Optimización de ensayos celulares para la detección de toxinas marinas responsables de intoxicaciones alimentarias. Aplicación en extractos lipofílicos de muestras naturales de *Mytilus galloprovincialis*

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3.4 Artículo 4

NG108-15 cell-based and protein phosphatase inhibition assays as alternative semiquantitative tools for the screening of lipophilic toxins in mussels. Okadaic acid detection

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RESUMEN

Presentamos la aplicación de un ensayo celular (cell-based assay; CBA) usando células de hibridoma NG108-15 y un ensayo de inhibición de proteína fosfatasa (protein phosphatase inhibition-based assay; PPIA) como herramienta toxicológica o funcional alternativa, respectivamente, para el rastreo de toxinas lipofílicas en mejillones (*Mytilus galloprovincialis*). Extractos acetónicos fueron directamente testados por el CBA y el PPIA pero se observaron severos efectos matriz. Como solución, un protocolo simple de 17 fracciones con cartuchos de extracción en fase sólida (solid-phase extraction; SPE) se optimizó para ser previamente aplicado en ambos el CBA y el PPIA. Se realizaron análisis mediante LC-MS/MS en paralelo para determinar el contenido de los extractos de mejillón en toxinas lipofílicas. La evaluación del protocolo con SPE, por LC-MS/MS, mostró una recuperación del ácido okadaico (OA) en torno al 90% e insignificantes efectos de la matriz de mejillón en el desarrollo de la SPE. El conjunto de los métodos proporciona límites de detección de 47 y 45 µg equivalentes de OA/ kg para el CBA y el PPIA, respectivamente. La estrategia combinada permite la identificación de la toxicidad producida por el OA en dos fracciones, y nos permite distinguir claramente entre muestras positivas y negativas, estas últimas siendo dopadas con OA o bien

muestras positivas naturales a niveles superiores o igual a los límites regulados. La combinación del fraccionamiento con el CBA o el PPIA permite la cuantificación de los efectos tóxicos y funcionales de las muestras en torno a esas concentraciones.

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ABSTRACT

We report the application of a cell-based assay (CBA) using NG108-15 a hybridoma cell strain and a protein phosphatase inhibition-based assay (PPIA) as alternative toxicological or functional semiquantitative tools, respectively, for the screening of lipophilic toxins in mussels (*Mytilus galloprovincialis*). Acetonic extracts were directly tested by CBA and PPIA but severe matrix effects were observed. As a solution, a simple 17-fraction protocol with solid-phase extraction (SPE) cartridges was optimised and applied as a previous step to the CBA or the PPIA. LC–MS/MS analyses were performed in parallel to determine the lipophilic toxins content in mussel extracts. Evaluation of the SPE protocol by LC–MS/MS showed okadaic acid (OA) recovery above 90% and negligible effects of mussel matrix on the SPE performance. The whole methods provided limits of detection of 47 and 45 μg OA equivalents/kg for CBA and PPIA, respectively. The combined strategy permitted the identification of OA toxicity in two fractions, and allowed us to clearly distinguish between negative and positive samples, the latter being either OA-spiked or naturally-contaminated samples at levels equal or above the regulatory limit. The combination of fractioning with CBA or PPIA allows the quantification of the toxic and functional effects of samples above these concentrations.

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1. Introduction

Marine toxins from toxic microalgal blooms can be accumulated in filter-feeding shellfish and produce toxic syndromes on humans through food-borne intoxication. The European Union supports a series of methods for the detection of marine toxins as alternatives or supplementary to animal testing methods, if their implementation provides an equivalent level of public health protection (Regulation (EC) 2074/2005). Functional methods, such as mammalian cell-based assays (CBA) or protein phosphatase inhibition-based assays (PPIA), could contribute to reduced animal testing as soon as they are validated to detect marine toxins at the regulatory limits (Regulation (EC) 853/2004).

Cell-based assays have been used as toxicological models for the evaluation of marine toxins (Garthwaite, 2000; Rossini, 2005). Their potency as toxicological tools depends on: cell type, method of toxicity evaluation, toxin exposure time, co-exposure with other toxins, etc. (Cañete and Diogène, 2008). When selecting a CBA for a routine toxicological screening, the use of simple and widespread tools for toxin detection, such as established cell lines and cell staining to estimate the proportion of living cells, may be advantageous in comparison to more sensitive but complex tools. Previous publications regarding the specific use of NG108-15 cells and the MTT cell proliferation assay for the evaluation of marine toxins, including okadaic acid (OA), illustrate their potency as toxicological screening models showing even more sensitive responses for other lipophilic toxins (dinophysistoxin-1 (DTX-1), pectenotoxin-2 (PTX-2), yessotoxin (YTX) and azaspiracid-1 (AZA-1)) than for OA (Cañete and Diogène, 2008, in press).

Okadaic acid and DTX-1 are potent inhibitors of protein phosphatases of type 1 (PP1) and type 2A (PP2A), two enzymes that play an important role in protein dephosphorylation in cells (Bialojan and Takai, 1988). This inhibitory effect has been exploited to develop functional assays for the detection and quantification of these marine toxins in mussels. These assays use different detection techniques to measure enzyme inhibition, namely colorimetry and fluorescence (Della Loggia et al., 1999; Tubaro et al., 1996a). Despite interest in the method, which gives an indication of the functional effect of a sample, this approach may suffer from false positives due to the inhibition of the enzyme by other compounds, e.g. matrix components. Another limitation of the assay is the high instability of the enzyme. To solve this problem, enzymes from different sources or obtained by genetic engineering are currently being explored (Allum et al., 2008).

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Apart from marine toxins, mussel lipophilic and hydrophilic extracts may contain other matrix compounds that may interfere with mammalian CBA (Malaguti et al., 2002; Nasser et al., 2008) and PPIA (Honkanen et al., 1996). In order to reduce this interference, different methodologies have been applied to mussel crude extracts previous to CBA and PPIA evaluation such as consecutive liquid–liquid fractioning (Bellocci et al., 2008; Della Loggia et al., 1999; Malaguti et al., 2002) and/or solid-phase fractioning (Bottein Dechraoui et al., 2005; Dickey et al., 1999). Liquid–liquid fractioning can contribute to the reduction of this interference with different cleaning steps and provides one final fraction used for the toxicological analysis. Solid-phase fractioning reduces interferences in individual fractions for their subsequent toxicological analysis.

Our aim was to develop toxicological and functional semiquantitative methods, alternative to MBA, to detect lipophilic marine toxins at the regulatory limits. To this end, two methods, one using a CBA and another using a PPIA were established and evaluated for OA detection.

In this work, NG108-15 CBA and PPIA were coupled to a SPE fractioning protocol and evaluated as possible routine tools for the screening of OA in mussel tissue at the present regulatory limit according to EU legislation. Both mussel extraction and fractioning purification processes were established to ensure lipophilic molecule recovery. First, crude extracts of mussels determined as negative according to the mouse bioassay (MBA) for Diarrhetic Shellfish Poisoning (DSP) toxins (Yasumoto et al., 1978) were analysed by CBA and PPIA. After observing interfering matrix effects of the crude extract in both assays, samples were fractionated to eliminate the matrix interferences. DSP negative samples (according to the MBA) were spiked with OA at a final concentration corresponding to the regulatory limit and subsequently analysed. Finally, contaminated mussels (positive by MBA for DSP toxins and/or liquid chromatography–tandem mass spectrometry, LC–MS/MS, analysis) were used to evaluate natural positive samples toxicity. LC–MS/MS was used in parallel to detect and quantify lipophilic toxins and to confirm the distribution of toxic fractions along the fractionated material.

2. Methods

2.1. Materials and samples

Certified solution for OA, YTX, gymnodimine (GYM), desmethyl spirolid C (SPX-1), PTX-2 and AZA-1 were obtained from the Institute for Marine Bioscience of the National Research Council (Halifax, Canada). In order to establish dose–response curves for OA with the CBA and PPIA, OA (Sigma Aldrich) solutions were prepared in methanol. A mussel tissue internal reference material containing OA and DTX-1 at high concentrations was used as positive sample A. Two positive (B and C) and three negative (1, 2 and 3) mussel samples (*Mytilus galloprovincialis*) according to the MBA for DSP toxins (Yasumoto et al., 1978) from the shellfish harvesting areas at the Ebre Delta bays (NW Mediterranean Sea), Spain, were obtained from the Monitoring Programme of shellfish harvesting areas of Catalonia.

NG108-15 neuroblastoma × glioma hybrid cell line was obtained from the American Type Culture Collection (ATCC, HB12317), University of Texas, Southwestern Medical Centre, Texas, USA. Dulbecco's modified Eagle's medium (DMEM), pyridoxine–HCl, hypoxanthine, aminopterin, thymidine, 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma and foetal bovine serum (FBS), L-glutamine solution (200 mM) and antibiotic solution (10 mg/ml streptomycin and 1000 U/ml penicillin) from Lonza.

Protein phosphatase 2A (PP2A) was obtained from Upstate Biotechnology (New York, USA). The activity of the stock solution was 8900 U/ml, 1 unit being defined as the amount of enzyme required to hydrolyse 1 nmol of *p*-nitrophenyl phosphate (*p*-NPP) in 1 min at 25 °C. Components of buffers and *p*-NPP were purchased from Sigma.

HPLC-grade acetonitrile, methanol, hexane, acetone and acetic acid were purchased from Merck (Darmstadt, Germany). All solutions were prepared using Milli-Q grade water obtained from a Millipore purification system (Bedford, USA), apart from CBA solutions which were prepared using HPLC-grade water.

2.2. Sample extraction

An acetone-based extraction of mussels (Yasumoto et al., 1978) was used in order to obtain mussel extracts comparable with those used regularly with the MBA. From approximately 2 kg fresh mussel sample, a homogenate of total flesh representative of the population was obtained. A portion of 7 g of mussel tissue was extracted in triplicate with 24 ml of acetone, combining the extracts. Only for sample A, extract was obtained from crude hepatopancreas. The acetone extract was evaporated to dryness at 40 °C using a rotary evaporator. The residue was redissolved in methanol to obtain an extract of 600 mg tissue equivalent/ml, which was further partitioned in triplicate with hexane in a 1:1 ratio of hexane:methanol. Finally, the crude methanolic phase was stored at −20 °C.

2.3. Chromatographic fractioning

In order to apply our CBA method to identify toxic positive samples according to current legislation for OA, a specific fractioning protocol was established to obtain OA containing fractions that would cause 50% mortality on NG108-15 cells when the OA content in the sample is 160 μg/kg mussel tissue. This protocol takes into consideration the sensitivity of NG108-15 cells to OA. Thus, 1.28 ml of mussel extract (crude methanolic phase) was evaporated and redissolved in 0.2 ml methanol and subsequently diluted to 2.0 ml with water 0.1% acetic acid (HAc). Samples were loaded on reversed-phase SPE cartridges Accubond C18 (Agilent Technologies, Santa Clara, USA) and fractioned on a vacuum Manifold in 17 fractions at constant flow rate (ca. 2 drops/s). A gradient elution with water:acetonitrile both containing 0.1% HAc (Table 1) was optimised to avoid matrix toxic effects either on NG108-15 cells or PP2A.

2.4. Toxic and inhibitory effect evaluation

Prior to exposure of NG108-15 cells or PP2A, crude extracts or fraction solutions were evaporated under a N_2 stream at 40 °C using a Turbovap (Zymark corp., Hopkinton, Massachusetts). Evaporated extracts were redissolved in 5% FBS NG108-15 culture medium (for the CBA) or 30 mM tris–HCl buffer, 30 mM $MgCl_2$, pH 8.4 (for the PPIA) and added to the corresponding wells of 96-well plates. Other concentrations were prepared by dilutions.

2.4.1. NG108-15 cell-based assay (CBA)

NG108-15 cells were maintained in 10% FBS/DMEM at 37 °C and 5.0% CO_2, as described by Cañete and Diogène (2008).

For CBA, 96-well plates (flat bottom) were prepared with cells obtained from a 90–100% confluent flask. Inocula of 200 μl cell suspension were added to each well. Cell densities were approximately 25000 cells/well. All wells had the same final volume (230 μl), which was adjusted with 5% FBS culture medium. Cells were grown without any treatment for 24 h, and then, cells were

Table 1
SPE 17-fraction protocol.

	Percentage (%)				Volume (ml)
	MeOH	ACN_AC	H2O_AC	ACN	
Column condition 1				100	6
Column condition 2		10	90		5
Sample	10		90		2
Fraction 1		10	90		5
Fraction 2		20	80		5
Fraction 3		30	70		5
Fraction 4		40	60		5
Fraction 5		45	55		5
Fraction 6		50	50		5
Fraction 7		60	40		5
Fraction 8		65	35		5
Fraction 9		70	30		5
Fraction 10		75	25		5
Fraction 11		80	20		5
Fraction 12		85	15		5
Fraction 13		90	10		5
Fraction 14		95	5		5
Fraction 15		100	0		6
Fraction 16		100	0		6
Fraction 17		100	0		6

Corresponding percentages of methanol (MeOH), acetonitrile or water with 0.1% HAc (ACN_AC and H2O_AC, respectively) and acetonitrile (ACN) from the total volume in any stage (column condition, sample loading or solvent addition for fraction elution) of chromatographic fractioning.

treated for 24 additional hours with the toxin or samples at different concentrations. The protocol was optimised with an exposure of 100 mg mussel tissue equivalents/ml in order to be able to detect OA concentrations in mussel tissue near the regulatory limit (160 μg/kg mussel tissue). Cell viability was evaluated using the MTT method, as described elsewhere (Manger et al., 1993). Absorbance values were measured at 570 nm on an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA). Assays were performed in triplicate with the exception of fractions exposures which were performed in duplicate. The OA dose–response curves obtained by CBA were analysed with the software Prism 4 (GraphPad, San Diego, California, USA). Non-linear regression for curve fit was applied using a sigmoidal dose–response curve (variable slope) of the Log *X*, *X* being toxin concentration. The ordinate axes of the dose–response curves represent the viability estimations using MTT reduction percentage to estimate absolute viability values. The OA equivalent estimations were performed with the use of an integrated dose–response curve obtained from several experiments as was recommended on Cañete and Diogène (in press).

2.4.2. Protein phosphatase 2A inhibition assay (PPIA)

The colorimetric PPIA protocol was similar to that described by Tubaro et al. (1996a): 50 μl of OA standard solution or crude mussel extracts at different concentrations were added in microtiter wells containing 100 μl of PP2A solution (3 U/ml). When fractioned samples were analysed, the protocol was optimised with an exposure of 10 mg mussel tissue equivalents/ml in order to be able to detect OA concentrations in mussel tissue near the regulatory limit. Then, 50 μl of 25 mM *p*-nitrophenyl phosphate (*p*-NPP) solution were added and after 1 h at room temperature, absorbance values were measured at 405 nm on an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA). PP2A and *p*-NPP were dissolved in 30 mM tris–HCl, 20 mM $MgCl_2$, 2 mM DTT, 0.2 mg/ml BSA, pH 8.4. Controls without PP2A, OA standard solution or mussel extract were always used. Assays were performed at least in triplicate. The OA dose–response calibration curves obtained by PPIA were analysed with SigmaPlot software package 8.0 (Systat Software, Inc., San José, California, USA) and fitted to a sigmoidal logistic four-parameter equation. 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 values mentioned above. Consequently, a calibration curve was always performed in parallel to each analysis of fractioned mussel sample.

2.5. LC–MS/MS analysis

Chromatographic separations were performed on an Agilent 1200 LC (Agilent Technologies, Santa Clara, USA) equipped with a Luna C8(2) column (50 × 1 mm, 3 μm particle size) and a Supelco-Guard C8(2) cartridge (4 × 2 mm, 3 μm) (Phenomenex, Torrance, USA). Separations were carried out at 30 °C and 0.2 ml/min using a binary gradient elution based on McNabb et al. (2005) and Villar-González et al. (2007), with modifications. Mobile phases consisted of 100% water (A) and 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM formic acid. The chromatographic gradient started at 90% A increasing up to 80% B over 6 min. Then, it increased to 90% B for 6 min and afterwards up to 100% B for 2 min, holding it for an additional time of 2 min. Finally, the gradient came back for 0.5 min and equilibrated for 8.5 min before the next run. Injection volume was 5 μl, and the syringe was washed for 4 s with methanol 100% at the flush port to avoid carry-over. The auto sampler was set at 4 °C. Mass spectrometry detection was carried out with a 3200 QTRAP mass spectrometer equipped with a TurboV™ ion source (Applied Biosystems, Foster City, CA, USA) in positive and negative mode. Gas/source parameters were set as follows: curtain gas: 20 psi; ion spray voltage: 5500 V (positive) and −4500 V (negative); temperature: 500 °C (positive) and 400 °C (negative); nebuliser gas: 50 psi; heater gas: 50 psi; collision gas: medium. Compound-dependent parameters were tuned on the mass spectrometer through direct infusion. Multiple Reaction Monitoring (MRM) analysis was performed with two *m/z* transitions for each compound as quantitative and qualifier ions, respectively (parent > daughter1/daughter2): ESI positive $[M+H \text{ or } M+Na]^+$, 508.2 > 202.2/160.2 for GYM, 692.5 > 444.2/426.3 for SPX-1, 881.6 > 539.5/569.5 for PTX-2, 899.5 > 557.5/587.5 for PTX-2 seco acid, 843.5 > 362.4/462.4 for AZA-1; ESI negative $[M-H \text{ or } M-2Na+H]^-$, 803.5 > 255.2/209.2 for OA and DTX-2 (DTX-2), 817.5 > 255.2/209.2 for DTX-1 (DTX-1), 1141.5 > 855.2/713.2 for YTX, 1157.5 > 871.2/729.2 for 45-OHYTX, 1155.5 > 869.2/727.2 for homoYTX, and 1171.5 > 885.2/743.2 for 45-OHhomoYTX. Analyst® software was used for the entire MS tune, instrument control, data acquisition and data analysis.

Additionally, alkaline hydrolysis of samples was also performed following the protocol described by Mountfort et al. (2001) with slight modifications, in order to investigate the presence of OA-ester derivatives.

3. Results

3.1. OA dose–response curves by CBA and PPIA

The OA dose–response curve obtained by CBA presented an $r^2 = 0.879$, using a sigmoidal dose–response curve (Fig. 1a: an example of OA dose–response curve obtained by CBA). Error in viability percentage estimations were always lower than ±5 (with a 95% confidence interval). The OA detection limit at a concentration of exposure equivalent to 100 mg mussel tissue/ml was defined to be 10% mortality in regard to fractions standard deviation of control mussel exposures (Section 3.4), which would correspond to a theoretical value of 47 μg OA/kg. For OA quantification the working range was defined to be between 20% and 80% mortality, which would correspond to theoretical values of 71 and 282 μg OA/kg, respectively.

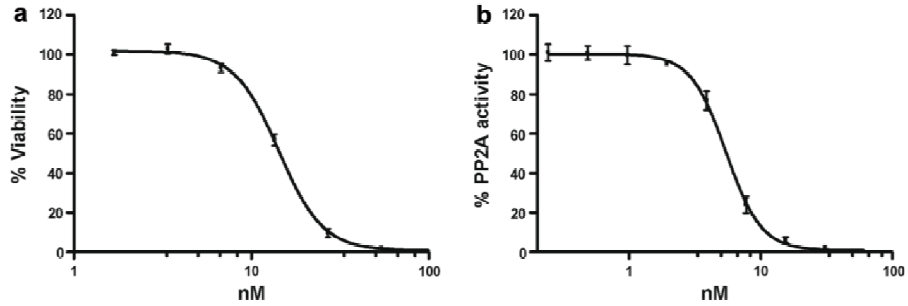

Fig. 1. Examples of dose–response curves of (a) NG108-15 CBA and (b) PPIA exposed to OA. In any graph an example of OA sigmoidal dose–response curve with r^2 superiors to 0.995. Any data point was performed with the average ± SD of three replicates in any OA concentration analysed.

The OA dose–response calibration curves obtained by PPIA presented correlation coefficients values of at least 0.999 (Fig. 1b: an example of OA dose–response curve obtained by PPIA). Standard deviation values were always lower than 5%. The OA detection range was defined to be between 5% and 95% inhibition (taking into account that the relative standard deviation values were always lower than 5%, including in fractions of control mussel exposures (Section 3.4)), which would correspond to theoretical values of 45 and 289 μg OA/kg, respectively. For OA quantification, the working range was defined to be between 10% and 90% inhibition, since this criteria is more restrictive than twice the relative standard deviation at both extremes, which would correspond to theoretical values of 57 and 222 μg OA/kg, respectively, at a concentration of exposure equivalent to 10 mg mussel tissue/ml.

3.2. Analysis of samples negative by MBA for DSP toxins (crude extracts)

Toxicity and inhibition of mussel extracts from three DSP negative samples (determined by MBA for DSP toxins within the Monitoring Programme of shellfish harvesting areas of Catalonia) was evaluated with CBA and PPIA, respectively.

In order to evaluate the applicability of the CBA to mussel samples, the effect of mussel matrix crude extracts was studied. According to our experimental protocol, when cells are exposed to a sample containing OA at the regulatory limit of 160 μg OA/kg, a concentration of exposure equivalent to 88 mg mussel tissue/ml would result in 50% mortality and would correspond to a concentration of OA equivalent of 17.5 nM (the EC50 of OA for NG108-15 cells under our conditions). NG108-15 cells directly exposed to control mussel crude extracts at 88 mg mussel tissue/ml resulted in 100% mortality (Fig. 2a).

In the PPIA, the 50% inhibition coefficient (IC50) for OA was around 1.4 nM. This OA concentration would be equivalent to 6.9 mg mussel tissue/ml in a sample containing OA at the regulatory limit. PP2A directly exposed to control mussel crude extract caused a 53% inhibition at 6.9 mg mussel tissue/ml, in the worst case (Fig. 2b).

Consequently, NG108-15 cells or PP2A directly exposed to mussel lipophilic crude extracts react strongly to the matrix which interferes with toxin evaluation. As a solution, our approach was to fractionate the mussel crude extract previous to the CBA and the PPIA.

3.3. Fractioning protocol evaluation

Recoveries of OA from fractioning were calculated for blanks of reagents and mussel matrix spiked with 160 μg/kg, by application of the SPE protocol in triplicate and analysing the resulting fractions by LC–MS/MS in quadruplicate. Results showed that OA eluted in fractions 5 and 6, with total recoveries of 91 ± 3% and 97 ± 13% in the fractioning of blank reagents and mussel matrix, respectively. Hence, it can be assessed that mussel matrix does not significantly affect the SPE fractioning performance.

3.4. Analysis of samples negative by MBA for DSP toxins (fractions)

In the CBA and PPIA, solvent fractions did not produce any toxic effect (data not shown). In the CBA, viability estimation of fractioned samples negative by MBA for DSP toxins (Figs. 3a, c and e; grey) was almost always around 100 ± 10%. Some mussels showed cell toxicity (30% mortality) in fraction 1 (samples 2 and 3). Slight toxicity (15% mortality) was also appreciated in fraction 13 of sample 2. A viability increase (15%) was also observed in fraction 8 of samples 1 and 3. These effects do not seem to be related to OA, DTX-1, SPX-1, PTX2-sa, YTX or homoYTX, as the LC–MS/MS analysis of the fractions indicates (results not shown). These may therefore be considered as light matrix effects, depending on the mussel matrix composition. Whereas interferences in fraction 1 can be neglected for lipophilic toxins analysis, since at this fraction only hydrophilic components of the matrix may appear, interferences in fractions 8 and 13 should be considered in the subsequent lipophilic toxicity determination.

In the PPIA, enzyme activity of fractioned samples negative by MBA for DSP toxins (Figs. 3b, d and f; grey) was around 100 ± 5% with the exception of fraction 6 of sample 3, where 14% inhibition was measured. This inhibition correlates with the OA detected (although below the quantification limit) in the LC–MS/MS analysis of fractioned sample 3 (results not shown).

LC–MS/MS analysis revealed that there were no quantifiable toxin concentrations in any of the three control samples, apart from very low amounts of SPX-1 in samples 1 and 3, and PTX-2sa in sample 3 (Table 2).

3.5. Analysis of OA-spiked samples (fractions)

Both CBA and PPIA showed the same trend in the evaluation of the spiking of solvents (data not shown), where toxic or inhibiting effect was concentrated in fractions 5 and 6. Exposure of NG108-15 cells to fractioned OA-spiked samples (Figs. 3a, c and e; black) resulted in cell mortality (between 20% and 50%) in fractions 5 and/or 6. The PPIA also showed PP2A inhibition (between 20% and 80%) in fractions 5 and/or 6 (Figs. 3b, d and f; black).

Whereas CBA provided OA maximum content levels around the 90% of the regulatory limit (average = 148 ± 16 μg/kg) (Table 3), results obtained by PPIA showed overestimated OA contents (aver-

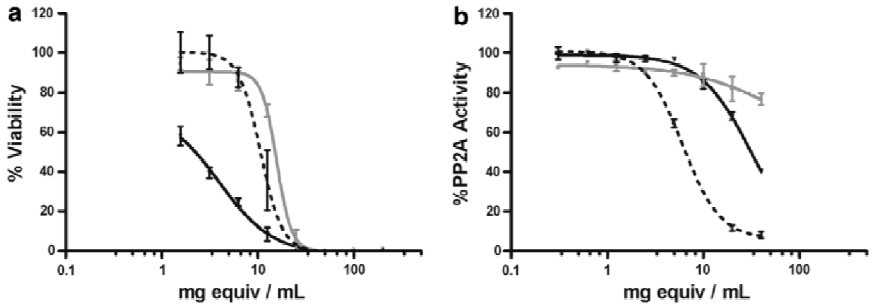

Fig. 2. Dose–response curves of (a) NG108-15 CBA and (b) PPIA exposed directly to crude extracts. In any graph, three curves are represented corresponding to three uncontaminated mussel samples: sample 1 (black), sample 2 (grey) and sample 3 (discontinuous). All curves corresponds to sigmoidal dose–response curves with r^2 superior to 0.980 with the exception of sample 2 in PPIA for which, at the concentrations tested, only an initial inhibition response was obtained. Any data point was performed with the average ± SD of three replicates in any concentration analysed.

age = 337 ± 24 μg/kg). This overestimation could be due to a synergistic effect of the toxin in the presence of fractioned mussel matrix, since the OA content quantified in the spiking of solvents was 151 ± 18 μg/kg. Consequently, a correction factor of 0.5 was applied in this work for the quantification of spiked and contaminated samples and to the limits of detection and quantification (Table 3). This factor will be the subject of further work using a wider spectrum of shellfish matrices.

As mentioned previously, LC–MS/MS analysis revealed OA elution in fractions 5 and 6.

3.6. Analysis of contaminated mussel samples (fractions)

Fractioned samples A, B and C were analysed by CBA, PPIA and LC–MS/MS analysis.

For sample A fractions, 64.9 and 6.49 mg mussel matrix equivalents/ml were used as exposure concentrations for CBA and PPIA, respectively. These concentrations correspond to a dilution 1/1.5 from the common exposures (100 and 10 mg mussel matrix equivalents/ml in CBA and PPIA, respectively). At these concentrations, more than 80% mortality or 90% PP2A inhibition would suppose levels higher than 434 and 223 μg/OA kg per fraction for CBA and PPIA, respectively. The CBA showed a high number of toxic fractions, from fraction number 5 to fraction 16 (Fig. 4a). Peaks of toxicity were observed in fractions number 5, 6, 7, 11 and 14, with toxicities from 60% to 100% mortality. In the PPIA, there was also a large number of toxic fractions (Fig. 4b), from fraction number 3 to fraction 11, and also fraction number 14. In these fractions, enzyme inhibition ranged from 20% to 100%. Since some fractions showed 100% mortality for the CBA or inhibition values higher than 100% for the PPIA, other exposure concentrations were tested in order to be able to provide quantitative values. Exposure concentrations of 0.7 and 0.07 mg mussel matrix equivalents/ml for CBA and PPIA, respectively, provided mortality and inhibition values between 20% and 80% (Figs. 4c and d). These concentrations correspond to a dilution 1/143 from the common exposures (100 and 10 mg mussel matrix equivalents/ml in CBA and PPIA, respectively). At these concentrations, the detection limit (10% mortality in CBA and 5% inhibition in PPIA) was about 6261 and 3218 μg OA equivalents/kg for CBA and PPIA, respectively. Estimation of the CBA toxic effect in fractions 5 and 6 was around 130 times higher than in OA-spiked samples (Table 3). The estimation of the inhibitory effect by PPIA was around 70 times higher, in fractions 5 and 6, than in OA-spiked samples (Table 3). LC–MS/MS analysis revealed high levels of OA and DTX-1 (Table 2), about 100 and 10 times, respectively, above the regulatory limit. Approximately the 80–85% of these OA and DTX-1 were free molecules, the rest of them being determined only after hydrolysis (Table 2). Okadaic acid mainly eluted in fractions 5 and 6 and DTX-1 in fraction 7 (further confirmed by LC–MS/MS). Consequently, DTX-1 seems to be responsible for the mortality and inhibition detected in fraction number 7 (Fig. 4a and b). The increase on the number of toxic fractions in both assays may be explained either by the high concentration of free DSP toxins (OA and DTX-1) that would elute in adjacent fractions or by the presence of OA and DTXs-ester derivatives. As an example, OA was detected by LC–MS/MS in fractions 13–15 only after hydrolysis of such fractions. Coupling chromatographic fractioning and toxicological models as CBA or PPIA could be a powerful tool in the study of ester derivatives toxicities of known toxins as OA and DTXs-ester derivatives.

For sample B fractions, 100 and 10 mg mussel matrix equivalents/ml were used as exposure concentrations for CBA and PPIA, respectively. The CBA provided a complex profile with several toxicity peaks in fractions number 1, 4, 7 and 13, which ranged from 30% to 50% mortality, and with slight toxicities (under 20% mortality) in fractions number 5, 6 and 8 (Fig. 4e). In the PPIA, fractions 5 and 6 showed slight inhibitions (Fig. 4f). It is necessary to note that, due to the high sensitivity of the assay and the unstable nature of the phosphatase, important differences in the inhibition values and calibration curves may appear from one analysis to another. Estimation of the CBA toxic effect in fractions 5 and 6 was in the range of OA-spiked samples (Table 3). The estimation of the inhibitory effect by PPIA was around half of that observed in OA-spiked samples (Table 3). LC–MS/MS analysis revealed a multi-toxin profile with presence of SPX-1, PTX2-sa, OA, YTX and homoYTX (Table 2). Although OA + PTX2-sa in samples B and C are below regulatory limit, it is well-known that the presence of other toxins may be responsible for false positives on MBA, in this case probably due to the presence of YTXs. Consequently, despite that the amount of OA estimated by PPIA in fractions 5 and 6 does not explain the DSP positive result obtained by MBA, it could be justified by the multi-toxin profile revealed by CBA and LC–MS/MS analysis. As we have previously mentioned, the acetonic extraction protocol used allows the extraction of not only OA and DTXs, but also other lipophilic toxins such as PTXs, YTXs and AZAs. The other lipophilic toxins present in this sample such as YTXs could elute in other fractions, as the CBA results suggest.

For sample C fractions, 100 and 10 mg mussel matrix equivalents/ml were used as exposure concentrations for CBA and PPIA, respectively. The CBA did not show a complex profile, only fraction 1 showing a 30% viability reduction and fractions 6 and 15 providing slight toxicities (under 20% mortality) (Fig. 4g). In the PPIA,

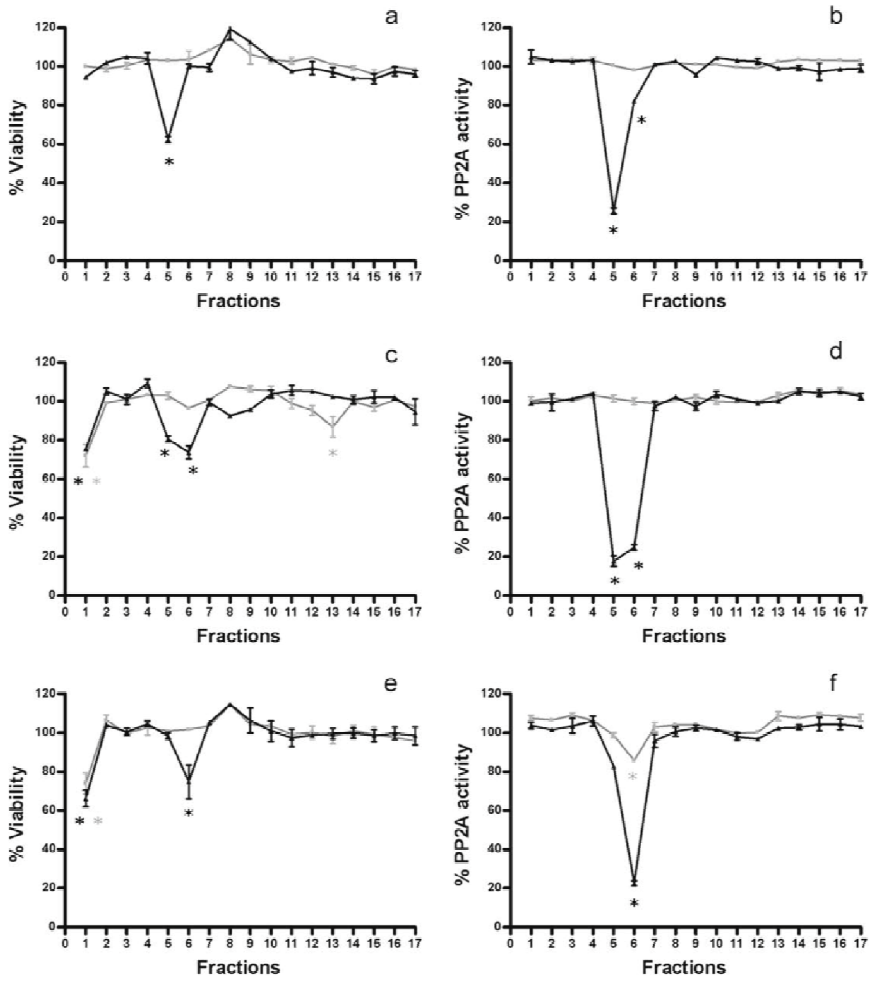

Fig. 3. Fraction toxic effect representations of uncontaminated mussel samples with and without OA spiking. Fraction toxic effects evaluated on (a, c, and e) NG108-15 CBA at 100 mg mussel tissue equivalents/ml, and (b, d and f) PPIA at 10 mg mussel tissue equivalents/ml exposed to uncontaminated mussel extracts: sample 1 (a and b), sample 2 (c and d) and sample 3 (e and f), without (grey) and with (black) OA spiking. Fraction toxic effects estimations were performed by the average ± SD of two (NG108-15 CBA) or three (PPIA) replicates. Those estimations which exceeded detection limits for any assay (10% mortality for CBA and 5% inhibition for PPIA) were labelled as significant toxic fractions *. In CBA, toxic effects in fraction 1 can be neglected for lipophilic toxins analysis, since at this fraction only hydrophilic components of the matrix may appear.

fractions 5 and 6 showed slight inhibition percentages (Fig. 4h). Estimations of the CBA toxic effect and the enzyme inhibition were lower than those obtained for OA-spiked samples (Table 3). Once again, LC–MS/MS analysis revealed a multi-toxin profile including SPX-1, PTX2sa, OA and YTX (Table 2), below the regulatory limit.

4. Discussion

Exploitation of toxicological and functional tools is highly desirable since they could contribute to reductions in animal testing while providing valuable toxicological information from the sample. In this respect, it is important to compare the results obtained with such tools with those provided by the official MBA and chemical analysis, to assess the level of public health protection that functional assays can provide.

In our work, we evaluated two semiquantitative methods, a toxicological assay and a functional assay, which could assess marine lipophilic toxin detection and be practically used for screening purposes. The application of the simple NG108-15 CBA with MTT viability estimation allows their use for the detection not only of OA but also of other lipophilic toxins, including DTX-1, PTX-2, YTX, AZA-1 and several neurotoxins (Cañete and Diogène, in press). The PPIA used is a simple and sensitive functional tool for the detection of protein phosphatases inhibitors such as OA or DTX-1.

Different protocols of mussel lipophilic toxin extractions based on methanol–water (Della Loggia et al., 1999; Leira et al., 2003; Tubaro et al., 1996b), heated methanol (Nasser et al., 2008) or acetone (Croci et al., 2001; Flanagan et al., 2001; Malaguti et al., 2002; Plakas et al., 2002) appropriate for use prior to CBA and PPIA have been proposed. In this work, we have selected acetonic mussel extraction, despite possible problems related to their matrix inter-

Table 2
LC–MS/MS analysis of lipophilic toxins.

Sample	Toxin concentration (average ± SD μg/kg)							
	Positive mode		Negative mode				Negative mode	
	SPX-1	PTX2-sa	Before hydrolysis				After hydrolysis	
			Free OA	Free DTX-1	YTX	homoYTX	Total OA	Total DTX-1
1	2 ± 0.5	nd	nd	nd	nd	nd	nd	nd
2	nd	nd	nd	nd	nd	nd	nd	nd
3	nq	4 ± 0.3	nd	nd	nd	nd	nd	nd
A[a]	nd	nd	13820 ± 46	1327 ± 12	nd	nd	15934 ± 49	1711 ± 11
B	nq	12 ± 2	nq	nd	183 ± 28	143 ± 36	18 ± 0.3	nd
C	nq	14 ± 1	25 ± 4	nd	172 ± 33	nd	56 ± 0.3	nd

Results for LC–MS/MS analysis of lipophilic toxins: desmethyl spirolid C (SPX-1), pectenotoxin-2 seco acid (PTX-2sa), okadaic acid (OA), dinophysistoxin-1 (DTX-1), yessotoxin (YTX), homoyessotoxin (homoYTX); determined in control samples (1, 2 and 3) and contaminated mussel samples (A, B and C). All analyses were performed in triplicate for positive and negative mode. The total amount of toxins from OA-group including acyl derivatives was determined after hydrolysis of the sample. Details of LC–MS/MS conditions are described in Section 2.5.
nd: Not detectable. Signal/noise (*S/N*) ratio was lower than 3.
nq: Not quantifiable. *S/N* ratio was between 3 and 10.
[a] Sample was quantified through a dilution 1:20 from original extract.

Table 3
Okadaic acid toxic effects estimations.

Sample	μg OA equivalents/kg					
	NG108-15 CBA			PPIA		
	Fraction 5	Fraction 6	Total estimated	Fraction 5	Fraction 6	Total estimated
1-OA	111 ± 3	nd	111–158	140 ± 6	41 ± 1	181
2-OA	nq	85 ± 8	132–156	90 ± 5	78 ± 2	168
3-OA	nd	83 ± 19	83–130	40 ± 1	116 ± 6	156
A (dil: 1/1.54)	>434	>434	>868	>223	>223	>446
A (dil: 1/143)	nd*	13249 ± 1502	13249–19510	6261 ± 200	5632 ± 1187	11893
B	nq	nq	94–142	44 ± 3	nq	67–73
C	nd	nq	47–118	35 ± 5	41 ± 1	76

Toxic effects estimations on OA-spiked uncontaminated mussel samples (1, 2 and 3) namely 1-OA, 2-OA and 3-OA, respectively, and three contaminated mussel samples (A, B and C) by CBA and PPIA. Partial (fractions 5 and 6) and total values (sum of fractions 5 and 6 effects) are presented. Fraction toxic effects estimations were performed by the average + SD of two (NG108-15 CBA) or three (PPIA) replicates. Minimum and maximum OA equivalents (total estimated) in any sample were estimated in regard to fractions 5 and 6 toxic effects estimations.
nd: Not detectable. Mortality percentage lower than 10%. Equivalence lower than 47 μg OA equivalents/kg.
nd*: Not detectable. Mortality percentage lower than 10% (in dilution 1/143). Equivalence lower than 6261 μg OA equivalents/kg.
nq: Not quantifiable. Mortality percentage between 10% and 20% for CBA and inhibition percentage between 5% and 10% for PPIA. Equivalence lower than 71 μg OA equivalents/kg in CBA and 57 μg OA equivalents/kg in PPIA.

ferences on mammalian CBA (Malaguti et al., 2002; Nasser et al., 2008) or PPIA, in order to ensure that the same lipophilic molecules were recovered as in the standard MBA methods currently implemented. In other studies, development of CBA-based screening methods for marine lipophilic toxins in mussel acetonic extracts were focused to discriminate between positive and negative samples determined by MBA (Croci et al., 2001; Flanagan et al., 2001), and matrix effects were not considered. Detection sensitivity of functional assays could be incremented by reducing mussel matrix effects. Longer protocols based on solid-phase fractioning coupled to CBA have been developed by other research groups, which are clearly more effective for the purification of bioactive compounds (Bottein Dechraoui et al., 2005; Dickey et al., 1999). Nevertheless, the protocol timings were inappropriate for screening purposes.

In our study, the fractioning protocol was optimised using a minimum number of fractions to reduce matrix effects on CBA and PPIA. This allowed us to quantify OA effects at the regulatory limit without interferences and ensured the elution of lipophilic molecules from the cartridge. Additionally, the application of SPE as a fractioning procedure did not require expensive analytical instrumentation. Evaluation of the 17-fraction SPE protocol by LC–MS/MS showed appropriate OA recoveries above 90% and negligible mussel matrix effects on the SPE performance. When working with OA-spiked samples, CBA provided maximum content levels close to OA recovery values. In the PPIA, a correction factor of 0.5 was applied to eliminate OA amount overestimations. Both CBA and PPIA clearly identified fraction numbers 5 and 6 as being toxic. This was further confirmed by LC–MS/MS. The 17-fraction protocol described enables the identification of samples containing OA at concentrations above 47 and 45 μg/kg (detection limits of OA for the CBA and the PPIA, respectively). The semiquantification of effects above these limits allows to clearly distinguish between negative and positive samples at levels of OA equal or above the regulatory limit. For the analysis of OA present in natural samples, both CBA and PPIA results showed similar values in relation to LC–MS/MS.

Comparing the toxicities of fractions determined by the CBA and PPIA with toxin analysis performed with LC–MS/MS, it is possible to identify known toxins but also to select those bioactive fractions without known toxins in order to identify new compounds that may have an impact on public health.

Both the accuracy on toxicity estimation and the identification of different bioactive compounds are potent properties attained in this study by the CBA and PPIA, which may increase our knowledge of marine toxins and better characterise food hazards that may have adverse effects on human health. Further work will consider a larger amount of samples for validation purposes. More

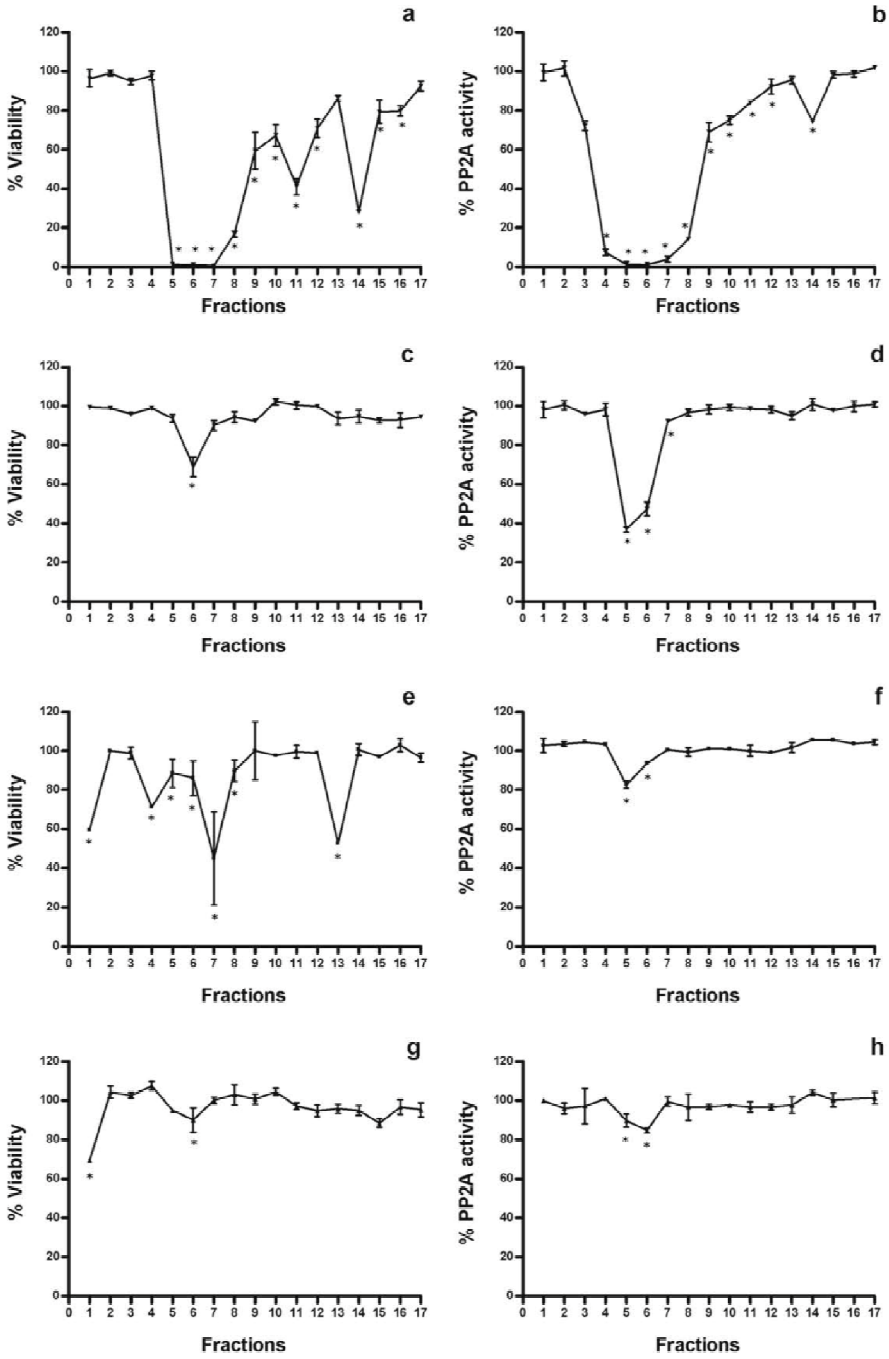

Fig. 4. Fraction toxic effect representations of contaminated mussel samples. Fraction toxic effects evaluated on (a, c, e and g) NG108-15 CBA and (b, d, f and h) PPIA exposed to three contaminated mussel extracts: sample A (a, b, c and d), sample B (e and f) and sample C (g and h). In CBA, exposure concentration were 64.9 (a), 0.7 (c) and 100 (e and g) mg mussel matrix equivalents/ml. In PPIA, exposure concentration were 6.4 (b), 0.07 (d) and 10 (f and h) mg mussel matrix equivalents/ml. Fraction toxic effects estimations were performed by the average ± SD of two (NG108-15 CBA) or three (PPIA) replicates. Those estimations which exceeded detection limits for any assay (10% mortality for CBA and 5% inhibition for PPIA) were labelled as significant toxic fractions *. In CBA, toxic effects in fraction 1 can be neglected for lipophilic toxins analysis, since at this fraction only hydrophilic components of the matrix may appear.

work focused on the effect of lipophilic toxins other than DSP has to be done to improve the agreement among the results obtained with MBA, toxicological and functional assays, and LC–MS/MS.

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