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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3.5 Artículo 5

Evaluation of a toxicological alternative tool for lipophilic toxicity screening in mussels. NG108-15 cell-based assay coupled to chromatographic fractioning; a case study for YTX contamination

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RESUMEN

Presentamos la evaluación de un ensayo celular (cell-based assay; CBA) con células NG108-15 acoplado a un protocolo de fraccionamiento como herramienta de rastreo de toxinas lipofílicas en mejillones como el ácido okadaico, la dinofisistoxina-1, la pectenotoxina-2, la yessotoxina (YTX) y el azaspirácido-1. El CBA fue evaluado en relación a los límites regulados para esas toxinas bajo dos condiciones experimentales en relación al tiempo de exposición.

El CBA permite una aproximación semicualitativa y semicuantitativa y fue desarrolllado en paralelo a los análisis de toxinas en las muestras mediante LC-MS/MS. Se puede asociar una o dos fracciones de elución para cada una de las toxinas estudiadas, las cuales presentan un comportamiento toxicológico característico en relación a las dos condiciones experimentales del CBA. El método permite detectar algunos derivados nuevos de las toxinas conocidas, otras moléculas bioactivas y demostró ser una herramienta útil en el descarte de falsos positivos del bioensayo ratón en muestras por contenido en YTXs. Para la validación y aplicación del método, se necesita más trabajo para establecer los límites de discriminación entre muestras positivas y negativas en contenido de toxinas lipofílicas marinas que supongan un riesgo para la salud pública.

ABSTRACT

We report the evaluation of a NG108-15 cell-based assay (CBA) coupled to a fractioning protocol as a tool for the screening of okadaic acid, dinophysistoxin-1, pectenotoxin-2, yessotoxin and azaspiracid-1 lipophilic toxins in mussels. The CBA was evaluated for these toxins at concentrations corresponding to the regulatory limits of these toxins, under two experimental conditions according to time of exposure.

The CBA allowed a semiqualitative and semiquantitative assay for these toxins and was developed in parallel with LC-MS/MS analyses of toxins. For all toxins tested, after fractioning the samples through SPE, toxicity was detected in one or two fractions. These toxins and presented a characteristic toxicity relation between both CBA experimental conditions. The method allowed to detect some new derivates for known toxins, other bioactive molecules and demonstrated to be a powerful tool in discarding false positive mouse bioassay YTX containing samples.

For method validation and application, further work is needed in order to establish CBA limits to discriminate positive from negative samples in dangerous lipophilic marine toxins.

Keywords

Mussel, fractioning, cell-based assay, lipophilic marine toxins, okadaic acid, dinophysistoxin-1, pectenotoxin-2, yessotoxin, azaspiracid-1.

INTRODUCTION

Since the association of a marine lipophilic toxin from suspected microalgal blooms to toxic diarrhetic symptoms by consumption of filter feeding shellfish by Yasumoto (1978), there has been an increment on mussel lipophilic extract studies. Initially, all lipophilic toxins seemed to be related to diarrhetic symptoms and in 1980 a dinoflagellate, *Dinophysis fortii*, was identified as the causative organism of a syndrome defined as Diarrhetic Shellfish Poisoning (DSP) (Yasumoto et al., 1980).

As studies on DSP toxins have been developed worldwide, DSP intoxication has demonstrated to have a wide geographical distribution with high number of episode descriptions in southwest Asia and in northwest Europe. Okadaic acid (OA) and its isomers (dinophysistoxins, DTXs), were subsequently described as the principal lipophilic DSP toxins with a mechanism of action related to protein phosphataseinhibition. Several lipophilic toxins with different mechanism of action have been described in lipophilic extracts, apart from protein phosphatase inhibitors, such as pectenotoxins (PTXs) (Yasumoto et al., 1985), yessotoxins (YTXs) (Murata et al., 1987) and azaspiracids (AZAs) (McMahon and Silke, 1996). Toxicities of these toxins expressed as 50% lethal dose (LD_{50}) obtained in intra-peritoneal injection mouse bioassay (i.p. MBA), supported by current Europe legislation methods (European Union Commission Regulation, 2005), are about 160-200 (OA and DTX-1), 219-411 (PTXs), 100-750 (YTX) and 110-200 (AZAs) µg/kg (Aune, 2008), and current regulatory limits are 160 µg of OA equivalent/kg in OA, DTXs and PTXs content, 160 µg of AZA equivalents/kg for AZAs and 1000 µg of YTX equivalent/kg for YTXs (European Union Commission Regulation, 2004). In areas with persistence of YTXs, an important problem exists with the application of the mouse bioassay (MBA) for lipophilic marine toxins, since evaluation of lipophilic toxins by the MBA may be difficult to assess in the presence of concentrations of YTXs below regulatory level. Although this difficulty can be overcome with the application of a modified MBA in order to eliminate interferences caused by YTXs when evaluating other lipophilic toxins (Fernández et al., 2002) evidence exists that this protocol may not be applicable to all YTXs interferences (Ciminiello et al., 2006). Taking into consideration the evidence that YTXs are distributed in many parts of the world, like Japan, Norway, New Zealand, Chile, Spain, Russia, Canada, United Kingdom and Italy (Paz et al., 2008) developing methods to identify them and eliminate their interference should improve monitoring programmes.

For ethical and practical reasons, current legislation enhances the replacement of MBA by other methodologies (European Union Council Directive, 1986).

Development of a toxicological tool, alternatively to the MBA, is justified according to this legislation but also as new analogues and toxins are being described for which no analytical tools may yet be developed. Additionally, considering the existing difficulties on availability of purified toxin standards and therefore limiting the application of analytical tools, toxicological assays may bring very useful information in order to assess toxicological risk.

Cell-based assays had been proved, by several research groups to be usefull toxicological tools for marine lipophilic toxin screening in shellfish (Rossini, 2005). Mussel acetonic extracts have been analysed by simple CBA methods obtaining qualitative (Croci et al., 2001; Flanagan et al., 2001) or semiquantitative (Cañete et al., 2010) toxicity estimations. An important issue when considering the evaluation of lipophilic toxins present in shellfish consists on defining the extraction procedures. Acetonic mussel lipophilic toxin extraction, currently implemented MBA methods, and selected for our study, ensures recovering more lipophilic toxin from samples but can also contribute to more matrix interferences on CBA (Malaguti et al., 2002; Nasser et al., 2008) than other methods based on methanol extraction.

The use of NG108-15 cells and the MTT cell proliferation assay, for viability estimation, has been demonstrated to be a sensitive and simple tool for lipophilic marine toxins detection (Cañete and Diogène, 2010). For YTX, and AZA-1, CBA using different cell type and/or different viability estimation assays (Pérez-Gómez et al., 2006; Twiner et al., 2005) it was necessary to expose cells during 48 hours in order to obtain maximum toxic effect and maintain repeatability of the assay (Cañete and Diogène, 2010).

In the present work, NG108-15 CBA was coupled to a 17-fractioning solid-phase-extraction (SPE) and evaluated as a possible routine tool for the screening of lipophilic toxins at the regulatory limit in mussel acetonic extracts. The method allows to conduct a semiqualitative estimation of the toxins present according to the position of the toxic fraction along the chromatographic separation and previous

understanding of the chromatographic behaviour of toxin standards and their toxicity in two experimental conditions (Semiqualitative approach). Additionally, the quantification of the toxic effect of the toxic fraction(s) with the CBA allows a semiquantitative estimation of the toxicity of the sample (Semiquantitative approach).

Diarrhetic Shellfish Poisoning negative mussel samples (according to the MBA), spiked samples with OA, DTX-1, PTX-2, YTX or AZA-1 and natural contaminated mussels (positive by MBA for lipophilic toxins and/or liquid chromatography-tandem mass spectrometry, LC-MS/MS analysis) were used to evaluate negative and positive samples toxicity in both CBA experimental conditions.

LC-MS/MS was used in parallel to detect and quantify lipophilic toxins and to confirm toxic fractions distribution along the fractioning protocol.

METHODS

Material and samples

Certified solution for OA, YTX, gymnodimine (GYM), desmethyl spirolid C (SPX-1), PTX-2 and AZA-1 were purchased from the Institute for Marine Bioscience of the National Research Council (Halifax, Canada). In order to establish CBA dose-response curves for OA and DTX-1, OA (Sigma Aldrich) and DTX-1 (Wako) 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. Seven positive natural mussel samples (B-H) and three negative (1, 2 and 3) by MBA for lipophilic 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 x 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. 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.

Sample processing

An acetone-based extraction of mussels (Yasumoto et al., 1978) was used in order to obtain mussel extracts comparable with those used regularly by laboratories implementing the MBA for the control of lipophilic toxins in shellfish samples. Acetonic extracts were fractioned in 17 fractions on reversed-phase C18 SPE cartridges with gradient elution using water and acetonitrile as described previously (Cañete et al., 2010).

Toxin spiking

Negative mussel samples (according to the MBA for lipophilic toxins) were spiked with OA, DTX-1, PTX-2, YTX or AZA-1 at concentrations in relation to current Europe regulatory limits (regulation (EC) 853/2004). Spiking concentration for DTX-1 and PTX-2 was established according to their toxicity relation to OA toxicity in i.p. MBA (Aune et al., 2007; Miles et al., 2004). For OA and AZA-1 spikings were carried at 160 µg /kg, while for DTX-1, PTX-2 and YTX, spiking were conducted at 163, 171 and 1000 µg /kg, respectively.

Toxic effect evaluation

NG108-15 cells were maintained in 10% FBS/DMEM at 37ºC and 5.0% CO_2 as described by Cañete and Diogène, 2008. For cell viability assays, 96-well plates (flat bottom) were prepared with cells obtained from a 90-100% confluent flask. Inoculates of 200 µL cell suspension (5% FBS/DMEM) were added to each well at an approximate density of 25,000 cells/well. During experimental work, all wells

had the same final volume (230 µl), which was adjusted with 5% FBS culture medium. For the whole study all exposures were performed in triplicate with the exception of fractions exposure which was performed in duplicate.

Experimental conditions for toxin and extract exposure

Two experimental conditions regarding growth time previous to toxin exposure and exposure time were used to study lipophilic mussel extracts by NG108-15 as described for marine lipophilic toxin assays in Cañete and Diogène, 2010:

In experimental condition 1, cells were grown without any treatment during 24 h, and after this time, cells were treated for 24 additional hours with the toxins or the extracts at different concentrations. Absorbance plate reading was performed after 24 h of toxin exposure.

In experimental condition 2, cells were grown without any treatment during 1 h, and after this time cells were treated for 48 additional hours with the toxins or the extracts at different concentrations. Absorbance reading was performed after 48 h of toxin exposure.

Previous to cell exposures, defined aliquots of solutions were evaporated under N_2 stream at 40 º C using a Turbovap (Zymark corp., Hopkinton, Massachusetts). Evaporated extracts were redissolved in 5% FBS NG108-15 culture medium and added to the corresponding wells in 96-well plates. Other concentrations were prepared by dilutions.

Cell exposures in both experimental conditions were performed at 100 and 50 mg mussel tissue equivalents/ml in order to be able to detect and quantify OA, DTX-1, PTX-2, YTX and AZA-1 concentrations in mussel tissue close to their regulatory limits. These exposure concentrations were established according to previous studies on purified toxin dose-response curves in NG108-15 cells (Cañete and Diogène, 2010). Exposures were almost always performed at 100 mg mussel tissue equivalents/ml, for experimental condition 1 (necessary for OA detection as was observed in previous works (Cañete et al., 2010), and enough for DTX-1 and PTX-2 in regard to expected results) and 50 mg mussel tissue equivalents/ml, for

experimental condition 2 (enough for YTX and AZA-1 in regard to expected results). For YTX and AZA-1 experimental condition 2 was performed too at 100 mg mussel tissue equivalents/ml. This assay allows a detection limit of 47 or 93 µg OA equivalents/kg at 100 and 50 mg mussel tissue equivalents/ml, respectively.

The exposure of 100 and 50 mg mussel tissue equivalents/ml allows quantification limits from 71 to 142 and from 282 to 564 µg OA equivalents/kg, respectively. When necessary, other dilutions were performed.

Cell response evaluation

Cell viability was evaluated using the MTT method, as described elsewhere (Manger et al., 1993). Absorbances were measured at 570 nm on an automated multi-well scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA). Purified toxins 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 OA equivalent estimations were performed with the use of a theoretical dose-response curve obtained from several experiments as was recommended on Cañete and Diogène, 2010.

Toxicities from spiked or natural samples were evaluated in OA equivalents/Kg discarding fraction number 1 which has been demonstrated to recover some matrix toxic components corresponding to an hidrophilic fraction (Cañete et al., 2010).

The detection limit of toxicity was defined to be 10% mortality. For quantification analysis, the working range was defined to be between 20 and 80% mortality as in previous studies (Cañete et al., 2010).

Values above 120% viability were considered as abnormal increments of viability.

Semiqualitative toxicity estimation in mussel samples was established according to elution of toxins with the experimental protocol and contrasted against standard elution time.

Semiquantitative toxicity estimation in mussel samples was established by all toxic fractions in the range of quantifiable toxicity (from 20 to 80% mortality) was

calculated according to the average of the quantified toxicity for any replicate ± SD. If sample presented no-quantifiable toxic fractions (from 20 to 10% mortality) minimum and maximum toxicity estimation were calculated by the sum of the individual values of minimum or maximum toxicity estimated in any fraction. For fractions with quantifiable toxicity, the minimum or maximum toxicity value was calculated by the rest or sum of the SD to the average value of replicates, respectively. For fractions with no-quantifiable toxicity, minimum or maximum toxicity value was estimated by the toxicity related to 10% mortality (equivalent to 47 or 93 µg OA/kg, on 100 or 50 mg mussel/mL exposures, respectively) or 20% mortality (equivalent to 71 or 142 µg OA/kg, on 100 or 50 mg mussel/ml exposures, respectively), respectively.

Estimations of the toxicities of OA, DTX-1, PTX-2, YTX and AZA-1- spiked uncontaminated lipophilic samples were calculated in regard to each purified toxin dose-response curves and relating these toxicity estimations to OA equivalents taking in to account corresponding errors in toxicity equivalence estimations (with a 95% confidence interval). All these purified toxin dose-response curves presented r^2 superiors to 0.861, using sigmoidal dose-response curves.

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 SupelcoGuard C8(2) cartridge (4×2 mm, 3 µm) (Phenomenex, Torrance, USA). Separations were carried out at 30 ºC and 0.2 ml/min using a binary gradient elution based on (McNabb et al., 2005; 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 additional 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 seconds with methanol 100% at the flush port to avoid carry-over. The auto sampler was set at 4 ºC. 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: 5500V (positive) and -4500 (negative); temperature: 500ºC (positive) and 400ºC (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.

RESULTS

Analysis of control samples: fractions of negative mussels for lipophilic toxins

Solvent fractions did not produce any toxic effect at any of the experimental conditions tested (data not shown). Under experimental condition 1 viability estimation of fractioned samples negative by MBA for lipophilic toxins was almost

always around 100 ± 10% (Cañete et al., 2010). Under experimental condition 2 these samples presented generally an increase in toxicity estimation (Table 1).

Control sample 1 demonstrated to be a good negative control even at high exposure concentrations (100 mg mussel tissue equivalent/ml) in experimental condition 2 (Fig. 1). For this reason, sample 1 was used for YTX and AZA-1 spikings (100 mg mussel tissue equivalent/ml in experimental condition 2).

Analysis of spiked samples (fractions)

Okadaic acid

As observed in previous studies using the same fractioning protocol, OA-spiked samples at 160 µg/kg could be detected and semiquantified by CBA at an exposure concentration of 100 mg mussel tissue equivalent/ml (Cañete et al., 2010). An exposure of 50 mg mussel tissue equivalent/ml would be insufficient for OA detection in mussel samples at experimental condition 1 to satisfy current legislation requirements. Okadaic acid elution, in spiked mussel matrix, was determined by LC-MS/MS to be around 97% in fraction 5 and 6 (Cañete et al., 2010).

When comparing experimental conditions 1 and 2 for the evaluation of the toxic effects, at a concentration of 50 mg mussel tissue equivalent/ml, some differences on OA-spiked samples were observed (Fig. 2).

Okadaic acid-spiked sample 1, showed a significant increment in toxicity in fraction 7 under experimental condition 2, but not in experimental condition 1 (Fig. 2b). Similar results were obtained for OA-spiked sample 3, where significant toxicity increment was obtained in fractions 5, 11 and 13 (data not shown). These toxicity increments were related with lower OA toxic estimations in fractions 5 and 6 in relation to expected values (155 -165µg/kg) (Table 1; experimental condition 1).

Okadaic acid spiked control sample 2 showed similar toxic profiles between both experimental conditions (data not shown); no increment in toxicity were obtained in

any other fraction at experimental condition 2. This result was related with more toxic effect quantification in fraction 5 and 6 (Table 1; experimental condition 1).

Dinophysistoxin 1

Dinophysistoxin 1-spiked sample (sample 3) presented toxicity mainly in fraction number 7 under experimental condition 1. LC-MS/MS analysis revealed quantifiable values (116 ± 9 µg/kg) only in fraction number 7. Comparison of experimental condition 1 and 2, at a concentration of 50 mg mussel tissue equivalent/ml (Fig. 3a), revealed toxicity increment in fraction number 11 and 12 in DTX-1-spiked sample in regard to non-spiked sample at experimental condition 2.

Expected toxic effect in DTX-1-spiked sample, was about 211-269 µg OA equivalents/kg. Estimated toxicity was in agreement with expected values at experimental condition 1, but higher toxicities were obtained at experimental condition 2 by adding fraction 11 and 12 toxicities (Table 1). Estimated toxicity quantifications have to be done at 50 mg/ml of exposure,in both experimental conditions.

Pectenotoxin 2

Pectenotoxin 2-spiked sample (sample 1) toxicity was equally distributed in fractions number 6 and 7 (Fig. 3b). Similar toxicities in both experimental conditions were observed (Table 1). LC-MS/MS analysis revealed quantifiable values in fraction number 6 (94 ± 15 µg/kg) and 7 (44 ± 2 µg/kg).

Expected toxic effect in PTX-2-spiked sample, distributed in two fractions, was superior to 1127 µg OA equivalents/kg (about 2321 - 2895 µg OA equivalents/kg). Estimated toxicity was lower than the expected toxicity in both experimental conditions (Table 1). Estimated toxicity quantifications had to be done at 50 mg/ml of exposure, in both experimental conditions.

Yessotoxin

Fractionated YTX-spiked sample (sample 1) toxicity was concentrated mainly in fractions number 3 and 4. Comparison of experimental condition 1 and 2, at a

concentration of 100 mg mussel tissue equivalent/ml (Fig. 3c), revealed an increment of toxicity in fractions 3, 4 at experimental condition 2.

Expected toxic effect in YTX-spiked sample, distributed in two fractions, was about 356 - 470 µg OA equivalents/kg, in experimental condition 1, and about 1834 - 2392 µg OA equivalents/kg, in experimental condition 2. Estimated toxicity was in agreement with expected values at experimental condition 1 and 2 (Table 1). Estimated toxicity quantification, at experimental condition 1, performed at 100 mg/ml of exposure was just in the limit of quantification. Under experimental condition 2 another dilution would be needed for toxicity quantification. LC-MS/MS analysis revealed quantifiable values in fraction number 3 (668 ± 67 µg/kg) and 4 (460 ± 57 µg/kg).

Azaspiracid 1

Azaspiracid 1-spiked sample (sample 1) toxicity was concentrated mainly in fraction number 3 at experimental condition 1(Fig. 3d). Under experimental condition 2, there was no toxicity in fraction 3 but toxicity increment was observed in fractions 8 and 9. In regard to purified toxin response (Cañete and Diogène, 2010), only toxicities of fraction 8 and 9 could be related to AZA-1 effect.

Expected toxic effect in AZA-1-spiked sample was about 117 - 141 µg OA equivalents/kg, in experimental condition 1, and 470 - 641 µg OA equivalents/kg in experimental condition 2. Estimated toxicity was slightly higher to expected values in experimental condition 1 and slightly lower in experimental condition 2 (Table 1). Estimated toxicity quantifications have to be done at 100 and 50 mg/ml of exposure under experimental condition 1 and 2, respectively.

The same fractions stored at 20º C were exposed on NG108-15 cells after one week fractioning and only a slight toxic response (around 71 µg OA equivalents/kg) was observed in experimental condition 2 (data not shown). No presence of AZA-1 was detected by LC-MS/MS one month after fractioning.

Analysis of contaminated samples

Comparing sample CBA results performed in the two experimental conditions, differences in fraction toxic profile (Fig. 4) and in general toxicity estimation (Table 1) were obtained for all samples. The classification of samples according to toxicity was: A > B > D > C > E under experimental condition 1, and A > D > E > B > C, under experimental condition 2. Samples F, G and H were out of this order because theirs abnormal increments of viability were not quantified with toxicity values.

Toxic effect evaluation on experimental condition 1

For sample A, fraction toxicities (5-7) were clearly related to OA and DTX-1 content (Cañete et al., 2010). For the rest of samples, toxic content was predominantly YTXs according to LC-MS/MS results (Table 2). As we have seen previously, YTX elute principally on fractions 3 and 4. Only samples B, D and G presented toxicity in these fractions and they were samples with the highest content on YTXs determined by LC-MS/MS (Table 2).

Slight OA concentration in sample C could justify no quantifiable toxicity obtained in fraction number 6.

Pectenotoxin-2sa presence in samples B, C and D determined by LC-MS/MS (Table 2) could not justify some toxic response because there were no fraction toxicity relations between these samples.

Toxicities in fractions 5-8 on sample B and D and 13 on sample B, cannot be related to the presence of known toxins with the analysis performed.

Abnormal increments on viability estimations could be observed in some sample fractions, mainly in fractions 8 and 9 (sample G and H) and slightly in fraction number 4 (sample F).

Toxic effect evaluation on experimental condition 2

Under this experimental condition, in comparison with experimental condition 1 (Fig. 4), increments in toxicity was obtained between fractions 8 and 13: in sample A (fractions 11 and 12), B (fractions 10-12), C (fractions 9 and 11), D (fractions 9, 11 and 13), E (fractions 8-12), F (fractions 10, 11 and 13) and H (fraction 12). No

increment in toxicity was observed in fractions 3 and 4 as was expected in regard to their content of YTXs in all samples except sample A.

For samples A and C, differences between experimental conditions could be related to OA (sample A and C) or DTX-1(only sample C) transformations as was observed on OA and DTX-1-spikings, previously. Differences between experimental conditions in the rest of samples could not be explained by toxin content analysed by LC-MS/MS (Table 2). Decrease of fraction toxicities in experimental condition 2 in regard to experimental condition 1 were observed in fraction 4 on sample B and G and in fraction 13 on sample B at the same concentrations, 50 mg/mL (data not shown).

Apart from sample G, all samples had an increment of global CBA toxicity estimation in experimental condition 2. Toxicities observed in fractions 3 and 4 on sample G seems to be related to their high concentration of homoYTX.

DISCUSSION

In our work, we evaluated a NG108-15 CBA which consists in two experimental conditions coupled to 17-fractioning protocol as a toxicological semiqualitative (multi-fraction toxic profile evaluation) and semiquantitative (general or fraction toxicity value estimation) method on lipophilic marine toxin detection in mussels for screening purposes.

Applying a 17-fractioning protocol for OA, YTX and AZA-1-spiked-samples at the regulatory limit and their toxic equivalence on DTX-1 and PTX-2 and exposing concentrations of 100 and 50 mg mussel tissue equivalents/ml for experimental condition 1 and 2, respectively, was adequate for their toxic effect detection and semiquantification with the exception of YTX analysis under experimental condition 2, where higher dilution would be needed for semiquantification.

In a semiqualitative analysis, the use of an additional exposure at a concentration of 100 mg/ml under experimental condition 2 or at 50 mg/ml under experimental condition 1 could be necessary in order to compare both experimental conditions

results at the same concentration of exposure. In regard to spiked-toxins elution, the method allows to associate 1 or 2 fractions for each of the studied toxins. Our results allowed us to associate toxin and fraction (fr.) number: OA (fr. 5-6), DTX-1 (fr. 7), PTX-2 (fr. 6-7), YTX (fr. 3-4) and probably for AZA-1 (fr. 8-9).

Estimated toxicities in spiked samples presented some differences from expected values for PTX-2 standard. PTX-2 estimations were lower (in both experimental conditions) to expected values. In order to relate toxicity semiquantification of mussel sample fractions and purified toxins, more work focused on different mussel matrix is needed in order to study potential synergistic (negative) effects of mussel matrix when conducting PTX-2 analyses.

After OA, DTX-1, and AZA-1 spiking of mussels, samples presented toxicity in fractions which could not be explained by purified toxin presence. For OA, toxicity increase of not-OA eluting fractions was related to a decrease of toxicity of fractions 5 and 6 (OA eluting fractions). For OA, DTX-1 and AZA-1 these new toxic fractions presented different toxicological characteristics according to mortality in both experimental conditions from those obtained with purified toxins. For AZA-1 fractions analysis before a storage time revealed important differences on toxicity estimations and no AZA-1 was found after one month of storage by LC-MS/MS. All these results suggest that there were toxin molecules transformations in presence of mussel matrix with different toxicological and physicochemical properties. The method allows the detection of some new derivates from purified toxins after spiking in control mussel samples.

Azaspiracid tissue reference material presents several difficulties to stabilise toxin content for a long-term storage and methyl esters of the toxin have been observed to appear in methanol extracts preserved at room temperature or higher for prolonged periods (several months)(Rehmann et al., 2008; Twiner et al., 2008). For OA and DTX-1 slight instability in storage mussel samples has been reported (Lawrence et al., 1996). It is possible that toxin transformations could generate other products with different bioactive properties as our results suggested.

According to our results obtained for AZA-1 spiking, the method allows the detection of sample toxicity but taking into consideration AZA-1 unstability, it will be necessary to further study the effect of fractioning natural contaminated samples or reference material on toxicity estimation according to our method.

Mussel Internal Reference material containing high concentrations of OA and DTX-1, sample A, presented a toxic fraction profile similar to that obtained for OA and DTX-1 from spiked-samples.

In regard to LC-MS/MS results, positive natural samples according to the MBA for lipophilic toxins analyzed in this work could be related to their content on YTXs. Yessotoxin, SPX-1 and PTX-2sa in samples were quantified in too low concentrations to explain NG108-15 CBA response.

The toxicity of a few fractions could be related to OA (and the possible presence of their analogues) or the presence of homoYTX in two samples, but the rest of fraction toxicities on CBA viability estimations could not be related to toxins content analyzed by LC-MS/MS. Abnormal increments on some fractions viability estimations could be explained by the presence of bioactive molecules which has to be considered in sample toxicological analysis. These abnormal increments on cell mitochondrial activity could not be related either to toxins content analyzed by LC-MS/MS.

In regard to NG108-15 CBA, only sample C had a toxic equivalence similar to negative control samples. Toxicities in the rest of samples have to be accurately studied in order to determine toxic molecules and their potential hazard to public health. Semiquantitative and semiqualitative CBA analysis could be a valuable tool in discarding false positive MBA results (determined by analytical methods) as is a reliable problem in YTX containing samples at the Ebre Delta Bays. The method allows also the detection of new bioactive compounds and allows to generate a stock of material for further studies in the characterization of new lipophilic toxins.

In regard to sample G fractions toxic profile, HomoYTX seems to elute on fraction 3 and 4 as it was observed for YTX. However, according to toxicity, we observe a

different toxic effect between YTX and homoYTX on NG108-15 cells: while YTX increments its toxicity in experimental condition 2 in regard to experimental condition 1, homoYTX does not. More purification steps on this purified fraction would be necessary to quantify homoYTX with LC-MS/MS and ensure toxin effect behaviour in experimental conditions 1 and 2. The method allows the toxicological study of known toxin derivatives for which no purified standards are available.

For method application it would be necessary to identify their own limits (semiquantitative limits depending on the semiqualitative approximation) to discriminate positive from negative samples according to dangerous concentrations of lipophilic marine toxins. These limits have to be established in regard to current legislation comparing CBA and official methods results of natural and spiked (with known toxins at the regulatory limits) samples. A statistical study will be needed in order to define the minimum number of samples in the analysis to ensure method validation.

New advances in marine toxin knowledge using CBA as toxicological tools coupled to chromatographic fractioning could contribute to animal testing reduction and provide valuable toxin information in natural samples in the aim of improving risk assessment for consumer health protection.

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TABLE CAPTIONS

Table 1: NG108-15 CBA general toxic effect estimation.

		µg OA equivalents/kg	
		24 h growth	1h growth
Sample	Spiking	24 h exposure	48 h exposure
1	-	nd	nd
2	-	47 -71	349 ± 6
3	-	nd	372 - 568
1	OA	111 ± 3	> 564
2	OA	124 -164	231 - 290
3	OA	83 ± 19	919 - 1277
3	DTX-1	235 ± 2	725 - 810
1	PTX2	734 ± 50	749 ± 60
1	YTX	467 ± 15	> 1128
1	AZA-1	213 - 251	309 ± 7
A	-	> 5871	> 9785
B	-	450 - 692	819 - 979
C	-	47 - 71	186 - 284
D	-	330 - 510	1133 - 1334
E	-	nd	1032 - 1203
F	-	nd*	624 - 974*
G	-	111*	nd*
H	-	nd*	93 - 142*

Toxic effect estimations on uncontaminated samples, with and without toxins spiking, and natural contaminated samples at experimental condition 1 and 2. Toxic estimations were performed at 100 (black), 50 (dark grey) or 14.4 (light grey; sample A) mg mussel tissue equivalents/ml of exposure. Toxicity values were expressed by the average ± SD of two replicates or by a range of toxicity

estimation when sample presented no-quantifiable toxic fractions. Details of CBA toxic estimations are described in section 2.4.3.

*Samples with abnormal increments in some fractions viability estimations.

nd: not detectable. Mortality percentage lower than 10% in all fractions. Equivalence lower than 47 or 93 µg OA equivalents/kg at 100 and 50 mg/mussel tissue equivalents/mL, respectively, in any fraction.

Table 2: LC-MS/MS analysis of lipophilic toxin in contaminated samples.

	Toxin concentration (Average ± SD µg/kg)											
	Positive mode					**Negative mode** Before hydrolysis					**Negative mode** After hydrolysis	
Sample	SPX-1	GYM	PTX-2	PTX2-sa	AZA-1	OA	DTX-1	YTX	OHYTX	homoYTX	OA	DTX-1
A[d]	nd	nd	nd	nd	nd	13820 ± 46	1327 ± 12	nd	nd	nd	15934 ± 49	1711 ± 11
B	nq	nd	nd	12 ± 2	nd	nq	nd	183 ± 28	nd	143 ± 36	18 ± 0.3	nd
C	nq	nd	nd	14 ± 1	nd	25 ± 4	nd	172 ± 33	nd	nd	56 ± 0.3	nd
D	nq	nd	nd	17	nd	nq	nd	141	46	223	16	nd
E	nq	nd	nd	nd	nd	nq	nd	nd	nd	217	nq	nd
F	nq	nd	nd	nd	nd	nq	nd	nd	nd	270	nq	nd
G	nd	nd	nd	nd	nd	nd	nd	nd	nd	607	nd	nd
H	nq	nd	nd	nd	nd	nd	nd	nd	nd	98	nd	nd

Results for LC-MS/MS analysis of lipophilic toxins: desmethyl spirolid C (SPX-1), gymnodimine (GYM), pectenotoxin 2 (PTX-2), pectenotoxin 2 seco acid (PTX-2sa), azaspiracid 1, okadaic acid (OA), dinophysistoxin 1 (DTX-1), yessotoxin (YTX), hydroxy yessotoxin(OHYTX), homoyessotoxin (homoYTX); determined in contaminated mussel samples (A-H). 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

[d]: sample was quantified through a dilution 1:20 from original extract.

nd: not detectable. Signal/noise (S/N) ratio was lower than 3.

nq: not quantifiable. S/N ratio was between 3 and 10.

FIGURE CAPTIONS

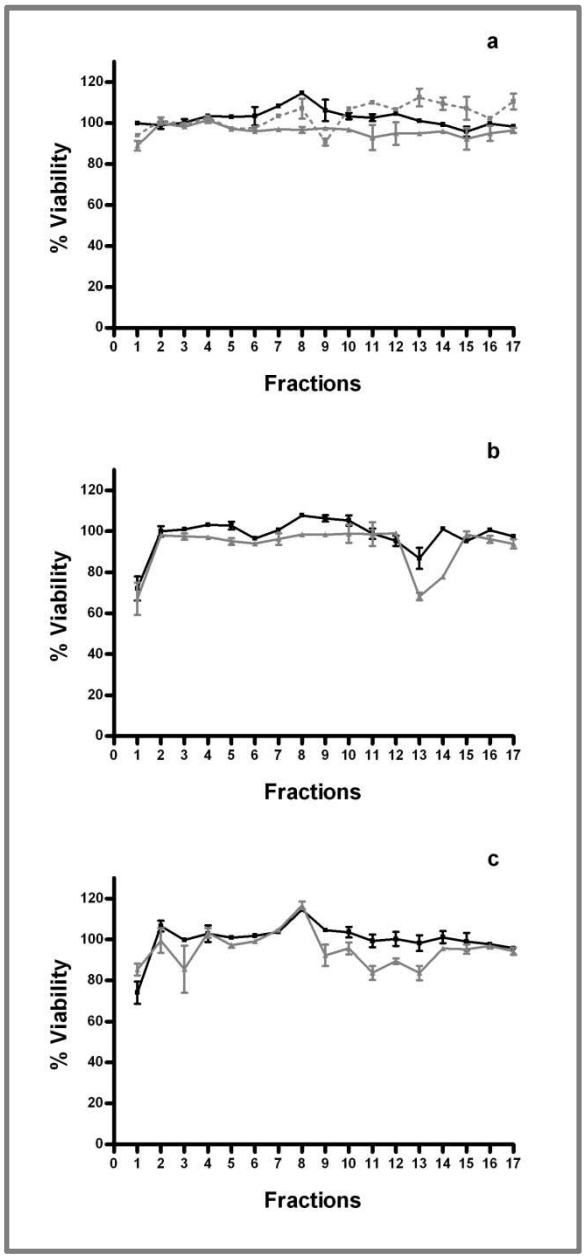
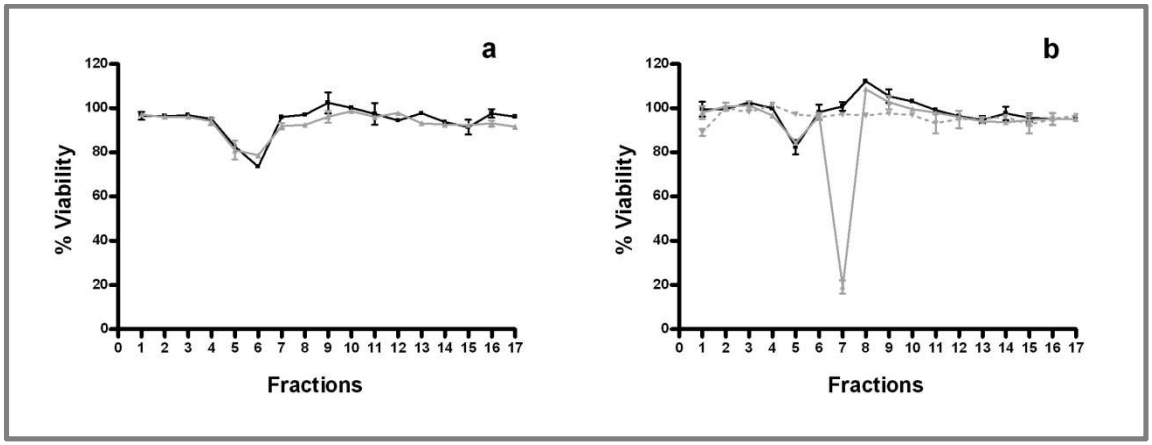


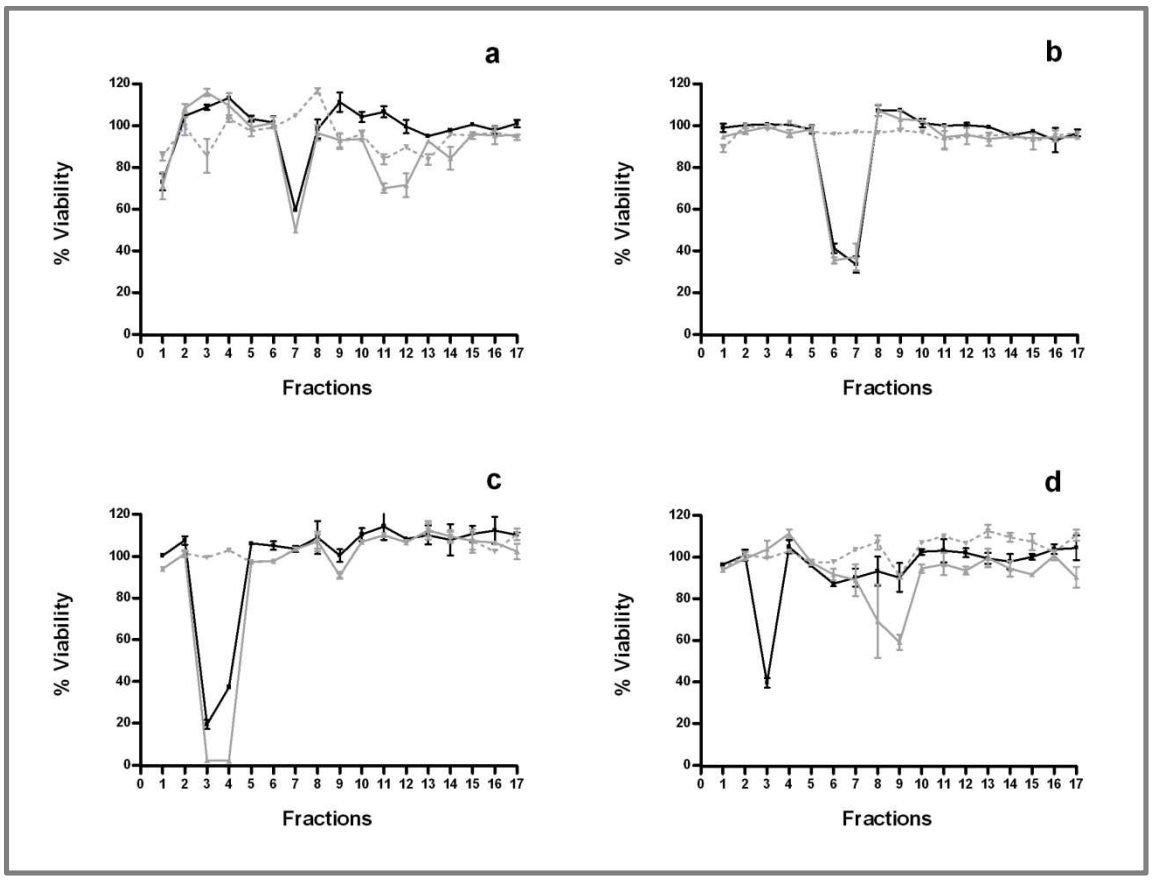
Figure 1: Fraction NG108-15 CBA toxic effect representations of negative control samples for lipophilic toxins. Fraction toxic effect representations of uncontaminated mussel (**a**) sample 1, (**b**) sample 2 and (**c**) sample 3 evaluated at 100 mg mussel tissue equivalents/ml at experimental condition 1 (black) and experimental condition 2 (grey, discontinuous line), or at 50 mg mussel tissue equivalents/ml under experimental condition 2 (grey, continuous line). Fraction toxic effects estimations were performed by the average ± SD of two replicates. Those estimations which exceeded assay detection limit (10% mortality) were labelled as significant toxic fractions *. Toxic effects in fraction 1 can be neglected for lipophilic toxins analysis, since at this fraction only hydrophilic components of the matrix may appear.

Figure 2: Fraction NG108-15 CBA toxic effect representations of samples with and without OA spiking.



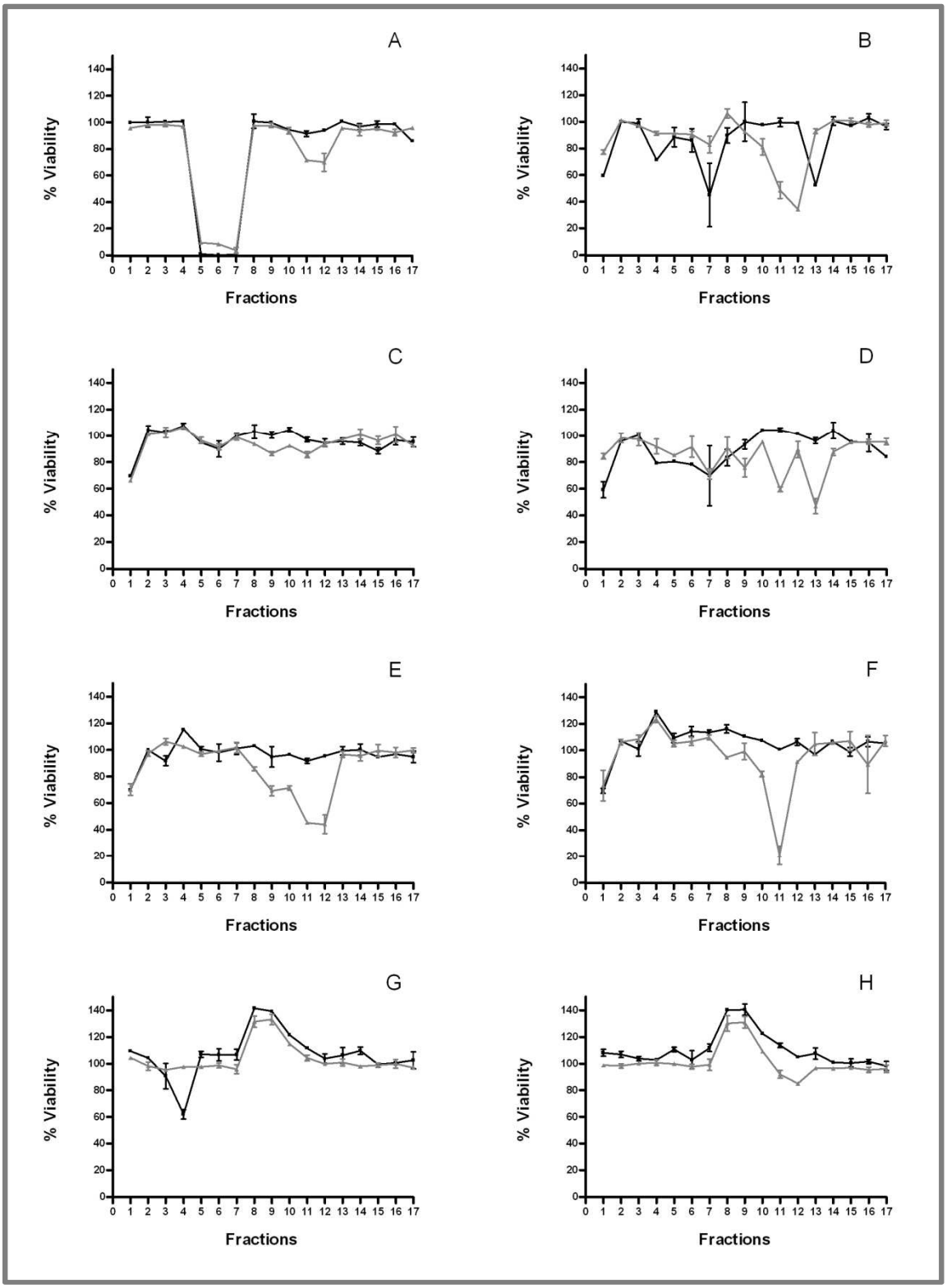
Fraction OA toxic effects evaluated at 50 mg mussel tissue equivalents/ml (**a**) without samples (only solvents) or with (**b**) uncontaminated mussel sample 1.Toxic effects on experimental condition 1 (black) were compared to those obtained for experimental condition 2 (grey). For mussel sample exposures at experimental condition 2, toxicities of OA spiked samples were compared to those obtained for unspiked (discontinous) samples. Fraction toxic effects estimations were performed by the average ± SD of two replicates. Those estimations which exceeded assay detection limit (10% mortality) were labelled as significant toxic fractions *. Toxic effects in fraction 1 can be neglected for lipophilic toxins analysis, since at this fraction only hydrophilic components of the matrix may appear.

Figure 3: Fraction NG108-15 CBA toxic effect representations of samples with and without DTX-1, PTX-2, YTX and AZA-1 spikings.



Fraction toxic effects of (a) DTX-1, (b) PTX-2, (c) YTX and (d) AZA-1spikings of (b, c and d) uncontaminated mussel samples 1and (a) 3 were evaluated at (a and b) 50 or (c and d) 100 mg mussel tissue equivalents/ml. Toxic effects on experimental condition 1 (black) were compared to those obtained for experimental condition 2 (grey). For mussel samples exposures at experimental condition 2 toxicities of spiked samples were compared to those obtained for unspiked samples (discontinuous). Fraction toxic effects estimations were performed by the average ± SD of two replicates. Those estimations which exceeded assay detection limit (10% mortality) were labelled as significant toxic fractions *. Toxic effects in fraction 1 can be neglected for lipophilic toxins analysis, since at this fraction only hydrophilic components of the matrix may appear.

Figure 4: Fraction NG108-15 CBA toxic effect representations of contaminated mussel samples.



Fraction toxic effects of (A-H) eight contaminated samples were evaluated for experimental condition 1 (black) at (A) 14.4 and (B-G) 100 mg mussel tissue equivalents/ml and for experimental condition 2 (grey) at (A) 7.2 and (B-G) 50 mg mussel tissue equivalents/ml. Fraction toxic effects estimations were performed by

the average ± SD of two replicates. Those estimations which exceeded assay detection limit (10% mortality) were labelled as significant toxic fractions *. Toxic effects in fraction 1 can be neglected for lipophilic toxins analysis, since at this fraction only hydrophilic components of the matrix may appear.