ASSAYS AND BIOSENSORS FOR THE DETECTION OF TOXINS FROM AQUATIC MEDIA

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DOCTORAL THESIS

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Supervised by Dr. Mònica Campàs i Homs

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CERTIFIES:

That the present work, entitled “Assays and biosensors for the detection of toxins from aquatic media” presented by Diana Garibo Ruiz for the award of the degree of Doctor, has been carried out under my supervision at Institue of Agri-food Research and Technology (IRTA), and that it fulfils the requirements to obtain the “European Doctor” Mention.

7th July 2014, Tarragona (Spain).

Dr. Mònica Campàs i Homs

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Abbreviations

OA	Okadaid Acid
MCs	Microcysitins
PPIA	Protein Phosphatase Inhibition Assay
DTXs	Dinophysistoxins
SPR	Surface Plasmon Resonance
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
DSP	Diarrheic Shellfish Poisoning
MBA	Mouse Bioassay
WHO	World Health Organization
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
IEF	Inhibitory Equivalency Factor
ELISA	Enzyme-Linked Immunosorbent Assay
DA	Domoic Acid
STX	Saxitoxin
Neo-STX	Neosaxitoxin
LOD	Limit of Detection
IC_{50}	50% Inhibition Coefficient
EFSA	European Food Safety Authority
MP	Magnetic Particle
MAb	Monoclonal Antibodies
MC-LR-HRP	MC-LR-Horseradish Peroxidase
EC_{50}	50% Effective Coefficient

Resumen

El objetivo de esta tesis ha sido el desarrollo de ensayos y biosensores para la detección de toxinas que están presentes en el medio acuático y el estudio de su aplicabilidad al análisis de muestras. Para lograr este objetivo, se han caracterizado proteínas fosfatasas recombinantes y posteriormente se han utilizado en el desarrollo de ensayos de inhibición de proteínas fosfatasas (PPIAs) para la detección de acido okadaico (OA) y sus análogos, así como de microcistinas (MCs). Además, se han explotado partículas magnéticas como soportes de inmovilización y transportadores de biomoléculas en el desarrollo de biosensores. Finalmente, se ha evaluado la aplicabilidad de ensayos y biosensores al análisis de muestras de marisco y cianobacterias, y se han establecido correlaciones con otras técnicas de análisis.

El Capítulo 1 es una introducción general sobre las toxinas de medios acuáticos, OA y análogos y MCs, y describe su estructura química, modo de acción, implicaciones en salud humana, directrices para su control, así como ensayos bioquímicos y biosensores para su detección.

El Capítulo 2 "*Ensayos de inhibición de la proteína fosfatasa para la detección de toxinas de medios acuáticos"* incluye tres artículos publicados. Se ha investigado la aplicabilidad de los PPIA con enzimas salvajes y recombinantes a la detección de toxinas marinas y de agua dulce. En el primer artículo, se evalúan los efectos de matrices de marisco y del protocolo de extracción de toxinas marinas (OA y ésteres correspondientes) en los PPIAs.

En el segundo artículo, se investiga la capacidad de los PPIAs de detectar varios derivados de toxinas marinas (dinofisistoxinas) y se propone la implementación de los ensayos en sistemas de vigilancia. En el tercer artículo, se describen las potencias inhibitorias de varias toxinas producidas por cianobacterias (variantes de MCs).

En el Capítulo 3 *"Partículas magnéticas como soportes de inmovilización y transportadores de biomoléculas en biosensores"* incluye tres artículos. Se ha explotado el uso de partículas magnéticas como soportes de inmovilización y

transportadores de biomoléculas en biosensores. Los dos primeros artículos muestran la factibilidad para usar partículas magnéticas como soportes de inmovilización de enzimas y anticuerpos en el desarrollo de ensayos colorimétricos y biosensores electroquímicos para OA y MCs. En el tercer artículo, se han utilizado partículas magnéticas como transportadores de anticuerpos en inmunosensores ópticos basados en resonancia de pasmón superficial (SPR) para la detección de OA.

En el Capítulo 4 se presenta una discusión general de los resultados obtenidos en los capítulos 2 y 3.

Finalmente, en el Capítulo 5 se resumen las conclusiones generales obtenidas de la experimentación y los resultados de esta tesis, así como el posible trabajo futuro.

Summary

The general objective of this thesis has been to develop assays and biosensors for the detection of toxins from aquatic media and to study their applicability to the analysis of samples. To achieve this goal, recombinant protein phosphatases have been characterised and used in the development of protein phosphatase inhibition assays (PPIAs) for the detection of okadaic acid (OA) and its analogues as well as microcystins (MCs). Moreover, magnetic particles have been exploited as biomolecule immobilisation supports and carriers in the development of biosensors. Finally, the applicability of assays and biosensors to the analysis of shellfish and cyanobacteria samples has been evaluated, and correlations with other analysis techniques have been established.

Chapter 1 is a general introduction about the toxins from aquatic media, OA and analogues and MCs, and describes their chemical structure, mode of action, human health implications, guidelines for their control, as well as the biochemical assays and biosensors for their detection.

Chapter 2 *"Protein phosphatase inhibition assays for the detection of toxins from aquatic environments"* includes three published articles. The applicability of PPIAs with wild and recombinant enzymes to the detection of marine and freshwater toxins has been investigated. In the first article, the effects of shellfish matrices and the marine toxins (OA and the corresponding esters) extraction protocol on the PPIAs are evaluated. In the second article, the ability of the PPIAs to detect several marine toxin analogues (dinophysistoxins) is investigated and the implementation of the assays in monitoring systems is proposed. In the third article, the inhibitory potencies of several cyanobacterial toxins (MCs variants) are described.

Chapter 3 *"Magnetic particles as biomolecule immobilisation supports and carriers in biosensors"* includes three published articles. The use of magnetic particles as biomolecule immobilisation supports and carriers in biosensors has been exploited. The first two articles show the feasibility to use magnetic particles as immobilisation supports for enzymes and antibodies in the development of colorimetric assays and electrochemical biosensors for OA and MCs. In the third

article, magnetic particles have been used as antibody carriers in optical surface plasmon resonance (SPR) immunosensors for OA.

Chapter 4 is a general discussion of the results obtained in chapters 2 and 3.

Finally, Chapter 5 summarises the general conclusions obtained from the experimentation and the results of this thesis, as well as the possible future work.

Articles published during the PhD thesis

1. Garibo, D., Devic, E., Marty, J.L., Diogène, J., Unzueta, I., Blázquez, Campàs, M. Conjugation of genetically engineered protein phosphatases to magnetic particles for okadaic acid detection. *J. Biotechnol.* **2012**,157, 89-95.

2. Campàs, M., Garibo, D., Prieto-Simón, B. Novel nanobiotechnological concepts in electrochemical biosensors for the analysis of toxins. *Analyst* **2012**, 137, 1055-1067.

3. Garibo, D., Dàmaso, E., Eixarch, H., de la Iglesia, P., Fernández.-Tejedor, M., Diogène, J., Pazos, J., Campàs, M. Protein phosphatase inhibition assays for okadaic acid detection in shellfish: Matrix effects, applicability and comparison with LC–MS/MS analysis. *Harmful Algae* **2012**, 19, 68-75.

4. Reverté, L., Garibo, D., Flores, C., Diogène, J., Caixach, J., Campàs, M. Magnetic particle-based enzyme assays and immunoassays for microcystins: From colorimetric to electrochemical detection. *Environ. Sci. Technol.* **2013**, 47, 471-478.

5. Garibo, D., de la Iglesia, P., Diogène, J., Campàs, M. Inhibition equivalency factors for dinophysistoxin-1 and dinophysistoxin-2 in protein phosphatase assays: Applicability to the analysis of shellfish samples and comparison with LC-MS/MS. *J. Agric. Food Chem.* **2013**, 61, 2572-2579.

6. Garibo, D., Campbell, K., Casanova, A., de la Iglesia, P., Fernández-Tejador, M., Diogène, J., Elliot, C.T., Campàs, M. SPR immunosensor for the detection of okadaic acid in mussels using magnetic particles as antibody carriers. *Sens. Actuators B* **2014**, 190, 822-828.

7. Garibo, D., Flores, C., Cetó, X., Prieto-Simón, B., del Valle, M., Caixach, J., Diogène, J., Campàs, M. Inhibition equivalency factors for micricystins variants in recombinant and wild-type protein phosphatase 1 and 2A assays. In press in *Environ. Sci. Pollut. Res.* **2014**.

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Chapter 1

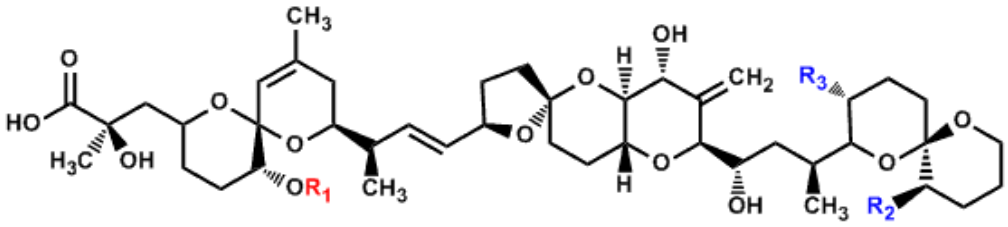
General introduction

This chapter is a general introduction about the toxins from aquatic media, OA and analogues and MCs, and describes their chemical structure, mode of action, human health implications, guidelines for their control, as well as the biochemical assays and biosensors for their detection.

1.1 Lipophilic marine toxins

Okadaic acid (OA) and the dinophysistoxins (DTXs) analogues are lipophilic marine toxins produced mainly by dinoflagellates of the genera *Dinophysis* and *Prorocentrum* (Steidinger, 1993). These toxins are accumulated in the digestive glands of shellfish, such as mussels, clam, oyster and scallops. The consumption of shellfish contaminated by OA and DTXs may result in diarrheic shellfish poisoning (DSP), which causes gastrointestinal disturbances, including diarrhea, nausea and vomiting (Yasumoto and Murata, 1993).

The chemical structure of OA and DTXs consists of a polyketide backbone, with furan and pyran-type ether rings and an *α*-hydroxycarboxyl function. The difference between OA, DTX-1 and DTX-2 lies only in the number or the position of the methyl groups. DTX-3 includes all acylated derivatives (Fig. 1.1).



	R_1	R_2	R_3
OA	H	H	CH_3
DTX-1	H	CH_3	CH_3
DTX-2	H	CH_3	H

Fig. 1.1. Chemical structure of OA and DTXs.

OA and DTXs are potent inhibitors of protein phosphatases (PP1 and PP2A), which play an important role in protein dephosphorylation in cells (Bialojan and Takai, 1998).

When these toxins bind to PPs, block their activity, and as a consequence they cause the hyperphosphorylation of proteins involved in sodium secretion control and of cytoskeletal or junctional moieties that regulate cell permeability, causing sodium release together with a subsequent passive loss of fluids, responsible for the diarrheic symptoms (Aune and Yndestad, 1993).

DSP toxic episodes are recurrent in European waters, Galicia and Catalunya being examples of very affected regions (Reguera et al., 2012). In order to assure food safety and protect the consumer health, monitoring programs for the control of lipophilic marine toxins have been implemented. The European Regulation (EC) No. 843/2004 has established a maximum permitted level (MPL) of 160 µg of OA equivalents/kg shellfish meat. Although it is possible to use the mouse bioassay (MBA), the Commission Regulation (EU) No. 15/2011 has recently established that liquid chromatography-tandem mass spectrometry (LC-MS/MS) should be applied as the reference method for the determination of lipophilic toxin contents in shellfish, starting from July 1, 2011, and completely replacing the MBA in 2015. Additionally, this regulation encourages the development of other chemical methods as well as immunoassays and functional assays, as alternatives or supplements to the LC-MS/MS method.

1.2 Microcystins

Microcystins (MCs) and nodularins are freshwater toxins produced by toxicogenic cyanobacteria such as *Microcystis aeruginosa* (Bouaïcha et al., 2002). Their presence in water supplies and recreational areas poses a threat to animal and human health because of their possible harmful effects, such as gastrointestinal disturbances but also tumour promotion and carcinogenicity (Eriksson et al., 1990).

The chemical structure of MCs consists of a cyclic peptide with five constant amino acids and two variable ones: cyclo-(D-Ala^{1}-X^{2}-D-MeAsp^{3}-Y^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}) (Fig. 1.2).

The name of MCs is defined according to the two variable L-amino acids at X and Y positions, e.g. MC-LR having leucine (L) at position X and arginine (R) at position Y. Nowadays, there are more than 85 MC variants with different toxicity reported in the literature (Rastogi et al., 2014). Among them, MC-LR is usually considered the most toxic and usually taken as a model.

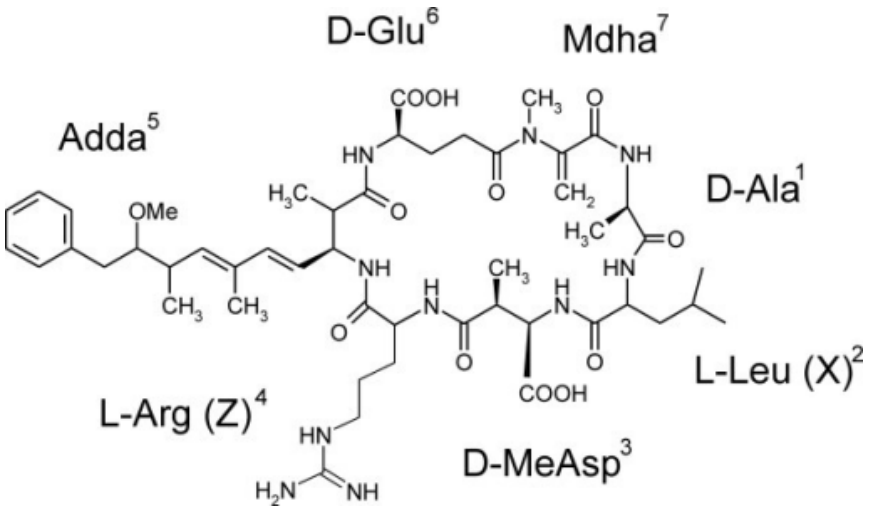


Fig. 1.2. Chemical structure of MCs (Fewer et al., 2007).

MCs are also potent inhibitors of PP1 and PP2A enzymes, but unlike lipophilic marine toxins the PP inhibition by MCs is reversible. Similarly to OA and DTXs, once the MCs have been ingested through the food chain or by drinking contaminated water, they can enter hepatocytes via the bile acid transport system, and disrupt the cytoskeleton; cells shrink, leading to haemorrhage of the liver (Falconer and Yeung, 1993).

Due to the serious human health and environmental implications, the World Health Organization (WHO) has proposed a provisional MPL of 1 µg/L of microcystin-LR in drinking water (WHO, 1998). This recommended value has been adopted by some countries, because of the lack of a European regulation. Chromatographic techniques coupled to different detection principles, e.g. LC-MS/MS, are very powerful because of their high sensitivity and the ability to detect different MC variants. Nevertheless, alternative detection methods based on biochemical principles are desired as alternative or complementary for both screening and quantification, because of their simplicity, low cost, short analysis times and possibility to perform high-throughput analysis.

1.3 Biochemical assays for the detection of toxins from aquatic media

Taking the mechanism of action of OA and MCs on PPs as a basis, protein phosphatase inhibition assays (PPIAs) have been developed for the detection of these toxins (for OA: Honkaenen et al., 1996; Tubaro et al., 1996; Della Loggia et al., 1999; Mountfort et al., 2001; Campàs and Marty, 2007a; Albano et al., 2009; Caillaud et al., 2010; Cañete et al., 2010; Sassolas et al., 2011a; Hayat et al., 2012b and for MCs: MacKintosh et al., 1990; An and Charmichael, 1994; Ward et al., 1997; Rivasseau et al., 1999; Heresztyn and Nicholson, 2001; Bouaïcha et al., 2002; Campàs et al., 2005; Campàs and Marty, 2007b; Campàs et al., 2007c; Ikehara et al., 2008; Sassolas et al., 2011b), slightly adjusting the protocol according to the type of toxin. The PP inhibition can be detected by different techniques, e.g. radioactivity, fluorescence and colorimetry. Although may be not the most sensitive detection method, colorimetry is the most commonly used because its simplicity and cost effectiveness. Fig.1.3 shows the detection principle in a colorimetric assay. If the toxin is not present, the PP is able to dephosphorylate the enzyme substrate, and the coloured enzyme product is detected. In the presence of toxin, the enzyme is inhibited and the absorbance decreases proportionally to the toxin concentration.

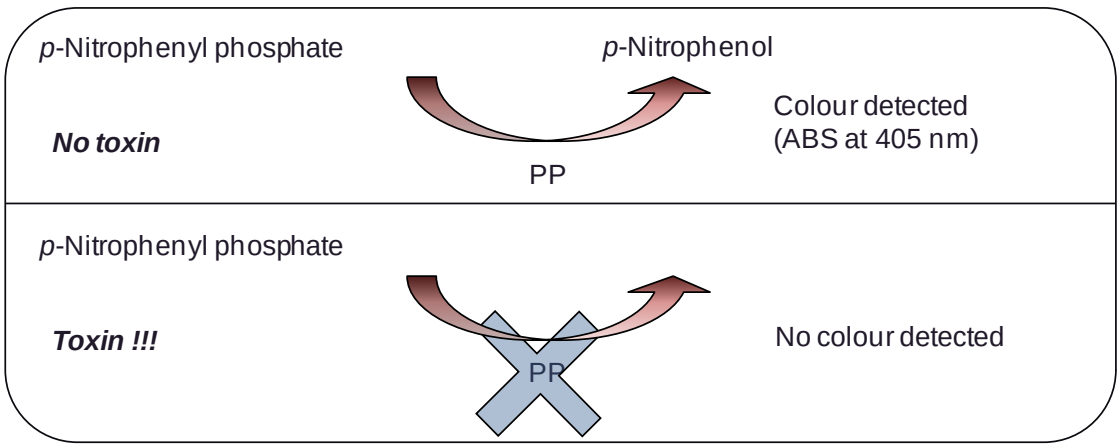


Fig. 1.3. Scheme of the chemical reaction of PP inhibition by aquatic toxins.

Apart from the enzyme substrate choice, several experimental parameters, e.g. enzyme concentration, temperature and reaction time, should be optimised in the development of a PPIA. But one of the most critical points is the enzyme stability. PPs suffer from fast deactivation rates, both under operation and storage, effect that may compromise the reliability of the assays. Consequently, the production of highly stable PPs is desired.

Once an appropriate PPIA has been developed, their applicability to the analysis of naturally-contaminated samples also requires exhaustive investigation. When dealing with natural samples, the effects of the sample matrix on the enzyme activity may also compromise the reliability of the assay, providing usually overestimations of the toxin contents (non-specific PP inhibition). Although less common, underestimation of the toxin contents may also occur due to the presence of exogenous PPs (Mountfort et al.,1999). The application of clean-up or purification protocols prior to the analysis of the sample could completely or partially solve these limitations. However, if the purpose of the assay is to provide a fast screening of samples, the time required for sample preparation is crucial and should be kept to the minimum. Consequently, the best choice would be to exactly understand the biorecognition event and to establish sample matrix loading limits.

In the validation of a PPIA, correlation of the obtained results with those provided by other techniques used as a reference, such as LC-MS/MS, is required. But it is necessary to keep in mind that the detection principle may be different. In that case, the establishment of inhibitory equivalency factors (IEFs) is very useful to understand the correlation, especially in the analysis of natural samples that contain more than one toxin analogue, derivative or variant, each one with different inhibition potency.

Immunoassays have also been exploited for the detection of toxins from aquatic media. Immunoassays are biochemical assays based on the affinity recognition between antibodies (polyclonal or monoclonal) and antigens (OA and analogues, or MCs).

Consequently, they are not functional but structural. The most commonly found format is the enzyme-linked immunosorbent assay (ELISA), where enzymes are used as labels to detect the interaction between antibodies and toxins. Several ELISAs have been developed for OA and analogues (Kreuzer et al., 1999; Campàs et al., 2008a; Dubois et al., 2010; Wang et al., 2011; Sassolas et al., 2013; Liu et al., 2014), and MCs (An and Carmichael, 1994; Chu et al., 1990; Ueno et al., 1996; Brooks and Codd 1998; Zeck et al., 2001; Pyo et al., 2004; Campàs and Marty, 2007b; Sheng et al., 2007), most of them based on colorimetric detection.

Similarly to the IEFs in PPIAs, cross-reactivity factors are crucial to understand the correlations with other techniques such as LC-MS/MS. Antibodies may be able to recognise different toxins on the same group, even though they are not toxic, since affinity and toxicity may not always be proportional. Nevertheless, immunoassays are useful for both screening and quantification of aquatic toxins due to the high sample throughput and the relative low cost, and because they do not require sophisticated instrumentation neither highly skilled personnel.

1.4 Biosensors for the detection of toxins from aquatic media

Biosensors are integrated devices composed of a biorecognition element (enzyme, antibody, oligonucleotide, cell or microorganism), which specifically recognises the target analyte, in direct contact with a transducer element, which converts the biorecognition event into a measurable signal (Fig 1.4). The signal is collected and processed by an associated electronic system, which displays the analyte content.

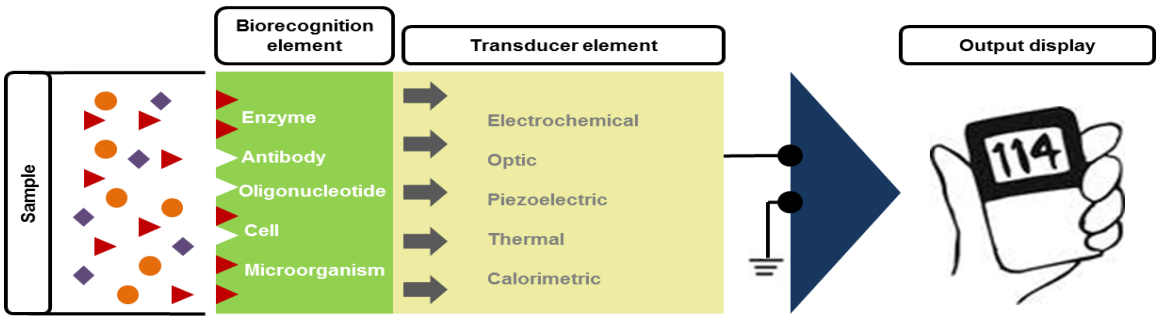


Fig. 1.4. Scheme of the biosensor principle.

Biosensors can be classified according to the biorecognition molecule (catalytic when using enzymes, and affinity when using for example antibodies, oligonucleotides and aptamers) or the transducer (electrochemical, optic, piezoelectric, thermal or calorimetric). Biosensors provide several advantages compared to other conventional techniques, such as high sensitivity, short analysis times, low cost and ease of use. Moreover, some of them can be miniaturised, leading to the development of integrated and portable devices for in situ measurements.

Biosensors are being considered as an appropriate approach for the screening and/or quantification of toxin contents in natural samples. In the last 15 years, biosensors have been developed for the detection of OA and analogues (Marquette et al., 1999; Tang et al., 2002; Campàs and Marty, 2007a; Campàs et al., 2008a; Steward et al., 2009a; Steward et al., 2009b; Campbell et al., 2011; Hayat et al., 2011; Dominguez et al., 2012; Hayat et al., 2012a; Hayat et al., 2012c; Llamas et al., 2007; McNamee et al., 2013) and MCs (Campàs et al., 2005; Campàs et al., 2007b; Campàs et al., 2007c; Campàs et al., 2008b; Dawan et al., 2011; Han et al., 2011; Herranz et al., 2010; Hu et al., 2009; Loyprasert et al., 2008; Szydlowska et al., 2006; Vinogradova et al., 2011). Regarding lipophilic marine toxins, although the PP inhibition has been detected by electrochemical techniques (Campàs et al., 2007a), the LODs achieved were not low enough to apply the enzyme sensors to the detection of OA and analogues, probably due to barrier created by the technique used for the enzyme immobilisation on the screen-printed electrodes (entrapment into a polymeric matrix). Much more efforts have been focused on immunosensors. Although piezoelectric (Tang et al., 2002) and chemiluminescence (Marquette et al., 1999) have been used as detection techniques, most immunosensors are electrochemical or based on Surface Plasmon Resonance (SPR). Electrochemical immunosensors for OA and analogues have usually attained appropriate LODs, and when necessary, enzyme recycling systems have been incorporated in order to amplify the electrochemical signals provided by the enzyme label (Campàs et al., 2008a). Some immunosensors have exploited the

use of magnetic particles as supports for the immobilisation of antibodies or immunospecies on the electrodes, using different reaction chemistries (Dominguez et al., 2012; Hayat et al., 2011; Hayat et al 2012c). The use of magnetic particles increases the sensitivity and decreases the LODs, and may reduce the effects from the sample matrix. SPR immunosensors have also demonstrated to be appropriate for the determination of OA and analogues in shellfish samples using competitive formats (Llamas et al., 2007; Steward 2009a). Moreover, some immunosensors have been able to detect, not only OA, DTX-1 and DTX-2, but also DTX-3 without the need of a hydrolysis step, which results in an optimisation of the assay time (Steward et al., 2009b). Finally, SPR systems also allow multiplex analysis, as it has been demonstrated by the works performed on the simultaneous detection of toxins, such as OA, domoic acid (DA), saxitoxin (STX) and neosaxitoxin (neo-STX) (Campbell et al., 2011; McName et al., 2013).

Regarding biosensors for the detection of MCs, enzyme sensors have been developed based on the PP inhibition, immobilising the enzyme by entrapment, and choosing the appropriate enzyme substrate, electrochemically active only after dephosphorylation (Campàs et al., 2007c). Again, enzyme recycling decreased the LODs down to 755 times (Campàs et al., 2008b). Electrochemical immunosensors have also been developed, using both monoclonal and polyclonal antibodies immobilised on the electrode by adsorption (Campàs and Marty, 2007). These immunosensors attained lower LODs than the enzyme sensors and were applied to the analysis of cyanobacteria blooms. Nanotechnological components have also been incorporated into the electrochemical immunosensors for MCs.

Loyprasert and collaborators (Loyprasert et al., 2008), in the development of a label-free capacitance immunosensor, immobilised the antibody on silver nanoparticles previously deposited on the electrodes. This strategy provided 1.7-fold higher sensitivity and a very low LOD (7.0 pg/L). A similar performance and efficiency has been obtained when using gold nanoparticles (Dawan et al., 2011).

Optical SPR immunosensors have also been developed for the detection of MCs, usually achieving LODs higher than the electrochemical ones (Herranz et al., 2010; Hu et al., 2009; Vinogradova et al., 2011). Nevertheless, SPR immunosensors have the advantages of providing real-time measurements, robustness to matrix effects, and chip regeneration and reutilisation. Again, the ability to detect different MC congeners will depend on the antibody performance.

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General objective

The general objective of this thesis is to develop assays and biosensors for the detection of toxins from aquatic media and to study their applicability to the analysis of samples.

Specific objectives

The specific objectives of this thesis are:

- To characterise recombinant protein phosphatases obtained by genetic engineering.

- To develop and optimise protein phosphatase inhibition assays for the detection of okadaic acid and its analogues as well as microcystins.

- To exploit magnetic particles as biomolecule immobilisation supports and carriers in biosensors for the detection of toxins from aquatic media.

- To study the applicability of colorimetric assays and electrochemical and optical biosensors to the analysis of shellfish and cyanobacteria samples.

- To better understand the correlation of assays and biosensors with other analysis techniques.

Chapter 2

Protein phosphatase inhibition assays for the detection of toxins from aquatic environments

This chapter includes three published articles. The applicability of PPIAs with wild and recombinant enzymes to the detection of marine and freshwater toxins has been investigated. In the first article, the effects of shellfish matrices and the marine toxins (OA and the corresponding esters) extraction protocol on the PPIAs are evaluated. In the second article, the ability of the PPIAs to detect several marine toxin analogues (DTXs) is investigated and the implementation of the assays in monitoring systems is proposed. In the third article, the inhibitory potencies of several cyanobacterial toxins (MCs variants) are described.

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Protein phosphatase inhibition assays for okadaic acid detection in shellfish: Matrix effects, applicability and comparison with LC–MS/MS analysis

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Abstract

The applicability of the protein phosphatase inhibition assay (PPIA) to the determination of okadaic acid (OA) and its acyl derivatives in shellfish samples has been investigated, using a recombinant PP2A and a commercial one. Mediterranean mussel, wedge clam, Pacific oyster and flat oyster have been chosen as model species. Shellfish matrix loading limits for the PPIA have been established, according to the shellfish species and the enzyme source. A synergistic inhibitory effect has been observed in the presence of OA and shellfish matrix, which has been overcome by the application of a correction factor (0.48). Finally, Mediterranean mussel samples obtained from Ría de Arousa during a DSP closure associated to *Dinophysis acuminata*, determined as positive by the mouse bioassay, have been analysed with the PPIAs. The OA equivalent contents provided by the PPIAs correlate satisfactorily with those obtained by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Keywords: recombinant protein phosphatase 2A (PP2A) catalytic subunit, protein phosphatase inhibition assay (PPIA), okadaic acid (OA), liquid chromatography-tandem mass spectrometry (LC-MS/MS), shellfish.

2.1.1 Introduction

Okadaic acid (OA) and the dinophysistoxins (DTXs) derivatives are lipophilic marine toxins produced by microalgae of the *Dinophysis* and *Prorocentrum* genera (Steidinger, 1993), usually found in shellfish. The consumption of shellfish contaminated by OA and some DTXs causes diarrheic shellfish poisoning (DSP), human illness characterised by gastro-intestinal symptoms including diarrhoea, nausea and vomiting (Yasumoto and Murata, 1993).

Diarrheic lipophilic toxins are known to inhibit the activity of several serine/threonine protein phosphatases (PPs) (Bialojan and Takai, 1988). These toxins bind to PP in the hydrophobic region near to the active site, blocking their activity. As a consequence, hyperphosphorylation of the proteins that control sodium secre- tion by intestinal cells and of cytoskeletal or junctional moieties that regulate solute permeability is favoured, causing a sodium release and a subsequent passive loss of fluids, responsible for the diarrheic symptoms.

DSP toxic episodes are recurrent in coastal waters of European countries. Specifically in Spain, Galicia and Catalonia are very affected regions (Reguera et al., 2012). In order to protect public health, the Commission Regulation (EC) No 853/2004 has established a maximum permitted level (MPL) of 160 µg of OA equivalents/kg in bivalve molluscs. Until recently, the official control method was the mouse bioassay (MBA) (Yasumoto et al., 1978). This method has been successful for the management of shellfish controls because it gives an indication of the total toxicity of a sample. However, because of its lack of specificity, and controversy regarding its application (reference), a European Commission regulation (EC No. 15/2011) has recently approved the use of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method as the reference method for the detection of lipophilic toxins in live bivalve molluscs. In Europe this new regulation has been applied since 1st July 2011, although the MBA can still be used until 31st December 2014. This Commission Regulation also allows a series of methods, such as other chromatographic techniques with

appropriate detection, immunoassays and functional (e.g. phosphatase inhibition) assays, as alternatives or supplementary to the LC-MS/MS method, provided that either alone or combined they can detect the required analogues, that they fulfil the corresponding method performance criteria, and that their implementation provides an equivalent level of public health protection.

Based on the OA mechanism of action, protein phosphatase inhibition assays (PPIAs) for the determination of DSP toxins have been developed. Although radioactivity (Honkanen et al., 1996) and fluorescence detection has been used (Tsuchiya et al., 1997; Vieytes et al., 1997; Mountfort et al., 1999; Leira et al., 2000;Mountfort et al., 2001; González et al., 2002), the colorimetric detection method has received more acceptance (Tubaro et al., 1996; Della Loggia et al., 1999; Campàs and Marty, 2007; Albano et al., 2009; Caillaud et al., 2010; Cañete et al., 2010).

This functional method has gained acceptance because of its advantages in terms of simplicity, multiple sample analysis, sensitivity and limit of detection (LOD), and short analysis time. As a consequence, researchers are trying to improve and refine PPIAs from different perspectives. Undesirable shellfish and microalgae matrix effects have been diminished by the use of sample purification strategies, such as a previous chromatographic fractionation protocol (Caillaud et al., 2010; Cañete et al., 2010). The use of recombinant enzymes (Ikehara et al., 2010), some of them with improved sensitivity (Zhang et al., 1994), has also been exploited. The establishment of toxicity equivalent factors (TEFs) in alternative methods to MBA for marine toxin detection is necessary to guarantee consumer protection in monitoring programmes, as they allow a better estimation of the toxic potential of a mixture of toxins with different potency (Botana et al., 2010). With regards to this, the inhibitory potencies of different OA derivatives should be investigated. Nevertheless, in this work OA has been taken as reference compound of the diarrheic lipophilic toxins group.

The development of a PPIA using a recombinant PP2A catalytic subunit has already been described in a previous work, where the genetically-engineered enzyme was compared with a commercial one in terms of activity, stability and inhibition by OA (Garibo et al., 2012). In the present work, an exhaustive evaluation of the matrix loading effect on the PP2A activity was performed in order to apply the developed PPIA to the determination of OA and its acyl derivatives in shellfish samples. Results have also been compared with those obtained with a commercial PP2A. Mediterranean mussel, wedge clam, Pacific oyster and flat oyster have been chosen as model shellfish species. Samples determined as negative by the MBA for DSP toxins (toxin content lower than 160 µg OA eq/kg) and by LC-MS/MS analysis (diarrheic lipophilic toxin content lower than the limit of quantification, LOQ = 30 mg OA/kg shellfish meat) have been used for the study of the matrix effects. Once the matrix loading limits were established, the PPIA was applied to the analysis of naturally-contaminated mussels and the results were compared to those obtained by LC-MS/MS analysis.

2.1.2 Materials and Methods

2.1.2.1 Reagents and materials

Certified Reference Material of okadaic acid (CRM-OA) in methanol was purchased from the Institute for Marine Biosciences of the National Research Council (Halifax, Canada). The genetically-engineered PP2A catalytic subunit was produced by Gene to Protein (GTP) Technology (Toulouse, France) and contains a hexa- His tail at the C-terminus. A commercial protein phosphatase 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from human red blood cells, was obtained from Upstate Biotechnology (NY, USA). The activity of the stock solutions was between 766 and 1364 U/mL for GTP Technology and 5660 U/mL for Upstate Biotechnology, 1 U being defined as the amount of enzyme required to hydrolyze of 1 nmol p-nitrophenyl phosphate (p-NPP) in one min at room temperature.

Components of buffers and p-NPP were purchased from Sigma (Tres Cantos, España). For LC-MS/MS analysis, gradient-grade methanol, formic acid and hyper-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Ammonium formate (≥99.995%), sodium hydroxide pellets (≥99%) and hydrochloric acid 37% for analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA), Riedel-de Haën (Seelze, Germany) and Panreac (Barcelona, Spain), respectively. All solutions were prepared using Milli-Q grade water obtained from a Millipore purification system (Bedford, USA).

2.1.2.2 Shellfish samples

Three negative Mediterranean mussel (*Mytilus galloprovincialis*) samples according to LC-MS/MS analysis were obtained from Ebro Delta, Catalonia (NW Mediterranean, Spain) in August, September and December 2008. Two negative wedge clam (*Donax trunculus*) samples according to MBA for DSP toxins and LC-MS/MS analysis were obtained from the shellfish monitoring programme of Catalonia (Ebro Delta and Vilanova i la Geltrú) in October and November 2009. Three negative Pacific oyster (*Crassostrea gigas*) samples according to MBA for DSP toxins and LC-MS/MS analysis were obtained from the shellfish monitoring programme of Catalonia (Ebro Delta) in January and February 2010. Four negative flat oysters (*Ostrea edulis*) were used, two of them provided by Ostres de la Badia (Santa Pola, Alicante, SW Mediterranean, Spain) in January and June 2010, according to LC-MS/MS analysis, and the other two obtained from the shellfish monitoring programme of Catalonia (Ebro Delta) in December 2009 and January 2010, negative according to MBA for DSP toxins and LC-MS/MS analysis.

Twelve positive Mediterranean mussel (*M. galloprovincialis*) samples according to MBA for DSP toxins (Yasumoto et al., 1978) and to LC-MS/MS analysis were provided by Amegrove (O Grove, Spain) from Galicia (NE Atlantic Ocean, Spain) in August 2010. These samples were obtained from 4 different rafts (C1,

C2, C3, C4) at 1, 5 and 10 m depth during a DSP closure in Ría de Arousa, inforced between July 2010 and November 2010 by INTECMAR (INTECMAR, 2010). The samples were available because of the Amegrove's own-checks on biotoxins. Integrated phytoplankton samples (0-15 m) were collected from stations A0 (42º28'54''N, 08º57'48''W), at the entrance of the Ría de Arousa, and A8 (42º29'48''N, 08º55'36''W), close to the rafts, using a PVC hose and preserved with Lugol's solution. The Utermöhl method was used for phytoplankton identification and quantification (Utermöhl, 1958): 25 mL of sample were settled during 12 h. The chamber was examined for quantification of *Dinophysis* species and total phytoplankton.

2.1.2.3 Lipophilic toxins extraction

Crude shellfish extracts were prepared by extracting 2g (out of 100 g) of shellfish homogenate with 9 mL of MeOH for 2 min at 17,500 rpm with an Ultra-Turrax® T25 Digital by IKA®from Rose Scientific Ltd. (Alberta, Canada). Extracts were centrifuged at 3000 rpm for 10 min in a Jouan centrifuge at room temperature. Supernatants were removed. A second extraction was performed by the addition of 5 mL of MeOH to the solid residue and 3-min agitation with a vortex. After centrifugation under the same conditions, the two supernatants were joined and passed through a 0.2-mm cut-off Whatman nylon membrane filter (Brentford, United Kingdom). Samples were directly injected into the LC- MS/MS system. For samples to be tested with the PPIA, extracts were evaporated in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen at room temperature, and the residues were resuspended in a buffer solution containing 30 mM Tris–HCl, 20 mM $MgCl_2$, pH 8.4.

2.1.2.4 Sample hydrolysis

The protocol for the hydrolysis of lipophilic toxins in mussels was based on that of Mountfort et al., 2001. Briefly, 125 mL of NaOH at 2.5 N were added to 1.25 mL of extract and homogenised for 10 s with a vortex.

Extracts were then incubated at 76ºC for 40 min in a Multi-Block®Heater from Lab-Line Instruments, Inc. (Maharashtra, India). After cooling, 125mL of HCl at 2.5N were added and hydrolysed extracts were passed through a 0.2-mm cut-off Whatman nylon membrane. Samples were directly injected into the LC-MS/MS system. For samples to be tested with the PPIA, extracts were evaporated in a Speed VAC concentrator under nitrogen at room temperature, and the residues were resuspended in a buffer solution containing 30 mM Tris–HCl, 20 mM $MgCl_2$, pH 8.4.

2.1.2.5 Colorimetric PPIA

The colorimetric PPIA was performed as follows: 50 µL of OA solutions (for the calibration curves), shellfish extracts (for the evaluation of the matrix effects from negative samples or the determination of toxins in positive samples) or both (for the evaluation of a possible synergistic effect) at different concentra- tions (starting at 100 µg/L for OA, 200µg/mL for negative shellfish samples and at the matrix loading limit for positive mussel samples, and diluting to the half) were added in microtitre wells containing 100 µL of enzyme solution at 1.25 U/mL). Then, 50 mL of 25 mM p-NPP solution were added and after 1-h incubation at room temperature in the dark, the absorbance at 405 nm was measured with an automated multi-well scanning spectropho- tometer (Biotek, Synergy HT, Winooski, VT, USA). OA and shellfish extract solutions were prepared in a buffer solution containing 30 mM Tris–HCl, 20 mM $MgCl_2$, pH 8.4. Enzyme and substrate solutions were prepared in the same buffer, also containing 2 mM DTT and 0.2 mg/mL BSA. Controls without PP2A, OA or mussel extract were always used. Assays were performed in triplicate.

It is necessary to mention that the sensitivity of the assay is strongly affected by the protein phosphatase activity, and small enzyme activity variations may significantly change the inhibition percentages. Consequently, an OA calibration curve was always performed in parallel to each positive mussel sample analysis for the precise toxin quantification.

The OA calibration curves obtained by PPIA were analysed with SigmaPlot software package 10.0 (Systat Software, Inc., San José, CA, USA) and fitted to sigmoidal logistic four-parameter equations.

2.1.2.6 LC-MS/MS analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/ MS) analysis were conducted on an Agilent 1200 LC (Agilent Technologies, Santa Clara, USA) coupled with a 3200 QTRAP mass spectrometer through a TurboV™ electrospray ion source (Applied Biosystems, Foster City, USA). Chromatographic separations were performed at 30 ºC and 0.2 mL/min on a Luna C8(2) column (50 mm ×1 mm, 3 mm) protected with a SupelcoGuard C8(2) cartridge (4 mm× 2 mm, 3 mm), both from Phenomenex (Torrance, USA). Other conditions followed the Harmonised Standard Operation Procedure (SOP) for LC-MS/MS analysis of lipophilic toxins proposed by the European Reference Laboratory (EURLMB, 2011), recently validated and recognised as the reference method for the analysis of this group of toxins. Acidic chromatographic elution was selected with mobile phases 100% water (A) and 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM formic acid. Other conditions related with MS/MS detection were optimised directly through direct infusion of the CRM-OA standard. For DSP toxins, multiple reaction monitoring (MRM) analysis was accomplished from the precursor ions 803.5 and 817.5 *m/z* for OA/DTX-2 and DTX-1, respectively. Product ions were common for all DSP toxins, with ions 255.2 *m/z* used for quantification and 113.1 or 209.2 *m/z* used for confirmatory purposes. The LOD and LOQ were at least 10 and 30 µg/kg OA in shellfish, respectively.

2.1.2.7 Statistical analyses

To evaluate differences in the matrix loading effect on the PP2A activity for the four shellfish species (non-hydrolysed and hydrolysed Mediterranean mussel, wedge clam, Pacific oyster and flat oyster) and the two enzymes (GTP and Upstate), the PP2A inhibition percentage was compared between both enzymes

for each species, and between species for each enzyme, using a paired *t*-test at the different shellfish matrix loading levels. The paired *t*-test was also used to evaluate differences in the PP2A inhibition when spiking buffer, non-hydrolysed and hydrolysed Mediterranean mussel samples, for both enzymes. Differences in the results were considered statistically significant at the 0.05 level. Prior to analysis, data were tested for normality; Wilcoxon matched-pairs signed-ranks test was used for non-normally distributed data sets instead of the paired *t*-test. One-way analysis of variance was used to detect possible differences between correction factors for each PP2A (GTP and Upstate) and Mediterranean mussel sample (non-hydrolysed and hydrolysed) pair. The two-way ANOVA test was also used to evaluate differences among sampling stations and depths in the free and total DSP contents determined by LC-MS/MS analysis. The SigmaStat software was used for the paired *t*-tests and the two-way ANOVA tests.

To describe how PP2A inhibition changes with DSP toxin content in shellfish samples, and to predict the DSP toxin content (that would be determined by LC-MS/MS) for a given value of PP2A inhibition, different non-linear regression models were tested using the program Statgraphics Centurion XVI. The models were adjusted to each of the 4 sets of results: free DSP and total DSP content for each of the 2 enzymes versus the value determined by LC-MS/MS. Each set of results contains 12 data points. The regression was considered statistically significant at the 95% confidence level.

2.1.3 Results

2.1.3.1 Shellfish matrix charge effect on the PP2A activity

Mediterranean mussel, wedge clam, Pacific oyster and flat oyster were chosen as model shellfish species for the applicability study. Samples determined as negative by LC-MS/MS analysis (diarrheic lipophilic toxin content lower than the LOQ = 30µg OA and derivatives/kg) and in some cases also by MBA for DSP toxins (toxin content lower than 160µg OA eq/kg) were chosen to evaluate the

effect of the sample matrix on the enzyme activity. Experiments were performed with a recombinant PP2A (from GTP) and a commercial PP2A (from Upstate) in order to compare their robustness to matrix effects. The PPIA was performed as normal, only changing the OA standard solution by shellfish matrix with no toxin content. Fig. 2.1.1 shows the PP2A inhibition percentage at different shellfish matrix concentrations. A 10% inhibition threshold was established, lower inhibition percentages being considered as non-significant.

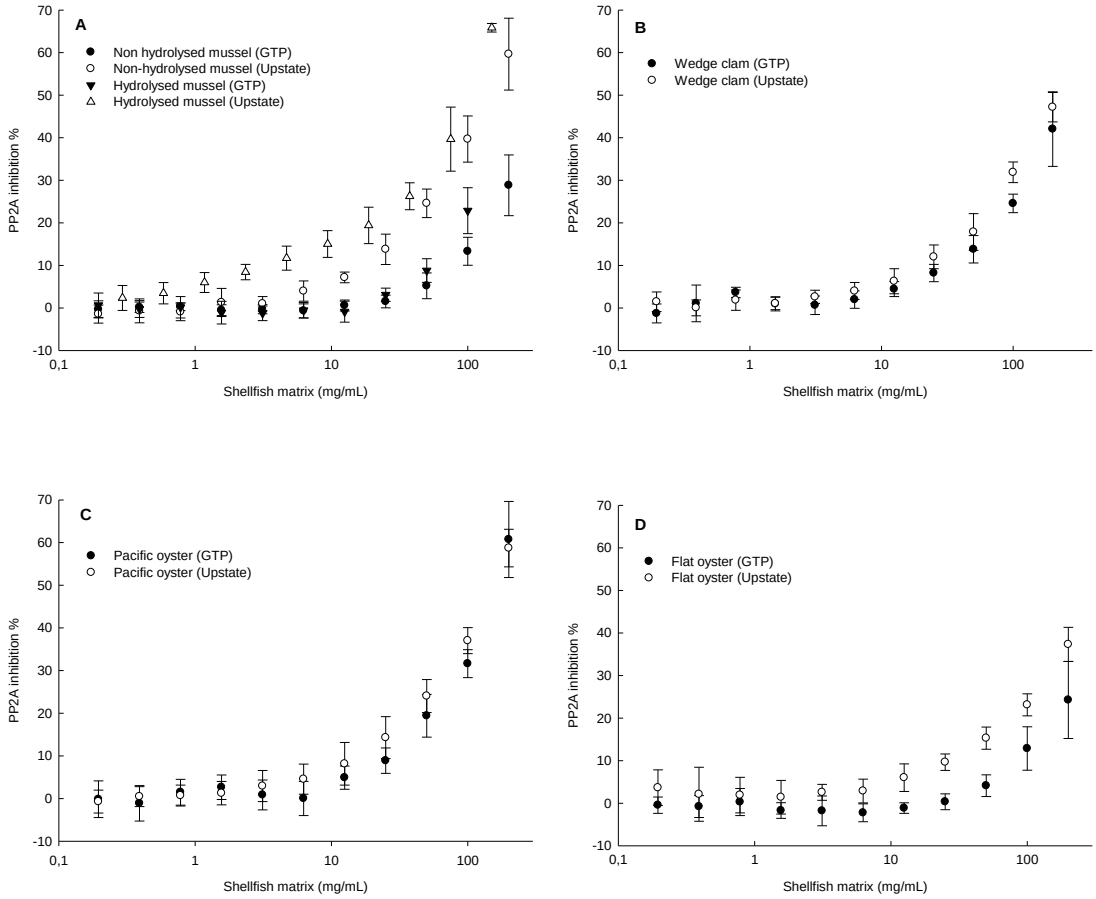

Fig. 2.1.1. PP2A inhibition percentage at different matrix concentrations of non-hydrolysed and hydrolysed Mediterranean mussel (A), non-hydrolysed wedge clam (B), non- hydrolysed Pacific oyster (C) and non-hydrolysed flat oyster (D) with PP2A from GTP and Upstate.

As expected, the same pattern was observed in all cases: the enzyme activity was not affected at low matrix concentrations and started to increase exponentially at high matrix concentrations. This matrix effect is not due to the colour of the shellfish samples (controls without enzyme are performed in parallel in order to subtract the corresponding absorbance value), but to the presence of compounds that alter the natural environment of the enzyme and thus its functionality.

There were statistically significant differences in the inhibition percentages between enzymes for each species (Mediterranean mussel: t = 2.6, p = 0.02, df =10; wedge clam: t = 2.9, p = 0.01, df = 10; Pacific oyster: t = 2.3, p = 0.04, df =10; flat oyster: t = 5.6, p = 0.001, df = 10), PP2A from GTP being able to operate under higher shellfish matrix concentrations.

There were statistically significant differences in the inhibition percentages between species for PP2A from GTP except for the wedge clam-Pacific oyster pair (Mediterranean mussel-wedge clam: t = -3.4, p = 0.01, df = 10; Mediterranean mussel-Pacific oyster: W = 56, p = 0.01; Mediterranean mussel-flat oyster: W = -64, p = 0.01; wedge clam-Pacific oyster: W = 24, p = 0.32; wedge clam-flat oyster: t = 3.7, p = 0.01, df = 10; Pacific oyster-flat oyster: t = 2.6, p = 0.03, df = 10). There were not statistically significant differences in the inhibition percentages between species for PP2A from Upstate except for the Mediterranean mussel-flat oyster pair (Mediterranean mussel-wedge clam: t = 1.3, p = 0.21, df = 10; Mediterranean mussel-Pacific oyster: t = -0.7, p = 0.47, df = 10; Mediterranean mussel-flat oyster: W = -18, p = 0.46; wedge clam-Pacific oyster: t = -1.9, p = 0.08, df = 10; wedge clam-flat oyster: t = 1.5, p = 0.16, df = 10; Pacific oyster-flat oyster: t = 1.8, p = 0.10, df = 10). The level of confidence used to determine significance was 95% (p < 0.05). Taking into consideration the inhibition percentages observed at the three highest matrix loadings, it is possible to list shellfish species from higher to lower matrix effect: ‘‘Pacific oyster > wedge clam > Mediterranean mussel > flat oyster’’ for PP2A from GTP and ‘‘Mediterranean mussel > Pacific oyster > wedge clam > flat oyster’’ for PP2A from Upstate.

In the case of the Mediterranean mussel, the effect of hydrolysed extracts was also evaluated in order to study the applicability of the developed PPIAs to the determination of total DSP toxin content (including OA acyl derivatives) (Fig. 2.1.1A). There were statistically significant differences between hydrolysed and non-hydrolysed samples for PP2A from Upstate (W = 66, p < 0.001) but not for PP2A from GTP (t = -1.1, p = 0.26, df = 9). There were statistically significant differences between enzymes for hydro- lysed (t = -4.6, p = 0.001, df = 9) and non-hydrolysed mussel samples (t = 2.6, p = 0.02, df = 10). Whereas the effect of hydrolysed mussel samples was minor for the recombinant PP2A, the commercial enzyme was drastically affected.

From results obtained in Fig. 2.1.1, standardised matrix loading limits in view of application in routine monitoring programmes were established depending on the enzyme source and the shellfish species (Table 2.1.1).

Table 2.1.1 Loading limits and LOD_{10} for OA in different shellfish matrices.

Shellfish sample	Enzyme	Loading limit (mg/mL)	OA LOD_{10} (µg/kg)
Non-hydrolysed mussel	GTP	50.0	22
	Upstate	12.5	111
Hydrolysed mussel	GTP	25.0	44
	Upstate	2.3	604
Non-hydrolysed wedge clam	GTP	12.5	88
	Upstate	12.5	111
Non-hydroliysed pacific oyster	GTP	12.5	88
	Upstate	6.3	222
Non-hydrolysed flat oyster	GTP	50.0	22
	Upstate	12.5	111

2.1.3.2 OA calibration curves by PPIA

As previously mentioned, a calibration curve was always performed in parallel to each positive mussel sample analysis, due to possible slight differences in the inhibition percentages from assay to assay. The OA calibration curves were described by the sigmoidal logistic four-parameter equation (SigmaPlot software package 10.0):

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

where a and $y_0$are the asymptotic maximum and minimum values, respectively, x_0 is the$x$ value at the inflection point and b is theslope at the inflection point. Taking into account the 10% inhibition values of OA calibration curves and the established loading limits, theoretical limits of detection (LODs) were calculated. In Table 2.1.1 the LODs for both enzymes combined with the different shellfish matrices, including hydrolysed mussel, are shown.

2.1.3.3 Analysis of positive mussel samples by PPIA and LC-MS/MS

The PPIAs with recombinant and commercial PP2A were applied to the determination of free and total DSP toxins in twelve non-hydrolysed and hydrolysed Mediterranean mussel samples, respectively. In the determination of OA equivalent contents in mussels, the IC_{50}values of the OA calibration curves determined from the sigmoidal logistic four-parameter equations and the IC_{50} values of the mussel extract dose-response curves determined from lineal regressions were used. The IC_{50} values of the mussel extract curves were determined from lineal regressions because some samples (those with low toxin contents) did not attain the sigmoidal shape (results not shown). In those samples where the highest mussel loading induced an inhibition percentage lower than 50%, the determination of OA equivalent contents was performed taking into account the inhibition percentage obtained at that highest mussel loading (and the corresponding inhibition percentage of the OA calibration curve). In the case of the analysis of hydrolysed mussel samples with PP2A from Upstate, where all concentrations tested were higher than the matrix loading limit, the determination of OA equivalent contents wasperformed taking into account the inhibition percentage obtained at the lowest mussel loading (and the corresponding inhibition percentage of the OA calibration curve). Table 2.1.2 shows the OA content estimates obtained by LC-MS/MS analysis and by the PPIA with both enzymes. According to LC-MS/MS analysis, there were not statistically significant differences among sampling rafts (free DSP: p = 0.49, total DSP: p = 0.23), nor among sampling depths (free DSP: p = 0.24, total DSP: p = 0.07). Inhibition enzyme assays slightly overestimated toxin content compared to

LC-MS/MS analysis, the overestimation being usually more evident with the Upstate enzyme.

Table 2.1.2 Free and total DSP toxin contents (µg/kg) in Mediterranean mussel samples determined by LC-MS/MS and PPIA with PP2A from GTP and Upstate (values non-corrected and corrected with the 0.48 factor). Relative standard deviation values were not shown for clarity of the results and were always below 10%.

		Free DSP				Total DSP			
		C1	**C2**	**C3**	**C4**	**C1**	**C2**	**C3**	**C4**
LC-MS/MS	1m	165	302	319	340	193	400	331	416
	5m	204	401	666	504	219	657	681	679
	10m	462	512	305	449	631	660	436	655
Non-corrected PPIA									
GTP	1m	235	511	410	425	307	416	498	490
	5m	214	928	972	710	313	1029	975	1094
	10m	1014	1146	405	602	1383	1367	544	1256
Upstate	1m	426	661	574	706	435	466	542	1027
	5m	356	1213	944	1358	413	1303	1002	2095
	10m	891	987	479	1210	1390	1417	688	3119
Corrected PPIA									
GTP	1m	113	245	197	204	147	200	239	235
	5m	103	445	467	341	150	494	468	525
	10m	487	550	194	289	664	656	261	603
Upstate	1m	205	317	276	339	209	224	264	493
	5m	171	582	453	652	198	638	481	1006
	10m	428	474	230	581	667	680	330	1497

This overestimation was investigated in detail. Fig. 2.1.2 shows the OA calibration curves obtained in the PPIA with both enzymes in the absence and presence of non-hydrolysed and hydrolysed Mediterranean mussel extracts.

The presence of mussel matrix, at a concentration below the matrix loading limit (12.5 mg/mL for PP2A from GTP and 6.25 mg/mL for PP2A from Upstate), increases the inhibition percentage due to the OA, shifting the calibration curves towards lower OA concentrations. When using PP2A from GTP there were statistically significant differences between the buffer and the non-hydrolysed mussel samples (W = 76, p < 0.001), and between the buffer and the hydrolysed mussel samples (W = 78, p < 0.001), but the differences were not statistically significant between hydrolysed and non-hydrolysed mussel samples (t = 1.1, p = 0.26, df = 12). In the same way, when using PP2A from Upstate there were statistically significant differences between the buffer and the non-hydrolysed mussel samples (W = 76, p < 0.001) and between the buffer and the hydrolysed mussel samples (t = -2.4, p = 0.03, df = 11), but the differences were not statistically significant between hydrolysed and non- hydrolysed mussel samples (t = 0.07, p = 0.93, df = 12).

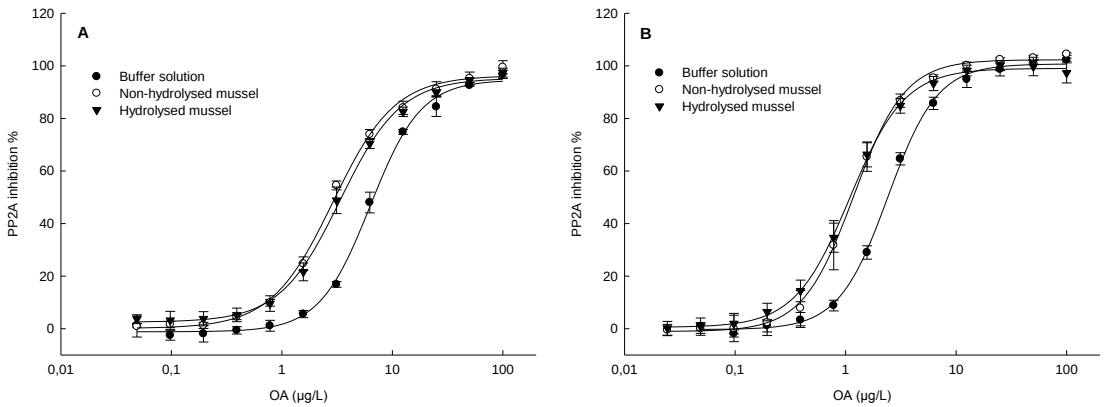

Fig. 2.1.2. PP2A inhibition percentage at different OA concentrations in buffer solution, non-hydrolysed and hydrolysed mussel with PP2A from GTP (A) and Upstate (B).

For the quantification of this synergistic inhibitory effect, the IC_{50} ratio between the OA calibration curve in the presence of matrix and the OA calibration curve in buffer was calculated. Since there were not statistically differences (p = 0.61) in the IC_{50} ratio for each PP2A (GTP and Upstate) and mussel sample (non-hydrolysed and hydrolysed) pair, the average IC_{50}ratio of 0.48 was applied as a correction factor for all enzymes and mussel samples to improve the OA quantifications (Table 2.1.2). The synergistic effect was also observed with non-

hydrolysed extracts of wedge clam, Pacific oyster and flat oyster (results not shown). For both enzymes, whereas Pacific oyster and flat oyster produce approximately the same synergistic effect than mussel, the effect of wedge clam is more important.

The corrected OA equivalent contents determined by the PPIA were correlated to the values obtained in the LC–MS/MS analysis. The best models for the regressions were ‘‘Double reciprocal’’ and ‘‘Square root-Y reciprocal-X’’ for the analysis of free and total DSP, respectively. The percentage of the variability in the predicted variable that has been explained by the models is between 75.62 and 95.01% for free DSP using the enzyme from Upstate and total DSP measured using the enzyme from GTP, respectively. Results are shown in Table 2.1.3.

Table 2.1.3 Regression equations, correlations and percentages of variability for the correlations between the corrected OA equivalent contents determined by the PPIA with GTP and Upstate enzymes and the values obtained in the LC-MS/MS analysis of mussel samples, in relation to free and total DSP toxin contents.

	Enzyme	Equation	Correlation	R^2
Free DSP	GTP	$y= 1/(0.000923724+0.474349/x)$	0.9354	87.50%
	Upstate	$y= 1/(0.000486659+0.826633/x)$	0.8696	75.62%
Total DSP	GTP	$y= 1/(29.6028-2238.44/x)^2$	-0.9747	95.01%
	Upstate	$y= 1/(28.8389-2664.06/x)^2$	-0.9163	83.97%

With regards to phytoplankton, *Dinophysis acuminata* was present in the area from April to October 2010 (Fig. 2.1.3). Other *Dinophysis* species present during the event were *D. caudata* and *D. rotundata* in very low abundances. Other dinoflagellate species known as potentially producers of DSP lipophilic toxins were absent.

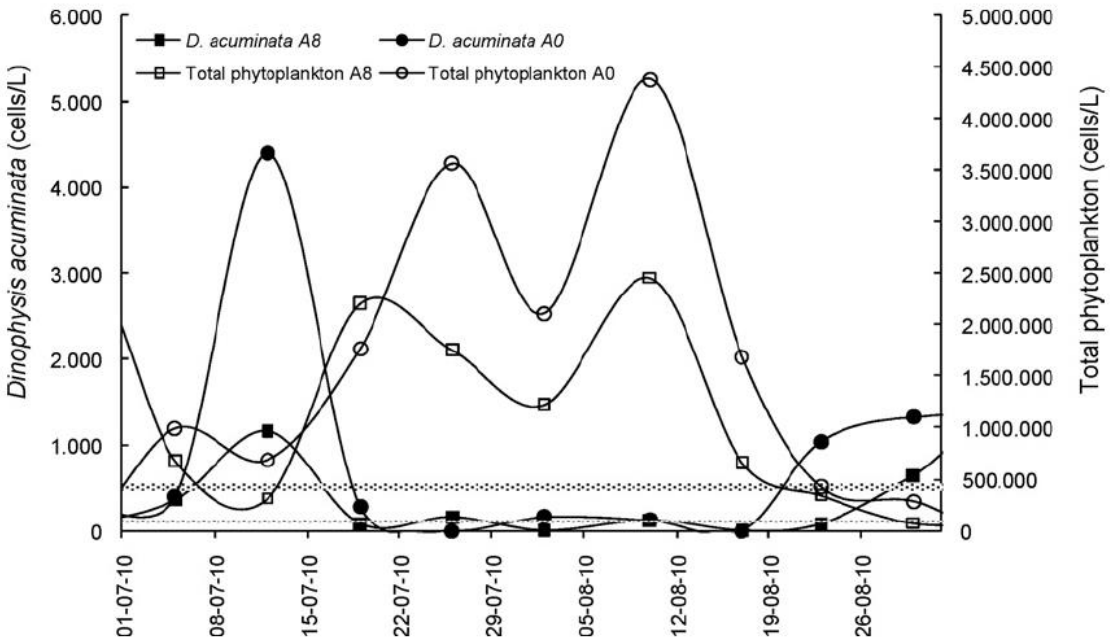

Fig. 2.1.3. Phytoplankton abundance (*Dinophysis acuminata* and total phytoplankton) in A0 and A8 stations near the rafts were shellfish samples were collected. Thick grey line at 500 cells/L indicates the alert value for *Dinophysis acuminata*. Thin grey line at 120 cells/L indicates the *Dinophysis acuminata* minimal abundance associated to DSP events in the Galician Rías.

2.1.4 Discussion

As expected, even if there is no OA in the shellfish sample, high concentrations of shellfish matrix interfere with the PPIA. This is not only due to the matrix colouration, effect that can be neglected by the use of appropriate controls, but presumably to the effect of matrix compounds other than diarrheic lipophilic toxins on the PP2A activity. Certainly, the presence of these compounds, when used in high concentrations, modifies the enzyme environment and may alter its functionality. Consequently, the establishment of matrix loading limits is necessary to guarantee the precision of the OA contents calculated from the PPIA.

Although the sensitivity of PPIAs may slightly vary from one day to another, in relation to both the OA inhibition and the matrix loading effect, results clearly

show that the recombinant PP2A from GTP is able to operate under higher shellfish matrix loadings than the PP2A from Upstate. In the case of hydrolysed mussel, the effect on the enzyme activity was minor when using the recombinant enzyme but it was drastic for PP2A from Upstate. Since no acyl derivatives of DSP toxins were detected in the LC-MS/ MS analysis of the samples used as the negative control, the stronger effect of hydrolysed matrix extracts must be due to the hydrolysis process itself (reagents and/or extract heating). The compounds used in the hydrolysis, even after evaporation of the solvents and re-dissolution in the appropriate buffer, may be inhibiting the PP2A activity. Or the heating step is somehow modifying the shellfish matrix, enhancing inhibition of the enzyme. The LODs calculated taking into account the OA calibration curves and the matrix loading limits indicate that, in principle, PP2A from Upstate could not be used for the determination of OA contents near the regulatory limit in Pacific oyster or hydrolysed Mediterranean mussel samples (nevertheless, OA contents in hydrolysed mussel samples have been determined with both enzymes in this work, as explained below and reported in Table 2.2.2).

In the application of the developed PPIAs to the determination of OA and its acyl derivatives in mussel samples, an overestimation in relation to LC-MS/MS analysis was observed, as we also mentioned in a previous work (Cañete et al., 2010). It seems that the overestimation has its origin in the shellfish matrix, since its presence, even at concentrations that should not inhibit, produces a synergistic effect to the OA inhibition. Nevertheless, it has not been possible to identify the reason. The presence of some compounds in the shellfish matrix, such as methanol-soluble lipids, has been hypothesised to exert an unspecific inhibitory effect on the PP (Honkanen et al., 1996). However, we consider that this hypothesis does not explain the synergistic effect, since the matrix presence would have inhibited the PP even in the absence of OA (and it did not at the matrix concentrations used in the assay). Neither are the possible presence of proteases a likely cause, since they would have also been detected

in the absence of OA (in any case, the use of protease inhibitors in the assay did not induce any significant change). The overestimation of PPIA in relation to LC-MS/MS has been previously reported and attributed to the possible presence of an unidentified interfering compound (Mountfort et al., 1999; González et al., 2002). These authors reported higher OA contents from the PPIA than from HPLC-FLD, which was attributed to the presence of some OA derivatives not detectable by HPLC-FLD (since no hydrolysis was performed) or non-OA-related inhibitors. Mountfort et al. (2001) also described the overestimation of PPIA in relation to HPLC-FLD analysis, although these authors described it as an underestimation of the HPLC-FLD analysis. In this case, they attributed the underestimation to possible losses in yield at critical stages of sample preparation during the extraction protocol, because they considered it unlikely that the PPIA overestimated the OA contents as yields in experiments with spiked shellfish closely matched theoretical yields. Consequently, we considered it appropriate to apply the correction factor established from the ratio between the IC_{50} ratio between the OA calibration curve in the presence of matrix and the OA calibration curve in buffer. In fact, it would have been possible to neglect the correction factor if a matrix-matched standard calibration had been performed.

Once the synergistic effect is overcome by the application of the correction factor to the quantitative results, the performance of each one of the enzymes in relation to the LC-MS/MS analysisestimation for non-hydrolysed and hydrolysed samples can be compared. Results obtained with PP2A from GTP correlate better with LC-MS/MS analysis than those obtained with PP2A from Upstate. This may be related to the previous observation about the higher robustness of PP2A from GTP compared to PP2A from Upstate. As expected, correlations for the analysis of total DSP were better than those for free DSP. This may be due to the fact that in the analysis of free DSP, LC-MS/MS is not detecting the presence of acyl derivatives (which are detected by LC-MS/MS in hydrolysed extracts), whereas they may be inhibiting the enzyme.

Consequently, the PPIA is interesting because it gives a measure of the total inhibitory potency of a sample, including that from the OA acyl derivatives, which are not detected by LC-MS/MS unless the hydrolysis step is performed.

In the application of the developed PPIA as screening tool, it is necessary to prevent false negative results. Looking at Table 2.1.2, two samples (C1-1 m and C1-5 m) were determined to have less than 160 µg OA eq/kg shellfish meat by the PPIA with the recombinant enzyme (if the correction factor is considered). In order to be safe, one can consider as appropriate the quantifications without the correction factors or assess a ‘‘suspicious area’’ for samples with toxin contents near the regulatory limit, which should be further analysed by LC-MS/MS. Only one ‘‘negative’’ mussel sample but with toxin contents lower than 160 mg/kg was analysed (not quantifiable free DSP toxins and 49 µg total DSP toxins/kg by LC-MS/MS). The PPIAs resulted in 14 µg/kg and 40 µg/kg (corrected values) with PP2A from GTP and Upstate, respectively. Although more ‘‘negative’’ mussel samples with toxin contents close to the MPL should be analysed, one can hypothesise that the ‘‘suspicious area’’ could be comprised between 80 and 160 µg/kg for PP2A from GTP and between 150 and 220 µg/kg for PP2A from Upstate. Moreover, although the purpose of this work was to perform an exhaustive study to better understand our system, in order to make the screening faster, only one shellfish sample concentration (dose that induces 50% PP2A inhibition for samples with toxin content of 160 µg/kg) should be analysed.

With regards to phytoplankton, one month before the collection of the shellfish samples, a maximum of *D. acuminata* was recorded. In July, the abundance of this species in the integrated samples of both stations was higher than 500 cells/L, recommended action limit for *D. acuminata*, which suggests closure or intensified monitoring (Anderson et al., 2001). In August (at the moment of sampling), however, the abundance of this species was lower than 500 cells/L. Nevertheless, the abundance was around 120 cells/L, and this concentration has already been associated to accumulation of lipophilic toxins in mollusks above the legal limit and thus requiring the area closure (Pazos and Moroño, 2008).

Consequently, the toxin profile of the positive Mediterranean mussel samples, corresponding to OA and its acyl derivatives, was likely due to the presence of *D. acuminata*, known as OA producer (Lee et al., 1989; FAO/IOC/WHO, 2004), and which moreover has been the main problem in the Spanish Rías (Reguera et al., 2012).

2.1.5 Conclusions

The applicability of PPIA assays to the determination of OA and its acyl derivatives, as reference compounds of the lipophilic toxins group, in shellfish samples has been investigated. Although in terms of sensitivity no differences were observed between enzymes, our recombinant PP2A catalytic subunit was less prone to inhibitory matrix effects, especially when analysing hydrolysed mussel samples. Shellfish matrix loading limits have been established in order to guarantee the accuracy in the determination of OA equivalent contents. A synergistic effect between shellfish matrix and OA has been observed and overcome by the establishment of a correction factor. Results obtained by the PPIAshave been compared to those obtained by LC-MS/MS. Good agreements have been observed, especially for PP2A from GTP and hydrolysed samples, where total DSP toxin contents were determined.

As a result, a useful analysis tool for the determination of OA equivalent contents in shellfish has been produced. This tool allows the analysis of a large number of samples simultaneously and with short analysis times. The low cost per sample and ease of the procedure make this tool useful in monitoring programmes to control shellfish toxicity, in parallel to LC-MS/MS analysis. For example, PPIA could be used to analyse hydrolysed shellfish samples (necessary to determine acyl ester derivatives of OA, DTX-1 or DTX-2, globally know as DTX-3), increasing the sample throughput, decreasing the demand of expensive instrumental equipment in control laboratories, with benefit for large monitoring programmes, and improving the efficiency of these monitoring programmes and public health protection due to the reduction of the response time. Additionally, the analysis of a higher number of shellfish samples (because of higher sampling

rates, larger geographic areas and/or different sampling depths) could be performed, favouring representativeness in surveillance systems. This may be of especial relevancy where oceanographic conditions (stratification, currents) or phytoplankton population dynamics enhance the risk of contamination.

Although the use of sample purification protocols after toxin extraction can be used to diminish the effect of the matrix on the enzyme activity, this was not our purpose. The work is aimed at evaluating the applicability of the developed PPIAs assays as fast analysis tools with minimum sample treatment. Nevertheless, such purification protocols may be envisaged in order to decrease the LODs and apply the developed PPIA to the analysis of low/trace toxin contents in phytoplankton or shellfish with research purposes.

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Inhibition Equivalency Factors for Dinophysistoxin-1 and Dinophysistoxin-2 in Protein Phosphatase Assays: Applicability to the Analysis of Shellfish Samples and Comparison with LC-MS/MS

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Supporting Information

Abstract

The protein phosphatase inhibition assay (PPIA) is a well-known strategy for the determination of diarrheic shellfish poisoning (DSP) lipophilic toxins, which deserves better characterization and understanding to be used as a routine screening tool in monitoring programs. In this work, the applicability of two PPIAs to the determination of okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), and their acyl ester derivatives in shellfish has been investigated. The inhibitory potencies of the DSP toxins on a recombinant and a wild PP2A have been determined, allowing the establishment of inhibition equivalency factors (IEFs) (1.1 and 0.9 for DTX-1, and 0.4 and 0.6 for DTX-2, for recombinant and wild PP2A, respectively). The PPIAs have been applied to the determination of OA equivalent contents in spiked and naturally contaminated shellfish samples. Results have been compared to those obtained by LC-MS/MS analysis, after application of the IEFs, showing good agreements.

***Keywords*:** protein phosphatase 2A, protein phosphatase inhibition assay, okadaic acid, dinophysistoxin-1, dinophysistoxin-2, liquid chromatography-tandem mass spectrometry.

2.2.1 Introduction

Okadaic acid (OA) and its analogues dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) are lipophilic phycotoxins produced mainly by dinoflagellates of the genera *Dinophysis* and *Prorocentrum*.[1] Their chemical structure is composed of a polyketide backbone containing furane and pyrane-type ether rings and an alpha-hydroxycarboxyl function, the difference between analogues being only the number or the position of the methyl groups.[2] When incorporated in shellfish, these phycotoxins are accumulated mainly in the digestive gland and are responsible for the diarrheic shellfish poisoning (DSP) syndrome, which causes gastrointestinal disturbances such as diarrhea, nausea, vomiting and abdominal pain.[3]

OA and DTXs are known inhibitors of protein phosphatases (PP1 and PP2A), enzymes that play an important role in protein dephosphorylation in cells.[4] These toxins bind to the receptor site of the enzyme, blocking its activity, and as a consequence they favour hyperphosphorylation of proteins that control sodium secretion and of cytoskeletal or junctional moieties that regulate solute permeability, causing sodium release and a subsequent passive loss of fluids responsible for the diarrheic symptoms.[5] Moreover, it has been demonstrated to be an additional tumour promoter in mouse skin carcinogenesis.[6]

Because of their implications on public health, the Regulation (EC) No. 853/2004 in Europe has established a maximum permitted level of 160 µg of OA equivalents/kg shellfish meat.[7] Although it is possible to use the mouse bioassay (MBA)[8] until December 31st, 2014, the Commission Regulation (EU) No. 15/2011 has recently established that liquid chromatography-tandem mass spectrometry (LC-MS/MS) should be applied as the reference method for the determination of lipophilic toxins contents in shellfish.[9] This Regulation was applied from July 1st, 2011, and the LC-MS/MS method will replace the MBA in 2015. These Regulations also accept the use of other chemical methods, as well as immunoassays and functional assays, such as the protein phosphatase

inhibition assay (PPIA), as alternatives or supplementary to the LC-MS/MS method, provided that they can determine OA, DTX-1, DTX-2 and their esters, that they fulfil the method performance criteria (they should be validated and successfully tested under a recognised proficiency test scheme), and that their implementation provides an equivalent level of public health protection.

The development of rapid, sensitive and low-cost methods for the detection of DSP toxins is necessary to guarantee shellfish safety and protect human health. The PPIA is an interesting method for the simple, fast, sensitive and robust determination of DSP toxins contents in shellfish. Colorimetric PPIAs using PP in solution have been developed.[10-17] In most of the works, OA has been used as model DSP toxin,[10,11] and only in few studies the inhibitory potential of OA analogues or derivatives has been evaluated and none of them has used high-quality certified reference materials.[12-17] The establishment and use of toxicity equivalent factors (TEFs) for toxic compounds of a same group in alternative methods for marine toxin detection is necessary to guarantee consumer protection in monitoring programs, as they allow a better estimation of the toxic potential of a mixture of toxins with different potency.[18-19]

Apart from the use of the PPIA for quantitative purposes, this assay is a promising screening tool to be run in parallel to the official control methods in monitoring programs. For example, PPIA could be used to screen DSP toxins in hydrolysed shellfish samples, reducing the instrumental analytical requirements and stillprotecting public health. Nevertheless, it requires in-depth characterisation and performance evaluation before its approval and routine use. With this aim, we have evaluated the practical application of the PPIA to the analysis of shellfish contaminated with DSP toxins. First, the different inhibitory potencies of OA, DTX-1 and DTX-2 on a recombinant and a wild PP2A have been determined and the corresponding inhibition equivalency factors (IEFs) have been established. Definition of IEFs is important to characterise the performance of the PPIA, but it is also crucial to get comparable results with the reference LC-MS/MS method.

Afterward, both PPIAs have been applied to the determination of DSP toxins contents in mussel samples spiked with OA, DTX-1 and/or DTX-2 and in naturally-contaminated shellfish (mussels, cockles, clams and razor clams) samples. Results have been compared with those obtained by LC-MS/MS analysis after the application of the IEFs.

2.2.2 Materials and Methods

2.2.2.1 Reagents and materials

Certified calibration solution of okadaic acid (NRC CRM-OA, 14,300 µg/L), dinophysistoxin-1 (NRC CRM-DTX1, 15,100 µg/L) and dinophysistoxin-2 (NRC CRM-DTX2, 7,800 µg/L) in methanol (MeOH) were kindly provided by the Institute for Marine Biosciences of the National Research Council (Halifax, Canada). The recombinant protein phosphatase 2A (PP2A) catalytic subunit was produced by Gene to Protein (GTP) Technology (Toulouse, France). Commercial protein phosphatase 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from human red blood cells, was obtained from Upstate Biotechnology (New York, USA). The activity of the stock solutions was between 766 and 1,025 U/mL for PP2Afrom GTP Technology and between 5,720 and 7,491 U/mL for PP2A from Upstate Biotechnology, 1 U being defined as the amount of enzyme required to hydrolyse of 1 nmol *p*-nitrophenyl phosphate (*p*-NPP) in 1 min at room temperature. Components of buffers and *p*-NPP were purchased from Sigma (Tres Cantos, España). For LC-MS/MS analysis, gradient-grade MeOH, formic acid and hyper-grade acetonitrile (ACN) for LC-MS were purchased from Merck (Darmstadt, Germany). Ammonium formate (≥99.995%), sodium hydroxide pellets (≥99%) and hydrochloric acid 37% for analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA), Riedel-de Haën (Seelze, Germany) and Panreac (Barcelona, Spain), respectively. All solutions were prepared using Milli-Q grade water obtained from a Millipore purification system (Bedford, USA).

2.2.2.2 Samples

Fourteen samples obtained from the European Union Reference Laboratory for Marine Biotoxins (EU-RL-MB) in Vigo, Spain, and that had been analysed for the collaborative Interlaboratory Validation Study of the "EU-Harmonised Standard Operation Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS" (EU-RL-MB SOP),[20] were used in this work. They corresponded to seven materials distributed as blind duplicates of different species of molluscs naturally contaminated: raw wedge shell clam homogenate (*Donax trunculus*), raw razor clam homogenate (*Ensis acuatus*), raw mussel homogenate (*Mytilus edulis*), raw stripped venus (*Chamelea gallina*), two cooked mussel homogenates (*Mytilus edulis*) and raw cockle homogenate (*Cerastoderma edule*).

2.2.2.3 Lipophilic toxin extraction

For lipophilic toxins extraction, the "EU-Harmonised Standard Operation Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS" (EU-RL-MB SOP) procedure was followed.[20] Firstly, tissue homogenate (2 g) was weighed in a 50-mL polypropylene centrifuge tube. 100% MeOH (9 mL) was added and sample was homogenised by vortex-mixing for 3 min at maximum speed level (~2500 laps/min). Extract was centrifuged at 2000*g* for 10 min at ca. 20 °C and the supernatant was transferred to a 20-mL volumetric flask. The extraction of the residual tissue pellet was repeated with 100% MeOH (9 mL) and sample was homogenised for 1 min with a high-speed homogeniser Ultraturrax T25 (IKA-Labortechnik). After centrifugation under the same conditions than previously applied, the supernatant was transferred and combined with the supernatant from the previous extraction, and the total extract was made up to 20 mL with 100% MeOH in a volumetric flask. Extracts were passed through 0.2 µm cut-off nylon syringe filters (Whatman), and were directly injected onto the LC-MS/MS system. For extracts to be tested with PPIA, samples were evaporated in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen at room temperature, and the residues were resuspended in the corresponding buffer.

2.2.2.4 Sample hydrolysis

In order to determine the total OA and DTX content, an alkaline hydrolysis was performed before LC-MS/MS analysis and PPIA.[20,21] For the hydrolysis, NaOH at 2.5 M (125 µL) was added to the extract (1.25 mL), mixture was homogenised in vortex for 0.5 min and heated at 76 °C for 40 min in a Multi-Block®Heater from Lab-Line Instruments, Inc. (Maharashtra, India). After cooling to room temperature, HCl at 2.5 M (125 µL) was added for neutralisation and the sample was homogenised by vortex-mixing for 0.5 min. The hydrolysed extract was then filtered through 0.2 µm cut-off nylon syringe filters (Whatman). As described for crude extracts, hydrolysed extracts were directly analysed by LC-MS/MS, while for PPIA they were evaporated under nitrogen and resuspended in the corresponding buffer to the desired concentration.

2.2.2.5 Colorimetric PPIA

The PPIA was performed as described before[17,22] but three different experiments were carried out: 1) evaluation of the inhibitory potencies of DSP toxins and the IEFs of DTXs, 2) determination of DSP toxins contents in spiked mussel samples, and 3) determination of DSP toxins contents in naturally-contaminated shellfish samples. To this purpose, 50 µL of blank mussel sample solution at 12.5 mg/mL spiked with OA, DTX-1 or DTX-2 standard solutions at different concentrations ranging from 1.6 to 100.0 µg/L (for experiment 1 and for OA calibration curves of experiments 2 and 3), 50 µL of blank mussel sample solution at 12.5 mg/mL spiked with OA at 160 µg/L, DTX-1 at 166 µg/L and/or DTX-2 at 176 µg/L (for experiment 2) or 50 µL of naturally-contaminated shellfish sample solution at different concentrations ranging from 1.6 to 12.5 mg/mL (for experiment 3) were added into microtiter wells containing 100 µL of PP2A solution (recombinant from GTP Technology or wild from Upstate Biotechnology) at 1.25 U/mL.

Then, 50 µL of 25 mM *p*-NPP solution were added and after 1-hour incubation at 22 °C in the dark, the absorbance at 405 nm was measured with an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA). Samples were prepared in a buffer solution containing 30 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.4. PP2A and *p*-NPP solutions were prepared in the same buffer, also containing 2 mM DTT and 0.2 mg/mL BSA. Assays were performed in triplicate. In the analysis of naturally-contaminated shellfish samples and in the evaluation of the inhibitory effect of DSP toxins mixtures, OA calibration curves using 12.5 and 6.3 mg/mL of mussel matrix, for recombinant and wild PP2A, respectively, were always performed in parallel for the precise toxin quantification.The DSP toxins calibration curves obtained by PPIA were analysed with SigmaPlot software package 10.0 and fitted to sigmoidal logistic four-parameter equations:

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point.

2.2.2.6 LC-MS/MS analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was applied following the "EU-Harmonised Standard Operation Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS" (EU-RL-MB SOP).[20] Analysis were conducted on an Agilent 1200 LC (Agilent Technologies, Santa Clara, USA) coupled with a 3200 QTRAP mass spectrometer through a TurboV™ electrospray ion source (Applied Biosystems, Foster City, USA). Chromatographic separations were performed at 30 °C and 0.2 mL/min on a Luna C8(2) column (50 mm × 1 mm, 3 µm) protected with a SupelcoGuard C8(2) cartridge (4 mm × 2 mm, 3 µm), both from Phenomenex (Torrance, USA).

Acidic chromatographic elution was selected with mobile phases 100% water (A) and 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM formic acid. For DSP toxins, multiple reactions monitoring (MRM) analysis was accomplished from the precursor ions 803.5 and 817.5 *m/z* for OA/DTX-2 and DTX-1, respectively. Product ions were common for all DSP toxins, with ions 255.2 *m/z* monitored for quantification and 113.1 *m/z* acquired for confirmatory purposes. Mass spectrometer operated in negative polarity, and compound-dependent parameters for MS/MS detection were tuned on the mass spectrometer through direct infusion of the CRM-OA standard: declustering potential -115 V, entrance potential -12 V (for 803.5>255.2) and -10.5 V (for 803.5>113.1), collision entrance potential collision energy -64 V (for 803.5>255.2) and -68 V (for 803.5>113.1), and collision cell exit potential -4 V. Gas/source parameters were also optimised (curtain gas: 20 psi; ion spray -4500 V, temperature 400 ºC, nebuliser gas: 50 psi, heater gas 50 psi). Under these conditions, the LOD and LOQ were 10 and 30 µg/kg OA in shellfish meat, respectively. The quantification curve obtained for OA was used also for the quantification of DTX-1 and DTX-2, since this approach was the recommended by the EU-RL-MB SOP.[20] Analyst software v1.4.2 was used for the entire MS tune, instrument control, data acquisition and data analysis.

2.2.2.7 Statistical analysis

To evaluate differences in the calibration curves for OA, DTX-1 and DTX-2 between recombinant and wild PP2A, the paired *t*-test was used (N = 10). Differences in the results were considered statistically significant at the 0.05 level. Prior to analysis, data were tested for normality; Wilcoxon matched-pairs signed-ranks test was used for non-normally distributed data sets instead of the paired *t*-test.

To evaluate the correlation between the OA equivalent contents in spiked mussel samples determined from the two PPIAs and the expected values, the linear regression model was used.

The linear regression model was also used to evaluate the correlation between the OA equivalent contents in naturally-contaminated shellfish samples determined from the two PPIAs and the values obtained from the LC-MS/MS analysis after application of the IEFs for each PP2A and the TEFs from EFSA. Differences in the results were considered statistically significant also at the 0.05 level.The SigmaStat software package 3.1 was used for the paired *t*-tests, the Wilcoxon matched-pairs signed-ranks tests and the linear regressions.

2.2.3 Results and discussion

2.2.3.1 Inhibitory Potencies of DSP Toxins and IEFs of DTXs

Dose-response curves with OA, DTX-1 and DTX-2 (Fig. 2.2.1) were performed in order to evaluate the inhibitory potencies of these DSP toxins on the activity of two PP2A enzymes from different origin (recombinant and wild). Toxin dilutions from stock solutions were prepared in a buffer solution containing blank mussel matrix at 12.5 mg/mL. This blank mussel did not contain DSP lipophilic toxins as determined by LC-MS/MS. The 12.5 mg/mL concentration had been previously established as equal to (for wild PP2A) or below (for recombinant PP2A) the maximum loading limit to use in the PPIA to avoid non-specific inhibition from the mussel matrix.[22]

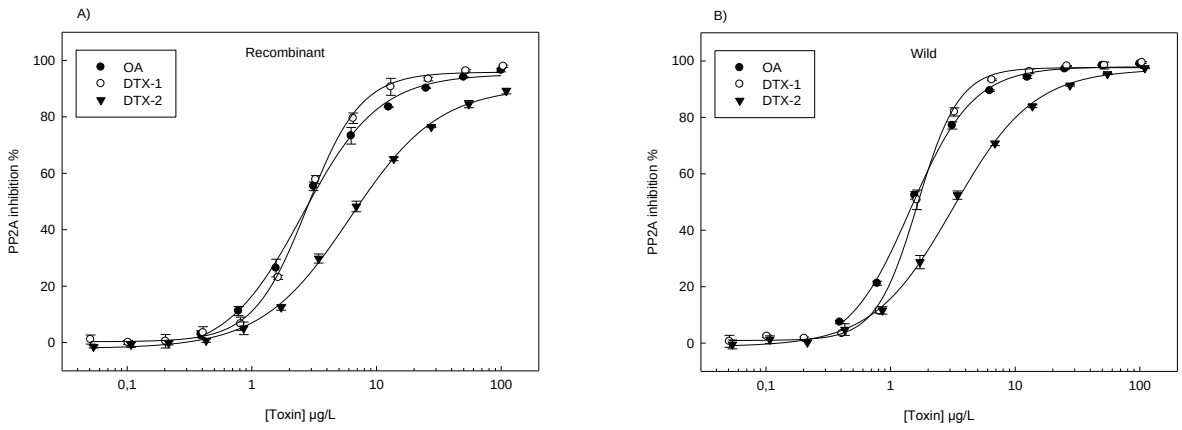

Figure 2.2.1. Dose-response curves for the inhibition of recombinant (A) and wild (B) PP2A by OA, DTX-1, and DTX-2. Inhibition is expressed as percentage of the control (no toxin); *x* values refer to initial toxin concentrations.

In Table 2.2.1, the 50% inhibition coefficient (IC_{50}) values, the inhibition equivalency factors (IEFs) and the working ranges (defined between IC_{20} and IC_{80}) are presented together with the equations and the corresponding R^2 values. The IEFs were calculated as the ratios of the IC_{50} for OA to the IC_{50} for DTX-1 or DTX-2, for each enzyme.

Table 2.2.1 Curve Parameters Derived from the Sigmoidal Logistic Four-Parameter Fitting for the Inhibition of PP2As by OA, DTX-1, and DTX-2.

Toxin	Enzyme	IC_{50} (µg/L)	IEF	Working range IC_{20} – IC_{80} (µg/L)	Equation	R^2
OA	Recombinant	2.93	1.0	1.2 – 8.4	$y=-2.0+(96.1/(1+x/2.7)^{-1.5})$	0.9994
	Wild	1.54	1.0	0.7 – 3.5	$y=-0.7+(98.4/(1+x/1.5)^{-1.8})$	0.9998
DTX-1	Recombinant	2.90	1.1	1.4 – 6.0	$y= 0.3+(95.5/(1+x/2.7)^{-2.0})$	0.9996
	Wild	1.66	0.9	0.9 – 2.8	$y= 0.9+(96.7/(1+x/1.6)^{-2.6})$	0.9996
DTX-2	Recombinant	7.54	0.4	2.2 – 29.7	$y=-2.1+(92.8/(1+x/5.6)^{-1.2})$	0.9996
	Wild	3.38	0.6	1.1 – 9.2	$y=-1.3+(98.4/(1+x/2.9)^{-1.4})$	0.9996

Comparing enzyme sources, the wild PP2A was significantly more sensitive to all DSP toxins than the recombinant one (t_{OA} = 3.957, p_{OA} = 0.003; W_{DTX-1} = 53, p_{DTX-1} = 0.004; t_{DTX-2} = 5.125, p_{DTX-2}< 0.001): 1.9-fold lower IC_{50} for OA, 1.7-fold lower IC_{50} for DTX-1 and 2.2-fold lower IC_{50} for DTX-2. Regarding the individual DSP toxins, DTX-1 inhibits both PP2As at approximately the same potency than OA ($t_{rec.}$ = 1.258, $p_{rec.}$ = 0.240; t_{wild} = 0.311, p_{wild} = 0.763), whereas DTX-2 inhibits both PP2As significantly less (respect to OA: $t_{rec.}$ = 4.502, $p_{rec.}$ = 0.001; t_{wild} = 3.610, p_{wild} = 0.006; respect to DTX-1: $t_{rec.}$ = 3.885, $p_{rec.}$ = 0.004; t_{wild} = 2.796, p_{wild} = 0.021).Although the IC_{50} values were different for each PP2A, the determined IEFs were similar because the trend was the same: OA ≈ DTX-1 > DTX-2.

Few works exist describing the inhibitory potencies of DTXs. Regarding DTX-1, Takai and collaborators[12] reported a 1.6-fold lower dissociation constant of DTX-1 compared to OA for a catalytic subunit PP2A from rabbit skeletal muscle (value comparable to our IEFs calculated from the IC_{50} values). Rivas and co-workers[23] also observed a lower IC_{50} value for DTX-1 respect to OA for a PP2A purified

from the mussel *Mytilus chilensis*, resulting in an IEF of 2.4. On the contrary, Mountfort and collaborators[21] observed a higher IC_{50} value for DTX-1 compared to OA for the same PP2A than the wild used in our work, which could be translated into an IEF of 0.6 for DTX-1. More recently, Ikehara and co-workers[16] obtained an IEF of 0.9 for DTX-1 with a catalytic subunit of recombinant human PP2A (also calculated from the IC_{50} values like herein), and Smienk and collaborators[24] obtained an IEF of 0.75 for DTX-1 with a PP2A purified from human red blood cells.

Regarding DTX-2, the lower inhibitory potency of DTX-2 respect to OA is comparable to its reduced acute intraperitoneal toxicity observed in mice, which allowed to establish a relative toxicity equivalency factor (TEF) of about 0.6.[18] This TEF has been adopted by the Panel on Contaminants in the Food Chain for regulated marine biotoxins.[25] The IEFs found in this work are also similar to the IEF reported by Aune and co-workers[18] of 0.48 with a PP2A from human red blood cells. The lower inhibitory potency of DTX-2 on PP2A has been suggested to be due to the 35-methyl group stereochemistry, which would be responsible for unfavourable interactions with Gln122 and His191 residues.[26,27] Nevertheless, the work from Smienk and collaborators[24] have recently reported equal toxicity for DTX-2 and OA.

Several reasons could explain the different IEFs reported in the literature. Differences in enzyme sources, enzyme concentrations, toxin standard purities, enzyme substrates and buffer compositions results in different IC_{50} values. Consequently, for a clear establishment of IEFs, PPIAs should be carefully controlled and performed simultaneously for all toxins under study.

Regarding the applicability of the PPIAs, taking into account the shellfish matrix loading limit and the attained LODs, the assays developed herein should be able to quantify 96 and 56 µg of OA/kg shellfish meat with recombinant and wild PP2A, respectively, far below the 160 µg of OA equivalents/kg shellfish meat, regulation level established by the EU 853/2004.[7] By applying the IEFs obtained in this work, the regulation levels for a sample containing only DTX-1 would be

145 and 178 µg of DTX-1/kg shellfish meat with recombinant and wild PP2A, respectively. The assays are able to quantify 88 and 72 µg of DTX-1/kg shellfish meat with recombinant and wild PP2A, respectively. For samples containing only DTX-2, the regulation levels would be 400 and 267 µg of DTX-2/kg shellfish meat with recombinant and wild PP2A, respectively. The assays are able to detect 176 and 88 µg of DTX-2/kg shellfish meat, with recombinant and wild PP2A, respectively. Consequently, in principle the developed PPIAs should be able to protect human health, regardless of the DSP toxins present in the shellfish sample.

2.2.3.2 Determination of DSP toxin contents in spiked mussel samples

To evaluate the applicability of the developed PPIAs to the analysis of shellfish samples with multi-DSP toxin profiles, an experiment was performed using different OA/DTX-1/DTX-2 combinations. In principle, the experiment was planned to spike 160 µg of each DSP toxin/kg mussel meat. However, preliminary reported DSP toxins concentrations were used in the PPIAs, which slightly varied at the moment of writing the present manuscript after LC-MS/MS confirmation. Nevertheless, the purpose of the assay is not compromised by these slightly higher concentrations (166 µg/kg for DTX-1 and 176 µg/kg for DTX-2). As in the previous experiment, a buffer solution containing blank mussel matrix at 12.5 mg/mL was used for the DSP toxins spiking and the OA calibration curve. In the determination of the DSP toxin contents (µg of OA eq/kg mussel meat), the inhibition percentage obtained for each spiked mussel sample and the corresponding IC value of the OA calibration curve determined from the sigmoidal logistic four-parameter equation were taken into account. Table 2.2.2 shows the DSP toxins combinations used in the spiking, the expected $[OA]_{eq}$, according to the theoretical spiked concentrations and the established IEFs, and the $[OA]_{eq}$ determined by the PPIAs with both enzymes. According to the spiked DSP toxins concentrations and the corresponding IEFs, combinations 7 and 8 were expected as “negatives”, combinations 1, 2, 3, 4 were expected as “positives”, and combinations 5 and 6 were expected as “suspicious”. Statistical analysis of all combinations as a whole revealed that, there were not significant differences in

the OA equivalent contents determined by the two PPIAs respect to the expected values ($PP2A_{rec.}$: $y = 1.191\ x - 25.131$, $R^2 = 0.817$, $p = 0.002$; $PP2A_{wild}$: $y = 1.298\ x - 42.204$, $R^2 = 0.910$, $p < 0.001$).

Table 2.2.2 DSP Lipophilic Toxin Spiking Combinations, OA Equivalent Contents (µg of OA equiv/kg mussel meat) Expected According to the Spiked Concentrations and the IEFs and Determined by PPIA with Recombinant and Wild PP2A[a].

Combination	DSP lipophilic toxin			expected $[OA]_{eq}$		determined $[OA]_{eq}$	
	OA	DTX-1	DTX-2	$PP2A_{rec.}$	$PP2A_{wild}$	$PP2A_{rec.}$	$PP2A_{wild}$
1	+	+	+	404	404	576 ± 3	569 ± 17
2	+	+	-	340	307	314 ±16	328 ± 3
3	+	-	+	224	257	277 ± 18	273 ± 1
4	-	+	+	244	244	187 ± 9	208 ± 4
5	+	-	-	160	160	172 ± 3	164 ± 1
6	-	+	-	180	147	101 ± 6	127 ± 9
7	-	-	+	64	97	n.d.[b]	63 ± 2
8	-	-	-	0	0	n.d.	n.d.

[a]The + symbol indicates 160, 166, and 176 µg/kg for OA, DTX-1, and DTX-2, respectively; the − symbol indicates 0. [b]n.d. = not detected: <96 µg/kg for $PP2A_{rec}$; <56 µg/kg for $PP2A_{wild}$.

Regarding “negative” combinations, the control sample without any DSP toxin (combination 8) did not induce any PP2A inhibition, thus confirming that the mussel matrix loading was appropriate for the developed PPIAs. Sample with only DTX-2 (combination 7) slightly inhibited the wild PP2A but it did not inhibit the recombinant PP2A. In fact, in the previous section we define 176 µg/kg as LOD of DTX-2 for recombinant PP2A; consequently, the toxin content of this sample was close to the corresponding LOD and thus difficult to detect. Nevertheless, the experimental design shows the suitability of the PPIAs as screening tools able to identify “negative” samples in a simple, fast, cheap and reliable way.

Both PPIAs indicated that combinations 1, 2, 3 and 4 are “positive”, as expected. One can appreciate that in the most toxic profile (combination 1), the OA equivalent contents determined by the PPIAs were higher than the expected ones, which may indicate a possible overestimation at high toxin levels. One cannot neglect that in this combination the three toxins are present and thus, a

synergistic effect could be present. Nevertheless, results indicate that PPIAs are able to identify “positive” samples. In an official monitoring program, a preventive closure of the shellfish harvesting area would be recommended in order to protect the consumer health; the sample would be also processed by LC-MS/MS to confirm the “positive” result.

Regarding “suspicious” combinations, quantifications derived from the PPIAs also agree with the expected values. Taking into account the obtained results and in order to use the PPIAs as screening tools, we define a “suspicious range” between 80 and 180 µg/kg of OA equivalent contents, which imply the analysis of the sample by the reference LC-MS/MS method for decision purposes. As we mentioned in the previous paragraph, a result above 180 µg/kg of OA equivalent contents would require the LC-MS/MS analysis for confirmatory purposes.

2.2.3.3 Determination of DSP Toxin Contents in Naturally Contaminated Shellfish Samples

The PPIAs with recombinant and wild PP2A were applied to the determination of DSP toxins in fourteen naturally-contaminated shellfish samples. They corresponded to seven samples analysed as duplicates: raw wedge shell clam homogenate (*Donax trunculus*), raw razor clam homogenate (*Ensis acuatus*), raw mussel homogenate (*Mytilus edulis*), raw stripped venus (*Chamelea gallina*), two cooked mussel homogenates (*Mytilus edulis*) and raw cockle homogenate (*Cerastoderma edule*). OA calibration curves were performed in parallel to each quantification analysis due to possible slight differences in the inhibition percentages between assays. As in the previous experiments, a buffer solution containing blank mussel matrix at 12.5 mg/mL was used for the OA calibration curves with recombinant PP2A, but a mussel matrix concentration of 6.3 mg/mL was used with wild PP2A because of the higher non-specific inhibition of hydrolysed mussel on the activity of this enzyme.[22]In the determination of the DSP toxins contents (µg of OA eq/kg shellfish meat), the IC_{50} values of the shellfish sample dose-response curves determined from lineal regressions and the IC_{50} values of the OA calibration curves determined from the sigmoidal logistic four-parameter equations were used. In Table 2.2.3, PPIA results are

compared to those determined by LC-MS/MS analysis, which provided individual OA, DTX-1 and DTX-2 contents. Additionally, total OA equivalent contents ($\Sigma[OA]_{eq}$) were calculated by applying the TEFs proposed by EFSA[25] and the IEFs obtained in this article for each PPIA to the individual toxin quantifications obtained by LC-MS/MS analysis. For those samples where the toxin content was not detected or not quantified, compromise values corresponding to the half of the thresholds were considered.

Table 2.2.3 OA, DTX-1, and DTX-2 Contents Determined by LC-MS/MS Analysis, Total OA Equivalent Contents Calculated by the Application of the Corresponding TEFs and IEFs Values to the Individual Toxin Quantifications, and Total OA Equivalent Contents Obtained by the PPIAs with the Recombinant and the Wild PP2A[a].

Sample	Number	LC-MS/MS			$\Sigma[OA]_{eq}$			$PP2A_{rec.}$	$PP2A_{wild}$
		OA	DTX-1	DTX-2	TE $value_{EFSA}$	IE $value_{rec.}$	IE $value_{wild}$		
Raw razor clam	1	n.d.	n.d.	n.q.	22	19	22	n.d.	n.d.
	1H	64	n.d.	n.q.	81	78	81	n.d.	157
	2	n.d.	n.d.	n.q.	22	19	22	n.d.	n.d.
	2H	55	n.d.	n.q.	72	69	72	n.d.	165
Raw cockle	3	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	3H	163	n.d.	63	206	194	205	139	188
	4	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	4H	152	n.d.	55	190	180	190	136	266
Raw stripped venus	5	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	5H	175	n.d.	50	210	201	210	175	307
	6	n.d.	n.d.	n.d.	13	13	13	n.d.	n.d.
	6H	201	n.d.	45	233	225	233	177	300
Raw wedge shell clam	7	30	n.d.	105	98	78	98	n.d.	162
	7H	172	n.d.	131	256	230	255	237	403
	8	30	n.d.	125	110	86	110	n.d.	161
	8H	151	n.d.	138	239	212	238	240	362

Raw mussel	9	36	133	n.d.	172	184	159	345	465
	9H	69	275	n.d.	347	374	320	368	499
	10	43	157	n.d.	203	218	187	333	408
	10H	63	187	n.d.	253	271	234	495	565
Cooked mussel	11	294	n.d.	n.q.	311	308	311	217	428
	11H	450	n.d.	n.q.	467	464	467	312	1229
	12	284	n.d.	n.q.	301	298	301	194	422
	12H	479	n.d.	n.q.	496	493	496	316	802
	13	178	107	168	386	363	375	289	581
	13H	388	129	252	668	631	655	420	1651
	14	186	135	162	418	399	405	254	443
	14H	430	120	224	684	652	672	459	1199

[a]All values are expressed as µg of toxin/kg mussel meat. [b]n.d. = not detected: <10 µg/kg for LC-MS/MS; <96 µg/kg for $PP2A_{rec}$; <56 µg/kg for $PP2A_{wild}$. [c]n.q. = not quantified: 10 µg/kg ≤ concentration ≤ 30 µg/kg for LC-MS/MS.

In general terms, results derived from the PPIAs agree with those obtained from the application of the TEFs and IEFs to the LC-MS/MS analysis. Moreover, PPIAs with both the recombinant and the wild PP2A provided similar results, and agreement is also observed between duplicates. As LC-MS/MS indicates, all samples contain acyl ester derivatives, detectable after hydrolysis. Since hydrolysed samples always provided higher toxins contents in the PPIAs, it is fair to suggest that the acyl ester derivatives inhibit PP2As less than the corresponding precursor toxins. Nevertheless, they may still be inhibiting thus contributing to the OA equivalent contents determination in non-hydrolysed samples.

Taking into account the different behaviour between non-hydrolysed and hydrolysed samples, statistical analysis of free and total DSP toxins contents was performed separately. The result obtained for sample 10H with the recombinant PP2A was considered discrepant and it was not included in the analysis. Figure 2.2.2 shows the linear regressions for the correlations between the OA equivalent contents obtained from the PPIAs and the IE values. As it can be observed, hydrolysed samples correlate better ($PP2A_{rec.}$: $y = 0.642\ x + 39.067$, $R^2 = 0.899$, $p < 0.001$; $PP2A_{wild}$: $y = 2.290\ x - 129.43$, $R^2 = 0.875$, $p < 0.001$) than non-hydrolysed ones ($PP2A_{rec.}$: $y = 0.674\ x + 46.771$, $R^2 = 0.664$, $p < 0.001$; $PP2A_{wild}$: $y = 1.338\ x + 36.691$, $R^2 = 0.850$, $p < 0.001$). As we mentioned above, the contribution from the acyl ester derivatives, which are inhibiting the enzymes although in a lower extent than the precursor toxins, may be responsible for the worse agreements in the free DSP toxins contents.

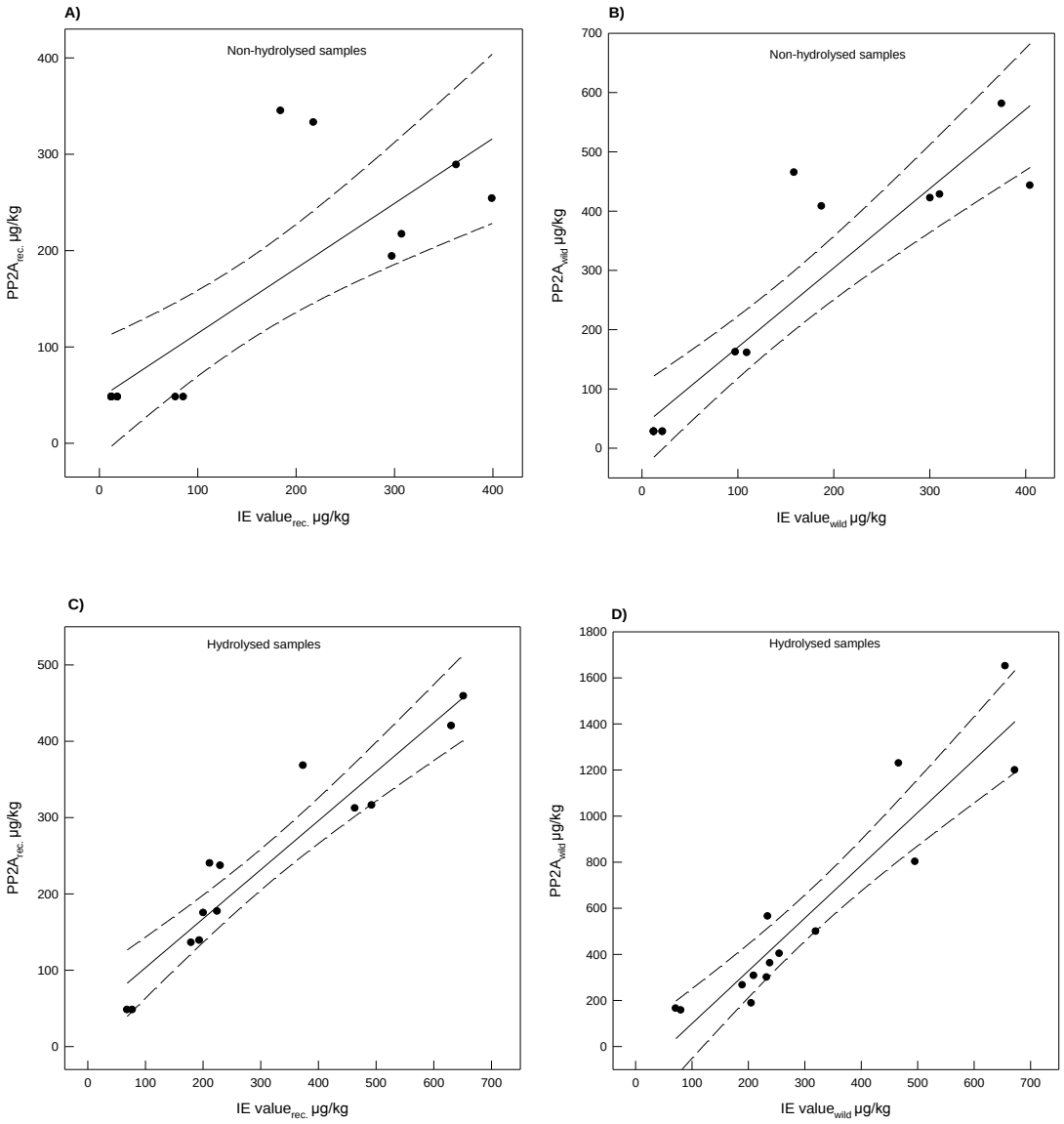

Figure 2.2.2. Linear regressions for the correlations of the PPIAs for non-hydrolyzed (A and B) and hydrolyzed (C and D) samples, with the recombinant (A and C) and wild (B and D) PP2As, with respect to the corresponding IE values.

The same behaviour was observed in the comparison of the PPIAs results with the TE values (Fig. 2.2.1S, Supporting Information), due to the similarity between the IEFs and the TEFs established by the EFSA.

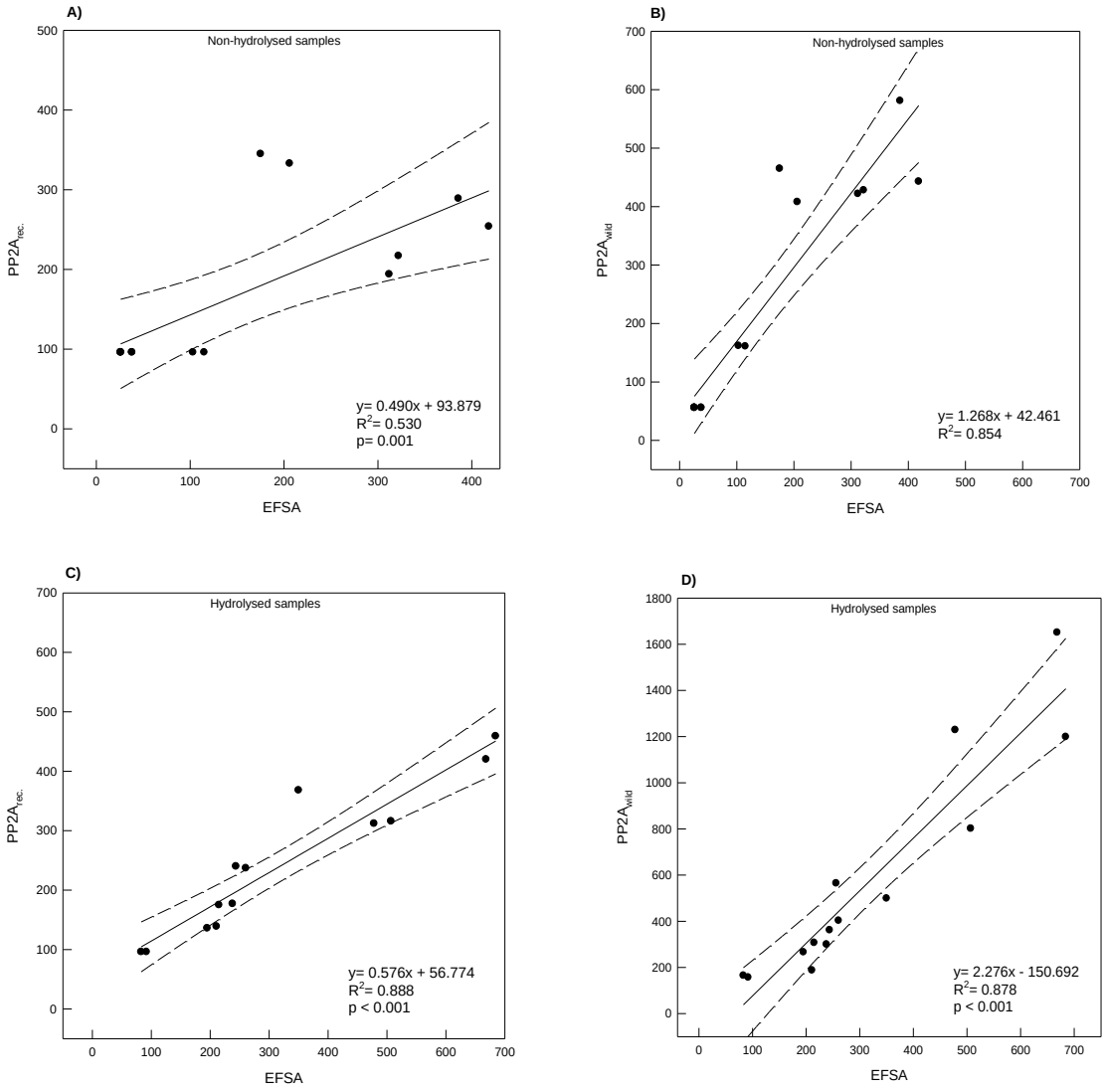

Figure 2.2.1S. Linear regressions for the correlations of the PPIAs for non-hydrolysed (A and B) and hydrolysed (C and D) samples, with the recombinant (A and C) and wild (B and D) PP2As, respect to the corresponding TE values from EFSA.

Comparing enzyme sources, whereas the tendency of the recombinant PP2A is to underestimate the OA equivalent contents, the tendency of the wild PP2A is to overestimate them. The underestimation from the recombinant PP2A together with the higher LODs compared to the wild PP2A would explain that the recombinant PP2A did not detect toxicity in samples 1H, 2H, 7 and 8, whereas the wild PP2A did. The overestimation from the wild PP2A is more evident in the analysis of hydrolysed samples than in the analysis of the non-hydrolysed ones. The hydrolysis procedure could be responsible for the overestimation, since non-

specific inhibition from blank hydrolysed samples is higher than from non-hydrolysed samples for this wild PP2A.[22]

Finally, no trends are observed in the OA equivalent contents determined by the PPIAs regarding the different DSP toxin profiles (OA, DTX-1 and/or DTX-2) of the samples, indicating the good performance of the assays in the analysis of shellfish samples with multi-toxin profiles. Furthermore, fit-for-purpose agreement with the reference LC-MS/MS method can be expected during their application within a monitoring program. Despite the under and overestimations, which require further investigation, results obtained with both PPIAs, especially in the analysis of hydrolysed samples, correlate well with LC-MS/MS analysis. Using the PPIAs as screening tools and taking into account the previously defined "suspicious" range, neither false "negative" nor false "positive" samples would have been identified, demonstrating that our strategy is, in principle, appropriate. Since the hydrolysis step is required only for the indirect determination of DSP toxins acyl ester derivatives (acyl esters of other toxins are not regulated up to date), hydrolysed extracts could be analysed exclusively with PPIA, which means a reduction by a half in the number of samples analysed by LC-MS/MS.

In summary, the inhibitory potencies of DTX-1 and DTX-2 on two PP2As, one recombinant and one wild, have been compared to that of OA allowing the establishment of the corresponding IEFs. Whereas the inhibition potency of DTX-1 is not significantly different from that of OA, DTX-2 inhibits PP2As markedly less. Regarding the enzyme source, the wild enzyme is slightly more sensitive than the recombinant one. Nevertheless, both PPIAs attain appropriate LODs, regardless of the nature of the enzyme. The developed assays have been applied to the determination of OA equivalent contents in mussel samples spiked with OA, DTX-1 and/or DTX-2. The experimental results have shown a good agreement with the expected OA equivalent contents, calculated from the theoretical toxin concentrations and the corresponding IEFs. Moreover, the experiment has allowed the establishment of a "suspicious range" between 80 and 180 µg/kg of OA equivalent contents, which would require samples to be

processed by LC-MS/MS prior to decision making. From our results, confirmatory analysis with the reference method would not even be essential above 180 µg/kg, the PPIA providing a fast response against severe DSP outbreaks. In the analysis of naturally-contaminated shellfish samples, OA equivalent contents determined by PPIAs have shown a good agreement with both TE and IE values, demonstrating that the assays can be used as reliable screening tools in monitoring programs.

The developed PPIAs with both recombinant and wild PP2A have shown the ability to detect DSP toxins with good performance. The assessment of the IEFs for the determination of the total OA equivalent contents has contributed to better understand the agreement with LC-MS/MS analysis. The PPIA is an interesting method for the simple, fast, sensitive, robust and reliable determination of DSP toxins contents in shellfish. The use of this functional assay in monitoring programs for the screening of a high number of samples would substantially decrease economic costs and save time. For example, although crude extracts would still require LC-MS/MS analysis for the quantification of non-DSP like lipophilic toxins, hydrolysed samples could be screened with this biochemical tool reducing the instrumental analytical requirements while providing equivalent protection level of public health. Moreover, PPIA can be yet considered an excellent tool for DSP toxins quantification and research purposes.

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RESEARCH ARTICLE

Inhibition equivalency factors for microcystin variants in recombinant and wild-type protein phosphatase 1 and 2A assays

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Abstract

In this work, protein phosphatase inhibition assays (PPIAs) have been used to evaluate the performance of recombinant PP1 and recombinant and wild-type PP2As. The enzymes have been compared using microcystins-LR (MC-LR) as a model cyanotoxin. Whereas $PP2A_{Rec}$ provides a limit of detection (LOD) of 3.1 µg/L, $PP1_{Rec}$ and $PP2A_{Wild}$ provide LODs of 0.6 and 0.5 µg/L respectively, lower than the guideline value proposed by the World Health Organization (1 µg/L). The inhibitory potencies of seven MCs variants (-LR, -RR, -dmLR, -YR, -LY, -LW and -LF) have been evaluated, resulting on 50% inhibition coefficient (IC_{50}) values ranging from 1.4 to 359.3 µg/L depending on the MC variant and the PP. The PPIAs have been applied to the determination of MC equivalent contents in a natural cyanobacterial bloom and an artificially-contaminated sample, with multi-MC profiles. The inhibition equivalency factors (IEFs) have been applied to the individual MCs quantifications determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and the estimated MC-LR equivalent content has been compared to PPIA results. PPIAs have demonstrated to be applicable as MC screening tools for environmental applications and to protect human and animal health.

Keywords: Microcystins (MCs); protein phosphatase 1 (PP1); protein phosphatase 2A (PP2A); protein phosphatase inhibition assay (PPIA); inhibition equivalency factors (IEFs); multivariate data analysis; liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.3.1 Introduction

Microcystins (MCs) and nodularins are potent hepatotoxic cyclic peptides produced by toxicogenic cyanobacteria (e.g. *Microcystis aeruginosa*) normally found in fresh and brackish water with low turbidity flow regimes (Carmichael, 1994). The general structure of MCs is cyclo-(D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha), X and Y being two variable L-amino acids. One of the main characteristics of MCs is the presence of the Adda chain (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (Rinehart et al., 1988). There are more than 80 MC variants, varying in the degree of methylation, hydroxylation, epimerization, peptide sequence and toxicity (Welker and von Döhren, 2006). Among them, MC-LR, L and R being leucine and arginine respectively, was originally the most frequent and toxic congener. Although recent works have described higher cytotoxic effects from other variants, such as MC-YR, MC-LW and MC-LF, compared to MC-LR (Monks et al., 2007; Fischer et al., 2010; Vesterkvist et al., 2012; Ufelmann et al., 2012; Perron et al., 2012), this variant is still considered the most representative of the group and is usually taken as a model. MCs inhibit serine/threonine protein phosphatases type 2A (PP2A) and type 1 (PP1), increasing the phosphorylation levels in the cells and inducing morphology changes on their cytoskeleton (Mackintosh et al., 1990; Yoshizawa et al., 1990; Dawson, 1998; Kuiper-Goodman et al., 1999).

The presence of MCs in water supplies poses a threat to animal and human health because of the possible harmful effects such as gastrointestinal disturbances and dermatitis, but also more severe chronic toxicity effects (neurotoxicity, hepatotoxicity and liver cancer promotion) and even death (Nishiwaki-Matsuchima et al., 1992; Pouria et al., 1998; Jochimsen et al., 1998; De Figueiredo et al., 2004). Due to the possible serious health implications, the World Health Organization (WHO) proposed a guideline value of 1 µg/L for MC-

LR in drinking water (WHO, 1998). In fact, several countries (e.g. Spain, France, Poland, Czech Republic, Norway, Brazil, Japan, Korea and New Zealand) have adopted the WHO recommended value in their regulations or the other national guideline variants that are based upon it (e.g. Canada and Australia) (Burch, 2008). This value, however, only covers MC-LR; thus, it was stated as "provisional" until more toxicity data for other cyanobacterial toxins will be generated. In order to assure water quality and protect human and animal health, the regulation should not be restricted to MC-LR, but it should consider the toxicity of the different variants. Consequently, the development of analysis methods that give an indication of the global toxicity of a sample is an evident necessity.

Several MCs analysis methods have been developed. The simplest detection method is the mouse bioassay, but its specificity is low and presents controversial ethical implications (Falconer, 1993; Campbell et al., 1994). Chromatographic techniques coupled to UV (Edwards et al., 1993; Lawton et al., 1994; Tsuji et al., 1994) or mass spectrometry detection (Spoof et al., 2001; Ortelli et al., 2008; Yang et al., 2009) are routinely used, providing high sensitivity and structural information on the different variants, but requiring expensive equipment and trained personnel. Also structural information is provided by enzyme-linked immunosorbent assays (ELISAs), which use monoclonal (Ueno et al., 1996; Zeck et al., 2001; Campàs and Marty, 2007; Pyo et al., 2004; Sheng et al., 2007) or polyclonal antibodies (Brooks and Codd, 1988; Chu et al., 1990; An and Carmichael, 1994) with high specificity towards several MCs and nodularin variants. The antibody cross-reactivity makes the discrimination of individual toxins impossible, but it can be considered as an advantage since all the toxins within the same structural family would be detected. Nevertheless, ELISAs do not distinguish between toxic and non-toxic variants.

The inhibition of PPs by MCs and nodularins has also been exploited to develop assays for the determination of these toxins (An and Carmichael, 1994; Heresztyn and Nicholson, 2001; Bouaïcha et al., 2002; Campàs et al., 2005; Ikehara et al., 2008). Unlike ELISAs, the protein phosphatase inhibition assays (PPIAs) is not based on structural information but provides a functional response, indicating the inhibitory potency of a toxin or mixture of toxins. Thus, although the PPIA cannot be used as an identification technique, it can be applied to detect the toxicity of a sample. Nowadays, colorimetric PPIAs are widely used because of their simplicity, low cost and sufficient sensitivity. However, more data on the inhibitory potencies of different MC variants, and their inhibition equivalency factors (IEFs) in relation to MC-LR, are necessary to better understand the toxicity of samples and to provide useful screening/detection tools.

In this work, the first step has been to test the enzyme activity of four PPs (PP1 and PP2A, recombinant and wild-type). Then, colorimetric PPIAs have been developed, using three enzymes and MC-LR as a model cyanotoxin, and the sensitivities and limits of detection (LODs) have been compared. Afterwards, the inhibitory potencies of seven MCs variants (-LR, -RR, -dmLR, -YR, -LY, -LW and -LF) on the PPs have been determined, and the corresponding IEFs have been established. Finally, PPIAs have been applied to the determination of MC equivalent contents in a natural cyanobacterial bloom sample and an artificially-contaminated sample. The IEFs have been applied to the individual MCs quantifications determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and the estimated MC-LR equivalent content has been compared to PPIA results.

2.3.2 Materials and Methods

2.3.2.1. Reagents and materials

Microcystin-LR (MC-LR), MC-RR, MC-YR, MC-LY, MC-LW and MC-LF were purchased from Alexis Biochemicals (San Diego, USA) and dissolved in 100%

methanol. 3-Desmethylmicrocystin-LR (MC-dmLR) in 100% methanol was purchased from DHI (Hørsholm, Denmark). The purity of MC standards was checked by LC-MS in full scan mode.

A recombinant protein phosphatase 1 catalytic subunit was purchased from New England BioLabs (Ipswich, MA, USA). $PP1_{Rec}$ consists of a 330 amino-acid catalytic subunit of the *α*-isoform, and it was isolated from a strain of *E. coli*. A recombinant protein phosphatase 2A catalytic subunit was purchased from GTP Technology (Toulouse, France). $PP2A_{Rec}$ consists of a 39 kDa human catalytic (C) subunit of the *α*-isoform with a hexa-His tail at the C-terminus, and it was isolated from SF9 insect cells infected by baculovirus. Wild-type PP1 and PP2A were purchased from Upstate Biotechnology (NewYork, USA). $PP1_{Wild}$ consists of a 42 kDa 375 amino-acid protein, and it was purified from rabbit skeletal muscle, $PP2A_{wild}$ consists of a heterodimer of 60 kDa and 36 kDa subunits, and it was purified from human red blood cells. Components of buffers and *p*-nitrophenyl phosphate (*p*-NPP) were purchased from Sigma (St. Quentin Fallavier, France).

In the LC-MS/MS analysis, all reagents used were of analytical grade or high-performance liquid chromatographic (HPLC) grade. Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany) and formic acid from Panreac (Montcada i Reixac, Barcelona, Spain).

2.3.2.2. Cyanobacterial bloom sample and MCs extraction

A sample of water with biomass (50 mL) from a cyanobacterial bloom that occurred in the Alcántara reservoir of the Tajo River (Cáceres, Spain) was collected in the summer of 2009. The sample was filtered through a Whatman GF/F filter (0.7 µm). The filter was then extracted three times by sonication with an Ultrasons-H sonicator set at 30% of full power (Abrera, Barcelona, Spain) for 15 min in 30 mL of acidified methanol (0.16% formic acid) according to Barco

and co-workers (Barco et al., 2005). The extract was evaporated to dryness, reconstituted in 8 mL of methanol, and directly injected into the LC-MS/MS system. For the extract to be tested with the PPIA, the methanol of an aliquot was evaporated in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen flux at room temperature, and the residue was resuspended in the corresponding buffer (in an equal volume to the evaporated aliquot).

2.3.2.3. PP activity

The PP enzyme activity was measured spectrophotometrically in a 1-mL cuvette by adding 10 µL of enzyme to 890 µL of 30 mM tris-HCl buffer solution, pH 8.4, with 20 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM 1, 4 dithiothreitol (DTT) and 0.2 mg/mL bovine serum albumin (BSA). After 5-min incubation, 100 µL of *p*-NPP solution at 100 mM (prepared in the same buffer) were added to the cuvette and the absorbance at 405 nm was measured during 1 min with a U-2001 UV/Vis spectrophotometer from Hitachi High-Tech (Krefeld, Germany). When using $PP1_{Rec}$, 1mM $MnCl_2$ was also added to the enzyme and substrate solutions, since this enzyme is Mn^{2+}-dependent. Assays were performed at 22 °C protected from light in triplicate. All solutions were prepared using Milli-Q water.

2.3.2.4. Colorimetric PPIA

The colorimetric PPIA was performed as follows: 50 µL of MC variants standard solutions (for the calibration curves, the study of the inhibitory potencies and the study of multi-MC profiles) at 10 different concentrations (starting at 100 µg/L and diluting to the half) or sample at different dilutions were added into microtiter wells containing 100 µL of enzyme solution at 1 U/mL for $PP1_{Rec}$ and $PP2A_{Wild}$ and 1.5 U/mL for $PP2A_{Rec}$ and incubated for 30 min. Then, 50 µL of 25 mM *p*-NPP solution were added, and after 1-hour incubation, the absorbance at 405 nm was measured with a KC4 automated microplate reader from Bio-Tek Instruments, Inc. (Bad Friedrichshall, Germany). MCs standard solutions were prepared in 30 mM tris-HCl buffer solution, pH 8.4, with 20 mM $MgCl_2$ and 2 mM EDTA. Enzyme

and substrate solutions were prepared in the same buffer solution also containing 2 mM DTT and 0.2 mg/mL BSA. When using $PP1_{Rec}$, 1mM $MnCl_2$ was also added to the enzyme and substrate solutions. Assays were performed at 22 °C protected from light and in triplicate. All solutions were prepared using Milli-Q water.

The calibration curves were described by the sigmoidal logistic four-parameter equation (SigmaPlot software package 10.0):

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point. The LOD was defined as the x value at 20% inhibition (IC_{20}). The working range was established between 20%and 80% inhibition (IC_{20}-IC_{80}).
The matrix effects on the PPIA were evaluated by observing the inhibition values from different concentrations of an extract of a non-MC producer microalgae culture, and also by comparing the inhibition values obtained when spiking 10 different concentrations (starting at 100 µg/L and diluting to the half) of MC-LR to buffer with the corresponding inhibition values obtained when spiking to the microalgae culture extract.

2.3.2.5. LC-MS/MS analysis

A triple quadrupole mass spectrometer (TSQ Quantum, Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source, a Surveyor MS plus pump and a HTC PAL autosampler were used for the LC-MS/MS analysis. The chromatographic separation was performed on a reversed-phase Kromasil C18 column (100 x 2.1 mm, 3.5 µm, Tracer, Teknokroma, Sant Cugat del Vallès, Spain). The mobile phase was composed of Milli-Q water as solvent A and acetonitrile as solvent B, both containing 0.08% (v/v) formic acid at a flow rate of 200 µL/min. The linear gradient elution program was from 0 to 10

min: 10-30% B, from 10 to 30 min: 30-35% B, from 30 to 45 min: 35-55% B, from 45 to 50 min: remains constant at 55% B, from 50 to 52 min: 55-90% B, from 52 to 55 min: remains constant at 90% B, and from 55 to 60 min: returns to initial conditions (10% B) for re-equilibration. The injection volume was 10 µL.

The analyses were carried out in positive ion mode with the spray voltage at 4.5 kV and the optimum tube lens voltage (TL) for each *m/z* (details are shown in supplementary information). The ion transfer tube temperature was set at 250 °C. Nitrogen (purity > 99.999%) was used as sheath gas, ion sweep gas and auxiliary gas at flow rates of 30 psi, 0 and 5 arb. unit, respectively. Data were acquired in full scan mode (*m/z* 400-1200, 1.2 sec/scan) for screening of MC variants. MS/MS experiments were performed for confirmation of MC identity when commercial standards were not available. The fragmentation pattern of MC in positive ionization gives a majority ion of *m/z* 135 characteristic of the amino acid Adda residue. High purity argon (Ar_1) was used as collision-induced gas at a pressure of 1.5 mtorr and the optimum collision energy (CE) for each fragmentation was selected. As there are no isotope-labelled MCs, nodularin was used as internal standard (added before the extraction). This toxin has physical and chemical properties very similar to MCs and its presence has not been reported in continental waters so far. The calculated recovery for nodularin was of the order of the MCs (75-90%) and the response factor of each MC variant regarding nodularin was acceptable and reproducible. The $[M+H]^+$ and $[M+2H]^{2+}$ were monitored for quantification purposes. Data acquisition was performed with Xcalibur 2.0.7 software (Thermo Fisher Scientific). Figure 2.3.1S (supplementary information) shows the chromatograms of the standards mixture containing the different MC variants and nodularin.

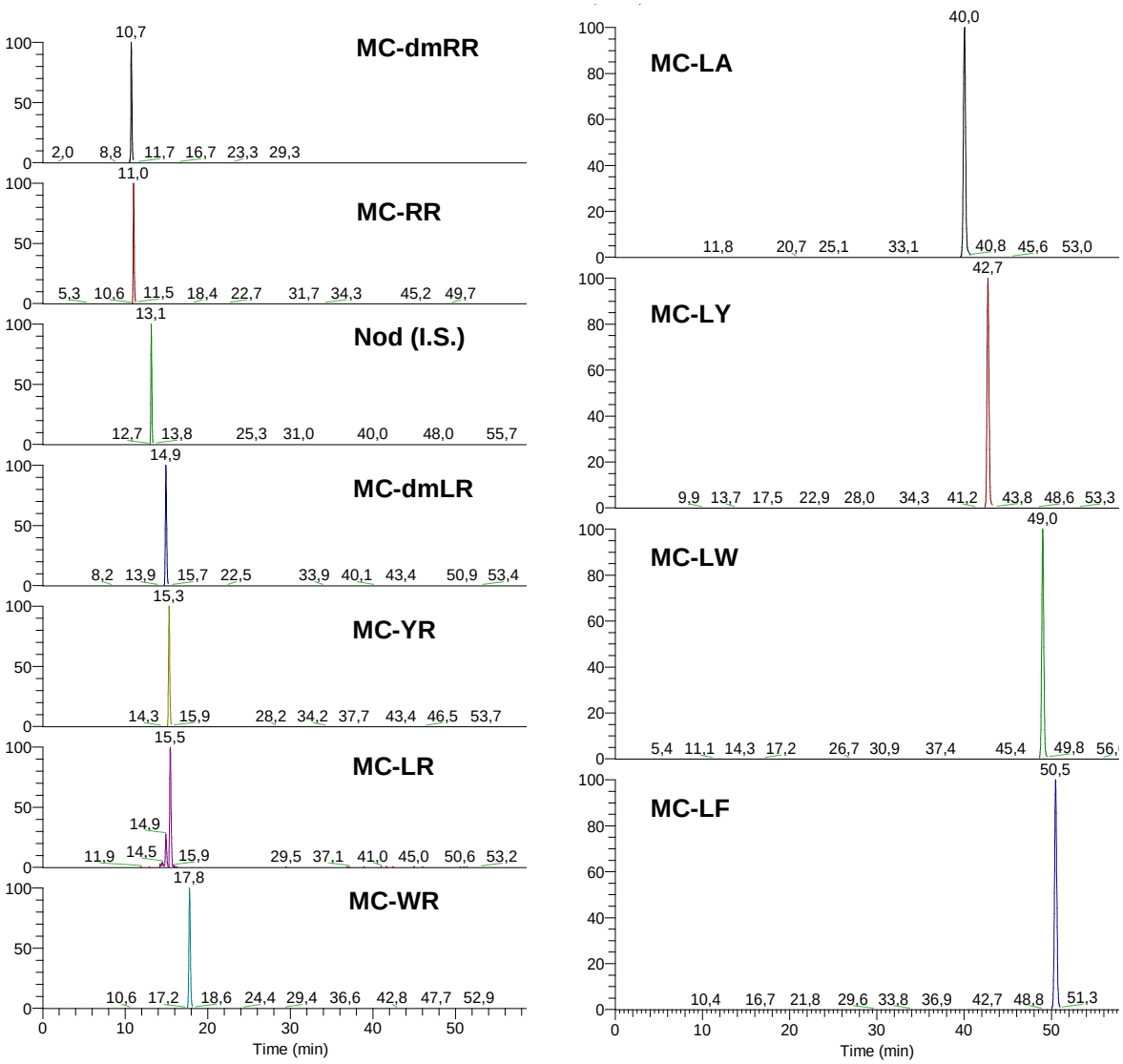

Figure 2.3.1S. Chromatogram of MC-dmRR, -RR, -YR, -LR, -dmLR, -WR, -LA, -LY, -LW, -LF and nodularin standard mixture.

The matrix effects on the LC-MS/MS analysis were evaluated by comparing the areas obtained when spiking three different concentrations (0.3, 20 and 500 µg/L) of MC-LR, MC-RR and MC-YR to an extract of non-MC producer natural bloom with the corresponding areas obtained when using methanolic MCs solutions.

2.3.2.6. Statistical analysis

The paired t-test was used to compare the IC_{20} and IC_{50} values for MC-LR and the different PPs and the MCs equivalent contents in the natural cyanobacterial bloom obtained by PPIAs (N = 3). Differences in the results were considered statistically significant at the 0.05 level. SigmaStat 3.1 software was used. Principal Component Analysis (PCA) was used to compare the IC_{50} values for all MC variants as a whole and for the different PPs. MATLAB 7.1 software was used for chemometric processing.

2.3.3 Results and discussion

2.3.3.1. MC-LR calibration curves by PPIAs

The enzyme activities of the stock solutions were 655 U/mL for $PP1_{Rec}$, 2 U/mL for $PP1_{Wild}$, 1140 U/mL for $PP2A_{Rec}$, and 8935 U/mL for $PP2A_{Wild}$, 1 U being defined as the amount of enzyme required to hydrolyse 1 nmol of *p*-NPP in 1 min at 22 °C. Since the activity of the $PP1_{Wild}$ stock solution was too low, it was not economically viable to perform the corresponding PPIA and thus $PP1_{Wild}$ was put aside. Colorimetric PPIAs were developed using $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$, and MC-LR as model toxin. The PPIAs demonstrated the inhibitory effect of MC-LR on the enzyme activity.

In Table 2.3.1, the 50% inhibition coefficient (IC_{50}) values and the working ranges are presented together with the equation and the corresponding correlation coefficient (*R*) values. To evaluate the PPs performance, the IC_{50} and IC_{20} values were compared. The calibration curves may differ not only in LOD (*x* displacement) but also in sensitivity (curve shape and slope at the linear range), and these two values may provide different information. Comparing the IC_{50} values, $PP1_{Rec}$ and $PP2A_{Wild}$ provided similar sensitivities (t = 4.221, p = 0.052), in both cases lower to $PP2A_{Rec}$ ($PP2A_{Rec}$ *vs.* $PP1_{Rec}$: t = 33.325, p < 0.001; $PP2A_{Rec}$ *vs.* $PP2A_{Wild}$: t = 31.059, p = 0.001). Regarding the LODs, $PP1_{Rec}$ and $PP2A_{Wild}$ provided similar values (t = 0.896, p = 0.465), both enzymes being able to detect the regulated guideline of 1 µg/L of MC-LR in drinking water. The $PP2A_{Rec}$ provided a LOD significantly higher than the other PPs ($PP2A_{Rec}$ *vs.*

$PP1_{Rec}$: t = 54.202, p < 0.001; $PP2A_{Rec}$ *vs.* $PP2A_{Wild}$: t = 15.878, p = 0.004). Although $PP2A_{Rec}$ would not detect 1 µg/L of MC-LR in drinking water, it could provide additional and/or complementary information to the other PPs (e.g. a specific MC variant could inhibit $PP2A_{Rec}$ without causing any inhibitory effect on other PPs). Consequently, it was still considered in next experiments.

Table 2.3.1 Curve parameters derived from the sigmoidal logistic four-parameter fitting for the inhibition of the PPs by MC-LR.

Enzyme	IC_{50} (µg/L)	Working range IC_{20}-IC_{80} (µg/L) IC_{20} – IC_{80}	Equation	*R*
$PP1_{Rec}$	2.1	0.8-4.1	$y = 15.2 + \frac{80.8}{1+(x/2.3)^{-2.5}}$	1.000
$PP2A_{Rec}$	7.3	3.1-18.7	$y = 1.6 + \frac{93.1}{1+(x/7.0)^{-1.7}}$	1.000
$PP2A_{Wild}$	1.4	0.6-2.8	$y = 11.1 + \frac{85.4}{1+(x/1.6)^{-2.4}}$	1.000

2.3.3.2. Inhibition equivalency factors (IEFs)

Calibration curves of MC-LR, -RR, -dmLR, -YR, -LY, -LW and -LF were performed to evaluate the inhibitory potencies of different MC variants on the activity of $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$. Figure 2.3.1 shows the calibration curves for MC-LR and MC-LY obtained when using $PP2A_{Rec}$, as an example. The IEFs were calculated as the ratios of the IC_{50} for MC-LR to the IC_{50} for the corresponding variant, for each enzyme.

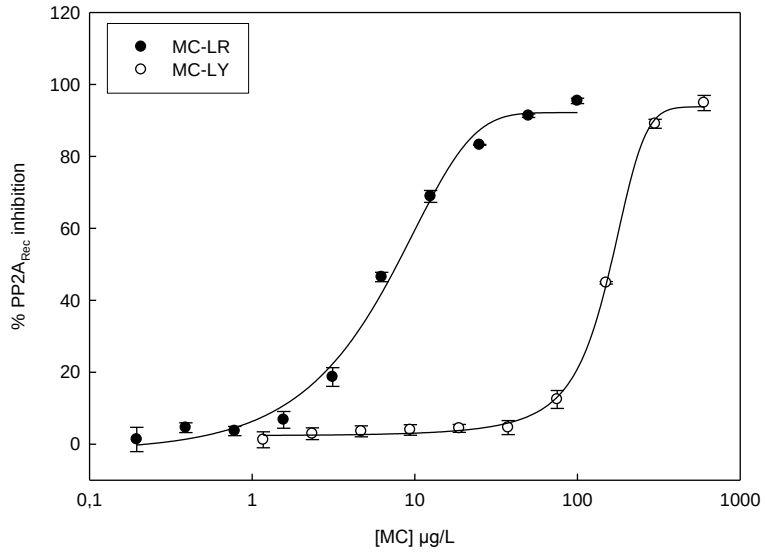

Figure 2.3.1. Doses-responses curves for the inhibition of recombinant PP2A by MC-LR and MC-LY. Inhibition is expressed as percentage of the control (no toxin); *x* values refer to initial toxin concentrations. *Errors bars* represent the standard deviation of the average ($N = 3$).

Table 2.3.2 shows the IC_{50} values as well as the IEFs for all variants except MC-dmLR.

Table 2.3.2. IC_{50} (µg/L) values derived from the sigmoidal logistic four-parameter fitting for the inhibition of the PPs by MC-LR, -RR, -YR, -LY, -LW and -LF, and corresponding IEFs calculated respect to MC-LR for each PP.

	PP1$_{Rec}$		**PP2A$_{Rec}$**		**PP2A$_{Wild}$**	
	IC_{50}	**IEF**	**IC_{50}**	**IEF**	**IC_{50}**	**IEF**
MC-LR	2.1	1.00	7.3	1.00	1.4	1.00
MC-RR	14.6	0.14	21.3	0.34	7.9	0.18
MC-YR	74.2	0.03	129.9	0.06	37.7	0.04
MC-LY	188.2	0.01	167.5	0.04	83.3	0.02
MC-LW	187.3	0.01	239.1	0.03	105.3	0.01
MC-LF	359.3	0.01	255.0	0.03	117.8	0.01

It is important to note that MC-LR standard solution contained 3% of MC-dmLR and MC-dmLR standard solution contained 9% of MC-LR. It is known that demethylation of an amino acid can alter the solubility, membrane permeability, and ionic or hydrogen bond formation at the binding site of MCs (Ikehara et al., 2009). Concerning MC-dmLR, whereas some authors report no or minor effects of the desmethylation of a variant on the inhibitory potency (Ufelmann et al., 2012; Blom and Jüttner, 2005; Hoeger et al., 2007), some others observe an evident reduction of the inhibitory potency (Ikehara et al., 2009). Consequently, the MC-dmLR content in the MC-LR standard is not supposed to substantially affect the IC_{50} of MC-LR but, on the contrary, the MC-LR content in the MC-dmLR standard certainly affects its IC_{50}. When correcting the MC-dmLR contents (taking into account the 91%), its IC_{50} values were 17.1, 65.4 and 26.6 µg/L for $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$, respectively. Nevertheless, the 9% of MC-LR would involve the presence of additional 1.7, 6.5 and 2.6 µg/L of MC-LR in the midpoint of each calibration curve. Since the IC_{50} values for MC-LR were 2.1, 7.3 and 1.4 µg/L for $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$, respectively, the content of MC-LR in the MC-dmLR standard is certainly causing an underestimation of the IC_{50} values for MC-dmLR, i.e. we suspect that MC-dmLR has a lower inhibitory potency than observed. Due to the effect of this impurity, MC-dmLR was put aside for the subsequent experiments.

In general terms, $PP2A_{Wild}$ provided the lowest IC_{50} values and $PP2A_{Rec}$ the highest ones. The regulatory subunit of the $PP2A_{Wild}$ may be mediating the MC binding and thus promoting inhibition compared to the $PP1_{Rec}$ and $PP2A_{Rec}$ catalytic subunits. As expected, MC-LR was the most potent variant for all enzymes. In all cases the variants with arginine in position 4 (-LR, -RR and -YR) show higher inhibition than the rest. Additionally, the trend was the same for all enzymes, i.e. MC-LR > -RR > -YR > -LY > -LW > -LF.

Compared to other works, differences in the IC_{50} values and trends are observed. The source and nature of the enzyme as well as the concentrations

and purity of the MC variants standard solutions, some of them not certified, may be responsible for the different observed inhibitory potencies. Nevertheless, in general terms the MC-LR > -RR > -YR inhibitory trend is also observed in other works (Blom and Jüttner, 2005; Heresztyn and Nicholson, 2001; Ikehara et al., 2008 and 2009).

A multivariate data analysis based on the PCA was applied to the IC_{50} values (triplicates) in order to evaluate the differences among MC variants and the capability of the PPs to distinguish them. PCA allows the projection of the information carried by the original variables onto a smaller number of underlying ("latent") variables called principal components (PCs) with new coordinates called scores, obtained after data transformation. Consequently, by plotting the PCs, it is possible to obtain information about the interrelationships between different samples and variables, and detect and interpret sample patterns, groupings, similarities or differences.

Figure 2.3.2 displays the distribution of the MCs in the new coordinates, showing an accumulated variance of 99.8% from the two first PCs, this high value indicating that nearly all the variance contained in the original information is represented by only these two new coordinates. As it can be observed, discrimination of the different MC variants can be achieved with this simple analysis of the scores, where PC1 seems to be related with the IC_{50} values of the different MC variants and PC2 with the different inhibitory potencies towards PP1 and PP2As.

Figure 2.3.2 also shows the loadings of the three PPs. This representation allows detecting uninformative or meaningless variables by evaluating the proximity to the centre (0,0), a criterion that might be used to discard non-useful variables. In our case, the wide distance of the different PPs to the centre indicates that all PPs provide useful information. Besides, it is necessary to take into account that, unlike the previous results, the PCA is considering the behaviour of all MC variants simultaneously. This is particularly important in the analysis of natural samples, where multi-MC profiles are almost always encountered.

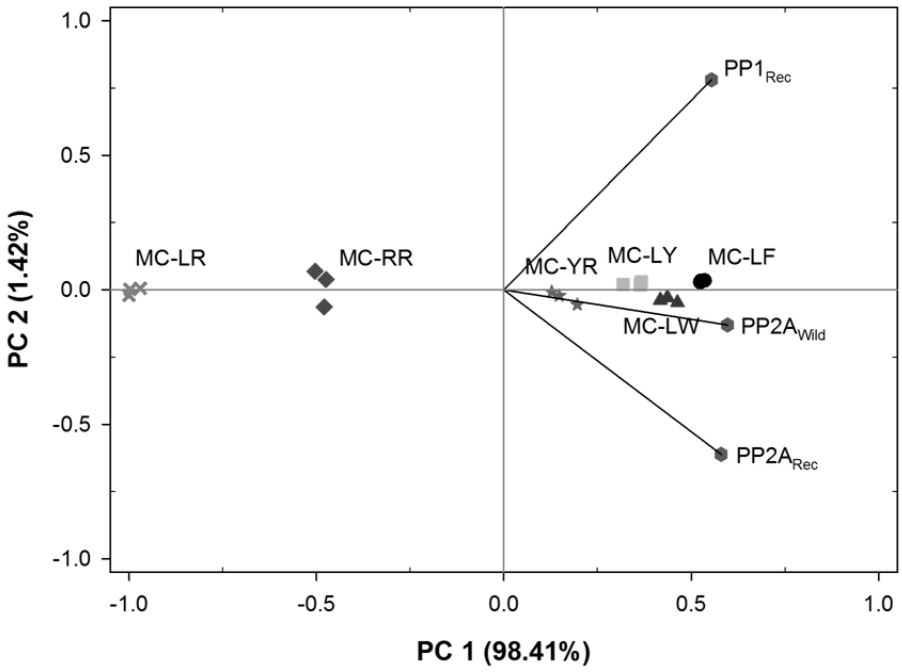

Figure 2.3.2. Scores and loadings plot of the first two components obtained after PCA analysis of the IC_{50} values (triplicates) from the inhibition of $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$ by MC-LR, -RR, -YR, -LY, -LW and -LF standards.

2.3.3.3. Application of the PPIAs to multi-MC profiles and comparison with LC-MS/MS analysis

The PPIAs with $PP1_{Rec}$, $PP2A_{Rec}$ and $PP2A_{Wild}$ were applied to the determination of MC-LR equivalent contents in the extract of the natural cyanobacterial bloom from Alcántara (intracellular fraction). The Alcántara bloom sample was diluted until the dose-response curve provided the corresponding sigmoidal shape. MC-LR calibration curves were performed in parallel to the quantification analysis due to possible slight differences in the inhibition percentages between assays. Results were provided as MC-LR equivalent contents (µg/L) of the extract, calculated from the IC_{50} values of the MC-LR calibration curves and the IC_{50} values of the sample dose-response curves, values determined from the sigmoidal logistic four-parameter equations. $PP1_{Rec}$ and $PP2A_{Wild}$ provided similar

MC-LR equivalent contents, 11292 ± 1685 and 13646 ± 442 µg/L respectively (*t* = 1.941, *p* = 0.192), and different to $PP2A_{Rec}$, 16482 ± 849 µg/L ($PP2A_{Rec}$ *vs.* $PP1_{Rec}$: *t* = 5.221, *p* = 0.035; $PP2A_{Rec}$ *vs.* $PP2A_{Wild}$: *t* = 5.594, *p* = 0.030).

LC-MS/MS analysis showed significantly lower MC contents, 7576 µg/L, the composition being 30% of MC-RR, 18% of MC-LR, 7% of MC-YR, 5% of MC-LW, 2% of MC-LY and 38% of minority and/or non-identified variants. Figure 2.3.2S (supplementary information) shows the chromatograms of the MCs found in the natural bloom sample.

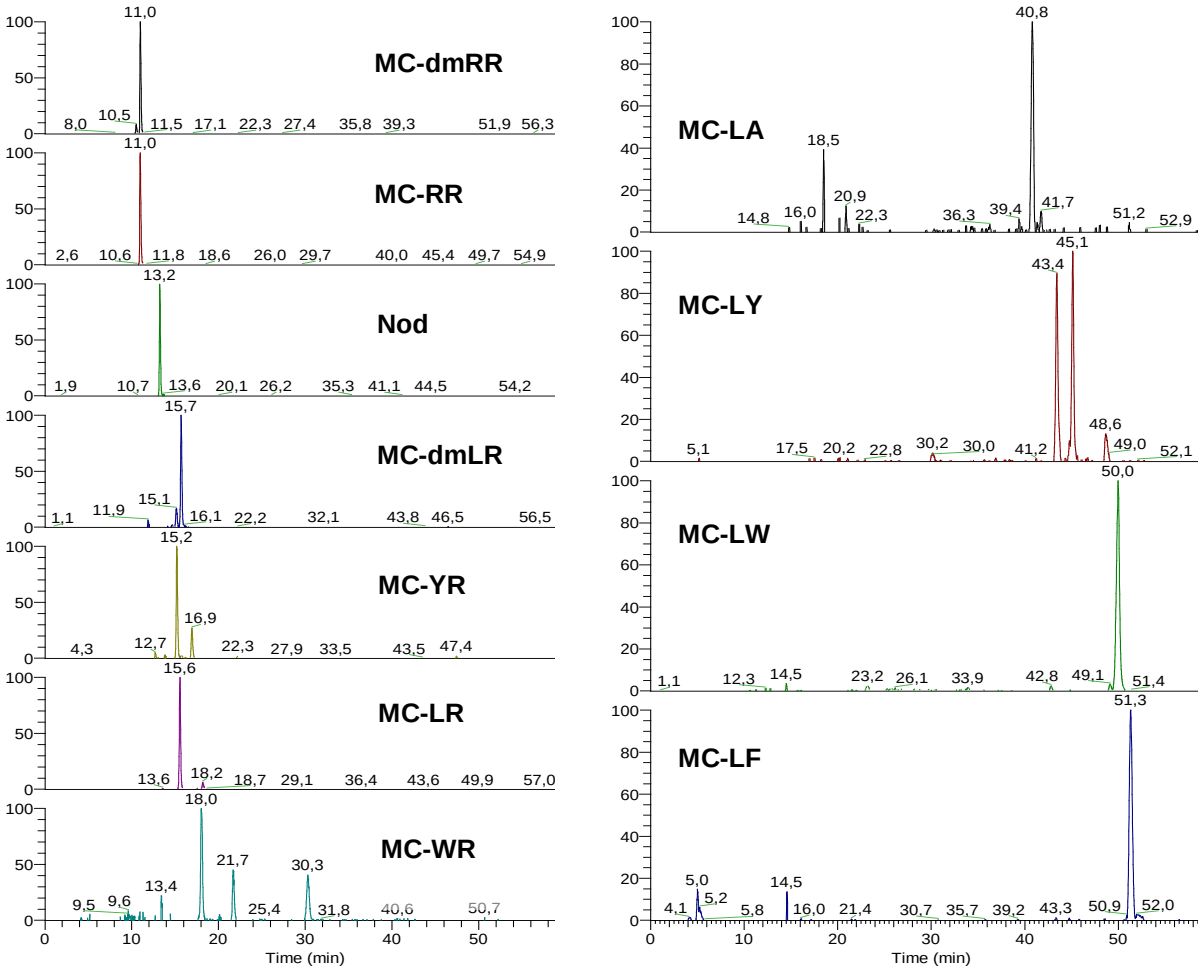

Figure 2.3.2S. Chromatogram of MCs detected in Alcántara bloom sample.

No matrix effects were observed in the LC-MS/MS analysis (chromatograms showed the same peak areas of the three MC spiking levels in methanol and natural bloom extract), neither in the PPIA (no PP inhibition was observed and, in the spiking experiment, the inhibition values were the same in buffer and microalgae culture extract). However, the Alcántara bloom could contain other natural compounds, which could be inhibiting the PPs (Heresztyn and Nicholson 2001). Additionally, these compounds could be inhibiting the PPs to a different extent depending on the enzyme source and nature, and thus could also be responsible for the different MC-LR equivalent contents reported by the three enzymes. The effect of the minority and/or non-identified variants on the inhibitory potency of the mixture could also be responsible for the overestimation by the PPIAs.

To study in depth the effect from the sample matrix and/or the multi-MC profile, a spiking experiment was performed using approximately the same MC composition found in the natural cyanobacterial bloom by setting up an artificially-contaminated sample, prepared by the spiking of buffer with MC standard solutions (extraction was not performed). As previously mentioned, the natural sample contained 38% of minority and/or non-identified variants, two extreme scenarios were established: considering that this 38% did not inhibit the PPs (minimum contents), and considering that it inhibited them at the same potency than MC-LR (maximum contents). Table 2.3.3 shows the MC-LR equivalent contents obtained by the PPIAs as well as the IE values calculated by the application of the corresponding IEFs to the individual MCs spiked concentrations (established from the LC-MS/MS analysis of the Alcántara bloom sample).

Table 2.3.3. MC-LR equivalent contents (µg/L) in a multi-MC profile artificially contaminated sample determined by the PPIAs with $PP1_{Rec}$, $PP2A_{rec}$ and $PP2A_{wild}$, and IE values calculated by the application of the corresponding IEFs to the individual MC quantifications reported by LC-MS/MS analysis. Minimum and maximum contents indicate that all minority and/or non-identified MCs have been considered as non-toxic or toxic at MC-LR potency, respectively.

Enzyme	Minimum content		Maximum content	
	PPIA	IE_{Value}	PPIA	IE_{Value}
$PP1_{Rec}$	1,862 ± 94	1,669	2,572 ± 226	4,568
$PP2A_{Rec}$	3,331± 78	2,180	4,865 ± 15	5,048
$PP2A_{wild}$	2,090 ± 60	1,783	3,540± 84	4,651

The highest MC-LR equivalent contents were those provided by $PP2A_{Rec}$, followed by $PP2A_{Wild}$ and finally $PP1_{Rec}$, following the same trend than in the natural bloom quantification. Minimum and maximum MC-LR equivalent contents determined by PPIAs also follow the appropriate trend. However, the contents determined by PPIAs slightly differ from the IE values, but still being of the same order of magnitude. Unexpectedly, the MC contents are lower than those reported in the quantification of the natural bloom. Although the presence of non-identified MC variants could be in part responsible for the MC equivalent contents overestimations by PPIAs in natural samples in relation to LC-MS/MS analysis, their usually smaller abundance percentage and the probably lower inhibitory potency would not justify it. It is more plausible that the presence of matrix compounds interfere on the PPIAs, despite the high dilutions used. As a future work, an exhaustive evaluation of the possible matrix effects of natural samples would be convenient. The removal of interfering compounds with purification protocols could also improve the results.

Nonetheless, the developed PPIAs can be used as tools to screen the presence of MCs in cyanobacterial samples. Unlike ELISA, PPIA is not based on a structural recognition, but gives a functional response. Although a cell-based assay would provide toxicological information of a sample, PPIA is easier to perform and can be considered to be indicative of that toxicity. In any case, the PPIAs developed in this work would not provide false negatives; only false positives could occur, which would not be of concern for public health. As with any other screening method, a high incidence of false positives would make the test unpractical for routine monitoring. Consequently, the PPIAs could be used as tools to screen the presence of MCs in the environment and, in the case of positive or suspicious results, samples should be addressed towards confirmatory analytical methods such as LC-MS/MS analysis.

2.3.4 Conclusions

In this work, colorimetric PPIAs have been developed for the determination of MCs, attaining low LODs for MC-LR, some of them even below 1 µg/L. Since natural blooms rarely contain a single MC variant, the estimation of inhibitory potencies and IEFs of MC variants contributes to better understand the PPIAs performance and the correlation with LC-MS/MS analysis. Nonetheless, the interpretation of the results provided in the analysis of samples with multi-MC profiles is not straightforward. It is crucial to understand the complexity of natural samples, due to the multi-MC profiles and to the presence of additional compounds that may be present in the extracts and interfere as matrix effects. Nonetheless, the developed PPIAs can be considered useful screening tools to monitor aquatic environments.

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Chapter 3

Magnetic particles as biomolecule immobilisation supports and carriers in biosensors

This chapter includes three published articles. The use of magnetic particles as biomolecule immobilisation supports and carriers in biosensors has been exploited. The first two articles show the feasibility to use magnetic particles as immobilisation supports for enzymes and antibodies in the development of colorimetric assays and electrochemical biosensors for okadaic acid and microcystins. In the third article, magnetic particles have been used as antibody carriers in optical Surface Plasmon Resonance immunosensors for okadaic acid.

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Conjugation of genetically engineered protein phosphatases to magnetic particles for okadaic acid detection

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Abstract

This work presents the functional characterisation of a protein phosphatase 2A (PP2A) catalytic subunit obtained by genetic engineering and its conjugation to magnetic particles (MPs) via metal coordination chemistry for the subsequent development of assays for diarrheic lipophilic marine toxins. Colorimetric assays with free enzyme have allowed the determination of the best enzyme activity stabiliser, which is glycerol at 10%. They have also demonstrated that the recombinant enzyme can be as sensitive towards okadaic acid (OA) (LOD = 2.3 µg/L) and dinophysistoxin-1 (DTX-1) (LOD = 15.2 µg/L) as a commercial PP2A and, moreover, it has a higher operational stability, which makes possible to perform the protein phosphatase inhibition assay (PPIA) with a lower enzyme amount. Once conjugated to MPs, the PP2A catalytic subunit still retains its enzyme activity and it can also be inhibited by OA (LOD = 30.1 µg/L).

Keywords: Genetically-engineered protein phosphatase 2A (PP2A); protein phosphatase inhibition assay (PPIA); magnetic particles (MPs); Ni-His affinity; marine toxin okadaic acid (OA); dinophysistoxin-1 (DTX-1).

3.1.1 Introduction

Okadaic acid (OA) and dynophysistoxins (DTXs), OA derivatives, are marine toxins produced by dinoflagellates of the genera *Dinophysis* and *Prorocentrum* (Steidinger, 1993). These phycotoxins are accumulated in the digestive glands of shellfish with no evidence of toxic effects on them. However, the consumption of contaminated seafood by humans may result in diarrheic shellfish poisoning (DSP), responsible for gastrointestinal disturbances such as diarrhea, nausea, vomiting and abdominal pain (Yasumoto and Murata, 1993). The action mechanism of OA and DTX-1 is based on the inhibition of protein phosphatases (PPs), enzymes that play an important role in protein desphosphorylation in cells. These toxins bind to the receptor site of the enzyme, blocking its activity. As a consequence, hyperphosphorylation of proteins that control sodium secretion by intestinal cells and of cytoskeletal or junctional moieties that regulate solute permeability is favoured, causing sodium release and a subsequent passive loss of fluids, responsible for the diarrheic symptoms (Aune and Yndestad, 1993).

The Commission Regulation (EC) No. 853/2004 of the European Community has established a maximum permitted level of 160 µg of OA equivalents/kg in bivalve molluscs. Numerous methods are reported in the literature for the detection of OA alone or in combination with DTXs. Until this year, the official control method was the mouse bioassay (MBA) (Yasumoto et al., 1978). This method is based on the administration of shellfish samples to mice and the evaluation of the lethal doses. Despite the low specificity and the controversial ethical implications, the assay is useful because it gives an indication of the total toxicity of a sample. However, very recently, the Commission Regulation (EC) No. 15/2011 has established that liquid chromatography-tandem mass spectrometry (LC-MS/MS) should be applied as the reference method. This new regulation has applied from 1st July 2011, being possible to use MBA until 31st December 2014. Nevertheless, this Commission Regulation allows a series of methods, such as other chromatographic techniques with appropriate detection, immunoassays and functional (e.g. phosphatase inhibition) assays, as alternatives or supplementary

to LC-MS/MS, provided that either alone or combined they can detect the required analogues, that they fulfil the corresponding method performance criteria, and that their implementation provides an equivalent level of public health protection. In the development of these alternative methods, the use of toxicity equivalent factors for different compounds of a marine toxin group will be very important to protect consumers and guarantee that toxins levels are below values established in the legislation (Botana et al., 2010).

Cell-based assays (CBAs) have also been used as toxicological models for the evaluation of OA (Cañete and Diogène, 2008; Cañete et al., 2010). The use of that simple and widespread tool may be advantageous in comparison to more sensitive but complex methods. Chromatographic techniques, such as high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) or to tandem mass spectrometry (LC-MS/MS), allow the separation of toxins and their sensitive quantification (de la Iglesia et al., 2008). However, they are laborious, time-consuming and require trained personnel. Immunoassays, based on the affinity interaction between monoclonal or polyclonal antibodies and OA and the use of enzymes as labels, have also been developed (Kreuzer et al., 1999; Campàs et al., 2008). Although enzyme-linked immunosorbent assays (ELISAs) may present the limitation of cross-reactivity, which makes impossible the discrimination of individual toxins, this feature may also be considered as an advantage since all the toxins within the same structural family could be detected. Like CBAs, immunoassays are a promising tool for routine detection and quantification, due to the simplicity, high sample throughput and relative low cost. Finally, another biochemical method for OA determination is the PP inhibition assay (PPIA) (Tubaro et al., 1996; Campàs and Marty, 2007). The PPIA detects DSP lipophilic toxins and it is interesting as a simple, cost-effective and rapid screening tool.

Biosensors have also been developed for marine toxin detection. Most biosensors for OA are immunosensors, i.e. based on immunoassays. The Quartz Crystal Microbalance (QCM) has been applied to the construction of a label-free

immunosensor (Tang et al., 2002), which has been able to detect 3.6 µg/L. Lower concentrations have been detected by Marquette et al. (1999) with a semi-automatic chemiluminescent immunosensor integrated into a flow-injection analysis (FIA) system. Their device had a limit of detection (LOD) of 2.5 µg/L, corresponding to 2 ng/g, with an overall measurement time of 20 min. Surface Plasmon Resonance (SPR)-based immunosensors have also been developed and applied to the analysis of shellfish extracts (Llamas et al., 2007; Stewart et al., 2009). The immunosensor was able to detect 2 µg/L of OA. Originally, the immunosensor was not able to detect DTX-1 and DTX-2 (Llamas et al., 2007). Nevertheless, the authors improved its performance using a new monoclonal antibody, able to detect not only DTX-1 and DTX-2 but also DTX-3 (Stewart et al., 2009). The use of electrochemical methods involves important advantages in terms of sensitivity, cost effectiveness, ease of handling and possibility of miniaturisation. Therefore, electrochemical immunosensors for OA and other phycotoxins have also been developed attaining LODs of 1.5 µg/L for OA (Tang et al., 2003; Kreuzer et al., 2002). Campàs et al., (2008) improved the LOD by combining the immunosensor with a diaphorase-based recycling system. This signal amplification allowed the immunosensor to detect as low as 0.03 µg/L of OA, more than one order of magnitude less than the same system without amplification (1.07 µg/L). Moreover, the working range was enlarged by approximately two orders of magnitude.

Only one enzyme sensor for OA detection, based on the inhibition of immobilised PP, has been reported (Campàs and Marty, 2007). Although the authors demonstrated the feasibility of that electrochemical biosensor and applied it to the detection of OA in microalgae extracts, the LOD was not low enough for specific applications, such as the determination of production of trace amounts of OA by dinoflagellates or the detection of this toxin in shellfish matrices. The authors entrapped PP2A into a photopolymeric matrix and on a screen-printed graphite electrode. Although this immobilisation method maintains the biomolecules in a flexible conformation and substantially retains the stability of the enzyme activity, critical limitation of PPs, it also creates a barrier, which limits accessibility to the

enzyme by both the substrate and the toxin. This suggests that the method for enzyme immobilisation is crucial to the performance of the biosensor. Other immobilisation methods are available, such as adsorption, covalent attachment, inclusion into composites, self-assembling and those based on affinity interactions. The affinity interaction between metals and amino acid residues has been exploited to develop acetylcholinesterase (AChE)-based biosensors for pesticides detection (Adreescu et al., 2001). The authors immobilised AChE to Ni-modified electrodes through a hexa-histidine (hexa-His) tag introduced to the enzyme by genetic engineering. The same research group combined this approach with the use of magnetic particles for biosensor development (Istamboulie et al., 2007). The authors attained LODs lower than those reported for a sensor based on entrapment of the enzyme into a poly (vinylalcohol) matrix. In fact, by carefully locating the hexa-His tail far away from active and inhibitory sites, it seems possible to immobilise the enzyme in such a way that access to those sites is not impaired. These oriented immobilisations together with the absence of immobilisation barriers are likely to produce assays with lower LODs.

In this work we propose the use of genetically-engineered PPs with hexa-His tails allowing conjugation to Ni-modified magnetic particles (MPs) and the development of colorimetric assays for OA detection. First, genetically-engineered PPs have been characterised in terms of activity, stability and inhibition by diarrheic lipophilic toxins and compared with the commercially available enzyme (Upstate Biotechnology). Then, recombinant PPs have been conjugated to MPs. The conjugation has been characterised and the stability of the PP-MP conjugates assessed. Finally, inhibition of the conjugates by OA has been tested. With the use of this immobilisation technique and when the electrochemical biosensors will be set up, we expect to attain lower LODs and to improve the currently available enzyme sensors for OA and derivatives.

3.1.2 Materials and methods

3.1.2.1 Reagents and materials

Okadaic acid (OA) (14,300 µg/L methanolic solution) was purchased from the National Research Council (Halifax, Canada). Dinophysistoxin-1 (DTX-1), from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan), was dissolved in ethanol (0.1 g/L) and subsequently diluted in a buffer solution at pH 8.4 containing 30 mM Tris–HCl, 20 mM $MgCl_2$ (buffer 1) prior to use. Commercial protein phosphatase 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from human red blood cells, was obtained from Upstate Biotechnology (NewYork, USA). The genetically-engineered PP2A catalytic subunit was produced by Gene to Protein (GTP) Technology (Toulouse, France) and contains a hexa-His tail at the C-terminus. The activity of the stock solutions was of 10,800 U/mL for Upstate Biotechnology and between 520 U/mL and 1,545 U/mL for GTP Technology, 1 U being defined as the amount of enzyme required to hydrolyse of 1 nmol p-nitrophenyl phosphate (p-NPP) in one min at 22 °C. Components of buffers and p-NPP were purchased from Sigma (St. Quentin Fallavier, France). All solutions were prepared using Milli-Q water. The Histidine Adem-kit was provided by Ademtech (Pessac, France).

3.1.2.2 Apparatus

HulaMixer™ Sample Mixer from Invitrogen (Leek, The Netherlands) was used in the PP-MP conjugation. A Titramax 1000 Vibrating Platform Shaker from Heidolph (Schwabach, Germany) was used for agitation of microtiter plates. Z5342 MagneSphere® Technology Magnetic Separation Stand (for twelve 1.5-mL tubes) and Z5410 PolyATtract® System 1000 Magnetic Separation Stand (for one 15- or 50-mL tube) from Promega Corporation (Madison USA) were used for magnetic separations in the PP-MP conjugation. Magnetic disks (4 mm diameter x 2 mm height) from Ademtech (Pessac, France) were used for magnetic separations in microtiter plates. Colorimetric measurements were performed with a U-2001 UV/Vis spectrophotometer from Hitachi High-Tech (Krefeld, Germany)

and an automated microplate reader KC4 from Bio-Tek Instruments, Inc. (Bad Friedrichshall, Germany).

3.1.2.3 Enzyme activity

The stock buffer solution for PP2A from Upstate Biotechnology contains 20 mM 3-(N, morpholino) propanesulfonic acid (MOPS), 500 mM NaCl, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM $MnCl_2$, 1mM DTT, 10% glycerol, 0.1 mg/mL BSA, pH 7.4. The initial lot 1 of PP2A from GTP Technology was produced in the initial stock solution containing 18 mM Tris-HCl, 150 mM NaCl, 54 mM 2-mercaptoethanol, 0.9 mM $MgCl_2$, 1mM EGTA, pH 7.5, therefore in order to find the best enzyme activity stabiliser, 10% glycerol (lots 2 and 3), 1 mg/mL BSA (lot 4), 5% sucrose and 0.1% dextran sulphate (lot 5), and 5% sucrose and 2% phosphoglycerate (PGA) (lot 6) were added to the initial stock solution and the activity of the enzyme solutions was evaluated. The PP2A enzyme activity was measured spectrophotometrically in a 1-mL cuvette by adding 10 µL of enzyme to 890 µL of buffer 2. Composition of buffer 2 was 30 mM Tris–HCl, 2 mM EDTA, 20 mM $MgCl_2$, 2 mM 1,4-dithiothreitol (DTT), 0.2 mg/mL bovine serum albumin (BSA), pH 8.4. After 5-min incubation, 100 µL of p-NPP solution at 100 mM in buffer 2 were added to the cuvette and the absorbance at 405 nm was measured for 1 min. Assays were performed in triplicate.

To evaluate the operational stability of PP2A enzymes, the activity was measured spectrophotometrically in a microtiter plate. In this case, 50 µL of buffer 1 were added into microtiter wells containing 100 µL of enzyme solution at different concentrations (between 30 and 100 U/mL) depending on the assay. 50 µL of 25 mM p-NPP solution were added and absorbance at 405 nm was measured every 5-10 min during 1-hour incubation at 22 or 37 °C in the dark. Enzyme and substrate solutions were prepared in buffer 2. Assays were performed in triplicate.

3.1.2.4 Colorimetric PPIA with free enzyme

The colorimetric PPIA was similar to that described by Tubaro et al. Brifely, 50 µL of OAstandard solutions at different concentrations ranging from 0.2 to 100 µg/L were added into microtiter wells containing 100 µL of enzyme solution at different concentrations (between 3 and 1.25 U/mL) depending on the assay. 50 µL of 25 mM p-NPP solution were added and after 1-hour incubation at 22 °C in the dark, absorbance at 405 nm was measured. Enzyme and substrate solutions were prepared in buffer 2 and OA solutions in buffer 1. Assays were performed in triplicate.

3.1.2.5 Conjugation of recombinant PP2A to MPs

The conjugation protocol was: (1) 15 µL of a MP suspension was added to a 1.5-mL microtube containing 150 µL of binding buffer (provided in the Histidine Adem-Kit); (2) the tube was placed on the magnetic device until supernatant clearing and the supernatant was then removed by pipetting; (3) 150 µL of binding buffer were added to the microtube and mixed by vortexing; (4) step (2) was repeated; (5) 500 µL of recombinant PP2A solution prepared in a buffer containing 30 mM Tris–HCl, 20 mM $MgCl_2$, 0.2 mg/mL BSA, pH 8.4 (buffer 3) were added and incubated for 15 min at 22 °C while shaking (800 rpm): (6) step (2) was repeated and free enzyme excess supernatant (SN) was removed from the microtube; (7) 220 µL of binding buffer were added and mixed with vortexing; (8) steps (2) and (7) were repeated thrice and three washing solutions were removed from the microtube (W1, W2 and W3). The resulting product consists on the PP-MP conjugate suspension.

3.1.2.6 Characterisation of the PP-MP conjugation

To characterise the PP-MP conjugation, a colorimetric assay was performed in microtiter plates. 110 µL of PP-MP conjugate suspension and 50 µL of buffer 3 were added into microtiter wells. Then, 50 µL of 25 mM p-NPP solution were added and incubated for 30 min while shaking (800 rpm) at 22 °C in the dark. After substrate incubation, PP-MPs were separated with magnets and 200 µL of

clear solution were taken for absorbance reading at 405 nm. Assays were performed in duplicate. To evaluate the effect of the number of washing steps, the absorbance values reached by the supernatant and washing solutions were also recorded.

Because the interaction of His with MPs is reversible and imidazole can compete with His residues for metal coordination positions, the PP-MP conjugation was also confirmed by removal of the bound PP2A by an excess of imidazole. In this case, after PP-MP conjugation the following steps were performed: (1) the tube containing the PP-MP conjugate suspension was placed on the magnetic device until supernatant clearing and the supernatant was then removed by pipetting; (2) 220 µL of elution buffer 1 (with 100 mM imidazole) provided in the kit were added and mixed while shaking (800 rpm) at 22 °C for 1 hour; (3) step (1) was repeated; (4) 220 µL of elution buffer 2 (with 500 mM imidazole) provided in the kit were added and mixed while shaking (800 rpm) at 22 °C for 1 hour; (5) step (1) was then repeated; (6) and finally, 220 µL of binding buffer were added. The colorimetric assay protocol for the “theoretically” imidazole-MP conjugate was the same as the previously described for PP-MP.

3.1.2.7 Storage stability of PP-MP conjugates

The storage stability of the PP-MP conjugates was tested at 4 °C and -20 °C. PP-MP suspensions were kept at these temperatures and the activity of the conjugates was measured at storage times of 0 (reference activity value), 24, 48 and 72 hours using the protocol described above for the characterisation of the PP-MP conjugation.

3.1.2.8 Colorimetric PPIA with PP-MP conjugates

50 µL of OA standard solutions at different concentrations ranging from 1.6 to 100 µg/L or shellfish (mussel, wedge clam, flat oyster and Pacific oyster) extracts at 25 mg/mL spiked with OA at 3200 µg/kg were added into microtiter wells containing 110 µL of PP-MPs at 50 U/mL in the conjugation (or 55 µL of PP-MPs at 100 U/mL in the conjugation). Then, 50 µL of 25 mM p-NPP solution were

added and incubated for 30 min while shaking (800 rpm) at 22 °C in the dark. After substrate incubation, PP-MPs were separated with magnets and 200 µL of clear solution were taken for absorbance reading at 405 nm. Assays were performed in duplicate.

3.1.3 Results and discussion

3.1.3.1 PP2A activity characterisation

The performance of recombinant PP2A catalytic subunit from GTP Technology was compared to that of the commercial PP2A from Upstate Biotechnology, used as an enzyme model. The inherent PP instability is one of the critical limitations of this enzyme and the derived assays. Several compounds were tested as possible enzyme activity stabilisers for stock solutions. Lyophilisation was also investigated as a possible procedure to maintain enzyme activity during storage. Table 3.1.1 reports the enzyme activity values of these stock solutions with different supposed stabilisers, determined immediately after synthesis and one week later.

Table 3.1.1 Enzyme activities (U/mL) of the stock solutions of lots of PP2A from GTP Technology with different stabilisers, as reported by GTP Technology after synthesis and determined experimentally at IRTA approximately one week later. Relative standard deviation values (%) are shown in parentheses.

Lot number	Stabiliser	Enzyme activity (U/mL)	
		Determined at IRTA	Reported by GTP
Lot 1	-	5 (0.1)	120
Lot 2	10% glycerol	302 (13.7)	329
Lot 3	10% glycerol	1473 (6.0)	1400
Lot 4	0.1% BSA	8 (6.5)	153
Lot 5*	5% sucrose / 0.1% dextran sulphate	45 (7.7)	115
Lot 6*	5% sucrose / 2% PGA	-	32

[a] Lyophilised stock enzymes.

The best enzyme activity stabiliser was 10% glycerol (lots 2 and 3), as used by Upstate Technology in their stock solutions. The difference in activity between lots 2 and 3 could be explained by the production process (different insect cell lines were used) leading to protein batches with different enzyme purity and specific activity. The other compounds were not appropriate stabilisers, probably due to enzyme inactivation during transport and storage. Nevertheless, since lot 5 was also lyophilised, it is not possible to determine if its low enzyme activity was due to the stabiliser or to the lyophilisation procedure. In the case of lot 6, it was not possible to redissolve it and enzyme activity could not be quantified.

Compared with PP2A from Upstate, the GTP enzyme was less active. Nevertheless, when the operational stability (ability to retain the enzyme activity during the PPIA) of both enzymes was compared at 22 and 37 °C, results showed that PP2A from GTP was more stable during the hour required for the assay (attaining higher absorbance values). In order to quantify this operational stability, the enzyme activity at the last 5 min was compared to the enzyme activity at the first 5 min and expressed in percentage. Whereas PP2A from Upstate retained 52 and 3% of the activity after 1 hour working at 22 and 37 °C, respectively, PP2A from GTP retained 100 and 24% of the activity at the same temperatures. This higher operational stability of PP2A from GTP implies that a lower enzyme amount can be used to reach an appropriate absorbance value and thus, the PPIA could be more sensitive than that performed with PP2A from Upstate. For the rest of the study, only lots 2, 3 and 5 of PP2A from GTP and PP2A from Upstate were used.

3.1.3.2 Colorimetric PPIA with free enzyme

The PPIA demonstrated the inhibitory effect of OA on the PP2A activity. The calibration curves were described by the sigmoidal logistic four-parameter equation (SigmaPlot software package 9.0):

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

Where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point.

In Table 3.1.2, the concentration of enzyme used in the assay, the absorbance value reached by the controls without toxin at the end point, the 50% inhibition coefficient (IC_{50}) values and the working ranges (defined between IC_{20} and IC_{80}) are presented together with the equations and the corresponding R values.

Table 3.1.2 Curve parameters derived from the sigmoidal logistic four-parameters fitting for the inhibition of PP2As by the marine toxins OA and DTX-1 in colorimetric assays.

Toxin	Enzyme	$[PP2A]_0$ (U/mL)	ABS_f (units)	IC_{50} (µg/L)	Working range (µg/L)	Sigmoidal logistic equation	R
	Upstate	3	0.433	4.0	1.8-9.9	$y= -5.8 + (102.4/(1 + (x/3.5)^{-1.6}))$	0.9986
	GTP lot 5	3	0.799	46.2	29.0-69.9	$y= 1.4 + (105.3/(1 + (x/48.6)^{-2.9}))$	0.9990
OA	GTP lot 2	3	0.863	22.9	14.8-39.8	$y= -0.9+ (91.4/(1 + (x/21.3)^{-3.2}))$	0.9988
	GTP lot 2	1.25	0.441	20.4	14.9-29.0	$y= 2.4+ (90.6/(1 + (x/20.0)^{-4.8}))$	0.9989
	GTP lot 3	1.25	0.400	5.3	2.3-12.9	$y= 1.8+ (92.7/(1 + (x/5.0)^{-1.8}))$	0.9995
DTX-1	GTP lot 3	1.25	0.406	27.0	15.2-46.8	$y= 2.9+ (96.3/(1 + (x/27.5)^{-2.5}))$	0.9988

The first colorimetric PPIA was performed at 3 U/mL concentration for PP2A from Upstate and for PP2A from lots 2 and 5 of GTP. Fig. 3.1.1 shows the normalised calibration plots. The calibration curves showed that lot 2 from GTP was more sensitive than lot 5: $LOD_{GTP\ lot\ 2}$ = 14.8 µg/L in front of $LOD_{GTP\ lot\ 5}$ = 29.0 µg/L (LOD was defined as the 20% inhibition coefficient (IC_{20}) value). This difference may be due to the specific activity of each one of the lots. Although both PPIAs were performed at 3 U/mL, this concentration may represent a lower number of enzyme molecules when using the stock solution with a 6.7-fold higher activity (lot 2) and thus, a higher sensitivity towards the toxin. However, both lots were less sensitive than PP2A from Upstate, which provided a $LOD_{Upstate}$ of 1.8 µg/L. Despite the higher LODs attained with PP2A from GTP, it was observed again that GTP enzymes were more stable during the 1-hour experiment (whereas an absorbance value of 0.4 is reached at the end point in the control wells without

toxin with PP2A from Upstate, 0.8-0.9 is reached with PP2A enzymes from GTP).

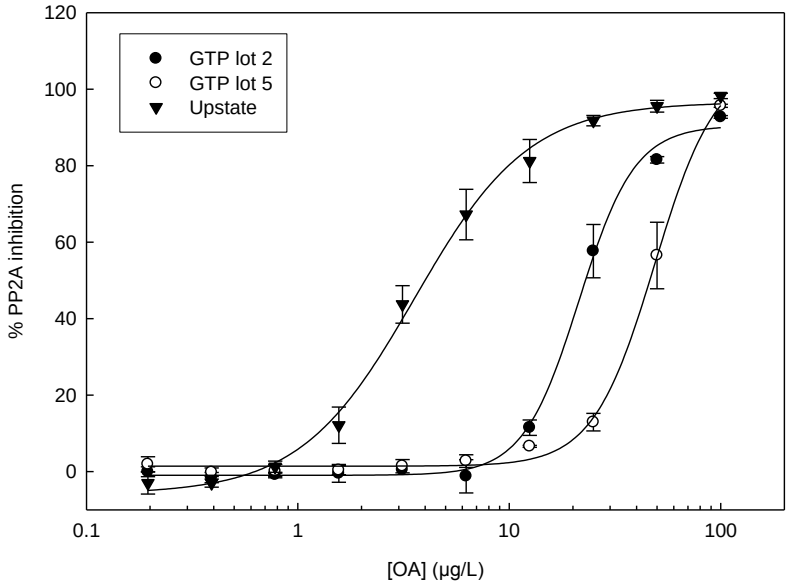

Fig. 3.1.1. Colorimetric calibration curves for the inhibition of PP2As (from Upstate Biotechnology and from lots 2 and 5 of GTP Technology at 3 U/mL) in solution by OA. Inhibition is expressed as percentage of the control (no OA). *x* values refer to initial OA concentrations.

With the aim of optimising the sensibility of the assay and taking into account the higher operational stability of PP2A from GTP, the PPIA was performed with lot 2 at 1.25 U/mL. Nevertheless, no significant differences were observed and the assay was still less sensitive than that performed with PP2A from Upstate. It seems that the 2.4-fold lower enzyme concentration in the assay was not enough to appreciate a significant improvement of the LOD.

The inhibition of lot 3 from GTP by OA was tested together with the inhibition of DTX-1 in order to compare both toxins (Fig. 3.1.2). As expected due to the higher activity of the stock solution, this lot resulted to be more sensitive to OA than lots 2 and 5, and practically as sensitive as enzyme from Upstate. Moreover, it was more stable during 1 hour of experimentation. DTX-1 showed a lower inhibitory power than OA (5.1-fold lower IC_{50}).

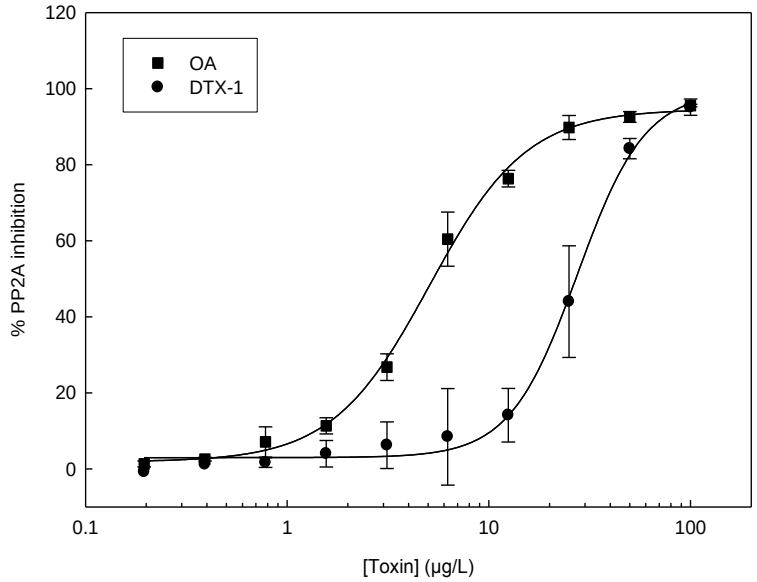

Fig. 3.1.2. Colorimetric calibration curves for the inhibition of PP2A (from lot 3 of GTP Technology at 1.25 U/mL) in solution by OA and DTX-1. Inhibition is expressed as percentage of the control (no toxin). *x* values refer to initial toxin concentrations.

3.1.3.3 Conjugation of PP2A to MPs

The conjugation of PP2A to MPs is based on the Ni^{2+} ability to bind strongly by chemical coordination to proteins containing His (or Cys) residues. On the one hand, the PP2A catalytic subunit produced by GTP Technology contains six consecutive His residues added to the C-terminus. On the other hand, MPs from

Ademtech have iminodiacetic acid (IDA) groups covalently bound to their surface and they are precharged with Ni. Three of the six sites of the Ni coordination sphere are occupied by the tridentate chelating group of IDA and the other three sites are occupied by three of the six His residues of the genetically-engineered enzyme.

In order to determine the optimum enzyme concentration to use in the conjugation of PP2A from GTP to MPs, several enzyme concentrations were tested. Fig. 3.1.3 shows the absorbance values attained by the PP-MP conjugates as well as their corresponding supernatants from the free enzyme excess (SN) and the three washing steps (W1, W2 and W3).The colour development observed in the colorimetric assays with PP-MP demonstrates that conjugations were successful. The absorbance attained in the assays increased proportionally to the enzyme concentration used in the conjugation protocol. No saturation plateau was observed, although it was not our purpose to fully coat the MPs with enzyme (as previously mentioned, too much enzyme could result in higher LODs). Looking at the absorbance from the SNs, free enzyme was started to be detected at 37.5 U/mL. Nevertheless, the absorbance attained with the PP-MP conjugate at this concentration was not enough to perform a PPIA. The PP-MP conjugation at 75 U/mL produced conjugates with appropriate catalytic responses but it was not economically efficient, since a high amount of enzyme was detected in the SN. A concentration of 50 U/mL was chosen in further conjugations.

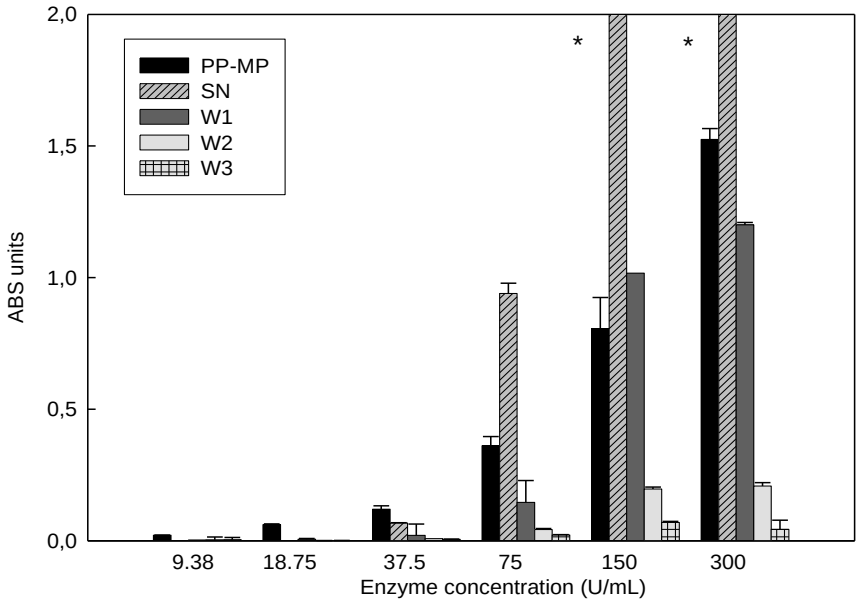

Fig. 3.1.3. Absorbance values attained in the colorimetric assay from PP–MP conjugates obtained using several initial enzyme concentrations (with lot 3 of GTP Technology) and their respective supernatants from the free enzyme excess (SN) and the three washing steps (W1, W2 and W3). *Absorbance values beyond the spectrophotometer interval range.

The effect of the number of washing steps on the PP-MP conjugates was characterised, since each washing could be compromising their enzyme activity. To this purpose, an experiment was performed stopping the conjugation protocol at different washing steps. Fig. 3.1.4 shows the absorbance values reached with the PP-MP conjugates, the first supernatant (SN) and the corresponding washings (W1, W2 and W3). The PP-MP bars show that the absorbance of the conjugates decreases with the number of washing steps. In order to know if the decrease is because of the removal of unbound PP (desired) or to the inactivation of the bound PP (undesirable), it is necessary to consider the sum of all the absorbance values for each set with washing steps with respect to the same value for the set without washing steps (in percentage values). Whereas the absorbance of the PP-MP conjugates decreases with the number of washing steps (96 ± 2, 80 ± 6 and 71 ± 3 %, for the conjugates washed 1, 2 and 3 times, respectively), in the global balances (∑PP-MP+SN+W), absorbance values do not significantly vary in the first (102 ± 4 %)

or the second (98 ± 6 %) washing step. In the third washing step the sum starts to decrease (84 ± 6 %), may be due to the inactivation of the conjugated enzyme. Nevertheless, in order to fully remove adsorbed enzyme and taking into account that the absorbance reached by the PP-MP conjugate is still appropriate, further experiments were performed with 3 washing steps.

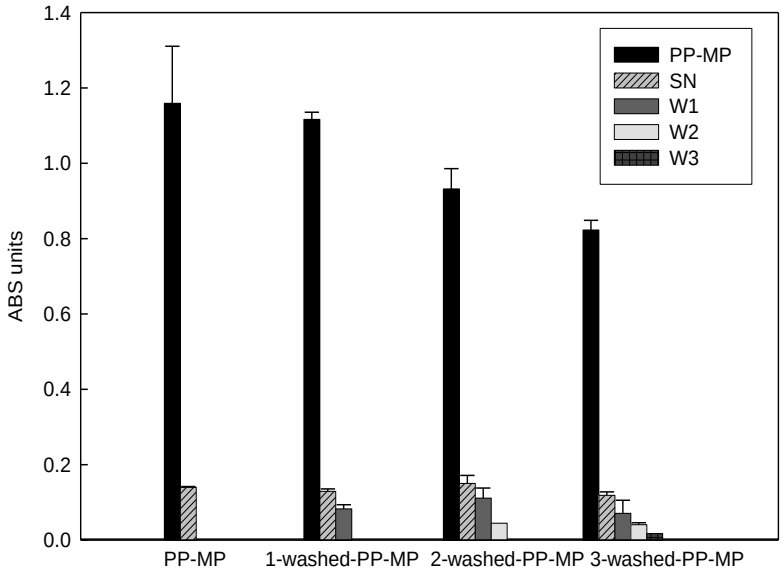

Fig. 3.1.4. Absorbance values attained in the colorimetric assay from PP–MPconjugates (synthesised at 50 U/mL with lot 2 of GTP Technology), from the free enzyme excess (SN) and from the washing steps (W1, W2 and W3) for non-washed, 1-washed, 2-washed and 3-washed PP–MP conjugates.

In order to verify the PP-PM conjugation, imidazole was added to the conjugate for the competition with His residues for metal coordination positions. After imidazole addition, the MPs did not show any significant absorbance value compared to the PP-MP conjugate, indicating that most of the enzyme was effectively conjugated and not adsorbed.

3.1.3.4 Storage stability of the PP-MP conjugates

In order to investigate if it is possible to store the PP-MP conjugates until use or if, on the contrary, they should be freshly prepared, their storage stability was determined at 4 °C and -20 °C. In Fig. 3.1.5, the activity percentages of the PP-MP conjugates after several times at different temperatures are presented.It is clearly observed that PP-MP was more stable when stored at -20 °C. Whereas after 72 hours at that temperature the activity was retained by 72%, at 4 °C only 56% of the activity was maintained. Nevertheless, it is interesting to mention that free PP2A can be only stored at -20 °C, its enzyme activity drastically decreasing when storing it at 4 °C. Therefore, the immobilisation of PP2A on MPs is increasing the stability of the enzyme activity, as others authors have also reported when using this (Sustrova et al., 2009) or other immobilisation techniques (Campàs and Marty, 2007).

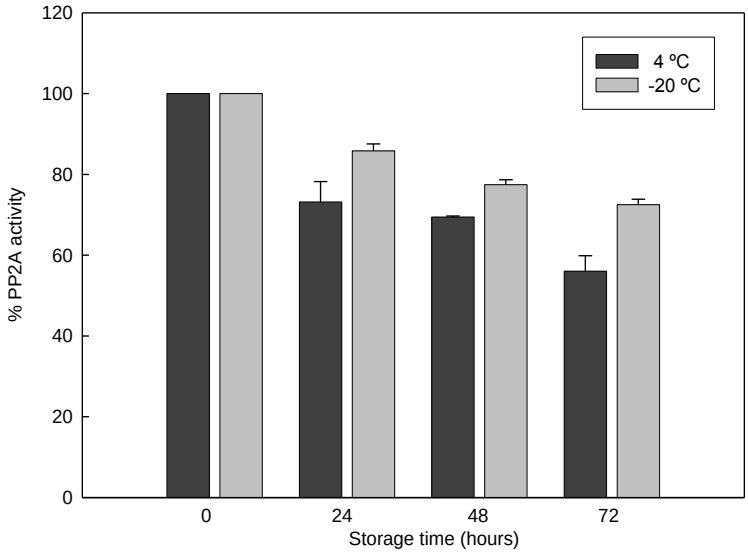

Fig. 3.1.5 Activity percentage from PP–MP conjugates (synthesised at 50 U/mL with lot 2 of GTP Technology) after several storage times at 4 ° C and −20 ° C.

3.1.3.5 Colorimetric PPIA with PP-MP conjugates

The inhibitory effect of OA on the enzyme activity of PP-MP conjugates was investigated. Since no significant differences were observed between 1-hour and 30-min incubation (data not shown), the shortest time was chosen for the colorimetric assay. Calibration curves with free and conjugated enzyme are shown in Fig. 3.1.6. Again the calibration curves were described by the sigmoidal logistic four-parameter equation.

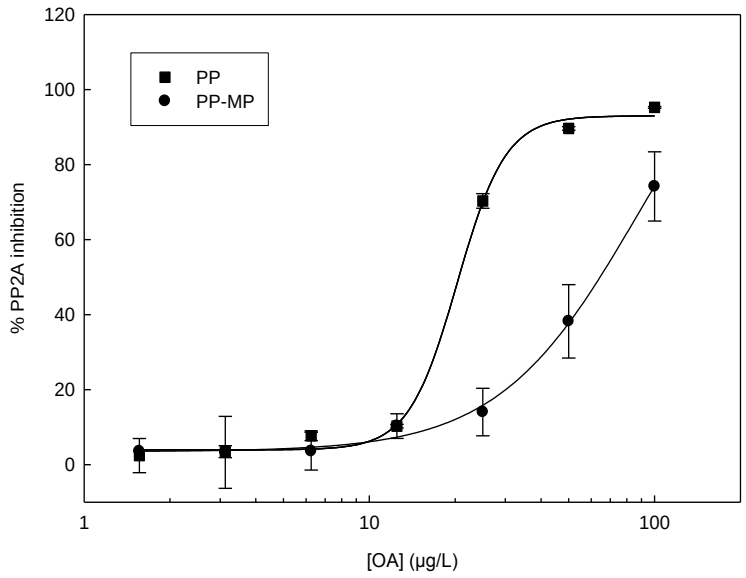

Fig. 3.1.6. Colorimetric calibration curves for the inhibition of PP2A (from lot 2 of GTP Technology at 1.25 U/mL) and PP–MP conjugates (synthesised at 50 U/mL with lot 2 of GTP Technology) in solution by OA. Inhibition is expressed as percentage of the control (no OA). *x* values refer to initial OA concentrations.

Table 3.1.3 reports the absorbance value reached by the controls without toxin at the end point, the IC_{50} values and the working ranges together with the sigmoidal logistic equations and the *R* values. The PPIA demonstrated that the PP-MP conjugates were inhibited by OA, although in a lower extent than free enzyme ($LOD_{PP\text{-}MP}$ was of 30.1 µg/L).

Table 3.1.3. Curve parameters derived from the sigmoidal logistic four-parameters fitting for the inhibition of PP2A and PP–MP conjugates (both with lot 2 of GTP Technology) in solution by OA in colorimetric assays.

Enzyme	ABS_f (units)	IC_{50} (µg/L)	Working range (µg/L)	Sigmoidal logistic equation	R
Free PP	0.441	20.4	14.9-29.0	$y = 2.4 + (90.6/(1 + (x/20.0)^{-4.8}))$	0.9989
PP-MP	0.407	64.4	30.1-111.2	$y = 3.4 + (126.1/(1 + (x/87.1)^{-1.7}))$	0.9991

The PPIA with PP-MP conjugates was applied to the determination of OA in spiked shellfish extracts. Recovery results were 83 ± 10%, 82 ± 5%, 83 ± 11% and 96 ± 7% for mussel, wedge clam, flat oyster and Pacific oyster, respectively. Although the toxin contents used for the spiking were very high (3200 µg OA eq/kg shellfish), results demonstrate the applicability of the approach. It is expected that the electrochemical detection to be used in the development of the biosensor will improve the sensitivity of the analysis and thus, shellfish samples with toxin contents near the maximum permitted levels (160 µg OA eq/kg shellfish) will be reliably detected.

3.1.4 Conclusions

This work characterises a recombinant PP2A (GTP) catalytic subunit in terms of activity, stability and inhibition by diarrheic lipophilic toxins, and compares it with a commercial PP2A (Upstate). Due to the instability inherent to PP, the effect of several enzyme activity stabilisers has been evaluated, glycerol being the most appropriate one. Moreover, the operational stability of the recombinant enzyme is higher than that provided by the commercial one. One of the lots of genetically-engineered PP2A has resulted to be as sensitive towards OA as the commercial enzyme. Another advantage of the genetically-engineered PP2A is the His tail, which has allowed the conjugation of the enzyme to Ni-modified magnetic particles via coordination chemistry. Colorimetric assays have demonstrated that the PP-MP conjugation is successful and that PP2A retains the enzyme activity. Removal of conjugated PP2A by imidazole has also demonstrated that the enzyme was effectively conjugated and not simply adsorbed. A colorimetric

inhibition assay with PP-MP conjugates has been developed, resulting in a detection limit of 30.1 µg/L of OA. Although the LOD is lower than those attained with free PP2A in solution, the electrochemical detection will probably provide with better sensitivities. Work is in progress to develop the corresponding electrochemical biosensor. It is expected that the high sensitivity inherent to the electrochemical detection together with the immobilisation through MPs will decrease the LODs compared to those achieved by colorimetric assays and previous electrochemical biosensors.

Acknowledgements

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Magnetic Particle-Based Enzyme Assays and Immunoassays for Microcystins: From Colorimetric to Electrochemical Detection.

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Abstract

In this work, magnetic particles (MPs) are used as supports for the immobilisation of biorecognition molecules for the detection of microcystins (MCs). In one approach, a recombinant protein phosphatase 1 (PP1) has been conjugated to MPs via coordination chemistry, and MC-LR detection has been based on the inhibition of the enzyme activity. In the other approach, a monoclonal antibody (MAb) against MC-LR has been conjugated to protein G-coated MPs, and a direct competitive enzyme-linked immunoparticle assay (ELIPA) has been then performed. Conjugation of biomolecules to MPs has been first checked, and after optimisation, MC detection has been performed. The colorimetric PPIA with PP1-MP and the best ELIPA strategy have provided limits of detection (LOD) of 7.4 and 3.9 µg/L of MC-LR, respectively. The electrochemical ELIPA has decreased the LOD to 0.4 µg/L, value below the guideline recommended by the World Health Organisation (WHO). The approaches have been applied to the analysis of a cyanobacterial culture and a natural bloom, and MC equivalent contents have been compared to those obtained by conventional assays and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results have demonstrated the viability of the use of MPs as biomolecule immobilisation supports in biotechnological tools for MCs monitoring.

3.2.1 Introduction

Cyanobacteria usually occur in lakes, ponds, reservoirs and rivers with low flow regimes. Not all cyanobacteria are toxicogenic, but those that are pose a serious threat to human health. Cyanobacteria blooms and cyanotoxins production depend on seasonal and climatic variations, such as water temperature, sunlight, wind, nutrients and flow currents.[1] Microcystins (MCs) and nodularins are the most commonly found cyanotoxins. They are hepatotoxins, with demonstrated potent tumor promoter and carcinogenic effects.[2] They contain an unusual aromatic amino acid, the Adda chain, whose hydrophobicity allows the entrance of the toxins into the hepatocytes.[3]Once into the cells, MCs and nodularinsinhibit protein phosphatases 1 (PP1) and 2A (PP2A),[4-7]two key enzymes in cellular regulation. The most frequent and toxic variant is MC-LR, which contains leucine (L) and arginine (R), and it is usually taken as a model.

The presence of cyanobacterial blooms and their associated toxins in drinking water supplies poses a serious threat to animal and human health.[7,8] Many cases of diseases, some of them resulting in liver cancer and even death, have been reported.[8-15] In order to assure water quality and to protect human health, the World Health Organisation (WHO) has proposed a provisional maximum permitted level of 1 µg/L of MC-LR in drinking water.[16] Although in Europe there is no legislation, several countries have adopted the WHO recommended value in their regulations.[17] Consequently, the development of fast, sensitive and reliable analysis methods, able to detect toxin contents below this value, is an evident necessity.

To date, several methods have been developed for MC detection. Chromatographic techniques coupled to different detection principles, e.g. Ultraviolet (UV), Fluorescence (FL) and Mass Spectrometry (MS), are very powerful because of their high sensitivity and the ability to detect different MC variants.[18-21] Nevertheless, analysis times are quite long, and skilled personnel

and expensive equipment are required.[22-24]Alternative techniques based on biochemical principles have emerged as quantitative and screening methods. This is the case of PP inhibition assays (PPIAs)[4,25-31] and immunoassays.[32-38] These two methods are based on different principles: whereas PPIA measures the inhibition effect of MCs on the enzyme activity, immunoassays, such as the enzyme-linked immunosorbent assay (ELISA), are based on the affinity recognition between antibodies and antigens. Thus, PPIA informs about the toxicity of the sample and ELISA detects structurally-related variants.

Based on these assays, some biosensors have been developed for the detection of MCs.[39-42] Although some improvements are still required, biosensors are interesting because of their high sensitivity, short analysis time, low cost and ease of use. Moreover, their miniaturisation may lead to the development of compact and portable analysis devices, able to operate *in situ*. Nanobiotechnology is certainly contributing to attain this challenge. The use of nanomaterials in the design of novel biosensors, specifically in the biorecognition element immobilisation and in the signal transduction strategy, may provide with advantages in terms of sensitivity, stability, analysis time, multiple analyte detection and automation.[43]

Magnetic particles (MPs) have been recently exploited as biomolecule immobilisation supports for their subsequent integration in automated microfluidic systems.[44-49] The use of MPs as biomolecule carriers is promising because they can be manipulated externally with magnets, assay kinetics are achieved more rapidly and matrix effects are diminished because of the improved washing steps.

In this work, two approaches involving MPs as supports for the immobilisation of a recombinant PP1 and a monoclonal antibody (MAb) against MC-LR for the detection of MCs have been investigated. Conjugation of the biorecognition molecules to MPs has been first checked. After optimisation, a colorimetric PPIA with PP1-MP conjugates and two enzyme-linked immunoparticle assays (ELIPAs) have been developed. The colorimetric ELIPA strategy that has provided the best limit of detection (LOD) has been chosen for the development

of the electrochemical ELIPA. All approaches have been applied to the analysis of a cyanobacterial culture and a natural bloom, and MC equivalent contents have been compared to those obtained by conventional PPIA and ELISA, as well as by liquid chromatography-tandem mass spectrometry (LC-MS/MS). To the best of our knowledge, no approaches have been reported on the detection of MCs with biochemical strategies that integrate MPs for future development of compact microfluidics devices.

3.2.2 Experimental section

3.2.2.1 Reagents and materials

A recombinant protein phosphatase 1 (PP1) catalytic subunit, with a hexa-His tail at the C-terminus, was obtained from CRITT-BioIndustries (Toulouse, France). The lyophilised PP1 was dissolved in a buffer solution at pH 8.4 containing 30 mM Tris–HCl, 2 mM EDTA, 20 mM $MgCl_2$, 0.2 mg/mL bovine serum albumin (BSA) and 10% glycerol. A protein phosphatase 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from human red blood cells, was obtained from Upstate Biotechnology (New York, USA). The enzyme activity of stock solutions was 365 U/mL for PP1 and 3,812 U/mL for PP2A, 1 U being defined as the amount of enzyme required to hydrolyse 1 nmol of *p*-nitrophenyl phosphate (*p*-NPP) in one min at 22 °C. Monoclonal antibody (MAb) raised in mouse against MC-LR (MC10E7) was purchased from Enzo Life Sciences (Lausen, Switzerland). This antibody recognises all 4-Arg MCs.

Superparamagnetic particles precharged with nickel and metal-chelating iminodiacetic acid (IDA) groups covalently bound to their surface (Histidine Adem-Kit, 04500) and superparamagnetic nanoparticles conjugated with protein G (Bio-Adembeads Protein G, 0433) were supplied by Ademtech SA (Pessac, France). MC-LR, MC-RR, MC-YR, MC-WR, MC-LA, MC-LF, MC-LW and MC-LY were obtained from Alexis Biochemicals (Lausen, Switzerland), and stock solutions were prepared in methanol. MC-dmRR was purchased from Cyano Biotech GmbH (Berlin, Germany) and MC-dmLR from DHI (Hørsholm, Denmark).

MC-LR-horseradish peroxidase (MC-LR-HRP) was purchased from Prof. W.W. Carmichael's laboratory (Wright State University, Dayton, USA). 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, *p*-NPP, BSA and components of buffers were purchased from Sigma (Tres Cantos, Spain). All solutions were prepared using Milli-Q water.

For LC-MS/MS analysis, all reagents used were of analytical or HPLC grade. Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany) and formic acid from Panreac (Montcada i Reixac, Spain). Solutions were prepared using Milli-Q water.

3.2.2.2 Equipment

A HulaMixerTM Sample Mixer from Invitrogen (Leek, The Netherlands) was used in the PP1-MP and MAb-MP conjugations.Magnetic separation was performed using a Z5342 MagneSphere® Technology Magnetic Separation Stand (for twelve 1.5-mL tubes) and a Z5410 PolyATtract® System 1,000 Magnetic Separation Stand (for one 15- or 50-mL tube), from Promega Corporation (Madison, USA). Magnetic disks (4 mm diameter × 2 mm height) from Ademtech (Pessac, France) were used for magnetic separations in microtiter plates. A Titramax 1000 Vibrating Platform Shaker from Heidolph (Schwabach, Germany) was used for plate agitation.

Colorimetric measurements were performed with a U-2001 UV/Vis spectrophotometer from Hitachi High-Tech (Krefeld, Germany) and a Microplate Reader KC4 from BIO-TEK® Instruments, Inc. (Vermont, USA).

Cyclic voltammetry and chronoamperometric measurements were performed with an AUTOLAB PGSTAT128N potentiostat. Data were collected and evaluated by General Purpose Electrochemical System (GPES) software. Disposable screen-printed three-electrode systems, with carbon as working and counter electrodes, and silver as reference electrode were obtained from Dropsens (Llanera, Spain).

3.2.2.3 Culture and natural bloom samples

A lyophilised biomass sample of a PCC 7820 *Microcystis aeruginosa* culturewas supplied by the *Institut Pasteur* (Paris, France). A sample of a natural MCs-producing microalgae bloom (50 mL of water with biomass) was collected in the *Alcántara* reservoir (Cáceres, Spain) in summer 2009 and filtered through Whatman GF/F filters (0.7 µm).

3.2.2.4 Microcystins extraction

The lyophilised biomass sample of the PCC 7820 culture (0.05 g) was extracted three times by sonication for 15 min with acidified methanol (0.16 % formic acid), according to Barco and co-workers.[21] Extract was evaporated to dryness and reconstituted in 1 mL of methanol. In the case of the natural bloom, the extract corresponding to the intracellular fraction was reconstituted in 8 mL of methanol.

Extracts were directly injected into the LC-MS/MS system. For extracts to be tested with the PPIA, samples were evaporated in a Speed VAC concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen at room temperature, and residues were resuspended in buffer.

3.2.2.5 Conjugation of recombinant PP1 to MPs

The conjugation protocol was: (1) 15 µL of a MP suspension were added to a 1.5-mL tube containing 150 µL of binding buffer (provided in the Histidine Adem-Kit); (2) the tube was placed on the magnetic separation stand until supernatant clearing and the supernatant was removed; (3) 150 µL of binding buffer were added and mixed by vortexing; (4) step (2) was repeated; (5) 500 µL of recombinant PP1 solution were added and incubated for 15 min at 22 °C with shaking on the Hulamixer; (6) step (2) was repeated and free enzyme excess supernatant was removed; (7) 220 µL of binding buffer were added and mixed with vortexing; (8) steps (2) and (7) were repeated three times, removing the washing solutions. The resulting product consists on the PP1-MP conjugate suspension. Assays were performed in duplicate.

3.2.2.6 PPIA with PP1-MP conjugates

The colorimetric PPIA with PP1-MP conjugates was performed similarly to the protocol for PP2A-MP conjugates and okadaic acid.[49]First, 50 µL of MC-LRstandard solution at different concentrations (for the calibration curve) or sample at different dilutions were added into microtiter wells containing 110 µL of PP1-MPs (at 25 U/mL for the conjugation) and incubated for 30 min at 22 °C. Then, 50 µL of a 25 mM *p*-NPP solution were added and incubated for 30 min under shaking at 22 °C in the dark. After substrate incubation, PP1-MPs were separated with magnetic disks and 200 µL of solution were taken for absorbance reading at 405 nm. Assays were performed in duplicate. The same protocol but using buffer instead of MC-LR standard solution or sample was used to optimise the PP1 concentration to be used in the conjugation.

3.2.2.7 Colorimetric enzyme-linked immunoparticle assay (ELIPA)

Two strategies, with differences in the MAb-MP conjugation and the competition between MC-LR-HRP and free MC-LR, were compared. In Strategy 1, the MAb was firstly conjugated to MPs, MC-LR was then pre-incubated and, finally, the MC-LR-HRP tracer was added. In Strategy 2, MC-LR was firstly pre-incubated with the MAb, the MC-LR-HRP tracer was then added and, finally, the MAb/MC-LR-HRP complex was conjugated to MPs. All incubation steps (unless otherwise stated) were performed at 22 °C under shaking on the Hulamixer.

*Strategy 1.*Colorimetric checkerboards were performed to optimise the MP amount, and the MAb and MC-LR-HRP dilutions. Firstly, (1) 0.5-mL plastic tubes were coated with BSA by incubation of PBS-BSA overnight at 4 °C in order to avoid non-specific adsorptions; (2) MPs were washed three times in PBS; (3) 100 µL of MAb at different dilutions in PBS were incubated with 4 µL of washed MPs for 30 min in the BSA-coated tubes; (4) tubes were placed on the magnetic separation stand until supernatant clearing and the supernatant was removed; (5) 100 µL of PBS were added and mixed with vortexing; (6) steps (4) and (5) were repeated twice; (7) 100 µL of MC-LR-HRP tracer at different dilutions in PBS

were added and incubated for 30 min; (8) steps (4) and (5) were repeated twice; (9) the content of each tube was resuspended in 100 µL of PBS, vortexed and the suspension was transferred to new tubes; (10) step 4 was repeated and 150 µL of TMB were incubated for 30 min; (11) tubes were placed on the magnetic separation stand and 100 µL of solution were taken for absorbance reading at 620 nm.

In the direct competition assay, the protocol was similar to that described for the checkerboard assay, with differences only in step (7), where the competition was performed (with 1:1,000 MAb dilution): 90 µL of MC-LR standard solution at different concentrations or sample at different dilutions in PBS were pre-incubated for 1 hour; then, 10 µL of MC-LR-HRP tracer at 1:1,600 dilution were added and competition was performed for 30 min. Assays were performed in triplicate.

Strategy 2. In the checkerboards, steps (3) to (7) of Strategy 1 were substituted by the following ones: 100 µL of MAb and 100 µL of MC-LR-HRP tracer were incubated together for 30 min in BSA-coated tubes; 4 µL of washed MPs were then added and incubated for 30 min. Washing steps were performed using 200 µL of PBS.

In the direct competition assay, 90 µL of MC-LR standard solution or sample were pre-incubated with 100 µL of MAb at 1:500 dilution during 30 min in BSA-coated tubes; then, 10 µL of MC-LR-HRP tracer at 1:800 dilution were added and competition was performed for 30 min. MPs were added afterwards and incubated for 30 min. Assays were performed in triplicate.

3.2.2.8 Electrochemical enzyme-linked immunoparticle assay (ELIPA)

Since the lowest LOD was attained with the Strategy 1 of ELIPA, this approach was chosen for further development. Firstly, cyclic voltammetry was used to investigate the bioelectrocatalysis from the HRP label of the ELIPA configuration using TMB as redox mediator. The protocol was the same as in the colorimetric

checkerboards with only minor changes: after incubation of the MAb-MP conjugates with the MC-LR-HRP tracer, the resulting MP-MAb/MC-LR-HRP complexes were resuspended in 20 µL of PBS for their emplacement on the working electrode, where they were trapped by placing a small magnet at the back side; then, 80 µL of TMB liquid substrate were added and after 5 min, cyclic voltammograms were recorded between 0.5 and -0.3 V at 10 mV/s. Controls without each one of the components were performed.

Once the electrochemical detection was demonstrated to be appropriate and the working potential was chosen, chronoamperometry (-0.2 V for 5 sec after the 5-min TMB incubation) was used to optimise the MAb dilution. To perform the competition assay, 4 µL of MPs, 1:8,000 MAb dilution and 1:1,600 MC-LR-HRP tracer dilution were chosen. Assays were performed in triplicate.

3.2.2.9 LC-MS/MS analysis

A triple quadrupole mass spectrometer (TSQ Quantum, Thermo Fisher Scientific, San José, CA, USA) equipped with an electrospray ionisation (ESI) source, a Surveyor MS plus pump and a HTC PAL autosampler were used for LC-MS/MS analysis. The chromatographic separation was performed on a reversed-phase Kromasil C18 column (100 x 2.1 mm, 3.5 µm, Tracer, Teknokroma, Sant Cugat del Vallès, Spain). The mobile phase was composed of Milli-Q water as solvent A and acetonitrile as solvent B, both containing 0.08% (v/v) formic acid at a flow rate of 200 µL/min. The linear gradient elution program was: 10-30% B 10 min, 30-35% B 20 min, 35-55% B 15 min, 55% B 5 min, 55-90% B 2 min, 90% B 3 min and return to initial conditions for re-equilibration (10% B 5 min). The injection volume was 10 µL.

The analyses were carried out in positive ion mode with the spray voltage at 4.5 KV and the optimum tube lens (TL) voltage for each m/z. The ion transfer tube temperature was set at 250 °C. Nitrogen (purity > 99.98%) was used as sheath gas, ion sweep gas and auxiliary gas at flow rates of 30 psi, 0 and 5 a.u. (arbitrary units), respectively. Data were acquired in full scan mode (400-1200

m/z, 1.2 sec/scan) for the screening of MC variants. MS/MS experiments were performed for confirmation of MC identity when commercial standards were not available. Its fragmentation pattern in positive ionisation gives a majority ion of 135 m/z characteristic of the amino acid Adda residue. High purity argon (Ar_1) was used as collision-induced gas with a pressure of 1.5 mtorr and the optimum collision energy (CE) for each fragmentation was selected. Data acquisition was performed with Xcalibur 2.0.7 software (Thermo Fisher Scientific).

3.2.3 Results and discussion

3.2.3.1 PP1 conjugation to MPs

The conjugation of PP1 to MPs is based on the Ni^{2+} ability to strongly bind to proteins containing His (or Cys) residues by chemical coordination. The IDA tridentate chelating group bound to the MP surface occupies three sites of the nickel coordination sphere. The other three sites are occupied by three His residues of the hexa-His tail of the recombinant PP1. Compared to enzyme sensors where PP was immobilised by entrapment,[41] the use of MPs favours an oriented enzyme immobilisation and eliminates the barrier created by the photopolymer.

In order to determine the optimum enzyme concentration to use in the conjugation to MPs, several concentrations were tested. The absorbance values attained by the conjugates, proportional to the PP1 concentration, demonstrate that conjugations were successful (Fig. 3.2.1S, Supporting Information).No saturation plateau was observed, indicating that MPs were not fully coated by PP1. Nevertheless, saturation was not our purpose, since the use of lower enzyme concentrations could provide more sensitive inhibition assays. Catalytic responses were appreciated at 12.5 U/mL PP1 concentration, but absorbance values were not high enough to perform the PPIA. A 25 U/mL PP1 concentration was chosen for subsequent experiments.

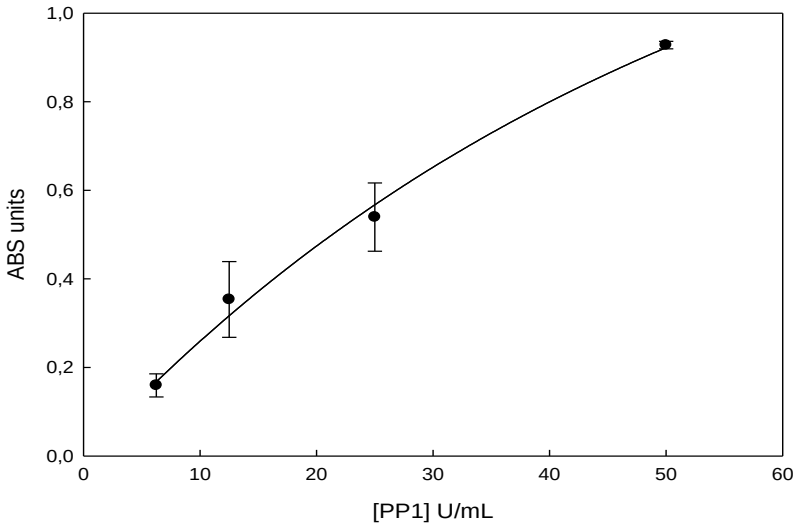

Figure 3.2.1S. Absorbance values attained in the colorimetric assay from PP1-MP conjugates obtained using several initial enzyme concentrations.

In order to verify the PP1-MP conjugation, imidazole was added to the conjugate for the competition with His residues for metal coordination positions. An imidazole excess would replace the coordinated PP1, if any. After imidazole addition, MPs did not show any significant absorbance compared to the PP1-MP conjugate, indicating that PP1 was effectively conjugated and not adsorbed.

3.2.3.2 MC-LR calibration curves by PPIA with PP1-MPs

The inhibition of MC-LR on the enzyme activity of PP1-MP conjugates was investigated. The MC-LR calibration curve (Fig. 3.2.1) was described by the sigmoidal logistic four-parameter equation:

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point.

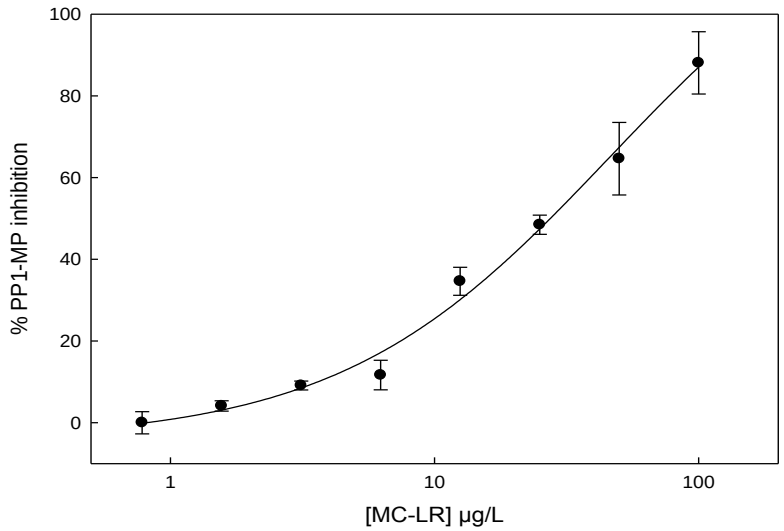

Figure 3.2.1. Calibration curve obtained in the colorimetric PPIA with PP1-MP conjugates for MC-LR. Inhibition is expressed as percentage of the control (no toxin). *x* values refer to initial toxin concentrations.

In Table 3.2.1, the 50% inhibition coefficient (IC_{50}) value and the working range (between IC_{20} and IC_{80}) are presented together with the equation and the corresponding *R* values.

Table 3.2.1 Curve Parameters Derived from the Sigmoidal Logistic Four-Parameter Fitting for the PPIA with PP1-MP Conjugates and For the ELIPA Strategies.

Strategy	**IC_{50}/EC_{50} (µg/L)**	**Working range IC_{20}–IC_{80} (µg/L) EC_{80}–EC_{20} (µg/L)**	**Equation**	***R***
PPIA with PP1-MP	27.5	7.4 - 77.3	$y = -4.4 + \frac{138.9}{1+(x/46.2)^{-0.9}}$	0.9957
Colorimetric ELIPA Strategy 1	4.9	3.9 - 6.4	$y = 4.2 + \frac{98.0}{1+(x/4.8)^{5.8}}$	0.9995
Colorimetric ELIPAStrategy 2	48.1	34.3 - 85.2	$y = 16.0 + \frac{84.5}{1+(x/44)^{4.5}}$	0.9997
Electrochemical ELIPA Strategy 1	2.0	0.4 – 20.0	$y = 7.1 + \frac{108.4}{1+(x/1.1)^{0.7}}$	0.9916

The PPIA demonstrated that the PP1-MP conjugates were inhibited by MC-LR. Compared to the calibration curve with free PP1 (Protocol S2, Supporting Information), the 50% inhibition coefficient (IC_{50}) with enzyme in solution was significantly lower (7.4 µg/L) than that provided by PP1-MP conjugates (27.5 µg/L), and slight differences were observed in the LOD (7.4 µg/L *vs.* 4.3 µg/L, LOD = IC_{20}). Two hypothesis could explain the lower sensitivity of PP1-MP conjugates: the conjugation of PP1 to MPs, although being performed through the hexa-His tail located far away from the active site, slightly decrease the enzyme sensitivity towards MC-LR due to steric impediments, or some PP1 molecules lose their enzyme activity once immobilised on the MPs and, in consequence, the sensitivity towards the toxin is "masked" (i.e. inactive PP1 molecules still recognise MC-LR but do not show any activity change). Nevertheless, although the use of MPs as enzyme immobilisation supports compromises the sensitivity of the assay, it is still appropriate and the approach could allow the integration of the components in flow systems for the subsequent development of compact analysis devices.

3.2.3.3 MAb conjugation to MPs

The conjugation of MAb to MPs is based on the ability of the recombinant protein G to bind to proteins containing Fc region. Compared to immunosensors where antibodies were immobilised by adsorption,[42] the use of MPs provides an oriented MAb immobilisation.

The amount of MPs was optimised, testing 1, 2 and 4 µL of MP per tube. Whereas the total system (without toxin) using 1 and 2 µL did not show significant absorbance values, 4 µL of MP provided appropriate values after 30 min of substrate incubation. Consequently, this amount was chosen for subsequent assays. Higher MP amounts were not tested since they would have implied the use of higher immunospecies concentrations and consequently higher LODs. The absorbance values found with Strategy 1 were higher than with Strategy 2 (2.7 *vs.* 1.7), using the same MC-LR-HRP dilution (1:16,000 in the tube). Although

one could thought that the pre-incubation of MAb with MC-LR-HRP could benefit from no steric impediments and thus provide higher absorbance values, it seems that the MAb/MC-LR-HRP complex compromises the conjugation to MPs. Consequently, the protein G/MAb interaction has more influence on the total response than the MAb/MC-LR-HRP interaction.

In order to determine if conjugations were successfuland, if they were, the optimum MAb and MC-LR-HRP concentrations, colorimetric checkerboards were carried out. In the preliminary checkerboards, non-specific adsorption of MC-LR-HRP was observed. Taking into account the possible adsorption of MC-LR and/or HRP on plastic, tubes were coated with 3% BSA overnight at 4 ºC prior to use. Moreover, conjugates were transferred to fresh tubes before the TMB incubation. These two actions decreased the non-specific adsorption to 4%, thus it was considered negligible.

Once the non-specific adsorption removed, the absorbance values obtained in the checkerboards showed appropriate trends according to the MAb and MC-LR-HRP tracer dilutions (*results not shown*), demonstrating the MAb conjugation to the MPs and the feasibility of both strategies. Absorbance values did not reach saturation plateaux, and 1:1,000 MAb and 1:16,000 dilutions (concentrations in the tubes), which provided appropriate absorbance values, were chosen.

3.2.3.4 MC-LR calibration curves by colorimetric ELIPA

In the competition assays, the signal is reported as the MC-LR-HRP binding. MC-LR calibration curves demonstrated that the competition of free toxin with MC-LR-HRP for MAb binding sites was successful (Fig. 3.2.2).

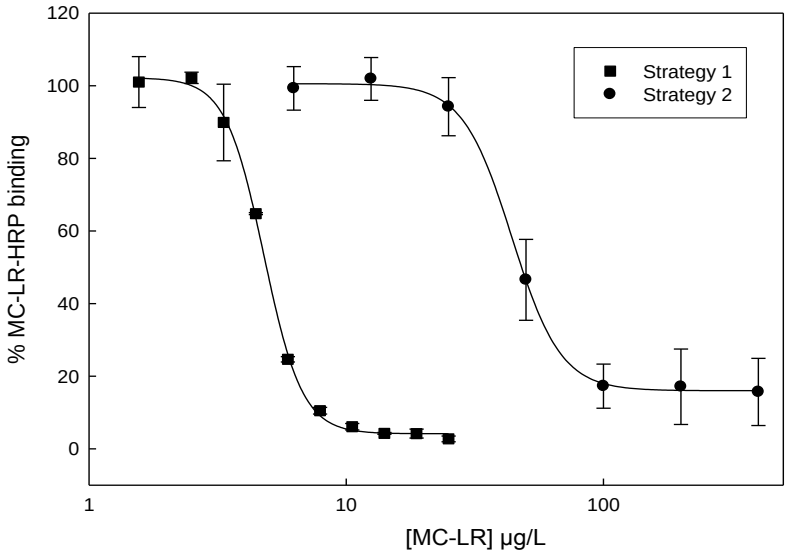

Figure 3.2.2. Calibration curves obtained in the colorimetric ELIPA for MC-LR with the two strategies. MC-LR-HRP binding is expressed as percentage of the control (no toxin). *x* values refer to initial toxin concentrations.

In Table 3.2.1, the 50% effective coefficient (EC_{50}) values and the working ranges (between EC_{80} and EC_{20}) for both strategies are presented together with the equations and the corresponding *R* values.

Strategy 1 provided a lower EC_{50} value than Strategy 2 (4.9 µg/L 1 *vs.* 48.1 µg/L). Contrarily to our purpose, the incubation of MAb with MC-LR and MC-LR-HRP in solution did not decrease the LOD. Probably, the higher EC_{50} and LOD values of Strategy 2 are due to the presence of higher amounts of MAb during the immunoaffinity interaction step. Although MAb molecules recognise the toxin, most or some of them are probably not conjugated to the MPs at the following step. Compared to the conventional ELISA (Protocol S3, Supporting Information), Strategy 1 and 2 provided 1.6 and 2.6 orders of magnitude higher EC_{50} values, respectively. This higher EC_{50} values could be due to the immobilisation of more MAb molecules on the MPs respect to ELISA plates and/or the incubation of higher tracer amounts. Nevertheless, results demonstrate the possibility to use MPs as MAb immobilisation supports for the subsequent development of flow system-based compact analysis devices.

3.2.3.5 MC-LR calibration curve by electrochemical ELIPA

As we previously mentioned, the final purpose of this work, beyond the tasks herein developed, is the use of MPs as immobilisation supports for their subsequent integration in automated microfluidic systems. The first step towards such development is the synthesis of the MP-based conjugates and their characterisation by colorimetric methods. Once demonstrated that conjugations are successful and that conjugates are able to recognise the analyte of interest, next step is to investigate if the electrochemical measurement is feasible. In this section, the complete ELIPA configuration (Strategy 1) was placed on a three-electrode system and cyclic voltammograms and chronoamperometries were recorded.

In order to investigate if the presence of 4 µL of MPs on the working electrode surface affects the electron transfer, cyclic voltammograms were recorded with the complete ELIPA configuration. Results demonstrate that MPs do not block the electron transfer and do not impede the electrochemical detection of the bioelectrocatalysis between the HRP label and the redox mediator (Fig. 3.2.4S, Supporting Information).

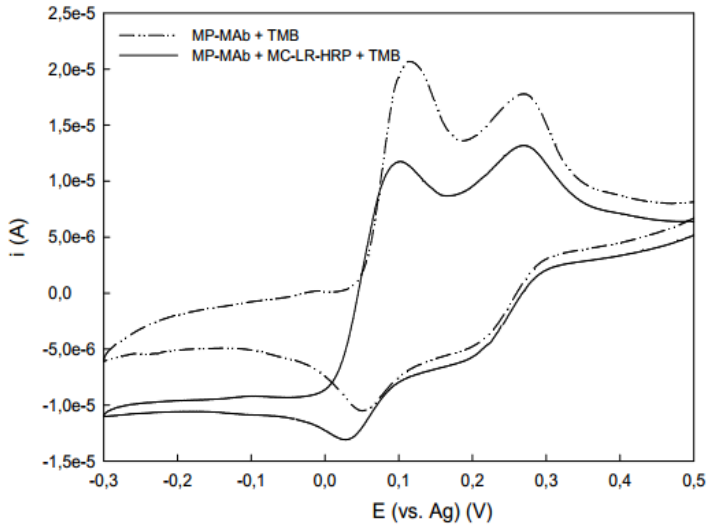

Figure 3.2.4S Supporting Information. Cyclic voltammograms of the complete ELIPA configuration (solid line) and the control without MC-LR-HRP tracer (dashed line) at 10 mV/s after 5-min TMB incubation.

Taking into account the inherent higher sensitivity of the electrochemical techniques and that the use of lower antibody concentrations could decrease the LOD, MAb dilutions from 1:1,000 to 1:8,000 were tested. Whereas the colorimetric detection did not allow the use of MAb dilutions higher than 1:1,000, chronoamperometric measurements showed significant differences between the complete configuration and the controls even at 1:8,000 MAb dilution. Moreover, non-specific adsorption was not observed.

The competition was then performed and the MC-LR calibration curve was fitted to a four-parameter logistic equation (Fig. 3.2.3).

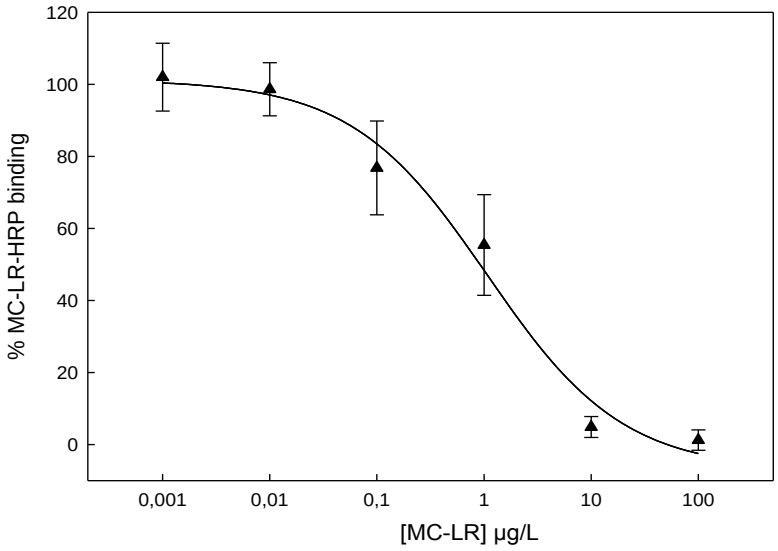

Figure 3.2.3. Calibration curve obtained in the electrochemical ELIPA for MC-LR with Strategy 1. MC-LR-HRP binding is expressed as percentage of the control (no toxin). *x* values refer to initial toxin concentrations.

Table 3.2.1 also shows the EC_{50} value, the working range, the equation and the corresponding *R* value for the electrochemical ELIPA. As envisaged, the electrochemical detection and the use of a lower MAb concentration decreased the LOD compared to the colorimetric ELIPA (0.4 vs 3.9 µg/L). Moreover, a wider working range was observed. Thus, the electrochemical detection has provided a LOD below the guideline value recommended by WHO and of the same order of magnitude of commercial ELISA tests. In conclusion, results demonstrate the

feasibility of the approach for the subsequent development of MP-based electrochemical analysis compact devices.

3.2.3.6 Analysis of culture and natural bloom samples

In order to demonstrate the applicability of the approaches, they were applied to the determination of MCs in a culture and a natural bloom of MC-producing cyanobacteria. MC-LR calibration curves were performed in parallel to sample analysis for the precise toxin quantification. Sample dose-response and calibration curves were analysed with SigmaPlot software package 10.0 and fitted to sigmoidal logistic four-parameter equations.Results were provided as MC-LR equivalent contents (µg/L), calculated from the IC_{50}/EC_{50} values of the MC-LR calibration curves and the IC_{50}/EC_{50} values of the sample dose-response curves. Table 3.2.2 shows the results obtained with PPIAs, ELISA and ELIPAs, as well as those reported by LC-MS/MS.

Table 3.2.2 MC-LR Equivalent Contents (µg/L) in the Culture and the Natural Cyanobacteria Bloom Determined by the Enzymatic and Immunochemical Approaches As Well as by LC-MS/MS.

Strategy	PCC 7820 culture	*Alcántara* bloom
PPIA (PP2A)	34072 ± 562	17187 ± 862
PPIA (PP1)	39310 ± 1467	14148 ± 707
PPIA (PP1-MP)	34533 ± 5015	13036 ± 302
ELISA	32560 ± 2295	12320 ± 1715
Colorimetric ELIPA (Strategy 1)	33237 ± 678	11731 ± 1147
Electrochemical ELIPA (Strategy 1)	33522 ± 1404	12343 ± 1551
LC-MS/MS*	37510 (17600)	7576 (1352)

* The first value provided is the sum of all MC variants concentrations; the value provided in parentheses is the MC-LR concentration.

The MC-LR equivalent contents determined by the PPIA with PP1-MP conjugates were similar to those determined by the PPIAs with free enzymes, indicating the feasibility of the approach. Compared to LC-MS/MS analysis, differences were observed according to the nature of the sample. In the analysis of the PCC 7820 culture, the MC-LR equivalent contents determined by the PPIAs were higher

than the MC-LR concentration and similar to the total MCs concentration determined by LC-MS/MS. The analysis by LC-MS/MS detected MC-LR as the majority variant (47%) and other identified variants were MC-LF (14%), [D-Glu(OCH3)6]LR (7%), MC-LY (7%), MC-LW (6%) and MC-dmLR (5%). In fact, some of the variants found in this sample inhibit both PP1 and PP2A less than MC-LR. It is also necessary to keep in mind that 20% of the total MCs concentration were variants detected but not identified or without inhibitory potency information. In the analysis of the *Alcántara* bloom, the MC-LR equivalent contents determined by the PPIAs were higher than both the MC-LR and total MCs concentrations quantified by LC-MS/MS analysis, which reported 30% of MC-RR, 18% of MC-LR, 7% of MC-YR, 5% of MC-LW, 2% of MC-LY, and 38% of other minority variants. Two hypothesis may explain this observation: or the enzymatic approach is overestimating the MC contents due to non-specific enzyme inhibitions coming from the sample matrix, effect more evident in natural blooms than in cultures, or the presence of 38% of unidentified MCs or MCs identified but without inhibitory potency information (more than 20% of the culture) could be responsible for the higher inhibitions detected.

The MC-LR equivalent contents determined by the colorimetric and the electrochemical ELIPAs were very similar between them and similar to those determined by ELISA, indicating the feasibility of the approach. Results were also similar to those reported by PPIAs, even despite the different recognition principles. In comparison with LC-MS/MS analysis, differences were also observed according to the nature of the sample. In the analysis of the PCC 7820 culture, the MC-LR equivalent contents determined by the immunochemical approaches were higher than the MC-LR concentration and only slightly lower than the total MCs concentration determined by LC-MS/MS. Once again, it is necessary to take into account that the cross-reactivity of some of the variants is lower (MC-LY, MC-LW and MC-LF) than the immunoaffinity of MC-LR. In this case, 25% of the total MCs concentration corresponded to variants detected but not identified, or detected but without provided cross-reactivity information. In the analysis of the *Alcántara* bloom, the MC-LR equivalent contents determined by

the PPIAs were higher than both the MC-LR and total MCs concentrations. Once again, the explanation may be related to the sample matrix, or to the presence of 38% of unidentified MCs and consequently without cross-reactivity information.

In conclusion, the use of MPs as supports for the immobilisation of biorecognition molecules for the detection of MCs is viable. Both approaches, with PP1 and MAb against MC-LR, have provided appropriate calibration curves, LODs and working ranges. In the case of the approach using MPs, MAb and electrochemical detection, an LOD below the guideline value recommended by the WHO has been attained. All MP-based approaches have allowed the determination of MCs contents in cyanobacterial cultures and natural blooms with high precision and accuracy. The comparison of the results with those provided by conventional biochemical assays and LC-MS/MS demonstrates the applicability of the biotechnological approaches as screening tools for fast and reliable detection of MCs, as well as the possible subsequent development of automated flow-systems and compact analysis devices.

Notes

The authors declare no competing financial interest.

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SPR immunosensor for the detection of okadaic acid in mussels using magnetic particles as antibody carriers

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Abstract

Protein G-coated magnetic particles (MPs) were used as immobilisation supports for an antibody against okadaic acid (MAb_{OA}) and carriers into a surface plasmon resonance (SPR) device for the development of a direct competitive immunosensor for okadaic acid (OA). SPR analysis of MAb_{OA}-MP conjugates demonstrated that conjugations were successful with complete immobilisation of all the antibody biomolecules on to the MPs. Moreover, MAb_{OA}-MP conjugates provided up to 11-fold higher SPR signals, compared to free MAb_{OA}. The use of conjugates in the direct competition assay provided a 3-fold lower LOD µg/L (2.6 µg of OA/L, equivalent to 12 µg of OA/kg mussel meat). The presence of mussel matrix did not interfere in the OA quantification as seen in the calibration curves. Mussel samples, obtained from Ebro Delta's bays (NW Mediterranean) during a diarrheic shellfish poisoning (DSP) event and in the presence of *Dinophysis sacculus*, an OA producer, in the shellfish production area, were analysed with the MP-based SPR immunosensor. The OA contents correlated with those obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (y = 0.984 x - 5.273, R^2= 0.789, p< 0.001) and by mouse bioassay (MBA).

Keywords: Okadaic Acid (OA), Monoclonal Antibody (MAb), Protein G-coated Magnetic Particles (MPs), Surface Plasmon Resonance (SPR), Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS), mussel.

3.3.1 Introduction

Surface plasmon resonance (SPR) immunosensors are powerful bioanalysis tools, which offer several advantages over conventional analysis methods such as stability, selectivity, sensitivity, real time monitoring, label free detection, small sample and reagent volumes and short analysis times [1,2]. They have been developed for the detection of many different molecules, covering a wide range of applications [3,4]. Thanks to the advantages of these biosensors and taking into account the recent development of small and portable SPR devices, SPR immunosensors are promising candidates for routine analysis in point-of-care diagnostics, environmental monitoring, food safety and human health protection programs.

When shifting from the development of biosensors to their applicability, main challenges include to avoid matrix interferences and to attain the required sensitivities. In SPR immunosensors, several strategies can be used to enhance the signal and to achieve lower limits of detection (LODs). The most frequently reported is the use of secondary antibodies. Magnetic micro and nanoparticles have also been exploited for signal amplification purposes, both as antibody labels in sandwich immunoassays [5,6] and as supports to immobilise antibodies on the SPR chips, which moreover could be magnetised to favour immobilisation [7-12]. Magnetic particles (MPs) have high surface-to-volume ratios leading to immobilisation of high densities of biomolecules. Moreover, they are available in a wide variety of functionalities, being possible to conjugate them to different biomolecules and in an easy and fast way.

Marine toxins are secondary metabolites produced by toxicogenic microalgae. These toxins may transfer through the trophic webs chain into shellfish and fishand thus have serious human health implications. In order to assure food safety and to protect the consumer, there is a need to develop powerful bioanalysis tools and to fully characterise their applicability to the analysis of natural samples. Taking lipophilic marine toxins as an example, the Regulation

(EC) No. 853/2004 in Europe has established a maximum permitted level of 160 µg of okadaic acid (OA) equivalents/kg shellfish meat [13]. Although it is possible to use the mouse bioassay (MBA) [14] until December 31st, 2014, the Commission Regulation (EU) No. 15/2011 has recently established that liquid chromatography-tandem mass spectrometry (LC-MS/MS) should be applied as the reference method for the determination of lipophilic toxins contents in shellfish [15]. Nevertheless, this Regulation encourages the use of other chemical methods, as well as immunoassays and functional assays, as alternatives or supplementary to the LC-MS/MS method, after proper validation.

With this aim, SPR immunosensors for the detection of paralytic shellfish poisoning (PSP) toxins [16], amnesic shellfish poisoning (ASP) toxins [17,18], diarrheic shellfish poisoning (DSP) toxins [19,20] as well as other toxins such as palytoxins [21] have been developed and in some cases also validated. One step beyond has been the development of a multiplex SPR analyser for the simultaneous detection of OA, domoic acid (DA), saxitoxin (STX) and neosaxitoxin (Neo-STX) [22].

Although antibodies against PSP toxins have been conjugated to MPs, the use of these conjugates has been applied to toxin extraction and purification [23,24]. In this work, we propose the conjugation of an antibody against OA (MAb_{OA}) to protein G-coated MPs, which are used as immobilisation supports and carriers in the development of a directive competitive SPR immunosensor for OA (Fig. 3.3.1).

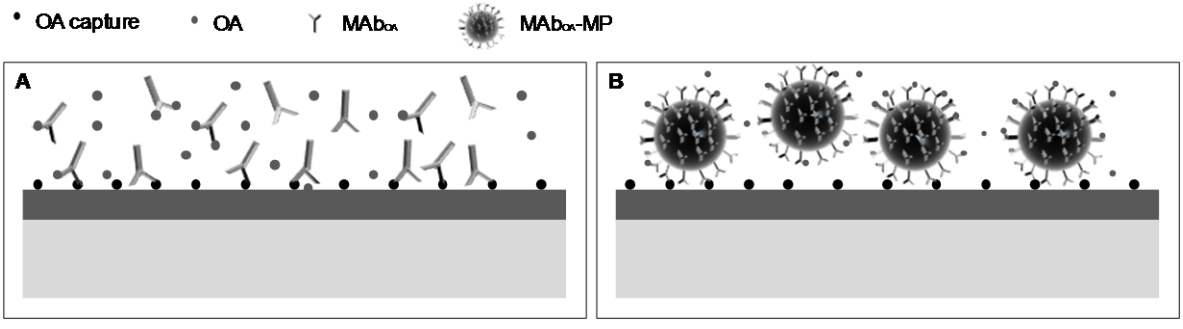

Fig. 3.3.1. A schematic representation of the direct competitive SPR immunosensor for OA detection using free MAbOA (A) and MAbOA–MP conjugates as antibody carriers (B).

First, MAb_{OA}-MP conjugates were synthesised, and their immunoaffinity and stability were characterised. Afterwards, conjugates were used in a competition assay between free and immobilised OA into an SPR device, and the LOD and working range were established. The possible effects from mussel matrix in the performance of the MP-based SPR immunosensors were evaluated. Finally, the immunosensor was applied to the analysis of mussel samples obtained from Ebro Delta's bays (NW Mediterranean) during a DSP event, and results were compared to those obtained by LC-MS/MS and MBA.

3.3.2 Materials and Methods

3.3.2.1 Reagents and materials

A certified solution of okadaic acid (NRC CRM-OA-c) at 14,300 µg/L in methanol (MeOH) was provided by the Institute for Marine Biosciences of the National Research Council (Halifax, Canada). The monoclonal antibody against OA (MAb_{OA}) was produced at IGFS, Queen's University. The production and characterisation of the antibody have been described previously [25]. The antibody was provided in 150 mM saline at a protein concentration of 1 mg/mL. Protein G-coated magnetic particles (Ø = 300 nm, Bio-Adembeads Protein G 0433) were supplied by Ademtech SA (Pessac, France). For SPR analysis, a CM5 sensor chip (research grade), HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4) and 1 M ethanolamine hydrochloride were supplied from Biacore AB (Uppsala, Sweden), and carbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS), sodium dodecyl sulphate (SDS) and hypergrade acetonitrile (ACN) from Sigma-Aldrich (Dorset, UK). For LC-MS/MS analysis, methanol and ACN were purchased from Merck (Darmstadt, Germany), and ammonium hydroxide solution ≥ 25% (eluent additive for LC-MS) was purchased from Fluka (Sigma-Aldrich, Tres Cantos, Madrid, Spain). All solutions were prepared using Milli-Q grade water obtained from a Millipore purification system (Bedford, MA, USA).

3.3.2.2 Mussel and phytoplankton samples

A confirmed negative mussel sample according to MBA and LC-MS/MS analysis was a gift from the National Reference Laboratory for Marine Biotoxins (UK), Agri-Food and Biosciences Institute (Northern Ireland).

Fifteen Mediterranean mussel (*Mytilus galloprovincialis*) samples were obtained from the shellfish monitoring programme of the Catalan littoral, specifically from the Ebro Delta (NW Mediterranean), during a DSP event that occurred from February to April 2012. Shellfish samples were collected from 4 different stations in Alfacs Bay (stations A, B, C and D) plus one station placed offshore in open Mediterranean waters (station E).

In Alfacs Bay, integrated water samples (0-6m) were collected weekly from other 4 stations plus additional surface and bottom samples at the central station. The samples were preserved immediately with Lugol's solution. The Utermöhl method was used for phytoplankton identification and quantification (Utermöhl, 1958): 50 mL of sample were settled during 24 h. The chamber was examined for quantification of *Dinophysis* species and total phytoplankton.

3.3.2.3 Lipophilic toxins extraction

Crude mussel extracts for SPR analysis were prepared according to the method of Stewart and co-workers [20], by weighing 1 g (out of 100 g) of mussel homogenate in glass bottlesand adding 10 mL of aqueous ACN (90%). Samples were vortexed for 5 s prior to 30-min roller mixing. Extracts were centrifuged at 3,500 rpm for 10 min in a Sorvall Legend *RT* centrifuge at 10 ºC. Supernatants were transferred into glass tubes and evaporated to dryness at 45 ºC under gentle stream of nitrogen (TurboVap LV, Caliper LifeSciences). Residues were reconstituted in 10 mL of HBS-EP buffer. Finally, samples were vortexed for 20 s and passed through a Millex-HV 0.22-µm cut-off polyvinylidene difluoride (PVDF) (Millipore, Ireland) membrane filters prior to SPR analysis.

Crude mussel extracts for LC-MS/MS analysis were prepared according to the method of the "EU-Harmonised Standard Operation Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS" (EU-RL-MB SOP) [26], by weighing 1 g (out of 100 g) of mussel homogenate in 15-mL polypropylene centrifuge tubes and adding 3 mL of methanol (100%). Samples were vortexed for 1 min. Extracts were centrifuged at 4,000 rpm for 5 min in a Thermo Scientific MR 23i centrifuge at 20 °C. The extraction steps were repeated twice. Supernatants were transferred into 10-mL plastic flasks and the volume was adjusted to 10 mL with methanol (100%). Finally, samples were passed through a Waltham 0.22-µm cut-off polytetrafluoroethylene (PTFE) (Thermo Fisher Scientific, Spain) membrane filters prior to LC-MS/MS analysis.

3.3.2.4 Conjugation of MAb_{OA} to protein G-coated magnetic particles (MPs)

The conjugation of MAb_{OA} to MPs is based on the protein G ability to bind to proteins containing Fc region. The MPs are modified with a recombinant form of protein G, expressed in *Escherichia coli*, that lacks albumin, Fab binding sitesand membrane-binding regions. The Fc receptor of this recombinant protein G is able to bind to the Fc region of IgGs, providing an oriented MAb immobilisation on the MP surface. The conjugation protocol was: (1) 10 µL of the MP suspension was pipetted into a plastic microtube; (2) the tube was placed on the magnetic support (Z5342 MagneSphere®) and the supernatant was pipetted off leaving beads undisturbed; (3) the microtube was removed from the magnet and the beads were resuspended in 100 µL of HBS-EP buffer, containing 0.65% Tween 20, pH 7.4; (4) steps (2), (3) and (2) were repeated; (5) 500 µL of MAb_{OA} solution in HBS-EP buffer, pH 7.4 (several concentrations from a protein concentration of 1 mg/mL to prepare 1/400 dilution and diluting to the half), was added to the tube and incubated for 30 minutes at room temperature while shaking on a HulaMixer™ Sample Mixer; (6) step (2) was repeated and free MAb_{OA} excess supernatant (SN) was kept for further analysis; (7) for the washing, steps (3) and (2) were performed twice with 250 µL of HBS-EP buffer, pH 7.4, and the corresponding supernatants (W1 and W2) were kept for further analysis; (8)

finally, 150 µL of HBS-EP buffer, pH 7.4, was added to the microtube containing the MAb_{OA}-MP conjugate.

3.3.2.5 OA immobilization onto a CM5 sensor chip surface

OA was immobilised onto a CM5 sensor chip surface as described by Llamas and co-workers [19]. First, the carboxymethylated surface of the chip was equilibrated to room temperature. Afterwards, 50 µL of an EDC/NHS mixture (1:1 *v/v*) was incubated on the chip surface for 30 min. The excess solution was removed, and 50 µL of freshly prepared 1 M ethylene diamine, pH 8.5, was added to the NHS-ester chip surface and incubated for 1 h. The surface of the chip was washed with HBS-EP buffer and the free carboxyl groups were blocked with 50 µL of 1 M ethanolamine hydrochloride, pH 8.5, for 20 min. Then, 9 mg of EDC and 4 mg of NHS were dissolved in 1 mL of 10 mM sodium acetate buffer, pH 4.5, and 10 µL of this mixture was added to 50 µg of OA, which had been previously dissolved in 10 µL of DMSO and 30 µL of 10 mM sodium acetate buffer, pH 4.5. 50 µL of OA-NHS derivative was then added to the amine chip surface and incubated for 4 h. The excess solution was removed, and the chip surface was washed with deionised water, dried under a gentle stream of nitrogen gas, and stored desiccated at 4 °C when not in use.

3.3.2.6 SPR analysis

A Biacore Q Surface Plasmon Resonance system with control and evaluation software (GE Healthcare, Uppsala, Sweden) was used. The SPR instrument was programmed in automatic injection mode. Firstly, the regeneration solution (100 mM HCl: 1% SDS; 1:1 *v/v*) was injected at a flow rate of 20 µL/min for three times. Between each regeneration step, a washing step with HBS-EP buffer was performed. Afterwards, 50 µL of a mixture of free MAb_{OA}, MAb_{OA}-MP, SN, W1, W2 and bare MPs with HBS-EP buffer, OA standard and mussel extract (1:1 *v/v*; combinations depending on the specific experiment and described in the “results and discussion” section) was injected at the same flow rate. Finally, the chip surface was regenerated and washed for the next experiment. The binding of the molecules to the sensor surface generates a response, which is proportional to

the bound mass, and is expressed in response units (RU). Assays were performed in duplicate.

3.3.2.7 LC-MS/MS analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were conducted on an Agilent 1200 LC (Agilent Technologies, Santa Clara, USA) coupled with a 3200 QTRAP mass spectrometer through a TurboV™ electrospray ion source (Applied Biosystems, Foster City, USA). Chromatographic separations were performed at 30 °C and 0.5 mL/min on Waters X-Bridge™ C8 guard and separation columns (10 mm and 50 mm length, respectively); both of 2.1 mm inner diameter and 3.5 µm particle size (Waters, Milford, MA). Alkaline chromatographic conditions at pH 11 [27] were applied with mobile phases 100% water (A) and 90% ACN (B), both containing 6.7 mM ammonium hydroxide. Gradient elution was programmed starting at 80% A, after injection of 10 µL of sample according to the conditions previously optimized [28]. The flow was diverted to waste by a 10-port Valco valve for the first 1 min of each run to keep clean the ion source. Detection windows were set to operate the mass spectrometer in negative and positive polarity aimed at covering all toxins regulated in the EU. For the specific case of DSP toxins, multiple reaction monitoring (MRM) analysis was accomplished from the precursor ions *m/z* 803.5 and 817.5 for OA/DTX-2 and DTX-1, respectively. Product ions at *m/z* 255.2 and 113.1 were monitored for quantification and confirmation purposes for the three DSP toxins. Mass spectrometer operated in negative polarity according to optimized parameters: curtain gas 20 psi, ion spray voltage -4500 V, auxiliary gas temperature 500 ºC, nebuliser gas 50 psi, heater gas 50 psi, collision-activated dissociation gas medium, declustering potential -115 V, collision energy -64 V (for 803.5/817.5>255.2) and -68 V for (for 803.5/817.5>113.1), channel electron multiplier 2400 V. Both quadrupole analyzers (Q1 and Q3) operated at unit resolution with a dwell time of 150 and100 ms for quantification and confirmation transitions, respectively, maintaining a total cycle time ≤1 s. All gases used during LC-MS/MS operation were high-purity N_2. Analyst software v1.4.2 was used for the entire MS tune, instrument control, data acquisition and data analysis. Under

these conditions, the LOQ was 12.5 µg OA/kg in mussel edible tissue. Analysis were performed in duplicate.

3.3.2.7 Statistical analyses

To evaluate the correlation between the OA contents in naturally-contaminated mussel samples determined by the SPR immunosensor and those provided by LC-MS/MS, a linear regression model (SigmaStat software package 3.1) was used. Differences in the results were considered statistically significant at the 0.05 level.

3.3.3 Results and discussion

3.3.3.1 Conjugation of MAb_{OA} to MPs

In order to optimise the conjugation protocol and the binding capacity of the conjugates, the different MAb_{OA}-MP conjugates and the corresponding supernatants from the free MAb_{OA} excess (SN) and the two washing steps (W1 and W2), as well as bare MPs, were injected over the OA-modified chip and the SPR signal was measured (Fig. 3.3.2). The response obtained from bare MPs was not significant (30 RU), indicating that there was not non-specific adsorption form the MPs. The comparison of the response attained with bare MPs respect to those obtained with the conjugates demonstrates that conjugations were successful. No significant responses (between 12 and 38 RU) were observed when injecting SN for none of the conjugates, suggesting that all MAb_{OA} molecules were conjugated to the MPs, even when using the highest antibody concentration. The response attained with the conjugates decreased from 1/6,400 to 1/25,600 MAb_{OA} dilution (dilutions taking into account the 1:1 mixing prior to injection into the SPR device), as expected because of the lower amount of MAb_{OA} molecules used in the conjugation. One could expect the same trend from 1/800 to 1/6,400 or an ABS plateau that would suggest saturation of the MPs with MAb_{OA} (although taking into account the theoretical binding capacity of

the MPs and the experimental MAb_{OA} dilutions, only the most concentrated MAb_{OA} solution would involve MP saturation). Nevertheless, the responses at these antibody concentrations were lower than expected. This behaviour is hypothesised to be due to steric hindrance arising from the high amount of MAb_{OA} molecules immobilised on the MPs, which could be impeding the correct antibody orientation for the interaction with the immobilised OA.

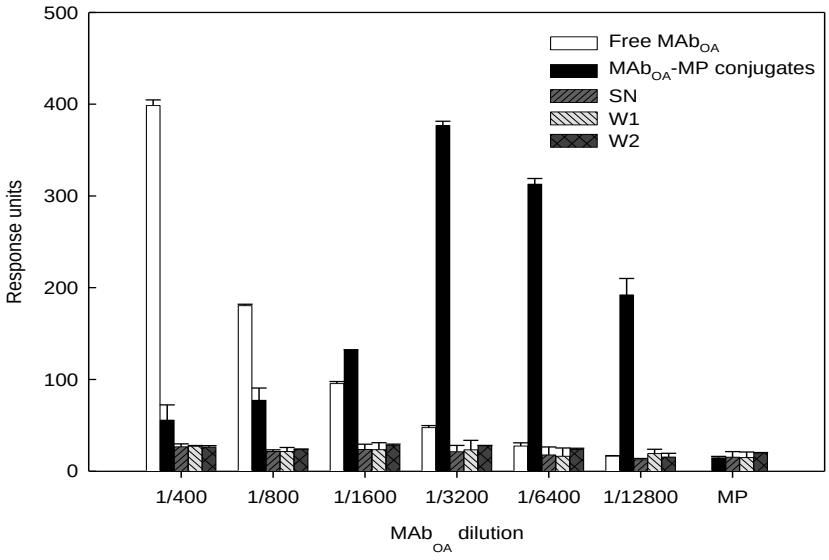

Fig. 3.3.2. Response units attained in the SPR analysis of free MAb_{OA} and MAb_{OA}–MP conjugates obtained using several initial antibody concentrations, as well as the supernatant from the conjugation (SN) and the two washing steps (W1 and W2).

When using free MAb_{OA}, the response trend was proportional to the antibody concentration, as expected. Comparing the responses obtained from free MAb_{OA} with those from the conjugates at the three lowest antibody concentrations (from 1/6,400 to 1/25,600), it is evident that the use of the MPs amplifies the SPR signal, being for example 11-fold higher at MAb_{OA} dilution 1/6,400. The SPR signal enhancement is certainly provided by the high refractive index and high molecular weight of the MPs. With conjugates it is possible to attain approximately the same response than with free MAb_{OA}, requiring an 8-fold lower MAb_{OA} concentration. The use of a lower MAb_{OA} amount represents an economisation of antibody and could turn into a decrease of the LOD. The

following experiments were performed with 1/800 dilution of free MAb_{OA} and 1/6,400 dilution of MAb_{OA} conjugated to MPs. The binding affinity of MAb_{OA}-MP conjugates synthesised different days showed a variability of 16%, indicating good conjugation reproducibility.

3.3.3.2 Storage stability of MAb_{OA}-MP conjugates

The stability of the MAb_{OA}-MP conjugates after several storage daysat 4 ºC and -20 ºC was tested in order to know if it is necessary to prepare them fresh. The storage stability was expressed as the percentage of the binding from MAb_{OA}-MP conjugates after several days respect to the binding at day 0 (freshly prepared conjugates). Conjugates were more stable at -20 ºC than at 4 ºC. Whereas conjugates stored at -20 ºC retained their affinity by 100, 97 and 84% after 10, 15 and 20 days, respectively, conjugates stored at 4 ºC retained their affinity by 31% after 10 days, disappearing completely after 15 days. Consequently, storage of the conjugates at -20 ºC (allowing them to attain room temperature prior to use) avoids the necessity to prepare them fresh, shorteningthe total analysis times. It is interesting to note that the free MAb_{OA} lost completely its affinity after 2 hours at 4 ºC, whereas when conjugated to MPs it was able to retain 31% after 10 days. We hypothesise that the binding is protecting the tridimensional structure of the antibody, preventing denaturation and subsequent loss of functionality.

3.3.3.3 OA calibration curves with free MAb_{OA} and MAb_{OA}-MP conjugates

The SPR immunosensor was based on a direct competitive immunoassay, where the conjugate was pre-incubated with the toxin (1:1 *v/v*) prior to their injection into the SPR system. Dose-response curves for OA were performed in order to investigate if the use of MAb_{OA}-MP conjugates, which enhances the SPR signal, decreased the LOD (defined as the 80% effective coefficient (EC_{80})) respect to free MAb_{OA}. The OA calibration curves obtained by the SPR biosensor were

analysed with BIAevaluation software package 4.1 and fitted to sigmoidal logistic four-parameter equations:

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

where a and y_0 are the asymptotic maximum and minimum values, respectively, x_0 is the x value at the inflection point and b is the slope at the inflection point. As it is shown in Table 3.3.1, the use of conjugates shifts the calibration curve to lower toxin concentrations, providing a 3-fold lower LOD (2.4 µg/L vs. 7.3 µg/L).

Table 3.3.1. Curve parameters derived from the sigmoidal logistic four-parameter fitting for the OA detection with the SPR immunosensor strategies.

MAb_{OA}	Calibration curve	EC_{50} (µg/L)	Working range EC_{80} – EC_{20} (µg/L)	Equation	R^2
Free	Mussel matrix	7.8	4.7 – 12.7	$y = -0.7+99.2/(1+(x/7.9)^{2.8})$	0.9995
	HBS-EP buffer	7.3	4.2 – 13.3	$y = 3.3+97.9/(1+(x/7.1)^{2.5})$	0.9998
Conjugated	Mussel matrix	2.6	1.2 – 5.3	$y = 0.3+98.3/(1+(x/2.6)^{2.8})$	0.9985
	HBS-EP buffer	2.4	1.4 – 14.9	$y = 9.6+90.1/(1+(x/2.2)^{2.6})$	0.9999

The higher standard deviation observed in the calibration curve with conjugates (Fig. 3.3.3) may be due to the settlement of those conjugates at the bottom of the well prior to their injection. Although the improvement in the LOD is not extensive, this experiment demonstrates the feasibility to use MPs as antibody carriers and signal amplification systems, as well as for MAb_{OA} economisation purposes.

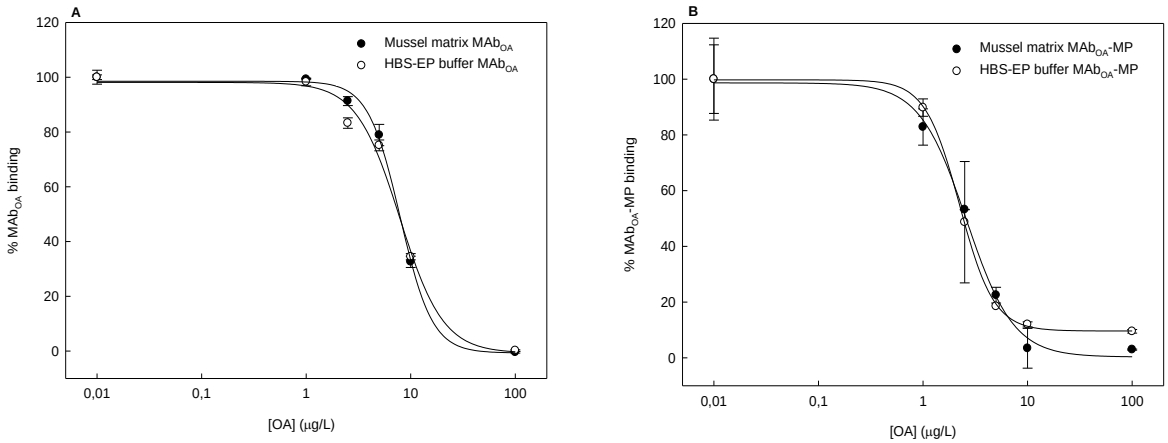

Fig. 3.3.3. MAb_{OA} binding percentage at different OA concentrations in HBS–EP buffer and in mussel matrix (without lipophilic toxins as determined by LC–MS/MS) using free MAb_{OA} (A) and MAb_{OA}–MP conjugates (B).

3.3.3.4 Mussel matrix effects on the SPR immunosensor

To evaluate if mussel matrix has an effect on the SPR response, an OA calibration curve in negative mussel sample at 106 mg/mL (53 mg/mL after 1:1 mixing) was compared to the OA calibration curve in buffer (Fig. 3.3.3). This negative mussel sample does not contain DSP toxins as determined by MBA neither by LC-MS/MS. As it can be seen, the presence of mussel matrix did not interfere in the OA detection using MAb_{OA}-MP conjugates, the experimental parameters being not significantly different (Table 3.3.1). Differences were neither found when using the conventional approach with free MAb_{OA}. Taking into account the attained LOD, the MP-based approach developed herein should be able to quantify 12 µg of OA/kg mussel meat, i.e. 3-fold lower toxin contents than with free MAb_{OA} (the LOD obtained herein for free MAb_{OA} in agreement with previous work [20]). In both cases, the obtained LOD is far below the 160 µg of OA equivalents/kg shellfish meat, regulation level established by the EU 853/2004 [13]. This high sensitivity of the method may be an advantage in order to identify the presence of low amounts of OA in shellfish early during a DSP episode.

3.3.3.5 Analysis of naturally-contaminated mussel samples by SPR and LC-MS/MS

The MP-based SPR approach was used in the determination of OA contents in fifteen mussel samples obtained during a DSP event in Alfacs Bay (Ebro Delta, NW Mediterranean Sea). OA calibration curves in negative mussel matrix were performed in parallel for the precise toxin quantification. The determination of OA equivalent contents was performed taking into account the binding percentage of MAb_{OA}-MP conjugates and the corresponding binding percentage of the OA calibration curve, and applying the previously obtained extraction recovery factors [20]. Samples causing less than 20% of binding were diluted until the obtained response was between 20 and 80%.

Table 3.3.2 shows the OA contents obtained by the MP-based SPR immunosensor and by LC-MS/MS analysis.

Table 3.3.2. OA contents in mussel samples (µg/kg) determined by the MP-based SPR immunosensor and LC–MS/MS analysis, as well as MBA result. Relative standard deviation values are not shown for clarity of the results and were always below 10%.

Number	Sample	SPR	LC-MS/MS	MBA
1	120220A	401	573	+
2	120220B	280	304	+
3	120227A	338	470	+
4	120227B	542	542	+
5	120315D	805	611	+
6	120320B	394	306	+
7	120322A	480	440	-
8	120326A	332	296	+
9	120326C	161	194	-
10	120328A	133	295	+
11	120402A	165	220	-
12	120402B	69	86	-
13	120402C	130	99	-
14	120402D	133	84	-
15	120315E	45	40	-

LC-MS/MS analysis indicated that no DTX derivatives were present in the mussel samples, being OA the only DSP toxin detected. Samples 1 to 11 were determined as positive (toxin content higher than 160 µg OA eq/kg) by LC-MS/MS. The SPR immunosensor also indicated that all these samples were positive, except sample 10, which was considered as negative. Samples 12 to 15 were determined as negative also by both techniques. Despite the discrepancy in sample 10, statistical analysis of the data shows that the correlation between techniques is acceptable (Fig. 3.3.4, y = 0.984 x - 5.273, R^2= 0.789, p< 0.001). Consequently, results confirm that the MP-based directive competitive SPR immunosensor is able to detect and quantify OA in mussel samples, providing toxin contents that correlate with those obtained by LC-MS/MS.

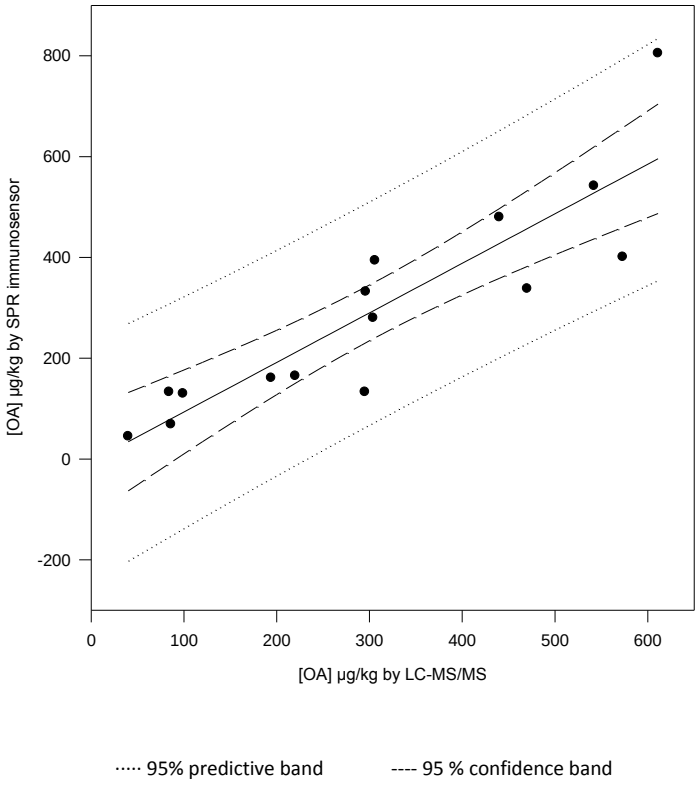

Fig. 3.3.4. Linear regression for the correlation between the OA contents in mussel samples (µg/kg) determined by the MP-based SPR immunosensor and those provided by LC–MS/MS analysis.

Comparing the results obtained with the SPR immunosensor to those provided by the MBA, discrepancies appear in samples 7, 9 and 10. But it is necessary to take into account that the MBA may suffer from specificity problems. In fact, LC-MS/MS analysis did not coincide with MBA in samples 7 and 9; and the OA contents of sample 9 determined by both SPR and LC-MS/MS were very close to the regulatory limit.

The overall examination of the results provided by the three methods invited us to establish a threshold "suspicious" area comprised between 130 and 180 µg/kg of OA contents determined by the SPR immunosensor, those samples requiring complementary analysis with other techniques.

Regarding the phytoplankton populations in Alfacs Bay, *Dinophysis sacculus* was present in the area from January to March at concentrations above the alert level (500 cells/L) (Fig. 3.3.5).

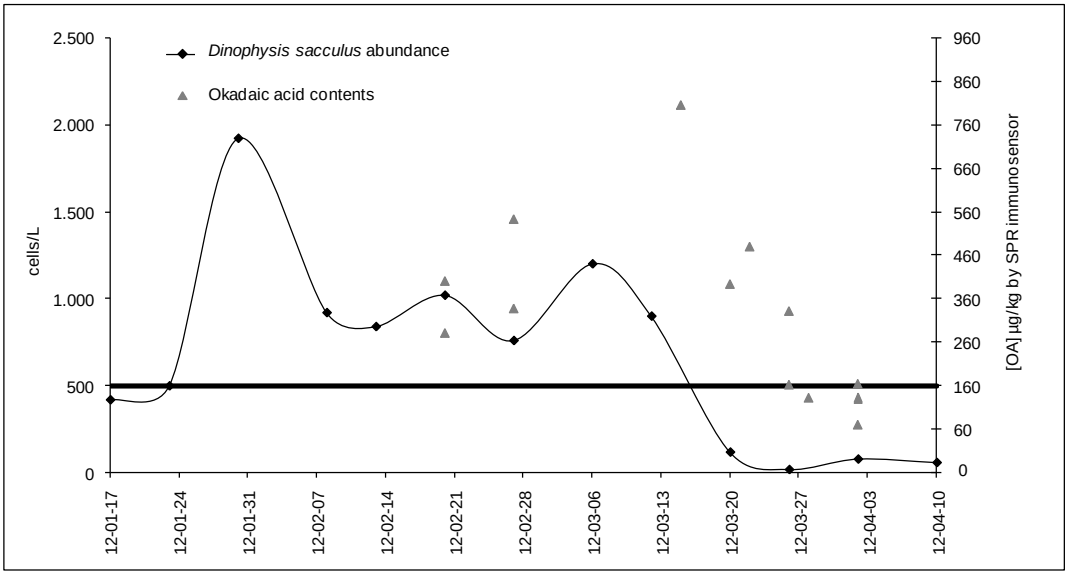

Fig. 3.3.5. Maximum phytoplankton (*Dinophysis sacculus*) abundance (cells/L) present from January to April in Alfacs Bay. Thick black line at 500 cells/L and at 160 µg of OA/kg mussel meat indicates the alert value for *D. sacculus* and positive mussel samples. Grey points present the OA contents in mussel samples (µg/kg) determined by the MP-based SPR immunosensor.

Other *Dinophysis* species present during the event, although in very low abundances, were *D. caudata* and *D. rotundata*. The abundance of *Dinophysis sacculus* resulted in an intensified monitoring, and after analysis of the mussel samples by MBA, closure of the production area due to OA levels above the maximum permitted level (MPL). These higher toxic microalgae densities observed from 12/01/30 to 12/03/12 correlate with the accumulation of OA above the legal limit in the mussel samples collected in that period and two weeks later. Consequently, the toxin profile of the positive mussel samples was likely due to the presence of *D. sacculus*, a known OA producer [29].

3.3.4 Conclusions

In this work, a direct competitive SPR immunosensor for the detection of OA has been developed integrating MPs as antibody carriers and signal amplification elements. SPR analysis has demonstrated that the conjugation of the MAb_{OA} to the MPs, via protein G, is successful and efficient at 100%. Compared to free MAb_{OA}, the conjugates enhance the SPR signal, being possible to economise antibody. Moreover, the conjugates work in the direct competition assay, providing a 3-fold lower LOD than when using free MAb_{OA}, the OA calibration curve being not affected by the mussel matrix presence. The lower LOD attained with our approach allows the use of more diluted extracts in the analysis of contaminated samples. This would be of interest in the analysis of other shellfish species that could present matrix effects. Nevertheless, to improve precision in the quantifications, the standard deviation should be decreased. This could be achieved by proper mixing automation of conjugates and samples prior to their injection into the SPR devices or the use of lighter and/or smaller particles. An example would be colloidal particles, although in this case their use would require a revaluation of the approach in order to know if the shift in the SPR angle is significant enough and if they are able to carry enough antibody molecules to promote signal enhancement.

Although other works report the use of MPs in SPR immunosystems, none of them has showed their applicability to the analysis of natural samples [5-12]. The analysis of fifteen naturally-contaminated mussel samples obtained from Alfacs Bay during a DSP event has demonstrated the viability of the approach. Good correlations have been observed in the comparison of the OA contents obtained with the SPR immunosensor and those provided by LC-MS/MS analysis. Significant toxin contents have been determined in the mussels until two weeks after the *Dinophysis sacculus* alert levels. The SPR immunosensor has been able to easily discriminate positive and negative samples (with OA contents as low as 45 µg/kg), being promising as a screening tool in monitoring programs. Nevertheless, a "suspicious" area comprised between 130 and 180 µg/kg of OA contents should be defined, those samples requiring complementary analysis with other techniques.

In conclusion, this work is the proof-of-concept of the use MPs as antibody immobilisation supports and carriers in the development of SPR immunosensors for the detection of OA. The immunochemical SPR approach has been demonstrated to provide specific and real-time monitoring of OA with minimal sample and reagent consumption and with short analysis times. Although more efforts should be undertaken to attain lower LODs, the conjugates already provide advantages in terms of SPR signal enhancement and antibody economisation. Moreover, the applicability of the MP-based SPR immunosensor to the determination of OA contents in mussels has been demonstrated.

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Chapter 4

General discussion

4.1 PPIAs for the detection of toxins: The biorecognition molecule

The first step towards the development of a biosensor is an exhaustive study of the biorecognition event in order to understand the phenomena that are taking place, regardless the detection technique. As a consequence of this investigation, colorimetric assays are obtained as intermediate product, which may provide specific advantages. PPs are very useful enzymes for the detection of some aquatic toxins such as OA and analogues, and MCs, both toxin groups inhibiting their enzyme activity. However, the inherent instability of PPs, both under storage and in operation, is one of the critical limitations of the derived assays and biosensors. Recombinant PPs were produced with the aim of attaining stable enzymes and convenient for immobilisation purposes. First of all, several chemical compounds as well as a lyophilisation procedure were tested as possible recombinant PP2A activity stabilisers for stock solutions. The highest stability yields were achieved with 10% glycerol. Although higher glycerol percentages could be even more stabilising, they could not be appropriate for subsequent immobilisations. Regarding the operational stability, the recombinant PP2A was more stable during the assay than the wild-type PP2A. Apart from the PP stability, it is important to know that enzyme activity may differ among production lots. Consequently, it is imperative to check the enzyme activity prior to the assay execution. Recombinant enzymes were also able to detect the toxins from aquatic environments but, whereas the recombinant PP1 and PP2A provided LODs and 50% inhibition coefficient (IC_{50}) values towards MC-LR and OA, respectively, similar or only slightly higher to those obtained with the wild-type enzyme, the recombinant PP2A provided significantly higher LODs and IC_{50} values towards MC-LR. In any case, recombinant PPs were thought to be advantageous because of their His tail, able to conduct oriented immobilisations on biosensors.

4.2 PPIAs for the detection of toxins: The sample matrix effects

As it is well known, the enzyme activity can be affected by compounds from the sample matrix. In order to apply the PPIA to the determination of OA and analogues in natural samples, the effects of shellfish matrices on the activity of wild-type and recombinant PP2As were studied in detail. As expected, even if there is no OA in the shellfish sample, high concentrations of shellfish matrix (mussel, wedge clam, pacific oyster and flat oyster) interfere with the PPIA. This is not only due to the matrix coloration, effect that can be neglected by the use of appropriate controls, but presumably to the effect of matrix compounds other than diarrheic lipophilic toxins on the PP2A activity. Certainly, the presence of these compounds, when used in high concentrations, modifies the enzyme environment and may alter its functionality. Consequently, loading limits were established for each matrix and each PP (Table 2.1.1), using samples with no toxin contents. In general terms, results show that the recombinant PP2A is able to operate under higher shellfish matrix loadings than the wild-type PP2A. The effect of hydrolysed mussel samples was also evaluated because this step is required for the determination of total DSP toxins content (including OA acyl derivatives). In this case, the effect on the enzyme activity was minor when using the recombinant enzyme but it was drastic for the wild-type one. Since no acyl derivatives of DSP toxins were detected in the LC-MS/ MS analysis of the samples used as negative controls, the stronger effect of hydrolysed matrix extracts must be due to the hydrolysis process itself (reagents and/or extract heating). This effect may compromise the applicability of the wild-type PP2A to the analysis of hydrolysed mussel samples.

Once established and controlled, PPIAs were applied to the determination of free and total DSP toxin contents in twelve non-hydrolysed and hydrolysed Mediterranean mussel samples, respectively. PPIAs slightly overestimated toxin contents compared to LC-MS/MS analysis, the overestimation being usually more evident with the wild-type enzyme. This overestimation was likely to be due to a synergistic effect observed in the presence of both shellfish matrix and OA (Fig.

2.1.2), which was overcome by the application of a correction factor (0.48). Results obtained with recombinant PP2A correlated better with LC-MS/MS analysis than those obtained with wild-type PP2A, probably due to the higher robustness of the recombinant enzyme. Correlations for the analysis of total DSP were better than those for free DSP, probably due to the fact that, in the analysis of free DSP, LC-MS/MS is not detecting the presence of acyl derivatives, whereas they may be inhibiting the PP2A. But in general terms, the developed PPIAs can be considered useful analysis tools for the determination of OA equivalent contents, with the advantages of providing a large number of results in short analysis times and at low cost.

4.3 PPIAs for the detection of toxins: The multi-toxin profiles

Although the previous twelve mussel samples did not contain acyl ester derivatives, fact that facilitates the comparison of the PPIA with LC-MS/MS, natural samples are prone to present multi-toxin profiles. Consequently, the establishment IEFs for toxins of the same group (in relation to OA in the DSP toxins group and to MC-LR in the MCs group) is useful to understand the correlations with other analysis techniques. In this direction, dose-response curves with OA and two analogues and the two PP2As showed the inhibition trend OA ≈ DTX-1 > DTX-2 (IEF of 1.1 and 0.9 for DTX-1, and 0.4 and 0.6 for DTX-2, for recombinant and wild-type PP2A, respectively), the wild-type enzyme being slightly more sensitive (Fig. 2.2.1). Different IEFs have been reported in the literature, probably due to differences in enzyme sources, enzyme concentrations, toxin standard purities, enzyme substrates and buffer compositions. Consequently, it is always necessary to fully characterise the PPIA prior to its applicability and correlation with other techniques. It is also necessary to mention that, contrarily to other works, one of the highlights of the present work was the use of certified high-purity DTX-1 and DTX-2 standards for the study. The application of the PPIAs to the analysis of mussel samples

spiked with different toxin profiles provided OA equivalent contents that correlated with the theoretical values (Table 2.2.2). Only in the most toxic profile, the OA equivalent contents were higher than expected, which may indicate a possible synergistic effect. Nevertheless, although positive and negative samples (according to the regulatory limit of 160 µg OA eq/kg mussel meat) were clearly distinguished, it is convenient to define a "suspicious range" (in this case between 80 and 180 µg OA eq/kg), which imply the analysis of the sample by the reference LC-MS/MS method for decision purposes. The application of the PPIAs to the analysis of seven naturally-contaminated shellfish samples (raw wedge shell clam homogenate, raw razor clam homogenate, raw mussel homogenate, raw stripped venus, two cooked mussel homogenates and raw cockle homogenate) with different toxin profiles provided OA equivalent contents that agreed with those resulting from the application of the IEFs to the individual OA, DTX-1 and DTX-2 contents determined by LC-MS/MS analysis (Table 2.2.3), and also those resulting from the application of the TEFs proposed by European Food Safety Authority (EFSA). In this case, as LC-MS/MS analysis indicates, all samples contained acyl ester derivatives, detectable after hydrolysis. Since hydrolysed samples always provided higher toxins contents in the PPIAs, it is fair to suggest that the acyl ester derivatives inhibit PP2As less than the corresponding precursor toxins. Nevertheless, they may still be inhibiting thus contributing to the OA equivalent contents determination in non-hydrolysed samples. Finally, it is important to note that the PPIAs led not only to the screening but also to the quantitative determination of the toxin equivalent contents.

With the same purpose, the IEFs for several MCs variants were established using recombinant PP1 and PP2A and wild-type PP2A (the activity of the wild-type PP1 stock solution was so low that experimentation with this enzyme were not viable). The inhibition trend was the same for all enzymes: MC-LR > -RR > -YR > -LY > -LW > -LF. MCs variants with arginine in position 4 (-LR, -RR and -YR) showed higher inhibition values than the rest. Nevertheless, different IEFs and trends can

be observed in other works. Again, the differences could be due the impurity of the MCs variants and to the enzyme source and nature. The PPIAs were applied to the analysis of a natural cyanobacterial bloom with a multi-MC profile. Recombinant PP1 and wild-type PP2A provided approximately similar MC-LR equivalent contents, but lower than the recombinant PP2A. All PPIAs overestimated the toxin contents compared to LC-MS/MS. This overestimation could due to the presence of other compounds in the sample, which could be inhibiting the PPs, and/or the effect of the minority and/or non-identified MC variants. In the analysis of an artificially-contaminated sample, prepared by the spiking of buffer with MC standard solutions, an appropriate agreement was observed between the contents provided by the PPIAs and those resulting from the application of the IEFs to the individual MC variants contents determined by LC-MS/MS analysis. Unexpectedly, the MC contents provided by the PPIAs were lower than those reported in the quantification of the natural bloom. Since non-identified MC variants were found in smaller abundance and probably were less inhibitory, matrix compounds are likely to be interfering in the analysis of the natural bloom.

In general terms, the establishment of IEFs contributes to better understand the agreement between PPIAs and LC-MS/MS analysis and allows us to affirm that PPIAs can be used as reliable screening tools in monitoring and research programs.

4.4 Magnetic particles as biomolecule immobilisation supports

In the development of a biosensor, the immobilisation of the biorecognition molecule on the transducer is crucial. In order to overcome the drawbacks arising from the immobilisation of enzymes by entrapment (random immobilisation, diffusion barriers and possible enzyme leakage), the use of magnetic particles (MPs) has been explored. MPs present large surface-to-volume ratio, high surface reaction activity for biomolecule loading and favour the oriented biomolecule immobilisation, without barriers. MPs have been used

as immobilisation supports for both enzymes and antibodies for the subsequent development of biosensors.

In the first approach, recombinant PP2A and PP1 were conjugated to nickel-modified MPs via coordination chemistry between nickel and the hexa-His tail introduced to the enzymes by genetic engineering, which provided an oriented enzyme immobilisation. Colorimetric experiments demonstrated that the conjugation was successful. Moreover, the conjugation increased the storage stability of PPs. However, the absolute enzyme activity decreases and thus higher PP amounts are required to perform the corresponding PPIA for OA or MC-LR (in relation to when using free PP), which decreases the sensitivity of the assay. Nevertheless, this approach is still applicable to the determination of toxin contents in highly contaminated samples, as the analysis of cyanobacteria cultures and blooms demonstrate.

In the second approach, monoclonal antibodies (MAbs) against MC-LR were conjugated to protein G-modified MPs by means of the ability of the recombinant protein G to bind to Fc antibody regions, which provided an oriented antibody immobilisation. Colorimetric experiments using a MC-LR-horseradish peroxidase (MC-LR-HRP) tracer demonstrated that the conjugation was successful. Additionally, when performing the competition assay with the conjugates, the calibration curves showed 50% effective coefficient (EC_{50}) values and working ranges higher than in the corresponding ELISA, but still appropriate (unexpectedly, the pre-incubation of the MAb with the MC and the tracer did not decreased the LOD). In this case, the step from a colorimetric measurement to an electrochemical one, using a redox mediator in solution, decreased considerably the EC_{50} value and the LOD, and enlarged the working range (Table 3.2.1). Thus, the developed electrochemical immunosensor was able to detect MC-LR at concentrations below the guideline value recommended by the WHO (1 µg/L) and of the same order of magnitude of commercial ELISA tests. Its applicability was demonstrated by the analysis of a

cyanobacteria culture and a natural bloom, which provided toxin contents similar to those obtained by PPIA, ELISA and LC-MS/MS (Table 3.2.2). Slight differences were observed in the comparison with the LC-MS/MS analysis of the natural bloom, which could be related to the sample matrix (as previous experiments indicate).

4.5 Magnetic particles as biomolecule carriers

MPs have also been exploited as biomolecule carriers in SPR flow systems for the development of optical biosensors. In this case, MAbs against OA were conjugated to protein G-modified MPs. SPR measurements demonstrated that the conjugation was successful and stable for 15 days at -20 °C (97% affinity retention), which indicates that there is no need to prepare the conjugates fresh. But the most important insight was that the use of conjugates amplified the SPR signal compared to when using free MAb, up to 11 times. When performing the competition assay with conjugates, the calibration curves provided a 3-fold lower LOD compared to the approach with free antibody). Moreover, the presence of mussel matrix did not interfere in the measurements (Table 3.3.1).

Taking into account the attained LOD, the MP-based approach should be able to quantify 12 µg of OA/kg mussel meat (so far below of the limit value established by the European commission). The analysis of fifteen mussel samples obtained from Ebro Delta's bays (NW Mediterranean) during a DSP event, and the good agreement with MBA and LC-MS/MS analysis (Table 3.3.2 and Fig. 3.3.4) demonstrate that the hypothesis was correct and that the SPR can be considered an appropriate tool to screen the presence of OA. In any case, it is safe to establish a threshold "suspicious" area for samples with OA contents comprised between 130 and 180 µg/kg, which would require complementary analysis with other techniques.

Chapter 5

Conclusions and future work

5.1 Conclusions

The results obtained during the PhD program lead to the following conclusions:

- Recombinant PPs, obtained by genetic engineering, have shown higher operational stability than wild-type PPs. The addition of 10% glycerol to stock recombinant PP solutions has maintained their storage stability. In the PPIAs, some of the recombinant PP production lots have been able to attain LODs of toxins similar to those obtained with wild-type PPs.

- Shellfish matrix loading limits for the PPIAs have been established, according to the shellfish species and the enzyme source, which favour minimisation of the non-specific shellfish matrix effects.

- A synergistic effect has been observed in the presence of OA and shellfish matrix, which has resulted in PP inhibition percentages higher than in the absence of shellfish matrix. The effect of this synergy on the determination of total OA equivalent contents has been overcome by the application of a correction factor.

- The inhibitory potencies of the DSP toxins on recombinant and wild-type PPs have been determined, and the IEFs for DTX-1 and DTX-2 have been established. The application of the IEFs to the individual toxin contents determined by LC-MS/MS analysis in samples with multi-toxin profiles has contributed to better understand the correlation with the OA equivalent contents provided by the PPIAs. In the application of the PPIAs to the screening of shellfish samples in monitoring programs, a “suspicious” range should be established, which would require samples to be analysed by LC-MS/MS prior to decision making.

- The inhibitory potencies of several MC variants on recombinant and wild-type PPs have been determined, and the corresponding IEFs have been established. The application of the IEFs to the individual toxin contents determined by LC-MS/MS analysis in samples with multi-toxin profiles has contributed to better understand the correlation with the MC-LR equivalent

contents provided by the PPIAs. Although a more exhaustive study on the matrix effects would be useful to reduce or avoid false positives, these PPIAs can be used as tools to screen the presence of MCs in cyanobacterial samples.

- PPs and MAbs towards MC-LR and OA have been successfully conjugated to MPs, providing an oriented biomolecule immobilisation, and retaining their functionality and ability to detect the corresponding toxins. Whereas the conjugation of PPs to MPs has resulted in LODs of toxins higher than required, the conjugation of MAbs to MPs has allowed the development of high-performance electrochemical immunosensors and their application to the analysis of cyanobacteria samples.

- The use of MPs as antibody carriers in SPR flow systems amplifies the optical responses, decreasing the LOD of OA. In the study of the applicability, the OA calibration is not affected by the mussel matrix presence, and the determination of OA equivalent toxin contents in naturally-contaminated mussel samples agrees with the MBA and LC-MS/MS analysis. In the screening of mussel samples with the optical immunosensor, a “suspicious” range should be established, which would require complementary analysis LC-MS/MS.

5.2 Future Work

Based on the results obtained in the PhD thesis, our future work and recommendations are:

- Whereas the applicability of the PPIA to the analysis of lipophilic marine toxins in shellfish has been studied in detail, more efforts should be made to assess the applicability of the assay to the analysis of MCs in cyanobacteria samples.

- Antibody cross-reactivity factors should be established to better understand the correlations of immunoassays and immunosensors with other analysis techniques.

- The conjugates based on antibodies and MPs should be integrated into flow systems for the development of compact analysis devices. Additionally, immobilised redox mediators should be incorporated in the designs.

- The use of conjugates based on antibodies and MPs in SPR systems is promising, but variability should be minimised. This could be achieved by proper mixing automation of conjugates and samples prior to their injection into the SPR devices and/or the use of lighter and/or smaller particles.