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 charge 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.
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 secretion 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 low specificity and the controversy of their application, the Commission Regulation (EC) No 15/2011 has recently approved 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 is applied since 1st July 2011 and the use of the MBA is possible 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 has received more acceptance (Tubaro et al. 1996; Della Loggia et al. 2000; Campàs and Marty, 2007; Albano et al., Rossini, 2009; Caillaud et al., 2010; Cañete et al., 2010).

This functional method has gained much interest because its advantages in terms of simplicity of the protocol, multiple-sample analysis, enough sensitivity and limit of detection (LOD) of the assay, 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 us 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 charge effect on the PP2A activity has been 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 µg OA/kg shellfish meat) have been used for the study of the matrix effects. Once the matrix charge limits established, the PPIA has been applied to the analysis of naturally-contaminated Mediterranean mussels and results have been compared to those obtained by LC-MS/MS analysis.
2. Materials and Methods

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 (New York, USA). The activity of the stock solutions was between 766 and 1364 U/mL for GTP Technology and 5,660 U/mL for Upstate Biotechnology, 1 U being defined as the amount of enzyme required to hydrolyze 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).

2.2 Shellfish samples

Three negative Mediterranean mussel (\textit{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 (*Mytilus 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 different 4 rafts (C1, C2, C3, C4) at 1, 5 and 10 m depth during a DSP closure in Ría de Arousa, enforced 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 (42°28’54”N, 08°57’48’’W), at the entrance of the Ría de Arousa, and A8 (42°29’48”N, 08°55’36’’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 hours. The chamber was examined for quantification of *Dinophysis* species and total phytoplankton.

### 2.3 Lipophilic toxins extraction

Crude extracts corresponded to shellfish samples (aliquot of 2 g from a flesh homogenate of 100 g) blended with a hand-held homogeniser and extracted 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 3,000 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-µm 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 MgCl₂, pH 8.4.

### 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 µL of NaOH at 2.5 N were added to 1.25 mL of extract and homogenised for 10 seconds with a vortex. Extracts were then incubated at 76 °C for 40 min in a Multi-Block®Heater from Lab-Line Instruments, Inc. (Maharashtra, India). After cooling, 125 µL of HCl at 2.5 N
were added and hydrolysed extracts were passed through a 0.2-µm 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 MgCl₂, pH 8.4.

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 concentrations (starting at 100 µg/L for OA, 200 mg/mL for negative shellfish samples and at the matrix charge limit for positive mussel samples, and diluting to the half) were added in microtiter wells containing 100 µL of enzyme solution at 1.25 U/mL. Then, 50 µL of 25 mM p-NPP solution were added and after 1-hour incubation at room temperature in the dark, the absorbance at 405 nm was measured with an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA). OA and shellfish extract solutions were prepared in a buffer solution containing 30 mM Tris-HCl, 20 mM MgCl₂, 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, California, USA) and fitted to sigmoidal logistic four-parameter equations.

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 °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). 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.

### 2.7 Statistical analyses

To evaluate differences in the matrix charge effect on the PP2A activity for the four shellfish species (non-hydrolys ed 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 charge 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.

3. Results

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. As previously mentioned, samples determined as negative by LC-MS/MS analysis (diarrheic lipophilic toxin content lower than the LOQ = 30 µg OA and derivatives/kg) and in some cases also by MBA for DSP toxins (toxin content lower than 160 µg 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 usually, only changing the OA standard solution by shellfish matrix with no toxin content. Figure 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.

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: $W = 24$, $p = 0.32$;
 washington 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 charges, 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) (Figure 1.A). 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 hydrolysed (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 Figure 1, standardised matrix charge limits in view of application in routine monitoring programmes were established depending on the enzyme source and the shellfish species (Table 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):

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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. Taking into account the 10% inhibition values of OA calibration curves and the established charge limits, theoretical limits of detection (LODs) were calculated. In Table 1 the LODs for both enzymes combined with the different shellfish matrices, including hydrolysed mussel, are shown.

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 charge 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 charge (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 charge limit, the determination of OA equivalent contents was performed taking into account the inhibition percentage obtained at the lowest mussel charge.
(and the corresponding inhibition percentage of the OA calibration curve). Table 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.

This overestimation was investigated in detail. Figure 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 charge 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 (\( t = 0.07, p = 0.93, df = 12 \)).
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). 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 3.

With regards to phytoplankton, *Dinophysis acuminata* was present in the area from April to October 2010 (Fig. 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.
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 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, the establishment of matrix charge 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 charge effect, results clearly show that the recombinant PP2A from GTP is able to operate under higher shellfish matrix charges 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 negative, the stronger effect of hydrolysed matrix extracts should be due to the hydrolysis step (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, making it inhibitorier to the enzyme. The LODs calculated taking into account the OA calibration curves and the matrix charge 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).

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 an origin on 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 the possible presence of proteases, 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 and co-workers (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 unlikely that the PPIA overestimated the OA contents as yields in experiments with spiked shellfish closely matched theoretical yields.

Consequently, we consider 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 analysis estimation 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, two samples (C1-1m and C1-5m) 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 µg/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, on 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; FAC/IOC/WHO, 2004), and which moreover has been the main problem in the Spanish Rías (Reguera et al., 2012).

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 are observed between enzymes, our recombinant PP2A catalytic subunit can operate under higher shellfish matrix charges than the commercial enzyme, especially when analysing hydrolysed mussel samples. Shellfish matrix charge 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 PPIAs have 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 known 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.

Acknowledgements
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References

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Figure 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.
Figure 2. PP2A inhibition percentage at different OA concentrations in buffer solution, non-hydrolysed and hydrolysed mussel with PP2A from GTP (A) and Upstate (B).
Figure 3. Phytoplankton abundance (*Dinophysis acuminata* and total phytoplankton) in A0 and A8 stations near the rafts were shellfish samples were collected. Thick gray line at 500 cells/L indicates the alert value for *Dinophysis acuminata*. Thin gray line at 120 cells/L indicates the *Dinophysis acuminata* minimal abundance associated to DSP events in the Galician Rías.
Table 1. Charge limits and LOD\textsubscript{10} for OA in different shellfish matrices.

<table>
<thead>
<tr>
<th>Shellfish sample</th>
<th>Enzyme</th>
<th>Charge limit (mg/mL)</th>
<th>LOD\textsubscript{10} (µg/kg)</th>
</tr>
</thead>
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<tr>
<td>Mussel</td>
<td>GTP</td>
<td>50.0</td>
<td>7222</td>
</tr>
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<td></td>
<td>Upstate</td>
<td>12.5</td>
<td>7231</td>
</tr>
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<td>Hydrolysed mussel</td>
<td>GTP</td>
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</tr>
<tr>
<td></td>
<td>Upstate</td>
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<td>604</td>
</tr>
<tr>
<td>Wedge clam</td>
<td>GTP</td>
<td>12.5</td>
<td>7258</td>
</tr>
<tr>
<td></td>
<td>Upstate</td>
<td>12.5</td>
<td>111</td>
</tr>
<tr>
<td>Pacific oyster</td>
<td>GTP</td>
<td>12.5</td>
<td>7268</td>
</tr>
<tr>
<td></td>
<td>Upstate</td>
<td>6.3</td>
<td>222</td>
</tr>
<tr>
<td>Flat oyster</td>
<td>GTP</td>
<td>50.0</td>
<td>7292</td>
</tr>
<tr>
<td></td>
<td>Upstate</td>
<td>12.5</td>
<td>711</td>
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</table>
Table 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%.

<table>
<thead>
<tr>
<th></th>
<th>Free DSP</th>
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<th>Total DSP</th>
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<tbody>
<tr>
<td></td>
<td>C1</td>
<td>C2</td>
<td>C3</td>
<td>C4</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1m</td>
<td>165</td>
<td>302</td>
<td>319</td>
<td>340</td>
</tr>
<tr>
<td>5m</td>
<td>204</td>
<td>401</td>
<td>666</td>
<td>504</td>
</tr>
<tr>
<td>10m</td>
<td>462</td>
<td>512</td>
<td>305</td>
<td>449</td>
</tr>
<tr>
<td>Non-corrected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1m</td>
<td>235</td>
<td>511</td>
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<td>425</td>
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<tr>
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<td>928</td>
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<td>1146</td>
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<td>Upstate</td>
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<tr>
<td>1m</td>
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<td>661</td>
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<tr>
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<td>987</td>
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<td>GTP</td>
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<tr>
<td>1m</td>
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<td>245</td>
<td>197</td>
<td>204</td>
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<tr>
<td>5m</td>
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<td>445</td>
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<td>341</td>
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<tr>
<td>10m</td>
<td>487</td>
<td>550</td>
<td>194</td>
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<tr>
<td>Upstate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1m</td>
<td>205</td>
<td>317</td>
<td>276</td>
<td>339</td>
</tr>
<tr>
<td>5m</td>
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<td>582</td>
<td>453</td>
<td>652</td>
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<tr>
<td>10m</td>
<td>428</td>
<td>474</td>
<td>230</td>
<td>581</td>
</tr>
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</table>
Table 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 Mediterranean mussel samples, in relation to free and total DSP toxin contents.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Equation</th>
<th>Correlation</th>
<th>R²</th>
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</thead>
<tbody>
<tr>
<td>Free DSP</td>
<td>GTP</td>
<td>y = 1/(0.0000923724 + 0.474349x)</td>
<td>0.9354</td>
</tr>
<tr>
<td></td>
<td>Upstate</td>
<td>y = 1/(0.000048669 + 0.829681x)</td>
<td>0.8696</td>
</tr>
<tr>
<td>Total DSP</td>
<td>GTP</td>
<td>y = (20.6028 - 2238.44x²)</td>
<td>-0.9747</td>
</tr>
<tr>
<td></td>
<td>Upstate</td>
<td>y = (28.8389 - 2664.06x²)</td>
<td>-0.9163</td>
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</table>