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Marine biotoxins in the Catalan littoral: could biosensors be integrated into monitoring programmes?

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Resum

Aquest article descriu els sensors enzimàtics i immunosensors electroquímics que s'han desenvolupat als nostres grups per a la detecció de la biotoxina marina àcid okadaic (OA), i discuteix la possibilitat d'integrar-los en programes de seguiment. Els sensors enzimàtics per a OA que es presenten es basen en la inhibició de la proteïna fosfatasa (PP2A) per aquesta toxina i la mesura electroquímica de l'activitat enzimàtica mitjançant l'ús de substrats enzimàtics apropiats, electroquímicament actius després de la seva desfosforació per l'enzim. Els immunosensors electroquímics descrits en aquest article es basen en un enzimoimmunoassaig sobre fase sòlida competitiu indirecte (ciELISA), amb fosfatasa alcalina (ALP) o peroxidasa (HRP) com a marcatges, i un sistema de reciclatge enzimàtic amb diaforasa (DI). Els biosensors presentats aquí s'han aplicat a l'anàlisi de dinoflagel·lats, musclos i ostres. Les validacions preliminars amb assaigs colorimètrics i LC-MS/MS han demostrat la possibilitat d'utilitzar les bioeines desenvolupades per al cribratge preliminar de biotoxines marines en mostres de camp o de cultiu, que ofereixen informació complementària a la cromatografia. En conclusió, tot i que encara cal optimitzar alguns paràmetres experimentals, la integració dels biosensors a programes de seguiment és viable i podria proporcionar avantatges respecte a altres tècniques analítiques pel que fa al temps d'anàlisi, la simplicitat, la selectivitat, la sensibilitat, el fet de poder ser d'un sol ús i l'efectivitat de cost.

Paraules clau: biosensor · floració algues nocives (FAN) · toxina marina · marisc · programa de seguiment · àcid okadaic

Abstract

This article describes the electrochemical enzyme sensors and immunosensors that have been developed by our groups for the detection of marine biotoxin okadaic acid (OA), and discusses the possibility of integrating them into monitoring programmes. The enzyme sensors for OA reported herein are based on the inhibition of immobilised protein phosphatase 2A (PP2A) by this toxin and the electrochemical measurement of the enzyme activity through the use of appropriate enzyme substrates, which are electrochemically active after dephosphorylation by the enzyme. The electrochemical immunosensors described in this article are based on a competitive indirect Enzyme-Linked ImmunoSorbent Assay (ciELISA), using alkaline phosphatase (ALP) or horseradish peroxidase (HRP) as labels, and an enzymatic recycling system with diaphorase (DI). The biosensors presented herein have been applied to the analysis of dinoflagellates, mussels and oysters. Preliminary validations with colorimetric assays and LC-MS/MS have demonstrated the possibility of using the developed biotools for the preliminary screening of marine biotoxins in field or cultured samples, offering complementary information to chromatography. In conclusion, although optimisation of some experimental parameters is still required, the integration of biosensors into monitoring programmes is viable and may provide advantages over other analytical techniques in terms of analysis time, simplicity, selectivity, sensitivity, disposability of electrodes and cost effectiveness.

Keywords: biosensor · Harmful Algal Bloom (HAB) · marine toxin · shellfish · monitoring programme · okadaic acid

Abbreviations

ACA: *Agència Catalana de l'Aigua*
AChE: Acetylcholinesterase
ALP: Alkaline Phosphatase
AOAC: Association of Analytical Communities
ASP: Amnesic Shellfish Poisoning
AZA: Azaspiracid
CSIC: *Consejo Superior de Investigaciones Científicas*
DA: Domoic Acid
DGPAM: *Direcció General de Pesca i Acció Marítima*
DI: Diaphorase
DSP: Diarrheic Shellfish Poisoning
DTX: Dinophysistoxin
EC: European Community
ELISA: Enzyme-Linked Immunosorbent Assay
GTX: Gonyautoxin

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HAB: Harmful Algal Bloom
HPLC-FLD: High Performance Liquid Chromatography Fluorescence Detection
HRP: Horseradish Peroxidase
IC: Inhibition Coefficient
IRTA: *Institut de Recerca i Tecnologia Agroalimentàries*
LC-MS: Liquid Chromatography Mass Spectrometry
LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry
LC-UV: Liquid Chromatography UltraViolet Detection
LOD: Limit of Detection
MBA: Mouse Bioassay
MC: Microcystin
MPMS: 5-Methyl-Phenazinium Methyl Sulphate
OA: Okadaic Acid
OVA: Ovalbumin
p-AP: *p*-Aminophenol
p-APP: *p*-Aminophenyl Phosphate
PaTX: Palytoxin
PbTx: Brevetoxin
PP: Protein Phosphatase
PSP: Paralytic Shellfish Poisoning
PTX: Pectenotoxin
SPR: Surface Plasmon Resonance
STX: Saxitoxin
TTX: Tetrodotoxin
XRAq: *Xarxa de Referència d'R+D+I en Aqüicultura*
YTX: Yessotoxin

1 Introduction

Biosensors are analytical devices incorporating a biological material (e.g. tissue, microorganisms, cells, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.) associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical. Biosensors usually yield a signal that is proportional to the concentration of a specific analyte or group of analytes. Electrochemical biosensors combine the high affinity of biochemical interactions with the inherent sensitivity of electrochemical techniques, providing low limits of detection. Electrochemical techniques enable miniaturisation, automation and portability of biosensors, making them interesting for *in situ* monitoring. Moreover, electrochemical biosensors are simple to use and cost effective, resulting in interesting bioanalytical tools for fast preliminary screenings.

This article describes the electrochemical enzyme sensors and immunosensors that the *Université de Perpignan* has developed and that are presently undergoing further development and validation at the *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) for the detection of marine biotoxins. It also presents the advantages and limitations of these biosensors and discusses the possibility of integrating them into water, shellfish and harmful phytoplankton monitoring programmes.

2 Marine biotoxins in Catalonia

The main marine biotoxins detected in the Mediterranean Sea are those related with Paralytic Shellfish Poisoning (PSP), intoxications due to lipophilic toxins previously known as Diarrheic Shellfish Poisoning (DSP) toxins, and Amnesic Shellfish Poisoning (ASP). Below, the characteristics of these toxins are briefly summarised:

- PSP toxins are water-soluble and thermostable tetrahydropurine compounds, e.g. saxitoxin (STX), gonyautoxins (GTXs) and derivatives, which block sodium channels, affecting the propagation of the action potential. They may cause slight tingling, numbness in the mouth and extremities, prickly sensations in the fingertips and toes, burning of the lips and skin, dizziness, floating sensations, headaches, ataxia, fever and even death by cardiorespiratory failure. The toxin producing dinoflagellate species *Alexandrium catenella* [1-4], *A. minutum* [5-16] and *A. tamarense* [3] have been identified in the Catalan littoral, although only the two former have been involved in toxicity episodes.
- Lipophilic toxins include okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins (YTXs), pectenotoxins (PTXs) and azaspiracids (AZAs). Among them, OA, DTXs, YTXs and PTXs are recurrently found in our waters. OA and DTXs are liposoluble compounds mainly present in bivalve hepatopancreas. Their mechanism of action is based on the inhibition of protein phosphatases (PPs), enzymes that play an important role in protein dephosphorylation in cells. These toxins bind to the receptorial site of PP1 and PP2A, blocking their activity. As a consequence, hyperphosphorylation of the proteins that control sodium secretion by intestinal cells and of cytoskeletal or junctional moieties that regulate solute permeability is favoured, causing a sodium release and a subsequent passive loss of fluids [17]. The main symptom of human intoxication with OA and DTXs is diarrhoea (this explains why they have also been called DSP toxins), although other effects are also relevant, such as nausea, vomiting and abdominal pain. Studies carried out with animals show OA and DTXs to be potent tumour promoters and possible mutagenic and immunotoxic agents. The main producer species registered in the Catalan littoral are: *Dinophysis sacculus* [3,18,19], *D. caudata* [3], *D. rotundata* [3], but only *D. sacculus* and *D. caudata* have been involved in DSP episodes. Another potential lipophilic toxin producing microalgae present in Catalonia is the benthonic dinoflagellate *Prorocentrum lima* (detected in the monitoring programme). YTXs are lipophilic toxins, although not implicated in DSP episodes in humans. They are mainly produced by the dinoflagellates *Lingulodinium polyedrum*, *Gonyaulax spinifera* and *Protoceratium reticu-*

latum, the latter having been detected in the monitoring programme. These toxins may interfere with the mouse bioassay (MBA) for other lipophilic toxins. In this case, their discrimination with specific bioassay and/or chemical methods is necessary.

- ASP toxins include domoic acid (DA), a potent neurotoxin water-soluble acidic amino acid, and its derivatives. They are kainoid excitatory neurotransmitters that bind to specific receptor proteins causing depolarisation of the neuronal cells and their subsequent rupture. Symptoms due to their ingestion are both gastrointestinal (nausea, vomiting, diarrhoea, abdominal cramps) and neurological (memory loss). These symptoms may be combined with optical problems, such as disconjugate gaze, diplopia and ophthalmoplegia. In cases of severe intoxication, major neurological deficits are usually described, involving confusion, mutism, seizures, autonomic dysfunction, lack of response to painful stimuli and uncontrolled crying or aggressiveness, sometimes leading to coma and death. Several diatom species, such as *Pseudo-nitzschia delicatissima* and *Pseudo-nitzschia pseudodelicatissima* have been identified as potential ASP toxin producers in the Mediterranean [20].

Apart from the previously described toxins, there are other toxins, such as spirolides, gymnodimines and palytoxins (PaTXs) that, although eventually marginal, deserve consideration in research projects in order to understand their importance in our waters. It is also necessary to take into account toxins such as ichthyotoxins that affect fauna, including shellfish, but can also cause fish mortality. In Catalan waters, two producer species have been registered, *Karlodinium veneficum* and *K. armiger* [21,22], both being involved in harmful episodes [3,9,23,24]. *Karlodinium* spp. were detected for the first time in winter 1994-1995 in Alfacs Bay and, since then, it has been proliferating variably, creating serious problems to shellfish aquaculture producers [25,26].

3 Biosensors for harmful algae and marine biotoxins

3.1 State-of-the-art

Only a few biosensors for harmful algae and marine biotoxins have been reported. Whereas most sensors for microalgae are based on the detection of the hybridisation of specific sequences by electrochemical [27,28] or gravimetric methods [29], most biosensors for marine biotoxins are based on the use of monoclonal or polyclonal antibodies as biorecognition molecules and electrochemical [30-33], gravimetric [34] or optical detection methods [35-38]. In this direction, biosensors for OA [30,32,34,36,38], DA [30,31,33,36], STXs [37], brevetoxin-3 (PbTx-3) [30], tetrodotoxin (TTX) [30] and GTXs [37] have been described. In the development of electrochemical biosensors for PSP toxins, sodium channels have also been exploited [39,40]. Recently, neuronal networks have been cultured over electrode arrays in order to detect TTX [41], PbTx-3, STX [42] and AZA-1 [43] by electrical methods.

The main objective of these studies was the development of the biosensor. Only a few biosensors have been validated by implementing the analysis of field samples. Micheli et al. [33] applied their electrochemical immunosensor to the analysis of DA in spiked mussels and certified material, and compared the results with those obtained by spectrophotometric ELISA and high performance liquid chromatography (HPLC), obtaining good agreement and comparable working ranges and limits of detection (LODs). Marquette et al. [35] also used spiked mussels for the analysis of OA with their chemiluminescent immunosensor and studied the stability and reproducibility of the measurements, although in this case they did not compare their biosensor with other analytical techniques. The biosensors that we have developed have been applied to the analysis of naturally contaminated shellfish samples and cultured dinoflagellate extracts. This represents an advantage with respect to the work performed by Micheli and Marquette, since the use of field samples, despite complicating the validation of the biosensor due to their multi-toxin profiles, provides a closer approach to the reality and may explain actual toxicity episodes. It is fair to mention the work performed by Cheun et al. [39,40], who have applied their sodium channel-based biosensor to the analysis of PSP toxins in naturally contaminated puffer fish and crab. However, this biosensor uses frog bladder membrane as a biorecognition element, with the consequent drawbacks characteristic of all tissue-based biosensors, in terms of diffusional limitations, long analysis times and low specificities.

Undoubtedly, the most interesting work was that done by Fonfría, Llamas et al. [37,38] on the analysis of shellfish samples by surface plasmon resonance (SPR)-based immunosensors. These studies assess the matrix effects produced by a high variety of shellfish species (mussels, clams, cockles, scallops and oysters) and toxin extraction methods, analyse a relevant number of naturally contaminated samples with the biosensors, use calibration curves constructed with mollusc extracts for the quantification and compare the results with those obtained by the MBA and chromatographic techniques. In our case, these studies are pending tasks that are being undertaken, together with purification of field samples by fractionation protocols. Once the applicability studies have been performed, our electrochemical biosensors may provide added advantages in terms of miniaturisation and portability of the devices, practically impossible aspects when using SPR devices, and attainable limits of detection (although this may not be relevant if maximum toxins levels permitted by current legislations are already detected).

3.2 Our contribution

IRTA and the *Université de Perpignan* have joined efforts to create a new research line for the development of biosensors for marine toxins. The *Université de Perpignan* is experienced in the development of electrochemical biosensors, whereas the expertise of IRTA lies in the treatment and analysis of natural samples of marine origin. This complementary approach enabled us to tackle integrated projects, achieving not only the development of analytical devices but also their validation with applicability studies involving field samples.

The *Université de Perpignan* has been devoted for many years to the development of electrochemical biosensors for the detection of pesticides, such as carbaryl, carbofuran and pirimicarb carbamate, using acetylcholinesterase (AChE) and genetically engineered AChE-derived mutants as biorecognition molecules [44]. More recently, the *Université de Perpignan* has developed PP inhibition-based sensors for the detection of microcystins (MCs), cyanobacterial toxins usually present in freshwater [45], and OA, a phycotoxin. These biotoxins and others, such as the mycotoxin ochratoxin A, have also been detected by electrochemical immunosensors, using monoclonal or polyclonal antibodies [46,47].

IRTA is responsible for the monitoring of the quality of waters in shellfish harvesting areas of Catalonia and this includes the evaluation of the presence of microbiological and chemical contaminants as well as marine toxins and harmful algal blooms (HABs). This activity has resulted in several scientific studies [26,48-55]. Other interests of the IRTA include the development and implementation of cytotoxicity assays as possible alternative methods to the MBA [56-59], the use of chromatographic and mass spectrometry techniques for the evaluation of toxin profiles [57-59], the study of the ecophysiology and toxicity of *Karlodinium* [21-26], *Prorocentrum* [58] and *Gambierdiscus* spp. [60,61], and the development of molecular tools for the identification of *Pseudo-nitzschia* spp. [62].

In the construction of a biosensor, the first step is the validation of the recognition event and the optimisation of the experimental parameters, usually performed by colorimetric assays. In our case, this refers to the ability of OA to inhibit PP2A and the ability of a monoclonal antibody to recognise OA. Once the biorecognition is demonstrated, the second step is the transfer from assay to biosensor, which is achieved by carefully choosing the supports where the biorecognition element will be immobilised and designing appropriate transduction schemes, both dependent on the choice of transducer system. Our efforts have been focused on sorting out immobilisation and transduction, which are two key points in the construction of electrochemical biosensors.

3.2.1 PP2A enzyme sensors for OA

Taking into consideration the analogy regarding the mechanism of action of MCs and OA and derivatives, both groups inhibiting PPs, the *Université de Perpignan* has recently developed an enzyme sensor for OA, which has been applied to the detection of this toxin in dinoflagellate extracts [63]. The strategy is based on the entrapment of PP2A into a polymeric network for its immobilisation onto the electrode support, the reversible inhibition of this enzyme by OA, and the recording of the residual activity by chronoamperometry using appropriate PP2A substrates, electrochemically active only after dephosphorylation by the enzyme (Figure 1). First, it was necessary to choose the appropriate phosphatase enzyme. Colorimetric assays demonstrated that PP2A was more sensitive to OA than PP1. Next, PP2A was immobilised on different supports and using different techniques. Among them, the best immobilisation yields were achieved by entrapment into a photopolymeric matrix and on screen-printed carbon electrodes. The enzyme immobilisation maintained the biomolecule in a flexible conformation and substantially retained the stability of the enzyme activity, usually a critical limitation of PP2A. Several enzyme substrates were evaluated, the laboratory-synthesised catechyl monophosphate providing background-subtracted currents higher than those obtained with *p*-aminophenyl phosphate (*p*-APP), at appropriate potentials (+450 mV vs. Ag/AgCl). Different amounts of enzyme were immobilised on the electrodes. The results showed that the higher the enzyme activity, the higher the electrochemical response and reliability of the measurement, but also the higher the limits of detection. The most sensitive biosensor had an LOD (here defined as 20% inhibition) for OA of 6.42 μg L^{-1}, a 50% inhibition coefficient (IC50) value of 22.19 μg L^{-1}, and a working range of 2.69 – 171.87 μg L^{-1}. The device was applied to the analysis of toxin contents in dinoflagellate extracts provided by IRTA. Comparison with the colorimetric PP inhibition assay and with Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis demonstrated the viability of the approach.

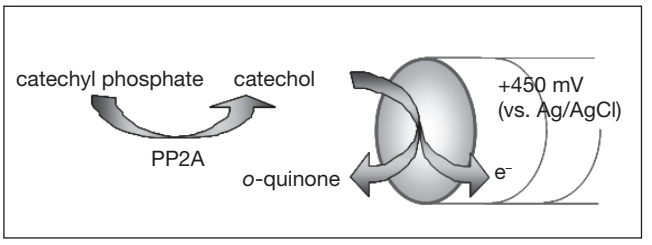

Figure 1. Enzymatic reaction between protein phosphatase 2A and catechyl phosphate and subsequent catechol detection on the electrode surface.

3.2.2 Immunosensors for OA

Electrochemical immunosensors for the detection of OA have also been developed jointly by the *Université de Perpignan* and IRTA [64]. These immunosensors are based on competitive indirect Enzyme-Linked ImmunoSorbent Assays (ciELISAs). An OA-ovalbumin (OA-OVA) conjugate was immobilised on screen-printed carbon electrodes and competition of a mono-

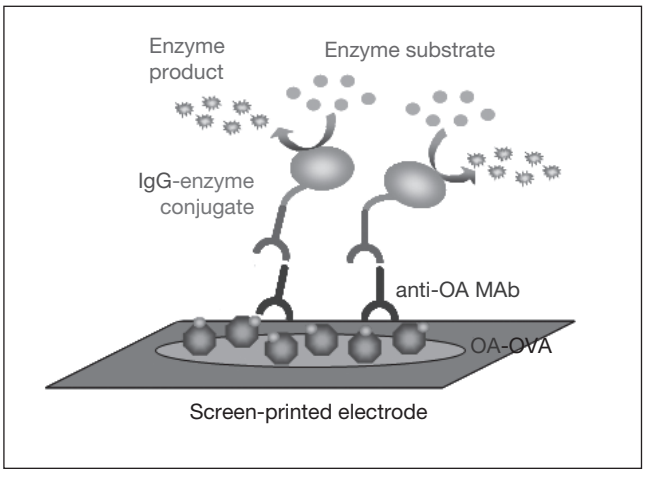

Figure 2. Competitive indirect immunosensor: competition between immobilised OA-OVA and free OA for the anti-OA MAb in solution is followed by incubation with enzyme-labelled IgG conjugate and enzyme label detection.

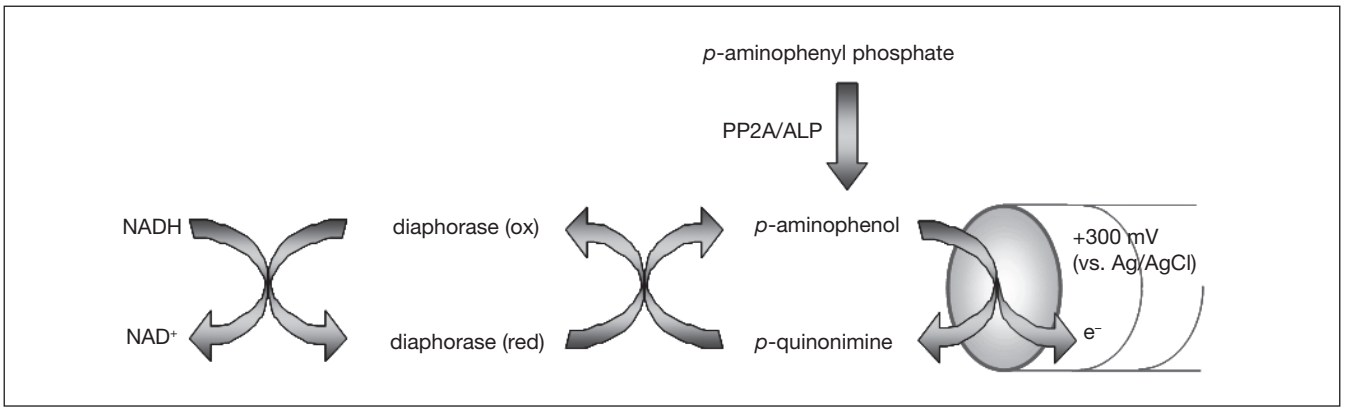

Figure 3. Reactions involved in the enzymatic signal amplification for the detection of the ALP or PP2A activity.

clonal antibody for free and immobilised OA was subsequently performed. Secondary antibodies labelled with alkaline phosphatase (ALP) or horseradish peroxidase (HRP) were used for signal generation (Figure 2). Electrochemical transduction depended on the label enzyme: whereas +300 mV vs. Ag/AgCl were applied in the detection of *p*-aminophenol (*p*-AP) produced by the reaction of *p*-APP with ALP, -200 mV vs. Ag/AgCl were used in the detection of 5-methyl-phenazinium methyl sulphate (MPMS), redox mediator in the HRP bioelectrocatalysis. The LODs (defined as 80% of MAb binding) for OA with standard solutions were approximately 1 and 2 µg L^{-1} when using ALP and HRP labels, respectively. It is necessary to mention that the limit for the ALP approach was lower than those reported for other electrochemical immunosensors [30-32], even though the assay was indirect. An electrochemical signal amplification system based on diaphorase (DI) recycling, already tested in the detection of MCs by PP2A [65], was integrated into the ALP-based immunosensor. The detection principle is based on the ability of DI to recycle *p*-AP, and thus to amplify the electrochemical signal arising from its oxidation (Figure 3). The amplification strategy decreased the LOD for OA to 0.03 µg L^{-1} and enlarged the working range by more than one order of magnitude. Mussel and oyster extracts from the Ebre Delta embayments were analysed with the immunosensor that integrated the amplification system, and results were compared with those obtained by the colorimetric immunoassay, the PP inhibition assay and LC-MS/MS, and correlations demonstrated the viability of the approach.

4 The monitoring programme

4.1 The purpose

As stated before, IRTA is responsible for the monitoring of the quality of waters in the shellfish harvesting areas of Catalonia, a public service commissioned by the *Direcció General de Pesca i Acció Marítima* (DGPAM), a body belonging to the *Generalitat de Catalunya,* and performed in collaboration with the *Consejo Superior de Investigaciones Científicas* (CSIC). The information provided by this monitoring programme has immediate impact on the administration of shellfish harvesting areas, but also on the productive sector and the international scientific community.

The monitoring programme was established to fulfil legislation on a Catalan, Spanish and European level. Nevertheless, the data obtained by the monitoring programme has also substantially contributed to the advancement of technical and scientific issues, and to fulfilling descriptive studies and long-term analysis, fundamental for the comprehension of the characteristics of the region.

4.2 Monitoring areas

The monitoring programme covers approximately 580 km of the Catalan coastline and focuses on shellfish production areas in beaches and bays. Additionally, recreational and industrial harbours are also monitored for the presence of harmful toxin producing algae, since these areas may be potential sources of HABs and associated toxins. Figure 4 shows the mollusc and other marine invertebrate production areas established in the Catalan littoral [66-67]. The number of production areas covering

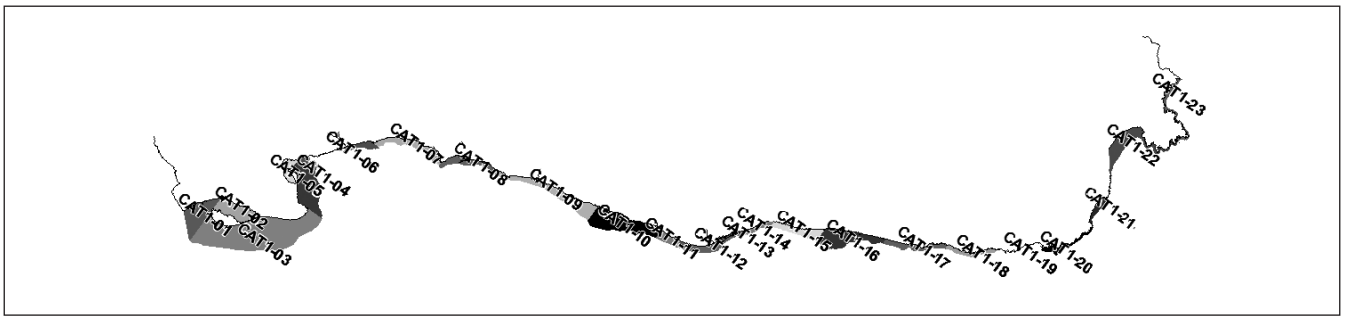

Figure 4. Map of the production areas in Catalonia (source: *Sistema d'Informació Geogràfica* of the *Direcció General de Pesca i Acció Marítima*, http://www.gencat.net/darp/c/pescamar/sigpesca/csig07.htm).

Table 1. Current practices in Catalonia regarding the management of toxin analysis methods in shellfish

Family	*Group*	*Primary Method**	*Confirmatory Method***
PSP	STX group	MBA[a]	LC-FLD[b]
Lipophilic DSP	OA group	MBA[c]	LC-MS/MS
	PTX group	MBA[c]	LC-MS/MS
Other lipophilic	YTX group	MBA[c]	LC/MS-MS
	AZA group	MBA	LC-MS/MS
ASP	DA group	LC-UV[d]	LC-MS/MS
Others	Spirolids and gymnodimines	MBA[c]	LC-MS/MS

* Primary method may be the reference test or a screen that is followed by a reference test

** Confirmatory method may be the reference test or validated alternatives

[a] [68]

[b] pre-column [69] and post-column [70]

[c] [71,72]

[d] [73-75]

the totality of the Catalan littoral is 23. The monitoring programme is an evolving platform adaptable to changes regarding not only the harvesting areas, but also changes due to new legislation and technical improvements. Changes that may affect the shoals and/or water quality, such as the construction or modification of sewers, arid movements and strategic changes in the culture and/or exploitation of species, are also taken into account.

In the production areas, the major sites for shellfish production include natural banks in open areas but also natural banks and aquaculture facilities for shellfish in coastal embayments. Among shellfish species in natural banks we find *Donax trunculus* (clam), *Cerastoderma edule* (cockle), *Tapes semidecussata* (Manila clam), *Callista chione* (smooth clam), *Bolinus brandaris* (purple dye murex). In semi-enclosed coastal embayments, aquaculture facilities are aimed at the production of *Mytilus galloprovincialis* (mussel) and *Crassostrea gigas* (oyster). The wide spectrum of harmful algae and associated toxins, and the variety of harvested shellfish species increases the complexity of technical strategies for toxin identification within the monitoring programme, and this has had an immediate impact on the development phases of the construction of applicable biosensors. Table 1 describes the current methods of analysis of the main marine toxins in shellfish flesh.

5 Could biosensors be integrated into monitoring programmes?

The use of biosensors to detect and quantify marine toxins and thus assess the quality of shellfish is *a priori* possible. The successful implementation of biosensors could reduce or avoid the use of mice in toxicity evaluation and would provide complementary information to chromatographic techniques. However, this situation is presently hypothetical and advancement in that direction is only achievable through elaborate processes for specific groups of toxins.

Results obtained by our research projects have led to the conclusion that biosensors constitute a promising approach for toxin evaluation but important experimental parameters still need to be optimised to guarantee their applicability to monitoring programmes. The variety of toxins encountered in nature (according to structure and mechanism of action) as well as the variety of monitored shellfish species increase the complexity of any innovative approach for toxin determination.

The main advantages and drawbacks, as well as part of the work required to integrate biosensors into the monitoring programme, are now discussed.

5.1 Limitations and progress in PP2A enzyme sensors for OA

The LOD of the PP2A inhibition-based biosensor for OA was defined as the toxin concentration that produces 20% inhibition, which corresponded to 6.42 μg L^{-1}. Although lower concentrations could be detected, the low reproducibility associated to the electrochemical detection (compared to the colorimetric one) obliged us to choose this value in order for the screening to be reliable. This low reproducibility was probably associated to the manual enzyme deposition on the electrode and/or to the fouling of the electrode by electropolymerisation of the phenoxy radicals that appeared during the enzymatic reaction [76,77]. Although the purpose of the device was to provide a screening tool for fast and preliminary tests, a precise and accurate bioanalytical device would also be highly desirable. In order to improve the reliability of the biosensor, we are setting up the instrumentation for automated enzyme deposition and studying the use of non-phenolic enzyme substrates.

The enzyme immobilisation technique could also be improved. The encapsulation technique provided the protein phosphatase with a friendly microenvironment that preserved its functional activity, a crucial aspect considering the inherent instability of this enzyme. Nevertheless, the diffusion barrier created by the photopolymer hindered the accessibility of the enzyme by both the substrate and the toxin, resulting in low LOD values (compared to the colorimetric approach). Work is in progress to eliminate this diffusion barrier by using immobilisation through coordination binding of the enzyme to magnetic particles and deposition onto magnetised electrode supports.

The enzyme sensor provided toxin contents in the dinoflagellates comparable to the other detection techniques, although always slightly higher, the effect being more evident in samples with lower toxin amounts. This overestimation could be due to the fouling of the electrode by phenoxy radicals or some of the extracted components, which would decrease the steady-state oxidation currents, simulating an enzymatic inhibition. Matrix effects should thus be evaluated in order to discard possible false positives. The methanol content in extracted samples is also critical, since it may directly inhibit the enzyme activity. These two problems could be circumvented by the careful design of the extraction processes and, if necessary, fractionation protocols. Nevertheless, the overestimation should not be considered a limitation, but rather a safety level in the screening of suspicious samples. Detection of toxic activity in natural samples by this screening tool should be followed by analysis with com-

plementary techniques in order to confirm toxicity (e.g. MBA) and identify the toxins involved (e.g. HPLC and LC-MS/MS).

5.2 Limitations and progress in immunosensors for OA

In what concerns the applicability of the immunosensor for OA, the LOD was satisfactory, but the reproducibility of the measurements still needs to be improved, probably by assessing the matrix effects. OA quantifications using the immunosensor correlated very well with those obtained with the immunoassay, although toxin estimations were slightly lower with the immunosensor. This underestimation may be due to the adsorption of some matrix compounds on the electrode surface and the subsequent non-specific binding of the anti-OA MAb/secondary Ab conjugate on them, or to the direct electro-oxidation of these adsorbed matrix compounds. As with the enzyme sensor, matrix interferences could eventually be minimised by rationally selecting appropriate extraction and fractionation protocols as purification steps prior to biosensor implementation.

OA quantification provided by the immunosensor and the immunoassay were lower than those obtained by the PP inhibition assay. This is not surprising as these two assays differ in the recognition event: whereas the immunosensor is based on structural recognition of OA (and probably some derivatives with an analogous structure) by the antibody, the PP2A enzyme sensor is based on the inhibition of the enzyme activity by some of the lipophilic toxins. A multi-toxin profile in field samples may justify the higher OA equivalent contents found by the PP2A enzyme sensor. Natural samples may contain enzyme inhibitors, other than OA or analogues, not recognised by the OA antibody. Studies reporting inhibition data for OA analogues, in parallel with chromatographic information on individual toxin contents in field samples, may help to explain the higher toxin contents reported by the enzymatic assay in relation to the immunochemical techniques.

When compared with LC-MS/MS lipophilic toxin analysis, the immunosensor and the immunoassay slightly underestimated the OA toxin content, probably because the chromatographic method also detected OA-related toxins such as DTX-2 and 7-hydroxy-2-methyl-hepta-2,4-dienyl okadaate. Nevertheless, the immunosensor was able to quantify lower toxin contents than this technique, due to the higher sensitivity inherent in both the biorecognition event and the electrochemical detection. Since chromatographic detection showed that samples have a multi-toxin profile, toxins other than OA could be contributing to the equivalent contents found by the immunological techniques. In this context, cross-reactivity immunological studies with OA derivatives would be useful to better compare both approaches.

It is important to mention that the lack of specificity of the MBA, the official method of analysis, is well-recognised, and positive results with the MBA might be due to the presence of a toxin other than OA or derivatives recognised by the antibody, such as YTXs. Compared to the MBA, the immunosensor has the advantage of the selectivity towards OA and derivatives, and this is also true for the PP2A enzyme sensor.

An important factor to be considered is analysis time. The time required for each of the steps in the construction of the biosensor has not been optimised and consequently, the assay takes a minimum of 7-8 hours. This analysis time can certainly be reduced, by carefully modifying the experimental parameters of the assay and compromising the performance of each step. In addition to the calibration curve, which is required each day, the manipulation of modified electrodes and the use of a mono-potentiostat for the electrochemical detection increase the processing time. A solution to reduce the analysis time would be the use of automated devices for the construction of the immunosensor and of multi-potentiostats for simultaneous electrochemical measurements, which are already commercially available.

6 Conclusions

The European Union Commission Regulation (EC) No 2074/2005 of 5 December 2005 [78] recognises a series of methods, such as HPLC with fluorimetric detection (HPLC-FLD), liquid chromatography with mass spectrometry detection (LC-MS), immunoassays and functional assays, such as the PP inhibition assay, as potential alternative or supplementary methodologies to the biological testing methods for the determination of lipophilic toxins, provided that these methods are not less effective than biological methods and that their implementation provides an equivalent level of public health protection. From our perspective, this regulation favours the use of biosensors for the preliminary screening of lipophilic marine biotoxins in water, microalgae and bivalve samples. Although the use of biosensors has been successful in the detection and quantification of lipophilic toxins in the laboratory, these promising biotools still need to be validated by high-throughput applicability studies with multiple samples in order to find out whether their integration into monitoring programmes is possible. These studies should include the assessment of the matrix effects using different shellfish species, the optimisation of the extraction and fractionation protocols, the establishment of calibration curves using negative controls instead of buffered solutions, the acquisition of supplementary data about the inhibitory effect of toxins on phosphatases and the cross-reactivity of antibodies, and the integration of the biosensors into compact and miniaturised microfluidic devices.

Regardless of these requirements, the first steps towards their real applicability have already been covered successfully, and high performance analytical devices for the fast and preliminary screening of lipophilic marine biotoxins in marine samples have been developed. These biotools may provide advantages over other analytical techniques in terms of analysis time, simplicity, selectivity, sensitivity, disposability of electrodes and cost effectiveness. The implementation of biosensors in monitoring programmes will depend on scientific efforts and on methodical and exhaustive validation studies. Additionally, the generic and versatile nature of biosensors means that simply by slightly modifying the protocols in accordance with the specific requirements, they could be extrapolated to other marine toxins or even other compounds of interest in food quality control, health protection and environment preservation.

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Margarita Fernández-Tejedor*, biologist (University of Barcelona, 1987), has been conducting research into marine biology since 1988 starting at CEAB (CSIC, Blanes, Spain). In 1989 she moved to the Laboratory of Marine Microbiology (CNRS, Marseille, France) participating in several projects within the Joint Global Ocean Flux Study (JGOFS) and the European River Ocean System (EROS 2000). In 1996 she moved to NatMIRC (Swakopmund, Namibia) where she participated in the environmental marine monitoring for the management of marine resources. Since 1998 she has been a researcher at IRTA, where she is involved in the identification of harmful microalgae, phytoplankton population dynamics, the evaluation of environmental parameters and the study of ichthyotoxic dinoflagellates and the effects of their toxins on marine fauna. She has participated in several projects for the implementation of shellfish sanitation mo-nitoring programmes in Namibia and Angola.*

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