though they may differ in their toxic potential. | (Fischer et al. 2001) |
| *PPINA* | Rapid (1h), cheap and sensitive. Detects total toxin bioactivity. Good correlation with other methods. | Non-specific. Cross reactivity of interference substances with toxin variants can sometimes occur. | (Carmichael and An 1999) (Mountfort et al. 2001) (Mountfort et al. 2005) |

### 2.3.3.1. Developments in the PPINA

Initial enzyme assay developments began with radioactive protein phosphatase involving $^{32}P$-labelled substrates (histone or phosphorylase), with the assay end-point being the inhibition of release of $^{32}P$ from the substrate by purified PP-2A (Honkanen *et al.*, 1996b). An alternative to the radioisotope method, was the development of a microplate format for high throughput use of the colourmetric substrate, *para*-nitrophenyl phosphate (*p*NPP), developed by Tubaro *et al.* (1996). *Para*-nitrophenyl phosphate is a commonly used colorimetric substrate for alkaline phosphatases (PP-2A and PP-1) as it is dephosphorylated by protein phosphatase enzymes and converted from a clear to a yellow solution (*para*-nitrophenol). The binding of MCs and DSP toxins to protein phosphatase enzymes prevents the production of *para*-nitrophenol. The level of toxin in the system is qualitatively measured by reading the absorbance of *para*-nitrophenol on a microplate spectrometer and comparing this to a standard inhibition curve. The detection limit for MC using the colorimetric assay is 0.5 μg/mL (below the WHO guideline of 1.0 μg/L drinking water) (Tubaro *et al.*, 1996).

The PPINA assay has been further modified using fluorescent substrates: fluorescein diphosphate (FDP), methylumbelliferyl phosphate (MUMP), difluoromethylumbelliferyl phosphate (DiFMUP) and dimethylacridinone phosphate (DDAO). The fluorescent PPINA is based on a similar method to the colorimetric PPINA, however it uses fluorimetric substrates. The hydrolysis of fluorimetric substrates is determined by fluorescence detection on a fluorescent microplate reader in 96-well microtiter plates. A comparative study by Leira *et al.* (2000) found no specific advantages of any of these PP-2A substrates as the sensitivity, reproducibility and recovery percentages were all similar. Similar findings using OA have also been obtained in other studies (Vieytes *et al.*, 1997; Mountfort *et al.*, 1999). Microcystins inhibit PP-1 and PP-2A with similar potency, with an $IC_{50}$ of 0.3 and 0.5 nM, respectively (Toivola *et al.*, 1994). Protein phosphatase type 2A however is believed to form a more stable complex with MC-LR relative to the PP-1 toxin complex (Toivola *et al.*, 1994). Nodularins are believed to inhibit PP-1 with similar potency, but inhibit PP-2A at a higher selectivity (van Dolah and Ramsdell, 2001). Diarrhetic shellfish toxins also have a differing binding affinity to protein phosphatase enzymes. Protein phosphatase type 2A is more strongly inhibited by OA (inhibition concentration of $OA_{50} \sim 0.2$nM) than PP-1 (inhibition concentration of $OA_{50} \sim 20$nM) (Holmes *et al.*, 1990). Consequently, PP-2A has been more commonly used to assay DSP toxins. The first

most commonly used PP-2A enzyme for these tests was sourced from rabbit skeletal muscle (Cohen *et al.*, 1989; Holmes *et al.*, 1990; MacKintosh *et al.*, 1990; Takai and Mieskes, 1991; Simon and Vernoux, 1994; Honkanen *et al.*, 1996a; Shimizu *et al.*, 1998). Later, Vieytes *et al.*, (1997) showed that PP-2A sourced from human red blood cells, yields 15-fold more activity (i.e. fluorescence) with the same amount of protein then the former PP-2A source. Thus, in recent years, PP-2A sourced from human red blood cells has become the enzyme of choice for use in the PPINA (Tubaro *et al.*, 1996; Mountfort *et al.*, 1999; Leira *et al.*, 2000; Mountfort *et al.*, 2001; Mountfort *et al.*, 2005). Protein phosphatase type 1 has generally been the enzyme of choice for MC analysis in the past, mainly because the cloned catalytic subunit of PP-1 has been commercially available (van Dolah and Ramsdell, 2001). Many recombinant forms of PP-1 have also been developed (Zhang *et al.*, 1992; An and Carmichael, 1994; Zhang, 1994; Heresztyn and Nicholson, 2001).

Fluorimetric methods ($IC_{50}$ for OA and PP2A 1.5 nM using MUMP) are inherently more sensitive than colorimetric methods ($IC_{50}$ for OA and PP2A 2.0 nM using substrate *p*-NPP), utilising similar preparation procedures (Mountfort *et al.*, 1999). Radioisotope assays have similar detection limits to fluorimetric substrates. However, they require expensive radioisotope facilities and purified protein phosphatases are more widely available. Unlike colorimetric substrates, fluorimetric substrates do not exhibit shifts in inhibitory activity as a result of differing enzyme dilutions (Mountfort *et al.*, 1999). Fluorimetric assays have also been shown to reduce the need for sample clean-up than absorbance-based methods (van Dolah and Ramsdell, 2001).

There has been considerable development of the PPINA to detect DSP toxins in shellfish at the Cawthron Institute, Nelson. The first published PPINA developed at the Cawthron Institute by Mountfort *et al.* (1999) utilised PP-2A with a number of colorimetric and fluorimetric substrates, for the determination of OA in mussels. Fluorimetric substrates, such MUMP proved to be more sensitive and accurate than colorimetric substrates. The assay compared favourably to results obtained with ELISA and LC-MS. The method was modified by Mountfort *et al.* (2001) to include a hydrolysis step that converted the diol esters of OA (acyl OA) and other DSP derivatives (DTXs) to a more detectable OA and DTX-1, as prior to utilising this step, the assay lacked sensitivity towards the ester derivatives of these toxins. The addition of the hydrolysis step during the preparation of samples increased the sensitivity of this assay to incorporate all

DSP toxins. These assays have typically incorporated bovine serum albumin (BSA) and $NiCl_2$ into the reaction mix as they are considered important cofactors in this type of assay system (Mountfort *et al.*, 1999; Mountfort *et al.*, 2003) (see Chapter 3 for more detail). Developments in the PPINA have now expanded to include detection of MCs (Mountfort *et al.*, 2005).

### 2.3.3.2 Method Specificity

The PPINA is a very attractive screening tool as it measures the biologically active components of the sample. The PPINA can detect all of the toxin variants that have affinity to protein phosphatase enzymes (60-odd variants of MCs), which is essentially the strength of the assay. The results of the assay can be confounded if either, other phosphatase enzymes are present within a sample, or if protein phosphatase inhibiting toxins other than the one in question is present within a sample.

Cyanobacteria have significant amounts of endogenous phosphorylase phosphatase enzymes which can mask (or add to) the inhibitory activity of MCs or DSP toxins (Carmichael, 1997). Shellfish also have significant amounts of protein phosphatase enzymes, which can confound the results of the assay. To overcome this, the samples are usually extracted with methanol which inhibits endogenous protein phosphatases before incorporation into the assay.

Co-occurring toxins present a different problem. Microcystins have been identified in the north-eastern Pacific Ocean, and east Canadian and European mussels (Chen *et al.*, 1993). Therefore, it is possible for MCs to co-occur with DSP toxins, especially in coastal and river inlets. The presence of OA in a sample of potable water is highly unlikely, so co-occurrence is not an issue for drinking water. However, it is possible for OA to co-occur with MCs in brackish water. The low specificity of the PPINA is therefore, both a disadvantage and an advantage. The PPINA would overestimate toxicity of the toxin in question, by detecting the toxicity of other protein phosphatase inhibiting toxins present in a sample. However, it would still be fulfilling its role of protecting the public by detecting all harmful toxins in the sample, unlike stoichiometric methods which would not detect other potentially harmful toxins. In addition, scientists at the Cawthron Institute who work with cyanobacteria and marine algae are not aware of blooms reported in co-existing areas within New Zealand (S. Wood and P. Holland, personal communication, 12 September 2005).

Other phosphatase inhibiting enzymes may also be present in a sample and potentially lead to false positive results, such as calyculin A, tautomyocin or motuparin (Carmichael, 1997). Calyculin A is a potent inhibitor of PP-1 and PP-2A, induces contraction of smooth muscle fibres and is a powerful tumour promoter (Ishihara *et al.*, 1989). It was first isolated from a marine sponge *Discodermia calyx*, collected in Japan (Chin *et al.*, 1995), although whether the toxin was produced from the sponge itself or if it was bioaccumulated has yet to be determined. Motuparin is another toxin present in the Papua New Guinea sponge *Theonella swihoei* and has a similar structure to NODLN. Again, the production of motuparin has yet to be determined. Tautomyocin is a soil bacterium that is an inhibitor of protein phosphatase. Although all of these toxins may share the same binding site on protein phosphatase molecules, it is doubtful if high concentrations of calyculin A, tautomyocin or motuparin required to inhibit these enzymes would exist naturally in the environment. Tautomyocin has been shown to be unstable in solution and has a higher $IC_{50}$ than that of OA and MC for PP-2A ($IC_{50}$ 0.28 and 7.51 nM, for OA and tautomyocin, respectively) (Honkanen *et al.*, 1994; Takai *et al.*, 1995). Thus, tautomyocin is unlikely to compromise the assay. Calyculin A also has a higher $IC_{50}$ than that of OA for PP-2A ($IC_{50}$ 1.5 and 12 nM, for OA (Mountfort *et al.*, 1999) and calyculin A (Malarvannan and Mountfort, unpublished), respectively). However, other studies have shown calyculin A to produce similar sensitivity to that of PP-2A ($IC_{50}$ 0.3 nM for both OA and calyculin A (Honkanen *et al.*, 1994)). The inhibiting concentration of motuparin is unknown (Carmichael, 1997). Whether the sponges, *Discodermia calyx* and *Theonella swihoei,* responsible for producing calyculin A and motuparin, are present where aquaculture or wild shellfish grow in coastal waters remains unknown. In addition, whether these toxins are capable of accumulating to such concentrations as to make the water toxic is unlikely, as such cases have not been reported. There have also been no reports of these sponges in New Zealand. Thus, calyculin A and motuparin are unlikely to compromise the assay.

## 2.4 Biosensors

### 2.4.1 Biosensor development

Biosensors are analytical devices that incorporate a biological element and are associated with, or integrated within, a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric or magnetic (Rogers and Gerlach, 1996).

Advantages of biosensors in comparison to bioanalytical assays include (Rogers and Gerlach, 1996):

- High specificity. The advantage of biological sensing elements is the ability to distinguish between the analyte of interest and similar substances.
- Rapid analysis. Products can be directly and instantaneously measured.
- Simplicity. The receptor and transducer are integrated onto one single sensor. This is in contrast to the conventional assay, in which many steps are used and each step may require a reagent to treat the sample.
- Continuous monitoring capability. Biosensors can regenerate and re-use the immobilised biological recognition element. For enzyme-based biosensors, an immobilised enzyme can be used for repeated assays. Hence, these devices can be used for continuous or multiple assays.

These characteristics, along with high sensitivity are all important components to a good biosensor design. In essence, biosensors can potentially offer the specificity and sensitivity of biological-based assays packaged into convenient devices which allow for rapid and multiple analysis on-site (Rogers and Gerlach, 1996).

With respect to algal toxins, biosensors could potentially play an important role in future monitoring programmes of toxic algae. An electrochemical immunosensor for OA and other seafood toxins responsible for ASP, NSP and pufferfish poisoning (domoic acid, brevetoxin and tetrodotoxin, respectively), has been developed by Kreuzer *et al.* (2002). The immunosensor is based on a disposable screen-printed electrode system (SPE) and alkaline phosphatase as the enzymatic label. The immunosensor for OA was shown to be accurate, rapid (<40 min), cost effective, disposable and sensitive (detection limit 4 ng/ml, $EC_{50}$ 32 ng/ml).

An immunosensor for OA using a quartz crystal microbalance (Tang *et al.*, 2002) and a semi-automated membrane chemiluminescent immunosensor for flow injection analysis of OA in mussels (Marquette *et al.*, 1999) have also been developed. The former immunosensor design showed a good long-term storage lifetime (38 days) however its sensitivity was not adequate for a biosensor (detection limit 1.9 μg/ml) (Tang *et al.*, 2002). The latter immunosensor design was rapid (20 min), had a low detection level (0.2 μg OA/100 g of homogenate), could be applied to crude mussel extracts and was reproducible (could perform more than 30 OA determinations) (Marquette *et al.*, 1999). However, all of these immunosensors have the disadvantage of detecting specific toxin congeners only.

An enzyme-inhibition bioelectrode biosensor test based on the capability of OA to inhibit the catalytic activity of acid phosphatase (AP) has been developed by Croci *et al.* (2001). The biosensor uses AP to catalyse glucose-6-phosphate into glucose and inorganic phosphate, and then uses glucose oxidase (GOD) enzymes to oxidise glucose into gluconolactone and hydrogen peroxide. The intensity of the current flow registered by hydrogen peroxide is proportional to the catalytic activity of AP, which is inhibited by OA. The biosensor showed high sensitivity and produced results that were rapid and compared well to other analytical methods. Unlike the immunosensor methods, the latter biosensor method had a broad toxin detection range for phosphatase inhibiting toxins, although whether this was applicable for MC detection was not discussed.

### 2.4.2 Application of the PPINA as a biosensor

Currently, the PPINA is limited to a central location (Mountfort *et al.*, 1999; Mountfort *et al.*, 2001; Mountfort *et al.*, 2005) and only a small number of environmental samples analysed in the laboratory are identified as toxin positive (Garthwaite, 2000). Thus, there is potential for the PPINA to be developed into a portable biosensor for on-site analysis of MCs and OA in marine- and fresh-water samples. The PPINA is based on a similar detection system as the enzyme-inhibition biosensor (section 2.4.1) and could be developed in a similar format. The assay is also capable of detecting all intracellular and extracellular phosphatase inhibiting toxins including possible unidentified phosphatase inhibitors. The PPINA is more powerful than stoichiometric methods as it is functionally based, responding to different toxin congeners depending on their toxicity (Mountfort *et al.*, 2005). A 'fill and flow' biosensor format similar to that shown in

Figure 2-5, could represent a feasible extension of the assay, providing that protein phosphatase enzyme is amendable to immobilisation, and retains its activity and sensitivity to inhibition in this format (see the caption on Figure 2-5 for details on the design of the system).

A protein phosphatase biosensor would avoid the time consuming and costly process of analysing multiple uncontaminated environmental samples in the laboratory. Some technical challenges would be present, as toxins are mostly cell-bound with little release into the environment until a bloom has collapsed (Watanabe *et al.*, 1988). Thus, it would need to be decided if water or algae should be tested. A method for homogenising algal cell samples in the field may need to be developed if the latter sample is to be measured. In the biosensor format, it is anticipated that the assay would have most value for use by regulatory authorities and other agencies concerned with toxin monitoring in potable water. It may also be applicable to the global market as a potentially simple technique able to be used by non-skilled personnel to ensure that the regulatory limit for MCs meets the WHO standards in potable water (1μg/l).

Monitoring of marine and freshwater microalgal toxins in New Zealand is clearly an important task to safeguard the health of the public, livestock and wildlife, and prevent economic loss to the shellfish industry. The range of current methods available for such analyses differs immensely in their methods of detection, strengths and weaknesses. If proven useful, the PPINA biosensor, could provide a rapid, on-site detection system for both fresh and marine water samples, serve as an early warning of toxin outbreaks (Mountfort *et al.*, 2001), and replace the mouse bioassay in routine analysis for MCs and DSP toxins.

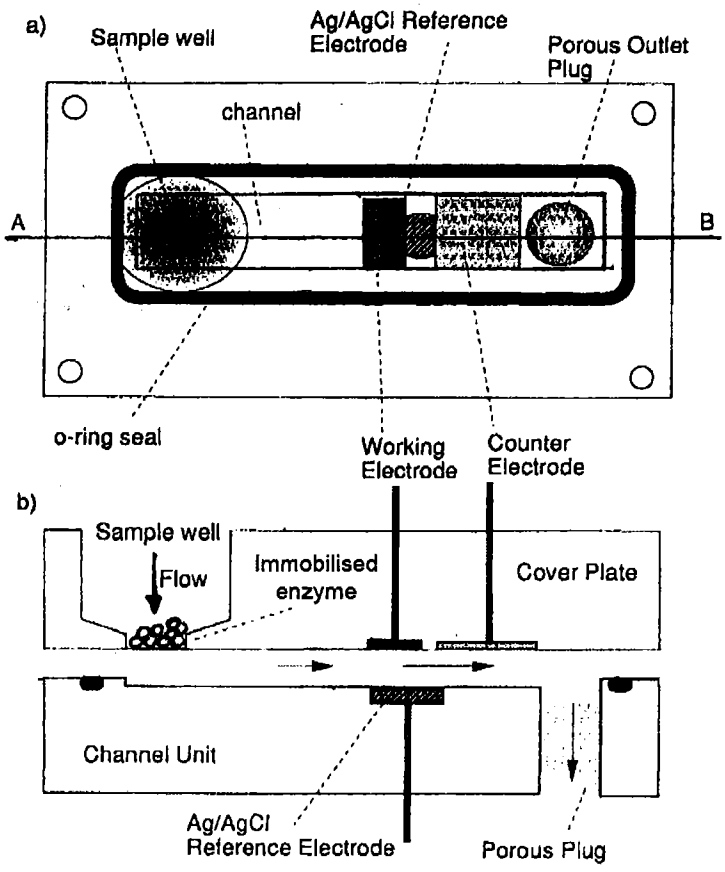



**Figure 2-5.** Schematic diagram of a typical 'fill and flow' channel biosensor. (a) Top view; (b) section view through cut A-B (Gooding and Hall, 1998). The sample well will contain a filter that retains the enzyme-bead complex. The biosensor is reminiscent of "dipstick" technology, but the biorecognition matrix (i.e. the immobilised enzyme beads) is located upstream of the detector electrode. Thus, the sample would not only fill the channel but also flow through it. The system would typically work as described: a small volume of the test solution (containing both the sample and assay components) is added in the well and the solution would flow by "gravity feed" through the channel and pass the electrode. The detector electrode current will be a function of the analyte concentration. Thus, the detection system would be based on a similar method as the PPINA, whereby the amount of substrate in the sample would indicate the presence of the toxin. This system would need to be optimised with regards to the flow rate, enzyme loading and channel dimensions to obtain the best response for the application. Refer to Gooding and Hall (1998) for more details regarding the development of this sytem.

# Chapter 3 Method Development

## 3.1 Introduction

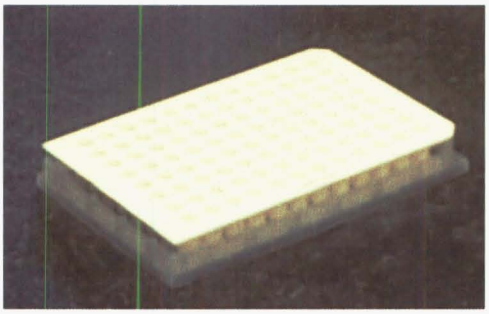
There have been significant developments in the PPINA to detect DSP toxins (OA and DTXs) and MCs (section 2.3.3.1). Recently, the PPINA method has evolved to test the suitability of a microfilter plate (Figure 3-1a) (Mountfort *et al.*, manuscript in preparation-a), as opposed to the standard microwell plates used in previous experiments (Figure 3-1b). The method employs an enzyme-immobilisation system, where PP-2A enzyme is immobilised onto sepharose microbeads prior to being used in the assay. The reaction system consists of an assay buffer (Tris), $NiCl_2$ (for enzyme stability), toxin, enzyme, and the substrate, MUMP, in a neutral medium. The assay is carried out in 96-well microfilter plates so that the enzyme can be retained in the filter after a test. The purpose of the enzyme immobilisation system, referred to as 'the immobilised PPINA', is that it can potentially serve as a vehicle towards the development of a prototype biosensor for onsite detection of DSP toxins and MCs in environmental samples. The immobilised PPINA is referred to as the pre-biosensor format and serves as an intermediate step in the process of development between the unimmobilised PPINA (i.e. the PPINA using unbound or free enzyme) (Mountfort *et al.*, 1999) and the prototype biosensor.

The immobilised PPINA has previously been tested using purified PP-2A obtained from human red blood cells (Mountfort *et al.*, manuscript in preparation-a). However, there is potential to apply the immobilised PPINA to enzymes other than PP-2A, such as protein phosphatase type 1 (PP-1). In September 1994, recombinant PP-1 (R.PP-1) became commercially available. The R.PP-1 was isolated from a strain of *E.coli* that carries the coding sequence for rabbit skeletal muscle PP-1 (□-isoform) (Zhang *et al.*, 1992). The R.PP-1 has appeal as it has low sensitivity for MCs (0.04 nM) (Zhang *et al.*, 1992) and is commercially available.

In this chapter, optimal conditions for the immobilisation procedure using PP-2A and R.PP-1 enzyme were determined so that both enzymes were shown to express maximum activity. Optimisation experiments were performed on unimmobilised R.PP-1 prior to the immobilised tests for this enzyme, as it had not been previously used in the laboratory. The purpose of all the optimisation experiments was to determine conditions that produced the highest enzyme activity,

as this enabled any change in activity to be reliably detected in the presence of a toxin inhibitor, such as OA or MC.

a)



b)

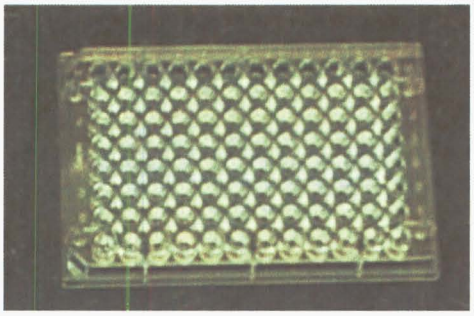


**Figure 3-1.** Photos of a 96-well microfilter plate (a), and a 96- microwell plate (b). The microfilter plate is used for the enzyme-immobilisation method (photo L. Allum).

## *3.2 Materials and Preparations*

### 3.2.1 Materials

#### 3.2.1.1 Chemicals

Activated sepharose beads (CN-Br) were purchased from Amersham Pharmacia Biotech AB, Sweden and albumin bovine fraction V (BSA) was obtained from BDH Lab Services, England. Four-methylumbelliferyl phosphate disodium salt (MUMP) and ascorbic acid 2-

phosphate were purchased from Sigma (Missouri, USA). All other chemicals were from the local commercial sources and of the highest possible grade.

#### 3.2.1.2 Enzymes

Protein phosphatase enzyme type 2A (PP-2A) obtained from Upstate Biotechnology Inc (New York, USA), was isolated from human red blood cells as a heterodimer of 60 kDa and 36 kDa subunits. Recombinant PP-1 obtained from New England Biolabs (Massachusetts, USA) was isolated from *E.coli* that carries the catalytic subunit (37.5 kDa) of the □isoform of PP-1 from rabbit skeletal muscle.

### 3.2.2 Preparations

#### 3.2.2.1 Substrate and BSA preparations

The reaction substrate MUMP, was prepared daily in 50 mM Tris buffer of pH 7.0 (5 mg/10 ml diluted 1:4). Stock solutions of 10 mg/ml BSA were prepared in distilled water and stored at -20°C.

#### 3.2.2.2 Enzyme preparations

Protein phosphatase type 2A was supplied with a dilution buffer containing 20 mM MOPS, pH 7.5, 150 mM NaCl, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $MnCl_2$, 1 mM DTT, 10% glycerol and 0.1 mg/ml BSA. Ten units (100 $\mu$l) of PP-2A were diluted in 1 ml of the supplied dilution buffer and 100 $\mu$l aliquots were stored at -20°C. Recombinant PP-1 was also supplied with a storage and reaction buffer containing 50 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, 2 mM dithiothreitol (DTT), and 0.025% Tween 20 (final pH 7.5) in water. However, R.PP-1 was stored undiluted in 20 $\mu$l aliquots (50 units) at -70°C. Thus, the reaction buffer for R.PP-1 was made up daily.

#### 3.2.2.3 Buffer preparations

*Coupling buffer*

The sodium phosphate buffer was prepared with 30.5 ml of 0.2 M of disodium hydrogen phosphate ($Na_2HPO_4$) and 19.5 ml of 0.2 M of sodium dihydrogen phosphate ($NaH_2PO_4$) and made up to 100 ml with distilled water (pH 7.0). The standard coupling buffer contained sodium

phosphate buffer, 0.05 M of potassium chloride (KCl) and 0.2 mM L-ascorbic acid 2-phosphate sesquimagnesium salt ($C_6H_9O_9P$) and was stored at 4°C.

*Tris assay buffer*

Tris buffer, 50 mM Tris (hydroxymethyl) methylamine (BDH Chemicals, Poole, England) containing 0.1 mM of calcium chloride ($CaCl_2$), was made up in 500 ml of distilled water and adjusted to pH 7.0 using 2.5 mM of hydrochloric acid (HCl) and stored at 4°C.

## 3.3 Assay and Immobilisation Methods for PP-2A

### 3.3.1 Protein phosphatase 2A inhibition assay

The PPINA was based on a hydrolysis reaction between serine/threonine protein phosphatase enzymes and the colourless substrate, MUMP. Protein phosphatase enzymes dephosphorylated MUMP and reduced it to the fluorescent compound, methylumbelliferone (MUM).

$$\text{MUMP} \xrightarrow{\text{PP}} \text{MUM} + \text{phosphate}$$

The resulting fluorescent product (MUM) was measured on a fluorescent microplate reader. Okadaic acid and MCs bind to protein phosphatase enzymes and inhibit binding of the enzyme to the substrate. Hence, toxin detection was based on the inhibition of product formation.

*Protocol for the PP-2A inhibition assay*

The protein phosphatase type 2A inhibition assay was based on the method of Mountfort *et al.* (1999). The assay was carried out with unimmobilised enzyme in Nunc Maxisorp™ 96-multiwell plates. The assay system for PP-2A contained 50 µl of 50 mM Tris buffer (pH 7.0) and 0.1 mM $CaCl_2$, 120 µl of buffer containing substrate (0.42 mM MUMP), 5 µl of 40 mM $NiCl_2$ (in distilled water), and 5 µl of BSA (1 mg/ml of distilled water) and 10 µl of PP-2A diluted 1:4 with Tris buffer (0.02 Units per assay, final concentration 1.5 nM) together with 10 µl of sample extract or purified toxin standard, diluted in 10% methanol. The total assay volume was 200 µl. Control wells did not contain the toxin or the sample extract but instead were

supplemented with additional 10% methanol to make up the final assay volume to 200 µl. Methanol was used as the solvent for the preparation of sample extracts (section 4.2.4) which was the reason it was included in the control. Blank wells did not contain the toxin, the sample extract or the enzyme but instead were supplemented with the same volume of 10% methanol as in the control and additional Tris buffer to bring it to the final assay volume of 200 µl. Samples were analysed in triplicate.

The reaction was started by automatic injection of the reaction substrate in a fluorimeter (Fluostar BMG™ Reader, BMG Lab Technologies, Offenberg, Germany), which was pre-warmed together with the assay mix at 37°C for 15 minutes. The substrate was added automatically after the first reading cycle. The incubation period for the enzyme reaction was approximately 1 hour at 37°C. The hydrolysis of MUMP was determined by the fluorescent detection on the automated microplate reader fluorimeter using real time kinetics (approximately 60 cycles with 10 flashes in each cycle), with settings for extinction at 355 nm and emission at 460 nm. Activity was determined by linear regression of the reaction curves and expressed as units/min.

### 3.3.2 Protocols for the immobilised PP-2A assay

The immobilised PP-2A inhibition assay has been developed by (Mountfort *et al.*, manuscript in preparation-a). The immobilised assay is based on the same process as the PP-2A inhibition assay described in section 3.3.1. However, prior to conducting the assay, the enzyme was immobilised onto activated sepharose microbeads, instead of adding it to the assay as a liquid. The immobilised assay also had a different assay volume and assay components (see next section). The procedure utilises the specific binding between activated sepharose and protein phosphatase enzymes. The immobilised assay was carried out in Millipore Multiscreen™ 96-well microfilter plates, instead of the standard 96-multiwell plate. Thus, the enzyme-bead complex is retained in the microfilter well for re-use.

*Enzyme immobilisation procedure*

Freeze-dried activated CN-Br sepharose beads (100 mg) were washed 15 times in distilled water (1 ml) to remove additives. Washing was by inverted mixing and pulse centrifugation (2054 x *g*), and the supernatant was removed in between washes by pasteur

pipette. Protein phosphatase type 2A (100 $\mu$l aliquots equivalent to 1 unit) was mixed with 1 ml of standard coupling buffer and added to 100 mg of washed beads. The enzyme-bead complex was spun overnight in microfuge tubes at 2-4°C on a rotating disc (approximately 60 rev/min). The enzyme was immobilised onto the beads for a minimum period of 20 hours.

The enzyme-bead complex was washed with 1 ml of 50 mM Tris buffer to remove unimmobilised enzyme from the solution. Washing consisted of slow inverted mixing and pulse centrifugation (2054 x *g*). The process was repeated for a total of 2 washes with the supernatant removed in between washes by pasteur pipette. The enzyme-bead complex equivalent to 50 mg of beads was transferred to wells of a 96-well Multiscreen™ Filter Plate (Millipore, MA, USA) in approximately 350 $\mu$l of buffer. The microfilter plate was vacuum filtered using a vacuum manifold (Millipore, MA, USA) attached to an Air Cadet® vacuum pressure station (Biolab Scientific Ltd, USA) to remove the liquid from the enzyme-bead complex (Figure 3-2). Tris buffer (100 $\mu$l) was added to each well, which was filtered a second time. The plate was then ready for use in the assay or for storage. Storage of the microfilter plate, consisted of adding 100 $\mu$l of Tris buffer to each well and storing the plate at 2°C.

*Immobilised PP-2A inhibition assay*

Preliminary experiments with the immobilised PP-2A assay did not include the toxin or the sample extract in the assay system. Assay conditions for the immobilised system followed conditions as for the unimmobilised enzyme (section 3.3.1) except that undiluted PP-2A was added to the system. Bovine serum albumin was also excluded from the assay as the intention was to keep the immobilised system as simple as possible for potential application as a biosensor. The immobilised assay system for PP-2A contained 0.5 units of PP-2A immobilised to 50 mg of beads (approximately 100 $\mu$l in volume), 25 $\mu$l of 50 mM Tris buffer (pH 7.0) and 0.1 mM $CaCl_2$, 120 $\mu$l of buffer containing substrate (0.42 mM MUMP) and 5 $\mu$l of 40 mM of $NiCl_2$. The total volume for the immobilised system was 250 $\mu$l (150 $\mu$l liquid volume). This differed to the unimmobilised assay which had a liquid volume of 150 µl. Thus, the immobilised assay was slightly more concentrated than the latter. Blank wells did not contain the enzyme or the sepharose beads but instead, were supplemented with 100 $\mu$l of Tris buffer to make up the final assay volume to 250 $\mu$l. The reaction was measured using the same procedure as previously described in section 3.3.1.

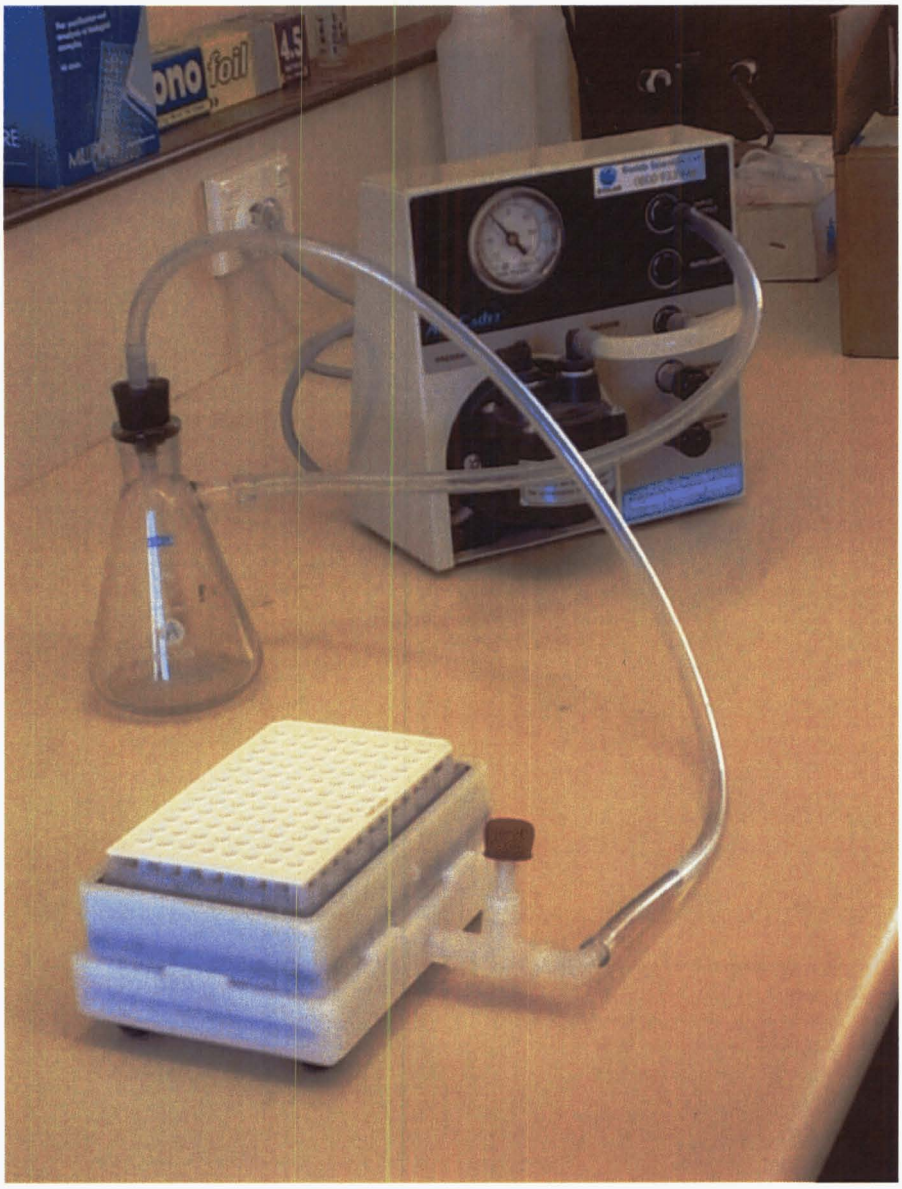



**Figure 3-2.** A photo of the filtration system using the 96-well microfilter plate. Liquid is vacuumed filtered through the microfilter plate and is deposited in the conical flask. The immobilised enzyme-bead complex is retained in the microfilter plate for re-use (photo L. Allum).

## *3.4 Experiments for Optimisation Studies*

### 3.4.1 Optimisation experiments for PP-2A

The immobilised PP-2A assay had previously undergone only limited preliminary testing. Thus, it was necessary to experiment with components in the immobilisation procedure and in the assay system, to determine if the activity of the enzyme could increase under different assay conditions. The following tests have been referred to as 'optimisation experiments' with the purpose of optimising conditions for the PP-2A assay.

#### 3.4.1.1 Optimal coupling buffer and stability of enzyme activity

Changes were made to components in the coupling buffer, to determine if additional enzyme can be immobilised onto the microbeads. Protein phosphatase type 2A was immobilised by the standard immobilisation procedure (1 unit per 100 mg of beads) (section 3.3.2) in 8 different coupling buffers, as shown in Table 3-1. The assay was run in duplicate for each coupling buffer according to the standard immobilised assay system for PP-2A (section 3.3.2). The degree of immobilisation would be reflected in enzyme activity measurements. Thus, the optimal coupling buffer would be the one that yields the highest enzyme activity (i.e. the test that exhibit the highest production of MUM).

In conjunction with the coupling buffer optimisation test, the length of time the activity of the PP-2A enzyme would be stable was determined, by conducting the assay repeatedly up to 1000 hours after immobilisation. This showed the trend in enzyme activity overtime, and revealed when the enzyme became stable and how long the enzyme was active in the immobilised system. Experiments with different assay conditions can only be performed on the immobilised enzyme once it becomes stable.

#### 3.4.1.2 Optimal assay pH

Changes were made to the assay pH to determine if pH influenced enzyme activity. Protein phosphatase type 2A was immobilised by the standard immobilisation procedure (section 3.3.2) using the optimal coupling buffer. The immobilised PP-2A assay was conducted in triplicate according to the standard assay protocols (section 3.3.2). The following pH combinations for PP-2A were tested 331 hours after immobilisation: 5.5, 6.5, 7.0, 7.5, 8.5 and distilled water (pH 5-6). The pH of Tris buffer in all assay components was adjusted prior to the

assay. In one test, replacing Tris buffer with distilled water, the substrate was diluted to the same concentration in distilled water, and water was added to the reaction mix in place of Tris buffer. Since the fluorimeter can inject only one substrate solution at one pH into each well in a variable pH test, 120 µl of substrate was added manually prior to incubation instead of being injected automatically after the first cycle by the fluorimeter. The optimal pH for the assay was determined by the system that produced the highest enzyme activity.

**Table 3-1: Layout of coupling buffer test for immobilised PP-2A**

| Coupling buffer components | Coupling buffer combinations | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| Sodium phosphate buffer (pH 7)[a] | + | + | + | + | - | - | - | - |
| 0.05 M KCl | + | + | - | - | + | + | - | - |
| 0.2 mM Ascorbic acid[b] | + | - | + | - | + | - | + | - |
| 50 mM Tris (pH 7) | - | - | - | - | + | + | + | + |

[a] see section 3.2.1 for preparation of buffer

[b] L-Ascorbic acid 2-phosphate sesquimagnesium salt ($C_6H_9O_9P$)

### 3.4.1.3 Optimal coupling buffer pH for immobilisation

Changes were made to the pH of the optimal coupling buffer (identified in section 3.4.1.2) to determine if additional enzyme can be immobilised onto the microbeads by shifting the pH. Protein phosphatase type 2A was immobilised by the standard immobilisation procedure (section 3.3.2) using the optimal coupling buffer adjusted to pH 7.0 and pH 8.0. The assay was conducted in duplicate at optimal assay pH, according to the standard assay protocols (section 3.3.2). The optimal coupling buffer was determined by the system that produced the highest enzyme activity.

### 3.4.1.4 Optimal assay cofactors

Changes were made to cofactors in the assay to determine if enzyme activity could be enhanced when exposed to different assay components. Bovine serum albumin and $NiCl_2$ were tested in the immobilised system by their addition or removal to the assay mix. Bovine serum albumin was tested at 5 µl of 10 mg/ml stock. This was a higher concentration than that used in the unimmobilised enzyme assay (section 3.3.1), because elevated levels of BSA in the assay have been shown to lessen the confounding effects when testing environmental samples (Mountfort *et al.*, manuscript in preparation-b). Thus, a higher concentration of BSA was tested. Nickel chloride was tested at the standard concentration of 1.3 mM by adding 5 µl of 40mM.

Protein phosphatase type 2A was immobilised by the standard immobilisation procedure (section 3.3.2) using the optimal coupling buffer at optimal pH. Assay combinations were tested in duplicate according to standard assay protocols (3.3.2), approximately 200 hours after immobilisation as shown in Table 3-2, with additional Tris buffer to supplement the volume to 250 µl as necessary. The optimal assay cofactor combinations were determined by the reaction mix that produced the highest enzyme activity.

**Table 3-2: Layout matrix for immobilised PP-2A cofactor test**

| **Cofactor** | **Cofactor combinations** | | | |
|---|---|---|---|---|
| | *1* | *2* | *3* | *4* |
| BSA | + | + | - | - |
| $NiCl_2$ | + | - | + | - |

### 3.4.1.5 Recovery of enzyme activity in immobilising procedures

Although 0.5 U of PP-2A are immobilised onto 50 mg of microbeads during the immobilisation procedure, not all the enzyme binds to the sepharose beads during this process. Preliminary studies (Mountfort *et al.*, manuscript in preparation-a) have shown that approximately 2% of PP-2A is retained in the system once the enzyme has reached stability. To validate these preliminary findings, the proportion of enzyme immobilised onto the microbeads

during the immobilisation procedure (i.e. recovery of enzyme) was determined by comparing the activity of the immobilised enzyme with the activity of unimmobilised enzyme, using a known amount of enzyme added to both systems. To ensure that both the unimmobilised and immobilised systems had the same assay conditions, both assays were conducted at pH 8, without BSA, and contained a liquid assay volume of 150 µl. The immobilised and unimmobilised PP-2A assays were conducted according to standard assay protocols (section 3.3.2 and 3.3.1, respectively), with the exception of those changes above.

### 3.4.2 Optimisation experiments for unimmobilised R.PP-1

The commercial availability of R.PP-1 (Zhang, 1994) allowed investigations of the suitability of this enzyme for toxin detection in this laboratory for the first time. Initial optimisation experiments for R.PP-1 were carried out with the unimmobilised enzyme to reduce the cost and time for analysis.

The R.PP-1 enzyme was supplied in a 200 µl volume, consisting of 500 Units (0.4 U per µl, as opposed to PP-2A which consisted of 0.01 U per µl). Thus, for the unimmobilised assay, R.PP-1 was diluted 1:50 with buffer (in comparison to a 1:4 dilution for PP-2A) and 10 µl of the diluted enzyme (0.5 U) was added to the assay mix. All optimisation experiments for unimmobilised R.PP-1 followed similar assay conditions as the unimmobilised PP-2A assay (section 3.3.1). However all optimisation experiments were carried out without toxin or sample extract in the reaction mix. Bovine serum albumin was added to the assay at a higher concentration (5 µl of 10 mg/ml, instead of 1 mg/ml) for the same reason as discussed in section 3.4.1.4.

The unimmobilised assay system for R.PP-1 contained 60 µl of Tris buffer (pH 7.5), 10 µl of diluted R.PP-1 (0.5 U, 10 µl R.PP-1 diluted to 500 µl in Tris buffer, pH 7.5), 120 µl of buffer containing substrate (0.42 mM MUP), 5 µl of 40 mM of $NiCl_2$, and 5 µl of 10 mg/ml BSA. All assays were carried out in a final assay volume of 200 µl. The assay was conducted at pH 7.5 as this was the assay pH used by the suppliers of the enzyme. All unimmobilised R.PP-1 enzyme tests were conducted based on this assay system unless otherwise stated.

### 3.4.2.1 Optimal storage, temperature and pH conditions

*Storage conditions and assay buffer*

Recombinant PP-1 was supplied with a storage and reaction buffer which was tested against the Tris buffer used for PP-2A, to determine a suitable storage and assay buffer. Storage conditions for R.PP-1 included:

1) R.PP-1 diluted with with reaction buffer (1:2.5), and stored at -20°C for approximately 48 hours

2) R.PP-1 stored undiluted at -20°C for approximately 48 hours

3) R.PP-1 stored undiluted at -70°C for approximately 48 hours

The unimmobilised PPINAs were carried out in triplicate with the R.PP-1 enzyme stored as above (three storage conditions) in a) Tris buffer, and b) reaction buffer, according to the assay system previously described (section 3.4.2). The amount of diluted enzyme added to the assay was corrected so that the same concentration of enzyme was tested across the three treatments.

*Temperature and pH*

Assay tests experimenting with different temperature and pH conditions were conducted to determine if the enzyme activity could be increased under different temperature and pH conditions. To test the effect of temperature and pH, the unimmobilised R.PP-1 assay was carried out with the optimal reaction buffer, according to the procedure described in section 3.4.2, at 30 °C and 37 °C, at pH 7.0, 7.5 and 8.0. All components of the assay were adjusted to the respective pHs. The assay was conducted with R.PP-1 stored under optimal conditions. Optimal temperature and pH conditions were determined by the reaction mix that produced the highest enzyme activity.

### 3.4.2.2 Optimal assay cofactors

*Assay cofactors*

To test the influence of assay cofactors on protein phosphatase activity, changes were made to the reaction mix to test the removal effect of BSA (5 $\mu$l of 10 mg/ml) or $NiCl_2$ (5 $\mu$l of 40 mM) from the system. The assay was carried out in triplicate with a) Tris buffer, and b) reaction buffer, as previously described in section 3.4.2. Both of these buffers were tested to determine if the performance of the assay could be increased by altering components in the

assay. The layout of the reaction matrix is shown in Table 3-3. All other assay components remained unchanged. The optimal assay cofactors were determined by the reaction mix that produced the highest enzyme activity.

**Table 3-3: Layout of matrix for unimmobilised R.PP-1 cofactor test**

| Cofactor | Cofactor combinations | | | |
|---|---|---|---|---|
| | *1* | *2* | *3* | *4* |
| BSA | + | + | - | - |
| $NiCl_2$ | + | - | + | - |

*Metal cofactors*

Recombinant PP-1 was supplied with 10 mM of manganese chloride ($MnCl_2$) as according to the suppliers' data sheet for R.PP-1 (product P0754L), enzyme activity is $MnCl_2$ dependant. To test the dependence of R.PP-1 on $MnCl_2$, phosphatase activity was tested with or without BSA (5 $\mu$l of 10 mg/ml) as shown in Table 3-4. In conjunction, another assay system was also tested substituting $MnCl_2$ with $NiCl_2$, so that the enzyme activity in both assay systems, (i.e. with either $MnCl_2$ or $NiCl_2$) could be compared with or without BSA. The assays were carried out simultaneously in Tris buffer (pH 8) according to the protocol previously described (in section 3.4.2) with the exception of changes made to the reaction mix as stated above. The optimal metal cofactors were determined by the reaction mix that produced the highest enzyme activity.

*Optimal BSA concentration*

The optimal concentration of BSA for the R.PP-1 assay was determined by testing BSA at several concentrations, ranging from 0.1- 30.1 µM (volumes ranged from 2 $\mu$l of 0.5 mg/ml to 20 $\mu$l of 15 mg/ml). The assay system was carried out in triplicate in Tris buffer (pH 8) as previously described (section 3.4.2) with the optimal metal cofactor.

*Optimal $MnCl_2$ concentration*

The optimal concentration of $MnCl_2$ for the R.PP-1 assay was tested at several concentrations ranging from 0.06-4.0 mM (volumes ranged from 5 $\mu$l of 1 mM to 15 $\mu$l of 40 mM). Two other assay conditions were also tested concurrently, one which substituted $MnCl_2$ with 5 $\mu$l of 40 mM $NiCl_2$ (1.3 mM), and the other without metal addition. The assay was carried out in duplicate under the three assay conditions as previously described (section 3.4.2) in Tris buffer (pH 8) with the optimal concentration of BSA.

**Table 3-4: Layout of matrix for unimmobilised R.PP-1metal cofactors**

| **Cofactor** | **Cofactor combinations** | | | |
|---|---|---|---|---|
| | *1* | | *2* | |
| Metal chloride[a] | Ni | Mn | Ni | Mn |
| BSA | + | + | - | - |

[a]Two tests were conducted simultaneously using a) $NiCl_2$ and b) $MnCl_2$

### 3.4.3 Optimisation experiments for immobilised R.PP-1

The optimal conditions for the unimmobilised R.PP-1 assay, determined in section 3.4.2, were applied to the immobilised assay procedure as a starting system to optimise the experimental conditions for the immobilised R.PP-1 assay. Optimisation experiments for the immobilised R.PP-1 enzyme followed the optimisation tests conducted for the immobilised PP-2A enzyme (described in section 3.4.1). Recombinant PP-1 was immobilised using the same immobilisation procedure for PP-2A (section 3.3.2), with the optimal R.PP-1 concentration for immobilisation determined as in section 3.4.3.1.

The immobilised assay system for R.PP-1 contained 25 $\mu$l of Tris buffer (pH 8.0), 120 $\mu$l of buffer containing substrate (0.334 mM MUMP), 5 $\mu$l of 40 mM of $MnCl_2$, and 100 $\mu$l of the enzyme-bead complex. All immobilised R.PP-1 tests were conducted based on this assay system unless otherwise stated. The total volume for the immobilised system contained 250 $\mu$l (150 $\mu$l liquid volume) and the assays were conducted without BSA for the same reasons as for the immobilised PP-2A (to keep the system as simple as possible). Blank wells did not contain the

enzyme or sepharose beads but instead were supplemented with 100 $\mu$l of Tris buffer to make up the final assay volume to 250 $\mu$l. Optimisation experiments did not involve the addition of the sample extract or the toxin standard. All experiments were carried out in duplicate.

### 3.4.3.1 Optimal enzyme concentration for immobilisation

The concentration of R.PP-1 required to give adequate phosphatase activity in the immobilised system was tested at several concentrations. The immobilisation procedure was carried out as previously described (section 3.3.2) with the following concentrations of undiluted R.PP-1 (stored at -70°C) immobilised onto 100 mg of beads: 10 Units (4 $\mu$l), 25 Units (10 $\mu$l), and 62.5 Units (20 $\mu$l). The immobilised assay procedure was carried out for R.PP-1 (section 3.4.3) with $NiCl_2$ replacing $MnCl_2$, as this test was initiated before it was discovered that $MnCl_2$ was a better cofactor than $NiCl_2$. The optimal enzyme concentration will be selected as the system that produced the highest activity with minimal enzyme input.

### 3.4.3.2 Optimal coupling buffer and stability of enzyme activity

Recombinant PP-1 was immobilised by the standard immobilisation procedure (3.3.2) with the optimal concentration of the enzyme in 8 different coupling buffers, as shown in Table 3-1. The assay was carried out according to the assay system described previously (section 3.4.3). The optimal coupling buffer and stability of enzyme activity was determined as previously described for PP-2A (section 3.4.1.1) up to 600 hours after immobilisation.

### 3.4.3.3 Optimal assay pH

Changes were made to the assay pH to determine if pH influenced enzyme activity in the immobilised system. Recombinant PP-1 was immobilised by the standard immobilisation procedure (3.3.2) with the optimal coupling buffer at pH 7. Optimal assay pH for R.PP-1 was tested 195 hours after immobilisation (i.e. when the enzyme reached stability) at pH 7 and 8, according to the assay system described previously (section 3.4.3), but with changes made to the assay pH. The Tris buffer in all assay components were adjusted for pH and the substrate delivery system changed to manual addition (see section 3.4.1.2) to accommodate variable pH in a single experiment. The optimal assay pH was determined as previously described for PP-2A (section 3.4.1.2).

### 3.4.3.4 Optimal coupling buffer pH

Recombinant PP-1 was immobilised by the standard immobilisation procedure (3.3.2) with the optimal coupling buffer adjusted to pH 7 or 8. The assay was conducted in duplicate according to the assay system described previously (section 3.4.3). The optimal coupling buffer pH was tested at 195 hours after immobilisation (i.e. when the enzyme reached stability) at assay pH 8. The optimal coupling buffer pH was chosen as the system that produced the highest enzyme activity.

### 3.4.3.5 Optimal assay cofactors

Recombinant PP-1 was immobilised by the standard immobilisation procedure (3.3.2) using the optimal coupling buffer at optimal pH. Changes were made to assay cofactors in the in the immobilised R.PP-1 system to test the addition or removal effect of BSA or $MnCl_2$ on phosphatase activity. Bovine serum albumin was tested at the optimal concentration range of BSA in the unimmobilised R.PP-1 assay. Magnesium chloride was tested at the standard concentration of 1.3 mM (5 µl of 40 mM). The R.PP-1 assay was conducted in duplicate according to the assay system described previously (3.4.3) with changes made to assay cofactors. Assay combinations were tested approximately 200 hours after immobilisation (i.e. when the enzyme reached stability) as shown in Table 3-2 (replacing $NiCl_2$ with $MnCl_2$).

### 3.4.3.6 Recovery of enzyme activity in immobilising procedures

The proportion of enzyme immobilised onto the microbeads during the immobilisation procedure (i.e. recovery of enzyme, section 3.4.1.5), was determined by comparing stable immobilised enzyme activity with unimmobilised enzyme activity, using a known amount of enzyme added to both systems. To ensure that both the unimmobilised and immobilised systems had the same assay conditions, both assays were conducted at pH 8 with $MnCl_2$ (5 µl of 40 mM), and contained a liquid assay volume of 150 µl. The only exception was that BSA (5 µl of 10 mg/ml) was added to the unimmobilised assay only (as BSA was required for adequate enzyme activity in this system). The immobilised and unimmobilised R.PP-1 enzyme assays were conducted according to standard assay protocols (section 3.4.3 and 3.4.2, respectively), with the exception of those changes above. The recovery of R.PP-1 in the immobilised system was compared to the recovery values for immobilised PP-2A.

### 3.4.4. Statistical analysis

Statistical analysis for all data was performed using 'R' software version 1.7.0. All optimisation experiments were analysed using ANOVA. Statistical analysis was only performed on enzymes when activity had become stable. When mean effects and interactions were significant, these were further explored using Fischers's least significant difference test. Tukey's honestly significant difference test was performed on data when multiple levels of a factor were compared (i.e. pH test for PP-2A). Linear regression was also performed on the pH test for PP-2A.

Only a limited number of data points were used for each test (some more than others), as the procedure was costly and subsequently, replication was limited. Therefore, in some cases the assumptions are probably not fulfilled (due to the lack of data points, departures from normality and heterogeneity of variances could not be adequately assessed). Thus, more weight was assigned to trends which showed differences in enzyme activity between treatments. Where outliers were evident, the data could not be eliminated due to lack of data points. All the data was well balanced and replicated equally. Any data that showed large deviation from normality were logged transformed prior to analyses to normalise variances.

## *3.5 Optimisation Results*

The results have been set out so that the optimisation experiments for each immobilised enzyme, PP-2A and R.PP-1, are displayed simultaneously. Although it does not follow the layout of the methods, it provides a more suitable structure in which to compare the activities of the two different immobilised enzymes. The optimisation experiments for the unimmobilised R.PP-1 will be displayed first, in the order that the experiments were conducted. This will be followed by the results for the optimisation experiments in the order that the experiments were conducted for both of the immobilised enzymes.

### 3.5.1 Optimisation experiments for unimmobilised R.PP-1

#### 3.5.1.1 Optimal storage conditions and optimal assay buffer, temperature and pH

Table 3-5 shows the rate of reaction of R.PP-1 utilising MUMP with different assay and storage buffers, and different storage methods. The data suggests that both the buffer and storage

conditions had an effect on enzyme activity, which was confirmed by a significant interaction detected between enzyme buffer and storage conditions ($p<0.05$).

**Table 3-5: Comparison of unimmobilised R.PP-1 reaction rates utilising different assay and storage conditions (pH 7.5).**

| Assay and storage buffer[a] | Storage method[b] | Rate[c] (nmol/ml/min) |
|---|---|---|
| Tris Buffer[1] | *diluted[e] stored at -20°C* | 0.111±0.032* |
| | *undiluted stored at -20°C* | 0.597±0.040^ |
| | *undiluted stored at -70°C* | 0.671±0.006^ |
| Supplied Reaction Buffer[d2] | *diluted[e] stored at -20°C* | 0.040±0.009* |
| | *undiluted stored at -20°C* | 0.033±0.004* |
| | *undiluted stored at -70°C* | 0.032±0.012* |

[a] pH 7.5

[b] The approximate duration of storage for all enzymes was less than 48 hours

[c] Values are means of triplicate determinations (subtracted from the blank)

[d] Blank 0.063 nmol/ml/min which is the mean of triplicate determinations

[e] Enzyme dilution 1:2.5, enzyme:buffer. The concentration of the enzyme in the diluted test was the same as the enzyme concentration in the undiluted tests (0.5 U per assay)

*^ For the Tris buffer and the supplied reaction buffer the means within each column followed by different symbols are significantly different ($p<0.05$)

[1,2] The rate of the reaction between the Tris buffer and the supplied reaction buffer is significantly different ($p<0.05$)

Figure 3-3, shows that the reaction rate (mean of enzyme activity) for the diluted enzyme (dotted line) is substantially lower compared to the rates for the undiluted enzymes (undiluted high and undiluted low) in a Tris storage buffer. The graph also reveals that enzyme activity is largely affected by assay buffer, with enzyme activity noticeably higher in a Tris buffer than in the supplied reaction buffer. The highest enzyme activity was with the undiluted enzyme stored at -70°C in the Tris assay buffer. Therefore, optimal storage conditions for R.PP-1 was stored undiluted at -70°C, and Tris buffer was selected as the optimal assay buffer.

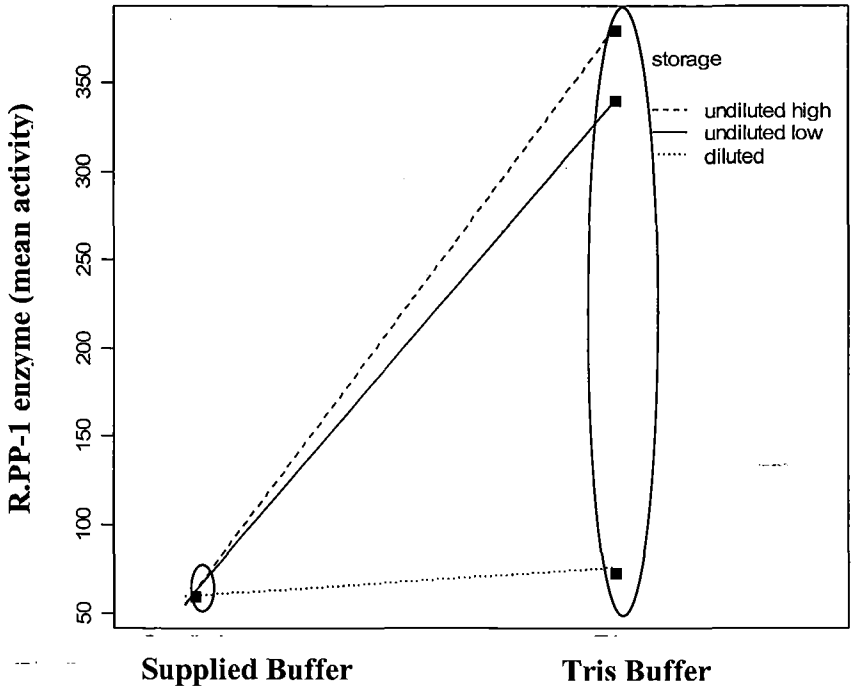



**Figure 3-3.** Interaction plot showing the influence of storage buffer and enzyme storage conditions on R.PP-1 activity. Enzyme activity is on the y-axis (units/min). Undiluted high, refers to enzyme stored undiluted at -70°C. Undiluted low, refers to enzyme stored undiluted at -20°C. Diluted, refers to the enzyme stored diluted with buffer at -20°C. The concentration of enzyme was the same for both the diluted and undiluted tests (0.5 U per assay). The duration of storage for all enzymes was less than 48 hours. The encircled areas indicate that enzyme activity is highest in Tris Buffer (as opposed to enzyme activity in the Supplied Buffer), and that enzyme activity in Tris buffer is affected by storage conditions.

Although the supplied reaction buffer was adequate as a storage buffer (there was virtually no difference in enzyme activity between storage methods for the supplied reaction buffer), the supplied reaction buffer was not suitable for use in the assay system proposed here. This was because certain components in the supplied reaction buffer were binding with the substrate in the assay, as the blank wells that contained no enzyme, were producing fluorescent products.

Table 3-6 shows the reaction of R.PP-1 utilising MUMP as a function of assay pH and temperature. A large significant difference was detected between assay pH ($p<0.05$) and assay temperature ($p<0.05$). The data was log transformed to normalise variances. This was consistent with the observed trend that showed optimal activity was obtained at a temperature of 37°C and at an assay pH of 8.0 (Figure 3-4).

**Table 3-6: Comparison of R.PP-1 reaction rates (nmol/ml/min) utilising different temperature and assay pHs.**

| pH | Temperature[a] | |
|---|---|---|
| | *30°C* | *37°C* |
| 7.0* | 0.034±0.010[b] | 0.113±0.033[b] |
| 7.5^ | 0.388±0.028[c] | 0.525±0.020[c] |
| 8.0# | 0.614±0.015[d] | 1.328±0.086[d] |

[a] Values are means of duplicate determinations.

[bcd] For the temperature test, the means within each column followed by different symbols are significantly different ($p<0.05$)

*^# The mean reaction rates for the pH test are all significantly different ($p<0.05$)

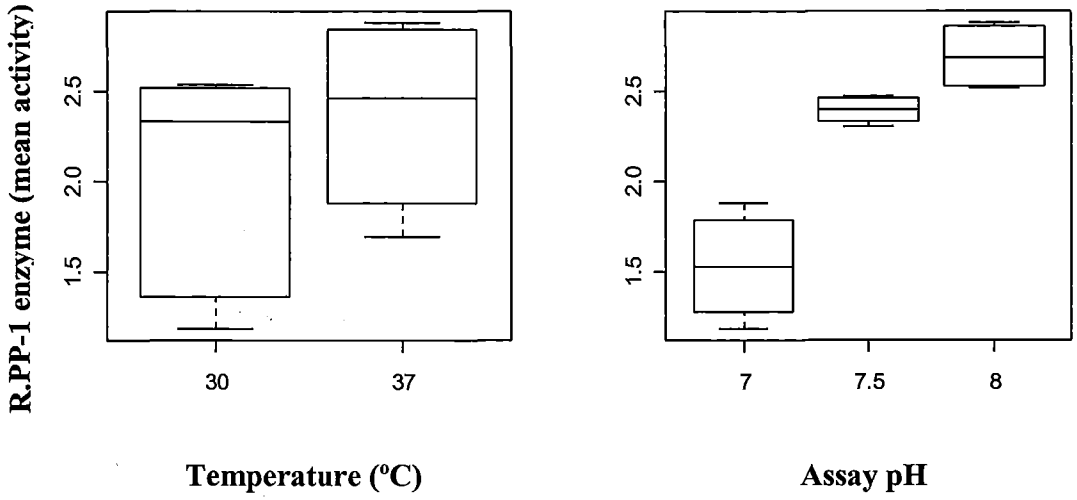



**Figure 3-4.** Boxplot of R.PP-1 activity (log transformed) against temperature (left, median and variance 0.388 and 0.29 nmol/ml/min, respectively) and pH (right, median and variance 0.53and 0.62 nmol/ml/min, respectively). Enzyme activity is on the y-axis (units/min). The boxplots indicate that R.PP-1 enzyme exhibits highest activity at a higher temperature (37°C) and a higher pH (pH 8). See Table 3-6 for

### 3.5.1.2 Optimal assay cofactors

Table 3-7 shows the reaction rate for unimmobilised R.PP-1 utilising MUMP with different assay buffers, and different assay cofactors. The data showed that the reaction rate using the supplied reaction buffer did not substantially change in response to the different assay cofactors and the activity remained low for all cofactor combinations, due to high activity in the blank wells (see section 3.5.1.1). Consequently, experiments with the supplied reaction buffer were concluded. In contrast, the reaction rate for R.PP-1 using Tris buffer increased in response to the assay cofactors. A significant difference was detected between cofactors treatments in the Tris Buffer ($p<0.05$). The highest enzyme activities were evident when BSA was incorporated in the reaction mix. However, a significant difference was not detected between BSA which had the highest activity, and $NiCl_2$ and BSA (second highest) when analysed using Tukey's HSD test. Thus, for the purpose of keeping $NiCl_2$ in the assay, the most optimal assay cofactors selected for R.PP-1 in a Tris buffer was with BSA and $NiCl_2$.

**Table 3-7: Comparison of R.PP-1 reaction rates (nmol/ml/min) using different assay cofactors and assay buffers.**

| Assay Buffer | Assay Cofactors[a] | | | |
|---|---|---|---|---|
| | *BSA & $NiCl_2$* | *BSA* | *$NiCl_2$* | *None* |
| Supplied reaction buffer | 0.011±0.047[b] | 0.00±0.055[b] | 0.009±0.037[b] | 0.014±0.028[b] |
| Tris buffer | 0.181±0.055[c] | 0.256±0.041[c] | 0.110±0.018[d] | 0.014±0.013[e] |

[a] Values are means of triplicate determinations.

[bcde] The means within each row followed by different symbols are significantly different ($p<0.05$)

Table 3-8 shows the reaction rate between R.PP-1 and MUMP utilising different metal cofactors. The data suggests that both BSA and metal chloride had an effect on enzyme activity, which was confirmed by a significant interaction detected between BSA and metal chloride treatments ($p<0.05$). Figure 3-6 shows that the enzyme activity is significantly higher with $MnCl_2$ than with $NiCl_2$ in the presence of BSA in the reaction mix. Hence, the most optimal assay cofactors selected for R.PP-1 was with BSA and $MnCl_2$.

**Table 3-8: Comparison of R.PP-1 reaction rates using different assay cofactors in Tris.**

| Metals[a] | Activity (nmol/ml/min)[b] | |
|---|---|---|
| | *Without BSA* | *With BSA[c]* |
| **$MnCl_2$** | 0.124±0.010[d] | 0.582±0.013[e] |
| **$NiCl_2$** | 0.076±0.030[f] | 0.264±0.042[g] |

[a] Concentration of metals was at 5 µl of 40mM (1.3mM)

[b] Values are means of duplicate determinations

[c] Concentration of BSA was at 5 µl of 10 mg/ml

[defg] The means within each column and row followed by different symbols are all significantly different ($p<0.05$)

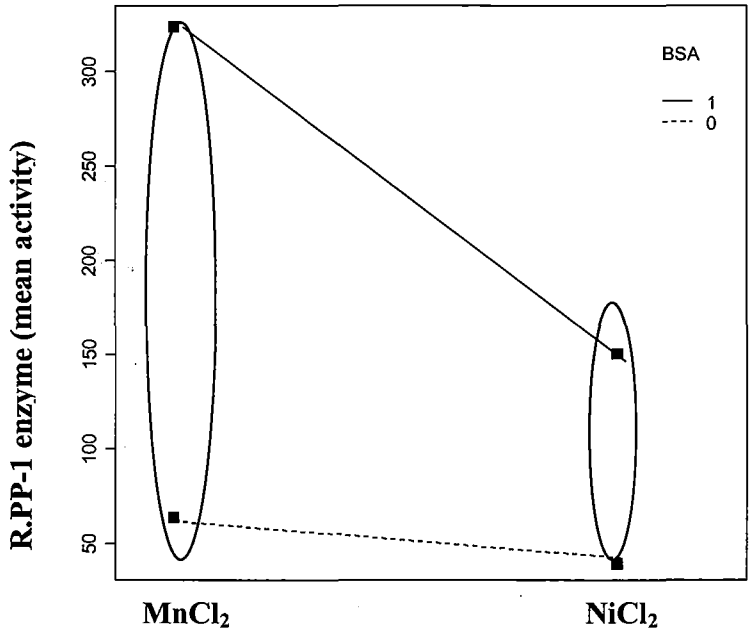



**Figure 3-5.** Interaction plot showing the influence of metal chloride and BSA on R.PP-1 activity. Enzyme activity is on the y-axis (units/min). BSA 1, refers to the assay with BSA (solid line). BSA 0, refers to the assay without BSA (dotted line). The concentration of BSA was at 5 µl of 10 mg/ml. The concentration of $MnCl_2$ and $NiCl_2$ was at 5 µl of 40mM (1.3mM). The encircled areas indicate that the R.PP-1 enzyme exhibits higher activity in the presence of $MnCl_2$, rather than $NiCl_2$, and that activity is higher when BSA is the assay.

Table 3-9 and Table 3-10 show the reaction rate for R.PP-1 utilising MUMP as a function of BSA and $MnCl_2$ concentration, respectively. The data on BSA and R.PP-1 revealed a parabolic relationship (Figure 3-7a) with an optimal concentration of BSA in the assay in the range of 100-300 µg/ml. Similar results were shown with $MnCl_2$ as the reaction rate of R.PP-1 was proportional to $MnCl_2$ concentration with near maximum activity at 1.3 mM (5 µl of 40 mM) (Figure 3-7b). The table and graph also show that the concentration range of $MnCl_2$ is near the optimum. In contrast to the results shown in Table 3-7, where R.PP-1 activity was higher with BSA and without $NiCl_2$, the results in Table 3-10 show that R.PP-1 activity is higher with BSA and $MnCl_2$ at all concentrations of the metal. This further verifys the importance of $MnCl_2$ for the R.PP-1 enzyme. Thus, the optimal assay cofactors for unimmobilised R.PP-1 are with BSA (5 µl of 5 mg/ml) and $MnCl_2$ (5 µl of 40 mM).

**Table 3-9: Comparison of R.PP-1 reaction rates using different concentrations of BSA.**

| BSA [amount per assay (ng)] | BSA (volume added to assay) | Activity (nmol/ml/min)[a] |
|---|---|---|
| 0 | 0 | 0.222±0.070 |
| 1.0 | 2 µl of 0.5 mg/ml | 0.883±0.002 |
| 2.5 | 5 µl of 0.5 mg/ml | 1.044±0.093 |
| 5.0 | 5 µl of 1 mg/ml | 1.095±0.080 |
| 25.0 | 5 µl of 5 mg/ml | 1.229±0.037 |
| 50.0 | 5 µl of 10 mg/ml | 1.199±0.073 |
| 150.0 | 10 µl of 15 mg/ml | 1.010±0.026 |
| 300.0 | 20 µl of 15 mg/ml | 0.825±0.027 |

[a] Values are means of duplicate determinations.

**Table 3-10: Comparison of R.PP-1 reaction rates using different concentrations of $MnCl_2$ in Tris buffer.**

| Metals (mM) | Molarity in assay (µM) | Activity[a](nmol/ml/min) |
|---|---|---|
| $MnCl_2$ | 0.06 | 0.572±0.034 |
| | 0.6 | 0.785±0.069 |
| | 1.3 | 0.863±0.044 |
| | 2.6 | 0.911±0.070 |
| | 4 | 0.921±0.048 |
| $NiCl_2$ | 1.3 | 0.409±0.026 |
| No Metals | 0 | 0.314±0.022 |

[a] Values are means of duplicate determinations.

### 3.5.2 Optimisation experiments for immobilised enzyme

#### 3.5.2.1 Optimal R.PP-1 concentration for immobilisation

Figure 3-7 shows the reaction rate of immobilised R.PP-1 with different enzyme concentrations as a function of time. The data revealed that there was a significant difference in activity between the three enzyme concentrations ($p<0.05$). However, a significant difference was not detected between the two higher enzyme concentrations ($p>0.05$). Thus, the optimal R.PP-1 concentration required to produce adequate activity overtime, with minimal enzyme input, was 12.5 Units per 50 mg of sepharose beads ($r^2$ value, 0.8959). This concentration of enzyme (12.5 Units) was equivalent to adding 10 µl of undiluted R.PP-1 to 100 mg of sepharose beads.

#### 3.5.2.2 Optimal coupling buffer and stability of enzyme activity

Figure 3-8 shows the reaction rate of immobilised (a) PP-2A and (b) R.PP-1, as a function of time utilising different coupling buffers during the immobilisation procedure (section 3.3.2). The results for PP-2A (Figure 3-8a) indicate that there was a large significant difference in enzyme activity between the two coupling buffers ($p<0.05$). The sodium phosphate buffers consistently produced a higher reaction rate in the immobilised PP-2A assays. Within the two buffer groups, there were no significant differences detected between buffers with or without KCl and/or ascorbic acid ($p>0.05$), which was consistent with the trend detected. Thus, the optimal coupling buffer for PP-2A was chosen as the original coupling buffer for which this procedure was initially designed, which was the sodium phosphate buffer with 0.05 M KCl and 0.2 mM ascorbic acid.

The results for R.PP-1 (Figure 3-8b) showed a similar result to that of PP-2A. A large significant difference in R.PP-1 activity was detected between the two coupling buffers ($p<0.05$), with the sodium phosphate buffer consistently producing a higher reaction rate. There were also no significant differences detected between buffers with or without KCl, and/or ascorbic acid ($p>0.05$), which was consistent with the trend detected. Hence, the sodium phosphate buffer with 0.05 M KCl and 0.2 mM ascorbic acid, was chosen as the optimal coupling buffer for the R.PP-1 enzyme.

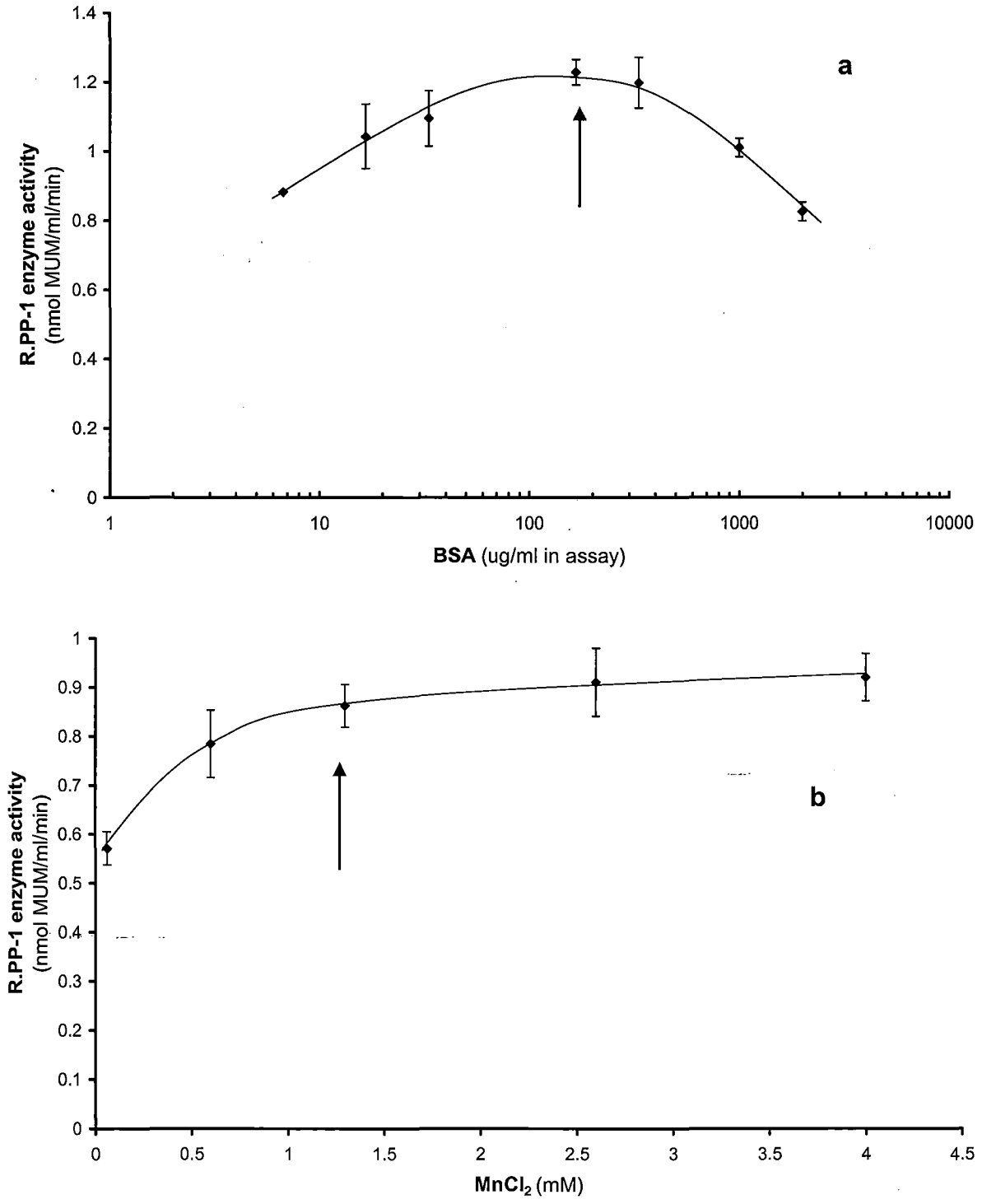



**Figure 3-6.** Reaction kinetics for R.PP-1 showing activity at different concentrations of BSA on a log scale (a) and $MnCl_2$ (b). Each value represents the mean of duplicate determinations ± standard deviation. Arrows indicate optimum concentration range.

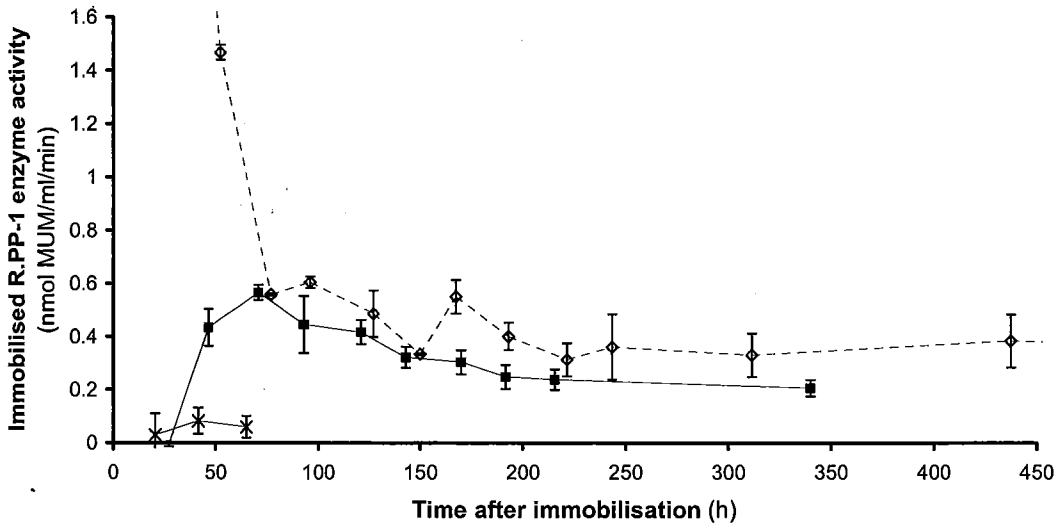



**Figure 3-7.** Reaction rate for immobilised R.PP-1 utilising different enzyme concentrations at various time intervals after immobilisation. Symbols: Δ, 31.25; ■, 12.5; X, 5 Units of enzyme per 50 mg of beads. R.PP-1 was immobilised by the standard procedure with activity determined under standard assay conditions for determination at 37°C at pH 8.0. Each value represents the mean of duplicate determinations ± standard deviation.

Figure 3-8 also revealed that PP-2A and R.PP-1 were relatively stable overtime. Preliminary tests (Mountfort *et al.*, manuscript in preparation-a) have shown that an immobilisation system has high enzyme activity initially, which declines around 150-200 hours after immobilisation and eventually levels off (i.e. activity becomes stable). Both enzymes were stable around 200 hours after immobilisation and remained as such for the full length of the experiments (i.e. 600 hours for R.PP-1 and 1000 hours for PP-2A). Thus, these results are consistent with preliminary findings. There were slight fluctuations in enzyme activity overtime with PP-2A. Natural fluctuations in enzyme activity have been shown in preliminary tests, particularly where there have been incomplete evacuations in washing the enzyme-bead complex from the previous run (Mountfort *et al.*, manuscript in preparation-a).

### 3.5.2.3 Optimal assay pH

Figure 3-9 shows the reaction rate of PP-2A as a function of time at different assay pHs. The results showed a trend between an increase in pH and an increase in enzyme activity over time ($r^2$ 0.9869). A significant difference was detected at all assay pH combinations, excluding assay pH 5.5 and 6.5 using Tukey HSD. Maximum enzyme activity was achieved at a pH of 8.5, which consistently produced higher enzyme activity overtime (0.6 nmol MUM/ml/min) (Figure 3-10). The pH test was primarily designed to see if the assay could run at pH 8.0, which is compatible with the pH of seawater (the assay has already been shown to be compatible with the pH of freshwater which is roughly around pH 7). Thus, an assay pH of 8 was chosen as the optimal assay pH for immobilised PP-2A.

Water was not included in the statistical analyses as it was not the intention of the design. Water was only included in the study to determine how the system could hold its pH, if for example, a 1 ml volume of lake water was added to the system to test for MCs. Enzyme activity in the wells containing water were maintained at a high level but fluctuated over time. This was not surprising as water has a poor buffering capacity. Further tests with water will be required if a large volume of either, sea or fresh water, is intended for the system.

Figure 3-11 shows the reaction rate of R.PP-1 as a function of time at different assay pHs. The results showed that an assay pH of 8 produced significantly higher activity than in an assay pH of 7 ($p<0.05$). Thus, an assay pH of 8 was chosen as the optimal assay pH for immobilised R.PP-1.

### 3.5.2.4 Optimal coupling buffer pH

Figure 3-12 shows the reaction rate of PP-2A as a function of time using the standard coupling buffer at pHs 7.0 and 8.0. The data for PP-2A revealed that the reaction rate between coupling buffer pH 7.0 and 8.0 was not statistically different ($p>0.05$), which was consistent with the trend observed. Therefore, the optimal coupling buffer for PP-2A remained as pH 7.0.

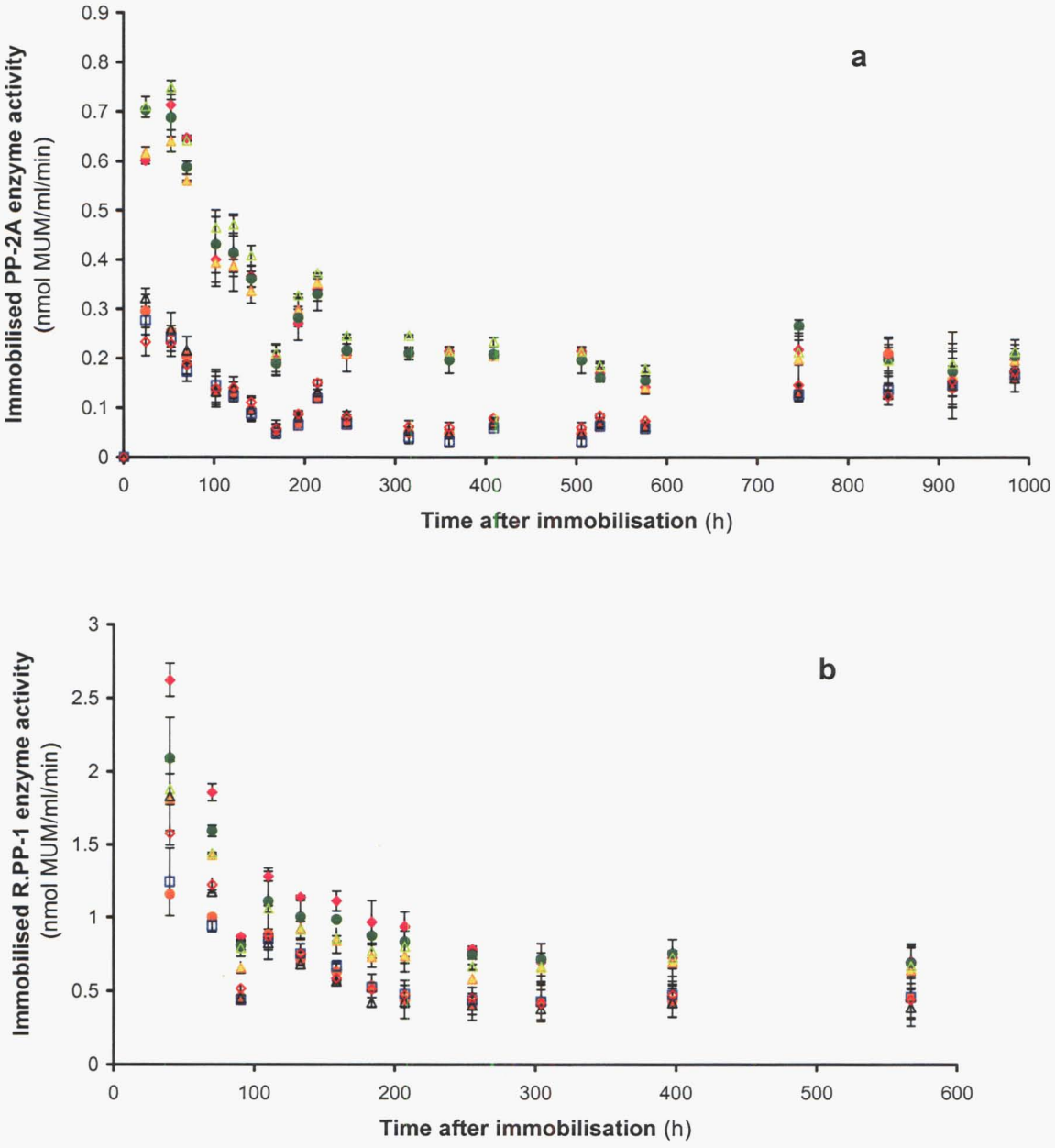



**Figure 3-8.** Reaction rate for immobilised PP-2A (a) and R.PP-1 (b) utilising different coupling buffers at various time intervals after immobilisation. Symbols donate coupling buffer composition: ♦, Na phosphate buffer, KCl and ascorbate; ▲, Na phosphate buffer and KCl; •, Na phosphate buffer and ascorbate; △, Na phosphate; •; Tris, KCl and ascorbate; Δ, Tris and KCl; ◊, Tris and ascorbate; □, Tris. PP-2A and R.PP-1 were immobilised by the standard procedure (1 U/12.5 U respectively per 100mg of beads) in 8 coupling buffers with activity determined under standard conditions for determination at 37°C at pH 7.0 for PP-2A, and pH 8.0 for R.PP-1. Each value represents the mean of duplicate determinations ± standard deviation.

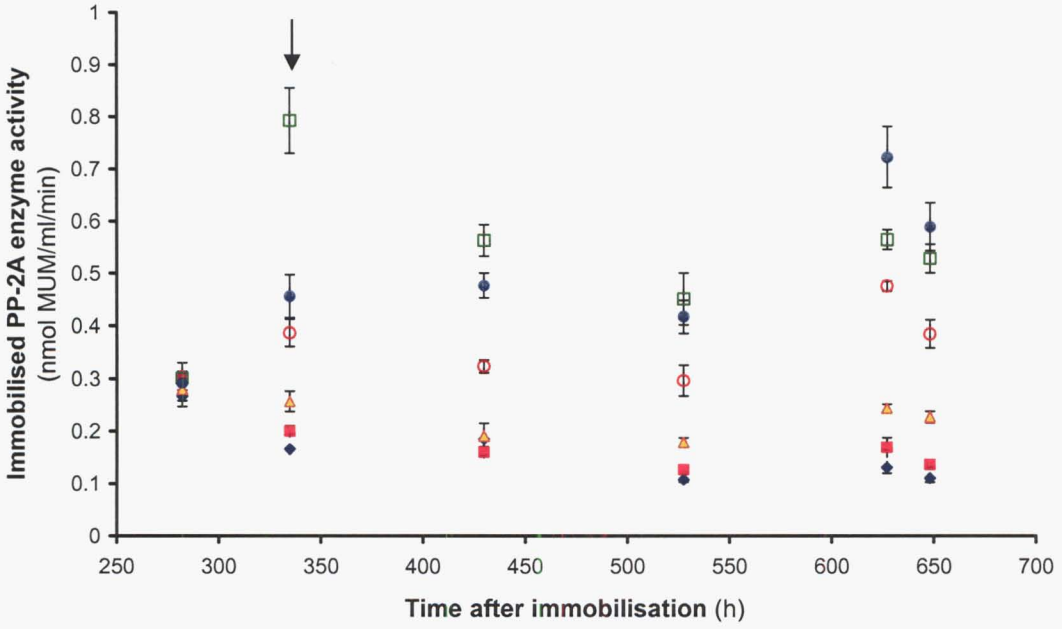



**Figure 3-9.** Reaction kinetics for immobilised PP-2A using a different assay pH. Assay pH was tested as indicated by the arrow at 331 hours after immobilisation. Symbols denote pH values: ◆, 5.5; ■, 6.5; ▲, 7.0; ○, 7.5; □, 8.5; ●, water (pH 5-6). PP-2A was immobilised by the standard procedure (1 Unit per 100mg of beads) in coupling buffer pH 7.0, with activity determined under standard conditions for determination of PP-2A at 37°C. Each value represents the mean of duplicate determinations ± standard deviation.

Figure 3-13 shows the reaction rate of R.PP-1 as a function of time using the standard coupling buffer at pHs 7.0 and 8.0. Coupling buffer was tested at approximately 195 hours after immobilisation (as indicated by the arrow on Figure 3-13), enzyme immobilised with a coupling buffer pH of 8.0, showed statistically higher activity than enzyme immobilised with a coupling buffer of pH 7.0 ($p<0.05$). Thus, for the R.PP-1 enzyme, a pH of 8.0 was selected as the optimal coupling buffer pH.

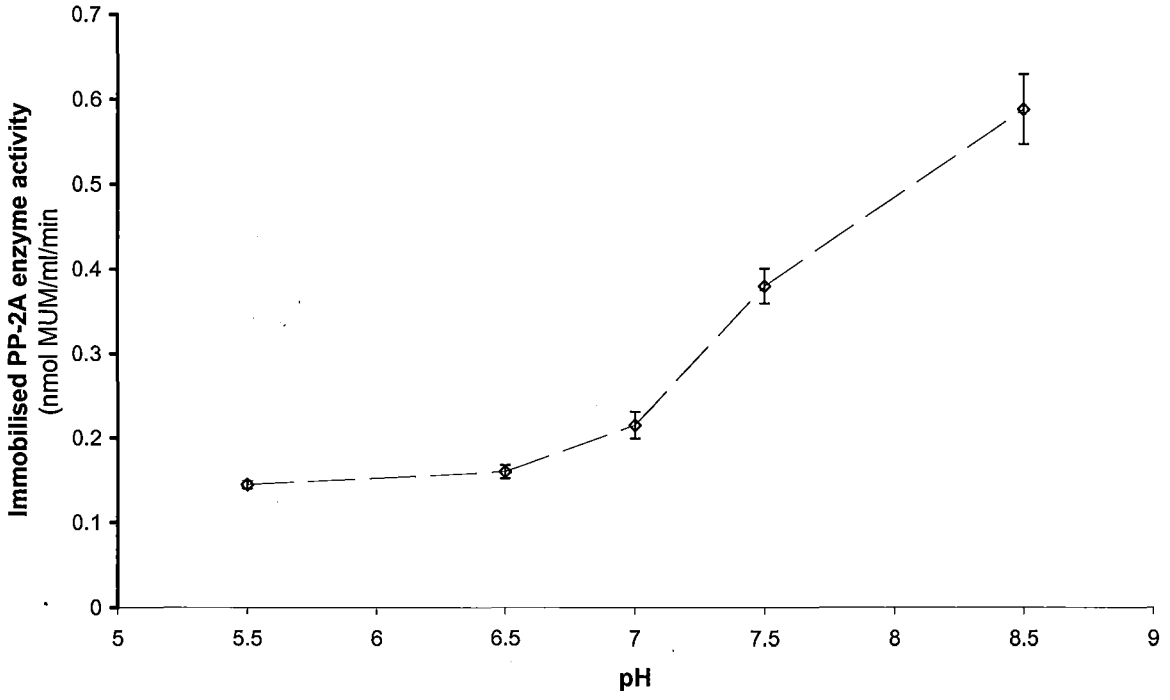



**Figure 3-10.** The mean reaction rate for immobilised PP-2A showing the influence of assay pH on phosphatase activity. Each value represents the mean of eight duplicate readings taken between 283 to 648 hours after immobilisation ± standard deviation.

### 3.5.2.5 Optimal assay cofactors

Figure 3-14 shows the reaction rate of PP-2A (a) and R.PP-1 (b) as a function of time utilising different assay cofactors. Protein phosphatase type 2A was tested with $NiCl_2$, while R.PP-1 was tested with $MnCl_2$. The results for PP-2A revealed that a large significant difference was detected between $NiCl_2$ and the other cofactor treatments ($p<0.05$). The results for R.PP-1 revealed that a large significant difference was detected for $MnCl_2$ only ($p<0.05$) which is consistent with the trend. The trend for both enzymes shows that the reaction rate was higher for the immobilised system containing metal chloride only. Thus, the optimal conditions for the immobilised system were selected as $NiCl_2$ and $MnCl_2$, for PP-2A and R.PP-1, respectively.

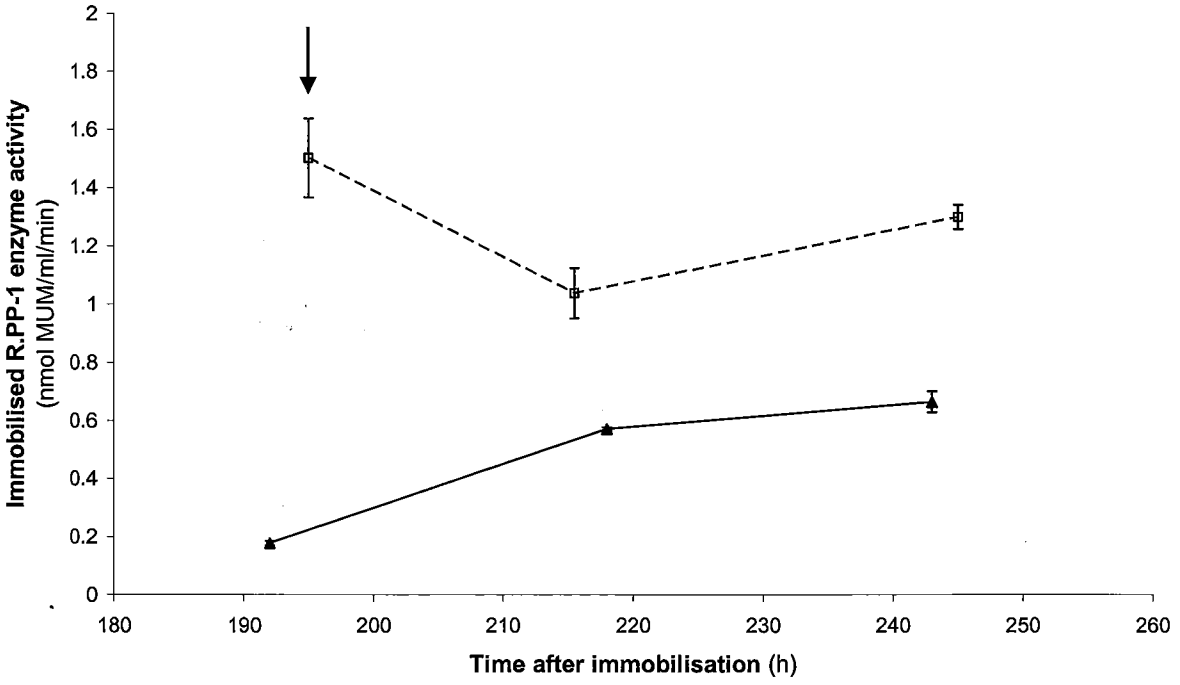



**Figure 3-11.** Reaction rate for immobilised R.PP-1 utilising different assay pHs at various time intervals after immobilisation. Assay pH was tested at 195 hours after immobilisation as indicated by the arrow. Symbols denote pH values in assay buffer: ▲, 7.0; □, 8.0. The same enzyme system was tested independently with two assay pHs, which is why the values are not aligned. R.PP-1 was immobilised by the standard procedure (25 Units per 100mg of beads) in coupling buffer pH 7.0 with activity determined under standard conditions for determination of R.PP-1 at 37°C. Each value represents the mean of duplicate determinations ± standard deviation.

### 3.5.3 Recovery of the enzymes in immobilising procedures

Table 3-11 shows the proportion of PP-2A and R.PP-1 immobilised onto the microbeads during the immobilisation procedure. Recombinant PP-1 had better recovery than PP-2A, as a higher percentage of the R.PP-1 enzyme was recovered during the immobilisation procedure, in comparison to recovery values for the PP-2A enzyme. Both enzymes however, showed higher recovery percentages to preliminary studies (around 2% recovery)(Mountfort *et al.*, manuscript in preparation-a), which suggests that the optimisation tests have been effective.

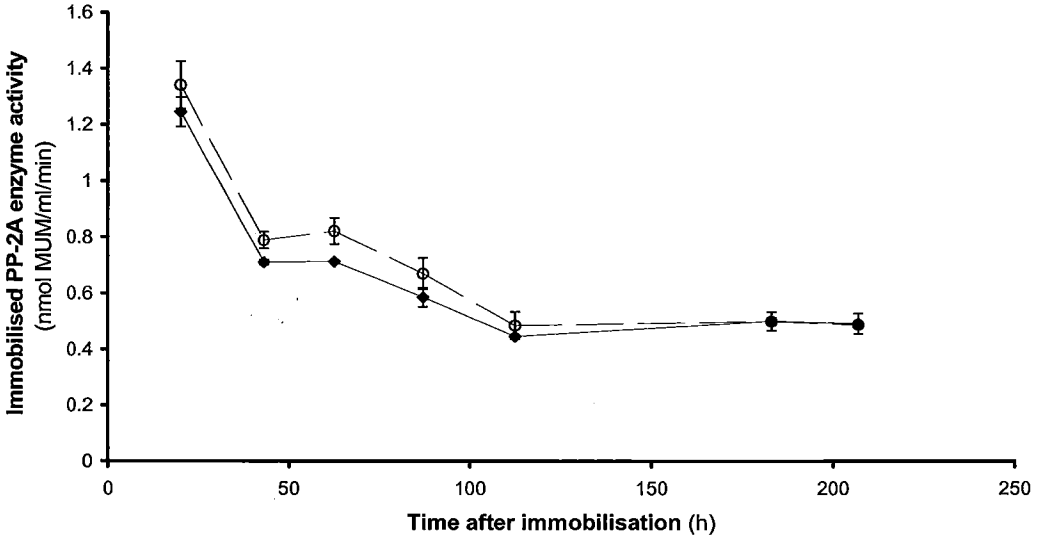



**Figure 3-12.** Reaction rate for immobilised PP-2A utilising different coupling buffer pHs at various time intervals after immobilisation. Symbols denote pH values: ♦, 7.0; ○, 8.0 for coupling buffer pH. PP-2A activity was determined at pH 8 under standard assay conditions. Each value represents the mean of duplicate determinations ± standard deviation.

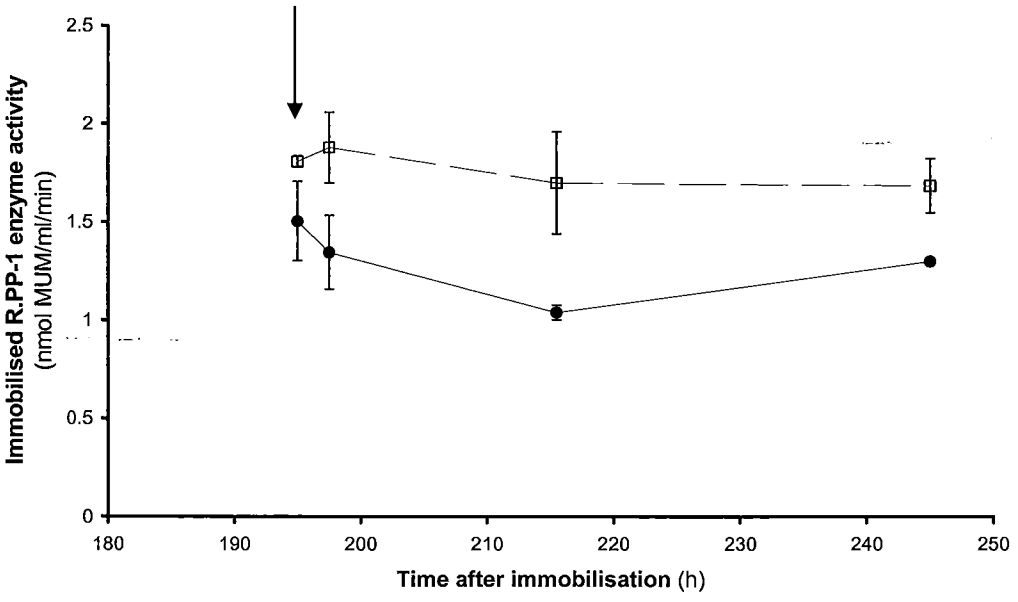



**Figure 3-13.** Reaction rate for immobilised R.PP-1 utilising a different coupling buffer pH at various time intervals after immobilisation. Symbols denote pH values: •, 7.0; □, 8.0 for coupling buffer pH. R.PP1 activity was determined under standard assay conditions at assay pH 8. Optimal coupling buffer was tested 195 hours after immobilisation as indicated by the arrow. Each value represents the mean of duplicate determinations ± standard deviation.

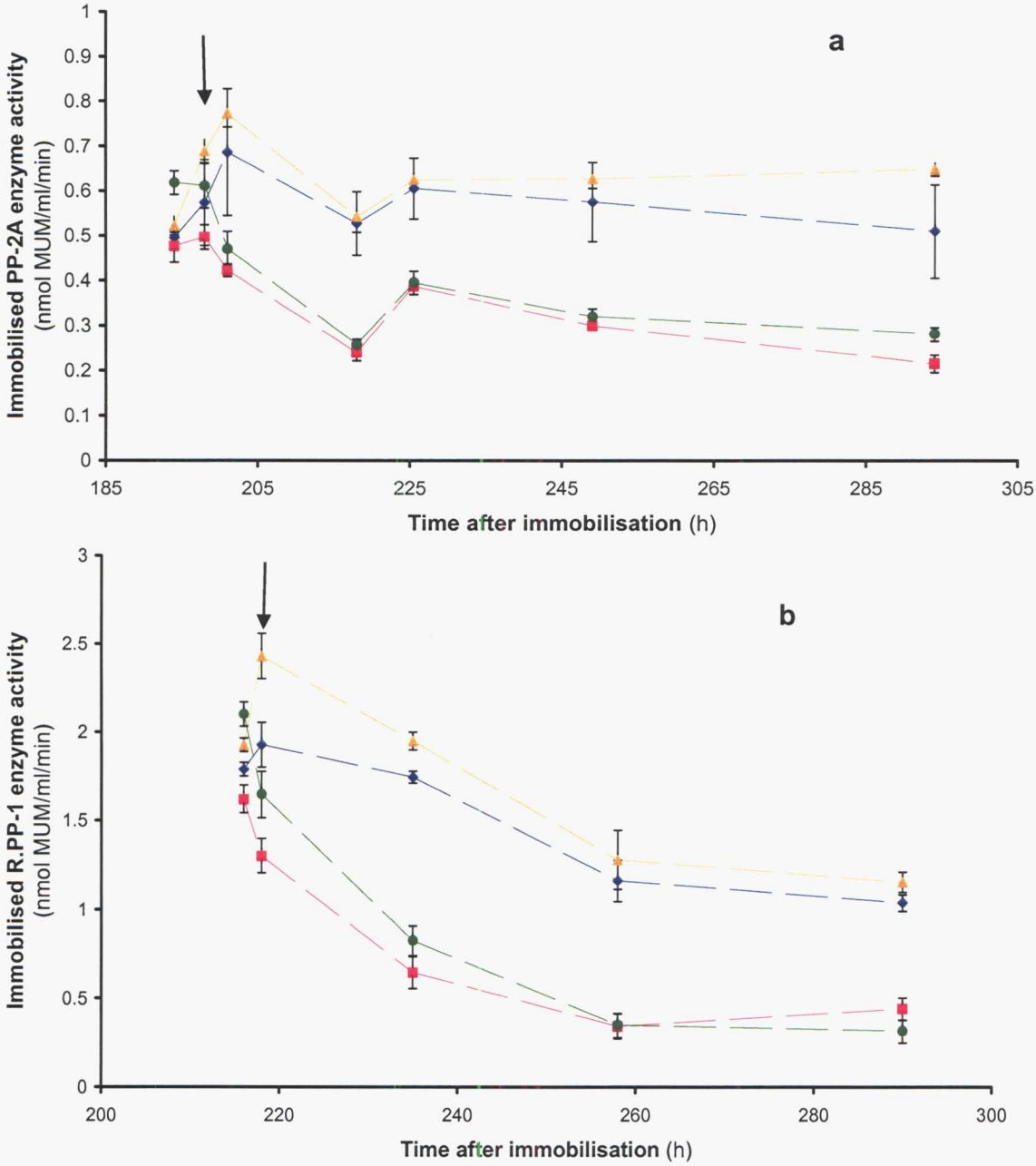



**Figure 3-14.** Reaction kinetics for immobilised PP-2A (a) and R.PP-1 (b) with different assay cofactors. Symbols represent assay cofactors: ◆, $NiCl_2$ or $MnCl_2$ and BSA; ■, BSA; ▲, $NiCl_2$ or $MnCl_2$; ●, No metals or BSA. Assay combinations were tested as indicated by the arrow approximately 200 hours after immobilisation. PP-2A was tested with 1.3 mM $NiCl_2$ and 5 $\mu$l of 10 mg/ml of BSA. R.PP-1 was tested with 1.3 mM $MnCl_2$ and 5 $\mu$l of 5 mg/ml of BSA. Both enzymes were immobilised by the standard procedure with activity determined under standard assay conditions for determination at 37°C. Each value represents the mean of duplicate determinations ± standard deviation.

**Table 3-11: The recovery of enzyme immobilised on microbeads.**

| *Enzyme* | *Activity of enzyme (nmol/ml/min)* | | *Activity of enzyme equivalent to that added to 100 mg beads* | | *% Recovery of immobilised enzyme* |
|---|---|---|---|---|---|
| | Immobilised enzyme [a] | Unimmobilised enzyme [b] | Immobilised enzyme [c] | Unimmobilised enzyme [d] | |
| PP-2A[e] | 0.45±0.05 | 0.63±0.02 | 0.99±0.10 | 31.65±1.05 | 3.15 |
| R.PP-1[f] | 1.52±0.06 | 1.55±0.05 | 3.03±0.12 | 77.45±2.55 | 3.91 |

[a] observed activity of immobilised enzyme in the standard immobilised system (0.5 and 12.5 Units per 50 mg of beads for PP-2A and R.PP-1, respectively).

[b] observed activity for unimmobilised enzyme (0.02 and 0.5 Units for PP-2A and R.PP-1, respectively) (10ul diluted in 500ul of buffer).

[c] and [d] amended activities assuming enzyme equivalent to 500 µl of enzyme added to100 mg of beads (equivalent to 1 and 25 U of PP-2A and R.PP-1, respectively, which is the amount of enzyme used for immobilisation).

[e] activity was determined under optimised conditions for unimmobilised and immobilised PP-2A (section 3.4.1.5). Assay cofactors included 1.3 mM $NiCl_2$ in an assay pH of 8 and coupling buffer pH 7.0.

[f] activity was determined under optimised conditions for unimmobilised and immobilised R.PP-1 (section 3.4.3.6). Assay cofactors included 1.3 mM $MnCl_2$ in an assay pH of 8 and a coupling buffer of pH 8.0. BSA (5 µl of 5 mg/ml) was added to the unimmobilised test only.

## 3.6 Discussion

The objective of the optimisation experiments was to develop the microfilter-plate technique to obtain the highest activity possible for the immobilised PP-2A and R.PP-1 enzymes, while keeping the system as simple as possible. The intent of this objective was fulfilled for both enzymes. A suitable system for the immobilised PP-2A and R.PP-1 enzyme was developed that can maintain good enzyme activity over a long period of time. High enzyme activity is important for a detection system, as marginal differences in the inhibition of enzyme activity (from toxins), can be detected.

The optimisation experiments for the unimmobilised R.PP-1 enzyme demonstrated a number of findings. Firstly, the supplied reaction and storage buffer was not suitable for the assay substrate, MUMP. Although the supplied buffer worked well as a storage buffer, by preserving the activity of the enzyme, there were one or more components in the buffer that were unspecifically binding to the substrate and causing the fluorescent product, MUM, to be produced. Secondly, the optimisation experiments for unimmobilised R.PP-1 demonstrated higher activity at higher pH (pH 8) and temperature (37°C). Thirdly, R.PP-1 was confirmed to be $MnCl_2$ dependant, as higher activity was observed with $MnCl_2$ than with $NiCl_2$ (0.58 and 0.26 nmol MUM/ml/min, respectively). This was consistent with the manufactures instructions (New England Biolabs, P0754S). Fourthly, unimmobilised R.PP-1 required BSA for adequate activity (0.58 and 0.12 nmol MUM/ml/min, with and without BSA, respectively). This differed to the findings for the immobilised R.PP-1 system, which showed higher activity without BSA (0.5 and 1.2 nmol MUM/ml/min, with and without BSA, respectively).

Similar trends were observed for PP-2A and R.PP-1 in relation to assay parameters (in both the immobilised and unimmobilised system), with the exception of the influence of BSA. For example, both enzymes showed higher enzyme activity at elevated pHs. Both enzymes also showed highest enzyme activity when metal chloride cofactors were present in the reaction mix (R.PP-1 required $MnCl_2$ for adequate activity, while PP-2A had adequate activity with $NiCl_2$). Protein phosphatase type 2A was not tested with $MnCl_2$. Therefore, it is not known if higher activity could be obtained with $MnCl_2$ as opposed to $NiCl_2$. This could potentially be addressed in future experiments, although it may not be necessary.

The optimisation experiments for the unimmobilised enzymes have also shown that the PP-2A enzyme was more effective at dephosphorylating MUMP, than the R.PP-1 enzyme. For example, in the optimised system, one unit of unimmobilised R.PP-1 typically released 3 nmol of phosphate/ml/min from 250 $\mu$M MUMP (final substrate molarity in assay), while one unit of unimmobilised PP-2A typically released 30 nmol of phosphate/ml/min from 250 µM MUMP. A similar pattern was shown in the immobilised enzyme systems. For example, 1 Unit of PP-2A applied to 100 mg of beads was sufficient to produce adequate activity (determined by Mountfort *et al.,* manuscript in preparation-a), while 25 Units of R.PP-1 were required to produce adequate enzyme activity in 100 mg of beads (concentrations tested in this study were 10, 25 and 62.5 U, $r^2$ value 0.8959). The different volumes of enzyme input would most likely explain the

differences in the amount of fluorescence produced between the two enzymes that were constantly shown throughout this chapter. For example, immobilised R.PP-1 consistently showed activity greater than 1 nmol MUM/ml/min, while PP-2A consistently showed enzyme activity less than 1 nmol MUM/ml/min when the enzyme was stable.

The activity of the R.PP-1 enzyme toward MUMP has been demonstrated here, possibly for the first time. Previous enzyme assays carried out with R.PP-1 have shown activity of R.PP-1 toward the following substrates, para-nitrophenyl (*p*-NPP) (An and Carmichael, 1994), $^{32}$P-labelled phosphorylase *a* (Zhang *et al.*, 1992; Zhang, 1994) and phosvitin (Heresztyn and Nicholson, 2001). Although R.PP-1 is not as effective at dephosphorylating MUMP as PP-2A, it still produced reasonable activity for the amount of enzyme input.

With the exception of the preliminary study carried out by (Mountfort *et al.*, manuscript in preparation-a) there have been no other studies that have used an immobilised method similar to the one described here for PP-2A and R.PP-1. Thus, a comparison of these results to other studies is limited. Both immobilised enzymes showed good stability overtime (over 600 hours) and generally reached stability between 100-200 hours after immobilisation. This concurs with preliminary results for PP-2A which showed enzyme stability for up to 1000 hours after immobilisation and generally approached stability within 100 hours (Mountfort *et al.*, manuscript in preparation-a). It should be noted that the length of 'time' for the enzyme to become stable may not be related to enzyme stability. Rather, the 'number of times' the plate had been run through an assay, may be a better indicator of the enzyme reaching stability. An example of this finding is discussed here. Usually 5-7 assays are performed on a microplate before enzyme stability is shown. These assays are performed to show the activity trend over time. Thus, they indicate when the enzyme has reached stable activity and can be used for toxin detection (i.e. the remaining enzyme in the system is strongly bound to the microbeads and thus, activity should not fluctuate over time). One experimental microplate with PP-2A was left for over 100 hours without being used in an assay, after the enzyme had been immobilised onto the beads. The activity of this enzyme revealed a lag effect, where the activity curve was shifted to the right and the enzyme reached stability at approximately 250 hours after immobilisation (6 assay cycles), in comparison to the standard enzyme stability profile which is normally reached after 150 hours after immobilisation (6 assay cycles) (see appendix 7.5). This lag effect has been observed in other studies (Goussain and Mountfort, unpublished). This potentially suggests that the stability

of the enzyme was more related to frequency of washing, rather than time. If this is the case, then the immobilisation procedure needs to be amended to include more than 2 washes of the enzyme-bead complex (section 3.3.2) before the beads are transferred to the microfilter plate, as this should reduce the period of time for the enzyme to reach stability. However, more experiments are required to adequately test this.

The results from the recovery table (Table 3-11) suggest that a higher proportion of R.PP-1 enzyme was recovered during the immobilisation procedure than that recovered with PP-2A. In other words, more R.PP-1 enzyme adhered to the sepharose beads during the immobilisation procedure in relation to enzyme input (recovery of immobilised enzyme was 3.91%), than the amount retained with PP-2A (recovery of immobilised enzyme was 3.15%). These enzyme recovery values are higher than those obtained in preliminary experiments with PP-2A which showed a recovery of approximately 2% of enzyme in the immobilised system (Mountfort *et al.*, manuscript in preparation-a). Thus, the R.PP-1 enzyme had a better ability to be retained in the system, than the PP-2A enzyme.

There were some differences between the levels of measurable activity between experiments. For example, in the stability experiment for PP-2A (Figure 3-8) enzyme activity plateaued (i.e. became stable) at around 0.2 nmol MUM /ml/min. In comparison, PP-2A activity in the cofactor experiment (with the same enzyme input) maintained stability at around 0.6 nmol MUM /ml/min under the same conditions (Figure 3-13). Natural fluctuations in enzyme activity between tests are normal and have been shown in preliminary tests with PP-2A (Mountfort *et al.*, manuscript in preparation-a). The reason for this cannot be explained. Natural enzyme variation should not be of concern as similar fluctuations occur in both control and treatment wells.

One of the drawbacks of the immobilised system is the cost associated with using a high concentration of enzyme when only approximately 3-4% is retained in the system during the immobilisation process. Ten units of PP-2A costs US$339.00 (Upstate cell signalling solutions, #26330), while 500 units of R.PP-1 costs US$420.00 (New England Biolabs, #P0754L). There are also high costs involved with shipping an enzyme that needs to be transported with dry ice, which can exceed the cost of the enzyme themselves (as is the case for R.PP-1 shipping to NZ). The total cost (including shipping) for obtaining PP-2A and R.PP-1 was approximately NZ$725 (10 units) and NZ$1400 (500 units), respectively. This translates into NZ$72.50 for one unit of PP-2A, and NZ$70.00 for 25 units of R.PP1 ($2.80 per unit), which is the minimal concentration

required for immobilisation (hence the minimal cost to perform a test on one sample). This is, however, the greatest expense as the cost to conduct the assay is minimal, once the fluorimeter has been purchased. Furthermore, the costs are quite low when compared to other methods. For example, the cost to run one sample through LC-MS to detect DSP toxins is $725 (A. Thompson, personal communication, 5 March 2006).

In summary, PP-2A and R.PP-1 can be successfully applied to the immobilised PPINA format. Both enzymes showed good activity and remained active over a long period. The unimmobilised method is time efficient, straightforward, and the whole procedure can be performed in less than 2 hours. In comparison, the immobilised PPINA is considerably more time consuming due to the lengthy immobilisation procedure (can take several hours depending on the number of samples being tested) and the period of time, or number of washes, for the enzymes to become stable (approximately 120 hours). However, once the enzyme is stable, it is possible to carry out multiple assays in a short period of time (results generally obtained within 1.5 hours). The washing procedure carried out between assay runs is simple and rapid. Both enzymes can maintain activity when stored at 4°C for at least one month. Although the costs to purchase the enzymes were high, the multiple assay procedure is a pathway to reaching to the final product, the prototype biosensor. Thus, the high cost involved with the enzyme can eventually be minimised. For example, other less expensive sources of enzymes have become available for testing, such as the Yasumoto R.PP-1. Costs can also be minimised by further optimising the immobilisation procedure so that more enzyme can be retained in the system and hence, less enzyme needs to be used. The microfilter-plate technique for PP-2A and R.PP-1 is now in an optimal format to test the feasibility of this method for MC and OA detection.

# Chapter 4 Feasibility Studies for Development of a Biosensor for Okadaic Acid and Microcystins

## 4.1 Introduction

Feasibility studies were carried out with the PP-2A and the R.PP-1 enzyme, to determine if the immobilised PPINA could potentially translate to a prototype biosensor for the detection of MC and DSP toxins (OA and DTXs) in the aquatic environment. Favourable features for a PPINA biosensor are high sensitivity (i.e. $IC_{50}$ below 10 nM for either toxin), a broad toxin detection range (i.e. <0.1 to >10 nM), and re-usability of the assay after toxin has been added to the system (i.e. residual toxin does not bind to the enzyme once washed from the system and inhibit enzyme activity). Optimal conditions for the immobilised PP-2A and R.PP-1 enzyme assays were determined in Chapter 3. These conditions were applied to the immobilised assay system to ascertain dose-response curves for each toxin with each enzyme. The re-usability of the assay system was also tested under those conditions. The robustness of the immobilised enzymes were tested by analysing environmental samples naturally contaminated with DSP toxins and MCs, and comparing the results with values obtained from other analytical methods. It was anticipated that at least one of the enzymes would fulfil the criteria of a biosensor for at least one of the toxins.

## 4.2 Materials and Preparations

### 4.2.1 Toxins

Okadaic acid (OA) (purity grade >96%) was obtained from the National Research Council, Institute for Marine Biosciences, Canada. Microcystin-LR (MC-LR) (purity grade ≥96%) in methanol was obtained from Alexis® Biochemicals, California, USA.

### 4.2.2 Preparation of algal extracts for PPINA and LC-MS

Water samples were collected from several lakes in New Zealand with dense cyanobacterial blooms that were naturally contaminated with MCs and NODLNs. The samples (V) were collected from the North Island by Susie Wood, Massey University, while the samples (E and L) were collected as part of the New Zealand Marine Biotoxin testing program on the North and South Island (refer to Table 4-4). Water samples were extracted by mixing with an equal volume of 0.2% formic acid in acetonitrile (breaks down algal cells), sonicating for 10 minutes and then centrifuging (12,000 x *g* for 10 mins). Aliquots of the supernatant were diluted with 80% methanol at variable dilutions depending on the algal concentration of the extract: V.432, 431 and 433 and E.329 diluted 1:9 (methanol/water), V.436 diluted 1:1 (methanol/water), and L.F diluted 1:3 (methanol/water).

### 4.2.3 Preparation of mussel extracts for PPINA

Mussels (*Perna canaliculus*) naturally contaminated with OA were collected as a part of the New Zealand Marine Biotoxin testing program. Whole mussel tissue (approximately 5 g wet weight) was homogenised for 1 minute using an Ultra Turrax blender (type T25B, IKA) with 90% methanol in the ratio 1 part by weight to 5 parts by volume (Mountfort *et al.,* 1999). Methanol was used to dissolve endogenous protein phosphatases. The suspension was centrifuged (4,355 x *g*) and the supernatant removed and stored at -20°C.

### 4.2.4 Preparation of mussel extracts for LC-MS

Mussel extracts were collected as described in section 4.2.3, and prepared for LC-MS by Paul McNabb, Cawthron Institute, by the method of MacKenzie *et al.* (2002). Whole mussel tissue was homogenised using a blender. The mussel homogenates were then extracted by blending 2 g homogenate with 18 ml methanol/water (9:1 v/v) for 3 minutes. The extract was centrifuged (3000 x *g* for 15 min) and 1 ml supernatant transferred to an autosampler vial for LC-MS analysis.

## 4.3 Methods

### 4.3.1 Protocol for the immobilised PPINA

The immobilised PPINA was carried out based on the final optimised experiments as described in chapter 3. Both the R.PP-1 and PP-2A enzymes were immobilised according to the protocol for the enzyme immobilisation procedure as detailed in section 3.3.2.

The optimised immobilised assay system for PP-2A based on the results in section 3.5.2, contained 0.5 Units of PP-2A immobilised to 50 mg of beads (approximately 100 $\mu$l in volume), 120 $\mu$l of reaction substrate (0.334 mM MUMP in 50 mM Tris buffer, pH 8.0), 5 $\mu$l of 40 mM of $NiCl_2$, 15 $\mu$l of 50 mM Tris buffer (pH 8.0), and 10 $\mu$l of purified toxin standard or sample extract.

. The optimised immobilised assay system for R.PP-1 based on the results in section 3.5.2, contained 12.5 Units of R.PP-1 immobilised to 50 mg of beads (approximately 100 $\mu$l in volume), 120 $\mu$l of reaction substrate (0.334 mM MUMP in 50 mM Tris buffer, pH 8.0), 5 $\mu$l of 40 mM of $MnCl_2$, 15 $\mu$l of 50 mM Tris buffer (pH 8.0) and 10 $\mu$l of purified toxin standard or sample extract.

The final assay volume for the immobilised system was 250 $\mu$l (150 $\mu$l liquid volume). Control wells did not contain the toxin or the sample extract, but instead were supplemented with 10 $\mu$l of distilled water (to derive a calibration curve) or 90% methanol/water mix (for sample extraction) to make up the assay liquid volume to 150 $\mu$l. Blank wells did not contain the enzyme, the sepharose beads, the toxin or the sample extract, instead the wells were supplemented with the same volume of water or 90% methanol/water mix as in the control and 100 $\mu$l of 50 mM Tris buffer to make up the final assay volume to 250 $\mu$l. The experiment was carried out in duplicate unless otherwise stated. The reaction rate was measured by the fluorimetric procedure as described in section 3.3.1.

### 4.3.2 Method for the unimmobilised PPINA

*Protein phosphatase type 2A*

The unimmobilised PP-2A inhibition assay was carried out as described in section 3.3.1, with the following changes to the assay mix: 5 $\mu$l of 10 mg/ml BSA (instead of 5 $\mu$l of 1 mg/ml), assay pH 8 (instead of pH 7), and a total assay volume of 150 µl (instead of 200 µl). These

changes were made so that the activity of the unimmobilised assay system could be directly compared to the activity of the immobilised assay system (section 4.3.1), under the same assay conditions. The only exception to this was that the unimmobilised system contained BSA while the immobilised system did not. This was because the unimmobilised assay has previously been shown to produce more optimal enzyme activity with BSA (Mountfort *et al.*, manuscript in preparation-b).

*Recombinant PP-1*

The optimised assay system for unimmobilised R.PP-1 was based on the final optimised method as described in section 3.5.1. The optimised assay system contained 10 $\mu$l of diluted R.PP-1 (0.5 Units) (diluted 1:50 with 50 mM Tris buffer, pH 8.0), 120 $\mu$l of buffer containing substrate (0.42 mM MUMP), 5 $\mu$l of 40 mM of $MnCl_2$, 5 $\mu$l of 10 mg/ml BSA and 10 $\mu$l of purified toxin standard or sample extract.

The final assay volume for the unimmobilised system was 150 $\mu$l. Control wells did not contain the toxin or the sample extract, instead were supplemented with 10 $\mu$l of distilled water (to derive a calibration curve) or 90% methanol/water mix (for sample extraction) to make up the final assay volume to 150 $\mu$l. Blank wells did not contain the enzyme, the toxin or the sample extract but instead were supplemented with the same volume of water or 90% methanol/water mix as in the control, and 10 $\mu$l of Tris buffer to make up the final assay volume to 150 $\mu$l. The experiment was carried out in triplicate unless otherwise stated. The reaction rate was measured as described in section 3.3.1.

## 4.3.3 Dose-Response Curves

### 4.3.3.1 Okadaic acid calibration curve

Okadaic acid dilutions for calibration curves were prepared from stock solution of OA in methanol (24.1 $\mu$g/ml). The stock solution was diluted with distilled water and added to the assay mixture to generate a dose-response curve for immobilised PP-2A and R.PP-1 in the range of 0.001-2000 and 0.1-2000 nM, respectively. These ranges were arrived at by noting the detection range for OA and PP-2A in other studies (Mountfort *et al.*, 1999). The immobilised enzyme assays were conducted according to assay conditions described in section 4.3.1. A dose-response curve was also generated for unimmobilised PP-2A and R.PP-1 (section 4.3.2) in the range of

0.0001-1000 and 0.01-2000 nM, respectively. The unimmobilised enzyme assays were conducted according to assay conditions described in section 4.3.2. The dose-response curves were expressed as % activity against the control (i.e. % activity test = (activity of test sample/ the activity of the control sample) x 100).

### 4.3.3.2 Microcystin-LR calibration curve

*Effect of pH*

The effect of pH on the MC-LR dose response curve was tested. This was because MCs are mostly found in freshwater, which has an approximate pH range of 7. This differs to the pH of seawater, which has a pH around 8. Therefore, it was preferable to change the assay pH when measuring MCs in freshwater, so that it was consistent with the sample pH. To determine if pH had an influence on inhibition by MC-LR, dose-response curves for unimmobilised PP-2A were generated at both pH 7.0 and 8.0. Although it would have been preferable to test the effect of pH in the immobilised system, there was only sufficient enzyme to test this in the unimmobilised system.

Microcystin-LR dilutions for calibration curves were prepared from stock solution of MC-LR in methanol (100 $\mu$g/ml). The stock solution was diluted with distilled water and added to the assay mixture to generate a dose-response curve for unimmobilised PP-2A in the range of 0.01-670 nM. These ranges were arrived at by noting the detection range for MC-LR and PP-2A in other studies (Mountfort *et al.,* 2005). The unimmobilised PP-2A assay was conducted at pH 7.0 and 8.0 according to assay conditions described in section 4.3.2 (with the exception of pH change). The dose-response curves were expressed as % activity of the control. If pH influenced the dose-response curves there would be a shift in the $IC_{50}$ and/or assay detection limit.

*Dose-response Curves*

Diluted stock solutions of MC-LR in water were added to the assay mixture to generate a dose-response curve for immobilised PP-2A and R.PP-1 in the range of 0.067-3350 nM. Based on the results of the previous test (section 4.3.3.2) and because MCs are mostly found in freshwater, all dose-response curves for MC-LR were conducted at pH 7.0. The immobilised enzyme assays were conducted according to assay conditions described in section 4.3.1 (with the exception of pH change). The dose-response curves were generated for unimmobilised PP-2A

and R.PP-1 in the range of 0.0067-3350 and 0.0067-670 nM, respectively. These ranges were arrived at by noting the detection range for MC-LR and PP-2A in other studies (e.g. Mountfort *et al.,* 2005). The unimmobilised enzyme inhibition assays were conducted according to assay conditions described in section 4.3.2 (with the exception of pH change). The dose-response curves were expressed as % activity of the control.

*Effect of Bovine serum albumin*

Bovine serum albumin was added to the unimmobilised assay system for two reasons. Firstly, BSA inclusion in the reaction mix produced higher enzyme activity, especially with the R.PP-1. Secondly, BSA addition eliminated the matrix effects of environmental samples by binding to non-specific components in the reaction mix (i.e. it allowed for specific binding between non-confounding components (e.g. toxin) and the enzyme and thus led to a more sensitive assay) (Mountfort *et al.*, manuscript in preparation-b). Bovine serum albumin was excluded from the immobilised system for two reasons. Firstly, the exclusion of BSA in the reaction mix lead to a higher enzyme activity in the immobilised system. Secondly, the intention of the immobilised system was to keep it as simple as possible for potential application as a biosensor.

Since the two assay systems were different with respect to BSA, the addition of BSA in the reaction mix was tested in the immobilised PP-2A assay to determine if it had an effect on the dose-response curve for MC-LR. Thus, dose-response curves for immobilised PP-2A were compared with or without BSA (5 $\mu$l of 10 mg/ml) at pH 7.0. The immobilised enzyme inhibition assays were conducted according to assay conditions described in section 4.3.2 (with the exception of pH change and BSA addition). The dose-response curves were expressed as % activity of the control. If BSA influenced the dose-response curves there would be a shift in the $IC_{50}$ and/or assay detection limit for PP-2A.

### 4.3.4 Recovery of immobilised enzyme activity after treatment with OA and MC-LR

To determine if the immobilised PPINA could be used as a re-usable biosensor, the immobilised assay was tested for re-usability after the toxin was added to the system. That is, if enzyme activity would recover to its original activity following treatment with the inhibiting

toxin. A re-usable assay would allow for toxin analyses of multiple environmental samples in the field. Essentially, enzyme re-usability was tested by filtering the enzyme with Tris buffer (referred to as washing) after toxin was added to the system and retesting the assay to determine if the enzyme activity had returned to its original activity. The number of times the enzyme had to be washed, before activity could be restored to pre-toxin levels, was a factor to be considered if the system had potential application as a re-usable biosensor. Thus, the enzyme recovery experiment tested whether the toxin permanently bound to the enzyme once added to the system, or if it was possible to remove the toxin from the system by washing with Tris buffer.

To determine if washing the enzymes removed the inhibitory effect of OA and MC-LR, each microwell was washed with 100 $\mu$l of 50 nM Tris buffer prior to conducting each assay. The washing process was repeated to show the level of residual toxin in the system after a given number of washes. For the immobilised assay system to be re-usable, washing should allow the enzyme activity to return close to its original activity. The recovery experiment was tested with immobilised PP-2A against OA and MC-LR, and R.PP-1 against MC-LR only. Recombinant PP-1 was not tested against OA because the calibration curves (section 4.3.3.1) revealed that it was unsuitable to detect this toxin.

### 4.3.5 Analysis of environmental samples using the modified PPINA

The robustness of the immobilised PP-2A and R.PP-1 enzyme assays were tested by adding environmental samples into the assay system and determining the accuracy of its measurement, by comparing it with other methods (e.g. LC-MS and other PPINAs). The performance of the PPINA was judged by the sensitivity of the assay to detect low toxin concentrations. Two types of environmental samples were tested, marine mussel extracts containing DSP toxins (OA and DTXs), and freshwater algal extracts containing MCs. Because of cost restrictions, only one immobilised enzyme assay was to be tested for each environmental sample. The immobilised enzyme that produced the most sensitive dose-response curve for OA or MC-LR was chosen to test for the relevant toxin in the sample extract. The dose-response curve for OA in the immobilised PP-2A assay system indicated greater sensitivity to OA than the R.PP-1 enzyme (Figure 4-1). Thus, the immobilised PP-2A system was tested using the mussel extracts. For comparative purposes, the unimmobilised PP-2A assay was also tested with the same mussel extracts. These mussel extracts have been tested previously with the PP-2A assay

and LC-MS analysis (Mountfort and Truman, 2001). The results from the current study were compared to these in terms of accuracy.

Although the immobilised PP-2A system was more sensitive with MC-LR than the immobilised R.PP-1 system, there was not sufficient PP-2A enzyme left to test the algal extracts using the immobilised system. Thus, the immobilised R.PP-1 enzyme was tested for MCs with the algal extracts. For comparative purposes, the same algal extracts were also tested using the unimmobilised R.PP-1 and PP-2A assay (uses much less enzyme than the immobilised system). These algal extracts have been previously tested with the unimmobilised PP-2A assay (Mountfort, unpublished) and LC-MS (Holland, unpublished), and therefore, the results from the current study were compared to these in terms of accuracy.

#### 4.3.5.1 Detection of okadaic acid in mussel extracts

*Undiluted mussel extract tests*

For determination of OA in naturally contaminated mussel extracts, the immobilised PP-2A assay was conducted in duplicate for five mussel samples (see section 4.2.3 for the preparation of mussel extracts) as described in section 4.3.1. The unimmobilised PP-2A assay was conducted in triplicate with the five mussel samples as described in section 4.3.2. The concentration of OA in each mussel extract, was expressed as the % of inhibition of enzyme activity compared to the control (i.e. % activity test = activity of test sample/ the activity of the control sample x 100). See appendix 7.3, for the calculation of OA in mussel samples. The concentration of OA was expressed as μg/kg of shellfish tissue.

*Diluted mussel extract tests*

Recent studies with the PPINA (Mountfort *et al.*, manuscript in preparation-b) have shown that when concentrated mussel extracts were added to the PP-2A assay, the results were unreliable as many interfering substances in the mussel tissue other than the toxin, prevented the binding of the substrate with the enzyme. This problem was overcome by diluting the mussel extracts with 16% methanol. Therefore, the unimmobilised PP-2A assay was tested with a range of diluted mussel extracts in order to identify levels giving inhibition in the linear portion of the curve. It was not possible to test a range of diluted mussel extracts in the immobilised system (although this would have been most useful) due to the small quantity of PP-2A available for

experimentation (the immobilised PP-2A system utilises 25 times more enzyme per test compared to the unimmobilised PP-2A assay). Thus, only undiluted extract was tested with the immobilised system.

Serial dilutions of each mussel extract were made in distilled water in a ratio of 1:4 (v/v extract/water). Five and 10 $\mu$l aliquots of each sample at 1:4 dilution were added to the unimmobilised PP-2A assay in triplicate as described in section 4.3.2. The two volumes (5 and 10 $\mu$l) of each sample were added to the assay to obtain a more thorough estimate, as the mean of these values were used in determining the toxin levels in each sample. Control wells contained the same dilution of methanol in water as in the experimental wells. For example, for 10 µl additions of the 1:4 extract in the experimental wells, the control wells were supplemented with 10 µl of 90% methanol diluted 1:4 with water. The concentration of OA was expressed as µg/kg of shellfish tissue.

The mussel extract assay was then repeated using a 1:2 dilution of extracts, because the 1:4 dilution produced results that were similar to the control values (i.e. very little toxin was detected in the extracts, as the samples may have been too diluted). Five and 10 $\mu$l aliquots of each sample at the 1:2 dilution were added to the unimmobilised PP-2A assay in triplicate as described previously for the 1:4 dilution.

### 4.3.5.2 Detection of microcystins in algal extracts

*Dilution of algal extracts*

Based on the previous results for the immobilised PP-2A mussel sample test (section 4.3.5.1), the sample extract was diluted before being added to the immobilised assay. Therefore, for determination of MCs in naturally contaminated algal extracts, serial dilutions of extracts were made in distilled water in a ratio of 1:4 (v/v extract/water, as described in section 4.2.2). Ten $\mu$l aliquots of the diluted extract were added to the immobilised R.PP-1 assay in duplicate, as described in section 4.3.1. Five and 10 $\mu$l aliquots of the diluted extract were added to the unimmobilised R.PP-1 assay (for reasons discussed in 4.3.5.1) in triplicate, as described in section 4.3.2. Control wells for both assays contained the same dilution of methanol in water as in the experimental wells. The concentration of MC in each algal extract was expressed as µg/L. The calculation of MCs in algal samples is shown in appendix 7.2.4.

*Further dilution of algal extracts*

The 1:4 diluted algal extracts strongly inhibited the unimmobilised enzyme (inhibited the enzyme by more than 90%). Thus, it was necessary to further dilute the 1:4 extracts to obtain sensible accurate readings from the calibration curve. Serial dilutions of the 1:4 diluted extracts were made in distilled water to a ratio of 1:39 (v/v extract/water) (see section 4.2.2 for the preparation of algal extracts). Five and 10 $\mu$l aliquots of the diluted extract were added to the unimmobilised R.PP-1 assay in triplicate, as described in section 4.3.2. The unimmobilised PP-2A assay was also tested with the diluted algal extracts to compare the sensitivities of the enzymes. Five and 10 $\mu$l aliquots of the diluted extract (1:39) were added to the unimmobilised PP-2A assay in triplicate as described in section 4.3.2. Control wells for both assays contained the same dilution of methanol in water as in the experimental wells. The concentration of MC in each algal extract was expressed as µg/L.

## 4.3.6 Analysis of environmental samples using other PP-2A inhibition assays

### 4.3.6.1 Detection of okadaic acid in mussel extracts

The mussel extracts that were tested in section 4.3.5.1, were tested for DSP toxins in 2001, based the PP-2A inhibition assay previously described by Mountfort *et al.* (1999) (section 3.3.1). The concentration of DSP toxins was expressed as µg/kg of OA equivalent in shellfish tissue. The calculation of DSP toxins in mussel samples is shown in appendix 7.3.

### 4.3.6.2 Detection of microcystin in algal extracts

The algal extracts described in section 4.3.5.2, were tested for MC in 2005, based on the PP-2A inhibition assay previously described (Mountfort *et al.,* 1999) (section 3.3.1). The concentration of MC was expressed as µg/L of MC-LR equivalents. The calculation of MC in mussel samples is shown in appendix 7.4.

### 4.3.7 Analysis of environmental samples using LC-MS

#### 4.3.7.1 Detection of okadaic acid in mussel extracts using LC-MS

Unhydrolysed mussel extracts were analysed for DSP toxins by Paul McNabb, Cawthron Institute, by the method of MacKenzie *et al.* (2002) using a Waters 2790 LC system and Quattro Ultima triple quadrupole mass spectrometer system (Micromass Ltd, UK). Chromatographic separation was performed using a Phenomonex Luna C18 column (150 x 2 $mm^2$) with 5 µm, packing. A gradient from 13% to 77% acetonitrile containing a constant concentration of buffer (4 mM ammonium hydroxide and 50 mM formic acid) was run between 2 and 10 min and held at 77% acetonitrile until 25 min. The electrospray ionisation interface (ESI) was operated in both positive and negative modes (capillary potential 3.0–3.5 kV, desolvation temperature 350 °C, source temperature 100 °C, desolvation gas flow 500 l $N_2$/h, cone gas flow 50 l $N_2$/h). The mass spectrometer was operated in MS–MS modes with collision cell gas pressure (argon) set at 1.2 x $10^{-3}$ T. For neutral loss and daughter ion scan experiments on YTX and derivatives, the cone voltage and collision energy were 100 V and 25 eV, respectively. For multiple reaction monitoring (MRM), the mass channels and optimal cone voltages and collision energies for each biotoxin were established from daughter ion studies with standards or extracts of contaminated shellfish. The MRM channels were monitored in windows that covered the elution of the compounds of interest (parent > daughter): ESI negative, capillary 3.0 kV, OA 803.5 > 255.0 and DTX1 817.5 > 255.0. The various toxin groups were quantified by comparison with authentic standards for the major parent compounds (OA and DTX-1).

#### 4.3.7.2 Detection of microcystin-LR in algal extracts using LC-MS

Microcystins were separated by LC (Alliance 2695, Waters Corp., MA) using a 150 x 2 mm Luna C18(2) 5 µm column (Phenomenex, CA) with water/methanol/acetonitrile gradient containing 0.15% formic acid (0.2 ml/min,10 µl injection). The Quattro Ultima TSQ mass spectrometer (Water-Micromass, Manchester) was operated in ESI positive with MRM using MS-MS channels set up for 13 MC and NODLN. The m/z 135 fragment from the protonated molecular cation was selected for each toxin (the doubly charged molecular species for MC-RR: the singly charged molecular species for all other toxin congeners). The instrument was calibrated with authentic standards of MC-RR, -YR, -LR and NODLN to give linear calibration

curves for concentrations over the range 5-200 ng/ml. The response factors for MC-RR and –LR were applied to other related toxins where no pure analytical standards were available.

### 4.3.8 Statistical Analysis

Statistical analysis for all data was performed using 'R' software version 1.7.0. Linear portions of the dose-response curves were analysed using linear regression. Only a limited number of data points were used for each test (some more than others), as the procedure was costly and subsequently, replication was limited. Therefore in some cases, the assumptions are probably not fulfilled (due to the lack of data points, departures from normality and heterogeneity of variances could not be adequately assessed). Where outliers were evident the data could not be eliminated due to lack of data points. However, all the data were balanced.

## *4.4 Results*

### 4.4.1 Dose-response curves

#### 4.4.1.1 Dose-response curves for OA

Figure 4-1 shows the dose-response for OA against unimmobilised and immobilised PP-2A and R.PP-1. The shape of the dose-response curve was similar for the unimmobilised and the immobilised PP-2A enzyme (Figure 4-1a). However there was a shift in the $IC_{50}$ values between the two different systems (Table 4-1), with the $IC_{50}$ for the unimmobilised and immobilised system 5.5 nM and 55 nM, respectively. Thus, the immobilised enzyme was approximately 10-fold less sensitive. The detection range for PP-2A against OA is shown in brackets in Table 4-1. A strong linear relationship was observed for the unimmobilised ($r^2$ 0.8772) and immobilised ($r^2$ 0.9552) PP-2A enzyme. The high $r^2$ value indicates that most of the data points were clustered close to the regression line ($r^2$ equals 1 for a perfect fit, while $r^2$ close to 0 indicates scattered data and a weak relationship). This implies that the assays were performed with some confidence.

A different trend was observed with the R.PP-1 enzyme, as activity was induced for both the immobilised ($r^2$ 0.8629) and unimmobilised ($r^2$ 0.7703) system between 10-200 nM and 1-100 nM of OA, respectively (Figure 4-1b). Consequently, very high $IC_{50}$ values were obtained

for both assay systems (Table 4-1). This indicates that the assay system used for R.PP-1 was unsuitable to test for OA.

### 4.4.1.2 Dose-response curves for MC-LR

*Effect of assay pH*

Figure 4-2 shows the dose-response curve for the inhibition of unimmobilised PP-2A against MC-LR at pH 7 ($r^2$ 0.9607) and pH 8 ($r^2$ 0.9807). The pH did not shift the $IC_{50}$ or change the shape of the dose-response curve for unimmobilised PP-2A. Freshwater has a pH close to 7. Thus, all tests analysing freshwater algae for MCs were assayed at pH 7.

*Dose-response Curves MC-LR*

Figure 4-3 shows the dose-response for MC-LR against PP-2A and R.PP-1 in both assay systems. The shapes of the dose-response curves were similar for the unimmobilised and immobilised PP-2A enzymes (Figure 4-3a). However, there was a shift in the $IC_{50}$ values between the two different systems (Table 4-1), with the immobilised enzyme at least 20-fold less sensitive. The detection range for both enzymes against MC-LR is shown in Table 4-1. The $IC_{50}$ for the unimmobilised PP-2A was similar to values obtained for MC-LR against PP-2A from other studies ($IC_{50}$ 2.2 nM (Mountfort *et al.*, 2005)). A linear trend was observed for the unimmobilised ($r^2$ 0.8344) and immobilised ($r^2$ 0.7206) PP-2A enzyme.

The shape of the dose-response curve and the $IC_{50}$ for the unimmobilised R.PP-1 enzyme (Figure 4-3b) compared well with PP-2A, and the enzyme showed a higher sensitivity toward MC-LR than PP-2A (Table 4-1). A different trend was observed for the immobilised enzyme, as the curve could not be used to detect MC-LR below 40% activity of the control. Consequently, a very high $IC_{50}$ value was obtained for the immobilised R.PP-1 enzyme against MC-LR (Table 4-1), which was approximately 200-fold less sensitive when compared to the unimmobilised R.PP-1 enzyme. A strong linear relationship was observed for the unimmobilised ($r^2$ 0.9845) and immobilised ($r^2$ 0.8961) R.PP-1 enzymes.

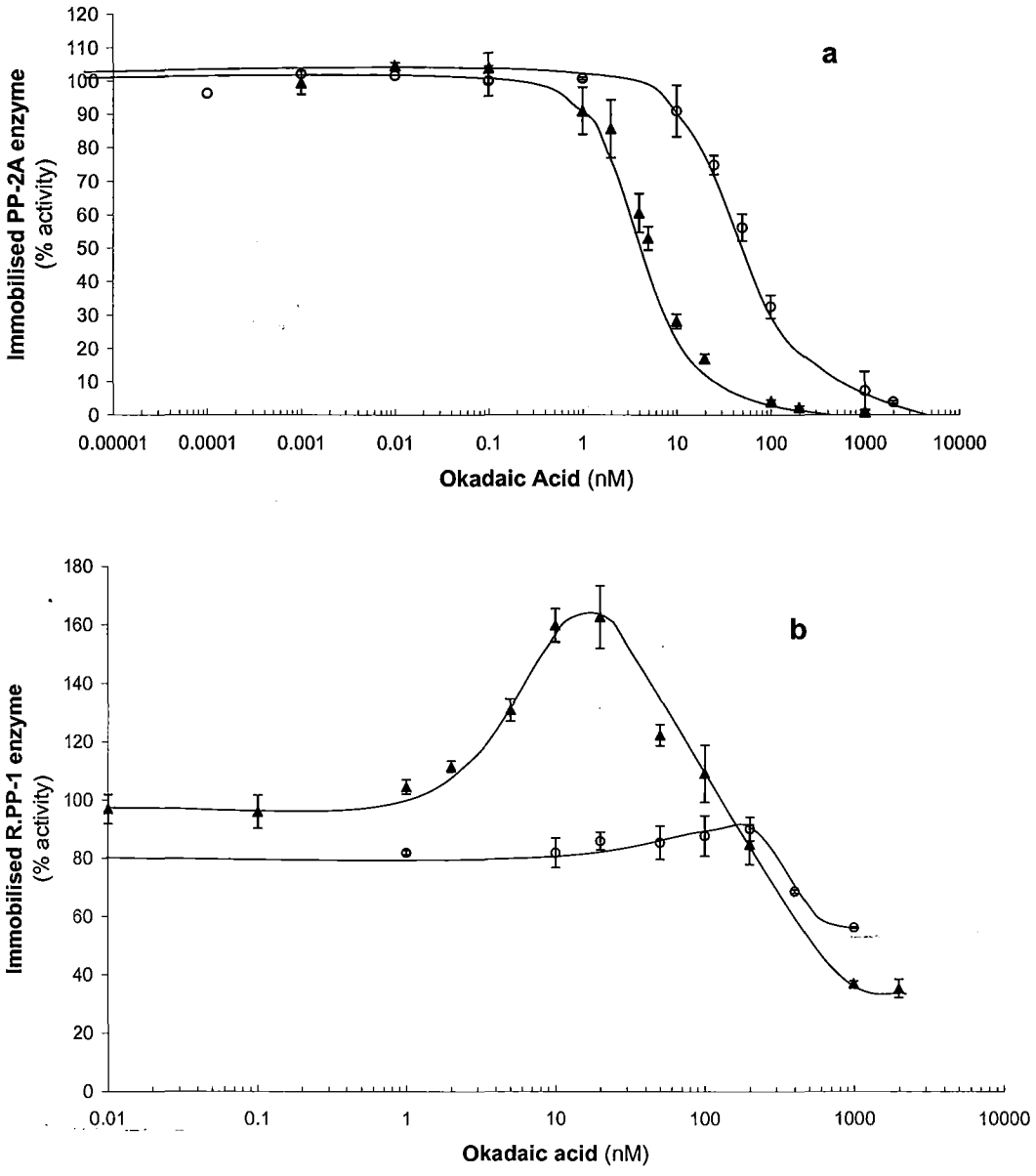



**Figure 4-1.** Dose-response curves for the inhibition/stimulation of PP-2A (a) and R.PP-1 (b) by OA. Symbols denote enzyme treatment: ▲, unimmobilised; ○, immobilised. In the immobilised system, each well contained enzyme immobilised onto 50 mg of beads at 0.5 and 12.5 Units for PP-2A and R.PP-1, respectively. In the unimmobilised system each well contained 0.02 and 0.5 Units of PP-2A and R.PP-1, respectively. Enzyme activity was determined at pH 8.0. Assay conditions were the same for both systems except that BSA was present in the unimmobilised system at 5 µl of 10 mg/ml and 5 mg/ml for PP-2A and R.PP-1, respectively. Each value represents the mean of at least duplicate determinations ± standard deviation.

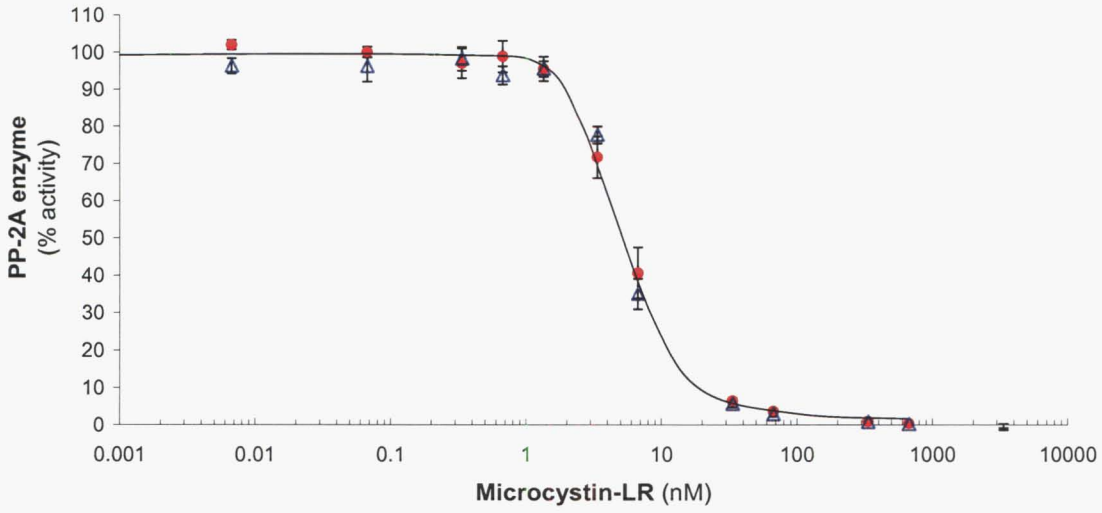



**Figure 4-2.** Dose-response curve for the inhibition of unimmobilised PP-2A by MC-LR at pH 7 (●) and pH 8 (Δ). The concentration of PP-2A was 0.02 U. Enzyme activity was determined with BSA (5.02 $\mu$M). Each value represents the mean of triplicate determinations ± standard deviation.

### 4.4.1.3 Dose-response curves for MC-LR utilising different assay conditions

Figure 4-4 shows the dose-response curve for MC-LR against immobilised PP-2A enzyme with ($r^2$ 0.9612), and without ($r^2$ 0.6853) BSA. The results indicated that the addition of BSA in the reaction mix did not shift the $IC_{50}$ or change the shape of the dose-response curve to any great extent. Although it would have been useful to test the effect of BSA on the immobilised system for the R.PP-1 enzyme, there was only sufficient enzyme to test this in the immobilised system for PP-2A. Thus, it was assumed that when comparing the unimmobilised and immobilised systems, the presence or absence of BSA in the reaction mix would not have much influence on the outcome of the dose-response curve for either toxin.

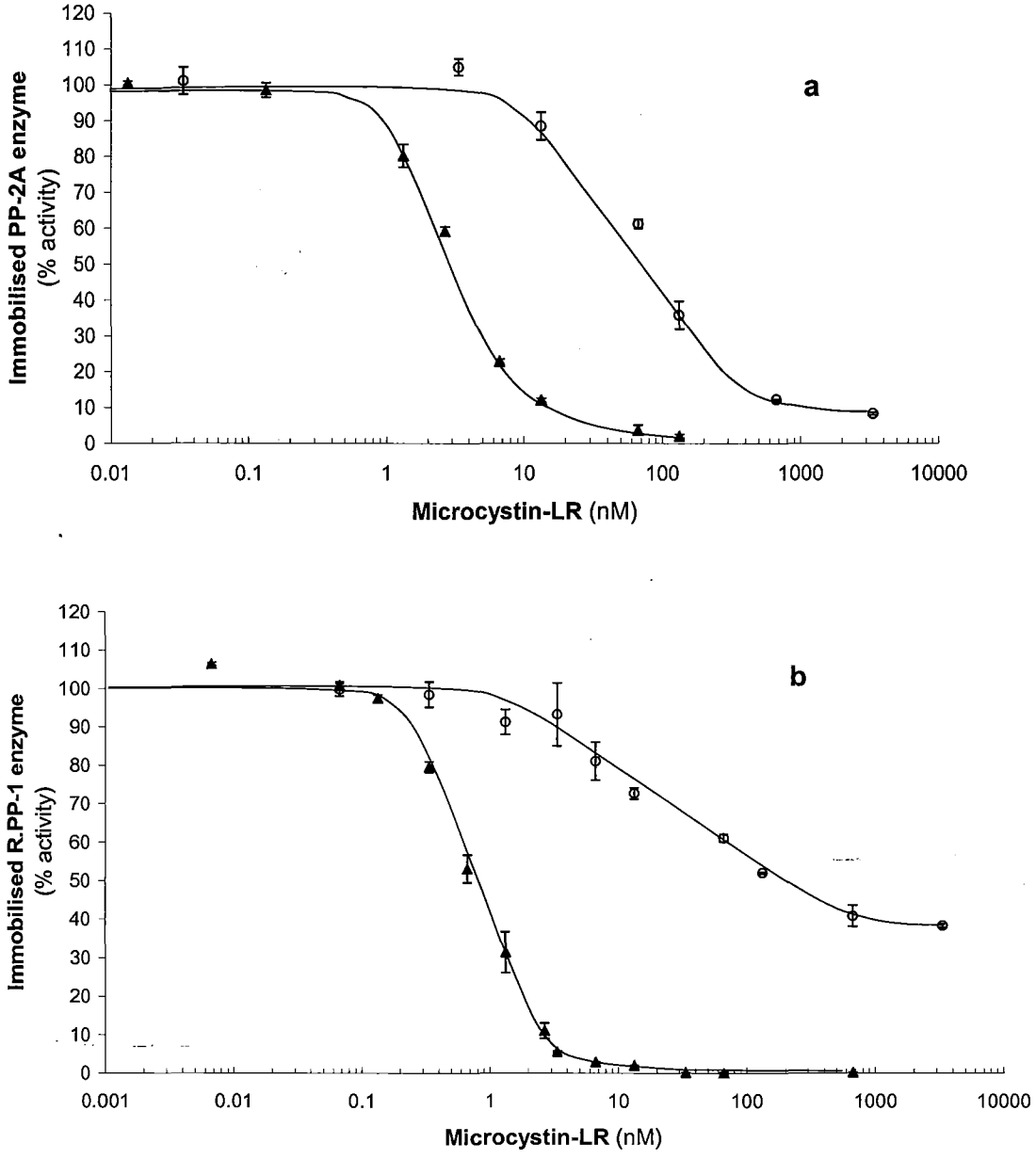



**Figure 4-3:** Dose-response curves for inhibition of MC-LR by PP-2A (a) and R.PP-1 (b). Symbols denote enzyme treatment: ▲, unimmobilised; ○, immobilised. In the immobilised system each well contained enzyme immobilised onto 50 mg of beads at 0.5 and 12.5 Units for PP-2A and R.PP-1, respectively. In the unimmobilised system, each well contained 0.02 and 0.5 Units of PP-2A and R.PP-1, respectively. Enzyme activity was determined at pH 7.0. Assay conditions were the same for both systems except that BSA was present in the unimmobilised system at 5 µl of 10 mg/ml and 5 mg/ml for PP-2A and R.PP-1, respectively. Each value represents the mean of at least duplicate determinations ± standard deviation.

**Table 4-1: Comparison of $IC_{50}$ (nM) values and toxin detection range for MC-LR and OA inhibition of, PP-2A and R.PP-1.**

| Enzyme | | $IC_{50}$(nM) (detection range)[c] | |
|---|---|---|---|
| | | *Microcystin-LR* | *Okadaic acid* |
| **PP-2A** | unimmobilised | 3.2 (1-10) | 5.5 (1.2-15) |
| | immobilised | 80 (10-300) | 55 (15-150) |
| | unimmobilised | 2.2[a] (0.2-10) | 1.5[b] (0.1-10) |
| **R.PP-1** | unimmobilised | 0.9 (0.2-2.5) | 600 (na) |
| | immobilised | 150 (2-300) | >2000 (na) |

[a] Value obtained by Mountfort et al. (2005)

[b] Value obtained by Mountfort et al. (1999)

[c] the detection ranges are an estimation only

na not applicable

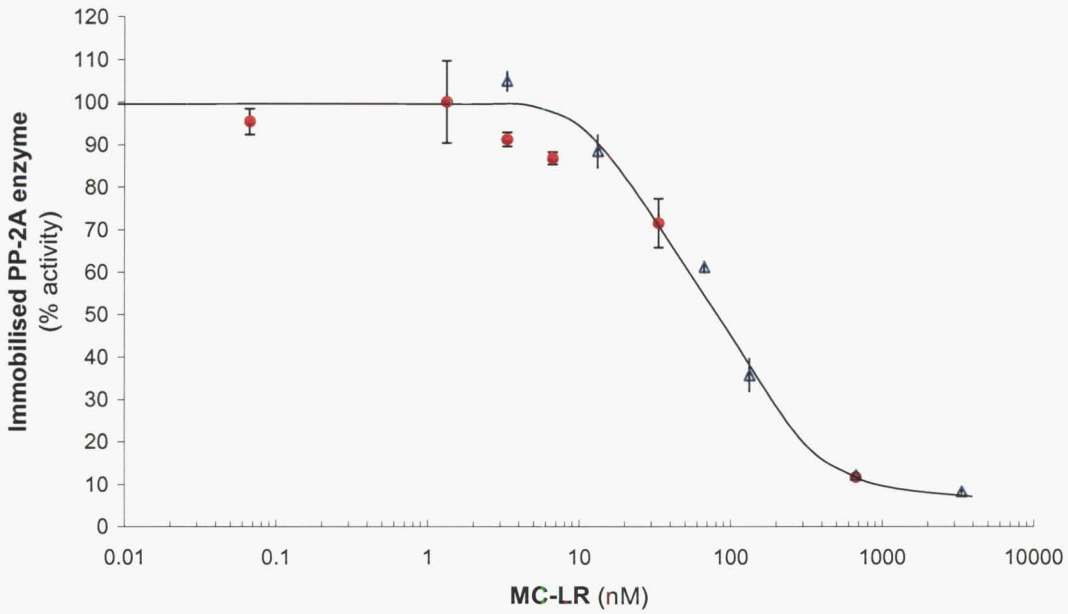



**Figure 4-4:** Dose-response curves for immobilised PP-2A with BSA at 5.02 $\mu$M (●) and without BSA (Δ) at pH 7.0. The concentration of PP-2A was 0.5 U. Each value represents the mean of at least duplicate determinations ± standard deviation.

### 4.4.2 Recovery of immobilised enzyme activity after testing with toxins

#### 4.4.2.1 Recovery of immobilised PP-2A activity after testing with OA and MC-LR

Figure 4-5 shows the recovery of immobilised PP-2A activity after testing with OA and MC-LR. Although enzyme activity increased as the number of washes increased, PP-2A activity did not reach the pre-toxin activity levels, even after the enzyme-bead complex had been washed 100 times. This indicated that the immobilised PP-2A system was not re-usable and that some form of permanent binding had occurred between the toxin and the enzyme.

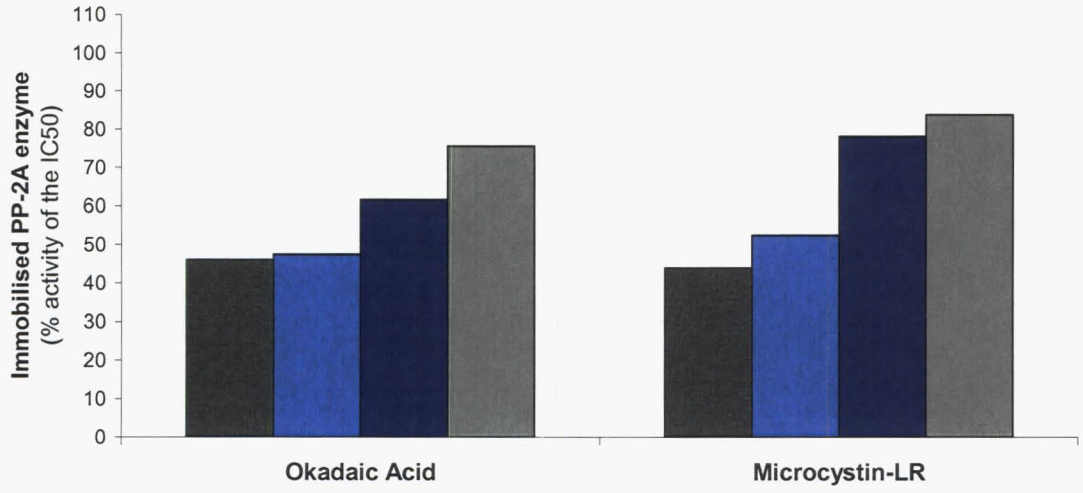



**Figure 4-5:** Recovery of immobilised PP-2A activity after treatment with toxins. Each colour represents the percent activity of the $IC_{50}$ after: toxin addition (■), and after 10 (■), 50 (■) and 100 (■) washes. Each value represents the mean of duplicate determinations ± standard deviation.

#### 4.4.2.2 Recovery of immobilised R.PP-1 activity after testing with MC-LR

Figure 4-6 shows the recovery of R.PP-1 activity after testing with MC-LR. Recombinant PP-1 was not tested for recovery against OA due to reasons discussed previously (section 4.3.4). Results for immobilised R.PP-1 showed good recovery of enzyme activity after washing. Enzyme activity recovered completely to its original activity within 20 washes. This recovery

was based on the $IC_{50}$ of 200 nM (Table 4-1). Therefore there is potential to use the immobilised R.PP-1 enzyme in a reusable biosensor method.

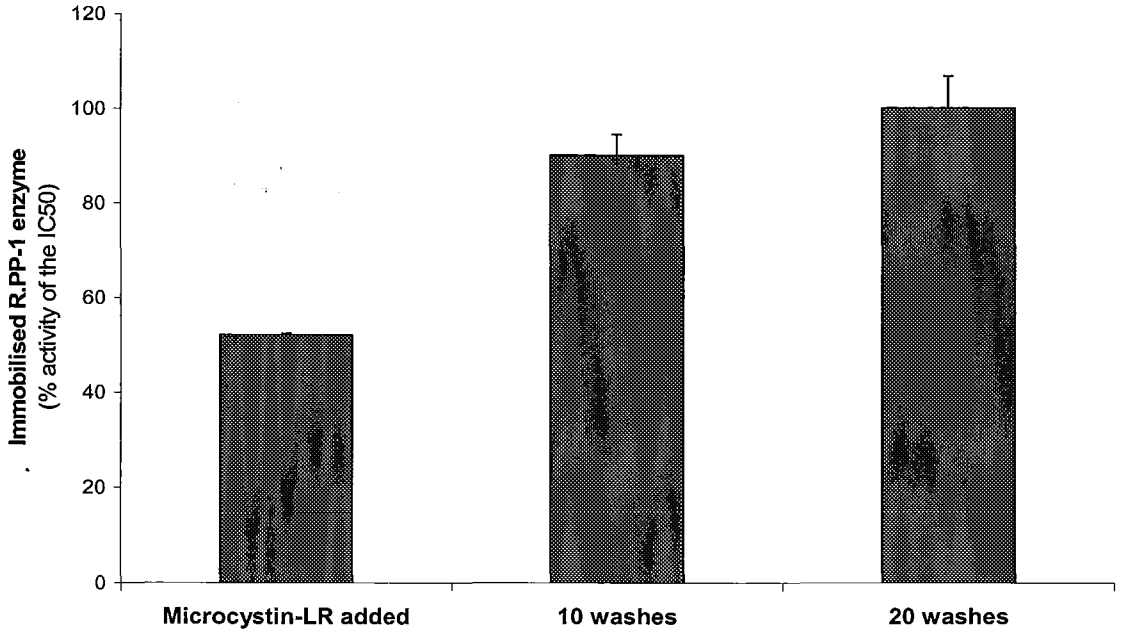



**Figure 4-6:** Recovery of immobilised R.PP-1 activity after washing following treatment with MC-LR. Each value represents the percent activity of the $IC_{50}$. Each value represents the mean of duplicate determinations ± standard deviation.

## 4.4.3 Determination of toxins in naturally contaminated samples

### 4.4.3.1 Determination of DSP toxins in naturally contaminated mussel extracts

Table 4-2 shows the concentration of DSP toxins (OA and DTXs) (nM) detected by the two assay systems (i.e. immobilised versus unimmobilised). The linear portion of the dose-response curve for unimmobilised and immobilised PP-2A against OA was used for calculation of these samples [i.e. the % activity of the control is read off the linear portion of the dose-response curve to obtain the concentration of toxin (nM) in the sample (appendix 7.3)].

**Table 4-2: The concentration of OA equivalents (nM) in the mussel samples using the immobilised and unimmobilised PP-2A enzyme assays** (measured off the immobilised and unimmobilised standard curves for PP-2A against OA).

| Mussel Sample | µl[a] | PP-2A | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Immobilised | | Unimmobilised | | | | | |
| | | *Undiluted* | | *Undiluted* | | *Diluted 1:2* | | *Diluted 1:4* | |
| | | % activity[b] | nM[d] | % activity[c] | nM[d] | % activity[c] | nM[d] | % activity[c] | nM[d] |
| **BM** | 5 | * | * | * | * | 121.03±7.67 | nd | 123.65±10.83 | nd |
| | 10 | 47.32±0.34 | 59 | 37.37±10.07 | 7 | 82.72±5.85 | 1.9 | 96.246.44 | 0.3 |
| **Y2** | 5 | * | * | * | * | 115.26±6.40 | nd | 112.99±4.25 | nd |
| | 10 | 60.33±0.74 | 40 | 38.02±6.00 | 6.8 | 93.09±4.21 | 0.85 | 96.28±6.81 | 0.3 |
| **X2.** | 5 | * | * | * | * | 103.56±6.52 | nd | 112.23±9.52 | nd |
| | 10 | 41.67±3.40 | 68 | 22.33±4.15 | 11 | 70.97±0.83 | 2.9 | 88.74±7.43 | 1.3 |
| **D38** | 5 | * | * | * | * | 105.78±6.13 | nd | 111.52±5.82 | nd |
| | 10 | 52.32±1.05 | 53 | 31.87±4.48 | 8.5 | 75.17±2.72 | 2.5 | 95.37±5.38 | 0.6 |
| **C7** | 5 | nd | nd | nd | nd | 87.24±4.86 | 1.5 | 103.64±6.75 | 0 |
| | 10 | 34.51±3.26 | 93 | 11.13±0.82 | 23 | 47.36±3.38 | 6.8 | 79.68±2.97 | 2.1 |

[a] volume of extract added to assay (µl)

[b] mean % activity of the control of duplicate determinations ± standard deviation

[c] mean % activity of the control of triplicate determinations ± standard deviation

[d] nM of OA equivalent, read off the dose-response curve

* no data

nd not detected

Table 4-3 shows the concentration of DSP toxins (OA and DTXs) in µg/kg of mussel tissue (assuming that 5 ml of extract is equivalent to 1 g shellfish tissue), detected in the unimmobilised and immobilised PP-2A assay systems. The results were compared to values obtained from LC-MS and other PP-2A inhibition assay methods (Mountfort and Truman, 2001). The results indicate that there was considerable variability in toxin detection based on the dilution of the sample for the unimmobilised PP-2A assay. The most diluted mussel extract gave the lowest measurement of DSP toxins, while the undiluted extracts gave the highest levels of toxin. This is consistent with other findings that non-specific binding can occur with undiluted

samples resulting in the enzyme being stimulated by components in the sample other than the substrate (Mountfort *et al.*, manuscript in preparation-b). Thus, it is shown in the unimmobilised assays that the matrix effect is gradually reduced by using highly diluted samples.

**Table 4-3: Comparison of DSP toxin (OA and DTXs) concentrations in mussels between the unimmobilised and immobilised PP-2A inhibition assay and LC-MS.**

| Mussel sample | OA (µg/kg mussel tissue) | | | | | |
|---|---|---|---|---|---|---|
| | Immobilised PP-2A[a] | Unimmobilised PP-2A[b] | | | PP-2A[cf] | LC-MS[df] |
| | *undiluted* | *undiluted* | *1:2*[e] | *1:4*[e] | *1:4* | |
| **BM** | 3562.1 | 422.6 | 344.1 | 90.6 | 0 | 0 |
| **Y2** | 2415.0 | 410.6 | 154.0 | 90.6 | 136.0±11 | 93.0 |
| **X2** | 4105.5 | 664.1 | 525.3 | 392.4 | 100.0±28 | 47.0 |
| **D38** | 3199.9 | 513.2 | 452.8 | 181.1 | 168.0±11 | 95.0 |
| **C7** | 5614.9 | 138.8.6 | 887.5 | 633.9 | 34.0±28 | 239.0 |

[a] 0.5 U of PP-2A immobilised to 50 mg of microbeads. Values are means of duplicate determinations.

[b] 0.02 U of PP-2A. Values are means of triplicate determinations.

[c] Values obtained by Mountfort *et al.* (2001) (Cawthron) ± SD.

[d] Values obtained by McNabb (Cawthron) (Mountfort *et al.*, 2001)

[e] Activity values for 5 µl additions of mussel extract were too near 100% to provide a valid reading from the dose-response curve. Thus, only the 10 µl additions were used here.

[f] detection level approximately 0.01 nM

The magnitude of the matrix effect can be seen in the substantial over-estimate of DSP toxins reported in the immobilised assay, which only tested undiluted samples. However, the magnitude of the difference is less than expected (10-fold) considering there was a 10-fold decrease in sensitivity between the immobilised and unimmobilised PP-2A system (as shown in the dose-response curve for DSP toxins and PP-2A, section 4.4.1.1). There were also some differences between the unimmobilised PP-2A assay carried out in this study compared to those values obtained from the PP-2A inhibition assay carried out by Mountfort and Truman (2001) for

the same assay dilution. This may have been the result of slight differences in the assay methods. The one concerning factor is the high amount of DSP toxins detected in the blank sample (BM), compared to the nil levels reported in by Mountfort and Truman (2001) and LC-MS. This difference may be attributable to an error during the dilution series where the control may not have received 16% methanol, as this discrepancy has been shown in other studies (Mountfort. pers.com).

While the unimmobilised PP-2A showed some effect of dilution, generally the values aligned to LC-MS particularly the 1:4 dilution. The results for LC-MS are not expected to be equal to the values obtained from the PPINA, as stoichiometric methods measures specific toxin congeners while the PPINA measures the total bioactivity of the sample.

### 4.4.3.2 Determination of MCs in naturally contaminated algal extracts

Table 4-4 shows the concentration of MCs (nM) detected by the two assay systems (i.e. immobilised versus unimmobilised). The linear portion of the dose-response curves for the unimmobilised and immobilised enzymes against MC-LR were used for calculation of these samples (as discussed in section 4.4.3.1).

Table 4-5 shows the concentration of MCs in μg/L detected in the unimmobilised and immobilised R.PP-1 assay system and the unimmobilised PP-2A assay. The results were compared to values obtained from the LC-MS (Holland, unpublished) and other PP2A inhibition assay methods (Mountfort, unpublished). The results indicate that there was variability in toxin detection based on the dilution of the sample for the unimmobilised R.PP-1 assay. The most diluted algal extracts usually gave the highest measurement of MCs, which is contrary to the trend observed for the mussel samples for PP-2A. This indicates that inhibitory confounding compounds may have been present in the algal cells, compared to stimulatory confounding compounds which were likely to have been present in the mussel extracts. These compounds can interfere with the enzyme-toxin binding and confound the results of the assay. Thus, sample dilutions in the unimmobilised assays were shown to gradually reduce the matrix effect. The optimal sample dilution was between 1:20 and 1:40. However, two of the seven samples tested in the 1:40 dilution (V.436 and V.432) detected a lower concentration of MCs than those detected in the 1:4 dilutions. Although these differences were marginal, it does suggest that the samples can be over diluted before incorporation in the assay.

**Table 4-4: The concentration of MC equivalents (nM) in the algal samples using the R.PP-1 and PP-2A enzyme assays** (measured off the immobilised and unimmobilised standard curves for R.PP-1, and unimmobilised standard curve for PP-2A, against MC-LR).

| Algal Sample | µl[a] | R.PP-1 | | | | | | PP-2A | |
|---|---|---|---|---|---|---|---|---|---|
| | | Immobilised | | Unimmobilised | | | | Unimmobilised | |
| | | *Diluted 1:4* | | *Diluted 1:4* | | *1:4 diluted 1:10* | | *1:4 diluted 1:10* | |
| | | % activity[b] | nM[d] | % activity[c] | nM[d] | % activity[c] | nM[d] | % activity[c] | nM[d] |
| **L.F1** | 5 | * | * | 2.45±1.12 | 5 | 2.4±0.24 | 6.2 | 18.57±3.40 | 8.5 |
| | 10 | 36.32±1.39 | 1.25 | 1.12±0.21 | 25 | 1.38±0.87 | 12 | 10.69±1.88 | 10.6 |
| **V.436** | 5 | * | * | 10.71±0.65 | 2.6 | 96.87±7.22 | 0.2 | 78.77±4.98 | 1.4 |
| | 10 | 74.96±4.70 | 0.44 | 6.66±0.36 | 3.2 | 86.4±5.13 | 0.32 | 85.35±7.61 | 1.2 |
| **V.432** | 5 | * | * | 7.35±1.12 | 3.1 | 96.68±4.94 | 0.2 | 80.33±4.40 | 1.4 |
| | 10 | 95.27±8.61 | 0.22 | 4.96±0.50 | 3.8 | 77.75±6.25 | 0.41 | 82.25±5.48 | 1.35 |
| **E.329** | 5 | * | * | 5.63±0.52 | 3.5 | 71.63±1.35 | 0.49 | 74.58±5.82 | 1.65 |
| | 10 | 61.73±1.17 | 0.64 | 3.84±0.72 | 4.9 | 32.66±3.46 | 1.3 | 73.15±1.51 | 1.75 |
| **V.431** | 5 | * | * | 5.48±0.57 | 3.6 | 39.41±2.24 | 1.1 | 71.22±5.70 | 1.9 |
| | 10 | 57.59±1.25 | 0.69 | 3.71±0.63 | 4.6 | 10.71±1.00 | 2.6 | 79.1±7.18 | 1.5 |
| **V.433** | 5 | * | * | 9.35±0.30 | 2.4 | 86.27±2.88 | 0.32 | 83.4±15.48 | 1.25 |
| | 10 | 83.76±3.96 | 0.34 | 7.92±0.38 | 3 | 83.06±3.70 | 0.35 | 94.52±6.93 | 0.86 |

[a] amount of extract added to assay (µl)

[b] mean % activity of the control of duplicate determinations ± standard deviation

[c] mean % activity of the control of triplicate determinations ± standard deviation

[d] nM of MC-LR equivalent, read off the dose-response curve

* no data

There was also an approximate 10-fold decrease in sensitivity of MC detected between the immobilised and unimmobilised R.PP-1 for the same sample dilution. This is surprisingly less than the 200-fold decrease in sensitivity between the immobilised and unimmobilised R.PP-1 system, as shown in the dose-response curve for MC and R.PP-1 (section 4.4.1.2). There is some degree of similarity between the R.PP-1 and PP-2A enzyme assays for the same sample dilution. Although in all samples, values are higher for the PP-2A assay. There is good

agreement between the PP-2A assay and PP-2A assay carried out by Mountfort (unpublished) for some of the samples tested (L.F1, E.329 and V.433). There is some agreement between the LC-MS and PP-2A assay for the 1:40 dilution. There is also similarity between the unimmobilised R.PP-1 assay and LC-MS, for one of the samples tested (V.431).

**Table 4-5: Comparison of MC toxin concentrations in algal extracts among the unimmobilised and immobilised R.PP-1 inhibition assay and LC-MS.**

| Algal sample | MC-LR equivalents (µg/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Immobilised | Unimmobilised | | | PP-2A[df] | | LC-MS[ef] |
| | R.PP-1[a] | R.PP-1[b] | R.PP-1[b] | PP-2A[c] | | | |
| | *1:4* | *1:4* | *1:40* | *1:40*[e] | *1:4* | *1:20* | |
| L. F1 | 93 | 1306 | 9104 | 10298 | 3385 | 13109 | 31050[g] |
| V. 436 | 33 | 313 | 269 | 1493 | 290 | 538 | 768 |
| V. 432 | 16 | 373 | 302 | 1548 | 318 | 620 | 2845 |
| E.329 | 48 | 444 | 851 | 1884 | 698 | 1996 | 80 |
| V.431 | 51 | 440 | 1791 | 1978 | 396 | * | 1629 |
| V.433 | 25 | 291 | 369 | 1254 | 10918 | 1354 | 2258 |

[a] 12.5 U of R.PP-1 immobilised to 50 mg of microbeads

[b] 0.05 U of R.PP-1. Values are means of triplicate determinations.

[c] 0.02 U of PP-2A. Values are means of triplicate determinations

[d] Values obtained by other PP-2A methods by Mountfort *et al.* (2001) (Cawthron) ± SD.

[e] Values obtained by LC-MS by Holland (Cawthron)

[f] detection level 0.01 nM (check this figure)

[g] mostly nodularin detected

* no data

## 4.5 Discussion

The objectives of the feasibility studies were to determine if the PP-2A and R.PP-1 enzymes could detect OA and MC in the immobilised, microfilter plate format and to assess whether the method was feasible for development into a prototype biosensor.

This study has showed that the PP-2A and R.PP-1 enzyme can detect both OA and MC in the immobilised format. However, the immobilised system showed a much lower sensitivity towards both toxins in comparison to the unimmobilised system, for both enzymes. Protein phosphatase type 2A against OA showed the smallest difference in sensitivity between the unimmobilised and immobilised format (10 fold) while the R.PP-1 enzyme against MC-LR showed the largest difference (>200 fold). The detection limits for the PP-2A and R.PP-1 enzymes in the unimmobilised format against MC and OA (excluding R.PP-1 with OA) were less than or close to 1 nM for both enzymes. In comparison, the detection limit for both enzymes in the immobilised system was less than or close to 10 nM for both toxins (excluding R.PP-1 with OA). This indicates that the detection limits for the immobilised systems were approximately 10 fold less sensitive, than the unimmobilised format. Recombinant PP-1 activity was stimulated at certain concentrations of the OA, rather than inhibited. This trend was observed in both the immobilised and unimmobilised system. This, together with the low sensitivities of the enzyme towards OA, indicates that the use of R.PP-1 should not be considered for detecting DSP. The dose-response test for unimmobilised PP-2A also revealed that assay pH does not change the dose-response curve or shift the $IC_{50}$ when tested with a MC-LR standard.

Enzyme recovery tests with pure toxin standard revealed that permanent binding between OA and MC-LR probably occurred with PP-2A, but not between the R.PP-1 enzyme with MC-LR. Recovery tests were not carried out for R.PP-1 and OA as this was an unsuitable enzyme for this toxin. Protein phosphatase type 2A activity cannot be recovered once OA and MC-LR have been added to the system, as enzyme activity remained below 80% of the control after 100 washes. Consequently, this precludes the use of non-replaceable PP-2A in a biosensor format. In contrast, R.PP-1 activity towards MC-LR showed that enzyme activity recovered to 100% after 20 washes (enzyme activity recovered to 90% of the control within 10 washes). This indicates that permanent binding between R.PP-1 and MC-LR did not occur. Thus, the R.PP-1 enzyme can be incorporated in a re-usable detection system for MC-LR, as 20 washes is not unreasonable for a biosensor design.

Toxin recovery tests for the environmental samples naturally contaminated with MCs and DSP toxins (OA and DTXs), revealed some problems with the immobilised, microfilter plate design. There was a significant over-estimate of the DSP toxins (OA and DTXs) when the

immobilised PP-2A assay was used to detect the toxins. Similarly, the results obtained using the unimmobilised PP-2A assay indicate that when the samples were either undiluted or diluted 1:2 there was considerable over-estimate of DSP toxins (OA and DTXs). However, when the sample extracts were diluted 1:4, as recommended in the published method (Mountfort *et al.,* 1999), the overestimate of toxin was largely reduced. The over-estimate suggests that stimulatory substances were present in the extract that confounded the assay. Previous studies by Mountfort *et al.* (manuscript in preparation-b) have shown that these confounding effects can been reduced by modifying the assay, through the addition of elevated levels of BSA (350 μg/ assay) and sodium cholate (10 μg/ assay) to the reaction mix. Thus, the high toxin levels reported in the immobilised assay may be explained by the low levels of BSA present, to prevent the matrix effect. Apart from dilution of the extract, modifying the assay should remove the matrix effect caused by these substances. Additionally, the assay system developed in this study was optimised for maximum enzyme activity, but it was not optimised for testing environmental samples. Thus, the immobilised enzyme assay was applied to the detection of DSP toxins without having been optimised for detection in extracts. Optimal conditions for testing environmental samples are currently being developed for the immobilised PP-2A assay (Goussain and Mountfort, unpublished).

The results from the toxin recovery test with the R.PP-1 enzyme detecting natural levels of MCs in homogenated algal cells, also highlighted the need for sufficient sample dilution, as there was variability in toxin detection based on the dilution of the sample. Samples diluted 1:4 tended to under estimate MCs, while samples diluted 1:20 and 1:40, produced MC levels that were more consistent with LC-MS. Thus, inhibitory substances were present in the less diluted samples that confounded the results of the assay. There was agreement between the unimmobilised PP-2A results of this study for most of the 1:40 diluted samples tested, to that of the method carried out by Mountfort and Truman (2001) for the 1:20 diluted samples. There was also some agreement between results of the unimmobilised R.PP-1 and PP-2A for the detection of MCs, although unimmobilised PP-2A consistently detected higher MC levels for the same sample dilution. The immobilised R.PP-1 system was less sensitive (approximately 10 fold) than the unimmobilised R.PP-1 system for the same sample dilution. This was expected as the immobilised system is less sensitive than the unimmobilised system for R.PP-1, as discussed

previously in section 4.5. The lower detection levels were most likely due to the low dilution of the sample and the lack of BSA in the system.

There was dissimilarity between results obtained for the PPINAs and LC-MS for both the mussel and algal extracts. For example, one algal sample analysed (L.F1) by LC-MS detected a very high concentration of NODLN, but detection with the PPINA was much less. It is possible that the LC-MS measurement was an over-estimate. Conversely, LC-MS and PPINA values may not be similar, as the latter method is measuring toxicity and does not detect each congener.

The results of the current study have shown some consistent trends with other studies. For example, the sensitivities of the unimmobilised enzymes were consistent with reports in the literature. The sensitivity of unimmobilised R.PP-1 toward MC-LR were consistent with published reports for the same enzyme source ($IC_{50}$ range: 0.3-0.9 nM depending on the substrate) (Zhang *et al.*, 1992; An and Carmichael, 1994; Heresztyn and Nicholson, 2001; Rapala *et al.*, 2002). Similarly, the sensitivity of unimmobilised PP-2A toward MC-LR was consistent with that reported in the literature from the same enzyme source ($IC_{50}$ 2.2 nM, Mountfort *et al.,* 2005). The sensitivity of R.PP-1 toward OA ($IC_{50}$ 600 nM) was approximately 10-fold less than that reported in the literature ($IC_{50}$ 60 nM). However, this was the result of one study only (Zhang *et al.,* 1992). Similarly, the sensitivity of PP-2A toward OA ($IC_{50}$ 5.5 nM) was slightly lower than those reported in the literature ($IC_{50}$ range: 0.1-1.5 nM) (Tubaro *et al.*, 1996; Vieytes *et al.*, 1997; Leira *et al.*, 2000; Mountfort *et al.*, 2001). The sensitivity of PP-2A towards MC-LR and OA varies considerably ($IC_{50}$ range: 0.006-1 and 0.2-2, respectively) (Cohen *et al.*, 1989; Holmes *et al.*, 1990; Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990; Takai and Mieskes, 1991; Simon and Vernoux, 1994; Toivola *et al.*, 1994; Ash *et al.*, 1995; Honkanen *et al.*, 1996a; Vieytes *et al.*, 1997), depending on the source of the enzyme. Thus only those sourced from human red blood cells were included in this discussion.

The dose-response curves also consistently produced a sigmoidal shape, similar to calibration curves obtained in other studies (Tubaro *et al.*, 1996; Vieytes *et al.*, 1997; Leira *et al.*, 2000; Mountfort *et al.*, 2001). The trends between experiments were also similar with the immobilised format consistently producing a curve with a shift to the left of the unimmobilised format. Thus, with dose-response curves, there can be some degree of certainty in the reproducibility of results. Hence, this should not affect the interpretation of the data. In contrast,

the results obtained from the environmental samples require further clarification. A single assay run with either PP-2A or R.PP-1 in this type of assay system is liable to yield results which could be questioned. Thus, the assay tests for algal and shellfish extracts should be repeated to verify the results obtained in this study.

A further finding in this study was the large variability in toxin detection based on the dilution of the sample. The study revealed that an increase in sample dilution in both the mussel and algal tests reduced the matrix effects and made the values approach those determined by LC-MS. This highlights the importance of determining an adequate sample dilution for toxin detection. Generally, a 1:4 sample dilution is appropriate for mussel extracts, while the algal extracts required a higher dilution of between 1:20 and 1:40.

Storage of the sample may have also affected the comparison of the results of this study, with other reports (Mountfort *et al.*, 2005; Mountfort *et al.*, manuscript in preparation-b). Okadaic acid is very stable when stored under suitable conditions and thus, should not degrade significantly over time. However, MCs are less stable and therefore, can degrade on storage. Thus, this could be one of the reasons for the lower MC values reported in the algal extract by Mountfort *et al.* (2005), as it was carried out several months after this study.

# Chapter 5 Discussion and Future Research

Biosensors have been developed for a wide range of study areas including the medical field, food analysis, defence/bioterrorism, and environmental applications (Kroger and Law, 2005). Many of these sensor formats have been commercialised and are applied in routine analysis, however, only a few have been targeted at the aquatic environment (Kroger *et al.*, 2002). Biosensors for algal toxins have received even less attention, and there appears to be only a small number of developments reported in the literature (Carter *et al.*, 1993; Cheun *et al.*, 1996; Cheun, 1998; Marquette *et al.*, 1999; Croci, 2001; Kreuzer, 2002; Tang *et al.*, 2002). Of these, most are concerned with the detection of OA. The monitoring of toxic algae is important to safe guard human health, prevent negative impacts on livestock and wildlife, and prevent the degradation of the aquatic environment. Currently, monitoring for algal toxins in New Zealand is limited to the laboratory, and is analysed using the mouse bioassay and LC-MS. There are several problems with the mouse bioassay, mostly concerned with its low efficiency, sensitivity and reliability. Liquid chromatography with mass spectrometry also has disadvantages, as it can only be carried out by specialised personnel and does not detect the total toxin bioactivity. The high cost of these procedures is also a problem. With the increasing occurrence and spread of toxic algae locally and globally, and the rigorous monitoring regimes that are required by law, there is now a demand to develop biosensors for rapid, on-site detection of algal and cyanobacterial toxins. Biosensors reduce the cost and time to conduct laboratory tests, as only toxin positive samples would be analysed in the laboratory for confirmation of toxicity. Biosensor assays can also potentially be carried out by untrained personnel, such as farm and aquaculture managers.

The results of this study form part of an intermediate phase to determine if the immobilised PPINA can translate into a pre-biosensor format for detection of MCs and DSP toxins (OA and DTXs). The method was optimised using two protein phosphatase enzymes, PP-2A and R.PP-1 against the substrate MUMP. Both of these enzymes produced activity curves that were consistent for an immobilised system and showed high activity and stability profiles. The total cost of the PP-2A and R.PP-1 enzymes (including shipping) were similar, when the effectiveness of the enzyme to dephosphorylate the substrate was considered. This study was

also the first to demonstrate the use of the fluorimetric substrate, MUMP for the R.PP-1 assay (in both the immobilised and unimmobilised format), as previous R.PP-1 assays have mainly utilised colorimetric substrates, namely *p*NPP and malachite green (Zhang *et al.,* 1992; An *et al.,* 1994; Heresztyn *et al.,* 2001; Rapala *et al.,* 2002).

The performance of the PP-2A and R.PP-1 enzymes in the pre-biosensor format were approximately 10-fold less sensitive for MC-LR and OA detection compared to the unimmobilised PPINA format. The detection limits of unimmobilised the PP-2A and R.PP-1 enzymes against MC-LR were 1 and 0.2 nM, respectively. These detection ranges are consistent with those of other PPINA investigations reported in the literature for MCs (Zhang *et al.,* 1992; An *et al.,* 1994; Heresztyn *et al.,* 2001; Rapala *et al.,* 2002; Mountfort *et al.,* 2005). This detection range also falls within the proposed safe WHO guideline value of 1 μg/L for MC-LR in drinking water (roughly equivalent to 1 nM) (World Health Organization, 2003). The detection limit for the immobilised the PP-2A and R.PP-1 enzymes against MC-LR however, are 10 and 2 nM, respectively, which is close to, but does not fall, within the safe regulatory guidelines.

The detection limit for unimmobilised PP-2A against OA was 1.2 nM, which is consistent with PPINA ranges reported in the literature for OA (Zhang *et al.,* 1992; Tubaro *et al.,* 1996; Leira *et al.,* 2000; Mountfort *et al.,* 2001). The detection limit for unimmobilised PP-2A falls within the guideline level of 160 μg/kg mussel meat set by the European Commission on Standards (European Commission, 2002). The detection limit of the immobilised PP-2A enzyme against OA was 15 nM. This detection limit is slightly higher than the current biosensor designs for OA and is outside the regulatory guidelines (Croci *et al.,* 2001; Kruezer *et al.,* 2002). Thus, the PP-2A and R.PP-1 assay limits in the pre-biosensor format do not conform to regulatory guidelines for MC-LR and OA, at this stage of the developmental phase. In addition, the R.PP-1 enzyme assay against OA was not considered suitable for OA detection as enzyme activity was induced for both the immobilised and unimmobilised assay system between 10-200 nM and 1-100 nM of OA, respectively.

The pre-biosensor format for the PP-2A and R.PP-1 enzyme was tested for re-usability by noting the activity of the enzymes once OA and MC had been washed from the system. Recombinant PP-1 could be applied to a reusable biosensor system as permanent binding did not occur between the enzyme and MC-LR (OA was not tested for re-usability with R.PP-1). This indicates that multiple assays could be performed using the same enzyme, which would reduce

the assay costs. In comparison, the immobilised PP-2A enzyme cannot be incorporated into a biosensor as a re-usable component, as permanent binding occurred between the enzyme and both toxins. Thus, for the PP-2A enzyme assay system to be applicable in a biosensor design, it must be in the form of a discardable system. For example, the enzyme-bead complex could be a discardable unit within the biosensor model, i.e. after an environmental sample has been tested the enzyme will need to be discarded. Most other biosensor designs for OA are also based on single-use systems (Croci *et al.,* 2001; Kruezer *et al.,* 2002). Single-use systems have the advantage over re-usable systems in that it avoids the problem of deterioration of the biosensor element when analysing environmental matrices (Baeumner, 2003).

The pre-biosensor format was also tested with environmental samples. Protein phosphatase type 2A and R.PP-1 enzymes were tested with mussel extracts naturally contaminated with DSP toxins (OA and DTXs) and with algal extracts containing natural levels of MCs, respectively. At this stage, the method is not designed to test samples in the presence of complicated matrices that exist within mussel and algal extracts. However, this exercise was carried out so that the robustness of the method could be compared to the results from other methods, such as LC-MS and other PPINA methods, and an assessment of modifications to the assay (if necessary) could be made based on these results.

The mussel and algal tests highlighted some important findings about the pre-biosensor method. Firstly, it showed how matrix effects in the assay, caused by confounding substances in the sample extract, can interfere with the enzyme-toxin binding. This highlights the constraints of the assay for use in testing samples with complex matrices, such as mussel and algal extracts. Secondly, the study emphasised the importance of sample dilution for maximising toxin detection. Matrix effects and sample dulitions are factors that need to be seriously taken into consideration when developing this type of assay system. Future studies modifying this technique need to address these issues. Goussain and Mountfort (unpublished) have recently demonstrated that modifying the immobilised PP-2A enzyme assay method by altering certain components, such as the immobilisation buffer and the assay volume, increased the detection limit for OA by a factor of 5 ($IC_{50}$ 12 nM). This detection range is adequate for a biosensor format for DSP toxins using PP-2A.

The intended outcome of this ongoing study is the development of a prototype biosensor for MC and DSP toxin detection. The method has not yet reached this stage. Further studies are

necessary to address the following issues: 1) increasing the performance and sensitivity of the immobilised enzyme to detect naturally occurring MCs within a detection range that is consistent with regulatory guidelines; 2) assessing other protein phosphatase enzymes that might be more suitable and sensitive for the biosensor design; 3) determining a more suitable method for washing to enhance the recovery of the immobilised enzyme for re-use and; 4) re-assessing assay conditions of the immobilised enzyme that best allow the assay to accurately determine DSP toxins and MCs. It is likely that in the future, such studies will be carried out with PP-2A, as most aspects of the experiments conducted in this thesis, have shown that the PP-2A enzyme is a better contender than the R.PP-1 enzyme.

A good biosensor design has the following characteristics: high sensitivity, easy to perform, rapid, high sample throughput, re-usable and high specificity (Baeumner, 2003). The method described in this study performs well to the aforementioned characteristics. The advantages of this method regarding the speed of analysis, simplicity of design and the detection of total toxin bioactivity, give this biosensor format great potential to have the leading edge over other laboratory based methods reported todate. It also has considerable potential to compete with current biosensor designs for DSP toxins which detect specific toxin congeners only (Marquette *et al.*, 1999; Kreuzer, 2002; Tang *et al.*, 2002). There have been no biosensors developed to test for MCs todate. With the global increase in marine and freshwater algal outbreaks, there is a need to develop an on-site detection system for MCs and DSP toxins in environmental samples.

In summary, this study has shown that: 1) PP-2A is the preferred enzyme over R.PP-1 for OA and MC detection, because of the enhanced sensitivity given by this enzyme to both toxins and, 2) the detection limit of the immobilised PP-2A enzyme towards MC-LR and OA is close to the levels prescribed by regulatory agencies. Thus, there is sufficient justification for the immobilised PP-2A enzyme method to be considered for translation into the prototype biosensor format. Further developments are currently underway with the PP-2A enzyme to increase the sensitivity of the system before incorporation into a prototype fill-flow biosensor (Goussain and Mountfort, unpublished).

# Chapter 6 References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# Chapter 7 Appendices

**Appendix 7-1: Common microcystin structures** [MCs identified from species and strains of *Anabaena, Hapalosiphon, Microcystins, Nostoc* and *Planktothrix* (Rinehart *et al.*,1994)]

| Microcystin (MC) | Molecular weight | Molecular formulae |
|---|---|---|
| MC-LA | 909 | $C_{46}H_{67}N_7O_{12}$ |
| MC-LAba | 923 | $C_{47}H_{69}N_7O_{12}$ |
| MC-AR | 952 | $C_{49}H_{66}N_{10}O_{12}$ |
| MC-YA | 959 | $C_{49}H_{65}N_7O_{13}$ |
| [D-Asp$^3$] MC-LR | 966 | $C_{47}H_{70}N_{10}O_{12}$ |
| [Dha$^7$] MC-LR | 980 | $C_{48}H_{72}N_{10}O_{12}$ |
| [DMAdda$^5$] MC-LR | 980 | $C_{48}H_{72}N_{10}O_{12}$ |
| MC-LF | 980 | $C_{48}H_{72}N_{10}O_{12}$ |
| MC-LR | 985 | $C_{52}H_{71}N_7O_{12}$ |
| [D-Asp$^3$,D-Glu(OCH$^3$)$^6$] MC-LR | 994 | $C_{49}H_{74}N_{10}O_{12}$ |
| [(6Z)$^1$-Adda$^5$] MC-LR | 994 | $C_{49}H_{74}N_{10}O_{12}$ |
| [L-Ser$^7$] MC-LR | 994 | $C_{49}H_{74}N_{10}O_{12}$ |
| MC-LY | 998 | $C_{48}H_{74}N_{10}O_{13}$ |
| MC-IHirR | 1001 | $C_{52}H_{71}N_7O_{13}$ |
| [D-Asp$^3$, ADMAdda$^5$] MC-LR | 1008 | $C_{50}H_{76}N_{10}O_{12}$ |
| [D-Glu-OCH$^6$] MC-LR | 1008 | $C_{49}H_{72}N_{10}O_{13}$ |
| [D-Asp$^3$, Dha$^7$] MC-RR | 1008 | $C_{50}H_{76}N_{10}O_{12}$ |
| [L-MeSer7] MC-LR | 1009 | $C_{47}H_{71}N_{13}O_{12}$ |
| [Dha$^7$] MC-FR | 1012 | $C_{49}H_{76}N_{10}O_{13}$ |
| [ADMAdda$^5$] MC-LR | 1024 | $C_{53}H_{70}N_{10}O_{12}$ |
| [D-Asp$^3$, ADMAdda$^5$] MC-LHar | 1022 | $C_{50}H_{74}N_{10}O_{13}$ |

| | | |
|---|---|---|
| [D-Asp$^3$] MC-RR | 1022 | $C_{50}H_{74}N_{10}O_{13}$ |
| [Dha$^7$] MC-RR | 1023 | $C_{48}H_{73}N_{13}O_{12}$ |
| MC-FR | 1023 | $C_{48}H_{73}N_{13}O_{12}$ |
| MC-M(O)R | 1028 | $C_{52}H_{72}N_{10}O_{12}$ |
| [Dha$^7$] MC-HphR | 1028 | $C_{48}H_{72}N_{10}O_{13}S$ |
| [Dha$^7$] MC-FR | 1028 | $C_{52}H_{72}N_{10}O_{12}$ |
| [D-Asp$^3$, Dha$^7$] MC-HtyR | 1030 | $C_{51}H_{70}N_{10}O_{13}$ |
| [Dha$^7$] MC-YR | 1030 | $C_{51}H_{70}N_{10}O_{13}$ |
| [D-Asp$^3$] MC-YR | 1030 | $C_{51}H_{70}N_{10}O_{13}$ |
| MC-YM(O) | 1035 | $C_{51}H_{69}N_7O_{14}S$ |
| [ADMAdda$^5$] MC-LHar | 1036 | $C_{51}H_{76}N_{10}O_{13}$ |
| MC-RR | 1037 | $C_{49}H_{75}N_{13}O_{12}$ |
| [(6Z)- Adda$^5$] MC-RR | 1037 | $C_{49}H_{75}N_{13}O_{12}$ |
| [D-Ser$^1$, ADMAdda$^5$] MC-LR | 1038 | $C_{50}H_{74}N_{10}O_{14}$ |
| [ADMAdda$^5$, MeSer$^7$] MC-LR | 1040 | $C_{50}H_{76}N_{10}O_{14}$ |
| [L-Ser$^7$] MC-RR | 1041 | $C_{48}H_{75}N_{13}O_{13}$ |
| [D-Asp$^3$, MeSer$^7$] MC-RR | 1041 | $C_{48}H_{75}N_{13}O_{13}$ |
| MC-YR | 1044 | $C_{52}H_{72}N_{10}O_{13}$ |
| [D-Asp$^3$] MC-HtyR | 1044 | $C_{52}H_{72}N_{10}O_{13}$ |
| [Dha$^7$] MC-HtyR | 1044 | $C_{52}H_{72}N_{10}O_{13}$ |
| MC-(H$^4$) YR | 1048 | $C_{52}H_{76}N_{10}O_{13}$ |
| [D-Glu-$OC_2H_3(CH_3)OH^2$] MC-LR | 1052 | $C_{52}H_{80}N_{10}O_{13}$ |
| MC-HtyR | 1058 | $C_{53}H_{74}N_{10}O_{13}$ |
| [L-Ser$^7$] MC- HtyR | 1062 | $C_{52}H_{74}N_{10}O_{14}$ |
| MC-YR | 1067 | $C_{54}H_{73}N_{11}O_{12}$ |
| [L-Melan$^7$] MC- LR | 1115 | $C_{52}H_{81}N_{11}O_{14}S$ |

**Appendix 7-2: Microcystin and Nodularin toxicity values** [toxicity of some MCs and NODLNs based upon an i.p. lethal dose 50% response ($LD_{50}$) in laboratory mouse or rat injections (Rinehart *et al.*,1994)].

| Toxin | LD50 (µg/kg) |
|---|---|
| Nodularin (NODLN) | 50 |
| [D-Asp$^3$] NODLN | 75 |
| [DMAdda$^3$] NODLN | 150 |
| [6(Z)- Adda$^3$] NODLN | >2000 |
| [D-Glu-OCH$^4$] NODLN DihydroNODLN | >1200 |
| [D-MeAbu$^5$] NODLN | 150 |
| [L-MeAbu$^5$] NODLN | 150 |
| MC-LR | 50 |
| MC-LA | 50 |
| MC-YR | 70 |
| MC-RR | 600 |
| MC-AR | 250 |
| MC-LY | 90 |
| MC-FR | 250 |
| MC-WR | 150-200 |
| MC-YM(O) | 56 |
| MC-M(O)R | 700-800 |
| MC-HtyR | 80-100 |
| MC-HilR | 100 |
| [D-Asp$^3$] MC-LR | 50 |
| [D-Asp$^1$] MC-RR | 250 |
| [Dha$^7$] MC-LR | 250 |
| [D-Asp$^3$] MC-LR | 150 |
| [L-MeLan$^7$] MC-LR | 1000 |
| [D-Glu-OCH$^6$] MC-LR | >1000 |

| | |
|---|---|
| [D-Glu-$OC_2H_3(CH_3)OH^6$] MC-LR | >1000 |
| [$DMAdda^5$] MC-LR | 90-100 |
| [$ADMAdda^5$] MC-LR | 60 |
| [D-$Asp^3$, $ADMAdda^5$] MC-LR | 160 |
| [$ADMAdda^5$] MC-LHar | 60 |
| [6(Z)-$Adda^5$] MC-LR | >1200 |
| [6(Z)-$Adda^5$] MC-RR | >1200 |
| [L-$MeAla^7$] MC-LR | 85 |
| [D-$MeAla^7$] MC-LR | 100 |

## Appendix 7-3: Calculation of DSP toxins in mussel extracts.

The procedure for determining the concentration of DSP toxins (OA and DTXs) in mussel samples follows the method of (Mountfort *et al.* 2003). The results are expressed as OA equivalents.

*Step 1:* Read off the nM of OA equivalents from the dose-response curve of % activity versus OA developed using the OA standard (= nM OA added) determined for each enzyme.

*Step 2:* Determine the pmol per assay volume (150 µl) (= nM OA added x [150/1000]).

*Step 3:* Determine the pg OA per assay (= step 2 x 805)
Note, the molecular weight of OA is 805 g/mol.

*Step 4:* Determine the pg per ml of extract added and correct for volume added
(= [step 3 x 1000]/amount of extract added to assay, i.e. either 5 or 10 µl.)

*Step 5:* Determine the µg per ml and correct for the extract dilution
(= [step 4/1000 000] x extract dilution).
Note, the dilution for mussel extracts were 1:2 and 1:4.

*Step 6:* Mean results from the 5 and 10 µl volumes of each extract added to the assay

*Step 7:* Determine µg per 5 ml of extract (= step 6 x 5)

*Step 8:* Calculate µg per 100 g of shellfish tissue assuming that 5 ml extract is equivalent to 1 g shellfish tissue (= step 7 x 100)

*Step 9:* Convert this figure to µg/kg of shellfish tissue.

## Appendix 7-4: Calculation of MCs in algal extracts.

The procedure for determining the concentration of MCs in algal samples follows the method of (Mountfort *et al.* 2003). The results are expressed as MC-LR equivalents.

*Step 1:* Read off the nM of MC-LR equivalents from the dose-response curve of % activity versus MC-LR developed using the MC-LR standard (= nM MC-LR added) determined for each enzyme.

*Step 2:* Determine the pmol per assay volume (150 µl) (= nM MC-LR added x [150/1000]).

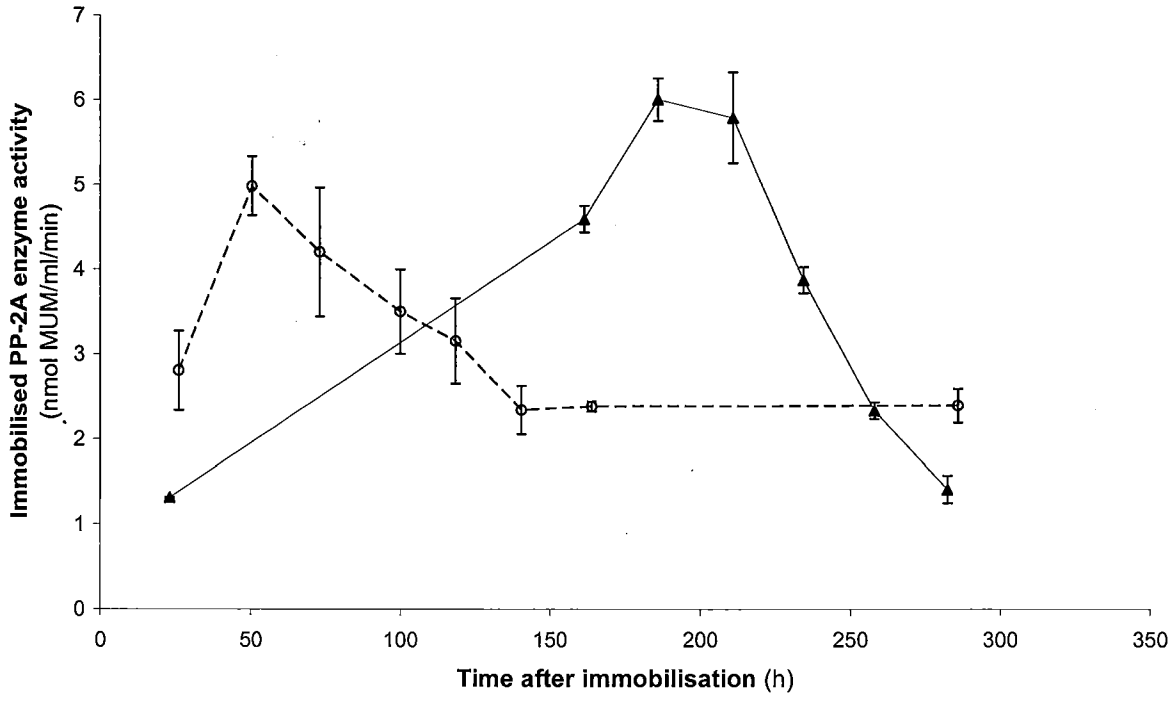
*Step 3:* Determine the pg MC-LR per assay (= step 2 x 995).
Note, the molecular weight for MC-LR is 995 g/mol.

*Step 4:* Determine the pg per ml of extract added and correct for volume added
(= [step 3 x 1000]/amount of extract added to assay, i.e. either 5 or 10 µl.)

*Step 5:* Determine the µg per ml and correct for the extract dilution
(= [step 4/1000 000] x extract dilution).
Note, the dilution for algal extracts was 1:4 and 1:9.

*Step 6:* Mean results from the 5 and 10 µl volumes of each extract added to the assay and express the results in µg/l.

## Appendix 7-5: Reaction kinetics for immobilised PP-2A showing the lag effect in enzyme stability as a result of washing frequency




Symbols donate the washing frequency overtime: ○, washing frequency at regular intervals; ▲, washing frequency at irregular intervals (an initial delay in time between the first and second wash of >100 hours). The concentration of PP-2A was 0.5 U. Each value represents the mean of triplicate determinations ± standard deviation.