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Self-assembled monolayer-based immunoassays for okadaic acid detection in seawater as monitoring tools

Sandra Leonardo[1], Anna Toldrà[1], Maria Rambla-Alegre[1], Margarita Fernández-Tejedor[1], Karl B. Andree[1], Laura Ferreres[1], Katrina Campbell[2], Christopher T. Elliott[2], Ciara K. O'Sullivan[3,4], Yolanda Pazos[5], Jorge Diogène[1], Mònica Campàs[1,*]

[1]*IRTA, Ctra. Poble Nou, km. 5.5, 43540 Sant Carles de la Ràpita, Spain*
[2]*Institute for Global Food Security, School of Biological Sciences, Queen's University, Stranmillis Road, Belfast, Northern Ireland, BT9 5AG*
[3]*Departament d'Enginyeria Química, Universitat Rovira i Virgili, Av. Països Catalans, 26, 43007 Tarragona, Spain*
[4]*Institució Catalana de Recerca i Estudis Avançats, Pg. Lluís Companys, 23, 08010 Barcelona, Spain*
[5]*INTECMAR, Peirao de Vilaxoán, s/n, 36611 Vilagarcía de Arousa, Spain*

e-mail: monica.campas@irta.cat

Abstract

Rapid and cost-effective methods to monitor the presence of diarrhetic shellfish poisoning (DSP) toxins in seawater samples in an easy and reliable manner are required to protect human health and avoid economic losses to shellfish industry. Immunoassays for the detection of okadaic acid (OA) and dinophysistoxin-1 and dinophysistoxin-2 are developed by immobilising OA on self-assembled monothiols or dithiols in an ordered and oriented way, providing an effective limit of detection of ~1 ng OA equiv./mL seawater. The immunoassays are applied to the analysis of the particulate fraction of seawater samples from two Catalan harbours (NW Mediterranean) and samples collected periodically from the Galician Rias (E Atlantic), as well as a reference mussel sample. Results are in agreement with LC-MS/MS and the certified values. OA concentration in seawater correlates with *Dinophysis* cell abundance, with a 1-2 weeks lag. The immunoassays provide powerful high-throughput analytical methods potentially applicable as alternative monitoring tools.

Keywords: algal blooms; okadaic acid; diarrhetic shellfish poisoning toxins; analytical techniques; immunoassay; seawater; phytoplankton; self-assembled monolayer; *Dinophysis*; monitoring.

1. Introduction

Harmful algal blooms (HABs), which may result in accumulation of marine toxins in shellfish, are a growing human health and economic concern in many coastal regions. Among the several illnesses caused by contaminated bivalve molluscs, diarrhetic shellfish poisoning (DSP) is the most significant problem in regions with well-developed aquaculture activities in temperate seas, e.g. Europe, USA, Chile, Japan and New Zeland (Eberhart et al., 2013; MacKenzie et al., 2005; Reguera et al., 2014). The DSP toxin group includes okadaic acid (OA) and analogues such as dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2), which are produced by dinoflagellates of the *Dinophysis* (and potentially by *Phalacroma rotundatum*, formerly *D. rotundata*) and *Prorocentrum* genera.

Current monitoring programs are based on the quantification of toxicity of shellfish tissue by LC-MS/MS as the reference method (European Comission, 2011), being 160 µg OA equiv./kg shellfish meat the maximum permitted level established by the European Commission (European Comission, 2004). The identification and quantification of microalgae in water as an early warning parameter is also performed on routine bases by monitoring programs, but DSP events are still very difficult to predict, among other reasons, because of the high variability of the toxin content in phytoplankton cells. Alternative monitoring methods have also emerged, for example, those based on molecular analysis, notable omics approaches such as trancriptomics and epigenomics, to monitor the presence of marine toxins in shellfish (González-Romero et al., 2017; Suárez-Ulloa, et al, 2015; Suárez-Ulloa, et al., 2013a; Suárez-Ulloa, et al., 2013b). However, other alternative strategies that facilitate the detection of DSP toxins before contamination of shellfish occurs are highly needed.

The product of *Dinophysis* abundance and cell toxin content have been demonstrated to be decisive for DSP toxicity in filter feeders such as mussels (Lindahl et al., 2007). Therefore, the measurement of toxin concentration in the particulate fraction of seawater provides a good indicator of mussel toxicity since both cell abundance and toxin content are included in the analysis (García-Altares et al., 2016). The ability to monitor the presence of marine toxins in seawater samples in a rapid, easy, robust and cost-effective manner would provide a powerful strategy within monitoring programs, facilitating important decisions to be taken prior to contamination of shellfish above legal limits occurs. Moreover, the development of these methods would clearly play a key role in understanding, among others, the relationship between phytoplankton population dynamics during a bloom and the presence of marine toxins, the toxin transfer within food webs, the definition of areas at risk according to time series studies and the

prevalence of toxins, and the identification of the possible impacts of climate change on the presence of marine toxins.

Immunoassays are promising sensitive rapid screening tools because, among other reasons, highly qualified personnel and expensive instrumentation are not required, in contrast to current analytical chemistry reference methods. Enzyme-linked immunosorbent assays (ELISAs) have been described for the detection of OA and its analogues, some of them being adapted for the development of immnosensors (Campàs et al., 2012, 2007; Leonardo et al., 2017b; Reverté et al., 2016; Sassolas et al., 2013b). It is well known that a key point in the development of immunoassays and immunosensors is the immobilisation of the antibody or antigen onto the surface. Most of the reported immunoassays require the conjugation of OA to protein carriers such as bovine serum album (BSA) or ovalbumin (OVA), but this strategy results in a random antigen immobilisation (Campàs et al., 2008; Dubois et al., 2010; Kreuzer et al., 1999; Kreuzer et al., 2002; Sassolas et al., 2013a). Thus, a new self-assembled monolayer (SAM)-based immunoassay for OA detection is proposed (Figure 1). The use of SAMs as building blocks in immunoassays enables to control the orientation, distribution and spacing of the immunospecies while reducing non-specific interactions (Fragoso et al., 2008). In this work, OA was immobilised on maleimide plates using monothiols (cysteamine) or dithiols (dithiol-alkane aromatic PEG6-$NHNH_2$) to covalently immobilise OA through their amine or hydrazide groups, respectively. OA calibration curves were obtained and cross-reactivity factors (CRFs) for DTX-1 and DTX-2 established. Both SAM strategies were applied to the analysis of the particulate fraction of seawater samples collected from different points of two Catalan harbours (NW Mediterranean) and seawater samples collected periodically from the Galician Rias (E Atlantic Ocean). In addition, DSP toxins were analysed in certified reference material of DSP-contaminated mussel. The correlation between the OA concentration and presence of *Dinophysis* in seawater samples was investigated. The potential application of the SAM-based immunoassays as a highly valuable tool in monitoring programs as well as research purposes was thus demonstrated.

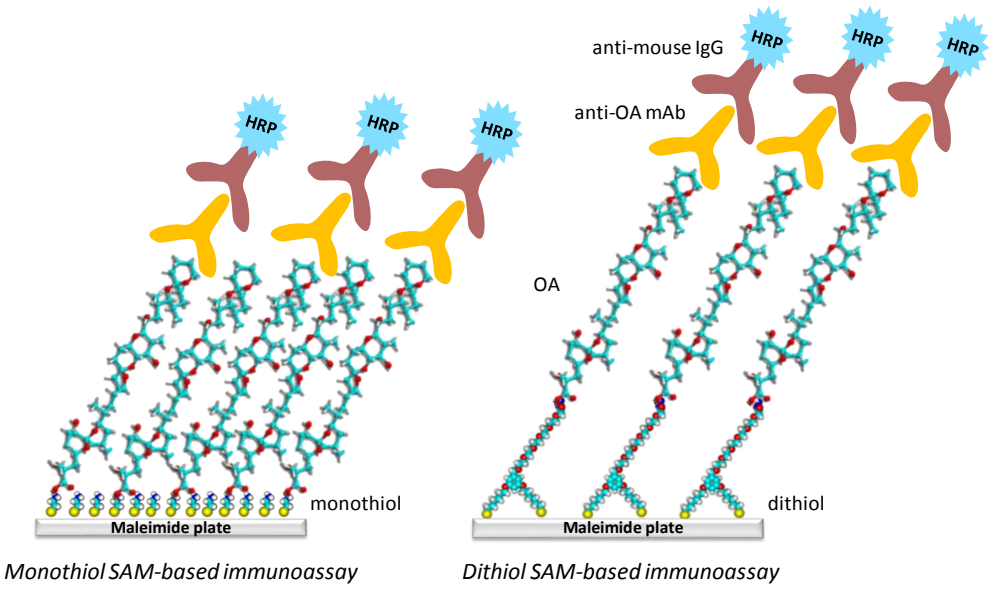

Figure 1. Schematic representation of the SAM-based immunoassays for the detection of OA.

2. Experimental

2.1. Reagents and Materials

Pierce maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain). Dithiol-alkane aromatic PEG6-$NHNH_2$ (dithiol) was purchased from Sensopath Technologies (Bozeman, USA). Cysteamine hydrochloride, okadaic acid potassium salt (OA) from *Prorocentrum concavum* (for immobilization on maleimide plates), potassium phosphate monobasic, potassium chloride, Tween®-20, ethylenediaminetetraacetic acid (EDTA), 2-(N-morpholino)ethanesulfonic acid hydrate (MES), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), anti-mouse IgG (whole molecule)–horseradish peroxidase antibody produced in rabbit (IgG-HRP) and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were supplied by Sigma–Aldrich (Madrid, Spain).

To perform calibration curves and LC-MS/MS analysis, certified reference materials (CRMs) of okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), yessotoxin (YTX), homoyessotoxin (hYTX), pectenotoxin-2 (PTX-2), azaspiracid-1 (AZA-1), azaspiracid-2 (AZA-2), azaspiracid-3 (AZA-3), 13-desmethylspirolide C (SPX-1), gymnodimine A (GYM-A) and pinnatoxin G (PnTX-G) were obtained from the National Research Council of Canada (NRC, Halifax, NS, Canada).

The anti-OA monoclonal antibody (mAb) OUB-OA-7 was produced and characterised for cross-reactivity in surface plasmon resonance assay development as described in Stewart et al. (2009a, 2009b).

2.2. Seawater and mussel samples

Seawater samples (n=14) from Sitges and Masnou harbours (NW Mediterranean Sea) were collected from 4 different sites at 3 different depths (surface, medium depth, between 1–2 m, and bottom, maximum depth = 2–4 m) in March and April 2016, respectively, coinciding with the presence of *Dynophysis* blooms (Figure 2). Seawater samples (1.5 L) were filtered under low vacuum onto 0.45-µm nylon filters. Particulate matter retained by the filter, which included phytoplankton, was analysed.

Seawater samples (n=20) from the Galician Rías (E Atlantic Ocean) were obtained weekly over a 5-month period (March to July 2015) as part of the Galician HAB monitoring program conducted by INTECMAR from a single sampling station located in Bueu (Figure 2). Seawater samples were collected from the water column using a PVC hose divided into 3 sections: 0-5, 5-10 and 10-15 m. An integrated water sample (0 to 15-m depth) was obtained by mixing equal volumes from each hose section. Seawater samples (1.5 L) obtained from the integrated profile were filtered through 47 mm diameter Whatman 2.7-µm GF/D and 0.7-µm GF/F filters. Phytoplankton passing through a GF/D, but retained onto GF/F filters was considered to represent the picoplankton fraction.

All filters were stored at -20 °C prior to extraction. Additionally, 50 mL of each sample were preserved with Lugol's iodine solution for phytoplankton identification and quantification. An additional subsample was retained from the Masnou and Sitges seawater samples for *Dinophysis* isolation.

Mussel tissue certified reference material for DSP toxins (CRM-DSP-Mus-c) and blank mussel tissue certified reference material (CRM-Zero-Mus) were obtained from the NRC (Halifax, NS, Canada). CRM-DSP-Mus-c contained certified values of OA (1.07 ± 0.08 mg/kg), DTX-1 (1.07 ± 0.11 mg/kg), DTX-2 (0.86 ± 0.08 mg/kg) and domoic acid (DA) and epiDA (11.8 ± 0.60 mg/kg), as well as non-certified values of fatty acid acyl esters of OA (2.4 mg/kg), DTX-1 (1.1 mg/kg) and DTX-2 (2.2 mg/kg).

Figure 2. Map of the Iberian Peninsula with sampling stations from the Catalan harbours (Sitges and Masnou) and from the Galician Rías (Bueu). Sampling stations are: P1B: 42°21'24.0"N 8°46'25.2"W; P1M: 41°28'32.9"N 2°18'31.0"E; P2M: 41°28'29.2"N 2°18'40.1"E; P3M: 41°28'33.7"N 2°18'50.2"E; P3M: 41°28'35.1"N 2°19'00.0"E; P1S: 41°14'03.6"N 1°49'17.6"E; P2S: 41°14'00.5"N 1°49'26.5"E; P3S: 41°13'57.8"N 1°49'30.8"E; P4S: 41°14'01.3"N 1°49'36.2"E.

2.3. Preparation of sample extracts

Filters retaining phytoplankton cells were extracted with 7 mL of methanol, vortex-mixed for 30 s and sonicated with a Vibra-Cell sonicator (Sonics & Materials Inc, Newton, USA) for 5 min at 37% amplitude with 3-s pulse cycles. The mixture was centrifuged at 3,000 rpm for 10 min and the supernatant was decanted and then filtered through 0.22-µm nylon membrane syringe filters. This extraction protocol was repeated twice. The extracts were combined, evaporated under nitrogen flow and reconstituted in 1 mL with methanol.

For mussel samples, a triple-step extraction with methanol (10 mL) was performed on whole homogenised tissues (1 g), according to the procedure proposed by Gerssen et al. (2009), which followed intra-laboratory validation by our group (García-Altares et al., 2013). The alkaline hydrolysis of the samples was performed according to the EURLMB Standard Operating Procedure (SOP) (EURLMB, 2011), based on the protocol developed by Mounfort et al. (2001).

2.4. Colorimetric self-assembled monolayer-based immunoassays

Cysteamine (monothiol) or dithiol-alkane aromatic PEG6-$NHNH_2$ (dithiol) were used for OA immobilisation on maleimide plates. The protocol was as follows: (1) maleimide-activated plates were rinsed three times with 200 µL of washing buffer (0.1 M potassium phosphate, 0.05% v/v Tween-20, pH 7.2); (2) 50 µL of 10 mM monothiol or 0.1 mM dithiol in binding buffer (0.1 M potassium phosphate, 10 mM EDTA, pH 7.2) were then added and incubated overnight at 4 °C for self-assembling; (3) previous to OA immobilisation, 2 µM OA in 0.1 M MES buffer, pH 5.0, was incubated with 20 mg/mL EDC and NHS (1:1) for 15 min to activate the OA carboxylic groups; (4) 50 µL of different concentrations (from 0.25 µM to 2 µM for protocol optimisation and 0.5 µM for the final competition assay) of the activated OA in 0.1 M MES buffer, pH 5.0, were added and incubated for 45 min to allow OA immobilisation through amine-carboxylic (monothiol approach) or hydrazide-carboxylic (dithiol approach) cross-linking reactions.

After OA immobilisation, (5) 25 µL of mAb diluted in binding buffer containing 1 % BSA (from 1/500 to 1/4,000 for protocol optimisation and 1/2,000 for the final competition assay) and 25 µL of OA, DTX-1 or DTX-2 standard solutions or samples, all diluted in binding buffer, were incubated for 45 min; (6) 50 µL of IgG-HRP dilution (1/1,000 and 1/2,000 for protocol optimisation and 1/2,000 for the final competition assay) in binding buffer containing 1% BSA were added to wells for 45 min; (7) finally, 100 µL of TMB liquid substrate were added and incubated, after 10 min absorbance was read at 620 nm with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA).

After each step, wells were rinsed three times with 200 µL of washing buffer and during incubations microtiter plates were placed on a plate shaker for mixing. With the exception of the thiol self-assembling step, which was performed at 4 °C, all steps were conducted at room temperature.

2.5. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis

For LC-MS/MS analysis of lipophilic marine toxins, our in-house validated method to determine lipophilic toxins in shellfish samples, described in García-Altares et al. (2013) was applied. Toxins were separated on a XBridge BEH C8 2.5µm 2.1x50mm column (Waters, Milford, MA, USA). Abinary gradient was programmed with ultrapure Milli-Q water (mobile phase A) and 90:10 v:v acetonitrile/water (mobile phase B), both containing 6.7 mM of ammonium hydroxide. Mobile phases were filtrated through 0.2 µm nylon membrane filters (Whatman, Springfield Mill, UK).

Chromatographic separation was performed on an Agilent 1200 LC system (Agilent Technologies, Santa Clara, CA). All runs were carried out at 30 °C using a flow rate of 500 µL/min. The injection volume was 10 µL and the auto-sampler was set at 4 °C. A triple quadrupole 3200 QTRAPmass spectrometer (MS) equipped with a TurboV electrospray ion source (Apllied Biosystems, Foster City, CA) was used to perform mass spectrometry.All regulated lipohilic toxins were analysed in both negative (-ESI) and positive polarity (+ESI) (García-Altares et al., 2013), selecting two product ions per toxin to allow quantification (the most intense transition) and confirmation; identification was supported by toxin retention time and the MRM ion ratios. Table S1 shows the MRM list used for DSP toxins quantification and for screening of the rest of regulated lipophilic toxins.

The quality control criteria stated by the EURLMB SOP (EURLMB, 2011) regarding resolution, limits of quantification (LOQs) and linearity was checked in every batch. Resolution (*Rs*) between the isomers OA and DTX-2 was calculated according to the following equation:

$$R_s = 2\,(t_{R(DTX\text{-}2)} - t_{R(OA)})/(W_{(OA)} + W_{(DTX\text{-}2)})$$

where t_R means retention time and W means peak width (both in min). The EURLMB request resolution between OA and DTX-2 to be greater than one (EURLMB, 2011).

Phytoplankton concentrates and the certified mussel sample were injected in duplicate. A preliminary screening was perfomed before accurate quantification. An external standard calibration curve was prepared with a six level curve (from 4 to 40 ng toxin/mL for OA, DTX-1 and DTX-2 and from 5-50 ng toxin/mL for PTX-2) to quantify samples with estimated OA, DTX-1, DTX-2 and/or PTX-2 below 40 ng/mL. Samples with concentrations above 40 ng toxin/mL for OA, DTX-1 and DTX-2 and above 50 ng PTX-2/mL were diluted to fall into the calibration range.

The correlation coefficients (r^2) of the quantification curves had to be greater than 0.98 to ensure linearity and the deviation of the slopes between consecutive calibration curves has to be lower than 25% to be considered as acceptable. The intermediate precision was expressed as the relative standard deviation (RSD in %) and could not exceed 25% among the injection replicates.

2.6. Phytoplankton identification and quantification

The taxonomic identification of phytoplankton according to morphology was performed by light microscopy. Phytoplankton cells were counted according to the Utermöhl method with a Nikon or Leica DMIL inverted microscope settling 50 mL in Hydrobios chambers (Utermöhl, 1931). The entire surface of the chamber was counted at 100-200x magnification to quantify microalgae cell abundance (cells/L).

2.7. Single-cell isolation, PCR amplification and DNA sequencing

Single cells of *Dinophysis* were isolated from seawater samples collected in Masnou (Catalonia) using a microcapillary pipette under an inverted microscope (Leica DMIL) and washed by repeatedly transferring to drops of sterile filtered seawater, following the procedure of Ki et al. (2004). Afterwards, DNA extraction based on proteinase K was performed using the Arcturus™ PicoPure™ DNA Extraction Kit (Applied Biosystems, CA, USA). Following the manufacturer's instructions, 10 µL of Extraction Solution were added to the samples, which were incubated at 65 °C for 3 hours, followed by 10 min at 95 °C to inactivate proteinase K.

The mitochondrial cytochrome oxidase (mt *cox1*) gene was amplified from all cells using the Dinocox1F/Dinocox1R (5-AAAAATTGTAATCATAAACGCTTAGG-3/5-TGTTGAGCCACCTATAGTAAACATTA-3) pair of primers (Raho et al., 2013). Each 25 µL reaction mixture contained 1mM dNTP, 3 mM $MgCl_2$, 1 µM of each primer, 1 U of Taq polymerase and 2 µL of template DNA. Amplifications were carried out in an Eppendorf Mastercycler nexus gradient as follows: an initial denaturation step of 5 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 55 °C and 3 min at 72 °C, and a final extension step of 10 min at 72 °C. Each PCR reaction was checked by agarose gel electrophoresis visualised with ethidium bromide. PCR products were purified with QIAquick PCR Purification Kit and bidirectionally sequenced (Sistemas Genómicos, LLC, Valencia, Spain), using the same primers as those applied in the amplification. Consensus sequences obtained from both reads for each strain were manually edited using BioEdit v7.0.5.2 to remove primer sequences and terminal artefacts (Hall, 1999). The mt *cox1* sequences obtained in this study were deposited in GenBank under accession numbers: KY849911-KY849921.

2.8. Phylogenetic analysis

Consensus sequences (mt *cox1*) were compared to those available in GenBank (NCBI) using the Basic Local Alignment Search Tool (BLAST) algorithm to compare similarity of sequences collected in this study to known sequences with a high similarity from GenBank for phylogenetic analysis. To obtain the phylogenetic tree, the obtained sequences were aligned using the ClustalW algorithm in BioEdit v7.0.5.2 with validated *Dinophysis* sequences from GenBank. Evolutionary analyses were conducted in MEGA v5 (Tamura et al., 2011). All codon positions were included and all ambiguous positions were removed for each sequence pair, using a final dataset of 864 nucleotide positions. The evolutionary history was inferred using the Maximum-Likelihood method (Felsenstein, 1981). The percentage of replicate trees in which the associated taxa

clustered together in the bootstrap test (1,000 replicates) was calculated (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter model and are in the units of the number of base substitutions per site (Kimura, 1980).

2.9. Data analysis

Measurements were performed in triplicate in immunoassay approaches and in duplicate in LC-MS/MS analysis. The immunoassay calibration curves were fitted using a sigmoidal logistic four-parameter equation. The linear regression model was used to evaluate the correlation between OA equiv. content in the particulate fraction of seawater samples determined with the monothiol and dithiol SAM-based immunoassays and the values obtained from the LC-MS/MS analysis, and to study the correlation between *Dinophysis* cell abundance and OA equiv. content. To evaluate differences between quantifications provided by the monothiol SAM-based immunoassay, the dithiol SAM-based immunoassay and LC-MS/MS analysis, a one-way analysis of variance was conducted. Prior to the analysis, normality and equal variance tests were performed. Differences in the results were considered statistically significant at the 0.05 level. SigmaStat software package 3.1 was used for statistical analysis.

3. Results and discussion

3.1. Monothiol and dithiol SAM-based immunoassay calibration curves

A competitive indirect immunoassay for OA was performed on maleimide plates, modifying the format reported by Reverté et al. for tetrodotoxin (Reverté et al., 2015). At a pH of 6.5-7.5, maleimides react with free sulfhydryl groups forming stable thioether linkages. Thus, monothiol or dithiol were used to covalently immobilise OA in an ordered and well-oriented manner through the formation of SAMs. Whereas the short monothiol provided a packed monolayer, the longer dithiol generated a spaced and more stable monolayer due to its multivalent mechanism of interaction (Fragoso et al., 2008). Checkerboard titrations and competitive immunoassays were first carried out to optimise the concentrations of OA, mAb and IgG-HRP in monothiol and dithiol approaches. The absorbance values obtained showed expected trends according to antigen and primary and secondary antibody concentrations. Dithiols provided higher absorbance values than monothiols, which can be explained by a higher spacing of the immobilised OA and a better recognition by the mAb. In both approaches, the lowest secondary antibody concentration tested provided the lowest non-specific adsorption values (e.g., 20 *vs.* 28 % in the monothiol approach,

and 10 *vs.* 25 % in the dithiol SAM-based immunoassay) while maintaining reasonable absorbance values. Thus, 1/2,000 IgG-HRP dilution was selected for subsequent experiments. Under these conditions, although both approaches provided acceptable values of non-specific adsorption from the secondary antibody, the formation of long-chain SAMs and the incorporation of the polyethylene glycol molecules in the dithiol molecules clearly reduced the non-specific binding as compared with the monothiols (Frederix et al., 2004).

To select OA and mAb concentrations, competitive immunoassays were performed using different immobilised OA/mAb ratios. Calibration curves demonstrated that the competition between free and immobilised OA for mAb binding sites was successful. The curves were corrected with respect to the controls with no mAb and fitted to sigmoidal logistic four-parameter equations (R=0.999 in the monothiol and R=1.000 in the dithiol approaches). Whereas the amount of immobilised antigen had a small effect on the competition assay, slightly decreasing the sensitivity with lower OA concentrations (e.g. half maximal inhibitory concentration (IC_{50}) values of 7.9 *vs.* 10.9 ng/mL for 1 and 0.25 µM OA, respectively), lower mAb concentrations provided higher sensitivity (e.g. IC_{50} values of 49.9, 26.0 and 13.9 ng/mL for 1/500, 1/1,000 and 1/2,000 mAb dilution, respectively). Optimal performance was achieved using 0.5 µM OA and a 1/4,000 mAb dilution, with the monothiol and dithiol approaches providing equal analytical performance (Figure 3). Standard deviations (SD) were always lower than 10%. Limits of detection (LODs) of 2.4 ± 0.3 and 2.1 ± 0.2 ng/mL OA equiv. and working ranges up to 17.1 ± 0.6 and 16.6 ± 1.4 ng/mL OA equiv. were achieved for the monothiol and dithiol SAM-based immunoassays, respectively. Results were in the same order of magnitude as other OA immunoassays previously reported (Campàs et al., 2008; Chin et al., 1995; Dubois et al., 2010; Hayat et al., 2011; Kreuzer et al., 1999; Sassolas et al., 2013a; Shestowsky et al., 1992). The SAM-based strategy provides an oriented, ordered and specific OA sensing platform, avoiding the need to conjugate the toxin to protein carriers, whilst favouring the antigen-antibody recognition.

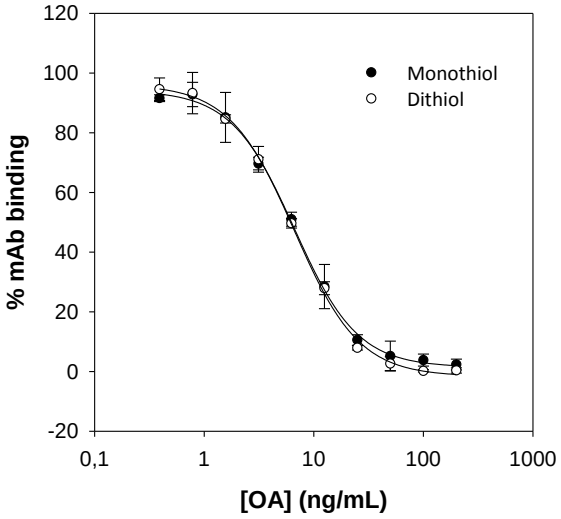

Figure 3. Calibration curves obtained using the monothiol and dithiol SAM-based immunoassays for OA detection. Inhibition is expressed as percentage of the control (no OA).

Besides detecting OA, an immunoassay for the detection of DSP toxins should also have the capability of recognising other OA group toxins that can also contribute to major contamination episodes. For this reason, the response to DTX-1 and DTX-2 with respect to OA was also evaluated. Cross-reactivity factors (CRFs) were calculated as the ratio of the IC_{50} value of the OA standard curve to the IC_{50} value of each analogue expressed in percentage. Although CRFs are mainly dependent on the intrinsic affinity of the antibody to recognise the different analogues, the immunospecies immobilisation strategy can also play an important role in the antigen-antibody interaction (Leonardo et al., 2017a). CRFs for DTX-1 and DTX-2 were 54 % and 52 % in the monothiol approach and 53 % and 50 % in the dithiol SAM-based immunoassay, respectively. It is important to take in mind that the reduction in immunoassay signal indicates competition, but does not indicate which toxin analogues are present in the samples. Thus, a global quantification of the sample relative to OA is provided by the immunoassays. The establishment of CRFs contributes to better understand the comparisons between the quantifications provided by the immunochemical tools and those obtained by other analytical methods based on different recognition principles.

3.2. Seawater sample analysis

3.2.1. Seawater matrix effects

Prior to the analysis of seawater samples, OA calibration curves using extracts of filtered seawater (phytoplankton free) were performed to evaluate matrix effects on the immunoassays. Calibration curves were performed with pure seawater extracts and with 1/2, 1/4 and 1/8 dilutions in buffer. Matrix effects (mAb binding inhibition) were observed with pure seawater extracts, but they decreased as the matrix dilution increased, and were negligible for 1/4 extract dilutions or lower. Correction factors (CFs) were established as the ratio of the IC_{50} value of the OA standard curve in each extract dilution to the IC_{50} value of the OA standard curve in buffer. CFs for pure, 1/2 and 1/4 matrix dilutions were 0.42, 0.71 and 0.92 for the monothiol SAM-based immunoassay, and 0.43, 0.81 and 0.96, for the dithiol configuration. Taking into account the CFs, the effective LOD (eLOD) was calculated to be 1.0 and 0.9 ng/mL OA equiv. for monothiol and dithiol SAM-based immunoassays, respectively.

3.2.2. Analysis of seawater samples from Catalan harbours (Masnou and Sitges).

Toxin content. The OA equiv. content in the particulate fraction of seawater samples collected at different depths in different points of Sitges and Masnou harbours was determined by the SAM-based immunoassays as well as by LC-MS/MS analysis. LC-MS/MS analysis revealed that OA was the only DSP toxin present in the samples (limit of quantification (LOQ) = 4 ng/mL). Quantifications provided by the immunoassays —after applying the CFs— were compared with those provided by the LC-MS/MS reference method. Both immunoassays showed good correlation with the LC-MS/MS method ($y_{\text{monothiol}} = 1.056\ x - 2.276$, $R^2 = 0.992$, $y_{\text{dithiol}} = 0.995\ x - 1.632$, $R^2 = 0.991$) (Figure 4). OA was detected in all samples by the three approaches, reaching values up to 38 ng/mL in Sitges and 45 ng/mL in Masnou.

The presence of other lipophilic toxins outside of the OA group of toxins was also evaluated by LC-MS/MS. YTX, hYTX, AZAs, SPX-1, GYM-A and PnTX-G were not present in the particulate fraction of seawater samples. However, all samples from Masnou and Sitges harbours contained high levels of PTX-2 in the particulate matter of seawater, in some cases more than 12-fold higher compared with OA concentration. Since no significant differences were observed in the OA quantifications of the samples determined by the monothiol SAM-based immunoassay, the dithiol SAM-based immunoassay and LC-MS/MS analysis (P = 0.358), it is safe to affirm the lack of interferences from other non-DSP lipophilic toxins, as expected from previous works (Stewart et al., 2009b).

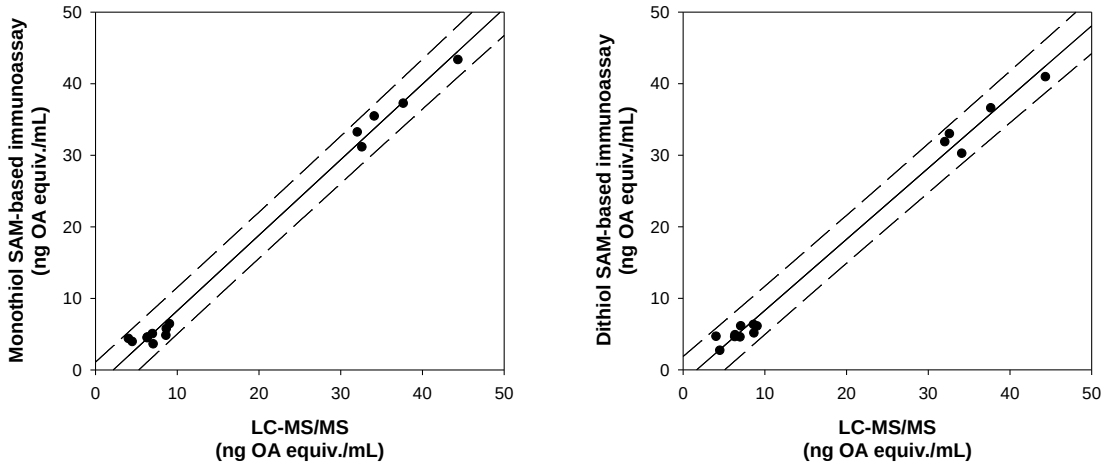

Figure 4. Linear regressions for the correlations between OA quantifications by the monothiol and dithiol SAM-based immunoassays and by LC-MS/MS analysis of seawater samples from the Catalan harbours. Dashed lines represent the 95% prediction intervals.

Phytoplankton identification. Cell contour –the size and shape of their large hypothecal plates– and the shape of the left sulcal lists and the ribs were the morphological features used for the taxonomic identification of *Dinophysis* species. Nevertheless, the morphological variability of *Dinophysis* species as a result of their polymorphic life cycle can lead to uncertain identification and quantification of phytoplankton samples, especially in those where two close species of *Dinophysis*, such as the pair *D. acuminata* and *D. sacculus*, co-occur (Reguera et al., 2012). On the Western Mediterranean coast, DSP events have been mainly related to blooms of *D. sacculus* (Giacobbe et al., 2000). Although no evidence of co-occurrence of *D. sacculus* with *D. accuminata* has been previously reported in the NW Mediterranean coast, *Dinophysis* cells with a wide range of different sizes and shapes were observed when analysing samples from Sitges and Masnou harbours. Most cells were attributed to *D. sacculus* by the comparison of morphologic formulae but some cells showed morphological characteristics close to *D. acuminata* species. Thus, genetic information was required to provide a reliable identification.

Dinophysis cells labelled as *D.* cf. *acuminata* (5 cells) and as *D.* cf. *sacculus* (6 cells) were isolated from samples from the Catalan coast. To date, most molecular markers for dinoflagellates are based on ribosomal DNA (rDNA) genes and their ITS regions. However, due to the low variability of rDNA genes among *Dinophysis* species, mt *cox1* mitochondrial marker was used to discriminate among *Dinophysis* species within the "*D. acuminata* complex", which includes *D. acuminata* (and its small cell *D. skagi*) (Escalera and Reguera, 2008), *D. sacculus* and *D. ovum*. The mt *cox1*

sequences isolated from the 11 *Dinophysis* cells showed high similarity. BLAST analyses showed that the sequences had a high degree of correlation with *D. sacculus* described in GenBank. The optimal mt *cox1* tree, including 19 nucleotide sequences, with the highest log likelihood (-1459.3415), is shown in Figure S1. The phylogenetic results confirm that the 11 isolated *Dinophysis* cells were clustered into a well-supported group, which corresponds to *D. sacculus*. Thus, *Dinophysis* cells initially labelled as *D.* cf. *acuminata* were actually *D. sacculus*, although intra-specific differences in morphology were identified by light microscopy.

From all samples, only one contained levels below the *Dinophysis* spp. abundance threshold of 500 cells/L established in the monitoring program of the Catalan coast as an early warning parameter. Whilst in Masnou values ranged from 280 cells/L to 5,160 cells/L of *D. sacculus*, in Sitges values ranged from 560 cells/L to 8,600 cells/L.

The dinoflagellate *Alexandrium minutum* was also detected in seawater samples from both harbours, with concentrations up to 687 cells/L in Sitges and from 238,980 cells/L to 3,785,000 cells/L in Masnou. *Pseudo-nitzchia* spp. cells were observed in high amounts, reaching values up to 53,703 cells/L in Masnou and 2,977 cells/L in Sitges.

Correlation between toxin content and cell abundance. OA concentration in the particulate fraction of seawater increased as the number of *D. sacculus* cells increased in seawater samples. Although *A. minutum* and diatom cells were in some cases detected at concentrations even higher than *D. sacculus*, the presence of these microalgae did not affect the OA detection (no significant differences were observed between the quantifications provided by the imunoassays and LC-MS/MS analysis). Strong correlations between the OA equiv. content determined by the monothiol ($R = 0.914$, $p < 0.0001$) and the dithiol ($R = 0.933$, $p < 0.0001$) immunoassays and *D. sacculus* cell abundance were observed (Figure S2). Concentrations of OA per cell during the *D. sacculus* bloom ranged from 1 to 11 pg OA/cell. A strong linear correlation between PTX-2 concentration (determined by LC-MS/MS) and the presence of *D. sacculus* was also observed ($R = 0.940$, $p < 0.0001$), with concentrations ranging from 20 to 96 pg PTX-2/cell. Results are in agreement with previous studies about toxin profiles of *D. sacculus* in the NW Mediterranean (García-Altares et al., 2016).

3.2.3. Analysis of seawater samples from the Galician Rias

Toxin content. The particulate fraction of seawater samples collected once a week from March to July 2015 in Bueu (Galicia) were analysed by the SAM-based immunoassays and LC-MS/MS to determine the concentration of OA in phytoplankton. The Galician Northern Rias are one of the largest mussel production areas in the world that suffers from high incidences of DSP toxins, leading to lengthy closures that affect shellfish producers and have a high economic impact. Thus, it was not surprising that OA equiv. were detected in all samples analysed, from trace amounts (~1 ng OA equiv./mL) to approximately 500 ng OA equiv./mL. Two OA equiv. content peaks were clearly observed, the biggest one coinciding with samples collected from mid-April to mid-May (and reaching 500 ng/mL of OA equiv. the 5th of May) and a smaller peak starting at the end of June 2015 and reaching its maximum level of 114 ng/mL the 6th of July 2015. LC-MS/MS analysis revealed that OA was the only regulated lipophilic toxin present in the sample. Excellent correlations of the quantifications provided by the monothiol ($y_{\text{monothiol}} = 1.03\ x - 0.983$, $R^2 = 0.999$) and the dithiol ($y_{\text{dithiol}} = 0.951\ x - 1.193$, $R^2 = 0.997$) SAM-based immunoassays with LC-MS/MS analysis were obtained (Figure 5). No significant differences were observed between the three strategies (P = 0.970).

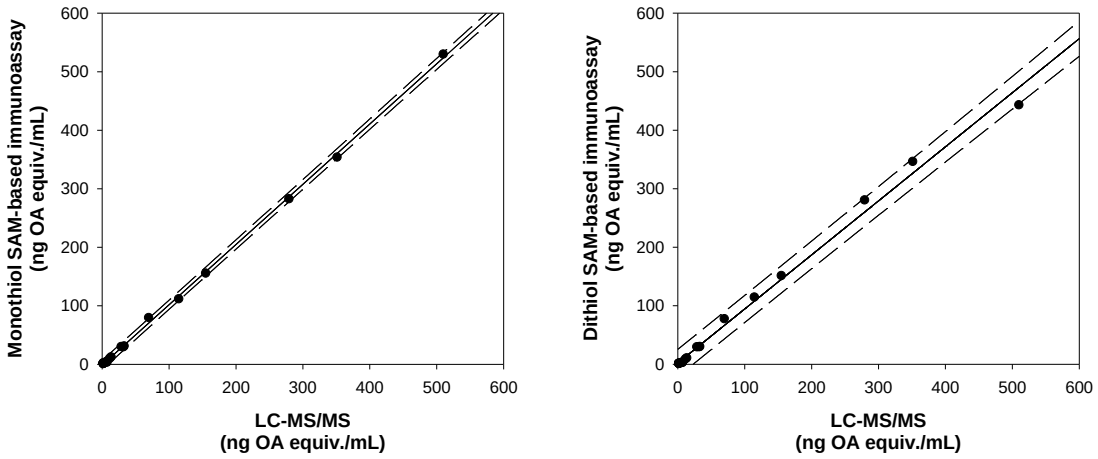

Figure 5. Linear regressions for the correlations between OA quantifications by the monothiol and dithiol SAM-based immunoassays and by LC-MS/MS analysis of seawater samples from the Galician Rías. Dashed lines represent the 95 % prediction intervals.

SAM-based immunoassays were also applied to the analysis of the picoplankton fraction (0.7-2.7 µm) of seawater samples. It is interesting to note that OA equiv. were detected in 5 out of the 20 samples analysed, coinciding with the two peaks observed in the analysis of phytoplankton particulate fraction (>2.7 µm), and representing up to 20% of the total OA equiv. content. Results were validated with LC-MS/MS analysis, showing non-significant differences with the results

obtained by the monothiol and dithiol SAM-based immunoassays (P = 0.997), and confirming the presence of OA (and none of its analogues or other lipophilic toxins). These results highlight the possible presence of OA associated with picoplankton and/or organic aggregates, making evident its contribution in the total DSP toxins content in seawater (Pizarro et al., 2013).

Phytoplanckton identification. As expected, *D. acuminata*, the main agent of DSP events on European Atlantic coasts (Reguera et al., 2014), was the principal phytoplankton species found in seawater samples from Galicia. Two main *D. acuminata* blooms were clearly evidenced, the largest one starting in mid-April to mid-May and reaching 10,760 cells/L the 27th of April, and the second bloom that lasted from mid to end of June and reached a maximum cell content of 3,080 cells/L. *D. skagi* and *P. rotundatum* were also detected in low abundance in a few samples. High amounts of other dinoflagellates (from 1,705 cells/L to 81,175 cells/L) and diatom cells (from 8,950 cells/L to 2,793,718 cells/L) were observed in all seawater samples.

Correlation between toxin content and cell abundance. In general, the OA concentration in the particulate fraction of sewater increased as total *Dinophysis* cell abundance in seawater samples increased. Appropriate correlations between OA content determined by the monothiol ($R = 0.874$, $p < 0.0001$) and the dithiol ($R = 0.879$, $p < 0.0001$) SAM-based immunoassays and total *Dinophysis* cells were obtained (Figure S3). When toxin content per cell was calculated, concentrations of OA ranged from less than 1 pg/cell to 466 pg/cell. The temporal representation of total *Dinophysis* cell abundance and OA content showed that OA peaks appeared 1-2 weeks later than *Dinophysis* blooms (Figure 6), explaining the variability in the OA content per cell (García-Altares et al., 2016). It is important to keep in mind that toxins are secondary metabolites that result from a balance between rates of toxin production, toxin excretion and division of *Dinophsysis* cells. Imbalances between these processes may lead to a very low accumulation rate of toxins (if either division or toxin release rates are high) or high accumulation rates (if division stops and toxin production continues).

Although setting *Dinophysis* alert levels could be efficient enough to warn against the presence of OA, it has been demonstrated that the risk of shellfish toxicity can persist even when cell abundance is below the threshold. Hence, OA detection in seawater samples provides an alternative in monitoring programs for early warning of DSP toxins.

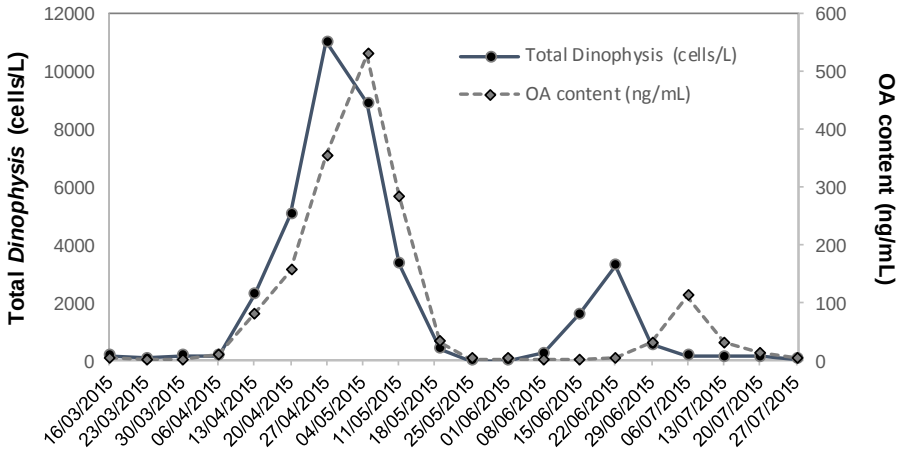

Figure 6. Temporal representation of total *Dinophysis* cell abundance and OA content in seawater samples from the Galician Rías.

3.3. Analysis of mussel samples

A certified reference material (CRM) from naturally contaminated mussel tissues containing certified contents of OA, DTX-1 and DTX-2 was analysed. The influence of matrix effects on the response was evaluated using 2.5 mg/mL mussel tissue certified to contain no DSP toxins, and the effect was observed to be negligible. Thus, the CRM-DSP-Mus-c extract was diluted to 2.5 mg/mL to obtain toxin values that fall into the calibration curve for its quantification. Taking into account the presence of DTX-1 and DTX-2 in the mussel sample in addition to the OA content, CRFs for both analogues were applied to the concentrations provided by the NRC certificate and LC-MS/MS analysis (Table 1). After the application of the corresponding CRFs to DTX-1 and DTX-2 individual concentrations, correlations between OA equiv. content obtained by the monothiol and dithiol SAM-based immunoassays and the values provided by NRC were 97 % and 99 %, respectively. When comparing the quantifications of the monothiol and dithiol approaches with those obtained in LC-MS/MS analysis, correlations were 104 % and 106 %, respectively.

Additionally to OA, DTX-1 and DTX-2, bivalve molluscs may contain esterified forms of the OA-group toxins (DTX-3), which are believed to be produced by the acylation of OA and analogues in the bivalves (Reguera et al., 2014). Thus, an alkaline reaction was performed on mussel extracts to hydrolyse the OA-group acyl esters and thus detect their possible presence. The hydrolysed sample was also diluted to 2.5 mg/mL. The prior analysis of the hydrolysed negative control discarded possible solvent effects. Again, CRFs were applied to post-hydrolysis NRC and LC-MS/MS values, and results were compared with those provided by the immunoassays (Table 1). In this

case, correlations were 105 % and 107 % for the monothiol and dithiol SAM-based immunoassays, respectively, compared with NRC values, and 99 % and 100 % when comparing with LC-MS/MS analysis.

Both SAM-based immunoassays provided OA equiv. contents in good agreement with the certified and LC-MS/MS values, both before and after sample hydrolysis. Thus, the lack of interference from other marine toxins such as domoic acid (DA) or epiDA, also present in the mussel tissues, was again demonstrated.

Table 1. DSP toxins concentrations (µg/kg mussel) of a mussel tissue certified reference material for okadaic acid and dinophysistoxins (CRM-DSP-Mus-c) and comparison between the reference values (NRC values), the quantifications provided by LC-MS/MS and those obtained by the dithiol and monothiol SAM-based immunoassays.

	NRC values					**LC-MS/MS**					**SAM-based immunoassays**	
	OA	DTX-1	DTX-2	OA equiv. (applying CRFs)		OA	DTX-1	DTX-2	OA equiv. (applying CRFs)		OA equiv.	
				Monothiol	Dithiol				Monothiol	Dithiol	Monothiol	Dithiol
Before hydrolisis	1070	1070	860	**2095**	**2067**	992	1026	776	**1950**	**1924**	**2024**	**2042**
Post-hydrolisis	2400	1100	2200	**4138**	**4083**	2655	927	2384	**4396**	**4338**	**4350**	**4357**

4. Conclusions

The self-assembling of both monothiols and dithiols on maleimide plates allows an ordered and optimally-oriented immobilisation of OA, providing colorimetric immunoassays with similar analytical performance. Whilst the use of dithiols enables a higher spacing of the immobilised immunospecies and reduces non-specific binding compared with monothiols, the use of monothiols such as cysteamine decreases the cost of the assay. The application of both immunoassays to the analysis of seawater samples and also contaminated shellfish samples has been successfully demonstrated. The SAM-based immunoassays provide powerful analytical tools that accomplish the major requirements to be implemented in monitoring programs as they are reliable, easy to perform and cost-effective methods that allow the detection of all DSP toxins in a selective way. Moreover, their high-throughput design allows multiple samples to be analysed in a matter of hours.

Although a good correlation between *Dinophysis* cell abundance and OA detection has been observed, it has been demonstrated that the poisoning risk can persist even when cell abundance is low. Thus, OA detection in the particulate fraction of seawater by SAM-based immunoassays constitutes a viable alternative to the monitoring of *Dinophysis* levels. The implementation of the SAM-based immunoassays to research programs will clearly contribute to set the basis for further investigation aimed to improve the allocation of monitoring efforts to protect the marine environment in production areas and guarantee seafood safety.

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References

Campàs, M., de la Iglesia, P., Le Berre, M., Kane, M., Diogène, J., Marty, J.-L., 2008. Enzymatic recycling-based amperometric immunosensor for the ultrasensitive detection of okadaic acid in shellfish. Biosensors & Bioelectronics 24, 716-722.

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

Campàs, M., Prieto-Simón, B., Marty, J.L., 2007. Biosensors to detect marine toxins: Assessing seafood safety. Talanta 72, 884-895.

Chin, J.D., Quilliam, M.A., Fremy, J.M., Mohapatra, S.K., Sikorska, H.M., 1995. Screening for okadaic acid by immunoassay. Journal of Aoac International 78, 508-513.

Dubois, M., Demoulin, L., Charlier, C., Singh, G., Godefroy, S.B., Campbell, K., Elliott, C.T., Delahaut, P., 2010. Development of ELISAs for detecting domoic acid, okadaic acid, and saxitoxin and their applicability for the detection of marine toxins in samples collected in Belgium. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment 27, 859-868.

Eberhart, B.-T.L., Moore, L.K., Harrington, N., Adams, N.G., Borchert, J., Trainer, V.L., 2013. Screening tests for the rapid detection of diarrhetic shellfish toxins in Washington State. Marine Drugs 11, 3718-3734.

Escalera, L., Reguera, B., 2008. Planozygote division and other observations on the sexual cycle of several species of Dinophysis (Dinophyceae, Dinophysiales). Journal of Phycology 44, 1425-1436.

EURLMB, 2011. European Union Reference Laboratory for Marine Biotoxins. Interlaboratory Validation Study of the EU-Harmonised SOP-LIPO-LC-MS/MS.

European Commission, 2004. Regulation (EC) No 853/2004. Off. J. Eur. Union L 226 (22).

European Comission, 2011. Regulation (EC) No 15/2011. Off. J. Eur. Union L 6 (3).

Felsenstein, J., 1985. Confidence-limits on phylogenies - An approach using the Bootstrap. Evolution 39, 783-791.

Fragoso, A., Laboria, N., Latta, D., O'Sullivan, C.K., 2008. Electron permeable self-assembled monolayers of dithiolatedaromatic scaffolds on gold for biosensor applications. Analytical Chemistry 80, 2556-2563.

Frederix, F., Bonroy, K., Reekmans, G., Laureyn, W., Campitelli, A., Abramov, M.A., Dehaen, W., Maes, G., 2004. Reduced nonspecific adsorption on covalently immobilized protein surfaces using poly(ethylene oxide) containing blocking agents. Journal of Biochemical and Biophysical Methods 58, 67-74.

García-Altares, M., Casanova, A., Fernández-Tejedor, M., Diogène, J., de la Iglesia, P., 2016. Bloom of Dinophysis spp. dominated by D. sacculus and its related diarrhetic shellfish poisoning (DSP) outbreak in Alfacs Bay (Catalonia, NW Mediterranean Sea): Identification of DSP toxins in phytoplankton, shellfish and passive samplers. Regional Studies in Marine Science 6, 19-28.

García-Altares, M., Diogène, J., de la Iglesia, P., 2013. The implementation of liquid chromatography tandem mass spectrometry for the official control of lipophilic toxins in seafood: Single-laboratory validation under four chromatographic conditions. Journal of Chromatography A 1275, 48-60.

Gerssen, A., Mulder, P.P.J., McElhinney, M.A., de Boer, J., 2009. Liquid chromatography-tandem mass spectrometry method for the detection of marine lipophilic toxins under alkaline conditions. Journal of Chromatography A 1216, 1421-1430.

Giacobbe, M.G., Penna, A., Ceredi, A., Milandri, A., Poletti, R., Yang, X., 2000. Toxicity and ribosomal DNA of the dinoflagellate Dinophysis sacculus (Dinophyta). Phycologia 39, 177-182.

Gonzalez-Romero, R., Suarez-Ulloa, V., Rodriguez-Casariego, J., Garcia-Souto, D., Diaz, G., Smith, A., Pasantes, J.J., Rand, G., Eirin-Lopez, J.M.., 2017. Effects of Florida Red Tides on histone variant expression and DNA methylation in the Eastern oyster *Crassostrea virginica*. Aquatic Toxicology 186, 196-204.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Hayat, A., Barthelmebs, L., Marty, J.-L., 2011. Enzyme-linked immunosensor based on super paramagnetic nanobeads for easy and rapid detection of okadaic acid. Analytica Chimica Acta 690, 248-252.

Ki, J.S., Jang, G.Y., Han, M.S., 2004. Integrated method for single-cell DNA extraction, PCR amplification, and sequencing of ribosomal DNA from harmful dinoflagellates Cochlodinium polykrikoides and Alexandrium catenella. Marine Biotechnology 6, 587-593.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. Journal of Molecular Evolution 16, 111-120.

Kreuzer, M.P., O'Sullivan, C.K., Guilbault, G.G., 1999. Development of an ultrasensitive immunoassay for rapid measurement of okadaic acid and its isomers. Analytical Chemistry 71, 4198-4202.

Kreuzer, M.P., Pravda, M., O'Sullivan, C.K., Guilbault, G.G., 2002. Novel electrochemical immunosensors for seafood toxin analysis. Toxicon 40, 1267-1274.

Lindahl, O., Lundve, B., Johansen, M., 2007. Toxicity of Dinophysis spp. in relation to population density and environmental conditions of the Swedish west coast. Harmful Algae 6 (2), 218-231.Leonardo, S., Rambla-Alegre, M., Samdal, I.A., Miles, C.O., Kilcoyne, J., Diogène, J., O'Sullivan, C.K., Campàs, M., 2017a. Immunorecognition magnetic supports for the development of an electrochemical immunoassay for azaspiracid detection in mussels. Biosensors and Bioelectronics 92, 200-206.

Leonardo, S., Toldrà, A., Campàs, M., 2017b. Trends and prospects on electrochemical biosensors for the detection of marine toxins. Comprehensive Analytical Chemistry.

MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P., Suzuki, T., Selwood, A., 2005. Pectenotoxin and okadaic acid-based toxin profiles in Dinophysis acuta and Dinophysis acuminata from New Zealand. Harmful Algae 4, 75-85.

Mountfort, D.O., Suzuki, T., Truman, P., 2001. Protein phosphatase inhibition assay adapted for determination of total DSP in contaminated mussels. Toxicon 39, 383-390.

Pizarro, G., Morono, A., Paz, B., Franco, J.M., Pazos, Y., Reguera, B., 2013. Evaluation of passive samplers as a monitoring tool for early warning of dinophysis toxins in shellfish. Marine Drugs 11, 3823-3845.

Raho, N., Rodríguez, F., Reguera, B., Marín, I., 2013. Are the mitochondrial cox1 and cob genes suitable markers for species of Dinophysis Ehrenberg? Harmful Algae 28, 64-70.

Reguera, B., Riobó, P., Rodríguez, F., Díaz, P., Pizarro, G., Paz, B., Franco, J., Blanco, J., 2014. Dinophysis toxins: Causative organisms, distribution and fate in shellfish. Marine Drugs 12, 394.

Reguera, B., Velo-Suárez, L., Raine, R., Park, M.G., 2012. Harmful Dinophysis species: A review. Harmful Algae 14, 87-106.

Reverté, L., de la Iglesia, P., del Rio, V., Campbell, K., Elliott, C.T., Kawatsu, K., Katikou, P., Diogène, J., Campas, M., 2015. Detection of tetrodotoxins in puffer fish by a self-assembled mono layer-based immunoassay and comparison with surface plasmon resonance, LC-MS/MS, and mouse bioassay. Analytical Chemistry 87, 10839-10847.

Reverté, L., Prieto-Simón, B., Campàs, M., 2016. New advances in electrochemical biosensors for the detection of toxins: Nanomaterials, magnetic beads and microfluidics systems. A review. Analytica Chimica Acta 908, 8-21.

Sassolas, A., Catanante, G., Hayat, A., Stewart, L.D., Elliott, C.T., Marty, J.L., 2013a. Improvement of the efficiency and simplification of ELISA tests for rapid and ultrasensitive detection of okadaic acid in shellfish. Food Control 30, 144-149.

Sassolas, A., Hayat, A., Catanante, G., Marty, J.-L., 2013b. Detection of the marine toxin okadaic acid: Assessing seafood safety. Talanta 105, 306-316.

Shestowsky, W.S., Quilliam, M.A., Sikorska, H.M., 1992. An idiotypic antiidiotypic competitive immunoassay for quantitation of okadaic acid. Toxicon 30, 1441-1448.

Stewart, L.D., Elliott, C.T., Walker, A.D., Curran, R.M., Connolly, L., 2009a. Development of a monoclonal antibody binding okadaic acid and dinophysistoxins-1, -2 in proportion to their toxicity equivalence factors. Toxicon 54, 491-498.

Stewart, L.D., Hess, P., Connolly, L., Elliott, C.T., 2009b. Development and single-laboratory validation of a pseudofunctional biosensor Immunoassay for the detection of the okadaic acid group of toxins. Analytical Chemistry 81, 10208-10214.

Suarez-Ulloa, V., Gonzalez-Romero, R., Eirin-Lopez, J.M., 2015. Environmental epigenetics: A promising venue for developing next-generation pollution biomonitoring tools in marine invertebrates. Marine Pollution Bulletin 98, 5-13.

Suarez-Ulloa, V., Fernandez-Tajes, J., Aguiar-Pulido, V., Rivera-Casas, C., Gonzalez-Romero, R., Ausio, J., Mendez, J., Dorado, J., Eirin-Lopez, J.M., 2013a. The CHROMEVALOA database: a resource for the evaluation of Okadaic Acid contamination in the marine enviornment based on the chromatin-associated transcriptome of the mussel Mytilus galloprovinciales. Marine Drugs 11, 830-841.

Suarez-Ulloa, V., Fernandez-Tajes, J., Manfrin, C., Gerdol, M., Venier, P., Eirin-Lopez, J.M., 2013b. Bivalve omics: state of the art and potential applications for the biomonitoring of harmful marine compounds. Marine Drugs 11, 4370-4389.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731-2739.

Utermöhl, V.H., 1931. Neue Wege in der quantitativen Erfassung des Planktons. (Mit besondere Beriicksichtigung des Ultraplanktons). Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie 5, 567-595.