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Analytical and Bioanalytical Chemistry

ISSN 1618-2642

Anal Bioanal Chem
DOI 10.1007/s00216-012-6029-0

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ORIGINAL PAPER

Collaborative study for the detection of toxic compounds in shellfish extracts using cell-based assays. Part II: application to shellfish extracts spiked with lipophilic marine toxins

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Received: 20 January 2012 / Revised: 30 March 2012 / Accepted: 6 April 2012
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Abstract Successive unexplained shellfish toxicity events have been observed in Arcachon Bay (Atlantic coast, France) since 2005. The positive mouse bioassay (MBA) revealing atypical toxicity did not match the phytoplankton observations or the liquid chromatography−tandem mass spectrometry (LC−MS/MS) investigations used to detect some known lipophilic toxins in shellfish. The use of the three cell lines (Caco2, HepG2, and Neuro2a) allows detection of azaspiracid-1 (AZA1), okadaic acid (OA), or pectenotoxin-2 (PTX2). In this study, we proposed the cell-based assays (CBA) as complementary tools for collecting toxicity data about atypical positive MBA shellfish extracts and tracking their chromatographic fractionation in order to identify toxic compound(s). The present study was intended to investigate the responses of these cell lines to shellfish extracts, which were either control or spiked with AZA1, OA, or PTX2 used as positive controls. Digestive glands of control shellfish were extracted using the procedure of the standard MBA for lipophilic toxins and then tested for their cytotoxic effects in CBA. The same screening strategy previously used with pure lipophilic toxins was conducted for determining the intra- and inter-laboratory variabilities of the responses. Cytotoxicity was induced by control shellfish extracts whatever the cell line used and regardless of the geographical origin of the extracts. Even though the control shellfish extracts demonstrated some toxic effects on the selected cell lines, the extracts spiked with the selected lipophilic toxins were significantly more toxic than the control ones. This study is a crucial step for supporting that cell-based assays can contribute to the detection of the toxic compound(s) responsible for the atypical toxicity observed in Arcachon Bay, and which could also occur at other coastal areas.

Keywords Cell-based bioassays · Collaborative study · Shellfish extracts · Cytotoxicity · Lipophilic phycotoxins

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Published online: 26 April 2012

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Introduction

Marine microalgae are known to produce a wide range of phycotoxins that may bioaccumulate in filter-feeding shellfish and produce human toxic syndromes as a result of food-borne poisoning. A certain number of phycotoxins are regulated within the European Union in order to protect consumers' health [1]. The health status of seafood intended for human consumption is checked by routine monitoring programs. On the 1st July 2011, a new regulation from the European commission came into force, requiring that the mouse bioassay be replaced by LC-MS/MS methods for monitoring the four families of regulated lipophilic phycotoxins [2]. These methods are the most sensitive and specific ones for monitoring the targeted phycotoxins, but they are not suitable for detecting atypical toxic compounds or providing information about any potential toxic effect of contaminated shellfish. In this context, cell-based assays (CBA) can be useful to investigate any shellfish toxicity event that is detected by animal bioassays performed in the context of a warning system. Indeed, CBA have been widely developed for research purposes in order to study several groups of marine toxins, by using various cell types and end-points (for a review, see [3, 4]). These studies concern the cellular effects of microalgal toxins, including their cytotoxicity, and the membrane alterations and cytoskeletal disorganization that they induce [5–7]. The cell types included epithelial cells [8], intestinal cells [9, 10], and neuroblastoma cells [11–18]. Most of these studies were designed to investigate either the effects of a single toxin on several different cell types (e.g., [6]), or the toxicity of various groups of toxins on a single cell type [9], but a few studies have investigated the cytotoxic effects of uncontaminated shellfish extracts [19]. Cell mortality resulting from the impairment of various essential cellular functions is generally considered to provide a relevant approach to detect the largest panel of toxic compounds including phycotoxins [4]. Thus, this parameter was employed to distinguish between mouse bioassay (MBA)-positive and MBA-negative shellfish samples for OA using kidney BGM cells [20] and, for AZAs using HepG2 and bladder ECV-304 cells [21].

In France, successive atypical toxic events have been reported in Arcachon Bay (Atlantic coast) since 2005, with positive MBA results revealing a toxicity that did not match the phytoplankton observations or the results of LC-MS/MS investigations for some well-known lipophilic toxins. A national research program was set up in 2008 to identify the compound(s) responsible for the Arcachon Bay atypical toxicity. As part of this program, CBA were proposed as a complementary tool for collecting toxicity data on atypical MBA-positive shellfish extracts. The first step in the study was to propose a screening strategy for characterizing and validating the responses of three cell lines representative of the main target organs of phycotoxins (intestinal Caco2, hepatic HepG2, and neuronal Neuro2a) when exposed to three lipophilic toxins (azaspiracid-1 (AZA1), okadaic acid (OA), and pectenotoxin-2 (PTX2)). Cyclic imines (such as spirolides) were not included in the study because of their low cytotoxic effect on different cell lines [22]. In part I, we demonstrated that the sensitivity and variability (repeatability and reproducibility) of the CBA responses with known lipophilic toxins on the three selected cell lines were similar within and between five participating laboratories.

The present work looked at the toxic effects of both control and spiked shellfish extracts on the same cell lines. The screening strategy proposed in Part I, including standard operating procedures (SOP) and rigorous raw data validation, was used to characterize the cytotoxic responses of shellfish extracts from two species (the mussel *Mytilus edulis* and the oyster *Crassostrea gigas*). The cytotoxic effects of control extracts prepared from shellfish of various geographical origins were investigated and were then compared with the same shellfish extracts spiked with each of the selected lipophilic toxins (AZA1, OA, and PTX2). The variabilities of these assays from three laboratories were determined and compared with those previously found for toxin standards. Investigating the responses of selected cell lines to control and spiked shellfish extracts is a crucial step in establishing the use of CBA to characterize the toxicity of unknown toxic compound(s) involved in atypical toxic events such as those observed in Arcachon Bay. Our original collaborative study based on the use of SOP is the first pre-validation study intended to characterize the cytotoxic effects of control and spiked shellfish extracts (mussels and oysters, from diverse geographical origins) on three different cell lines. To pick up the toxicity arising from a wide diversity of contaminants (natural or anthropogenic) that may be present in these atypical shellfish extracts, our strategy cannot rely on specific target assays but rather includes a non-specific cytotoxicity assay.

Experimental section

Study design

The same batches of cell lines, culture media, sera, certified reference calibration solutions of marine toxins, and shellfish extracts were used in all the laboratories. Cell maintenance and cytotoxicity assays were conducted according to the previously established SOP (part I).

Each assay was passed through a decision tree devised for assay validation and data modeling (part I). All the data (experimental conditions, raw data, and data analysis) obtained from each assay performed were added to a common Excel database.

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The shellfish matrix effects were first investigated by testing control shellfish extracts (mussel and oyster; i.e., with a negative mouse bioassay) under our experimental conditions. The cytotoxic effects of the same shellfish extracts spiked with one of the lipophilic toxins (AZA1, OA, or PTX2) were then investigated. Data analysis was based on the shape of the dose–response curves and on the relative IC_{50}, which is defined as the concentration that causes a response midway between the minimum (bottom) and the maximum (top) observed viability [23].

The repeatability of the cell-based assays was first assessed for the three cell lines (HepG2, Caco2, and Neuro2a) in a single laboratory (Lab.1). The reproducibility was then determined for a single cell line (HepG2) in three laboratories (Lab.1 to 3).

Cell line maintenance

The three cell lines were routinely grown in 75-cm^2 flasks at 37 °C in an atmosphere enriched with 5 % CO_2 as described in part I.

Origin and preparation of the control shellfish extracts

As the geographic origin can influence the chemical composition of shellfish [24], mussels (*M. edulis*) and oysters (*C. gigas*) were obtained from several different geographic areas along the French coast (Table 1).

The preparation of extracts was carried out in accordance with the official protocol for the extraction of lipophilic toxins [25] with a few modifications. For each sample, a 20-g test-portion of a homogenate of digestive glands (DG) was extracted three times with 50 mL of acetone. The supernatants were pooled and dried by rotary evaporation. The residual aqueous phase was partitioned using 50 mL of dichloromethane (DCM). This procedure was repeated twice. The DCM phases were pooled, and rinsed twice with 15 mL water to remove any hydrophilic compounds. The DCM extract was then evaporated to dryness. For CBA, the residue was resuspended in 4 mL methanol to produce a shellfish extract with a matrix concentration of 5 g DG/mL.

Table 1 Geographic origin of control shellfish extracts (with a negative mouse bioassay) tested in the study

	Reference	Geographic origin
Oyster	Oy-1	Charente-Maritime, France
	Oy-2	Brittany, France
	Oy-3	Brittany, France
	Oy-4	Charente-Maritime, France
Mussel	Mu-1	Lower Normandy, France
	Mu-2	Brittany, France

The extracts were filtered through 0.22-μm nylon filters, and then stored at −80 °C in glass vials before delivery to the different labs where they were further aliquoted and stored at -20 °C prior to performing the CBA.

The test solutions were prepared for each experiment by serial twofold dilutions of shellfish extracts in serum-free cell culture medium and tested on cell lines at concentrations ranging from 0.02 to 50 mg DG/mL.

Preparation of spiked shellfish extracts

The control methanolic shellfish extracts (5 g DG/mL) were spiked with certified toxins (AZA1, OA, or PTX2) purchased from the National Research Council (Halifax, NS, Canada). Test solutions were prepared just before use. To this end, 7 μL of filtered methanolic shellfish extract was gently mixed with 35 μL of undiluted toxin solution (1.47 μM for AZA1, 17.7 μM for OA and 10 μM for PTX2). The volume was completed to 700 μL with serum free-cell culture medium. A range of 12 final concentrations (equivalent to between 0.02 and 50 mg DG/mL) was prepared by serial twofold dilutions in serum-free cell culture medium from 0.04 to 73.5 nM for AZA1, from 0.4 to 885 nM for OA, and from 0.2 to 500 nM for PTX2. Thus, if we assume that the digestive glands correspond to 20 % of the total flesh of shellfish, the highest toxin concentrations in the spiked extracts corresponded to 250 μg AZA1, 2,850 μg OA, and 1,720 μg PTX2, respectively, per kilogram of whole shellfish flesh.

Procedure for the cytotoxicity assays

The cells were seeded in complete medium at 30,000 cells/well (for Caco2) and 20,000 cells/well (for HepG2 and Neuro2a) into 96-well plates to form a non-confluent monolayer. Peripheral wells were filled with culture medium; but without adding any cells. After 24 h, the medium was removed, and the cells were exposed in triplicate to 12 concentrations of control or spiked shellfish extracts (100 μL/well). The template was designed as follows: one column of 6 wells was used as the control (100 μL medium), one column on the left (VeC_1) and one on the right (VeC_2) were used as the vehicle control (VeC/methanol at a maximum final concentration of 6 % (*v*/*v*), which did not induce more than 20 % of cytotoxicity (data not shown)) and one column was used as an internal quality control (OA at 50 nM).

HepG2 and Caco2 cells were exposed for 48 h while Neuro2a cells were only treated for 24 h, except for shellfish extracts spiked with AZA1, which were incubated for 48 h. At the end of the exposure period, the medium was removed before adding 100 μL of medium containing 0.5 mg mL^{-1} of 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT;

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Sigma-Aldrich, France) to each well. The MTT assay was used to measure the mitochondrial reductase activity as a surrogate for the cell number [26].

The plates were incubated for 2 h at 37 °C. Finally, the MTT was discarded, and isopropanol acidified with 0.1 N HCl was added to each well to dissolve the formazan. The absorbance was read at 570 nm, and was expressed as the percentage of the VeC mean absorbance (100 % viability).

To perform the analyses, the mean percentages of viability (±SD) were plotted on a graph using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Data analysis and assay validation

For each assay, data (experimental conditions, raw data, analytical results, and acceptance criteria) were collected in a shared Excel database. Each assay was evaluated by means of a decision tree (part I) following quality and modeling criteria (part I), which were set up after an iterative process based on guidance documents [23, 27].

Statistical analyses

At least three independent experiments were validated using the decision tree for each of the experimental conditions tested. Data were expressed as mean±standard deviation. The differences between groups were tested with Kruskal–Wallis test with Dunn's post-test. A p value of <0.05 was considered as significant. Statistical analyses were performed with GraphPad Prism version 5.

Results and discussion

The responses of the three cell lines (Caco2, HepG2, and Neuro2a) exposed to control or spiked shellfish extracts were first studied in one laboratory (Lab.1). The collaborative study involving three laboratories (Lab.1 to 3) was set up in order to study the response variability of one selected cell line (HepG2) exposed to the same shellfish extracts (control or spiked).

Toxicity of control shellfish extracts

Cytotoxicity assays in Lab.1

A total of 80 assays were performed in Lab.1 with six shellfish extracts (Table 1) showing no toxicity according to the MBA for lipophilic toxins and were subsequently considered as being control extracts. After assessment using the decision tree, 76 assays were validated according to the quality and modeling criteria (95 %). The percentages of acceptance were roughly equal from one cell line to another. The Neuro2a cell line had the lowest validation percentage (90 %) while 96 % and 100 % of the assays were validated for Caco2 and HepG2 cells, respectively (data not shown).

Eighty-two percent of the data could be described by a sigmoid dose–response curve (Fig. 1), and only 18 % showed an incomplete sigmoid dose–response curve without a bottom plateau. Typically, cytotoxicity was induced by shellfish extracts at concentrations of more than 5 mg DG/mL for Caco2 and HepG2 cells (exposed for 48 h), and 1 mg DG/mL for Neuro2a cells (exposed for 24 h). For all three cell lines, the highest concentration of the extract tested (50 mg DG/mL) induced up to 90 % cytotoxicity.

For the four oyster extracts, the IC_{50} values were similar for the Caco2 and HepG2 assays, ranging from 8.6 to 12.2 mg DG/mL for Caco2 cells, and from 7.8 to 11.3 mg

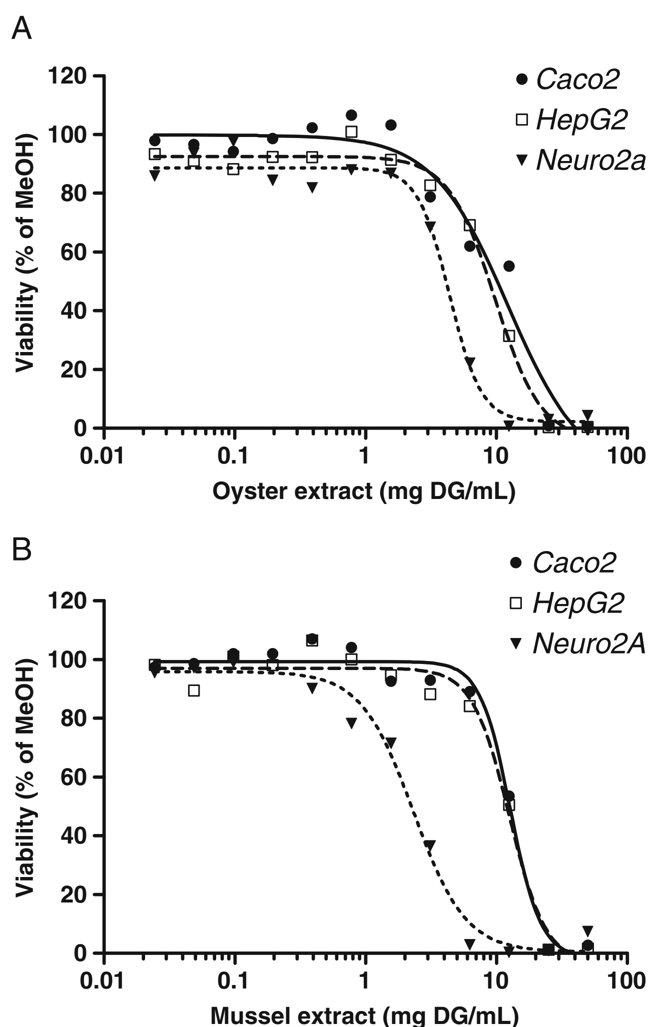

Fig. 1 Representative dose–response curves obtained for the control extracts: (**A**) Oy-1 oyster extract and (**B**) Mu-1 mussel extract on Caco2 (*solid lines*), HepG2 (*broken lines*), and Neuro2a (*dotted lines*) cells. Caco2 and HepG2 cells were exposed for 48 h while Neuro2a cells were only exposed for 24 h. *DG* digestive glands

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DG/mL for HepG2 cells (Table 2). The IC_{50} values for the Neuro2a cell line were lower, ranging from 3.3 to 4.6 mg DG/mL. A significant difference ($p<0.05$) was only observed with the Oy-1 extract on the Neuro2a cells, versus the Caco2 and HepG2 cell lines (Fig. 2).

With regard to the mussel extracts, the IC_{50} values ranged from 9.1 to 13.6 mg DG/mL on Caco2 cells, and from 11.4 to 16.7 mg DG/mL on HepG2 cells (Table 2). On Neuro2a cells, the IC_{50} values were lower (between 1.9 and 3.5 mg DG/mL; Table 2). There was no significant difference between the Caco2 and HepG2 responses, whatever the mussel extract. The Neuro2a response was statistically different ($p<0.05$) from those of the other two cell lines for the mussel extract Mu-1 only (Fig. 2).

Regarding the responses of the three cell lines, Neuro2a cells (exposed for 24 h) were significantly more susceptible to the matrix effect (as shown by lower IC_{50} values) than either HepG2 and Caco2 cells (both exposed for 48 h), irrespective of the shellfish species.

For the detection of known lipophilic toxins such as OA, using CBA-based methods, several protocols have been described for shellfish extraction using solvents such as saline buffer [28], methanol–water [29], and acetone [20, 21, 30]. In most cases, studies focused on distinguishing between MBA-positive and MBA-negative samples and matrix effects were not taken into consideration (e.g., [20, 21]). In this study, the same extraction protocol for shellfish digestive glands (an acetone extraction followed by a liquid/liquid partitioning with DCM/water) was performed for the CBA as well as for MBA and LC-MS/MS analysis, to ensure that the unknown lipophilic toxic compounds were recovered.

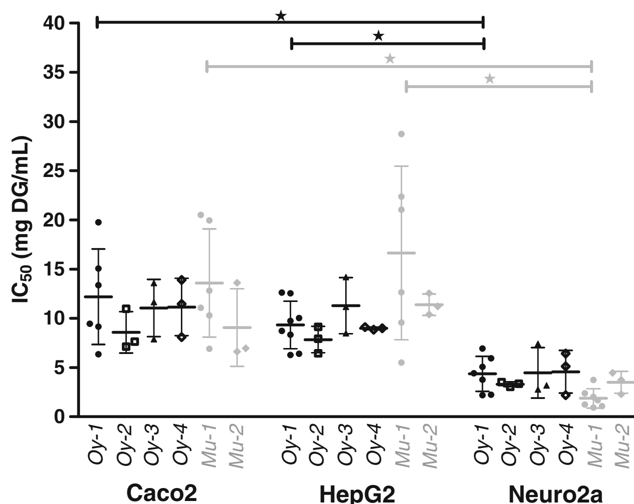

Fig. 2 Distribution of IC50 values (expressed in milligrams DG per milliliter) in Lab.1 of the four control oyster extracts (Oy-1 to Oy-4, in *black*) and the two mussel extracts (Mu-1 and Mu-2, in *grey*) tested on the three cell lines: Caco2, HepG2, and Neuro2a. Only data validated according to the decision tree are shown. *Short horizontal lines* represent the mean value, and *vertical lines* the standard deviation. For each type of extract, any differences between the cell lines were considered statistically significant when $p<0.05$ (*stars*). *DG* digestive glands

Effects of geographic origin and shellfish species

As the chemical composition of shellfish may vary depending on the species (mussel or oyster) or their geographic origin [24], several extracts of various geographic origins were selected (Table 1) and their IC_{50} values determined for each cell line (Table 2). When considering the response of each cell line individually, no statistically significant difference was observed whatever the origin of shellfish (Fig. 2) nor was any statistically significant difference observed between the oyster and mussel extracts, whatever the cell line (Fig. 2). Therefore, under our experimental conditions, neither the species (mussel or oyster) nor the geographic origin of the shellfish influenced the cytotoxic effects observed on the three cell lines.

The control extracts ($n=6$) from two species of shellfish (*M. edulis* and *C. gigas*) collected from different geographical areas exhibited cytotoxic effects on all three cell lines.

Table 2 Relative mean IC_{50} values (expressed in milligrams DG per milliliter) of control shellfish extracts tested on the three cell lines Caco2, HepG2, and Neuro2a with the MTT assay

	Caco2		HepG2		Neuro2a	
	Mean	CV%	Mean	CV%	Mean	CV%
Oy-1	12.2 (n=6)	40	9.3 (n=8)	26	4.4 (n=7)	41
Oy-2	8.6 (n=3)	24	7.8 (n=3)	17	3.3 (n=3)	7
Oy-3	11.0 (n=3)	26	11.3 (n=3)	25	4.5 (n=3)	58
Oy-4	11.2 (n=3)	26	9.4 (n=3)	8	4.6 (n=3)	47
Mu-1	13.6 (n=6)	40	16.7 (n=6)	53	1.9 (n=7)	52
Mu-2	9.1 (n=3)	44	11.4 (n=3)	10	3.5 (n=3)	31

Results are expressed as the mean of validated assays performed in Lab.1 (number of validated assays (n)) with the variability expressed as a coefficient of variation (CV%)

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Table 3 Comparison of the relative IC_{50} values (expressed in milligrams DG per milliliter) determined in each laboratory for the control shellfish extracts Oy-1 and Mu-1 tested on HepG2 cells

	Lab.1		Lab.2		Lab.3		Mean intra-laboratory CV%	Inter-laboratory mean	
	Mean	CV%	Mean	CV%	Mean	CV%		Mean	CV%
Oy-1	9.3 (*n*=8)	26	20.1 (*n*=5)	39	12.1 (*n*=5)	54	40	13.1	53
Mu-1	16.7 (*n*=6)	53	22.3 (*n*=4)	25	9.4 (*n*=5)	59	46	15.8	53

Results are expressed as the mean of validated assays performed in each laboratory (number of validated assays (*n*)) with the variability expressed as a coefficient of variation (CV%)

The observed cytotoxicity may not only have resulted from plasma membrane alteration due to the abnormal lipid content of the medium, but also from the impairment of housekeeping, regulatory and differentiating functions, as shown in breast cancer MCF-7 cells exposed to control mussel extracts [19]. The cytotoxic effects were observed for matrix concentrations in the culture media of more than 1 mg DG/mL. Similar results had previously been reported for MCF-7 cells [19] and also for neuroblastoma NG108-15 cells [30]. However, in contrast to the latter study, we did not observe any significant difference between the cytotoxic effects exerted by the six different extracts, irrespective of the cell line. Cañete et al. [30] used whole flesh to prepare shellfish methanolic extracts, whereas we performed the extraction solely on the digestive glands, and so we may have obtained more uniform extracts than Cañete et al.

Intra-laboratory variability

The coefficient of variation determined in Lab.1 ranged from 7 to 58 % for oyster extracts, and from 10 to 53 % for mussel extracts (Table 2). However, it should be noticed that when low variability was observed (CV≤10 %), the three validated assays were only performed during two different runs of experiments, thus minimizing the inter-day variability. If these lowest values were set aside, the mean intra-laboratory variability for the Caco2 and HepG2 cell lines were similar (33 and 30 %, respectively), whereas it was higher for the Neuro2a cell line (46 %), irrespective of the type of shellfish.

Inter-laboratory variability

The HepG2 cell line, previously selected for the collaborative study of standard lipophilic toxins (part I), was also selected in this study for comparing the variability of responses to two control shellfish extracts (Oy-1 and Mu-1) between the three laboratories (Lab.1 to 3).

A total of 41 assays were performed in the three labs with these two extracts. According to the decision tree, 85 % of the assays with the oyster extract and 71 % of the assays with the mussel extract were validated (data not shown).

The IC_{50} values ranged from 9.3 to 20.1 mg DG/mL for the oyster extract, and from 9.4 to 22.3 mg DG/mL for the mussel extract (Table 3). No statistically significant difference was observed between the three labs, except for the oyster extract between Lab.1 and 2 (Fig. 3). The mean inter-laboratory relative IC_{50} values were 13.1 and 15.8 mg

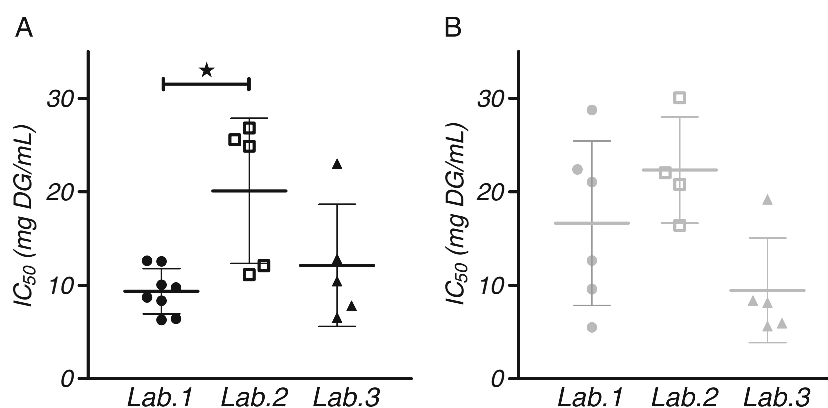

Fig. 3 Distribution of IC_{50} (expressed in mg DG/mL) obtained in the three labs on HepG2 cells for (**A**) the control oyster extract, Oy-1 and (**B**) the control mussel extract, Mu-1. The data shown had been validated according to the decision tree. The IC_{50} values are represented as the mean (*short horizontal lines*) and standard deviation (*vertical lines*). Differences were considered significant when $p<0.05$ (*stars*) (Kruskal–Wallis test with Dunn's multiple comparison post-test). *DG* digestive glands

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DG/mL, with a CV equal to 53 %, for both the oyster and the mussel extracts (Table 3). Within each laboratory, no statistically significant difference was observed between the two types of extracts ($p<0.05$).

Toxicity of shellfish extracts spiked with lipophilic toxins

In part I, we characterized the sensitivity and reproducibility of selected CBA towards the three pure toxins. We showed that each toxin could be individually distinguished by combining the results (IC_{50} values and shape of the dose–response curves) obtained for the three cell lines. However, it is more challenging to use CBA for complex biological matrices, because of the own matrix cytotoxicity. The challenge was to build up a method (based on CBA) that could distinguish between unknown toxic compound (anthropogenic or natural) and other shellfish compounds. We therefore compare the cytotoxic responses of three cell lines exposed to control shellfish extracts and shellfish extracts spiked with three selected lipophilic toxins

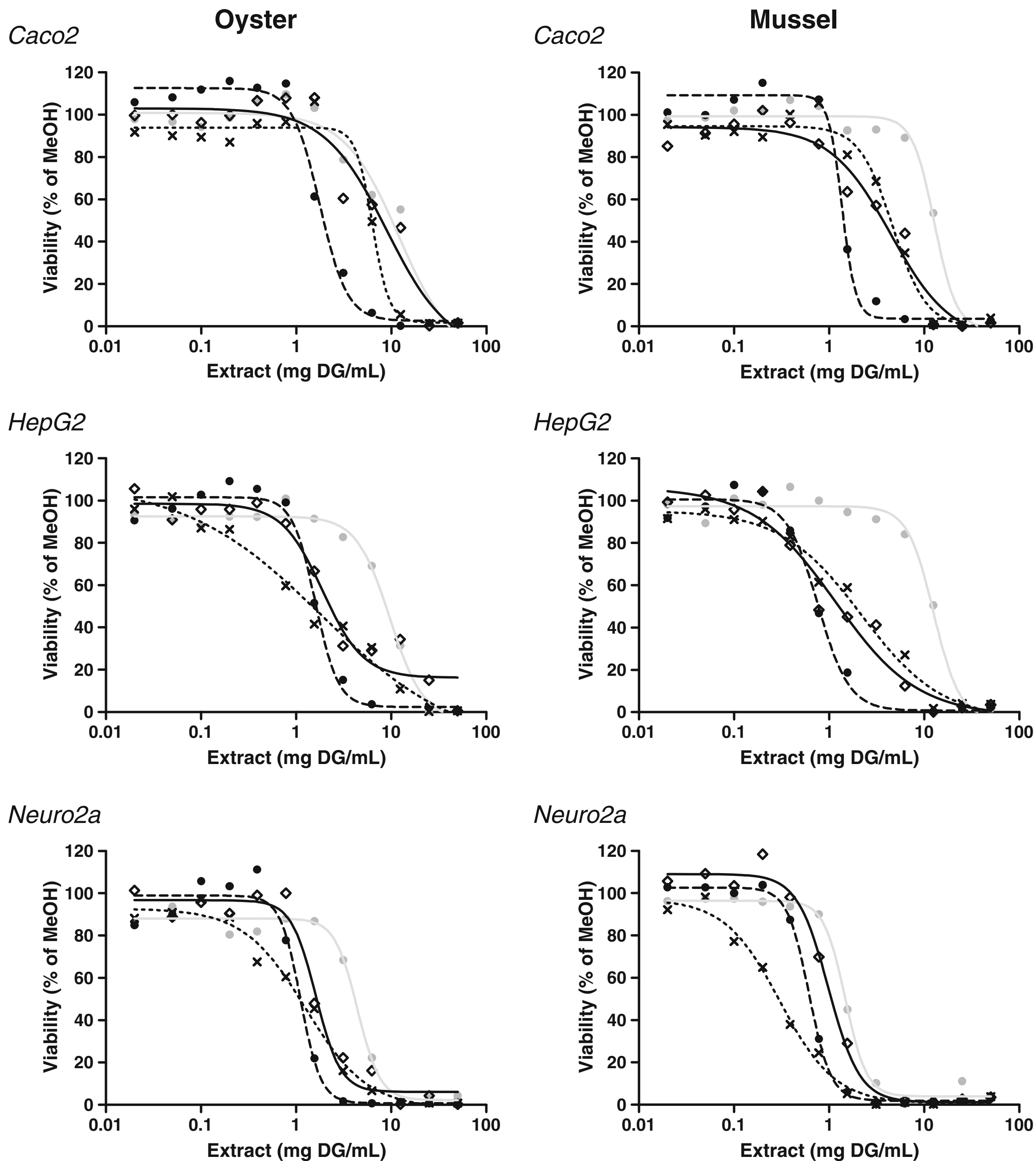

Fig. 4 Representative dose–response curves (in milligrams DG per milliliter extract) obtained using Caco2, HepG2, and Neuro2a cells for a control extract (*filled grey circles*), and for the same extract spiked with pure lipophilic toxins AZA1 (*empty diamonds*), OA (*filled black circles*), PTX2 (*error marks*). *DG* digestive glands

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(AZA1, OA, and PTX2) according to the atypical toxicity observed with lipophilic shellfish extracts from Arcachon bay. The range of toxin concentrations in the spiked extracts included the regulatory level (160 µg toxin/kg of whole shellfish) if we take into consideration the fact that the digestive glands correspond to 20 % of shellfish flesh.

The responses of the three cell lines exposed to shellfish extracts (Oy-1 and Mu-1) spiked with the lipophilic toxins (AZA1, OA, and PTX2) were compared with the control shellfish extracts. The representative dose–response curves are illustrated in Fig. 4.

Cytotoxicity of spiked shellfish extracts

A total of 73 assays were assessed in Lab.1 to determine the cytotoxicity of the spiked shellfish extracts. According to the criteria of the decision tree, 79 % of these assays were validated. The Neuro2a cell line showed the lowest percentage of validation (74 %), compared with HepG2 (81 %) and Caco2 cells (85 %). The assays performed with spiked mussel extracts exhibited a slightly higher percentage of validation (84 %) than those performed with spiked oyster extracts, with only 76 % of validated assays (data not shown).

Overall, under our experimental conditions, shellfish extracts spiked with any of the three lipophilic toxins exhibited a greater cytotoxic effect than the control extracts, whichever cell line was used.

On Caco2 cells, extracts spiked with OA exhibited the highest toxic effect, with a mean IC_{50} close to 1.5 mg DG/mL (equivalent to 27 nM OA) (Table 4), resulting in a statistically significant difference between the spiked and the control extracts (Fig. 5).

A high cytotoxic effect on HepG2 cells was observed for extracts spiked with any of the toxins, with IC_{50} lower than 3 mg DG/mL (Table 4). Significant statistical differences were observed for extracts spiked with AZA1 or OA, when compared with the control extracts (Fig. 5).

Overall, the differences between spiked extracts and control ones were less pronounced for Neuro2a cells than for Caco2 or HepG2 cells, partly because the control extracts were more toxic towards Neuro2a cells than towards the two other cell lines. Nevertheless, statistically significant differences were found for Oy-1 extracts spiked either with OA or PTX2, when compared with control Oy-1. A significant difference was also observed between Mu-1 spiked with PTX2 and the control Mu-1 (Fig. 5).

For each of the three tested toxins, no statistically significant difference was observed between the nature of shellfish spiked extracts (oyster or mussel), whatever the cell line.

Table 4 Relative IC_{50} values (in milligrams DG per milliliter and in nanomolars) expressed as the mean and the coefficient of variation (CV%) for shellfish extracts spiked with the pure lipophilic toxins AZA1, OA, or PTX2 and determined in Lab.1 on the three cell lines Caco2, HepG2, and Neuro2a by means of an MTT assay

	Caco2			HepG2			Neuro2a		
	Mean IC_{50} (mg DG/mL)	Mean IC_{50} (nM)	CV%	Mean IC_{50} (mg DG/mL)	Mean IC_{50} (nM)	CV%	Mean IC_{50} (mg DG/mL)	Mean IC_{50} (nM)	CV%
Oy-1	12.2	na	40	9.3	na	26	4.4	na	41
Mu-1	13.6	na	40	16.7	na	53	1.9	na	52
AZA1	na	NE (73)	–	na	4.3	71	na	6.8	62
Oy-1+ AZA1	7.8	11.5	55	1.3	1.9	77	3.7	5.5	62
Mu-1+ AZA1	4.7	7	26	0.8	1.2	14	1.1	1.6	22
OA	na	49	47	na	30	49	na	41	14
Oy-1+ OA	1.6	27	20	1.3	22	15	1.0	19	14
Mu-1+ OA	1.5	27	21	0.8	15	13	0.6	10	8
PTX2	na	202	50	na	67	80	na	36	42
Oy-1+ PTX2	7.3	89	53	3.0	30	34	1.0	10	53
Mu-1+ PTX2	5.9	59	50	2.2	22	58	0.3	3.3	26

Three to five replicates were performed for each condition. Relative IC_{50} values for toxin standards are detailed in Part I. The relative IC_{50} values obtained for the control extracts Oy-1 and Mu-1 are shown in Table 2

na not applicable, *NE* no effect (the highest concentration tested is shown in parentheses), *DG* digestive glands

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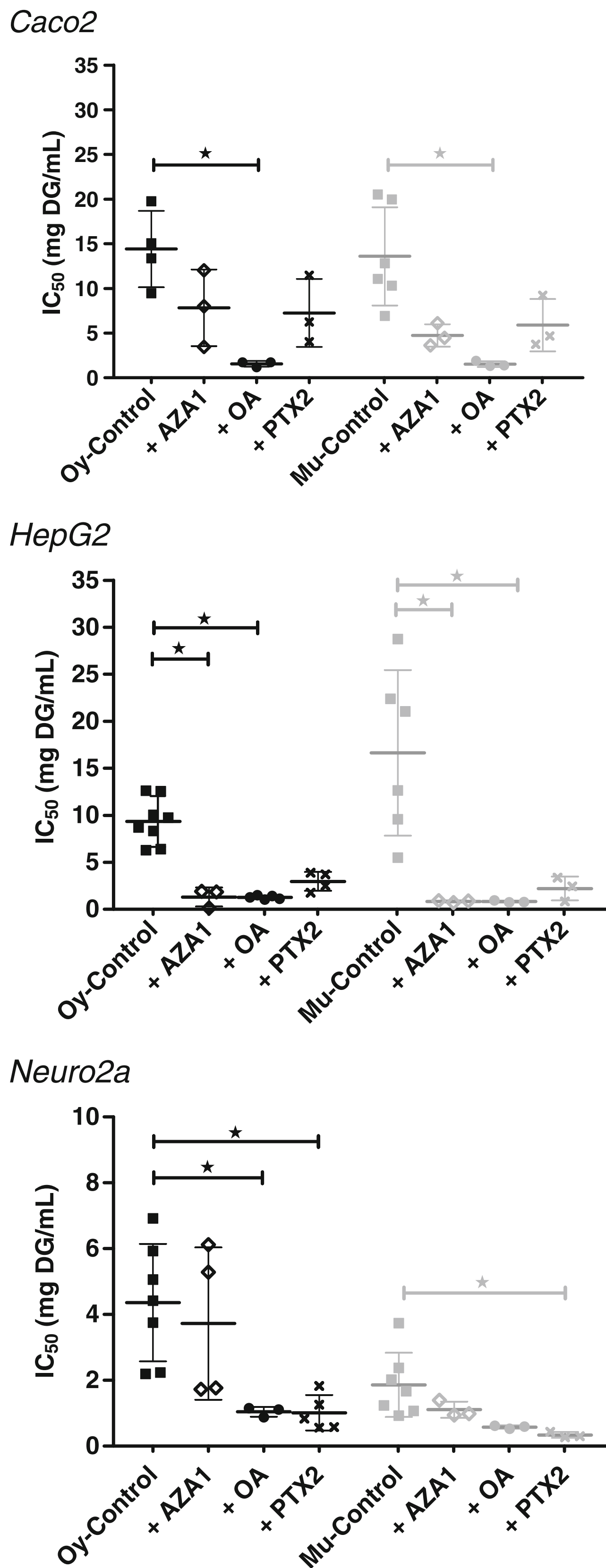

Fig. 5 Distribution of IC_{50} values (expressed in milligrams DG per milliliter) in Lab.1 for the control and spiked oyster extract Oy-1 (in *black*) and mussel extract Mu-1 (in *grey*) tested on the three cell lines Caco2, HepG2, and Neuro2a cells using the MTT assay. The data shown have been validated according to the decision tree. The IC_{50} values are represented as the mean (*short horizontal lines*), with the standard deviation (*vertical lines*). Differences were tested with Kruskal–Wallis test with Dunn's multiple-comparison post-test, and considered significant when $p<0.05$ (*stars*). *DG* digestive glands

Statistically significant differences were observed between the IC_{50} values of the control extracts and of the OA-spiked extracts for all three cell lines. Okadaic acid has previously been shown to inhibit both type I and type 2A serine–threonine phosphatases, which are ubiquitous intracellular targets [31]. This may explain the similarity of responses observed with OA as well as with OA-spiked shellfish extracts in the three cell lines.

As reported in Part I, the HepG2 cell line was very sensitive to AZA1. Here, the cytotoxic effects caused by AZA1-spiked shellfish extracts were significantly statistically greater than those caused by the control extracts only on the HepG2 cell line. Under our conditions at least, therefore, this cell line appears to be the most promising for distinguishing between control and AZA1-spiked extracts.

While the Neuro2a cell line was the most sensitive to control matrix effect, the IC_{50} values for PTX2-spiked shellfish extracts were statistically significantly different from the values determined for control extracts on this cell line only.

Thus, depending on the toxin added, the three cell lines appeared to have different sensitivities with regard to their ability to distinguish between control and spiked shellfish extracts. The use of cell lines representative of several cell types enhances the ability to detect a large panel of cytotoxic compounds. Therefore, the potential of cell-based assays to investigate atypical toxicity should be further characterized by assessing other toxins using a wider range of cell lines.

One of the main drawbacks of such CBA-MTT assay is that they are not specific enough to identify one definite group of toxins. However, this first step is necessary to follow-up the toxicity of a large panel of unknown-toxic compounds. In a second step, after identification of the compound(s) involved in the cytotoxicity, their mechanism of toxicity could be further investigated using a panel of functional related biomarkers (oxidative stress, actin depolymerization, etc.). Functional CBA have also been developed in the past decades. In most cases, they rely on the binding of toxins to their receptor (e.g., saxitoxins STXs, ciguatoxins CTXs, brevetoxins PbTxs acting on voltage-gated sodium channels, and palytoxins PlTxs acting on the Na^{+},K^{+}-ATPase pump), or on detecting the accumulation in the cytosol of an E-cadherin immunoreactive fragment after exposing MCF-7 cells to yessotoxins, and have made it

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possible to semi-quantify specific toxins present in shellfish or finfish extracts [15, 32–35]. In the case of neurotoxins (STXs, CTXs, PbTxs, and PlTXs), the use of agonists and/or antagonists has made it possible to detect the binding of specific toxins to their receptor. In functional assays of this type, even if a matrix effect occurs when cells are exposed to a complex extract, its effect on cell viability can easily be distinguished from the effects caused by the binding of toxins to their receptor, thus leading to the specific detection of the targeted toxins. However, in the case of atypical toxicity events, the mechanism of action of the putative compound is unknown, which makes it functional assays inappropriate to be run in the primary steps of the identification process.

Intra-laboratory variability

In Lab.1, we observed variability ranging from 8 (Neuro2a: Mu-1+OA) to 77 % (HepG2: Oy-1+AZA1) (Table 4). The lowest CV value (8 %) corresponded to the three validated assays for Mu-1+OA that were performed on Neuro2a cells on just two different runs, which contributed to minimizing the inter-day variability. The highest CV value (77 %) appeared to be clearly linked to an "outlier" value amongst the three IC_{50} values determined on HepG2 cells for Oy-1+AZA1 (2.8; 2.7 and 0.2 mg DG/mL).

Thus, regarding the intra-laboratory variability determined for Lab.1 on the three cell lines using control and spiked extracts, the coefficients of variation are widely scattered (from 8 to 77 %). No evidence that high intra-laboratory variability was attributable to a particular shellfish species or cell line can be identified from our data sets.

The global intra-Lab.1 variability for both control and spiked shellfish extracts is close to 35 % (data not shown), which is lower than the value reported for the same laboratory for standard toxins (CV=52 %) (part I). An improvement in technical experience between these two sets of experiments could partly explain this.

In part I and the present study, our goal was to compare the responses obtained for the three selected cell lines with standard toxins, and with shellfish extracts. The SOP and decision tree therefore remained unchanged.

Inter-laboratory variability

The HepG2 cell line was selected to evaluate the inter-laboratory variability for IC_{50} values determined for shellfish

Table 5 Comparison of the relative IC_{50} values (expressed in milligrams DG per milliliter and in nanomolars) determined in each laboratory for shellfish extracts Oy-1 and Mu-1 spiked with AZA1, OA, or PTX2 and tested on HepG2 cells

	Lab.1			Lab.2			Lab.3			Mean intra-laboratory variability	Inter-laboratory mean		
	Mean IC_{50} (mg DG/mL)	Mean IC_{50} (nM)	CV%	Mean IC_{50} (mg DG/mL)	Mean IC_{50} (nM)	CV%	Mean IC_{50} (mg DG/mL)	Mean IC_{50} (nM)	CV%		Mean IC_{50} (mg DG/mL)	Mean IC_{50} (nM)	CV%
Oy-1	9.3	na	26	20.1	na	39	12.1	na	54	40	13.1	na	53
Mu-1	16.7	na	53	22.3	na	25	9.4	na	59	46	15.8	na	53
AZA1	na	4.3	71	na	10.1	90	na	8.4	35	65	na	7.1	91
Oy-1+AZA1	1.3	1.9	77	0.5	0.8	33	5.6	6.2	15	42	2.5	3.6	88
Mu-1+AZA1	0.8	1.2	14	0.5	0.8	21	1.0	1.5	113	49	0.8	1.2	77
OA	na	30	49	na	27	9	na	52	35	31	na	37	49
Oy-1+OA	1.3	22	15	1.0	20	23	0.9	16	2	13	1.1	19	22
Mu-1+OA	0.8	15	13	1.6	29	21	0.8	13	16	17	1.1	19	43
PTX2	na	67	80	na	nd	–	na	107	39	60	na	94	50
Oy-1+PTX2	3.0	30	34	7.0	70	19	3.7	37	63	39	4.3	43	53
Mu-1+PTX2	2.2	22	58	3.5	36	28	3.0	30	16	34	2.8	29	35

Three to five replicates were performed for each experimental condition. Values are represented as the mean with variability (expressed as coefficient of variation). The inter-laboratory means of IC_{50} values (expressed in milligrams DG per milliliter and in nanomolars) for each condition tested, and the corresponding inter-laboratory variabilities are also presented. The IC_{50} values (expressed in nM) obtained in each lab on HepG2 cells with standard toxins AZA1, OA and PTX2, as well as the inter-laboratory IC_{50} values and their associated variability are detailed in Part I

na not applicable, *nd* not determined, *DG* digestive glands

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extracts (Oy-1 and Mu-1) spiked with AZA1, OA, or PTX2. A minimum of three assays, each run in triplicate, was performed in Lab.1 to 3, for each condition tested. Overall, 76 assays were performed in the three labs, and 76 % of these assays were validated according to the criteria of the decision tree. Only minor variations were observed between the percentages of assays accepted at each laboratory (data not shown). However, only 66 % of the assays for spiked oyster extracts were validated, whereas 93 % of the assays with mussel extracts were validated according to the criteria of the decision tree.

In all three laboratories, the IC_{50} values obtained for the spiked extracts were lower than those obtained for the control extracts for all the toxins and types of shellfish extracts (Table 5). No statistical difference ($p<0.05$) could be observed between the three labs (Fig. 6).

When AZA1-spiking was used, the intra-laboratory variability for the three laboratories was less than 35 %, except in two cases (Lab.1: 77 % for the oyster extract and Lab.3: 113 % for the mussel extract) (Table 5). As already pointed out, these high variability values were due to "outlier" values among the three values validated according to the decision tree (data not shown). The dispersion of the IC_{50} values in the three labs led to an inter-laboratory variability for the spiked oyster and mussel extracts of 88 % and 77 %, respectively (Table 5).

For the OA-spiked extracts, the IC_{50} values were quite similar in all three laboratories (Fig. 6; Table 5). The mean inter-laboratory IC_{50} values were the same for the spiked oyster and mussel extracts (1.1 mg DG/mL), however the inter-laboratory variability was higher for the mussel extract (43 %) than for the oyster extract (22 %).

With PTX2-spiking, the IC_{50} values ranged from 2.2 to 7.0 mg DG/mL (Table 5). The mean intra-laboratory variability was less than 40 % for both types of extracts. The inter-laboratory variability was close to this value for the mussel extract (35 %) whereas, for the oyster extract, the inter-laboratory variability was slightly higher (53 %).

Interestingly, the mean intra-laboratory variability for each toxin was similar for both types of extract. The lowest

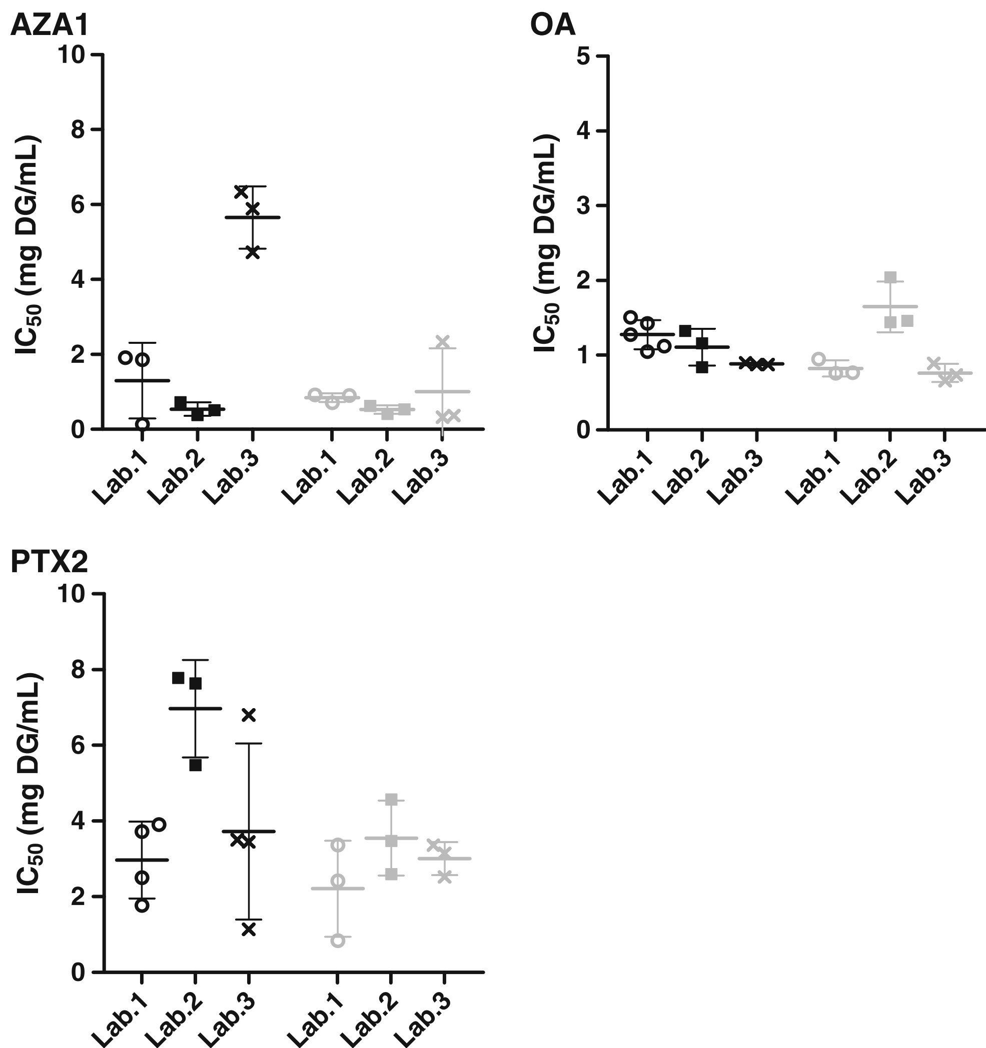

Fig. 6 Distribution of the IC_{50} values (expressed in milligrams DG per milliliter) obtained in the three labs on HepG2 cells for the oyster extract Oy-1 (in *black*) and the mussel extract Mu-1 (in *grey*) spiked with the lipophilic toxins AZA1, OA, or PTX2. The data shown have been validated according to the decision tree. The IC_{50} values are represented as the mean (*short horizontal lines*), with the standard deviation (*vertical lines*). No significant differences were found with Kruskal–Wallis test ($p<0.05$) with Dunn's multiple comparison post-test. *DG* digestive glands

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one was observed for OA (13 and 17 % for oyster and mussel extracts, respectively), whereas it reached 39 and 34 % for PTX2 and, 42 and 49 % for AZA1 (oyster and mussel extracts, respectively).

The collaborative study conducted in three laboratories on the HepG2 cell line demonstrated that the inter-laboratory variability (53 and 55 % for the control and spiked extracts, respectively) is higher than the mean intra-labortaory variability (43 % for control extracts and 33 % for spiked extracts, respectively). While every attempt was made to comply exactly with the SOP in performing the assays in each of the participating labs, the inter-laboratory variability remained quite high. However, it should be noticed that the inter-laboratory variability is mostly linked to the high CV obtained for the shellfish extracts spiked with AZA1 (88 and 77 %, for the spiked oyster and mussel extracts, respectively). A fairly high inter-laboratory variability was obtained during the collaborative study involving five labs for the pure toxin AZA1 (48 %), compared with OA (37 %) and PTX2 (39 %) (Part I). In fact, as explained previously, the high variability observed for AZA1-spiked extracts was linked to the presence of "outlier" values, which demonstrated that it is necessary to strengthen the quality and modeling criteria of the decision tree in order to avoid this situation.

The variability of cell-based assays could be explained by (1) differing physiological states of the cells, (2) the variability inevitable between different experimenters, (3) the hydrophobic properties of the compounds tested, and/or (4) the adsorption of the compounds to plastic labware.

Conclusions

This work was designed to complete the collaborative study performed on three lipophilic standard toxins. The use of SOP and the validation of the data through the decision tree clearly strengthened the integrated cytotoxicity assays. Our data sets provide the cytotoxic responses of three cell lines representative of the main organs targeted by phycotoxins, following exposure to control and spiked shellfish extracts different by their nature (mussels and oysters) and their geographical origin. This study enabled to determine the sensitivity and reproducibility of selected CBA exposed to control and spiked shellfish extracts. If considering that the primary objective of toxicological assays is to achieve similarity between the size of effects, rather than to determine absolute values of IC_{50} [36], our data sets achieved this goal.

These steps are necessary to support the use of unspecific CBA as a tool to study atypical toxicity events such as those observed in Arcachon Bay. Thus, CBA constitute a promising approach for detecting toxic compounds (e.g., anthropogenic or natural compounds*)* in shellfish extracts and a useful tool for selecting toxic chromatographic fractions during the identification process. In further work, the mechanisms of toxicity of the identified compounds should be investigated using functionally oriented biomarkers as well as functional assays.

Acknowledgements This work was carried out as part of the Arcachon National Program funded by the General Directorate for Food and the Directorate for Sea Fisheries and Aquaculture (Ministry of Agriculture) and by the General Directorate for Health (Ministry of Labor, Employment and Health). The authors wish to thank Stéphanie Magny, of the Food Safety Laboratory of the French Agency for Food, Environmental and Occupational Health and Safety, for preparing and sending the shellfish extracts to the different labs involved in the study.

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