Universitat Autònoma de Barcelona

Integrating new immunoassays, biosensors and cytotoxicity assays in the detection and quantification of the emerging marine toxins tetrodotoxins and ciguatoxins

Laia Reverté Calvet

Doctoral thesis

Integrating new immunoassays, biosensors and cytotoxicity assays in the detection and quantification of the emerging marine toxins tetrodotoxins and ciguatoxins

Laia Reverté Calvet

Supervisors: Dr. Mònica Campàs i Homs and Dr. Jorge Diogène Fadini

Tutor: Dr. Francisco Valero Barranco

Sant Carles de la Ràpita, Tarragona

June 2017

“Investigar es ver lo que todo el mundo ha visto,
y pensar lo que nadie más ha pensado”

Albert Szent-Györgyi

Acknowledgements

No voldria començar els agraïments sense dir que la realització d'aquesta tesi ha estat possible gràcies a la contribució de moltes persones que d'una manera o altra han format part d'aquest camí ple de reptes i entrebancs. Per això, espero no deixar-me a ningú.

En primer lloc, voldria agrair a l'IRTA de Sant Carles de la Ràpita per donar-me l'oportunitat de fer el doctorat amb el grup de Seguiment del Medi Marí, i concedir-me la beca finançada pel projecte **ECSafeSEAFOOD** (FP7/2007-2013, grant agreement nº 311820).

Tot va començar al 2007, quan **Ana Roque**, investigadora de l'IRTA de Sant Carles de la Ràpita em va acollir per fer les practiques de la carrera. Amb 19 anys, ni tan sols era conscient de què llavors començaria a agafar forma el meu futur en el món de la investigació. De fet, com ella sempre em recorda anecdòticament: "era una adolescent que només pensava en les festes del poble". I, qui havia de dir que avui, després de 10 anys, (sí, 10 anys!), amb 30 anys, presento la tesi doctoral. Gràcies per dipositar la teva confiança en mi des de el principi i per la teva visió crítica i constructiva fins al final d'aquest camí.

A continuacio, vull dedicar una paràgraf a la persona que em va empentar, va confiar des de el primer dia en mi i que, fins i tot quan jo no volia fer el doctorat, va creure en les meves possibilitats i em va convèncer per arribar fins aquí. I, aquesta, com no podia ser d'altra, és **Mònica Campàs**. No tinc prou espai per expressar tot el que signifiques per a la meva vida. No obstant, ho intentaré. Gràcies per ser com ets, però no només com a investigadora, com ets com a persona i com ets amb mi. Gràcies per ensenyar-me el món dels BIOSENSORS i per transmetre'm amb tanta il·lusió el molt que t'agrada la teva feina. Gràcies per tot el que he aprés amb tú, tant a nivell personal com a nivell professional i gràcies per acompanyar-me en aquest camí, ajudar-me i valorar-me com només tú saps fer. No podria estar més agraïda per tot el que has fet per mi i estic ben segura que això és el principi d'una gran amistat.

Juntament amb Mònica, voldria agrair especialment a **Jorge Diogène** per donar-me l'oportunitat de portar a terme aquest doctorat i per la supervisió i direcció d'aquesta tesi. Voldria agrair-te el teu esperit crític i la llibertat que m'has donat per prendre decisions durant aquests 4 anys que m'han fet créixer i madurar com a persona i investigadora. Donar-te les gràcies per recolzar-me, motivar-me i transmetre'm tranquil·litat en els moments de més estrés, així com per ensenyar-me que a la vida les coses si es "modulen" i "coordinen" sempre van millor.

De **l'IRTA de la Ràpita**, vull agrair a tothom, des de l'administració per l'enviament de paquets i tot tipus de gestions (**David, M. Jesús, Romina..**), a **Mari Pau,** per tenir tot el material de laboratori net i a punt tant aviat com ho he necessitat, als de manteniment (**Davidet, Xavi i Iván**), per la seva col·laboració en quan algun equip no funcionava i, a **Josep**, pel suport informàtic i pels nostres cigarrets acompanyats d'una bona conversa. Però en especial, tinc que agrair als que m'han fet sentir com de la família, al meu grup, la **SMM**. **Patró,** gràcies perquè sense els teus "piropos" dels dilluns res hagués estat igual i, perquè ens "estàvem fumant damunt a tota hora"! Han passat moltes persones per aquest grup i, cadascú, ha deixat una petjada: **Núria** (un immens gràcies perquè les cèl·lules no estarien vives si no m'haguessis ajudat i recolzat quan pensava que estava tot

perdut), **Pablito** (pel teu bon humor amb frases com "no està el forn per a pastissets"), **Esther** ("pels ratets al microscopi i els menús japo"), **Laura Aza** ("per les fideuas al poblet"), **Carles** (o Joan, per la teva alegría i positivisme)**, Alexis** (el nostre "caveret")**, Marga** (per ensenyar-me "bitxets")... i com no, **Vane Castàn** perquè..."no t'equivòquigues", això no és final! Agrair-te el fàcil que m'ho vas posar tot quan vaig arribar a la SMM, perquè em vas obrir les portes a l'IRTA i a la teva vida, perquè hem celebrat moltes coses juntes i les que queden per celebrar. Gràcies per ajudar-me pel lab quan no podia més i, per les teves brometes que sempre em treuen un somriure, per preguntar-me sempre com estic i animar-me quan ja acabava. Els nous, que no per això sou menys: **Maria**, **Laura, Maria i Jose Luis.**..Mil gràcies a tots, sou genials! Com no agrair també a la gent de pràctiques per la vostra ajuda, que sense vosaltres no hagués acabat mai: **Clara, Núria**, **Ferran, Pol, Carlos** i a la meva **Giada**, gràcias por querer tanto o más que yo a los *Gambierdiscus* y cuidar tan bién de ellos cuando yo no estaba, un gran GRÀCIES. També hi ha altra gent de l'IRTA que no ha passat desapercebuda com **David Mateu,** amb el qual hem celebrat molts moments junts i **Miriam**, sense els teus massatgets i les converses transcendentals les meves cervicals no ho haguessin aguantat!

A la meva segona família, el despatx del pis de dalt, els BECARIS, on he passat gran part del temps de l'IRTA, i amb els quals he compartit alegria, desesperació, locura, preocupacions, cansament, rises, dubtes, incerteses, treball i, així, mil coses més! **Maria**, **Lucía, Núria, Kruno, Diego, Zohar, Núria, Anna, Cindy, Àngels:** moltes gràcies per fer la ciència més divertida i ensenyar-me que hi ha vida social fóra de l'IRTA! I com no, **Olgueta,** per les mil aventures que hem viscut i les que ens falten, per ensenyar-me que amb esforç tot es pot aconseguir. Sempre "on fire max", no ho oblidaré mai!! Gràcies per valorar-me tant, ets una gran persona i una gran profesional. Estic segura que arribaràs allí on et proposes, però la próxima no fa falta que sigui tant lluny! Gràcies per la teva gran amistat. **Elvira,** la veïna del despatx, amb la qual vam conectar tan sols coneixe'ns, i amb la que hem compartit vides gairebé paral·leles i hem crescut juntes des de el primer fins a l'últim dia a l'IRTA. Donar-te les gràcies per la teva alegria, pels teus comentaris aberrants que sempre em treuen un somriure estigui com estigui i, sobretot, pels moments de locura que hem viscut juntes. En descriuria algun, però no seria adequat per a la tesi, tú ja m'entens! No canvies mai, ets única!

I què pensàveu que m'he oblidat de vosaltres? Impossible! A les meves BIOSENSOR GIRLS! **Diana**, **Sandra** i **Anna,** perquè no es pot tenir un millor equip de treball i pel qual n'estic més que orgullosa. A mi **xamaki** wey, porqué eres única, porqué siempre has confiado en mi, porqué has estado a mi lado en todos los momentos, pero sobretodo gracias por compartir tu vida conmigo y dejarme quedar en ella. ¿Y que más? Quiero verte ya! **Sandreta,** gràcies per transmetre'm tranquil·litat quan més ho he necessitat, per alegrar-te pels èxits però encara més, per animar-me quan tot estava fosc. Vull donar-te les gràcies per ser tant bona amb mi, per escoltar-me, per soportar les meves queixes que no han sigut poques. Per tot lo que m'has ajudat durant aquests anys i per moltes coses més. Com t'enlloraré! Ara, em toca a mi animar-te perquè això ja casi ho tens i perquè aconseguiràs tot el que et proposis i molt més! I **Anna,** gràcies per la teva serenitat, perquè amb tú tot sembla que sigui més fàcil i que "xales" i "te'n rigues" durant i fins al final d'aquest camí.

A **Carmen**, la meva veïna de despatx. Perquè no hem aconseguit fer un foradet a la paret, però això no ens ha impedit entaular una gran amistat dins i fóra de l'IRTA. A tú, gràcies, gràcies per entendre'm en els

moments més durs del doctorat, gràcies per aguantar les incansables conversacions i deixar-me desfogar amb tú i, gràcies, perquè sense els cafès/tes de bon de matí res hauria tingut sentit. Ets un gran apoio per a mi i espero que poguem celebrar molts dels moments que encara ens queden per viure juntes!

I a la meva **Vane** (Vane 2), que vas començar sent una nova companya de feina i ara ets com la meva germana. Gràcies per ser la meva amiga, consellera i aliada, i per…molt més! Gràcies per ser un pilar a la meva vida, per la teva ajuda i apoio incondicional, per entendre'm només amb una mirada, per comprendre'm, per estar al meu costat SEMPRE, per compartir una infinitat d'aventures juntes i, perquè com diu el nostre estimat Sabina: "Nos sobran los motivos" per a continuar! Estiguis on estiguis i vagis on vagis SEMPRE estaras al meu camí i saps que estic al teu costat.

I would also like to say thanks to **Katrina Campbell** for giving me the opportunity to stay at Queen's Univeristy for 3 months and to learn a lot more than Irish. Thanks to **Sara McNamee** for your warm welcome in the lab and for your help in all I need. I, agrair també a **Celia, Anna, Ester, Irene** i **Gema** per fer endolcir-me les estades amb la vostra companyia i amistat.

A part de la gent relacionada amb l'IRTA hi ha unes personetes que sempre han estat al meu costat. Les amigues, la família que no es tria, la millor que mai m'hagués pogut tocar. Gràcies a totes i cadascuna de vosaltres: **Berta, Vero, Anna, Idoia (Ane), Joana i Maria (Chloe)** i al meu amic **Albert.** Per confiar en mi, per animar-me quan pensava que no ho aconseguiria, per alegrar-me els caps de setmana i ajudar-me a desconnectar, per preocupar-vos per mi i per ser com sou: les millors!

A les meues "xurritas" de la carrera i amigues: **Noe**, **Maria, Laura y Mai.** Gracias por entrar en mi vida, por ser una gran fuente de motivación, por cargarme de energía en cada encuentro y por animarme y confiar en mí desde el primer día. Gracias por seguir en mi camino y espero que Alex sea una de muchas alegrías que tengamos en un futuro.

Però a qui de tot cor he d'agrair que aquesta tesi hagi arribat a bon port és a la meva família: **mama, papa** i germana **Gemma**. Vosaltres sou els qui m'heu impulsat i encoratjat perquè faci a la vida allò que més m'agradi i m'heu ensenyat que amb esforç, dedicació i sacrifici tot és pot aconseguir. Gràcies per la vostra paciència, per aguantar les meves tonteries i mal humor, per la plena confiança que sempre heu dipositat en mi, per ajudar-me a aixecar quan més enfonsada estava i, sobretot, pel vostre amor incondicional. Per això i per molt més: mil milions de gràcies! A la resta de **família**, per estar sempre preguntant si els experiments havien sortit bé. A les meves **iaies,** perquè sense entendre gaire bé que és això del doctorat m'heu acompanyat en tots els moments i heu estat orgulloses de tenir una neta "investigadora". Sempre recordaré amb un gran somriure les vostres preguntes:"com están els musclos enguany, podem menjar-ne? I les cèl·lules, están vives?" A **Karla,** gràcies per fer-me molts dels dibuixets de la tesi, molts d'ells a contrarellotge.

També voldria agrair a la meva família política: **Alejandrina, Toni** i a la "cunyis": **Angels**, **Pablo** i la meva filloleta **Elia,** pel seu carinyo i apoio durant aquesta anys i per fer-me sentir especial i com una més de la família. Estic encantada de tenir-vos.

I, finalment, al meu **Toninet**, a qui juntament amb els meus pares i germana, dedico aquesta tesi. El qui realment ha aguantat els moments més incerts d'aquests anys, qui m'ha animat en els mals moments, qui ha celebrat els bons moments, qui m'ha estimat i recolzat infinitament per a que aconsegueixi el que vulgui, qui m'ha motivat perquè continués. I, el qui ha esperat amb paciència i comprensió que s'acabi aquesta etapa per construir el nostre futur junts. Per això i per molt molt més un infinit gràcies perquè sense tú no hagués sigut possible!

Summary

Considering the food safety risk emerging marine toxins such as tetrodotoxins (TTXs) and ciguatoxins (CTXs) present in seafood may pose to consumers, there is a real need to develop new methods capable of detecting these toxins in food, and thus, ensure consumers protection.

To achieve this goal, this thesis has been mainly focused on the development of immunoassays, cytotoxic assays and immunosensors for the detection of emerging marine toxins implicated in seafood safety: TTXs and CTXs. The rapid, cost-effective, sensitive and reliable bioanalytical tools described herein are promising early warning tools alternative to the reference methods for the screening and quantification of TTXs and CTXs in fish, shellfish and microalgae.

Chapter 1 consists of a general introduction about the current state of art of the emerging marine toxins TTXs and CTXs. This chapter describes the problematic issues derived from the presence of these toxins in seafood products for human health. It also describes how relevant is the development of methods for their detection.

Chapter 2 includes the general and specific objectives of the thesis.

Chapter 3 comprises two parts. The first one gives a detailed overview of the chemical properties, mechanisms of action, human health implications of several emerging marine toxins, and the different alternative biochemical methods for their detection. In the second part, the advances that offer the integration of bionanotechnology materials in the development of electrochemical immunosensors for the detection of toxins are described.

Chapter 4 includes 5 scientific contributions. In the first work, an enzyme-linked immunosorbent assay based on the use of self-assembled monolayers for the TTX immobilisation on maleimide plates (mELISA) is developed. The applicability of the mELISA to the analysis of puffer fish samples is evaluated. In the second work, the mELISA is modified in order to decreased the assay in time and cost and adapted to enable the quantification of TTXs in oysters and mussels. In the third work, the SAM-based immobilisation approach employed in the mELISA is used for the development of an electrochemical immunosensor, and the applicability of the later to the analysis of puffer fish is investigated. The fourth work describes the development of an optical planar waveguide

immunosensor based on the immobilisation of TTX on nanoarrayed scaffolds. The applicability of the biosensor to the analysis of puffer fish is also studied. Finally, the fifth contribution shows the usefulness of combining the mELISA as screening tool with the LC-MS/MS analysis as confirmatory technique for the quantification of TTXs contents in puffer fish from the Western Mediterranean coast.

Chapter 5 includes two scientific contributions. In the first one, a multi-disciplinary approach combining the use of cell-based assays (CBA), the mouse bioassay (MBA) and high-resolution mass spectrometry (HRMS) is described for the identification and quantification of CTXs in a shark implicated in a fatal food poisoning event in Madagascar. The second work demonstrates the suitability of the Neuro-2a CBA to identify and quantify the production of CTXs and maitotoxins (MTXs) in *Gambierdiscus australes* extracts from the Macaronesian Islands.

Finally, Chapter 6 summarises the general conclusions and future work of the thesis.

Resum

Degut al risc que suposa la presència de les toxines marines emergents tetrodotoxines (TTXs) i ciguatoxines (CTXs) en peix i marisc per a la seguretat alimentària i als consumidors, és realment necessari desenvolupar mètodes capaços de detectar aquestes toxines en menjar, i conseqüentment, garantir la protecció dels consumidors.

Per tal d'assolir aquest objectiu, aquesta tesi s'ha centrat principalment en el desenvolupament d'immunoassaigs, assaigs citotòxics i immunosensors per a la detecció de toxines marines emergents implicades en seguretat alimentària: TTXs i CTXs. Les eines bioanalítiques descrites són ràpides, assequibles econòmicament, sensibles i fiables i, a més a més, són prometedores com a eines de detecció precoç alternatives als mètodes de referència per al cribatge i quantificació de TTXs i CTXs en peix, marisc i microalgues.

El capítol 1 consisteix en una introducció general sobre l'estat actual de les toxines marines emergents TTXs i CTXs, i descriu la problemàtica derivada de la presencia d'aquestes toxines en peix i marisc per a la salut humana, així com també sobre la rellevància de desenvolupar mètodes per a la seva detecció.

El capítol 2 inclou els objectius generals i específics d'aquesta tesis.

El capítol 3 es comprèn de dues parts. La primera dóna una descripció detallada de les propietats químiques, els mecanismes d'acció i les implicacions en la salut humana de les toxines marines emergents, així com també els diferents mètodes bioquímics alternatius per a la seva detecció. En la segona part, es descriuen els avenços que ha representat la integració de materials biotecnològics en el desenvolupament d'immunosensors electroquímics per a la detecció de toxines.

El capítol 4 inclou 5 contribucions científiques. En el primer treball, es desenvolupa un immunoassaig basat en l'ús de monocapes autoensamblades (SAMs) per a la immobilització de TTX sobre plaques de maleimida, anomenat mELISA. S'ha avaluat l'aplicabilitat del mELISA per a l'anàlisi de mostres de peix globus. En el segon treball, es modifica el mELISA per tal de disminuir el temps i el cost de l'assaig i s'adapta per tal de quantificar el contingut en TTX de musclos i ostres. En el

tercer treball, l'estratègia d'immobilització del mELISA basat en l'ús de SAMs es va utilitzar per al posterior desenvolupament d'un immunosensor electroquímic, i s'investiga la seva aplicabilitat per a l'anàlisi de peixos globus. En el quart treball, es desenvolupa un immunosensor basat en la tecnologia d'ones planes basat en la immobilització de TTX sobre plataformes de "nanoarrays". L'aplicabilitat del biosensor per a l'anàlisi de peix globus també va ser estudiada. Finalment, el cinqué treball mostra la utilitat de combinar el mELISA com a eina de cribatge amb la cromatografia líquida acoblada a espectrometria de masses com a tècnica de confirmació per a la quantificació dels continguts de TTXs en peixos globus de l'oest de la costa Mediterrània.

El capítol 5 inclou 2 contribucions científiques. En la primera, es descriu una estratègia multidisciplinari que combina l'ús d'assajos cel·lulars (CBA), el bioassaig ratolí (MBA) i l'espectrometria de masses d'alta resolució (HRMS) per a la identificació i quantificació de CTXs en un tauró implicat en un cas d'intoxicació alimentària a Madagascar. El segon treball, demostra que l'assaig cel·lular Neuro-2a és apte per a la identificació i quantificació de la producció de CTXs i maitotoxines (MTXs) en extractes de *Gambierdiscus australes* de les Illes Macaronèsia.

Finalment, el capítol 6 resumeix les conclusions generals i el treball futur de la tesi.

Table of contents

Chapter 1

General introduction

1. General introduction

1.1 Emerging marine toxins

Marine biotoxins are natural substances produced by several organisms, such as microalgae and bacteria among others, that cause a toxic effect on living organisms. Specifically, CTXs are lipophilic toxins produced by dinoflagellate microalgae of the genera *Gambierdiscus* (Yasumoto et al., 1977) and *Fukuyoa* (Gómez et al. 2015). TTXs are hydrophilic compounds that are accumulated in ovaries, gastrointestinal tract, skin and liver of puffer fish and are produced in symbiosis with some bacteria (of the species *Vibrio* and *Alteromona* among others) (Noguchi et al. 2008 and Praathepa et al. 2013). Although the mechanism through which TTX is produced still remains unclear, microalgae of the genera *Prorocentrum minutum* has also been recently associated with the production of TTX (Rodriguez et al., 2017). Further characteristics of each toxin are detailed in the corresponding chapter (see chapter 3 for general information of these marine toxins).

Along with the global warming, certain environmental (e.g. ocean acidification and high-nutrient conditions) and anthropogenic factors (such as ship-ballast water and chemical pollution from agricultural activities), promote the formation of the phenomenon known as harmful algal bloom (HAB) (Anderson et al., 2012). HABs are natural events which have occurred throughout the history, consisting on the proliferation of hazardous microalgae that can have negative impacts on the economy of fishery industries, on the marine environment, and more importantly, on human health (Hallegraeff et al., 2010). As for toxins of microalgal origin, bacteria producing TTXs can also proliferate due to specific conditions, expanding the presence of TTXs in the marine environment. Bacteria pose a serious threat to the environment, since they can adapt to changes even better than the microalgae (Botana et al., 2016). The resulting toxins are thus introduced into the food webs whereby they are bioaccumulated within each trophic level and transferred to other marine organisms, being biotransformed and eventually reaching humans. Hence, the consumption of seafood contaminated with these marine toxins poses a risk to human health and may lead to severe illnesses. Human clinical symptoms derived from seafood poisoning vary depending on the toxin group and the concentration of the hazardous substance ingested, ranging from gastrointestinal to neurological effects and can be even lethal in the most severe cases.

In order to assess this issue, European organisations such as the European Food Safety Agency (EFSA) evaluate the risk that these toxins may suppose and suggest possible prevention measures. If there is enough evidence of risk and the toxicological levels of concern for consumers is well-

documented from poisoning reports, following EFSA recommendations the European Comission establishes regulatory limits and reference methods for the detection of the selected marine toxins to protect public health and the environment. While levels of some marine toxins are effectively controlled through monitoring programs (e.g. Diarrheic shellfish poisoning (DSP) toxins) and determined by well-established chromatographic techniques, neither regulatory limits nor official methods have been specifically set for the detection of toxins such as CTX and TTX. The little toxicological information, the incomplete knowledge regarding the mechanism leading to the production of these toxins and the scarce availability of standard analogues explain the lack of the establishment of such safety levels in food for the aforementioned toxins. Accordingly, the concept "emerging marine toxins" was defined to include newly discovered toxins (e.g. cyclic imines) (EFSA 2010a), already known toxins that appear in locations or fishery products that were previously absent (e.g. CTX in Macaronesian Islands) and non-regulated toxins that may constitute a threat to human health but for which stronger evidence supporting their presence and toxicity is required to establish further regulations (e.g. TTX in Europe) (EFSA 2017).

Among multiple marine toxins, of special concern for this work are CTXs and TTXs. Along with international seafood trade and travel, current changes in global climate such as sea warming and ocean acidification are affecting distribution of phytoplankton species and bacteria (Lloret et al., 2016). These factors together with the lack of a legal framework, make CTXs and TTXs strong candidates for expansion worldwide. In case of CTX, their presence and the associated foodborne ciguatera through consumption of fish have always been reported in tropical and subtropical areas, but CTXs were then found in the Canary Islands (Spain) and Madeira (Portugal) (Perez-Arellano et al., 2005 and EFSA, 2010b). Additionally, the occurrence of HABs and ciguateric incidents in both endemic and non-endemic regions seems that it will increase in frequency and intensity in the future (Friedman et al., 2017). With regard to TTX, the opening of the Suez Canal together with the global warming favor the expansion of Indo-Pacific species such as puffer fish to more temperate waters. This invasion leads to the spreading of toxic puffer fish in the Mediterranean coast along with poisoning incidents following the consumption of puffer fish in several countries (Bentur et al., 2008, Katikou et al., 2009, Kheifets et al., 2012, Rodriguez et al., 2008). Recently, the presence of TTX has also been extended to European shellfish (Turner et al., 2015 and Vlamis et al., 2016), probably as a consequence of the distribution of phytoplankton species worldwide triggered by the climate change together with ship-ballast waters and international seafood trade. Despite being clear signs confirming the expanding profile of these toxins worldwide and the efforts that have

been made by seafood agencies and researchers to mitigate the risk, accidents still occur and thus, they can unavoidably reach the market.

1.2. Methods for the detection of emerging marine toxins

In the past decades, the official method to monitor the levels of toxins (e.g. lipophilic toxins) in seafood was the mouse bioassay (MBA), but this was replaced in 2015 when liquid chromatography tandem-mass spectrometry (LC-MS/MS) became the reference analysis method (European Commission 2011). The change to instrumental analysis techniques permitted overcoming the lack of sensitivity of the MBA and the toxin misidentification, while avoiding the ethical problems derived from the use of animals. The instrumental techniques provide unequivocal toxin identification, with high precisision and sensitivity. In many cases, the MBA is still employed to gather the toxicological information arising from the symptomatology of the event, and then, instrumental analysis techniques are used for confirmatory purposes. Instrumental analysis techniques are useful and perfectly suitable; however, they require high tech equipment, qualified personnel and expensive toxin standards which are not always available. To address this issue, other bioanalytical tools alternative to the MBA and instrumental analytical techniques have emerged because of the advantages they offer in terms of rapidity, ease to use, cost and sensitivity. Indeed, in the recent years, the straightforward implementation of these alternative methods in monitoring programs for the quantification of toxins is in the spotlight of the seafood safety and environmental research fields.

1.2.1 Immunoassays and immunosensors for the detection of TTXs

Among the different biorecognition molecules available for the specific detection of TTXs, the use of antibodies has been the most extensively exploited because of the robustness, specificity and sensitivity they confer for the development of immunoassays and immunosensors.

Several antibodies have been successfully raised against TTX, and consequently, immunoassays have been described in the literature (Raybould et al., 1992 and Tao et al., 2010, among others). Immunoassays are biochemical assays based on the affinity recognition between a specific antibody and the analyte (toxin), being enzyme-linked immunosorbent assay (ELISA) the most commonly used format. Depending on the antigen size, sandwich or competitive-ELISA is used. In case of TTX,

its small size only allows using a competitive ELISA, and a sandwich strategy is not possible. For this reason, the current assays for TTX are based on this format.

One of the most critical points for the success in the development of immunoassays for the detection of small analytes such as TTX is the immobilization strategy. Thus, the stability and the orientation of the toxin in this step are key factors for the proper biorecognition event to take place. For this reason, this needs to be carefully designed.

Another important issue to consider when developing immunochemical tools is the characterisation of the mAb binding affinities towards other analogues similar to the target compound, in order to understand correlations between different techniques in the analysis of samples with multi-toxin profiles. The mAb affinity is determined by the establishment of the cross-reactivity factors, and these need to be applied to the individual contents determined by the instrumental analysis technique for further comparison with the quantifications obtained by the imunoassay. However, due to the lack of comercial TTX analogues this issue is not widely studied in the literature.

As it is well-known, the affinity of the biorecognition between the antibody and the antigen can be affected by the presence of matrix compounds present in biological samples. The presence of these interferences in immunoassays can hamper the proper identification of the toxin in samples. Thus, the higher the matrix loading the systems allows, the higher the capability of the method to detect low levels of toxin. Thereby, the study of seafood matrix effects needs to be studied in detail.

Overall, after carefully optimising all the parameters mentioned above, immunoassays permit the high sample throughput analysis and have been demonstrated to be useful techniques for the screening and quantification of toxins such as TTX, being cost-effective, rapid, selective, sensitive and easy-to-use tools.

Accordingly, biosensors have also demonstrated to have the potential to achieve this goal and have been extensively used throughout the history for many practical applications (Lee et al., 2008). A biosensor consists of an integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biorecognition element which is in direct spatial contact with a transducer element (Thevenot et al., 1999). While the biorecognition element (receptor, antibody, aptamer or enzyme) recognises the analyte (complementary DNA, enzyme

substrate, analyte), the transducer element has the ability to convert a physical or biological event resulting from the interaction between the biorecognition element and the analyte into a measurable signal. The intensity of generated signal is directly or inversely proportional to the analyte concentration. According to the principle of the transuction system, biosensors can be classified into electrochemical, optical, piezoelectric and thermal sensors. Biosensors using antibodies as biorecognition elements (immunosensors) together with electrochemical and optical as transduction detection techniques are the main type of biosensors developed up to date for the detection of marine toxins. These devices have demonstrated their powerfulness, by providing rapid responses, excellent sensitivity, stability, low-cost, easy-of-use and even more important, portability.

As was true for immunoassays, the study of matrix effects for the application to the analysis of biological samples is of utmost importance in the development of immunosensors. Indeed, final validation of immunosensors relies on demonstrating their applicability to the analysis of samples, and this may facilitate them to reach the market.

1.2.2 Cell-based assays for the detection of CTXs

Given the well-known mode of action of CTX on nerve cells as a basis, cell-based assays (CBA) are the most suitable and commonly used method for the detection of CTXs up to date. The CBAs using murine neuroblastoma cells (so called Neuro2a) have been widely exploited for determining CTX in fish (Manger et al., 1993 and Caillaud et al., 2012), in shellfish and in *Gambierdiscus* culture extracts (Caillaud et al., 2011), which allow to detect CTX at the pg level. In addition to the detection of CTX, the usefulness of Neuro2a CBA has also been proved for the quantification of maitotoxin (MTX), which is a potent toxin also produced by *Gambierdiscus* sp. and that usually coexist with CTX in fish and in microalgae cultures extracts (Caillaud et al., 2010). The latter method has been developed by only slightly changing the protocol and using a different solvent in the toxin extraction step due to the different nature of the toxins. While instrumental analysis techniques are based on structural recognition, these CBAs measure the toxicological effect of toxins on cells but with higher sensitivity and selectivity than the MBA, thus, are based on a functional principle. They generally consist of colorimetric assays in multi-well plate format whereby the higher the concentration of the toxin exposed to the cells, the lower the percentage of viable cells obtained. Thus, CBAs enable to obtain a rapid and accurate response for multiple samples and analytes. An interesting issue to consider when using CBAs is the toxicity equivalency factors (TEFs) of the analogues of the toxin analysed,

molecules that can coexist in samples and culture extracts with the toxin and may have a toxicological effect on cells different than CTX. Furthermore, the final success in the validation of the CBAs lies in the study of matrix effects of biological samples.

Although CBAs are appropriate for the sensitive detection of CTXs, comparison and further confirmation of the results with those provided by instrumental analysis techniques such as liquid-chromatography tandem mass spectrometry (LC-MS/MS) is always required. As explained in detail in the following chapters, to understand the correlation between results obtained by different techniques, the distinct detection principles need to be considered. Even CBAs have been extensively exploited, more efforts are still necessary to understand the complex spreading profile of ciguatera and the *Gambierdiscus* species and better manage the risk in non-endemic areas. Moreover, the further applicability of the current methods to other species of fish potentially containing CTX is highly desired for the implementation of these techniques in daily life.

Considering the current situation of the emerging toxins CTXs and TTXs in European waters and seafood and in order to ensure seafood and human safety, there is evidence for the need to develop new bioanalytical tools or improve the existing ones by extending their applicability to the analysis of other seafood matrices. In chapter 3 the properties of emerging marine toxins as well as the current alternative methods for their detection are first reviewed. The second part of chapter 3 describes the progress in the development of electrochemical immunosensors by the integration of bionanotechnological materials. Taking advantage of the strengths of the current alternative techniques for the detection of emerging marine toxins, and with the aim of overcoming the weaknesses they present, chapter 4 and chapter 5 attempt to fill this gap by providing rapid, specific, sensitive, cost-effective and reliable bioanalytical tools alternative to convential analytical techniques for the screening and quantification of TTXs and CTXs. Chapter 4 initially exploites the use of self-assembled monolayers as immobilisation strategy for the subsequent development of immunoassays and electrochemical immunosensors for the detection of TTX in puffer fish samples and shellfish. The second part of chapter 4 attempts to validate the proposed methods by demonstrating their applicability to the analysis of biological samples and further comparison with reference analytical methods. In the last part of this chapter, an immobilization approach for the immobilization of TTX on nanoarrayed scaffolds is designed for the subsequent development of a planar waveguide biosensor for TTX quantification in puffer fish. Moving to CTX, chapter 5 aims at optimising the current CBA for two main applications. On one hand, the CBA is intended to be

adapted to the identification of CTXs in sharks and, on the other hand, to the determination of the cytotoxicity effects of the microalgae producer of CTXs: *Gambierdiscus* sp.

Overall, the main contribution of this thesis to the field of emerging marine toxins is that it provides simple, reliable and sensitive tools complementary to the reference instrumental analysis methods. The powerful benefits they present make them excellent candidates as early warning tools for the straightforward implementation in in-situ monitoring routine programs for the screening and quantification of emerging marine toxins. Moreover, they can play an important role in research programs by helping to better understand the transfer of the toxins within food-webs and to identify the risk they suppose in an area of study before they become a threat.

1.3 References

Anderson, D. M., A. D. Cembella and G. M. Hallegraeff (2012). Progress in Understanding Harmful Algal Blooms: Paradigm Shifts and New Technologies for Research, Monitoring, and Management. Annual Review of Marine Science, Vol 4. C. A. Carlson and S. J. Giovannoni. Palo Alto, Annual Reviews. 4: 143-176.

Bentur, Y., J. Ashkar, Y. Lurie, Y. Levy, et al. (2008). Lessepsian migration and tetrodotoxin poisoning due to Lagocephalus sceleratus in the eastern Mediterranean. Toxicon 52(8): 964-968.

Botana, L. M. (2016) Toxicological perspective on climate change: aquatic toxins. Chemical research in toxicology 29, 61-625.

Caillaud, A., P. de la Iglesia, E. Barber, H. Eixarch, et al. (2011). Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to Gambierdiscus pacificus in culture. Harmful Algae 10(5): 433-446.

Caillaud, A., H. Eixarch, P. de la Iglesia, M. Rodriguez, et al. (2012). Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment 29(6): 1000-1010.

Caillaud, A., T. Yasumoto and J. Diogene (2010). Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell based assay. Application to the screening of maitotoxin-like compounds in Gambierdiscus spp. Toxicon 56(1): 36-44.

European Comission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. Off. J. Eur. Union. 2011;L6:3–6

European Food Safety Authority (EFSA). Scientific Opinion on marine biotoxins in shellfish: Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins) EFSA J. 2010;8 doi: 10.2903/j.efsa.2010.1628.

European Food Safety Authority (EFSA). Scientific Opinion on marine biotoxins in shellfish—Emerging toxins: Ciguatoxin group. EFSA J. 2010;8 doi: 10.2903/j.efsa.2010.1627

European Food Safety Authority (EFSA). Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods, EFSA J., 15:4 (2017): 4752.
Friedman, M. A., M. Fernandez, L. C. Backer, R. W. Dickey, et al. (2017). An Updated Review of Ciguatera Fish Poisoning: Clinical, Epidemiological, Environmental, and Public Health Management. Marine drugs 15(3).
Gomez, F., Qiu, D., Lopes, R.M., Lin, S., (2015). Fukuyoa paulensis gen. et sp nov., a new genus for the globular species of the dinoflagellate Gambierdiscus (Dinophyceae). Plos One 10.
Hallegraeff, G. M. (2010). Ocean climate-change, phytoplankton community responses, and harmful algal blooms. A formidable Journal of phycology 46 (2), 220-235.
Katikou, P., D. Georgantelis, N. Sinouris, A. Petsi, et al. (2009). First report on toxicity assessment of the Lessepsian migrant pufferfish Lagocephalus sceleratus (Gmelin, 1789) from European waters (Aegean Sea, Greece). Toxicon 54(1): 50-55.
Kheifets, J., B. Rozhavsky, Z. Girsh Solomonovich, R. Marianna, et al. (2012). Severe Tetrodotoxin Poisoning after Consumption of Lagocephalus sceleratus (Pufferfish, Fugu) Fished in Mediterranean Sea, Treated with Cholinesterase Inhibitor. Case reports in critical care 2012: 782507-782507.
Lee, T. M.-H. (2008). Over-the-counter biosensors: past, present, and future. Sensors 8:5535-5559. Doi 10.3390/s8095535.
Lloret, J., H.-J. Raetz, J. Lleonart and M. Demestre (2016). Challenging the links between seafood and human health in the context of global change. Journal of the Marine Biological Association of the United Kingdom 96(1): 29-42.
Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., (1993). Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium-channels - semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal. Biochem. 214, 190-194.
Noguchi, T., Arakawa, O. Tetrodotoxin - Distribution and accumulation in aquatic organisms, and cases of human intoxication, Mar. Drugs, 6 (2008) 220-242.
Perez-Arellano, J. L., O. P. Luzardo, A. P. Brito, M. H. Cabrera, et al. (2005). Ciguatera fish poisoning, Canary Islands. Emerging Infectious Diseases 11(12): 1981-1982.
V., Pratheepa, V. Vasconcelos, Microbial diversity associated with tetrodotoxin production in marine organisms, Environ. Toxicol. Pharm., 36 (2013) 1046-1054.
Raybould, T. J. G., G. S. Bignami, L. K. Inouye, S. B. Simpson, et al. (1992). A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. . Journal of Clinical Laboratory Analysis 6(2): 65-72.
Rodriguez, P., A. Alfonso, C. Vale, C. Alfonso, et al. (2008). First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell Charonia lampas lampas in Europe. Analytical Chemistry 80(14): 5622-5629.
Rodriguez, I., Alfonso, A., Alonso, E., Rubiolo, J. A., Roel, M., Vlamis, A., Katikou, P., Jackson, S. A., Menon, M. L., Dobson, A and Botana, L. M. (2017). The association of bacterial C_9-based TTX-like compounds with *Prorocentrum minimum* opens new uncertainties about shellfish seafood safety. Scientific reports, 7:40880. 10.1038/srep40880
Tao, J., W. J. Wei, L. Nan, L. H. Lei, et al. (2010). Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment 27(11): 1589-1597.

Thevenot, D. R., K. Toth, R. A. Durst and G. S. Wilson (1999). Electrochemical biosensors: Recommended definitions and classification - (Technical Report). Pure and Applied Chemistry 71(12): 2333-2348.
Turner, A. D., A. Powell, A. Schofield, D. N. Lees, et al. (2015). Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance 20(2): 2-8.
Vlamis, A., P. Katikou, I. Rodriguez, V. Rey, et al. (2015). First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate Prorocentrum minimum. Toxins 7(5): 1779-1807.
Yasumoto, T., I. Nakajima, R. Bagnis and R. Adachi (1977). Finding of a dinoflagellate as a likely culprit of ciguatera. Bulletin of the Japanese Society of Scientific Fisheries 43(8): 1021-1026.
.

Chapter 2

Objectives

2.1 Objectives

The aims of the present thesis have been defined according to the state of the art on marine toxins explained above and the current available methods for the study of marine toxins implicated in seafood poisoning events.

In the first part of my thesis the general objective is the development of immunoassays and biosensors for the screening and quantification of TTXs in puffer fish and shellfish. The second part of the work aims at evaluating the applicability of the cytotoxicity assays to the determination of CTXs in the analysis of fish and microalgae.

The **specific objectives** of this thesis are:

- To exploit the use of SAMs as immobilisation platforms for the development of immunoassays to determine TTX and its analogues in puffer fish samples.
- To extend the applicability of the immunoassay for TTX to the analysis of shellfish samples.
- To develop an electrochemical immunosensor incorporating SAMs as immobilisation strategy for the determination of TTXs in puffer fish samples.
- To quantify the TTX contents in puffer fish caught along the Mediterranean coast of Spain.
- To develop an immunosensor based on planar waveguide technology and demonstrate its applicability to the determination of TTXs in several species of puffer fish.
- To evaluate and quantify the presence of CTXs in a bull shark involved in a fatal food poisoning event in Madagascar by cell-based assays.
- To characterise which species of *Gambierdiscus sp.* are present in a specific geographical area.
- To determine if certain *Gambierdiscus* strains are potential producers of CTXs and MTXs and to quantify the toxins levels by CBAs.
- To contribute to provide data on the risk of ciguatera in the area of study from the toxicological results obtained in *Gambierdiscus* strains.

Chapter 3

State of art and application of biochemical assays, cell-based assays and biosensors to the determination of toxins

Mar. Drugs **2014**, *12*, 5719-5763; doi:10.3390/md12125719

OPEN ACCESS

marine drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

Review

Alternative Methods for the Detection of Emerging Marine Toxins: Biosensors, Biochemical Assays and Cell-Based Assays

Laia Reverté, Lucía Soliño, Olga Carnicer, Jorge Diogène and Mònica Campàs *

IRTA, Carretera Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Spain;
E-Mails: laia.reverte@irta.cat (L.R.); lucia.solino@irta.cat (L.S.);
olga.carnicer@irta.cat (O.C.); jorge.diogene@irta.cat (J.D.)

* Author to whom correspondence should be addressed; E-Mail: monica.campas@irta.cat;
Tel.: +34-977-745-427; Fax: +34-977-744-138.

3.1 Alternative methods for the detection of emerging marine toxins: Biosensors, biochemical and cell-based assays

3.1.1 Abstract

The emergence of marine toxins in water and seafood may have a considerable impact on public health. Although the tendency in Europe is to consolidate, when possible, official reference methods based on instrumental analysis, the development of alternative or complementary methods providing functional or toxicological information may provide advantages in terms of risk identification, but also low cost, simplicity, ease of use and high-throughput analysis. This article gives an overview of the immunoassays, cell-based assays, receptor-binding assays and biosensors that have been developed for the screening and quantification of emerging marine toxins: palytoxins, ciguatoxins, cyclic imines and tetrodotoxins. Their advantages and limitations are discussed, as well as their possible integration in research and monitoring programs.

3.1.2 Introduction

Marine toxins constitute a heterogeneous group of complex chemical compounds, produced as secondary metabolites by bacteria and microalgae (e.g. dinoflagellates, diatoms, cyanophyceae). Although not fully understood, specific environmental conditions and biological cycles can modulate microalgal population dynamics and may cause the appearance of harmful algal blooms (HABs) and their toxins, which may affect the ecosystems. Among other organisms, fish or shellfish can accumulate marine toxins produced by microalgae entering in the food webs and occasionally posing a threat to human consumers. Other routes of human exposure to marine toxins, apart from oral consumption of contaminated seafood, are respiration and skin contact.

The concept “emerging marine toxins” is quite subjective. It has been used by regulatory organisms such as the European Commission and the European Food Safety Authority (EFSA) (2004a; 2004b; EFSA, 2009, 2010a, **b**) to focus on recently discovered marine toxins (e.g. some cyclic imines like pinnatoxins), but also those known marine toxins that appear in waters and seafood where they were previously absent (e.g. ciguatoxins, recently appearing in the eastern Atlantic, Macaronesia). “Emerging marine toxins” has also been applied to non-regulated known marine toxins that are

considered as a possible matter of concern but for which additional toxicological evidence is needed before establishing further regulations (e.g. palytoxins and brevetoxins).

The little or no toxicological information available, the structural complexity of marine toxins, and the scarcity of purified standards have hindered the development of methodologies for their detection and their subsequent regulation by the establishment of maximum permitted levels (MPLs). Mouse bioassays (MBAs) have been for years important tools to manage seafood safety and prevent risk situations for the consumers. For example, nowadays, the application of the MBA for the management of Paralytic Shellfish Poisoning (PSP) toxins may be in some situations the safest approach since other methods may not assure in time a good comprehension of the risk due to the complexity of this group of toxins. In addition, MBAs, among other bioassays, may be of interest to understand the toxic potential and mechanism of action of new marine toxins and may also contribute to unexplained toxicity of seafood. Nevertheless, European authorities, having specific regulations that forbid the use of laboratory animals when equivalent alternative methods exist, encourage and facilitate the development of alternative methods. Moreover, being the MBAs non-specific methods, they do not allow the quantification of individual toxins in seafood.

As for other contaminants, instrumental analysis methods have been applied to detect and quantify some marine toxins in order to fulfil regulations. For example, Amnesic Shellfish Poisoning (ASP) toxins present in seafood are quantified using high-performance liquid chromatography (HPLC-UV). In the EU, a liquid chromatography-coupled to tandem mass spectrometry (LC-MS/MS) method is currently the reference method for the quantification of lipophilic marine toxins, replacing completely the MBA by 2015. For PSP toxins, although the reference method in the EU is a MBA, the Lawrence HPLC method using pre-column derivatisation and fluorescence detection is also an official control method, but depending on the PSP toxins profile this method is not always applicable. Other HPLC methods for PSP toxins using post-column derivatisation are also available (van de Riet et al., 2009). Instrumental analysis approaches have also been contemplated for emerging marine toxins. For example, cyclic imines such as gymnodimines, pinnatoxins and spirolides, can be identified and quantified by LC-MS/MS methods (Fux et al., 2007; Gerssen et al., 2009; McNabb et al., 2005; Miles, 2010; Takada et al., 2001). Ciguatoxins can also be identified by LC-MS/MS, but the application of the method in routine is difficult due to the lack of certified material, the structural complexity of the toxins group, and the low sensitivity of the current instrumentation, since these toxins are extremely powerful and may be hazardous at concentrations in fish that are difficult to detect (Hamilton et al., 2002a; Lewis et al., 1998a).

Although the instrumental analysis approach is certainly a good strategy to evaluate emerging marine toxins in food, alternative methods that can provide a higher sensitivity or that may have other advantages such as shorter analysis times or lower cost may be used as screening or quantification tools to facilitate the evaluation of multiple samples in due time and at regulatory levels. Nevertheless, for some emerging marine toxins, the combination or coupling of instrumental analysis techniques with alternative methods could be the most appropriate approach to quantify them in seafood and evaluate their risk.

In this review, we present a detailed overview of methods alternative or complementary to the MBAs and instrumental analysis techniques for the detection of some emerging marine toxins. The advantages and drawbacks of biosensors, biochemical and cell-based assays for the detection of palytoxin (PLTX), ciguatoxin (CTX), tetrodotoxin (TTX) and cyclic imines (CIs) are discussed.

3.1.3 Biosensors, biochemical and cell-based assays

Biosensors, biochemical and cell-based assays are promising tools to overcome MBA drawbacks and to complement instrumental analysis techniques, because of their selectivity, sensitivity, ease-of-use and low cost. In this review, the term biochemical assay includes both immunoassays and receptor-binding assays (RBAs). For each toxin group, immunoassays are first described, followed by cell-based assays (CBAs) and finally RBAs (the latter sometimes being based on the same mechanism of action than CBAs but using receptors instead of whole cells). Since biosensors can be based on any of the assays, they are described at the end of each corresponding section.

Immunoassays are biochemical assays based on the immunological affinity between an antibody (Ab) and its antigen. They have been successfully used in both the screening and precise quantification of some marine toxins, because of their high selectivity and sensitivity. It is necessary to keep in mind that immunoassays are based on a structural recognition, thus they do not provide toxicological information. Moreover, Abs may have the ability to detect different toxin analogues or derivatives, if they share a structurally similar fragment, although may be in a different extent. This cross-reactivity may be advantageous or not, depending on whether the purpose is to detect the whole family of toxins (not all of them necessarily having the same toxicological potency) or just a specific one. The most common immunoassay format is the enzyme-linked immunosorbent

assay (ELISA), which relies on the use of specific Abs against the target analyte, and enzymes as labels. ELISAs can be direct or indirect. The indirect format involves the use of a labelled secondary Ab against the primary Ab, and the direct one implies the labelling of the primary Ab. While the indirect assays require more steps and thus longer analysis times, the direct approach provides higher sensitivities and shorter analysis times, but the primary Ab labelling may not be straightforward. ELISAs can also be competitive or sandwich. Usually in competitive assays the free and immobilised toxins compete for the Ab, whereas in sandwich assays the target analyte is sandwiched between two Abs, the capture Ab, which is usually immobilised and recognises the analyte of interest, and the detector Ab, which also recognises the antigen but not at the same antigenic site, and that may be labelled to allow the detection. Consequently, in the development of sandwich immunoassays, only large molecules with different antigenic epitopes can be targeted. Immunostrip or immunostick tests are specific types of immunoassay, which commonly use paper pads as immobilisation supports, characterised by their rapidity, portability and ease of use. Compared to other more sophisticated techniques, they are less sensitive but allow the screening of toxins *in situ* in only a few minutes by simple visual reading.

Cell-based assays (CBAs) are assays based on the toxicological effect of toxins on cells. Marine toxins usually produce a change in the physiology, the morphology or the viability of cells, which can be measured and quantified. Most CBAs require the presence of agonists or antagonist, e.g. the drugs veratridine and ouabain, in order to counteract or emphasise the action of those toxins. Veratridine is a well-known activator of the voltage-gated sodium channels (VGSCs), which binds to these channels and blocks them in an open position. Ouabain binds to the Na^+/K^+-ATPase pump and blocks it in a closed position, thus impeding the flux of sodium from the interior of the cells. Toxins acting on these channels and pumps, in the presence or absence of ouabain and veratridine at appropriate concentrations, will involve a specific response on cells. In this case, different toxins or analogues sharing the same mechanism of action may act on the cells to a different extent and therefore may have different toxic potency. It is necessary to differentiate those assays implementing primary cultures from those performed with established immortal cell lines. Primary cell cultures are obtained from tissues some hours or days prior to the execution of the assay. They present the advantage of reflecting, to a larger extent, the properties that the cells have in the organism, for example in regard to the presence and amount of membrane receptors where the toxins act. In that sense, these models could be more appropriate to study some mechanisms of action of the toxins and could be more sensitive than immortal cell lines. However, the use of primary cells may be more complex than immortal cell lines, as they may involve the use of

laboratory animals. In addition, primary cell cultures may present a higher variability than immortal cell lines regarding their physiology and functional properties, which are related to the organism source and the cell isolation process. Despite the advantages of primary cultures in terms of mechanism of action and high sensitivities, their use in CBAs for the determination of emerging toxins has not been extensively exploited.

The haemolytic test is a specific CBA based on the lysis of red blood cells (RBCs) in the presence of compounds that alter the osmotic equilibrium. Rather than a primary culture, RBCs should be considered as tissue samples since they lack a nucleus and are terminally differentiated. RBCs contain haemoglobin in their cytoplasm. When lysis occurs after exposure of RBCs to the toxins, haemoglobin is released and its absorbance can be measured. The haemolytic test can be applied to the detection of specific marine toxins that have the ability to bind to specific ion channels located in the RBCs membranes. Like other CBAs, in order to gain specificity an antagonist is needed. Haemolytic assays may be defined taking into account the toxin mechanism of action and the RBCs origin, since variability in the response may exist depending on the source of the cells (species, population, individual). As for any toxicological assay, the time of exposure, among other parameters, should be clearly defined.

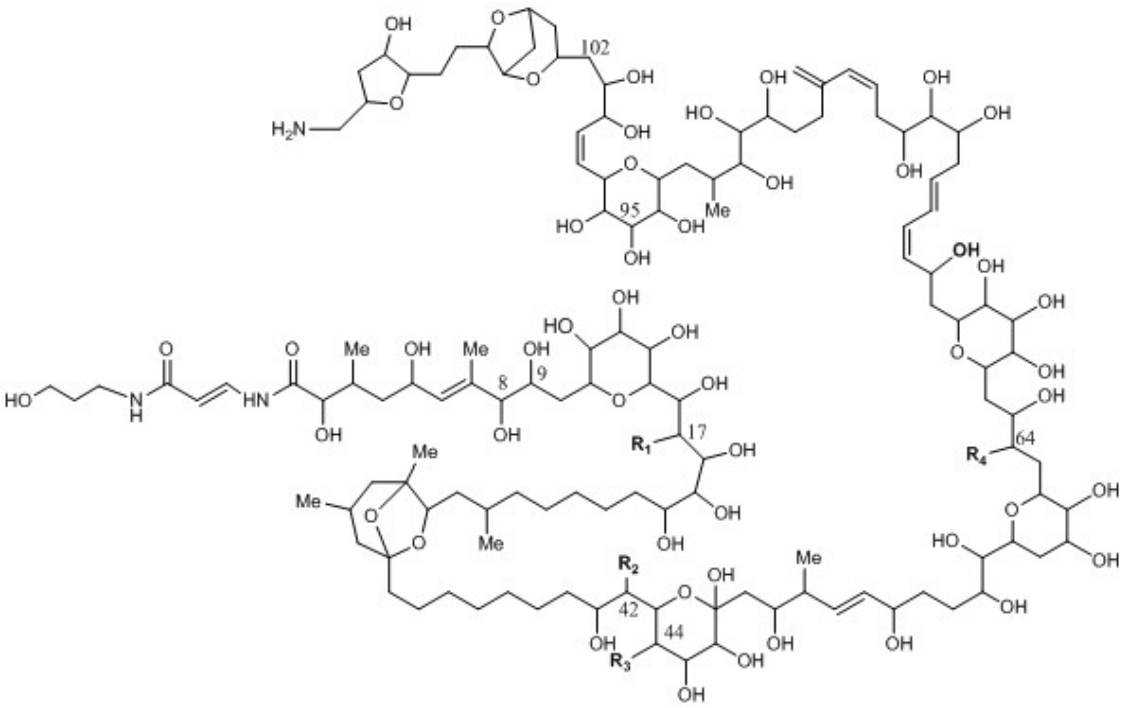
Receptor-binding assays (RBAs) are assays based on the ability of cellular receptors to bind to a specific ligand. In these assays, the competition between a labelled toxin and the toxin present in the sample for the receptor is usually carried out. Originally, ligands were labelled with radioactive moieties, but in the later years, fluorescence and chemiluminescence labels have been exploited, avoiding hazardous waste and attaining also very low limits of detection. Like in immunoassays, cross-reactivity from structurally-related toxins may exist. Since RBAs use biomolecules that have been isolated from cells, these may help to better understand the mechanism of action of toxins.

Biosensors are bioanalytical devices consisting of a biorecognition element, which specifically recognises the analyte of interest, in intimate contact with a transducer, which converts the biorecognition event into a measurable signal. Their specificity, sensitivity, simplicity, ease of use and low cost, together with the possibility to be developed for multiplex detection and to be miniaturised for portability purposes, make the development of biosensors for marine toxins highly desirable. Most biosensors for emerging marine toxins are surface plasmon resonance (SPR) immunosensors, this optical technique allowing the detection of the toxin of interest in real time

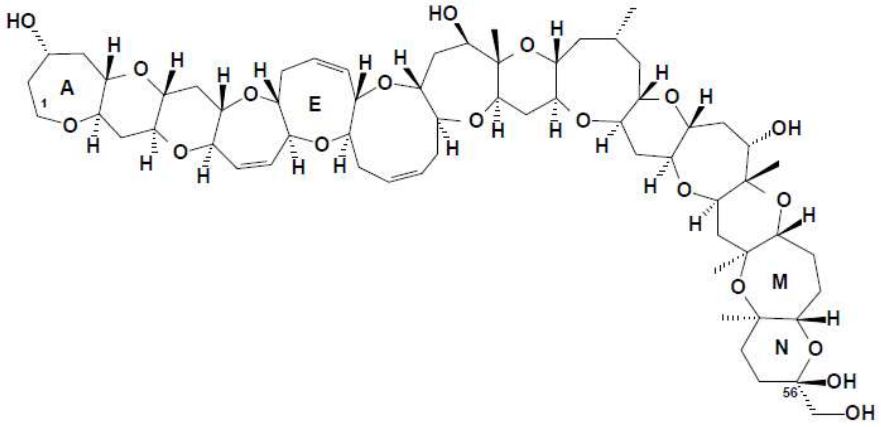
and without the need of labels. Fluorescence, fluorescence polarisation (FP), electrochemiluminescence (ECL) and electrochemical detection have also been exploited.

Figure 3.1.1 shows the chemical structure of some representative toxins of each toxin group, which are described in detail in the following sections.

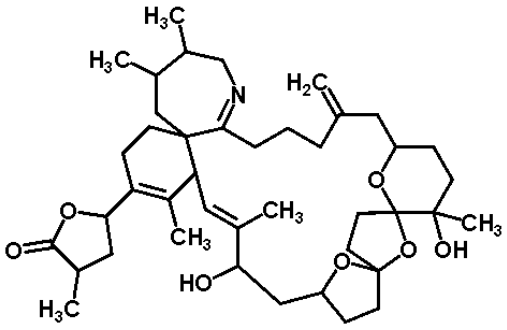
(A)

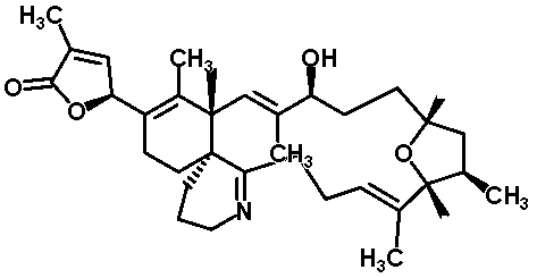


(B)

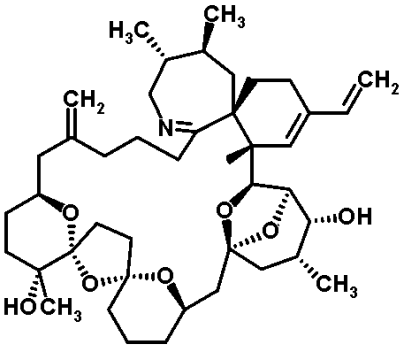


(C) **(D)**





(E)



(F)

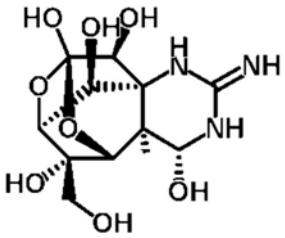


Fig. 3.1.1. Structures of (**A**) palytoxin (PLTX); (**B**) Caribbean ciguatoxin 1 (C-CTX-1); (**C**) gymnodimine A (GYM-A); (**D**) 13-desmethyl spirolide C (13-DesMeC SPX) and (**E**) pinnatoxin G (PnTX-G); and (**F**) tetrodotoxin (TTX).

Table 3.1.1. Main features of PLTX, CTX, CIs and TTX.

Toxin	Principal Derivatives	Producer Organism	Vectors	Syndromes and Effects in Humans	Endemic Areas
PLTX	Palytoxins Putative PLTX Ovatoxins Ostreotoxins Mascarenotoxins	*Palythoa* *Ostreopsis* cf. *ovata* *Ostreopsis lenticularis* *Ostreopsis mascarenensis*	Fish, Molluscs, Crustaceans, Echinoderms	Clupeotoxism Rhabdomyolisis Respiratory illness Skin illness Skeletal muscle injury Digestive distress Cardiac distress	Australia, New Zealand, French Polynesia, Cook Islands, Japan, Indonesia, Micronesia, Malaysia, Vietnam, Philippines, Singapore, Hawaii, Caribbean, Mexico, Brazil, Madagascar, Reunion Island, Mediterranean coastline of Europe
CTX	Ciguatoxins Gambiertoxins Maitotoxins	*Gambierdiscus* sp.	Fish	Gastrointestinal effects Neurological effects Cardiovascular effects	French Polynesia, Cook Islands, Hawaii, Japan, Mexico, Tokelau, North Marianna, Tuvalu, Marshall Islands, Fiji, New Caledonia, Tonga, Vanuatu, Samoa, Kiribati, Australia, Micronesia Sri Lanka, Hong Kong, Vietnam, Indonesia, Madagascar, Reunion Island, Maldive Islands, Gulf of Mexico, Guadeloupe, Florida, Virgin Islands, Puerto Rico, Brazil, Canary Islands, Madeira
CIs	Gymnodimines Spirolids Pinnatoxins Pteriatoxins Prorocentrolides	*Gymnondinium selliformis* *Alexandrium ostenfeldii* *Vulcanodinium* sp. unknown *Prorocentrum* sp.	Shellfish	Not reported	Canada, New Zealand, Japan, Denmark, Ireland, Norway, Spain, Italy, Tunisia
TTX	Tetrodotoxins	*Vibrio* sp. *Pseudomonas* sp. *Alteromonas* sp.	Pufferfish, Starfish, Blue-ring Octopus, Xanthid Crabs, Gastropods, Flatworm, Frogs, Goby, Newt Taricha	Ichthyosarcotoxism Numbness Respiratory paralysis Gastrointestinal effects	Japan, China, Taiwan, Madagascar, Australia, New Zealand, Korea, India, New Hampshire, New York, Pennsylvania, Viriginia, Chicago, California, Hawai, Bangladesh, Thailand, Norway, Meditaerranian region (Israel, Egypt and Greece), Spain (fish caught in Portugal)

3.1.3.1 Palytoxins

Palytoxins (PLTXs) are one of the most poisonous non-protein marine toxins. They have an acute toxicity in mice, specifically toxic by intravenous injection (lethal dose (LD_{50}) ranging from 0.15 to 0.73 µg/kg) (Munday, 2011). Despite their high toxicity in animals, few cases of human poisoning have been reported. PLTX was originally isolated from soft coral *Palythoa* sp. (Moore and Scheuer, 1971), which belongs to the family of *Zoanthidae*. This zoanthid was subsequently identified as *Palythoa toxica* (Walsh, 1971). Nearly a decade later, the structure of PLTX was described by two groups independently (Moore and Bartolini, 1981; Uemura et al., 1981). Later on, PLTX analogues named homopalytoxin, bishomopalytoxin, neopalytoxin, deoxypalytoxin and 42-hydroxypalytoxin (Ciminiello et al., 2009) were isolated from *Palythoa tuberculosa* (Uemura et al., 1985). PLTX-group toxins are large and complex molecules with a polyhydroxylated and partially unsaturated aliphatic backbone, which contains 64 chiral centers and presents both lipophilic and hydrophilic regions (Inuzuka et al., 2008). The molecular weights of PLTX and its analogues differ from 2659 to 2680 Da.

Furthermore, other PLTX analogues were described in different species belonging to the benthic dinoflagellate *Ostreopsis* in tropical areas: ostreotoxins 1 and 3 in *O. Lenticularis* (Mercado et al., 1994), ostreocin-D in *O. Siamensis* (Ukena et al., 2002; Ukena et al., 2001), and mascarenotoxins (Lenoir et al., 2004) in *O. mascarenensis*. In the last decades, several studies have associated *O.* cf. *ovata* seasonal blooms occurred in the Western Mediterranean with respiratory illness in humans (Tubaro et al., 2011) and adverse effects in marine organisms (Milandri et al., 2010) (e.g. sea urchins lost their spines and died). Based on these events, PLTX-like compounds have been identified in the Mediterranean strains, such as putative palytoxin (pPLTX) (Ciminiello et al., 2008), ovatoxins-a (Ciminiello et al., 2008), -b, -c, -d (Ciminiello et al., 2010) and -f (Ciminiello et al., 2012). PLTXs congeners have also been identified worldwide in echinoderms, molluscs (shellfish and cephalopods), crustacean and fish (Aligizaki et al., 2011; Munday, 2008).

Great efforts have been made to clearly understand the mechanism of action of PLTX. At a cellular level, PLTX blocks the Na^+/K^+-ATPase pump (Hilgemann, 2003). The activity of this transmembrane protein is essential for the maintenance of the cell homeostasis, which actively transports 3 Na^+ out of the cell and 2 K^+ in, by hydrolysing ATP under normal conditions. PLTX binds to the extracellular part of the Na^+/K^+-ATPase, inhibiting the active ion exchange by converting the pump into a permanently open ion channel, eventually causing cell lysis (Artigas and Gadsby, 2003). Moreover, an increase in the cytosolic Ca^{2+} caused by the action of a Na^+-Ca^{2+} exchange pump is observed

(Satoh et al., 2003; Shimahara and Molgó, 1990), also associated with cell death, apart from other secondary effects including cytoskeleton, cardiac and muscles contraction.

The potential routes of human exposure to PLTXs are seafood consumption and dermal and inhalation exposure to aerosols from *Ostreopsis* sp. blooms (Tubaro et al., 2011). The most commonly reported symptoms of PLTX poisoning include: rhabdomyolisis, skeletal muscle injury, decrease in miocytes content in blood plasma, bitter/metallic taste, abdominal cramps, nausea, vomiting, diarrhea, paresthesia, bradycardia, renal failure, cyanosis and respiratory distress, and even death in the worst cases (Deeds and Schwartz, 2010). Additionally, clupeotoxism is a rare occurring highly fatal form of human intoxication in tropical areas due to ingestion of clupeoid fish (some species of sardines and herrings), which was described for the first time in 1994 and attributed to the presence of pPLTX (Onuma et al., 1999).

The MPLs of PLTX-group toxins in shellfish are not established. Nevertheless, in 2005 the European Union Reference Laboratory for Marine Biotoxins (EU-RLMB) set a provisional limit of 250 µg/kg of PLTX in shellfish (CRLMB, 2005b). Later on, EFSA suggested to decrease the limit to 30 µg/kg of the sum of PLTX and ostreocine-D in meat (EFSA, 2009). However, these are only recommendations and not regulations. Nowadays, there is no recognised official method for the determination of PLTX-group toxins.

3.1.3.1.1 Immunoassays and biosensors for palytoxins

Contrarily to TTX and CIs but similarly to CTX, the large size of PLTX is an advantage for Ab production via animal immunisation, thereby simplifying the development of immunoassays. In that sense, highly specific ABs have been raised by the immunisation of mice against PLTX conjugated to carrier proteins such as keyhole limpet hemocyanin (KLH) (Bignami et al., 1992) and bovine serum albumin (BSA) (Frolova et al., 2000; Levine et al., 1987; Zamolo et al., 2012), through the amine group of the toxin. The specificity of the produced Abs has been assessed by the development of immunoassays using both direct and indirect strategies. Two indirect competitive ELISAs have been reported so far (Bignami et al., 1992; Frolova et al., 2000). While Bignami et al. (Bignami et al., 1992) used the classical PLTX-BSA conjugate as a coating agent, Frolova et al. (Frolova et al., 2000) took advantage of its large size and immobilised bear PLTX directly on the microtiter plate surface. The IC_{50} values obtained were 6.2 and 20 ng/mL, respectively. The applicability of these methods has been demonstrated by the analysis of naturally-contaminated samples of the coral *P. Tuberculosa* (Bignami et al., 1992) and bacterial (*Aeromonas sp. and Vibrio*

sp.) extracts from sea organisms including sponges, mussels and echinoderms (Frolova et al., 2000). Using the same Abs, Bignami et al. (Bignami et al., 1992) developed two direct competitive immunoassays with the aim of reducing time and costs, using alkaline phosphatase (ALP)-labelled monoclonal antibodies (mAbs) or PLTX. Both strategies showed good sensitivity towards PLTX in spiked shellfish samples (IC_{50} of 3.5 and 10.1 ng/mL for mAb-ALP and PLTX-ALP, respectively), which allowed to decrease the analysis time from 4 to 2 hours approximately.

Sandwich immunoassays using capture and detection Abs have also been developed for the detection of PLTX. In this direction, a direct (Bignami et al., 1992) and two indirect (Bignami et al., 1992; Boscolo, 2013) approaches have been reported. The direct strategy involved an ALP-labelled polyclonal antibody (PAb) and provided an IC_{50} of 4.8 ng/mL of PLTX. Interestingly, the same author developed one of the two indirect sandwich assays, which resulted in longer analysis times but, unexpectedly, higher sensitivity (IC_{50} of 0.6 ng/mL). In the development of the second indirect assay, the affinity of the antibody towards PLTX and the analogue 42-OH-PLTX was first characterised by SPR. The affinity of the Ab seemed to depend on the method used to immobilise PLTX on the sensor chip. The assay provided an IC_{50} of 7.6 ng/mL and a limit of detection (LOD) of 1.1 ng/mL. Whereas Bignami's assay (Bignami et al., 1992) was applied to the analysis of the coral *P. tuberculosa*, Boscolo and co-workers (Boscolo, 2013) applied it to PLTX-spiked mussels, microalgae and seawater samples, matrices that implied only a slight shifting in the sensitivity of the assay.

Beyond the traditional method for mAb production, Garet and co-workers (Garet et al., 2010) reported the first production of highly specific recombinant Abs against PLTX using recombinant hybridoma technology. Compared to the traditional methodology, this technology presents advantages in terms of production of affinity Ab fragments without the need for animal immunisation neither antigen coupling to a carrier protein, thus reducing time and costs. In this work, the selected phage-antibody clones against PLTX were first characterised, and the most specific one was used to develop the indirect competition assay. The recombinant Ab was able to recognise free and immobilised PLTX with the best specificity ever reported up to date, even using an indirect strategy (LOD = 0.5 pg/mL). The applicability of the assay was demonstrated by the analysis of spiked samples of mussels and clams, without sample purification steps, which showed good recoveries and no or little matrix effects.

Regarding biosensors for PLTX detection, a sandwich immunoassay combined with ECL detection has been developed (Zamolo et al., 2012). The immunosensor consisted of doubly amino-functionalised multi-walled carbon nanotubes (MWCNTs): CNT sidewalls were linked to mAb anti-

PLTX (capture Ab) and CNT tips to the surface of an optical transparent electrode covered by an electrochemical polymer layer. The electroluminescent detection was achieved by labelling the anti-PLTX PAb (detecting Ab) with a luminescence ruthenium complex. Firstly, the affinity of the immunoconjugate (CNT-mAb) towards PLTX was evaluated by SPR. Afterwards, the electrochemiluminescent immunoassay was carried out and its applicability was demonstrated using PLTX-spiked mussels and microalgae samples. The biosensor attained an LOD of 0.07 ng/mL and a limit of quantification (LOQ) of 0.24 ng/mL, and it was also suitable for shellfish and microalgae quantification, with LODs of 0.05 and 0.06 ng/mL and LOQs of 0.22 and 0.23 ng/mL, respectively. The CNT layer enhanced the electrochemiluminescent signal, the nanocomponents favouring the electron transfer and increasing the amount of immobilised mAb compared to the system without them.

An SPR biosensor has been developed based on a direct immunoassay (Yakes et al., 2011). After characterisation of mAb kinetics and optimisation of the experimental parameters, the appropriate performance of the optical immunosensor was demonstrated. This biosensor allowed the determination of PLTX with LODs of 0.52, 2.8 and 1.4 ng/mL in buffer, grouper and clam samples, respectively.

3.1.3.1.2 Cell-based assays for palytoxins

CBAs for PLTX detection are based on the ability of PLTX to bind to the Na^+/K^+-ATPase pump, inhibiting its activity and converting it into a permanently open ion channel. As a consequence, a rapid release of K^+ from cells is produced. In order to confer selectivity to the assay, ouabain, a specific inhibitor of the pump, is added to the system. Nevertheless, PLTX does not exactly mimic ouabain action and may not stimulate the same signalling pathways (Wattenberg, 2011). Actually, the mechanism of action is poorly understood and, at experimental level, some contradictory results have been observed.

The first CBA for PLTX was developed by Bellocci and collaborators (Bellocci et al., 2008) using MCF-7 cells and ouabain as an antagonist, and measuring the reduction of cytosolic lactate dehydrogenase (LDH) after cytolysis. After optimisation of the experimental parameters, the assay provided an EC_{50} of 530 pM of PLTX. Subsequent works (Kerbrat et al., 2011; Ledreux et al., 2009; Pawlowiez et al., 2013) have used 3-(4,5-dimethylthiadol-2-yl)-2,5diphenyltetrazolium(MTT) to measure the mitochondrial activity and quantify the cell viability, because it does not involve transferring supernatants and thus increases the reproducibility of the assay.

Comparing CBAs developed with the mouse neuroblastoma (N2a) cell strain, different findings have been reported. Ledreux and co-workers (Ledreux et al., 2009) observed that pre-incubation with ouabain augmented cell viability, but simultaneous addition of PLTX and ouabain increased cell death. Kebrart and collaborators (Kerbrat et al., 2011), however, claimed increased cell death in both situations. Pawlowiez and co-workers (Pawlowiez et al., 2013) also observed amplification of the PLTX effect after pre-incubation with ouabain. Finally, Cañete and Diogène (Cañete and Diogène, 2008) reported that cell treated with ouabain and veratridine were more sensitive to PLTX than without treatment, both in N2a and neuroblastoma/glioma hybrid (NG108-15) cells. Nonetheless, and despite the differences in sensitivity, all CBAs attained EC_{50} values in the pM range. In fact, genetic differences between cells or between the subunits of the Na^+/K^+-ATPase pump, and nuances in the experimental protocol could be responsible for the encountered differences, taking into account the complexity of the mechanism of action of PLTX and its antagonist, not yet fully understood (Bellocci et al., 2011; Rossini and Bigiani, 2011).

An interesting approach is that reported by Espiña and co-workers (Espiña et al., 2009), who have developed a dynamic assay using rat hepatocytes (clone 9) and human neuroblastoma BE(2)-M17 cells. In this case, the authors used Alamar Blue, a fluorescent dye, which allowed continuous measurement of cell viability. The preventive action of ouabain on the decrease of viability caused by PLTX was more evident in human N2a cells than in hepatocytes.

Regarding the applicability, these CBAs have been useful to detect PLTX-like toxicity in *Ostreopsis* sp. extracts (Bellocci et al., 2008; Espiña et al., 2009; Ledreux et al., 2009; Pawlowiez et al., 2013), and even in the marine cyanobacteria *Tricodhesmium* (Kerbrat et al., 2011). The CBA has also been applied to the determination of PLTX in spiked and naturally-contaminated seafood samples (Espiña et al., 2009; Ledreux et al., 2009; Pawlowiez et al., 2013).

In the early 80s, Habermann and co-workers (Habermann et al., 1981a; Habermann and Chhatwal, 1982; Habermann et al., 1981b) described delayed haemolytic action of PLTX on mammal red blood cells (RBCs). Taking as a basis this effect, and a previous work with the mAb against PLTX (Bignami et al., 1992), Bignami and collaborators (Bignami, 1993) developed the first haemolytic assay for PLTX using mouse blood, considered more sensitive to the PLTX effect. As previously mentioned, to verify that haemolysis is specifically due to PLTX, an antagonist should be used. Ouabain was an appropriate antagonist when using human erythrocytes; nevertheless, when using mouse blood, the anti-PLTX mAb seemed to be more effective (Taniyama et al., 2003).

Subsequent assays have derived from Bignami's test (Bignami, 1993), just with slight modifications in the origin of RBCs and some experimental parameters (Onuma et al., 1999; Riobo et al., 2008; Taniyama et al., 2002). Particularly interesting is the work performed by Riobó and co-workers (Riobo et al., 2008). Taking into account that the haemolytic effect may not be linear according to the toxin dose, and in an attempt to provide a more reliable assay, they modelled the toxicological dynamics and kinetics of haemolysis by PLTX. The model provided optimal working conditions, indicating the necessity to operate at a moderate temperature (25 °C) and to use ouabain as an antagonist.

Haemolytic assays have been applied to demonstrate the production of PTLX-like compounds by *Ostreopsis* sp. (Aligizaki et al., 2011; Lenoir et al., 2004; Nascimento et al., 2012; Penna et al., 2010; Pezzolesi et al., 2012; Rhodes et al., 2000; Rhodes et al., 2002; Rhodes et al., 2010b; Riobo et al., 2006; Vidyarathna and Graneli, 2011; Vidyarathna and Graneli, 2013), as well as the presence of PLTX analogues in marine organisms such as corals, echinoderms, crustaceans and cephalopods (Bire et al., 2013; Brissard et al., 2014; Gleibs and Mebs, 1999; Gleibs et al., 1995; Huang et al., 2011; Onuma et al., 1999; Rhodes et al., 2000; Rhodes et al., 2002; Seemann et al., 2009; Taniyama et al., 2003; Taniyama et al., 2002; Wachi and Hokama, 2001; Wachi et al., 2000) and cyanobacteria (Pagliara and Caroppo, 2011). Due to the scarcity of PLTX standards availability, some authors (Vidyarathna and Graneli, 2011; Vidyarathna and Graneli, 2013) have decided to quantify the toxicity in relation to the saponin haemolytic activity, based on Igarashi's method for other dinoflagellates (Igarashi et al., 1999).

Recently, an innovative method has been reported by Volpe et al. (Volpe et al., 2014) to measure the haemolytic activity of PLTX. The method is based on the release of LDH from sheep erythrocytes to the medium, caused by the haemolytic activity of PLTX, and the subsequent amperometric detection of the enzyme activity on 8-screen-printed electrode strips. Ouabain was used to ensure the specificity of the assay for PLTX. The optimisation of the electrochemical detection indicated that the best approach was the use of pyruvate and NADH as enzyme substrates and phenazine methosulfate (PMS^+). This compound reacts with the remaining NADH to produce PMSH, subsequently reacting with hexacyanoferrate (III) and producing hexacyanoferrate (II), which is oxidised on the electrode. The LOD depended on the haemolysis time, being 0.007 and 0.16 ng/mL for 24 and 4 h, respectively. Since both values are far below the proposed provisional limit, the shortest time was chosen. Compared to the spectrophotometric detection, this method is faster, cost effective and allows portability.

3.1.3.1.3 Receptor-binding assays and biosensors for palytoxins

Several works have developed assays for PLTX using the Na^+/K^+-ATPase pump. In a recent study, Na^+/K^+-ATPase has been labelled with a fluorescent molecule to develop a fluorescence polarisation (FP) assay (Alfonso et al., 2012). The binding between PLTX and the fluorescent pump was detected by measuring the degree of polarisation of the fluorescence light. After protocol optimisation using ouabain as a model, the assay was demonstrated to be useful for PLTX quantification (LOD = 2nM; LOQ = 10 nM), the FP decreasing proportionally to the toxin concentration. The assay was successfully applied to the analysis of mussel and dinoflagellates cultures extracts, previously cleaned up to minimise matrix interferences.

Optical biosensors, similar to the previous fluorescent assay, have been reported (Alfonso et al., 2014; Vale-Gonzalez et al., 2007). In these works, Na^+/K^+-ATPase pumps were immobilised on dextran-modified resonant mirrors (Vale-Gonzalez et al., 2007) or SPR chips (Alfonso et al., 2014), and the PLTX binding was recorded in real time with the corresponding detector. Although the resonant mirror showed a concentration-dependent ouabain binding, no response was observed with PLTX. Since PLTX was neither able to displace bound ouabain, the authors suggested that PLTX and ouabain do not share the same binding site. The lack of response could be justified by steric impediments or the necessity of having the Na^+/K^+-ATPase pump inserted into the cell membrane for the PLTX binding to occur. When using SPR, the same authors immobilised the Na^+/K^+-ATPase via thiol coupling instead of amino coupling, with the aim of favouring PLTX binding. In this case, not only ouabain but also PLTX bound to the pump with high affinity (LOD = 3.73 pg; LOQ = 11.20 pg). The biosensor was applied to the analysis of PLTX in *Ostreopsis siamensis* cultures, demonstrating the viability of the approach.

Table 3.1.2 summarizes the biosensors, biochemical assays and cell-based assays that have been developed for the detection of PLTX.

Table 3.1.2. Biosensors, biochemical assays and cell-based assays for the detection of PLTX.

Assay/Biosensor	Detection Technique	Sample	Reference(s)
Immunoassay	Colorimetry	P. tuberculosa and spiked shellfish	(Bignami et al., 1992)
		-	(Levine et al., 1987)
		Aeromonas sp. and Vibrio sp.	(Frolova et al., 2000)
		Spiked mussels, microalgae and seawater	(Boscolo, 2013)
		Spiked mussels and clams	(Garet et al., 2010)
Immunosensor	ECL	Spiked mussels and microalgae	(Zamolo et al., 2012)
	SPR	Grouper and clams	(Yakes et al., 2011)
CBA	Colorimetry	Ostreopsis sp.	(Bellocci et al., 2008; Ledreux et al., 2009; Pawlowiez et al., 2013)
		Spiked and naturally-contaminated seafood	(Ledreux et al., 2009; Pawlowiez et al., 2013)
		Cyanobacteria trichodesmium	(Kerbrat et al., 2011)
		-	(Cañete and Diogène, 2008)
	Fluorescence	Spiked and naturally-contaminated seafood and Ostreopsis sp.	(Espiña et al., 2009)
Hemolytic assay	Colorimetry	Corals (Palythoa sp. and Zoanthus sp.)	(Bignami, 1993; Gleibs and Mebs, 1999; Gleibs et al., 1995)
		Cyanobacteria	(Pagliara and Caroppo, 2011; Seemann et al., 2009)
		Ostreopsis sp.	(Aligizaki et al., 2011; Lenoir et al., 2004; Nascimento et al., 2012; Penna et al., 2010; Pezzolesi et al., 2012; Rhodes et al., 2000; Rhodes et al., 2002; Rhodes et al., 2010b; Riobo et al., 2006; Vidyarathna and Graneli, 2011; Vidyarathna and Graneli, 2013)
		Fish	(Huang et al., 2011; Onuma et al., 1999; Taniyama et al., 2003; Taniyama et al., 2002; Wachi and Hokama, 2001; Wachi et al., 2000)
		Sponges, soft coral, gorgonians, crustaceans	(Gleibs and Mebs, 1999)
		Mouthed rock shells, sea urchin, mullet, sea-brams	(Brissard et al., 2014)
		Crabs	(Gleibs et al., 1995)
		Clams	(Aligizaki et al., 2011)
		Mussels	(Aligizaki et al., 2011; Gleibs and Mebs, 1999; Rhodes et al., 2002)
	Electrochemistry	Mussels	(Volpe et al., 2014)
RBA	FP	Mussels and Ostreopsis sp.	(Alfonso et al., 2012)
Receptor-based biosensor	SPR	Ostreopsis sp.	(Alfonso et al., 2014; Vale-Gonzalez et al., 2007)

3.1.3.2 Ciguatoxins

Ciguatoxins (CTXs) are potent polyether neurotoxins with 13-14 rings linked by ether groups into a rigid ladder-like structure (Scheuer et al., 1967). They are odourless, tasteless and relatively heat-stable molecules that remain toxic after cooking and freezing, and exposure to mild acidic and basic conditions (Lange, 1994). CTXs production has been associated to benthic dinoflagellates of the genus *Gambierdiscus*. The first species described was *Gambierdiscus toxicus,* originally considered as a producer of maitotoxin (MTX) and gambierols, precursors of CTXs (Bagnis et al., 1980; Bagnis et al., 1979; Yasumoto et al., 1977). Significant variation in toxin production occurs within the genus *Gambierdiscus* (Sperr and Doucette, 1996). Later taxonomic studies have led to the organisation of the genus into several species, reaching the conclusion that several species within this genus would produce CTXs (Litaker et al., 2010), being *G. polynesiensis* a major CTX producer species (Chinain et al., 2010b).

CTXs can enter into the food webs through the consumption of dinoflagellates by herbivorous fish and their subsequent consumption by carnivorous fish (Mills, 1956). Humans would be intoxicated by consumption of herbivorous and carnivorous fish containing CTXs. CTX precursors accumulate in fish tissue (mainly in viscera, but also in the muscle or other parts (Vernoux et al., 1985)) and may afterwards be metabolised into different CTX forms, which are responsible for human intoxication. Around 30 analogues from the Pacific Ocean (P-CTXs) (Lehane and Lewis, 2000; Lewis et al., 1991; Murata et al., 1989; Murata et al., 1990; Satake et al., 1998; Satake et al., 1993b; Satake et al., 1997; Satake et al., 1993c), from the Caribbean Sea (C-CTXs) (Lewis et al., 1998b) and from the Indian Ocean (I-CTXs) (Hamilton et al., 2002a; Hamilton et al., 2002b) have been identified.

CTXs bind to VGSCs in cell membranes, blocking them in an open state, and cause membrane excitability, release of neurotransmitters, increase of intracellular calcium and blockage of voltage potassium channels (Benoit et al., 1996; Bidard et al., 1984; Mattei et al., 1999; Molgo et al., 1990; Molgo et al., 1993). This induced depolarisation of nerve cells is believed to cause some of the neurological signs associated with Ciguatera fish poisoning (CFP) (Lewis, 2000). CFP is the foodborne illness caused by consumption of CTXs-containing fish, responsible for the highest reported incidence of human poisoning from seafood consumption worldwide (Caillaud et al., 2010; Lehane and Lewis, 2000). This complex syndrome is characterised by a wide variety of symptoms such as gastrointestinal (e.g. vomiting, diarrhoea, nausea), neurological (e.g. tingling, itching) and cardiovascular (e.g. hypotension, bradycardia) effects. In severe cases the symptoms may begin in 30 minutes after ingestion of contaminated fish, while in milder cases they may be delayed for 24

to 48 hours. Fatalities may occur due to cardiorespiratory failure. At present, an estimation indicates that between 10,000 and 50,000 people are suffering around the world from this disease annually (EFSA, 2010a). CFP is endemically found in indo-pacific and Caribbean areas. However, in recent years CTXs are appearing in countries not expected for their latitude, such as waters close to European and African continents, e.g. in the Canary Islands (Spain) (Perez-Arellano et al., 2005) and Madeira (Portugal) (Gouveia, May 2009).

No regulatory limits exist for CTXs in fish in Europe, but the legislation requires that no fish products containing CTXs are placed on the market. The United States Food and Drug Administration (US FDA) has proposed guidance levels of ≤0.1 µg/kg of C-CTX-1 equivalents and ≤0.01 µg/kg of P-CTX-1 (initially named CTX-1B, both names being currently in use) equivalents (2009). In addition, since the unique existing certified standard is for P-CTX-1, toxicity equivalency factors (TEFs) for CTXs congeners have been established by acute toxicity in mice (LD_{50}) as follows: P-CTX-1 = 1, P-CTX-2 = 0.3, P-CTX-3 = 0.3, P-CTX-3C = 0.2, 2,3-dihydroxy P-CTX-3C = 0.1, 51-hydroxy P-CTX-3C = 1, P-CTX-4A = 0.1, P-CTX-4B = 0.05, C-CTX-1 = 0.1 and C-CTX-2 = 0.3. These TEFs should be applied to express individual analogues identified with quantitative detection methods as P-CTX-1 equivalents (EFSA, 2010a).

3.1.3.2.1 Immunoassays and immunosensors for ciguatoxins

The production of specific Abs for the development of immunoassays for CTXs has been hampered by the scarcity, toxicity and chemical complexity of CTXs. The first work on this subject (Hokama et al., 1977) reported the production of sheep PAbs against partially purified CTX-1B, which were subsequently radiolabelled to be used in a radioimmunoassay (RIA). Although the specificity and sensitivity were not fully optimised, this work paved the way towards the development of immunoassays for the detection of CTX in fish. These PAbs were also labelled with horseradish peroxidase (HRP) and used in a competitive ELISA (Hokama et al., 1983). The application of the assay to the analysis of CFP-related fishes provided results similar to those obtained by the previous RIA and also MBA. Two years later, this ELISA was improved in terms of time and simplicity by the development of a colorimetric immunostick test (Hokama, 1985), which was applied to the screening of fish tissue, cooked fish, soup or gravy from CFP outbreaks. The validation of this stick test was carried out with fishes caught from CFP areas (Hokama et al., 1987). The test, marketed as Cigua-Check™, correlated well with the previous immunoassays. Additionally, the authors observed the presence of *Gambierdiscus toxicus* cells by microscopy in positive fishes. Besides the Cigua-Check™, another immunostrip test for CTX detection, named as Ciguatect kit, was developed in 1995 by Park (Park, 1995). However, both commercial kits raised controversy regarding their

performance, as disagreement with the MBA for the evaluation of CTXs was evidenced, (see comments on (Bienfang, 2011; Dickey, 1994; Ebesu and Campora, 2012)), probably due to the use of the PAb against CTX previously developed by Hokama et al., which showed high cross-reactivity with OA.

In order to overcome this limitation, Hokama and co-workers (Hokama et al., 1989) produced the first mAbs against CTX, reducing the cross-reactivity towards OA to only 16%. Moreover, they developed the ELISA on oil-based paint-coated beads in order to favour the extraction and enhance the adherence of CTX. These mAbs were also exploited in a stick test format, demonstrating the ability to detect both Pacific and Caribbean congeners (P-CTX-1, P-CTX-2, P-CTX-3 and C-CTX-1) (Campora et al., 2006).

Due to the lack of pure CTXs and their high toxicity, synthetic fragments from different parts of CTX have been recently used for the development of Abs and their subsequent use in competitive (Pauillac et al., 2000) and sandwich (Campora et al., 2008a; Campora et al., 2008b; Oguri et al., 2003; Tsumuraya et al., 2010, 2014; Tsumuraya et al., 2006; Tsumuraya et al., 2012) immunoassays. The rings of CTX are designated from A to M. Abs are usually produced against the right or the left ring fragments, being possible to rationally synthesise them with different lengths (different number of rings). The combination of Abs against different fragments has been an important advantage in the development of sandwich immunoassays, where at least two antigenic sites are needed, one Ab being used as a capture and the other one being labelled with HRP for the direct detection. Moreover, the rational design allows choosing the best strategy depending on the targeted congener. These assays have improved the LODs, being able to detect CTX at nano and even sub-nanomolar levels. Some of these sandwich immunoassays have been applied to the analysis of fish tissues from CFP outbreaks, the results correlating well with those obtained with N2a CBAs (Campora et al., 2008a; Campora et al., 2008b).

3.1.3.2.2 Cell-based assays for ciguatoxins

CBAs for CTX detection are based on the capability of CTXs to bind to the VGSCs of cells and block them, in an open state. On excitable cells, this fact results in an influx of sodium ions and cell depolarisation causing spontaneous action potential. If cells are not able to counteract this extra-sodium influx, it concludes in cell death. In the assay, veratridine (which activates the VGSCs to initiate sodium channel gating) and ouabain (which inhibits the Na^+/K^+-ATPase pump) are added in order to avoid cells to compensate for sodium ion flux. Cell viability is usually quantified by means

of the MTT test, which measures the mitochondrial activity. CBAs for CTX have been developed using a range of mammalian tumour cell lines, being the N2a cells the most widely used (Manger et al., 1993). Other models, such as NG108-15 cells (Cañete and Diogène, 2010), have also been proved to be effective for CTX detection. Fairey and Ransdell (Fairey et al., 1999) have developed a reporter gene assay for N2a to detect VGSC toxins such as CTXs. In this assay, veratridine was used as an agonist of CTX and cfos gene as a biomarker. The intracellular signal was transduced into a light signal and the luciferase assay was used to measure the luminescence generated in each well. Nonetheless, the tendency is the application of N2a CBA with MTT due to its suitability, easy handling and resistance.

Brevetoxin (PbTx) and C-CTXs can co-occur in some areas such as the Caribbean and Gulf of Mexico, although they are usually considered responsible for different kinds of intoxication although they share the same mechanism of action. On one hand, blooms of *Karenia brevis* (PbTx producer) have been associated with massive fish kills as well as marine mammals, turtles and sea birds mortalities (Landsberg et al., 2009); in humans, PbTx intoxication has been mostly due to shellfish consumption (McFarren et al., 1965; Watkins et al., 2008) although planktivorous fish can also accumulate this toxin (Woofter et al., 2005). On the other hand, CTX is responsible for CFP after finfish consumption. Latest improvements of the CBAs have been focused on discriminating CTX from PbTx. In this direction, Bottein-Dechraoui and co-workers (Dechraoui et al., 2005b) combined the CBA with a sodium channel RBA and achieved discrimination, due to the different relative potencies of the toxins and the opposite sensitivity of both assays (C-CTX-1 was always more potent than brevetoxins, but whereas for C-CTX-1 the CBA was more sensitive, for brevetoxins the RBA was more sensitive). The assay was successfully applied to the analysis of barracuda from Florida Keys, CTX being the present toxin. Interestingly, differences in the affinity of PbTx and CTX towards VGSCs from different tissues and species (rat and fish) have been shown by this group (Bottein Dechraoui et al., 2006).

Manger and co-workers (Manger et al., 2014) have recently developed an innovative variant of the N2aCBA using flow cytometry. Unlike the traditional CBA, which uses a horizontal seeding N2a culture, this assay is performed with cells in suspension, and only veratridine is used as an agonist. Moreover, the membrane potential caused by sodium currents resulting from VGSC activation is measured at the end point of the assay using fluorescent voltage-sensitive dyes and a flow cytometer. The response of cells to CTX can be observed even without veratridine as enhancer,

although an approximate 1000-fold increase in sensitivity is observed in its presence. This assay reduces the analysis time to minutes and provides more direct mechanistic data.

The applicability of the CBAs to the analysis of different matrices is also under investigation. Toxin extraction and clean-up steps are decisive to obtain a good toxin recovery and avoid matrix interferences. When analysing flesh fish, the most commonly used protocol for toxin extraction is a modification of that for MBA (Scheuer et al., 1967). All protocols are based in acetone extraction, followed by evaporation and two solvent partitions with methanol:water and hexane, and ethanol:water and diethyl ether, to remove fatty acid and polar compounds (Campora et al., 2010; Lewis, 2003; Lewis et al., 1991; Yasumoto et al., 1995). Additional steps with solid-phase extraction (SPE) cartridges can be also performed for further purification (Dickey et al., 2008) or instead solvent partition for rapidity (Darius et al., 2007; Wu et al., 2011). Some examples of matrices (apart from flesh or viscera fish) where CTX has been detected by CBAs are clams urchins (Pawlowiez et al., 2013), mouse dry and fresh blood (Bottein Dechraoui et al., 2007; Bottein Dechraoui et al., 2005a), rat plasma, urine, faeces and tissues (Dechraoui Bottein et al., 2011; Ledreux and Ramsdell, 2013), and barracuda blood (O'Toole et al., 2012). The application of different extraction and clean-up protocols appropriate for each sample type results in recovery values as high as 90-96% (in case of mouse and rat blood (Bottein Dechraoui et al., 2007; Dechraoui Bottein et al., 2011; Ledreux and Ramsdell, 2013) and quantification limits of the order of 0.4 pg P-CTX/mL blood (Ledreux and Ramsdell, 2013). These examples illustrate the importance of the implementation of extraction and purification steps prior to the assay in order to gain specificity. They also demonstrate that the CBA may be appropriate to quantify CTXs in a wide range of tissues, providing LOQs under the values recommended for P-CTX-1.

When analysing microalgae, cells are usually broken by sonication and toxins are double extracted with methanol and methanol:water (50:50) (Fraga et al., 2011). Like in flesh fish, the protocol can include additional purification steps such as solvent partition (Chinain et al., 2010a; Rhodes et al., 2010b; Satake et al., 1993a) and SPE clean-up (Pawlowiez et al., 2013; Rhodes et al., 2010b). These procedures have allowed the detection of CTXs in *Gambierdiscus* species and even cyanobacteria (Kerbrat et al., 2011; Pawlowiez et al., 2013). Furthermore, CBA has been successfully applied to detect CTXs dissolved in water by means of absorption resins for lipophilic toxins, without observing any resin matrix interferences (Caillaud et al., 2011). In this work, additionally, fractioning of samples by SPE is proposed as a method to achieve discrimination of toxins.

CTX has also been demonstrated to have haemolytic capacity (Shimojo and Iwaoka, 2000). However, much work should be performed to obtain optimal LODs in the corresponding assay.

Holland and co-workers (Holland et al., 2013) proposed a haemolytic assay, which applied to the analysis of different *Gambierdiscus* sp. extracts. They observed that MTX could be the principal responsible of the haemolytic activity in those microalgae samples. This response may be attributed to the different mechanism of action in Na^{+}/Ca^{2+} channels among both toxins.

3.1.3.2.3 Receptor-binding assays for ciguatoxins

PbTXs have often been used in the development of RBAs for the detection of CTXs because of their similar mode of action (ability to bind to VGSCs) and availability. These RBAs are based on the competition of labelled and free toxin for the sodium channel receptor, PbTX being used as tracer and/or competing free toxin for the construction of the dose-response curve. These assays have contributed to elucidate the toxic potencies of CTX derivatives, although different findings have been observed. The fist RBA was developed by Lewis and co-workers (Lewis et al., 1991), who observed differences between C-CTX-1, C-CTX-2 and C-CTX-3 in their competition with titrated PbTX-3. Afterwards, Poli and co-workers (Poli et al., 1997), who used titrated PbTX-9 in the development of their assay, observed that both C-CTX-1 and PbTX-3 were approximately equipotent. This RBA was applied to the analysis of fish tissue (from a CFP outbreak in Haiti occurred in 1995), which allowed the quantification of 20 ng PbTx-3 eq/g fish tissue content, confirmed to be C-CTX-1 by LC-MS. Afterwards, as mentioned in the previous section, Dechraoui et al. (Dechraoui et al., 2005b) used CBA and RBA in parallel in order to differentiate C-CTX-1 from PbTX. It is important to note that, contrarily to the work reported by Poli et al. (Poli et al., 1997), C-CTX-1 exhibited an 8-fold higher potency in the RBA than brevetoxins. The variability may be due to the receptor source and isolation process. Afterwards, Darius et al. (Darius et al., 2007) applied the RBA to the analysis of fish and *Gambierdiscus* populations from CFP areas in two islands of French Polynesia. In this case, a threshold toxin content was established to differentiate positive from negative fishes. Interestingly, while in risky areas (where ciguatera cases are reported annually) 94% of the fishes were positive by RBA, *Gambierdiscus* cells were absent. Contrarily, in the area supposed to be safe, fewer fishes were positive by RBA (74%) but *Gambierdiscus* cells were present. The RBA has also been useful to confirm the P-CTX-3C production by *Gambierdiscus polynesiensis* cultures and to assess variations in the toxic potency of different clones (Chinain et al., 2010b). Despite the relevance of RBA as useful tool for CTX determination, the need for radiolabelled compounds has hampered the progress of these assays. Attempts have been performed to label PbTXs with other moieties such as a chemiluminescent acridinium dye (Yasumoto, 2008) or different fluorescent ligands (McCall et al., 2014), resulting in promising approaches to avoid constraints associated with radioactivity.

Table 3.1.3 summarizes the biochemical assays and cell-based assays that have been developed for the detection of CTX.

Table 3.1.3. Biochemical assays and cell-based assays for the detection of CTX.

Assay	Detection Technique	Sample	Reference(s)
Immunoassays	Colorimetry	Fish	(Campora et al., 2008a; Campora et al., 2008b; Hokama et al., 1983)
		-	(Oguri et al., 2003; Pauillac et al., 2000; Tsumuraya et al., 2010, 2014; Tsumuraya et al., 2006; Tsumuraya et al., 2012)
	Radioactivity	Fish	(Hokama et al., 1977)
Immunostick test	Colorimetry	Fish	(Campora et al., 2006; Hokama, 1985; Hokama et al., 1989; Hokama et al., 1987; Park, 1995)
CBA	Colorimetry	-	(Cañete and Diogène, 2010)
		Fish	(Bienfang, 2011; Caillaud et al., 2012; Campora et al., 2008a; Dechraoui et al., 2005b; Fraga et al., 2011; Manger et al., 1993; O'Toole et al., 2012; Pawlowiez et al., 2013; Robertson et al., 2014; Wu et al., 2011)
		Gambierdiscus sp.	(Caillaud et al., 2011; Pawlowiez et al., 2013; Rhodes et al., 2010b)
		Clams sea urchins and cyanobacteria	(Pawlowiez et al., 2013)
		Rat/mouse blood and urine	(Bottein Dechraoui et al., 2007; Bottein Dechraoui et al., 2005a; Dechraoui Bottein et al., 2011; Ledreux and Ramsdell, 2013)
		Cyanobacteria	(Kerbrat et al., 2010)
	Luminescence	-	(Fairey et al., 1999)
	Fluorescence	fish	(Manger et al., 2014)
Hemolytic assay	Colorimetry	*Prorocentrum* sp. and fish	(Shimojo and Iwaoka, 2000)
		Gambierdiscus sp.	(Holland et al., 2013)
RBA	Radioactivity	-	(Lewis et al., 1991)
		Fish	(Bottein Dechraoui et al., 2006; Darius et al., 2007; Dechraoui et al., 2005b; Poli et al., 1997)
		Gambierdiscus sp.	(Darius et al., 2007) (Chinain et al., 2010b)
	Fluorescence	-	(McCall et al., 2014)
	Chemiluminiscence	*Gambierdiscus* sp.	(Yasumoto, 2008)

3.1.3.3 Cyclic imines

Cyclic imines (CIs) are a heterogeneous group of marine lipophilic phycotoxins including: spirolides (SPXs), gymnodimines (GYMs), pinnatoxins (PnTXs), pteriatoxins (PtTXs), prorocentrolides and spiro-prorocentrimines (Chatzianastasiou et al., 2011; Otero et al., 2011a). They are macrocyclic compounds with imine (carbon-nitrogen double bond) and spiro-linked ether moieties. They have been grouped together because of their common imine group as a part of a cyclic ring, which confers the pharmacological and toxicological activity, and due to their similar acute "fast acting toxicity" in mice (EFSA, 2010b). CIs are produced by different dinoflagellates to a different extent: SPXs are mainly produced by *Alexandrium ostenfeldii* also known as *A. peruvianum* (Cembella et al., 2000; Touzet et al., 2008), GYMs are produced by *Karenia selliformis*, also known as *Gymnodinium selliforme* (Seki et al., 1995). The PnTXs-producing organism has been described as peridinoid dinoflagellate (Rhodes et al., 2010a) and recently identified as *Vulcanodinium rugosum* (Nezan and Chomerat, 2011), prorocentrolides have been isolated from *Prorocentrum lima* (Torigoe et al., 1988), spiro-prorocentrimines are suggested to be produced by *Prorocentrum* species (Lu et al., 2001) and PtTXs have only been detected in shellfish and no producing organism has been identified (EFSA, 2010b).

Nowadays, the growing CIs family found in dinoflagellates and/or in shellfish includes 31 members: 4 GYMs (Miles et al., 2003; Van Wagoner et al., 2011), 14 SPXs (among which, E and F are non-toxic due to the lack of imine ring) (Gueret and Brimble, 2010), 7 PnTXs (Selwood et al., 2010), 3 PtTXs, 2 prorocentrolides and 1 spiro-prorocentrimine (Gueret and Brimble, 2010; Molgó et al., 2008). Apart from these analogues, some fatty acid acyl esters derivatives, products of shellfish metabolism, have been identified: 21 SPXs (Aasen et al., 2006) and 26 PnTXs (McCarron et al., 2012). Among CIs, SPXs are the largest group and together with GYMs are the best characterised. PnTXs and PtTXs are almost structurally identical and they are the CIs most closely related to SPXs. Moreover, PtTXs, prorocentrolides and spiro-prorocentrimine have been recently identified as biotransformation products of PnTxs in shellfish (Selwood et al., 2010).

The first evidences indicating the presence of SPXs, GYMs and PnTxs were reported in the early 1990s during routine monitoring of bivalve molluscs in Canada (Hu et al., 1996), New Zealand (MacKenzie, 1996) and Japan (Uemura et al., 1995), respectively. The cyclic imine group in the molecule is assumed to be responsible for the neurotoxicity of these toxins. The mechanism of action of SPXs and GYMs is based on their inhibition of both muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs) in the central and peripheral nervous system (Bourne et al., 2010; Munday, 2008). Nevertheless, while GYMs effect on these receptors is reversible, SPXs binding seems to be irreversible (Molgó et al., 2008). As regards to PnTXs, it has recently been

demonstrated that they also bind to nAChRs (Selwood et al., 2010). Prorocentrolides and spiro-prorocentrimine molecular targets are still unknown (Molgó et al., 2008).

Only SPXs, GYMs and PnTXs have been detected in Europe. In addition, there is limited information about the absorption, distribution, metabolism and excretion of CIs in animals or humans, and no intoxication cases have ever been reported (EFSA, 2010b). This fact explains the absence of regulatory limits and official analysis methods for CIs in shellfish. However, the working group of the EU-RLMB has proposed a guidance level for the sum of SPXs of 400 µg/kg in shellfish meat (CRLMB, 2005a; Pigozzi et al., 2008).

3.1.3.3.1 Cell-based assays for cyclic imines

Even though no CBAs have been developed so far for CIs, there are some studies about mechanism of action, which will set the basis to develop those functional tests in a near future. One of the first evidences that GYM targets muscular and neuronal nAChRs was demonstrated by Kharrat et al. (Kharrat et al., 2008). In this work, frog and mouse nerve muscle preparations were used for electrophysiological studies by means of axonal clamp and tension experiments. GYM-A blocked the twitch response when nerve was stimulated, suggesting that this toxin should be a nAChRs blocker in muscles. Compared to tubocurarine, a 135-fold higher concentration was needed of this toxin to obtain a similar degree of GYM-A blocking. Furthermore, this blocking was concentration- and time-dependent. In order to confirm these findings in native receptors, primary cell cultures of *Xenopus laevi* embrios myocytes were used in patch clamp experiments, as these myocites express nAChRs. Extracellular perfusion of GYM-A reduced nicotinic currents elicited by the neurotransmitter in a reversible manner, thus confirming the results obtained in nerve muscles. In addition, studies on how GYM-A affected receptors and subunits affinity were performed. Homomeric neuronalhuman α-7 nAChR were expressed in *Xenopus laevis* oocytes for electrophysiological studies (patch clamp). Moreover, neuromuscular human heteropentameric and neuronal human chimeric receptors of α-7 nAChR were expressed in HEK293 cells for competition studies. These studies revealed that GYM-A is more effective on chimeric nAChRs neuronal alfa7-5ht3 than on muscular ones, but it was 30 times less effective on homomeric α-7 nAChR expressed in *Xenopus* oocytes. The inhibitory of GYM-A was similar on different animal tissues but dependent on the nAChR receptor subtype and subunits comprising the receptor.

Dragunow et al. (Dragunow et al., 2005) studied the effects of synthetic GYM, GYM acetate and GYM carbonate on N2a cells. The toxic effect on the viability, although significant compared to the control, was very low. Nevertheless, the authors observed that pre-exposure of cells to these toxins

sensitised them to okadaic acid, suggesting that GYM and its analogues may be rendering cells more susceptible to the effects caused by other toxins. However, GYM acetate did not produce stress proteins such as c-Junk, ATF-2 or ATF-3 neither proliferation, thus other mechanisms may be involved in the sensitisation of these toxins.

Another interesting study was performed by Geiger et al. (Geiger et al., 2013), which used several cytotoxicity assays in order to investigate the mechanisms of action of PnTx-G. No viability inhibition was found on N2a, KB and CaCo2 cells even after 72 hours of exposure of the toxin. Nevertheless, fractions obtained from *V. rugosum* extracts were found to be toxic for these cell lines, suggesting that other compoundscan be implied in the toxicity of *V. rugosum*.

Finally, Hellyer et al. (Hellyer et al., 2014) have recently synthesised a fluorescent PnTx-F and have applied to muscle sections from thy1-YFP-H transgenic mice, which express yellow fluorescent protein (YFP) in motor nerves, to allow visualisation of interactions of this toxin with nicotinic receptors. The addition of spacers and the fluorescent moiety to PnTx-F showed reduced in vitro neuromuscular-blocking potency and in vivo toxicity compared to unmodified PnTx-F. But despite this reduced potency, the fluorescent toxin efficaciously labelled endplates of mouse muscle motor nerves, retained neuromuscular blocking abilities in vitro, and displayed in vivo toxicity. Consequently, this study is an important step not only to provide with fluorescence assays for toxin detection, but also to understand how toxins and derivatives interact with nicotinic receptors.

Tatters and co-workers (Tatters et al., 2010) performed a haemolytic study based on Eschbach erythrocyte lysis assay (Eschbach et al., 2001) for ichthyotoxic algae, and quantified the response in relation to saponin. A weak haemolytic activity was observed for the GYM standard, whereas *Karenia selliformes* reported a high response. Haemolytic assay appeared to be a non-sensitive method for GYM detection.

3.1.3.3.2 Receptor-binding assays for cyclic imines

The birth of biochemical methods for the detection of CIs is very recent. The first work was a FP indirect assay in solution for the detection of GYM-A and 13-desmethyl C (13-DesMeC) SPX (Vilariño et al., 2009). The authors developed a competitive inhibition assay based on the affinity of these toxins towards nAChRs, purified from *Torpedo marmorata* electrocyte membranes, and using fluorescent α-bungarotoxin (BTX) as a competitive tracer. The assay provided IC_{50} values of 390 nM and 108 nM for GYM-A and 13-desmethyl C (13-DesMeC) SPX, respectively. Matrix effects were

evaluated using mussel samples, which only shifted slightly the IC_{50} values to 281 and 129 nM, respectively. The higher sensitivity, and thus affinity, of SPX towards the receptors compared to GYM, suggests an also higher toxicity. The same authors also demonstrated the suitability of this assay to the quantification of 13,19-didesmethyl C (13,19-diDesMeC) SPX, with a sensitivity similar to that of 13-DesMeC SPX, and negligible matrix effects (Fonfria et al., 2010b). The applicability of this assay to clams, cockles and scallops has also been demonstrated (Fonfria et al., 2010a). Spiked shellfish extracts provided IC_{50} values of around 100 nM for SPXs and between 300 and 400 nM for GYMs. Otero et al. (Otero et al., 2011b) labelled nAChRs with a derivative of fluorescein to develop a FP direct assay for the quantification of SPX. The approach provided appropriate LODs for 13-DesMeC SPX and13,19-diDesMeC SPX (25 and 150 nM, respectively). The shift from indirect to direct recognition implied a significant improvement of sensitivity and rapidity.

Based on the same sensing principle, solid-phase RBAs with different detection techniques have been developed (Araoz et al., 2012; Rodriguez et al., 2011; Rodriguez et al., 2013b; Rodriguez et al., 2013a). Rodríguez and co-workers (Rodriguez et al., 2011) developed an indirect assay based on a competition between biotin-labelled α-BTX and CIs for their binding to nAChRs in solution, and the subsequent immobilisation of the α-BTX-receptor complex on streptavidin-coated surfaces. The amount of immobilised receptor was measured by using an anti-nAChR Ab and an HRP-labelled secondary Ab. The use of three different substrates for HRP allowed adapting the assay to chemiluminescence, fluorescence and colorimetric detection techniques. The three end-points yielded similar calibration curves for 13-DesMeC SPX ($IC_{50} \approx 35$ nM and LOD (IC_{20}) ≈ 10 nM), although the chemiluminescence substrate seem to be slightly better. Moreover, no matrix interferences were observed when using cockle extracts. The chemiluminescence approach was also applied to the detection of GYM, being 10 times less sensitive than with 13-DesMeC, possibly due to the lower GYM toxicity. The same authors developed a similar assay, but first immobilising the biotin-labelled α-BTX on streptavidin-coated plates and then carrying out the competition step and the primary and secondary Ab incubations (Rodriguez et al., 2013b). The assay was successfully applied to the determination of 13-DesMeC SPX, 13,19-diDesMeC and 20-MetG (IC_{50} = 38, 18 and 55 nM and LOD (IC_{10}) = 11, 8 and 16 nM, respectively), the first one also tested in scallops. Additionally, the assay was optimised in 384-well plates for high-throughput screening.

The immobilisation of the receptors instead of the α-BTX has also been exploited for the development of two assays (Araoz et al., 2012; Rodriguez et al., 2013a). In the first work, the competition takes place between biotin-labelled α-BTX and 13,19-diDesMeC, 13-desMeC, PnTX-G, PnTX-A, GYM-A or AK-PnTX-A for binding to nAChRs immobilised on microtiter plates, and the

colorimetric detection is measured using streptavidin-HRP (Araoz et al., 2012). In order to address the lack of selectivity of the assay, the toxins bound to nAChRs were eluted from the wells and analysed by mass spectrometry to determine their chemical structure. In the second work, nACHRsor AChBPs (binding proteins) were immobilised on carboxylated microspheres and the subsequent binding competition between biotin-labelled α-BTX and 13-DesMeC was measured by flow cytometry using fluorescent phycoerythrin (PE)-labelled streptavidin (Rodriguez et al., 2013a). Both receptors allowed the detection of 13-desMeC SPX, 13,19-DesMeC SPX and 20-MetG SPX and GYM with different sensitivities but always in the nanomolar range, attaining even an LOD as low as 0.05 nM for 13-desMeC SPX in scallops.

Table 3.1.4 summarizes the biochemical assays and cell-based assays that have been developed for the detection of CIs.

Table 3.1.4. Biochemical assays and cell-based assays for the detection of CIs.

Assay	Detection Technique	Sample	Reference(s)
CBA	Colorimetry	-	(Dragunow et al., 2005)
		Vulcanodinium rugosum	(Geiger et al., 2013)
	Patch clamp (electrophisiology)	-	(Kharrat et al., 2008)
Hemolytic assay	Colorimetry	Karenia selliformis	(Tatters et al., 2010)
RBA	Colorimetry	Cockles	(Rodriguez et al., 2011)
		Clams, oysters, scallops and mussels	(Araoz et al., 2012)
	Fluorescence	Cockles	(Rodriguez et al., 2011)
	Fluorescence (coupled to flow cytometry)	Scallops	(Rodriguez et al., 2013a)
	FP	Mussels	(Fonfria et al., 2010b; Otero et al., 2011b; Vilariño et al., 2009)
		Clams, cockles and scallops	(Fonfria et al., 2010a)
	Chemiluminiscence	Cockle	(Rodriguez et al., 2011)
		Scallops	(Rodriguez et al., 2013b)

3.1.3.4 Tetrodotoxins

Tetrodotoxin (TTX) is one of the most potent low-molecular-weight marine neurotoxins (319 Da). Its chemical structure was described as a cage-like polar molecule with a cyclic guanidinium moiety fused to a dioxy-adamantane skeleton embellished by six hydroxyl groups. To date, 29 different analogues of TTX have been reported. Their degree of toxicity varies among analogues, although

not much is known about them (Yotsu-Yamashita et al., 1999). An important recognised feature is that the deoxy analogues of TTX are less toxic than TTX, while the hydroxyl analogues are more toxic than TTX. (Bane et al., 2014).

TTX was first discovered in 1909 by Tahara in ovaries of globefish (Tahara and Hirata, 1909) and its structure was more understood in 1950 with the isolation of the crystalline form of TTX from the ovaries of puffer fish (*Fugu rubripes*) (Yokoo, 1950). However, the complete structure was only known through the findings of three independent groups (Goto et al., 1965; Tsuda et al., 1964; Woodward and Gougoutas, 1964). Puffer fish seems to accumulate the toxin in the gonads, liver and skin (Noguchi et al., 2006). Later on, the presence of TTX has been reported in many invertebrate species, such as starfish, blue-ring octopus, xanthid crabs, gastropod and flatworm, and in vertebrate species, including frogs, goby and newt Taricha (Chau et al., 2011). TTX is produced by certain endo-symbiotic bacteria, such as *Vibrio* sp., *Pseudomonas* sp. and *Alteromonas* (Pratheepa and Vasconcelos, 2013).

Like saxitoxin (STX), TTX has the ability to selectively bind to VGSCs, blocking both nerve and muscle action potentials. Contrarily to CTX, TTX closes the channels. This blocking affects the passive influx of sodium ions, resulting in numbness, respiratory paralysis, mild gastrointestinal effects, and even death of human consumers. Consequently, bioaccumulation of TTX in seafood and subsequent entrance in the human food chain poses a real risk to human safety.

The Japanese government has established a regulatory limit of 2 mg/kg of TTX equivalents in food, due to “fugu” consumption in Japan. In contrast, no regulatory limits have been set in Europe because TTX poisoning had not been a problem. However, in 2008 and 2010, the first toxic European episodes were reported in the Mediterranean, caused by ingestion of trumpet shells and gastropods, respectively (Fernández-Ortega et al., 2010; Rodriguez et al., 2008). Since then, puffer fish with high levels of TTX in skin, liver and muscle, were found migrating from the Suez Channel to Greek waters (Bentur et al., 2008), and other seafood species such as molluscs and echinoderms in the Portugal coast have also been described to contain TTX (Silva et al., 2012). Therefore, European seafood is endangered of being contaminated with this hazard toxin, which highlights the need to have a regulation and to develop unambiguous, fast and reliable methods for specifically detect and quantify TTX in order to protect human health.

3.1.3.4.1 Immunoassays and immunosensors for tetrodotoxins

Like with CIs, in the development of immunoassays for TTX, production of Ab is a bottleneck because of the small size of the toxin and the need to conjugate it to a carrier protein in order to get animal immunisation. Ideally, the carrier protein to be used as a coating agent in the ELISA should be different from that used for animal immunisation in order to avoid cross-reactivity of the Ab against the protein (Kawatsu et al., 1997). As an example, TTX-BSA conjugate has been synthesised for mAb production and TTX-OVA conjugate has been used as a coating agent in ELISA (Kawatsu et al., 1997), and vice versa (Raybould et al., 1992; Zhou et al., 2009). The developed competitive ELISAs have attained proper LODs, e.g. 2 (Kawatsu et al., 1997; Neagu et al., 2006; Raybould et al., 1992), 5 (Tao et al., 2010) and 10 ng/mL (Stokes et al., 2012), regardless the experimental conditions and formats.

Another important feature in immunoassays is the cross-reactivity towards analogues. Not much work has been performed in this direction, due to the fact that standards are not commercially available. Although some TTX derivatives may be chemically synthesised or extracted and purified from naturally-contaminated material, the cross-reactivity values may not be accurate. Nevertheless, useful qualitative information has been provided. Kawatsu and co-workers (Kawatsu et al., 1997) reported no cross-reactivity of their Ab towards gonyautoxins or tetrodonic acid, and minor cross-reactivity towards anhydro-tetrodotoxin. In fact, the cross-reactivity of an Ab depends on the functional group used as a site to conjugate the toxin to the carrier protein. The observed results are consistent with the fact that the guanidinium group was used to conjugate TTX to BSA.
Some direct ELISA formats have been developed in order to reduce analysis times. Neagu and co-workers (Neagu et al., 2006) produced a TTX-ALP tracer to use in an ELISA with immobilised anti-TTX mAb. The Ab was still able to recognise the tracer, indicating that the conjugation did not affect much the affinity event. Nevertheless, it is necessary to mention that, even thought the detection was direct, a secondary Ab (non-labelled) was used to improve the anti-TTX mAb orientation and, therefore, the analysis time was still compromised. Other works have conjugated HRP (Kawatsu et al., 1997; Wang et al., 2014) or ALP (Kreuzer et al., 2002; Raybould et al., 1992) to the anti-TTX antibody to avoid the use of a labelled secondary Ab. Despite the advantages of using conjugates, their stability (of the linkage and of the enzyme activity) still is a pending task. Recombinant Ab fragments have also been synthesised through phage display technology, and used in the development of ELISAs for TTX determination (Wang et al., 2014). However, in order to achieve high sensitivities, more work needs to be undertaken.

Some of these direct ELISA configurations have been used in the development of electrochemical immunosensors on screen-printed electrodes. In the approach with the TTX-ALP tracer (Neagu et al., 2006), differential pulse voltammetry (DPV) was used to measure the α-naphthol produced by the reaction between the substrate α-naphthyl phosphate and the enzyme label, decreasing the LOD from 2 to 1 ng/mL (compared to the colorimetric approach). In the approach with labelled primary Ab (Kreuzer et al., 2002), based on the amperometric detection of the *p*-aminophenol at +300 mV *vs.* Ag/AgCl, product of the reaction between *p*-aminophenyl phosphate and ALP, an LOD of 0.016 ng/mL was attained, one of the lowest limits ever reported.

Immunochromatographic strips have also been developed with the aim of providing a rapid visual test for the screening of TTX (Thattiyaphong et al., 2013; Yu et al., 2010; Zhou et al., 2010). These assays, prepared on an absorbent pad to favour lateral flow, are based on the competition between the TTX from the sample and the TTX-BSA immobilised on the test line for a colloidal gold-labelled anti-TTX Ab. The visual LOD attained with this format using spiked puffer fish samples (muscle and gonad) was 40 ng/mL of TTX (Yu et al., 2010; Zhou et al., 2010). Although this method is not accurate, it has the advantages of simplicity, ease of use, no sophisticated equipment requirement and short analysis times (10 min). This test has also been used in the screening of fish, establishing an action limit of 2 mg/kg (Thattiyaphong et al., 2013). The comparison with LC-MS/MS analysis has demonstrated the applicability of strip tests to the analysis of naturally-contaminated fish samples.

Several SPR immunosensors for the detection of TTX based on direct approaches have been reported (Campbell et al., 2013; Taylor et al., 2008; Taylor et al., 2011; Vaisocherova et al., 2011; Yakes et al., 2011; Yakes et al., 2014). Due to the small size of the toxin, it is very difficult to directly detect its interaction with immobilised Ab and, consequently, the appropriate configuration involves antigen immobilisation and detection of the Ab interaction. In the first works (Taylor et al., 2008; Taylor et al., 2011; Vaisocherova et al., 2011; Yakes et al., 2011), the gold SPR chip was functionalised with a mixed self-assembled monolayer (SAM) of hydroxy- and amino-terminated oligo-ethylene glycol alkanethiols (OEG-ATs), since the ethylene glycol units were known to minimise non-specific protein adoption. Whilst amino-terminated OEG-ATs were used to covalently link the TTX to the surface with formaldehyde, hydroxy-OEG-ATs were used as spacer molecules to avoid cross-linking between amino-OEG-ATs. TTX was also linked using reductive alkylation but, as expected, higher Ab recognition yields were observed when using formaldehyde because the reaction site (the guanidine group) was the same as in the Ab production. TTX has also been immobilised directly on a carboxymethylated chip, which simplifies the protocol and still retains the

performance (Campbell et al., 2013). Like in colorimetric competition immunoassays, the mAb concentration was optimised taking into account that surface saturation is not desired, since low TTX concentrations would be more difficult to detect. These optical immunosensors attained a LOD (IC_{20}) as low as 0.3 ng/mL (Taylor et al., 2008). It is evident that the most exhaustive applicability studies have been performed with SPR immunosensors. The analysis of matrix effects from pufferfish liver and muscle, gastropods, and urine, is of utmost importance in food safety and clinical applications. Although the presence of a natural matrix may increase the LOD, this effect can be minimised by properly modifying the assay running buffer (Taylor et al., 2011). Several experimental parameters, such as pH, concentration and BSA presence, can be modulated to avoid non-specific adsorption while maintaining an appropriate Ab interaction and LOD. The analysis of naturally-contaminated fish has always shown appropriate correlations with LC/MS analysis (Taylor et al., 2011; Yakes et al., 2011). An additional advantage of these systems is the ability to regenerate the surface for continuous TTX determinations. A pre-validation study was performed in parallel by three independent laboratories using spiked and naturally-contaminated pufferfish samples (Vaisocherova et al., 2011). Although the method could be improved and the comparison was only a proof of concept, it is reasonable to conclude that SPR immunosensors are very promising as analysis tools for TTX quantification.

The SAM immobilisation strategy has also been used in the development of another optical immunoassay for TTX (Yakes et al., 2010). In this case, the chip was inserted into a microfluidic cell coupled to a microscope and a camera, and the interaction was monitored by imaging using a microbead-coated secondary Ab. Controlled fluidic forces were applied to remove non-specifically bound beads by fluidic force discrimination (FFD). The LOD for TTX was 15 ng/mL and a large dynamic range (4-5 orders of magnitude) was obtained.

Recently, Yakes and co-workers (Yakes et al., 2014) have developed a non-competitive SPR immunosensor, immobilising anti-TTX mAb on the surface of the SPR chip instead of TTX. This new configuration permits the direct detection of free TTX, which is a challenge taking into account its low molecular weight. This has been possible thanks to the recent advances in SPR instrumentation: lower noise detection systems, improved fluidics with stronger vacuum pumps and lower noise valves. Antibody kinetics and cross-reactivity studies towards other co-occurring toxins with a similar mode of action were performed, which demonstrated the selectivity of the antibody, since no competitive or additive effects were observed. This strategy has allowed lowering the LOD with

respect to the competitive SPR biosensors to 0.09 ng/mL of TTX and could, in a future, be extended to the detection of other small molecules.

3.1.3.4.2 Cell-based assays and biosensors for tetrodotoxins

The first N2a CBA for TTX (and also STX) detection was developed by Kogure and co-workers (Kogure et al., 1988), and was initially named tissue culture-based assay (TCBA) because of the cell tissue layer created on the well. Previous studies had suggested that TTX blocked conduction of nerve and muscle through selective inhibition of the sodium-carrying mechanism (Catterall and Nirenberg, 1973; Narahashi et al., 1964). The assay was thus based on the ability of TTX to block VGSCs and rescue cells from the effect of ouabain (which inhibits the Na^+/K^+-ATPase pump) and veratridine (which causes sodium ion influx into the cells), counteracting the toxic antagonist effect of veratridine and increasing the viability of cells. In that assay, viability was measured by the morphological changes of the cells with an optical microscope, attaining an LOD of 3 nM of TTX. The assay was applied to the analysis of strains of bacteria from marine sediments. The applicability of the assay to freshwater sediments was also demonstrated (Do et al., 1993), indicating that TTX can be produced by freshwater bacteria and accumulate in lakes and ponds sediments. The assay has also been useful to screen the TTX production by bacteria isolated from the ovaries of pufferfish (Wu et al., 2005).

Improvements to this assay have been performed in order to get more accurate quantifications. Specific staining of the cells with dyes is useful to estimate the relative abundance of living cells by absorbance measurements. In this direction, neutral red (Gallacher and Birkbeck, 1992) and tetrazolium salts such as WST-1 (Hamasaki et al., 1996a) and MTT (Hamasaki et al., 1996b) have been used, these salts avoiding the washing steps thus providing greater reproducibility. The IC_{50} values attained with these dyes were 50, 6.6 and 12.9 nM, respectively.

When investigating the applicability of the CBA for TTX, it is necessary to keep in mind that this assay is not specific for TTX but can also detect other VGSC blockers such as STX and gonyautoxins, which could co-occur with TTX in the same sample (Jang and Yotsu-Yamashita, 2006; Jen et al., 2007; Kodama et al., 1983). Nevertheless, this CBA has demonstrated to be useful as a screening method. Also of interest is the application of CBAs to assess the toxicity of TTX analogues (Yotsu-Yamashita and Mebs, 2003).

Following the same principle but a different measurement technique, a biosensor for TTX detection has been developed (Cheun et al., 1996). This biosensor consisted of a sodium electrode covered

with frog bladder membrane integrated within a flow cell. The concentration of TTX was measured from the inhibition ratio of the sensor peak output. When naturally-contaminated puffer fish samples were analysed, linear correlations were obtained in the comparison with MBA, the biosensor being able to detect TTX at concentrations far below the LOD of the MBA (1 μg/g). Again, the biosensor was also able to detect STX and GTX analogues (Cheun et al., 1996).

In a more sophisticated work, a portable microelectrode array incorporating neuronal networks has been developed for TTX detection (Charkhkar et al., 2012; Pancrazio et al., 2003). The system was able to monitor extracellular potentials from spinal cord cells cultured on electrodes and exposed to TTX, attaining an IC_{50} of 4 nM of TTX. Similar is the work performed by Mohan and co-workers (Mohan et al., 2006), who used patch clamp electrophysiology to record the action potentials caused by the effect of TTX on NG108-15 cells. In this case, experimental data were used to create a computer model. Despite the fact that the toxic effect of TTX was observed, more efforts are needed to provide a proper calibration curve and to refine the mathematical model. Comparison between electrophysiological recordings and a CBA with a fluorescent dye and has been performed (Weiser, 2004), using recombinant subunits expressed by Chinese hamster ovary (CHO) cells. There was a linear relationship between the log IC_{50} values obtained by the two methods for different VGSC modulators.

Like for CTX, the haemolytic capacity of TTX has been exploited for the development of a screening assay (Shimojo and Iwaoka, 2000), based on the N2a CBA for CTX (Manger et al., 1993) and the heamolytic assay for PLTX (Bignami, 1993). In this test veratridine was used as TTX antagonist. The haemolytic assay was performed with red tilapia RCBs. This fish has the capacity of regulate his body ion concentration to a wide range of salinities. More experimentation is required to find minimal concentration of TTX needed to inhibit haemolysis reactive concentrations for a valid detection.

Table 3.1.5 summarizes the biosensors, biochemical assays and cell-based assays that have been developed for the detection of TTX.

Table 3.1.5. Biosensors, biochemical assays and cell-based assays for the detection of TTX.

Assay/Biosensor	Detection Technique	Sample	Reference(s)
Immunoassay	Colorimetry	-	(Neagu et al., 2006; Stokes et al., 2012; Wang et al., 2014; Zhou et al., 2009)

		Puffer fish	(Kawatsu et al., 1997; Raybould et al., 1992; Tao et al., 2010)
Immunosensor	Electrochemistry	-	(Kreuzer et al., 2002; Neagu et al., 2006)
	SPR	-	(Taylor et al., 2008; Yakes et al., 2014)
		Pufferfish	(Taylor et al., 2011; Vaisocherova et al., 2011; Yakes et al., 2011)
		Sea snail	(Campbell et al., 2013)
		Human urine	(Taylor et al., 2011)
		Milk and apple juice	(Yakes et al., 2011)
	FFD	-	(Yakes et al., 2010)
Immunostick test	Visual	Spiked puffer fish	(Yu et al., 2010; Zhou et al., 2010)
		Fish	(Thattiyaphong et al., 2013)
CBA	Colorimetry	-	(Kogure et al., 1988)
		Bacteria from freshwater sediments	(Do et al., 1993)
		Bacteria from pufferfish	(Wu et al., 2005)
		Bacterial culture supernatants	(Gallacher and Birkbeck, 1992; Hamasaki et al., 1996a)
		Spiked E. coli	(Hamasaki et al., 1996b)
		Newts	(Yotsu-Yamashita and Mebs, 2003)
	Fluorescence	-	(Weiser, 2004)
	Patch clamp (Electrophysiology)	-	(Charkhkar et al., 2012; Mohan et al., 2006; Pancrazio et al., 2003; Weiser, 2004)
		Pufferfish	(Cheun et al., 1996)

3.1.4 Conclusions and future perspectives

When available, the instrumental analysis approach for the quantification of emerging toxins is suitable for the unambiguous quantification of analytes. However, if the potency of each analogue has not been described (i.e. toxicity factors between analogues are not well established), the instrumental analysis methods may not be the right solution to estimate toxicity and risk. Being MBAs controversial, alternative methods providing functional or toxicological information have a role to play in the identification of risks, as a complement to instrumental analysis approaches. Alternative methods based on structural recognition can also contribute to quantify overall presence of analogues of a group of toxins. Therefore, alternative methods, once validated, may become excellent screening tools and even contribute to quantify toxins or restricted groups of

toxins. Instrumental analysis methods may be applied to obtain more precise quantifications or toxin profiles in positive samples identified by screening assays.

Whereas some alternative methods for the detection of classical marine toxins have even reached the market (e.g. protein phosphatase inhibition assay (PPIA) for okadaic acid and ELISA for domoic acid), those for emerging marine toxins, although successful, have not been optimised neither validated, and those few who have reached the market, such as the immunostrip tests for CTXs, have suffered from inefficiencies and controversy. A reason for this may be that not enough is known about the toxicity and mechanism of action of some emerging toxins, this being especially important when considering families of toxins with numerous analogues. This scarce information, together with the lack of certified standards and reference materials, are responsible for the underdevelopment of analysis methods. As a consequence, official methods and MPLs of these toxins in seafood may not have been established yet. Additionally, for some of these toxins, toxicological evidence is not available yet and thus, regulatory institutions cannot adopt appropriate regulations.

Each alternative method has advantages and drawbacks. The interaction of toxins with antibodies is based on a structural recognition, thus not necessarily related with toxicity. But still antibodies are robust biorecognition molecules, with high affinity and sensitivity towards their analytes, sometimes being even able to detect different analogues of the same group of toxins. Moreover, their easy handling and manipulation allow their integration into different assay formats, being adaptable to the different end users. Like antibodies, receptors specifically bind to their corresponding ligands on a structural recognition basis. The replacement of the commonly used radioactive labels by optical ones has favoured the spreading of RBAs. However, they still suffer from complex setups and the requirement of the isolation of receptors from a variety of animals. Cells provide an indication of the overall toxicity, and have the advantage to be closer to animal models than antibodies and receptors. The use of human cell models can be even more useful to extrapolate some effects on human tissues. However, working with “live” material implies certain degree of variability, both inter and intra cell lines. Environmental conditions, cell status or cell-cycle stage can also lead to variations in the responses. Another limitation is the impossibility or difficulty to identify or discriminate compounds that share the same mechanism of action. The effects from the seafood matrices on the assay or biosensor are also of concern in any detection method.

It is evident that the alternative methods described in this review are promising tools to identify and quantify emerging marine toxins. Some of them have even been demonstrated to be highly performing and applicable to the analysis of naturally-contaminated samples, and when limitations have been identified, efforts have been made to overcome them. As an example, sample fractioning can separate toxins according to their chromatographic retention time and thus, contribute to discriminate them. As another example, matrix effects can be minimised by modifying toxin extraction protocols or adding previous sample clean-up steps. However, attempts to optimise, validate and standardise these methods have been scarce, so far. Now, it's about time to make efforts to solve the pending tasks. The combination of screening tools and confirmatory instrumental analysis methods should be the solution to achieve highly specific, sensitive and fast routine monitoring of emerging marine toxins. Additionally, research has to pursue to better describe mechanisms of action of emerging toxins, to identify or produce further recognition molecules, and to develop sample purification methods in order to promote the implementation of alternative methods. It is also necessary to take into account that these alternative methods will be used by laboratories performing environmental and food safety controls, food producers, fishermen and other end-users. Consequently, their formats should be friendly and easy to use. The development of kits is certainly a sound and reachable goal, as it has happened with some regulated toxins (PSP, ASP and some lipophilic toxins). However, for some experimental approaches such as CBAs, the development of kits may not be appropriate, and in this case the priority would be the coordination of laboratories in order to harmonise and validate the methodologies. Biosensors are a very promising approach since they have demonstrated to be very specific and robust, to attain very low limits of detection, and to conduct analyses in short times. Depending on the type and the format, they may provide additional advantages such as real-time responses and, when miniaturisation is possible, device portability for *in situ* measurements. Multi-disciplinary and cooperative work would certainly speed up and strengthen the progress in this area.

3.1.5 References

Aasen, J.A., Hardstaff, W., Aune, T., Quilliam, M.A., 2006. Discovery of fatty acid ester metabolites of spirolide toxins in mussels from Norway using liquid chromatography/tandem mass spectrometry. Rapid communications in mass spectrometry 20, 1531-1537.

Alfonso, A., Fernandez-Araujo, A., Alfonso, C., Carames, B., Tobio, A., Louzao, M.C., Vieytes, M.R., Botana, L.M., 2012. Palytoxin detection and quantification using the fluorescence polarization technique. Anal. Biochem. 424, 64-70.

Alfonso, A., Pazos, M.-J., Fernández-Araujo, A., Tobio, A., Alfonso, C., Vieytes, M.R., Botana, L.M., 2014. Surface Plasmon Resonance Biosensor Method for Palytoxin Detection Based on Na^+, K^+-ATPase Affinity. Toxins 6, 96-107.

Aligizaki, K., Katikou, P., Milandri, A., Diogène, J., 2011. Occurrence of palytoxin-group toxins in seafood and future strategies to complement the present state of the art. Toxicon 57, 390-399.

AOAC Official Method 2005.06. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid cromatography with fluorescence detection: first action. J. AOAC Int. 88, 1714-1732.

CDC (Centers for Disease Control and Prevention). Cluster of ciguatera fish poisoning, 2007. Morbidity and Mortality Weekly Report (MMWR), North Carolina, pp. 283-285.

CEN/TC 275. 2008. Foodstuffs—Determination of domoic acid in shellfish and finfish by RP-HPLC using UV detection. CEN/TC 275 WI 00275xxx:2008 (E).

European Comission. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Off. J. Eur. Union, L226, pp. 83-127.

European Commission. Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bibalve molluscs, Off. J. Eur. Union, L6; pp. 3-6.

European Comission. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Off. J. Eur.·Union, L139; pp. 22-82.

Araoz, R., Ramos, S., Pelissier, F., Guerineau, V., Benoit, E., Vilarino, N., Botana, L.M., Zakarian, A., Molgo, J., 2012. Coupling the Torpedo Microplate-Receptor Binding Assay with Mass Spectrometry to Detect Cyclic Imine Neurotoxins. Analytical Chemistry 84, 10445-10453.

Artigas, P., Gadsby, D.C., 2003. Na^+/K^+-pump ligands modulate gating of palytoxin-induced ion channels. Proceedings of the National Academy of Sciences 100, 501-505.

Bagnis, R., Chanteau, S., Chungue, E., Hurtel, J.M., Yasumoto, T., Inoue, A., 1980. Origins of ciguatera fish poisoning - a new Dinoflagellate, Gambierdiscus-Toxicus Adachi and Fukuyo, definitively involved as a causal agent. Toxicon 18, 199-208.

Bagnis, R., Hurtel, J.M., Chanteau, S., Chungue, E., Inoue, A., Yasumoto, T., 1979. Dinoflagellate Gambierdiscus - Toxicus Adachi et Fukuyo - probable cause of ciguatera. Comptes Rendus Hebdomadaires Des Seances De L'Academie Des Sciences Serie D 289, 671-674.

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A., Furey, A., 2014. Tetrodotoxin: Chemistry, toxicity, source, distribution and detection. Toxins 6, 693-755.

Bellocci, M., Ronzitti, G., Milandri, A., Melchiorre, N., Grillo, C., Poletti, R., Yasumoto, T., Rossini, G.P., 2008. A cytolytic assay for the measurement of palytoxin based on a cultured monolayer cell line. Anal. Biochem. 374, 48-55.

Bellocci, M., Sala, G.L., Prandi, S., 2011. The cytolytic and cytotoxic activities of palytoxin. Toxicon 57, 449-459.

Benoit, E., Juzans, P., Legrand, A.M., Molgo, J., 1996. Nodal swelling produced by ciguatoxin-induced selective activation of sodium channels in myelinated nerve fibers. Neuroscience 71, 1121-1131.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z.S., Litmanovich, M., Golik, M., Gurevych, B., Golani, D., Eisenman, A., 2008. Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. Toxicon 52, 964-968.

Bidard, J.N., Vijverberg, H.P.M., Frelin, C., Chungue, E., Legrand, A.M., Bagnis, R., Lazdunski, M., 1984. Ciguatoxin is a novel type of Na^+ channel toxin. Journal of Biological Chemistry 259, 8353-8357.

Bienfang, P., DeFelice S., Dowling, A., 2011. Quantitative Evaluation of Commercially Available Test Kit for Ciguatera in Fish. Food and Nutrition Sciences 2, 594-598.

Bignami, G.S., 1993. A Rapid and Sensitive Hemolysis Neutralization Assay for Palytoxin. Toxicon 31, 817-820.

Bignami, G.S., Raybould, T.J.G., Sachinvala, N.D., Grothaus, P.G., Simpson, S.B., Lazo, C.B., Byrnes, J.B., Moore, R.E., Vann, D.C., 1992. Monoclonal Antibody-Based Enzyme-Linked Immunoassays for the measurement of palytoxin in biological samples. Toxicon 30, 687-700.

Bire, R., Trotereau, S., Lemee, R., Delpont, C., Chabot, B., Aumond, Y., Krys, S., 2013. Occurrence of palytoxins in marine organisms from different trophic levels of the French Mediterranean coast harvested in 2009. Harmful Algae 28, 10-22.

Boscolo, S., 2013. Sandwich ELISA Assay for the Quantitation of Palytoxin and Its Analogs in Natural Samples. Environmental Science & Technology 47, 2034-2042.

Bottein Dechraoui, M.-Y., Wacksman, J.J., Ramsdell, J.S., 2006. Species selective resistance of cardiac muscle voltage gated sodium channels: characterization of brevetoxin and ciguatoxin binding sites in rats and fish. Toxicon 48, 702-712.

Bottein Dechraoui, M.-Y., Wang, Z., Ramsdell, J.S., 2007. Optimization of ciguatoxin extraction method from blood for Pacific ciguatoxin (P-CTX-1). Toxicon 49, 100-105.

Bottein Dechraoui, M., Wang, Z., Turquet, J., Chinain, M., Darius, T., Cruchet, P., Radwan, F.F., Dickey, R.W., Ramsdell, J.S., 2005a. Biomonitoring of ciguatoxin exposure in mice using blood collection cards. Toxicon 46, 243-251.

Bourne, Y., Radi´c, Z., Aráoz, R., Talley, T.T., Benoit, E., Servent, D., Taylor, P., Molgó, J., Marchot, P., 2010. Structural determinants in phycotoxins and AChBP conferring high affinity binding and nicotinic AChR antagonism. Proceedings of the National Academy of Sciences 107, 6076-6081.

Brissard, C., Herrenknecht, C., Sechet, V., Herve, F., Pisapia, F., Harcouet, J., Lemee, R., Chomerat, N., Hess, P., Amzil, Z., 2014. Complex toxin profile of French Mediterranean Ostreopsis cf. ovata strains, seafood accumulation and ovatoxins prepurification. Marine Drugs 12, 2851-2876.

Caillaud, A., de la Iglesia, P., Barber, E., Eixarch, H., Mohammad-Noor, N., Yasumoto, T., Diogene, J., 2011. Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to *Gambierdiscus pacificus* in culture. Harmful Algae 10, 433-446.

Caillaud, A., de la Iglesia, P., Darius, H.T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain, M., Diogene, J., 2010. Update on Methodologies Available for Ciguatoxin Determination: Perspectives to Confront the Onset of Ciguatera Fish Poisoning in Europe. Marine Drugs 8, 1838-1907.

Caillaud, A., Eixarch, H., de la Iglesia, P., Rodriguez, M., Dominguez, L., Andree, K., Diogene, J., 2012. Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. Food Additives & Contaminants: Part A 29, 1000-1010.

Campbell, K., Barnes, P., Haughey, S., Higgins, C., Kawatsu, K., Vasconcelos, V., Elliott, C., 2013. Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. Anal. Bioanal. Chem. 405, 7753-7763.

Campora, C.E., Dierking, J., Tamaru, C.S., Hokama, Y., Vincent, D., 2008a. Detection of ciguatoxin in fish tissue using sandwich ELISA and neuroblastoma cell bioassay. J. Clin. Lab. Anal. 22, 246-253.

Campora, C.E., Hokama, Y., Ebesu, J.S.M., 2006. Comparative analysis of purified pacific and Caribbean ciguatoxin congeners and related marine toxins using a modified ELISA technique. J. Clin. Lab. Anal. 20, 121-125.

Campora, C.E., Hokama, Y., Tamaru, C.S., Anderson, B., Vincent, D., 2010. Evaluating the risk of Ciguatera Fish Poisoning from reef fish grown at marine aquaculture facilities in Hawai'i. Journal of the World Aquaculture Society 41, 61-70.

Campora, C.E., Hokama, Y., Yabusaki, K., Isobe, M., 2008b. Development of an enzyme-linked immunosorbent assay for the detection of ciguatoxin in fish tissue using chicken immunoglobulin Y. J. Clin. Lab. Anal. 22, 239-245.

Cañete, E., Diogène, J., 2008. Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma-glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. Toxicon 52, 541-550.

Cañete, E., Diogène, J., 2010. Improvements in the use of neuroblastomaxglioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins. Toxicon 55, 381-389.

Catterall, W.A., Nirenberg, M., 1973. Sodium uptake associated with activation of action potential ionophores of cultured neuroblastoma and muscle cells. Proceedings of the National Academy of Sciences 70, 3759-3763.

Cembella, A., Lewis, N., Quilliam, M., 2000. The marine dinoflagellate *Alexandrium ostenfeldii* (Dinophyceae) as the causative organism of spirolide shellfish toxins. Phycologia 39, 67-74.

Charkhkar, H., Knaack, G.L., Gnade, B.E., Keefer, E.W., Pancrazio, J.J., 2012. Development and demonstration of a disposable low-cost microelectrode array for cultured neuronal network recording. Sensors and Actuators B: Chemical 161, 655-660.

Chatzianastasiou, M., Katikou, P., Zacharaki, T., Papazachariou, A., McKevitt, A., 2011. Cyclic imines, as emerging marine toxins: Chemical properties, distribution, toxicological aspects and detection methods. Journal of the Hellenic Veterinary Medical Society 62, 240-248.

Chau, R., Kalaitzis, J.A., Neilan, B.A., 2011. On the origins and biosynthesis of tetrodotoxin. Aquatic Toxicology 104, 61-72.

Cheun, B., Endo, H., Hayashi, T., Nagashima, Y., Watanabe, E., 1996. Development of an ultra high sensitive tissue biosensor for determination of swellfish poisoning, tetrodotoxin. Biosensors and Bioelectronics 11, 1185-1191.

Chinain, M., Darius, H., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S., 2010a. Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. Toxicon, in press.

Chinain, M., Darius, H.T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S., 2010b. Growth and toxin production in the ciguatera-causing dinoflagellate Gambierdiscus polynesiensis (Dinophyceae) in culture. Toxicon 56, 739-750.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., Florio, C., Lorenzon, P., De Bortoli, M., 2009. Stereostructure and biological activity of 42-hydroxy-palytoxin: a new palytoxin analogue from Hawaiian Palythoa subspecies. Chemical research in toxicology 22, 1851-1859.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., Guerrini, F., Pistocchi, R., 2010. Complex palytoxin-like profile of Ostreopsis ovata. Identification of four new ovatoxins by high-resolution liquid chromatography/mass spectrometry. Rapid communications in mass spectrometry 24, 2735-2744.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Tartaglione, L., Battocchi, C., Crinelli, R., Carloni, E., Magnani, M., Penna, A., 2012. Unique Toxin Profile of a Mediterranean Ostreopsis cf. ovata Strain: HR LC-MSn Characterization of Ovatoxin-f, a New Palytoxin Congener. Chemical research in toxicology 25, 1243-1252.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Tartaglione, L., Grillo, C., Melchiorre, N., 2008. Putative palytoxin and its new Analogue, Ovatoxin-a, in *Ostreopsis ovata* collected along the Ligurian Coasts during the 2006 toxic outbreak. Journal of the American society for mass spectrometry 19, 111-120.

CRLMB (Community Reference Laboratory for Marine Biotoxins), 2005a. Report on toxicology working group meeting, Cesenatico, Italy, 24-25.

CRLMB (Community Reference Laboratory for Marine Biotoxins), 2005b. Minutes of the 1rst Meeting of Working Group on Toxicology of the National Reference Laboratories (NRLs) for Marine Biotoxins, 24-25.

Darius, H.T., Ponton, D., Revel, T., Cruchet, P., Ung, A., Fouc, M.T., Chinain, M., 2007. Ciguatera risk assessment in two toxic sites of French Polynesia using the receptor-binding assay. Toxicon 50, 612-626.

Dechraoui Bottein, M.-Y., Wang, Z., Ramsdell, J.S., 2011. Toxicokinetics of the ciguatoxin P-CTX-1 in rats after intraperitoneal or oral administration. Toxicology 284, 1-6.

Dechraoui, M., Bottein, Y., Tiedeken, J.A., Persad, R., Wang, Z., Granade, H., Dickey, R.W., Ramsdell, J.S., 2005b. Use of two detection methods to discriminate ciguatoxins from brevetoxins: application to great barracuda from Florida Keys. Toxicon 46, 261-270.

Deeds, J.R., Schwartz, M.D., 2010. Human risk associated with palytoxin exposure. Toxicon 56, 150-162.

Dickey, R.W., Granade H. R., McClure, F. D., 1994. Evaluation of the Ciguatect solid-phase immunobead assay for the detection of ciguatera-related biotoxins in Caribbean fishfish Proceedings of the International Workshop on Ciguatera Management Memoirs of the Queensland Museum. Brisbane, Australia, pp. 481-488.

Dickey, R.W., Granade, H.R., Jester, E.L.E., Abraham, A., El Said, K.R., Plakas, S.M., 2008. A tiered method for determination of Caribbean and Pacific ciguatoxins in fish and formulation of regulatory advisory levels, Ciguatera and Related Biotoxins Workshop, Noumea, New Caledonia.

Do, H., Hamasaki, K., Ohwada, K., Simidu, U., Noguchi, T., Shida, Y., Kogure, K., 1993. Presence of tetrodotoxin and tetrodotoxin-producing bacteria in freshwater sediments. Applied and environmental microbiology 59, 3934-3937.

Dragunow, M., Trzoss, M., Brimble, M.A., Cameron, R., Beuzenberg, V., Holland, P., Mountfort, D., 2005. Investigations into the cellular actions of the shellfish toxin gymnodimine and analogues. Environmental Toxicology and Pharmacology 20, 305-312.

Ebesu, J.S., Campora, C.E., 2012. Comment on" Quantitative Evaluation of Commercially Available Test Kit for Ciguatera in Fish". Food & Nutrition Sciences 3.

EFSA, 2009. Scientific opinion on marine biotoxins in shellfish-palytoxin group. EFSA Journal 7, 1-38.

EFSA, 2010a. Scientific Opinion on marine biotoxins in shellfish - Emerging toxins: Ciguatoxin group. EFSA Journal 8, 1627: 1638.

EFSA, 2010b. Scientific Opinion on marine biotoxins in shellfish: Cyclic imines (spirolides, gymnodimines, pinnatoxins and pteriatoxins). EFSA Journal 8, 1628-1887.

Eschbach, E., Scharsack, J.P., John, U., Medlin, L.K., 2001. Improved erythrocyte lysis assay in microtitre plates for sensitive detection and efficient measurement of haemolytic compounds from ichthyotoxic algae. Journal of applied toxicology 21, 513-519.

Espiña, B., Cagide, E., Louzao, M., Fernandez, M., Vieytes, M., Katikou, P., Villar, A., Jaen, D., Maman, L., Botana, L., 2009. Specific and dynamic detection of palytoxins by in vitro microplate assay with human neuroblastoma cells. Bioscience reports 29, 13-23.

Fairey, E.R., Edmunds, J., Deamer-Melia, N.J., Glasgow Jr, H., Johnson, F.M., Moeller, P.R., Burkholder, J., Ramsdell, J.S., 1999. Reporter gene assay for fish-killing activity produced by *Pfiesteria piscicida*. Environmental health perspectives 107, 711.

Fernández-Ortega, J.F., Santos, J.M., Herrera-Gutiérrez, M.E., Fernández-Sánchez, V., Loureo, P.R., Rancaño, A.A., Téllez-Andrade, A., 2010. Seafood intoxication by tetrodotoxin: First case in Europe. The Journal of emergency medicine 39, 612-617.

Fonfria, E.S., Vilarino, N., Espina, B., Carmen Louzao, M., Alvarez, M., Molgo, J., Araoz, R., Botana, L.M., 2010a. Feasibility of gymnodimine and 13-desmethyl C spirolide detection by fluorescence polarization using a receptor-based assay in shellfish matrixes. Analytica Chimica Acta 657, 75-82.

Fonfria, E.S., Vilarino, N., Molgo, J., Araoz, R., Otero, P., Espina, B., Carmen Louzao, M., Alvarez, M., Botana, L.M., 2010b. Detection of 13,19-didesmethyl C spirolide by fluorescence polarization using Torpedo electrocyte membranes. Anal. Biochem. 403, 102-107.

Fraga, S., Rodríguez, F., Caillaud, A., Diogène, J., Raho, N., Zapata, M., 2011. *Gambierdiscus excentricus sp*. nov. (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). Harmful Algae 11, 10-22.

Frolova, G., Kuznetsova, T., Mikhailov, V., Elyakov, G., 2000. An enzyme linked immunosorbent assay for detecting palytoxin-producing bacteria. Russian Journal of Bioorganic Chemistry 26, 285-289.

Fux, E., McMillan, D., Bire, R., Hess, P., 2007. Development of an ultra-performance liquid chromatography-mass spectrometry method for the detection of lipophilic marine toxins. J. Chromatogr. A 1157, 273-280.

Gallacher, S., Birkbeck, T., 1992. A tissue culture assay for direct detection of sodium channel blocking toxins in bacterial culture supernates. FEMS microbiology letters 92, 101-107.

Garet, E., Cabado, A.G., Vieites, J.M., Gonzalez-Fernandez, A., 2010. Rapid isolation of single-chain antibodies by phage display technology directed against one of the most potent marine toxins: Palytoxin. Toxicon 55, 1519-1526.

Geiger, M., Desanglois, G., Hogeveen, K., Fessard, V., Lepretre, T., Mondeguer, F., Guitton, Y., Herve, F., Sechet, V., Grovel, O., Pouchus, Y.F., Hess, P., 2013. Cytotoxicity, Fractionation and Dereplication of Extracts of the Dinoflagellate Vulcanodinium rugosum, a Producer of Pinnatoxin G. Marine Drugs 11, 3350-3371.

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. J. Chromatogr. A 1216, 1421-1430.

Gleibs, S., Mebs, D., 1999. Distribution and sequestration of palytoxin in coral reef animals. Toxicon 37, 1521-1527.

Gleibs, S., Mebs, D., Werding, B., 1995. Studies on the origin and distribution of palytoxin in a Caribbean coral reef. Toxicon 33, 1531-1537.

Goto, T., Kishi, Y., Takahashi, S., Hirata, Y., 1965. Tetrodotoxin. Tetrahedron 21, 2059-2088.

Gouveia, N., Delgado, J., Vale P., May 2009. Primeiro registo da ocorrência de episódios do tipo ciguatérico no arquipélago da Madeira, Abstract book of X Reuniao Oberica, Fitoplancton Toxico e Biotoxinas, Lisbon, Portugal.

Gueret, S.M., Brimble, M.A., 2010. Spiroimine shellfish poisoning (SSP) and the spirolide family of shellfish toxins: Isolation, structure, biological activity and synthesis. Natural Product Reports 27, 1350-1366.

Habermann, E., Ahnerthilger, G., Chhatwal, G.S., Beress, L., 1981a. Delayed hemolytic action of palytoxin. General characteristics. Biochimica Et Biophysica Acta 649, 481-486.

Habermann, E., Chhatwal, G.S., 1982. Ouabain inhibits the increase due to palytoxin of cation permeability of erythrocytes. Naunyn-Schmiedebergs Arch. Pharmacol. 319, 101-107.

Habermann, E., Chhatwal, G.S., Hessler, H.J., 1981b. Palytoxin raises the nonspecific permeability of erythrocytes in an ouabain-sensitive manner. Naunyn-Schmiedebergs Arch. Pharmacol. 317, P374-P374.

Hamasaki, K., Kogure, K., Ohwada, K., 1996a. A biological method for the quantitative measurement of tetrodotoxin (TTX): tissue culture bioassay in combination with a water-soluble tetrazolium salt. Toxicon 34, 490-495.

Hamasaki, K., Kogure, K., Ohwada, K., 1996b. An improved method of tissue culture bioassay for tetrodotoxin. Fisheries Sciencie 62, 825-829.

Hamilton, B., Hurbungs, M., Jones, A., Lewis, R.J., 2002a. Multiple ciguatoxins present in Indian Ocean reef fish. Toxicon 40, 1347-1353.

Hamilton, B., Hurbungs, M., Vernoux, J.P., Jones, A., Lewis, R.J., 2002b. Isolation and characterisation of Indian Ocean ciguatoxin. Toxicon 40, 685-693.

Hellyer, S.D., Selwood, A.I., van Ginkel, R., Munday, R., Sheard, P., Miles, C.O., Rhodes, L., Kerr, D.S., 2014. In vitro labelling of muscle type nicotinic receptors using a fluorophore-conjugated pinnatoxin F derivative. Toxicon 87, 17-25.

Hilgemann, D.W., 2003. From a pump to a pore: How palytoxin opens the gates. Proceedings of the National Academy of Sciences of the United States of America 100, 386-388.

Hokama, Y., 1985. A rapid, simplified enzyme-immunoassay stick test for the detecction of ciguatoxin and related polyethers from fish-tissues. Toxicon 23, 939-946.

Hokama, Y., Abad, M.A., Kimura, L.H., 1983. A rapid enzyme-immunoassay for the detection of ciguatoxin in conatimnated fish-tissues. Toxicon 21, 817-824.
Hokama, Y., Banner, A.H., Boylan, D.B., 1977. Radioimmunoassay for the detection of ciguatoxin Toxicon 15, 317-&.
Hokama, Y., Honda, S.A.A., Kobayashi, M.N., Nakagawa, L.K., Asahina, A.Y., Miyahara, J.T., 1989. Monoclonal-antibody (MAb) in detection of ciguatoxin (CTX) and related polyethers by the stick-enzyme immunoassay (S-EIA) in fish-tissues associated with ciguatera posioning.
Hokama, Y., Shirai, L.K., Iwamoto, L.M., Kobayashi, M.N., Goto, C.S., Nakagawa, L.K., 1987. Assessment of a rapid enzyme-immunoassay stick test for the detection of ciguatoxin and related polyether toxins in fish-tissues. Biological Bulletin 172, 144-153.
Holland, W.C., Litaker, R.W., Tomas, C.R., Kibler, S.R., Place, A.R., Davenport, E.D., Tester, P.A., 2013. Differences in the toxicity of six Gambierdiscus (Dinophyceae) species measured using an in vitro human erythrocyte lysis assay. Toxicon 65, 15-33.
Hu, T., deFreitas, A.S., Curtis, J.M., Oshima, Y., Walter, J.A., Wright, J.L., 1996. Isolation and structure of prorocentrolide B, a fast-acting toxin from *Prorocentrum maculosum*. Journal of natural products 59, 1010-1014.
Huang, K.M., Liu, S.M., Huang, Y.W., Huang, K.L., Hwang, D.F., 2011. Food Poisoning Caused by *Sunfish Masturus* lanceolatus in Taiwan. Journal of Food and Drug Analysis 19, 191-196.
Igarashi, T., Aritake, S., Yasumoto, T., 1999. Mechanisms underlying the hemolytic and ichthyotoxic activities of maitotoxin. Natural Toxins 7, 71-79.
Inuzuka, T., Uemura, D., Arimoto, H., 2008. The conformational features of palytoxin in aqueous solution. Tetrahedron 64, 7718-7723.
Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish *Fugu pardalis*. Toxicon 48, 980-987.
Jen, H.-C., Lin, S.-J., Lin, S.-Y., Huang, Y.-W., Liao, I., Arakawa, O., Hwang, D.-F., 2007. Occurrence of tetrodotoxin and paralytic shellfish poisons in a gastropod implicated in food poisoning in southern Taiwan. Food Additives and Contaminants 24, 902-909.
Kawatsu, K., Hamano, Y., Yoda, T., Terano, Y., Shibata, T., 1997. Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. Japanese Journal of Medical Science & Biology 50, 133-150.
Kerbrat, A.-S., Darius, H.T., Pauillac, S., Chinain, M., Laurent, D., 2010. Detection of ciguatoxin-like and paralysing toxins in Trichodesmium spp. from New Caledonia lagoon. Marine Pollution Bulletin 61, 360-366.
Kerbrat, A.S., Amzil, Z., Pawlowiez, R., Golubic, S., Sibat, M., Darius, H.T., Chinain, M., Laurent, D., 2011. First evidence of palytoxin and 42-hydroxy-palytoxin in the marine cyanobacterium *Trichodesmium*. Marine Drugs 9, 543-560.
Kharrat, R., Servent, D., Girard, E., Ouanounou, G., Amar, M., Marrouchi, R., Benoit, E., Molgo, J., 2008. The marine phycotoxin gymnodimine targets muscular and neuronal nicotinic acetylcholine receptor subtypes with high affinity. Journal of Neurochemistry 107, 952-963.
Kodama, M., Ogata, T., Noguchi, T., Maruyama, J., Hashimoto, K., 1983. Occurrence of saxitoxin and other toxins in the liver of the pufferfish *Takifugu pardalis*. Toxicon 21, 897-900.
Kogure, K., Tamplin, M.L., Simidu, U., Colwell, R.R., 1988. A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. Toxicon 26, 191-197.

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

Landsberg, J., Flewelling, L., Naar, J., 2009. *Karenia brevis* red tides, brevetoxins in the food web, and impacts on natural resources: Decadal advancements. Harmful Algae 8, 598-607.

Lange, W.R., 1994. Ciguatera Fish Poisoning. American Family Physician 50, 579-584.

Ledreux, A., Krys, S., Bernard, C., 2009. Suitability of the Neuro-2a cell line for the detection of palytoxin and analogues (neurotoxic phycotoxins). Toxicon 53, 300-308.

Ledreux, A., Ramsdell, J.S., 2013. Bioavailability and intravenous toxicokinetic parameters for Pacific ciguatoxin P-CTX-1 in rats. Toxicon 64, 81-86.

Lehane, L., Lewis, R.J., 2000. Ciguatera: recent advances but the risk remains. International Journal of Food Microbiology 61, 91-125.

Lenoir, S., Ten-Hage, L., Turquet, J., Quod, J.P., Bernard, C., Hennion, M.C., 2004. First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in Southwestern Indian Ocean. J. Phycol. 40, 1042-1051.

Levine, L., Fujiki, H., Gjika, H.B., Vanvunakis, H., 1987. Production of antibodies to palytoxin - Neutralization of several biological properties of playtoxin Toxicon 25, 1273-1282.

Lewis, R.J., 2000. Ion channel toxins and therapeutics: From cone snail venoms to ciguatera. Therapeutic Drug Monitoring 22, 61-64.

Lewis, R.J., 2003. Detection of toxins associated with ciguatera fish poisoning, in: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris (France), p. 267.

Lewis, R.J., Jones, A., Vernoux, J.-P., 1998a. HPLC/Tandem Electrospray Mass Spectrometry for the Determination of Sub-ppb Levels of Pacific and Caribbean Ciguatoxins in Crude Extracts of Fish. Analytical Chemistry 71, 247-250.

Lewis, R.J., Sellin, M., Poli, M.A., Norton, R.S., MacLeod, J.K., Sheil, M.M., 1991. Purification and characterization of ciguatoxins from moray eel (*Lycodontis javanicus*, Muraenidae). Toxicon 29, 1115-1127.

Lewis, R.J., Vernoux, J.P., Brereton, I.M., 1998b. Structure of Caribbean ciguatoxin isolated from Caranx latus. Journal of the American Chemical Society 120, 5914-5920.

Litaker, R.W., Vandersea, M.W., Faust, M.A., Kibler, S.R., Nau, A.W., Holland, W.C., Chinain, M., Holmes, M.J., Tester, P.A., 2010. Global distribution of ciguatera causing dinoflagellates in the genus Gambierdiscus. Toxicon 56, 711-730.

Lu, C.K., Lee, G.H., Huang, R., Chou, H.N., 2001. Spiro-prorocentrimine, a novel macrocyclic lactone from a benthic *Prorocentrum sp* of Taiwan. Tetrahedron letters 42, 1713-1716.

MacKenzie, L., A.; Haywood, A. J.; Adamson, J.; Truman, P.; Till, D.; Seki, T.; Satake, M; Yasumoto, T. 1996. Gymnodimine contamination in shellfish in New Zealand, In: Harmful and toxic algal blooms. Eds.: Yasumoto, T.; Oshima, Y.; Fukuyo, Y. The Intergovernmental Oceanographic Commission of UNESCO, Paris, France, 97-100.

Manger, R., Woodle, D., Berger, A., Dickey, R.W., Jester, E., Yasumoto, T., Lewis, R., Hawryluk, T., Hungerford, J., 2014. Flow Cytometric-Membrane Potential Detection of Sodium Channel Active Marine Toxins: Application to Ciguatoxins in Fish Muscle and Feasibility of Automating Saxitoxin Detection. Journal of Aoac International 97, 299-306.

Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., 1993. Tetrazolium-Based Cell Bioassay for neurotoxins active on voltage-sensitive sodium channels: Semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal. Biochem. 214, 190-194.

Mattei, C., Dechraoui, M.Y., Molgo, J., Meunier, F.A., Legrand, A.M., Benoit, E., 1999. Neurotoxins targetting receptor site 5 of voltage-dependent sodium channels increase the nodal volume of myelinated axons. Journal of Neuroscience Research 55, 666-673.

McCall, J.R., Jacocks, H.M., Niven, S.C., Poli, M.A., Baden, D.G., Bourdelais, A.J., 2014. Development and Utilization of a Fluorescence-Based Receptor-Binding Assay for the Site 5 Voltage-Sensitive Sodium Channel Ligands Brevetoxin and Ciguatoxin. Journal of Aoac International 97, 307-315.

McCarron, P., Rourke, W.A., Hardstaff, W., Pooley, B., Quilliam, M.A., 2012. Identification of pinnatoxins and discovery of their fatty acid ester metabolites in mussels (*Mytilus edulis*) from Eastern Canada. Journal of Agricultural and Food Chemistry 60, 1437-1446.

McFarren, E., Tanabe, H., Silva, F., Wilson, W., Campbell, J., Lewis, K., 1965. The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. Toxicon 3, 111-123.

McNabb, P., Selwood, A.I., Holland, P.T., 2005. Multiresidue method for determination of algal toxins in shellfish: Single-laboratory validation and interlaboratory study. Journal of Aoac International 88, 761-772.

Mercado, J.A., Rivera-Rentas, A.L., Gonzalez, I., Tosteson, T.R., Molgó, J., Escalona De Motta, G., 1994. Neuro- and myo-toxicity of extracts from the benthic dinoflagellate *Ostreopsis lenticularis* is sensitive to μ-conotoxin. Soc. Neurosci. Abstr. 20, 718, 303.717.

Milandri, A., Ceredi, A., Riccardi, E., Gasperetti, L., Susini, F., Casotti, M., Faiman, L., Pigozzi, S., 2010. In: Impact of *Ostreopsis ovata* on marine benthic communities: accumulation of palytoxins in mussels, sea urchins and octopuses from Italy, Proceedings of ICHA 14th Conference, Crete, Grece, 1-5.

Miles, C.O., Rundberget, T., Sandvik, M., Aasen, J. and Selwood, AI., 2010. The presence of pinnatoxins in Norwegian mussels. National Veterinary Institute: Oslo, Norway, 2010.

Miles, C.O., Wilkins, A.L., Stirling, D.J., MacKenzie, A.L., 2003. Gymnodimine C, an isomer of gymnodimine B, from *Karenia selliformis*. Journal of Agricultural and Food Chemistry 51, 4838-4840.

Mills, A.R., 1956. Poisonous fish in the South Pacific. The Journal of tropical medicine and hygiene 59, 99-103.

Mohan, D.K., Molnar, P., Hickman, J.J., 2006. Toxin detection based on action potential shape analysis using a realistic mathematical model of differentiated NG108-15 cells. Biosensors and Bioelectronics 21, 1804-1811.

Molgo, J., Comella, J.X., Legrand, A.M., 1990. Ciguatoxin enhances quantal transmitter release from frog motor-nerve terminals British Journal of Pharmacology 99, 695-700.

Molgo, J., Gaudrytalarmain, Y.M., Legrand, A.M., Moulian, N., 1993. Ciguatoxin extracted from poisonous moray eels gymnothorax-javanicus triggers acetylcholine-release from torpedo cholinergic synaptosomes via reversed $NA^{+}/Ca2^{+}$ exchange. Neuroscience Letters 160, 65-68.

Molgó, J., Girard, E., Benoit, E., 2008. 18 Cyclic Imines: An insight into this emerging group of bioactive marine toxins. In Phycotoxins: chemistry and biochemistry; Botana, L. M., Ed.; Wiley-Blackwell: Ames, IA, USA, 319-335.

Moore, R.E., Bartolini, G., 1981. Structure of palytoxin. Journal of the American Chemical Society 103, 2491-2494.

Moore, R.E., Scheuer, P.J., 1971. Palytoxin: a new marine toxin from a coelenterate. Science 172, 495-498.
Munday, R., 2008. Occurrence and toxicology of palytoxins. Food Science and Technology 173, 693.
Munday, R., 2011. Palytoxin toxicology: Animal studies. Toxicon 57, 470-477.
Murata, M., Legrand, A.M., Ishibashi, Y., Yasumoto, T., 1989. Structures of ciguatoxin and its congener. Journal of the American Chemical Society 111, 8929-8931.
Murata, M., Legrand, A.N., Ishibashi, Y., Fukui, M., Yasumoto, T., 1990. Conformations of ciguatoxin and related polyethers. Abstracts of Papers of the American Chemical Society 200, 54-AGFD.
Narahashi, T., Moore, J.W., Scott, W.R., 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. The Journal of general physiology 47, 965-974.
Nascimento, S.M., Correa, E.V., Menezes, M., Varela, D., Paredes, J., Morris, S., 2012. Growth and toxin profile of *Ostreopsis cf. ovata* (Dinophyta) from Rio de Janeiro, Brazil. Harmful Algae 13, 1-9.
Neagu, D., Micheli, L., Palleschi, G., 2006. Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. Anal. Bioanal. Chem. 385, 1068-1074.
Nezan, E., Chomerat, N., 2011. Vulcanodinium rugosum gen. nov., sp. nov. (Dinophyceae): a new marine dinoflagellate from the French Mediterranean coast. Cryptogam. Algol. 32, 3-18.
Noguchi, T., Arakawa, O., Takatani, T., 2006. TTX accumulation in pufferfish. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 1, 145-152.
O'Toole, A.C., Dechraoui Bottein, M.-Y., Danylchuk, A.J., Ramsdell, J.S., Cooke, S.J., 2012. Linking ciguatera poisoning to spatial ecology of fish: A novel approach to examining the distribution of biotoxin levels in the great barracuda by combining non-lethal blood sampling and biotelemetry. Science of the Total Environment 427, 98-105.
Oguri, H., Hirama, M., Tsumuraya, T., Fujii, I., Maruyama, M., Uehara, H., Nagumo, Y., 2003. Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. Journal of the American Chemical Society 125, 7608-7612.
Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., Ratsimaloto, M., Naoki, H., Yasumoto, T., 1999. Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37, 55-65.
Otero, A., Chapela, M.-J., Atanassova, M., Vieites, J.M., Cabado, A.G., 2011a. Cyclic Imines: Chemistry and Mechanism of Action: A Review. Chemical research in toxicology 24, 1817-1829.
Otero, P., Alfonso, A., Alfonso, C., Araoz, R., Molgo, J., Vieytes, M.R., Botana, L.M., 2011b. First direct fluorescence polarization assay for the detection and quantification of spirolides in mussel samples. Analytica Chimica Acta 701, 200-208.
Pagliara, P., Caroppo, C., 2011. Cytotoxic and antimitotic activities in aqueous extracts of eight cyanobacterial strains isolated from the marine sponge *Petrosia ficiformis*. Toxicon 57, 889-896.
Pancrazio, J.J., Gray, S.A., Shubin, Y.S., Kulagina, N., Cuttino, D.S., Shaffer, K.M., Eisemann, K., Curran, A., Zim, B., Gross, G.W., 2003. A portable microelectrode array recording system incorporating cultured neuronal networks for neurotoxin detection. Biosensors and Bioelectronics 18, 1339-1347.
Park, D.L., 1995. Detection of ciguatera and diarrheic shellfish toxins in finfish and shellfish with ciguatect kit. Journal of Aoac International 78, 533-537.

Pauillac, S., Sasaki, M., Inoue, M., Naar, J., Branaa, P., Chinain, M., Tachibana, K., Legrand, A.M., 2000. Characterization of mice antisera elicited with a ciguatoxin tetracyclic synthetic ring fragment (JKLM) conjugated to carrier proteins. Toxicon 38, 669-685.

Pawlowiez, R., Darius, H.T., Cruchet, P., Rossi, F., Caillaud, A., Laurent, D., Chinain, M., 2013. Evaluation of seafood toxicity in the Australes archipelago (French Polynesia) using the neuroblastoma cell-based assay. Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment 30, 567-586.

Penna, A., Fraga, S., Battocchi, C., Casabianca, S., Giacobbe, M.G., Riobo, P., Vernesi, C., 2010. A phylogeographical study of the toxic benthic dinoflagellate genus *Ostreopsis* Schmidt. J. Biogeogr. 37, 830-841.

Perez-Arellano, J.L., Luzardo, O.P., Brito, A.P., Cabrera, M.H., Zumbado, M., Carranza, C., Angel-Moreno, A., Dickey, R.W., Boada, L.D., 2005. Ciguatera fish poisoning, Canary Islands. Emerging Infectious Diseases 11, 1981-1982.

Pezzolesi, L., Guerrini, F., Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E., Forino, M., Tartaglione, L., Pistocchi, R., 2012. Influence of temperature and salinity on *Ostreopsis cf. ovata* growth and evaluation of toxin content through HR LC-MS and biological assays. Water Research 46, 82-92.

Pigozzi, S., Bianchi, L., Boschetti, L., Cangini, M., Ceredi, A., Magnani, F., Milandri, A., Montanari, S., Pompei, M., Riccardi, E., 2006. First evidence of spirolide accumulation in northwestern Adriatic shellfish. In: Proceedings of the 12th ICHA, (Eds.) Moestrup, O. et al., ISSHA and IOC of UNESCO: Copenhagen, Denmark, 2008. 319-322.

Poli, M.A., Lewis, R.J., Dickey, R.W., Musser, S.M., Buckner, C.A., Carpenter, L.G., 1997. Identification of Caribbean ciguatoxins as the cause of an outbreak of fish poisoning among US soldiers in Haiti. Toxicon 35, 733-741.

Pratheepa, V., Vasconcelos, V., 2013. Microbial diversity associated with tetrodotoxin production in marine organisms. Environmental Toxicology and Pharmacology 36, 1046-1054.

Raybould, T.J.G., Bignami, G.S., Inouye, L.K., Simpson, S.B., Byrnes, J.B., Grothaus, P.G., Vann, D.C., 1992. A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. J. Clin. Lab. Anal. 6, 65-72.

Rhodes, L., Adamson, J., Suzuki, T., Briggs, L., Garthwaite, I., 2000. Toxic marine epiphytic dinoflagellates, *Ostreopsis siamensis* and *Coolia monotis* (Dinophyceae), in New Zealand. N. Z. J. Mar. Freshw. Res. 34, 371-383.

Rhodes, L., Smith, K., Selwood, A., McNabb, P., van Ginkel, R., Holland, P., Munday, R., 2010a. Production of pinnatoxins by a peridinoid dinoflagellate isolated from Northland, New Zealand. Harmful Algae 9, 384-389.

Rhodes, L., Towers, N., Briggs, L., Munday, R., Adamson, J., 2002. Uptake of palytoxin-like compounds by shellfish fed *Ostreopsis siamensis* (Dinophyceae). N. Z. J. Mar. Freshw. Res. 36, 631-636.

Rhodes, L.L., Smith, K.F., Munday, R., Selwood, A.I., McNabb, P.S., Holland, P.T., Bottein, M.-Y., 2010b. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. Toxicon 56, 751-758.

Riobo, P., Paz, B., Franco, J.M., 2006. Analysis of palytoxin-like in Ostreopsis cultures by liquid chromatography with precolumn derivatization and fluorescence detection. Analytica Chimica Acta 566, 217-223.

Riobo, P., Paz, B., Franco, J.M., Vazquez, J.A., Murado, M.A., 2008. Proposal for a simple and sensitive haemolytic assay for palytoxin. Toxicological dynamics, kinetics, ouabain inhibition and thermal stability. Harmful Algae 7, 415-429.

Robertson, A., Garcia, A.C., Quintana, H.A.F., Smith, T.B., Castillo, B.F., Reale-Munroe, K., Gulli, J.A., Olsen, D.A., Hooe-Rollman, J.I., Jester, E.L.E., Klimek, B.J., Plakas, S.M., 2014. Invasive Lionfish (Pterois volitans): A Potential Human Health Threat for Ciguatera Fish Poisoning in Tropical Waters. Marine Drugs 12, 88-97.

Rodriguez, L.P., Vilarino, N., Molgo, J., Araoz, R., Antelo, A., Vieytes, M.R., Botana, L.M., 2011. Solid-Phase Receptor-Based Assay for the Detection of cyclic imines by chemiluminescence, fluorescence, or colorimetry. Analytical Chemistry 83, 5857-5863.

Rodriguez, L.P., Vilarino, N., Molgo, J., Araoz, R., Botana, L.M., 2013b. High-throughput receptor-based assay for the detection of spirolides by chemiluminescence. Toxicon 75, 35-43.

Rodriguez, L.P., Vilarino, N., Molgo, J., Araoz, R., Carmen Louzao, M., Taylor, P., Talley, T., Botana, L.M., 2013a. Development of a Solid-Phase Receptor-Based Assay for the Detection of Cyclic Imines Using a Microsphere-Flow Cytometry System. Analytical Chemistry 85, 2340-2347.

Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., Botana, L.M., 2008. First toxicity report of tetrodotoxin and 5, 6, 11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe. Analytical Chemistry 80, 5622-5629.

Rossini, G.P., Bigiani, A., 2011. Palytoxin action on the Na^+, K^+-ATPase and the disruption of ion equilibria in biological systems. Toxicon 57, 429-439.

Satake, M., Fukui, M., Legrand, A.M., Cruchet, P., Yasumoto, T., 1998. Isolation and structures of new ciguatoxin analogs, 2,3-dihydroxyCTX3C and 51-hydroxyCTX3C, accumulated in tropical reef fish. Tetrahedron letters 39, 1197-1198.

Satake, M., Ishimaru, T., Legrand, A., Yasumoto, T., 1993a. Isolation of a ciguatoxin analog from cultures of *Gambierdiscus toxicus*, in: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York, pp. 575-579.

Satake, M., Ishimaru, T., Legrand, A.M., Yasumoto, T., 1993b. Isolation of a ciguatoxin analog from cultures of *Gambierdiscus toxicus*.

Satake, M., Morohashi, A., Oguri, H., Oishi, T., Hirama, M., Harada, N., Yasumoto, T., 1997. The absolute configuration of ciguatoxin. Journal of the American Chemical Society 119, 11325-11326.

Satake, M., Murata, M., Yasumoto, T., 1993c. The structure of CTX3C, a ciguatoxin congener isolated from cultured *Gambierdiscus toxicus*. Tetrahedron letters 34, 1975-1978.

Satoh, E., Ishii, T., Nishimura, M., 2003. Palytoxin-induced increase in cytosolic-free Ca^{2+} in mouse spleen cells. European journal of pharmacology 465, 9-13.

Scheuer, P., Takahaski, W., Tsutsumi, J., Yoshida, T., 1967. Ciguatoxin: isolation and chemical structure. Science 155, 1267-1268.

Seemann, P., Gernert, C., Schmitt, S., Mebs, D., Hentschel, U., 2009. Detection of hemolytic bacteria from *Palythoa caribaeorum* (Cnidaria, Zoantharia) using a novel palytoxin-screening assay. Antonie Van Leeuwenhoek 96, 405-411.

Seki, T., Satake, M., Mackenzie, L., Kaspar, H.F., Yasumoto, T., 1995. Gymnodimine, a new marine toxin of unprecedented structure isolated from New-Zeland oysters and the dinofalgellate *Gymnnodinium sp.* Tetrahedron letters 36, 7093-7096.

Selwood, A.I., Miles, C.O., Wilkins, A.L., van Ginkel, R., Munday, R., Rise, F., McNabb, P., 2010. Isolation, structural determination and acute toxicity of pinnatoxins E, F and G. Journal of Agricultural and Food Chemistry 58, 6532-6542.

Shimahara, T., Molgó, J., 1990. Palytoxin enhances quantal acetylcholine release from motor nerve terminals and increases cytoplasmic calcium levels in a neuronal hybrid cell line. Life Sci Adv Pharmacol 9, 785-792.

Shimojo, R.Y., Iwaoka, W.T., 2000. A rapid hemolysis assay for the detection of sodium channel-specific marine toxins. Toxicology 154, 1-7.

Silva, M., Azevedo, J., Rodriguez, P., Alfonso, A., Botana, L.M., Vasconcelos, V., 2012. New gastropod vectors and tetrodotoxin potential expansion in temperate waters of the atlantic ocean. Marine Drugs 10, 712-726.

Sperr, A.E., Doucette, G.J., 1996. Variation in growth rate and ciguatera toxin production among geographically distinct isolates of Gambierdiscus toxicus, in: *Harmful and Toxic Algal Blooms.* (Ed.) Yasumoto, T., Oshima, Y., Fukuyo Y. IOC of UNESCO, Paris, France 309-312.

Stokes, A.N., Williams, B.L., French, S.S., 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. Biological procedures online 14, 1-5.

Tahara, Y., Hirata, Y., 1909. Studies on the puffer fish toxin. J. Pharm. Soc. Jpn 29, 587-625.

Takada, N., Umemura, N., Suenaga, K., Chou, T., Nagatsu, A., Haino, T., Yamada, K., Uemura, D., 2001. Pinnatoxins B and C, the most toxic components in the pinnatoxin series from the Okinawan bivalve Pinna muricata. Tetrahedron letters 42, 3491-3494.

Taniyama, S., Arakawa, O., Terada, M., Nishio, S., Takatani, T., Mahmud, Y., Noguchi, T., 2003. Ostreopsis sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. Toxicon 42, 29-33.

Taniyama, S., Mahmud, Y., Terada, M., Takatani, T., Arakawa, O., Noguchi, T., 2002. Occurrence of a food poisoning incident by palytoxin from a serranid *Epinephelus sp* in Japan. Journal of Natural Toxins 11, 277-282.

Tao, J., Wei, W., Nan, L., Lei, L., Hui, H., Fen, G., Jun, L., Jing, Z., Rong, J., 2010. Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China. Food Additives and Contaminants 27, 1589-1597.

Tatters, A.O., Muhlstein, H.I., Tomas, C.R., 2010. The hemolytic activity of *Karenia selliformis* and two clones of *Karenia brevis* throughout a growth cycle. J. Appl. Phycol. 22, 435-442.

Taylor, A.D., Ladd, J., Etheridge, S., Deeds, J., Hall, S., Jiang, S., 2008. Quantitative detection of tetrodotoxin (TTX) by a surface plasmon resonance (SPR) sensor. Sensors and Actuators B-Chemical 130, 120-128.

Taylor, A.D., Vaisocherová, H., Deeds, J., DeGrasse, S., Jiang, S., 2011. Tetrodotoxin detection by a surface plasmon resonance sensor in pufferfish matrices and urine. Journal of Sensors 2011, 10.

Thattiyaphong, A., Unahalekhaka, J., Mekha, N., Nispa, W., Kluengklangdon, P., Rojanapantip, L., 2013. Efficiency of a rapid test for detection of tetrodotoxin in puffer fish. Journal of Immunoassay and Immunochemistry 35, 111-119.

Torigoe, K., Murata, M., Yasumoto, T., Iwashita, T., 1988. Prorocentrolide, a toxic nitrogenous macrocycle from a marine dinoflagellate, *Prorocentrum lima*. Journal of the American Chemical Society 110, 7876-7877.

Touzet, N., Franco, J.M., Raine, R., 2008. Morphogenetic diversity and biotoxin composition of Alexandrium (Dinophyceae) in Irish coastal waters. Harmful Algae 7, 782-797.

Tsuda, K., Tachikawa, R., Sakai, K., Tamura, C., Amakasu, O., Kawamura, M., Ikuma, S., 1964. On the structure of tetrodotoxin. Chemical & pharmaceutical bulletin 12, 642-645.

Tsumuraya, T., Fujii, I., Hirama, M., 2010. Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Toxicon 56, 797-803.

Tsumuraya, T., Fujii, I., Hirama, M., 2014. Preparation of Anti-Ciguatoxin Monoclonal Antibodies Using Synthetic Haptens: Sandwich ELISA Detection of Ciguatoxins. Journal of Aoac International 97, 373-379.

Tsumuraya, T., Fujii, I., Inoue, M., Tatami, A., Miyazaki, K., Hirama, M., 2006. Production of monoclonal antibodies for sandwich immunoassay detection of ciguatoxin 51-hydroxyCTX3C. Toxicon 48, 287-294.

Tsumuraya, T., Takeuchi, K., Yamashita, S., Fujii, I., Hirama, M., 2012. Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA. Toxicon 60, 348-357.

Tubaro, A., Durando, P., Del Favero, G., Ansaldi, F., Icardi, G., Deeds, J.R., Sosa, S., 2011. Case definitions for human poisonings postulated to palytoxins exposure. Toxicon 57, 478-495.

Uemura, D., Chou, T., Haino, T., Nagatsu, A., Fukuzawa, S., Zheng, S.Z., Chen, H.S., 1995. Pinnatoxin A. A toxic amphoteric macrocycle from the okinawan bivalve *Pinna Muricata*. Journal of the American Chemical Society 117, 1155-1156.

Uemura, D., Hirata, Y., Iwashita, T., Naoki, H., 1985. Studies on palytoxins. Tetrahedron 41, 1007-1017.

Uemura, D., Ueda, K., Hirata, Y., Naoki, H., Iwashita, T., 1981. Further studies on palytoxin. II. Structure of palytoxin. Tetrahedron letters 22, 2781-2784.

Ukena, T., Satake, M., Usami, M., Oshima, Y., Fujita, T., Naoki, H., Yasumoto, T., 2002. Structural confirmation of ostreocin-D by application of negative-ion fast-atom bombardment collision-induced dissociation tandem mass spectrometric methods. Rapid communications in mass spectrometry 16, 2387-2393.

Ukena, T., Satake, M., Usami, M., Oshima, Y., Naoki, H., Fujita, T., Kan, Y., Yasumoto, T., 2001. Structure elucidation of ostreocin D, a palytoxin analog isolated from the dinoflagellate *Ostreopsis siamensis*. Bioscience, biotechnology, and biochemistry 65, 2585-2588.

Vaisocherova, H., Taylor, A.D., Jiang, S., Hegnerova, K., Vala, M., Homola, J., Yakes, B.J., Deeds, J., DeGrasse, S., 2011. Surface plasmon resonance biosensor for determination of tetrodotoxin: Prevalidation study. Journal of Aoac International 94, 596-604.

Vale-Gonzalez, C., Pazos, M., Alfonso, A., Vieytes, M., Botana, L., 2007. Study of the neuronal effects of ouabain and palytoxin and their binding to Na, K-ATPases using an optical biosensor. Toxicon 50, 541-552.

van de Riet, J.M., Gibbs, R.S., Chou, F.W., Muggah, P.M., Rourke, W.A., Burns, G., Thomas, K., Quilliam, M.A., 2009. Liquid Chromatographic Post-Column Oxidation Method for Analysis of Paralytic Shellfish Toxins in Mussels, Clams, Scallops, and Oysters: Single-Laboratory Validation. Journal of Aoac International 92, 1690-1704.

Van Wagoner, R.M., Misner, I., Tomas, C.R., Wright, J.L., 2011. Occurrence of 12-methylgymnodimine in a spirolide-producing dinoflagellate *Alexandrium peruvianum* and the biogenetic implications. Tetrahedron letters 52, 4243-4246.

Vernoux, J.P., Lahlou, N., Elandaloussi, S.A., Riyeche, N., Magras, L.P., 1985. A study of the distribution of ciguatoxin in indiviual Caribbean fish. Acta Tropica 42, 225-233.

Vidyarathna, N.K., Graneli, E., 2011. Influence of temperature on growth, toxicity and carbohydrate production of a Japanese Ostreopsis ovata strain, a toxic-bloom-forming dinoflagellate. Aquatic Microbial Ecology 65, 261-270.

Vidyarathna, N.K., Graneli, E., 2013. Physiological responses of *Ostreopsis ovata* to changes in N and P availability and temperature increase. Harmful Algae 21-22, 54-63.

Vilariño, N., Fonfria, E.S., Molgo, J., Araoz, R., Botana, L.M., 2009. Detection of Gymnodimine-A and 13-Desmethyl C Spirolide Phycotoxins by Fluorescence Polarization. Analytical Chemistry 81, 2708-2714.

Volpe, G., Cozzi, L., Migliorelli, D., Croci, L., Palleschi, G., 2014. Development of a haemolytic-enzymatic assay with mediated amperometric detection for palytoxin analysis: application to mussels. Anal. Bioanal. Chem. 406, 2399-2410.

Wachi, K.M., Hokama, Y., 2001. Diversity of marine biotoxins in the near-shore ocean area: presence of a palytoxin-like entity at Barber's Point Harbor, Oahu. Journal of natural toxins 10, 317-333.

Wachi, K.M., Hokama, Y., Haga, L.S., Shiraki, A., Takenaka, W.E., Bignami, G.S., Levine, L., 2000. Evidence for palytoxin as one of the sheep erythrocyte lytic in lytic factors in crude extracts of ciguateric and non-ciguateric reef fish tissue. Journal of Natural Toxins 9, 139-146.

Walsh, G.E.B., Ralph L, 1971. A review of Hawaiian zoanthids with descriptions of three new species. Zoological Journal of the Linnean Society 50, 161-180.

Wang, R., Huang, A., Liu, L., Xiang, S., Li, X., Ling, S., Wang, L., Lu, T., Wang, S., 2014. Construction of a single chain variable fragment antibody (scFv) against tetrodotoxin (TTX) and its interaction with TTX. Toxicon: official journal of the International Society on Toxinology 83, 22-34.

Watkins, S.M., Reich, A., Fleming, L.E., Hammond, R., 2008. Neurotoxic shellfish poisoning. Marine Drugs 6, 431-455.

Wattenberg, E.V., 2011. Modulation of protein kinase signaling cascades by palytoxin. Toxicon 57, 440-448.

Weiser, T., 2004. A novel toxicity-based assay for the identification of modulators of voltage-gated Na^+ channels. Journal of neuroscience methods 137, 79-85.

Woodward, R., Gougoutas, J.Z., 1964. The structure of tetrodotoxin. Pure Appl. Chem 9, 49-74.

Woofter, R.T., Brendtro, K., Ramsdell, J.S., 2005. Uptake and elimination of brevetoxin in blood of striped mullet (*Mugil cephalus*) after aqueous exposure to *Karenia brevis*. Environmental health perspectives, 11-16.

Wu, J.J., Mak, Y.L., Murphy, M.B., Lam, J.C.W., Chan, W.H., Wang, M., Chan, L.L., Lam, P.K.S., 2011. Validation of an accelerated solvent extraction liquid chromatography- tandem mass spectrometry method for Pacific ciguatoxin-1 in fish flesh and comparison with the mouse neuroblastoma assay. Anal. Bioanal. Chem. 400, 3165-3175.

Wu, Z., Xie, L., Xia, G., Zhang, J., Nie, Y., Hu, J., Wang, S., Zhang, R., 2005. A new tetrodotoxin-producing actinomycete, *Nocardiopsis dassonvillei*, isolated from the ovaries of puffer fish *Fugu rubripes*. Toxicon 45, 851-859.

Yakes, B., DeGrasse, S., Poli, M., Deeds, J., 2011. Antibody characterization and immunoassays for palytoxin using an SPR biosensor. Anal. Bioanal. Chem. 400, 2865-2869.

Yakes, B.J., Deeds, J., White, K., DeGrasse, S.L., 2011

Evaluation of Surface Plasmon Resonance Biosensors for Detection of Tetrodotoxin in Food Matrices and Comparison to Analytical Methods. Journal of Agricultural and Food Chemistry 59, 839-846.

Yakes, B.J., Etheridge, S.M., Mulvaney, S.P., Tamanaha, C.R., 2010. Fluidic Force Discrimination Assays: A New Technology for Tetrodotoxin Detection. Marine Drugs 8, 565-576.

Yakes, B.J., Kanyuck, K.M., DeGrasse, S.L., 2014. First Report of a Direct Surface Plasmon Resonance Immunosensor for a Small Molecule Seafood Toxin. Analytical Chemistry.

Yasumoto, T., Search for ciguatoxin producing clones of *Gambierdiscus toxicus* on Okinawan coastal reefs. In Abstract book of the Ciguatera and related biotoxins worshop, Institut de recherché pour le développement (IRD). Noumea, New Caledonia 2008.

Yasumoto, T., Fukui, M., Sasaki, K., Sugiyama, K., 1995. Determinations of marine toxins in foods. J AOAC Int. 78, 574-582.

Yasumoto, T., Nakajima, I., Bagnis, R., Adachi, R., 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bulletin of the Japanese Society of Scientific Fisheries 43, 1021-1026.

Yokoo, A., 1950. Chemical studies on pufferfish toxin (3)-separation of spheroidine. Nippon Kagaku Zasshi 71, 590-592.

Yotsu-Yamashita, M., Mebs, D., 2003. Occurrence of 11-oxotetrodotoxin in the red-spotted newt, *Notophthalmus viridescens*, and further studies on the levels of tetrodotoxin and its analogues in the newt's efts. Toxicon 41, 893-897.

Yotsu-Yamashita, M., Sugimoto, A., Takai, A., Yasumoto, T., 1999. Effects of specific modifications of several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. Journal of Pharmacology and Experimental Therapeutics 289, 1688-1696.

Yu, C.-H., Yu, C.-F., Tam, S., Hoi-Fu Yu, P., 2010. Rapid screening of tetrodotoxin in urine and plasma of patients with puffer fish poisoning by HPLC with creatinine correction. Food Additives and Contaminants 27, 89-96.

Zamolo, V.A., Valenti, G., Venturelli, E., Chaloin, O., Marcaccio, M., Boscolo, S., Castagnola, V., Sosa, S., Berti, F., Fontanive, G., Poli, M., Tubaro, A., Bianco, A., Paolucci, F., Prato, M., 2012. Highly Sensitive Electrochemiluminescent Nanobiosensor for the Detection of Palytoxin. Acs Nano 6, 7989-7997.

Zhou, Y., Li, Y., Lu, S., Ren, H., Li, Z., Zhang, Y., Pan, F., Liu, W., Zhang, J., Liu, Z., 2010. Gold nanoparticle probe-based immunoassay as a new tool for tetrodotoxin detection in puffer fish tissues. Sensors and Actuators B: Chemical 146, 368-372.

Zhou, Y., Li, Y., Pan, F., Liu, Z., Wang, Z., 2009. Identification of tetrodotoxin antigens and a monoclonal antibody. Food chemistry 112, 582-586.

Analytica Chimica Acta 908 (2016) 8–21

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Review

New advances in electrochemical biosensors for the detection of toxins: Nanomaterials, magnetic beads and microfluidics systems. A review

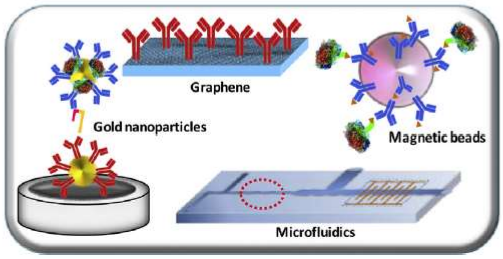
Laia Reverté [a], Beatriz Prieto-Simón [b], Mònica Campàs [a, *]

[a] IRTA, Carretera Poble Nou km. 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain
[b] ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Future Industries Institute, University of South Australia, SA 5095, Australia

HIGHLIGHTS

- Nanomaterials improve the performance of electrochemical biosensors.
- Carbon nanomaterials can act as electrocatalysts or label supports in biosensors.
- Metal nanomaterials can act as nanostructured supports or labels in biosensors.
- Magnetic beads are exploited as immobilisation supports and/or label carriers.

GRAPHICAL ABSTRACT



3.2 New advances in electrochemical biosensors for the detection of toxins: Nanomaterials, magnetic beads and microfluidics systems. A review

3.2.1 Abstract

The use of nanotechnology in bioanalytical devices has special advantages in the detection of toxins of interest in food safety and environmental applications. The low levels to be detected and the small size of toxins justify the increasing number of publications dealing with electrochemical biosensors, due to their high sensitivity and design versatility. The incorporation of nanomaterials in their development has been exploited to further increase their sensitivity, providing simple and fast devices, with multiplexed capabilities. This paper gives an overview of the electrochemical biosensors that have incorporated carbon and metal nanomaterials in their configurations for the detection of toxins. Biosensing systems based on magnetic beads or integrated into microfluidics systems have also been considered because of their contribution to the development of compact analytical devices. The roles of these materials, the methods used for their incorporation in the biosensor configurations as well as the advantages they provide to the analyses are summarised.

3.2.2 Introduction

Over the past decade the number of publications describing the incorporation of nanomaterials in biosensors has exponentially increased. Several reviews on nanomaterial-based biosensors have recently been published with focus on certain types of target analytes, detection techniques and applications (Barsan et al., 2015; Campas et al., 2012; Dai and Ju, 2012; Handford et al., 2014; Justino et al., 2013; Malhotra et al., 2014; Marin and Merkoci, 2012; Pedrero et al., 2012; Sundramoorthy and Gunasekaran, 2014; Tothill, 2011; Wang and Yu, 2013; Wang et al., 2010). The analysis of the published literature reveals the well-established use of nanotechnology in biomedical applications, and the increasing interest of its application in the agro-food sector and the environmental field.

This paper aims to critically review research work that exploits nanotechnology for the development of biosensors. Due to the highly productive publication records in the field, the scope of the review is limited to electrochemical biosensors for the detection of toxins of interest in agro-food and environmental applications. Biosensing systems based on the use of magnetic beads and/or microfluidics are also considered due to their key contribution towards the development of

compact analytical devices. Tables 3.2.1, 3.2.2 and 3.2.3 summarise the electrochemical biosensors for mycotoxins, aquatic toxins and other analytes, respectively, reviewed according to the biorecognition element, electrochemical technique, performance parameters and analysed matrix.

3.2.3 Incorporation of nanomaterials in biosensors

The International Organisation for Standardisation (2011) defines the term "nanomaterial" as a material with an external dimension, or an internal or surface structure in the nanoscale (1–100 nm). Nanomaterials have unique optical and electrical characteristics that make particularly interesting their incorporation in biosensor configurations by providing advantages, such as high sensitivity, low limits of detection (LODs) and reduced matrix effects. The features of certain nanomaterials, such as their biocompatibility, conductivity, catalytic activity or stability, show them an attractive choice for certain purposes: they are widely used as label supports and signal enhancers, as they increase the electroactive surface area, and might favour the electron transfer and amplify the electrochemical signals. Carbon and metal nanomaterials have often been incorporated in electrochemical biosensor configurations. Next, these works are reviewed and classified according to the purpose of their use in the biosensor configuration as well as the method used for the nanomaterial incorporation.

3.2.3.1 Carbon nanomaterials

The unique physical and chemical properties of carbon nanomaterials, such as ultra lightweight, high mechanical strength, excellent electrical and thermal conductivity, highly ordered structure and high surface area, are responsible for the increasing interest in their incorporation in biosensor configurations. Carbon nanotubes (CNTs), carbon nanospheres (CNSs), carbon nanohorns (CNHs), graphene oxide (GO) and graphene nanoribbons (GNRs) have been immobilised onto electrodes, sometimes the carbon nanomaterial being modified with biomolecules prior or after the immobilisation step, to amplify the electrochemical signal and decrease the limit of detection in biosensing systems. Another role of carbon nanomaterials in biosensors is to act as supports for the label in competition and sandwich assay formats, also enhancing the electrochemical response.

3.2.3.1.1 Carbon nanomaterials-modified electrodes

The modification of electrodes with carbon nanomaterials provides advantages such as larger surface areas that can lead to higher number of immobilised bioreceptors and enhanced electrochemical signals, which as a consequence result in higher sensitivities and lower limits of

detection. Several techniques, alone or in combination, have been used for the immobilisation of carbon nanomaterials: adsorption, entrapment into polymers, covalent binding, electrochemical techniques and chemical vapour deposition. Below, examples of carbon nanomaterials-modified electrodes are described and organised according to the immobilisation method.

3.2.3.1.1.1 Adsorption

Due to its simplicity, adsorption has been widely used as immobilisation technique in biosensor development. Adsorption of multi-walled CNTs (MWCNTs) onto a gold electrode was used by Yao et al. (Yao et al., 2006) to develop an electrochemical biosensor for the detection of the mycotoxin sterigmatocystin (ST), a precursor of aflatoxin B_1 (AFB_1). The enzyme sensor prepared by covalent binding of aflatoxin–detoxifizyme (ADTZ), the enzyme responsible for the detoxification of AFB_1 and ST, to activated MWCNTs showed wider working range (0.04 – 1.33 µg/mL) and lower LOD (42 ng/mL) than those obtained by an equivalent sensor based on the adsorption of ADTZ on adsorbed MWCNTs (Yao et al., 2004). The improved performance of the former relies on the active centre protection undertaken during the immobilisation procedure: ADTZ was saturated with its substrate ST to protect the active centre from being chemically modified and thus to maintain its activity. Similarly, Li and co-workers (Li et al., 2011) developed an enzyme sensor for AFB_1 detection that incorporates MWCNTs adsorbed onto the electrode surface. The enzyme aflatoxin oxidase (AFO), embedded in a silica sol-gel, was covalently bound to the activated MWCNTs previously adsorbed. The sol-gel matrix provides the biocompatibility required to retain enzyme activity and prevents enzyme leakage. The biosensor exhibited a linear range from 1 to 225 ng/mL of AFB_1 with an LOD of 0.5 ng/mL. Upon CNTs adsorption, different approaches have been suggested to incorporate both bioreceptors and electron transfer mediators onto the already modified surface, aiming to improve stability and electron transfer. As an example, Fang et al. (Fang et al., 2014) reported the immobilisation of MWCNTs on glassy carbon electrodes, followed by the subsequent incorporation of Prussian Blue (PB), chitosan and glutaraldehyde. Chitosan provides the proper environment for the immobilisation of anti-*Clostridium difficile* toxin B antibody and avoids leakage of MWCNTs and PB, thus increasing the sensor stability. After toxin binding, graphene oxide (GO) conjugated with horse-radish peroxidase (HRP) and secondary HRP-labelled antibody was subsequently incubated as part of a multienzyme amplification step. The system showed a linear working range from 3 pg/mL to 320 ng/mL with an LOD of 0.7 pg/mL. This low LOD was attributed to the high amount of immobilised antibody, the excellent electrical conductivity of MWCNTs and improved electron transfer mediated by PB, and the signal amplification provided by the use of GO as enzyme/antibody carrier.

A particular study is that reported by Wang et al. (Wang et al., 2009) on a CNT-modified paper electrode for the detection of microcystin-LR (MC-LR). Paper impregnated with single-walled CNTs (SWCNTs) and anti-MC-LR antibody was shown as a simple and low-cost method of biosensor design. The change in conductivity of the paper electrode in the presence of MC-LR allowed the detection of MC-LR at an LOD of 0.6 ng/mL and a linear range up to 10 ng/mL.

Apart from CNTs, other carbon nanomaterials have also been adsorbed on electrodes. Srivastava et al. (Srivastava et al., 2014) developed an impedimetric immunosensor for the detection of AFB_1 incorporating GO. Anti-AFB_1 antibodies were conjugated to GO previously adsorbed on gold electrodes. The large available surface area and numerous carboxyl groups on the GO sheets provided a high loading of anti-AFB_1 antibodies, resulting in a wide linear detection range (0.5 – 5 ng/mL) and a low LOD (0.23 ng/mL). Zhang et al. (Zhang et al., 2010) described the development of an immunosensor for the detection of MC-LR based on a competitive immunoassay, where MC-LR was covalently conjugated to carboxyl groups on the single-walled carbon nanohorns (SWCNHs) previously adsorbed on a glassy carbon electrode. The biosensor exhibited a wide linear response, 0.05 – 20 ng/mL, an LOD of 30 pg/mL and, in the analysis of polluted water, good agreement with the values obtained using high performance liquid chromatography (HPLC) as reference analytical method.

3.2.3.1.1.2 Entrapment and/or covalent binding

Carbon nanomaterials have also been immobilised onto the electrode surface via covalent binding and/or entrapment into polymers. Chen and co-workers (Chen et al., 2010) developed an enzyme sensor for ST by co-entrapment of AFO and SWCNTs in a chitosan film adsorbed on a gold electrode. The nanocomposite matrix provided a favourable environment for the enzyme, which preserves the enzyme activity and promotes the direct electron transfer between the enzyme active site and the electrode surface. As a result, an LOD of 3 ng/mL was attained. Also based on carbon nanomaterials entrapment into a chitosan film, Kaushik et al. (Kaushik et al., 2010) developed an immunosensor for the detection of ochratoxin A (OTA): chitosan-MWCNTs nanobiocomposites were adsorbed on indium tin oxide (ITO) electrodes for the subsequent co-immobilisation via adsorption of immunoglobulin G (IgG) and bovine serum albumin (BSA). The high antibody loading together with increased electroactive surface area enhanced the electron transport, providing a linear range between 25 and 600 pg/mL and an LOD of 25 pg/mL. MWCNTs have also been embedded in Nafion for the ultrasensitive detection of cholera toxin (CT) (Viswanathan et al., 2006).

Anti-CT antibodies were entrapped in a poly(3,4-ethylenedioxythiophene) (PEDOT) film electropolymerised onto the MWCNT-modified Nafion layer, providing high conductivity and a large number of recognition sites. Voltammetric measurements using the sandwich immunosensor, which exploits liposomic magnification, provided a linear range of 10 fg/mL – 100 ng/mL and an LOD of 1 fg/mL. This low value was certainly achieved because of the cooperative functions of Nafion, MWCNTs, PEDOT and the liposomes used as labels.

A particular case is that in which CNTs were exploited as supports for the surface imprinting of plastic antibodies, also known as molecularly imprinted polymers (MIPs), for the detection of MCs (Queiros et al., 2013). The molecularly imprinted MWCNTs were integrated as ionophores into a polyvinylchloride (PVC) selective membrane on a carbon electrode for the development of a potentiometric sensor. Results showed similar behaviour for all tested MCs (MC-LR, MC-YR and MC-RR), with an LOD below 1 ng/mL, and non-significant interfering effects from sulphate, iron and ammonium ions, chloroform and tetrachloroethylene.

Conductive polymers have also been used to covalently bind carbon nanomaterials on the electrode surface. Such is the case of the immunosensor for the detection of Anthrax protective antigen described by Huan et al. (Huan et al., 2011). Firstly, aniline monomers were electropolymerised onto the surface of a glassy carbon electrode to form a polyaniline (PANI) film. Afterwards, activated MWCNTs were covalently bound to PANI. Remaining carboxylic groups of MWCNTs were then conjugated to a specific peptide used as a biorecognition molecule. An LOD of 33 pg/mL was attained, which was 13-fold lower in comparison to that of a sensor in which a self-assembled monolayer was used for the immobilisation of the same peptide (Huan et al., 2009). The use of a PANI layer not only allowed the nanomaterial immobilisation, but also provided good electrochemical conductivity. Similarly, Wang et al. (Wang et al., 2015) entrapped reduced GO (rGO) during the electrochemical co-polymerisation of polypyrrole (PPy) and pyrrolepropylic acid (PPa). Anti-AFB_1 antibodies were covalently bound to the carboxyl groups of the PPa. The authors reported a working range of 10 fg/mL–10 pg/mL and a very low LOD of 10 fg/mL. The improved electroactivity provided by PPy, and the electric conductivity, rigidity and high stability rendered by rGO contributed to the excellent performance of the biosensor.

Although not being a polymer, the use of ionic liquids for nanomaterial entrapment deserves to be mentioned in this section. Yu et al. (Yu et al., 2015) developed an impedance-based immunosensor for AFB_1, where a nanobiocomposite film based on a mixture of MWCNTs and ionic liquids was used

for the entrapment of anti-AFB_1 antibodies on glassy carbon electrodes. The enhanced electron transfer provided by the presence of MWCNTs and the ideal environment for the antibody created by the 3D-structured composite film, allowed the detection of AFB_1 between 0.1 and 10 ng/mL, with an LOD of 0.03 ng/mL. The applicability of the developed immunosensor was demonstrated by the analysis of AFB_1 in spiked olive oil extracts.

Inclusion of carbon nanomaterials into carbon pastes to fabricate screen-printed electrodes is of special interest. Suresh et al. (Suresh et al., 2010) mixed MWCNTs with paraffin oil and graphite powder to develop a sandwich-type immunosensor for the detection of ricin. The presence of MWCNTs widened the linear working range obtained with an equivalent biosensor without MWCNTs (0.625 – 25 ng/mL and 2.5 – 25 ng/mL, respectively). In another example, Zachetti et al. (Zachetti et al., 2013) mixed MWCNTs with mineral oil, HRP and ferrocene, for the development of an amperometric biosensor for the quantification of citrinin (CIT). The LOD was 63 pg/mL and good recoveries were obtained in the analysis of rice samples. This type of immobilisation provides good stability, preventing possible leakage of biosensor components. The success of the incorporation of carbon nanomaterials in carbon pastes, led to the commercialisation of screen-printed electrodes already modified with carbon nanomaterials. Commercial graphene-modified carbon screen-printed electrodes have been used in the development of an electrochemical immunosensor for okadaic acid (OA) (Eissa and Zourob, 2012). The authors electrografted a carboxyphenyl monolayer on the printed electrodes for the subsequent covalent immobilisation of anti-OA antibodies. OA and OA-ovalbumin (OA-OVA) in solution compete for their binding to the immobilised antibody. A decrease of the reduction current of $[Fe(CN)_6]^{4-/3-}$ was observed for lower OA concentrations, caused by the blocking effects of bound OA-BSA which hindered the electron transfer. The immunosensor exhibited good sensitivity and an LOD of 19 pg/mL, attributed to the electrochemical properties of graphene and the stability of the carboxyphenyl layer. Additionally, the application of the immunosensor to the quantification of OA in certified mussel samples showed no significant matrix interferences.

3.2.3.1.1.3 Electrochemical methods

Carbon nanomaterials have also been immobilised on electrodes using electrochemical techniques. The simplest electrochemical approach is based on electrophoretic deposition (EPD), which allows the deposition of positively charged material on the anode. Following this approach, Srivastava et

al. (Srivastava et al., 2013) immobilised rGO modified with Mg2+ ions on ITO electrodes (Fig. 3.2.1). Then, they covalently bound anti-AFB1 antibodies to the carboxyl groups on the rGO. The presence of AFB1was directly related to the increase in the anodic peak observed by cyclic voltammetry, which was suggested to be the result of the improved electron transfer induced by the toxin. The LOD was as low as 0.15 ng/mL, probably because of both the large area of the GO sheets, which provided high loading of antibodies, and their excellent electrical conductivity.

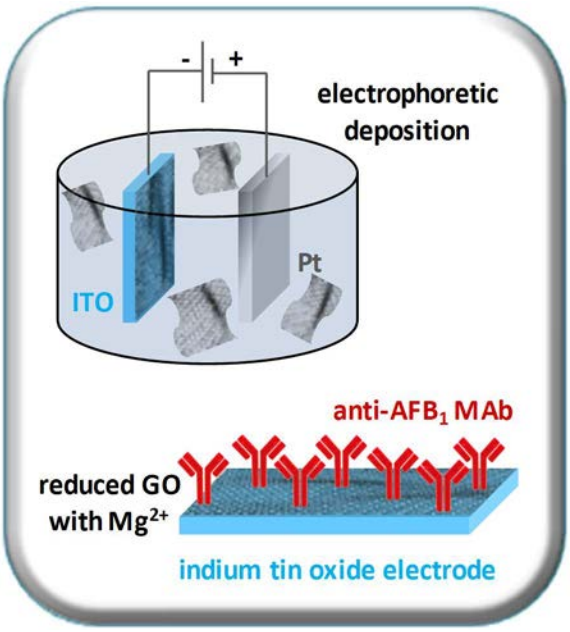


Fig. 3.2.1. Example of biosensing strategy based on rGO as antibody immobilisation support.

3.2.3.1.1.4 Chemical vapour deposition

Vertically aligned MWCNTs have been grown on patterned Si substrates via water-assisted chemical vapour deposition (Han et al., 2013). These MWCNTs were functionalised and modified with MC-LR, providing an electrode array used in a competitive-type immunoassay. This immunosensor was able to detect this cyanobacterial toxin in a concentration range from 0.05 to 20 ng/mL.

3.2.3.1.2 Carbon nanomaterials as label supports

As previously mentioned, carbon nanomaterials can also play a role as label supports when incorporated in biosensors. In this case, apart from being conjugated to the label, they have also been modified with the detection antibody or the antigen, depending on the assay format. Such mixed conjugates have been incorporated in sandwich and competition configurations providing

advantages such as higher number of labels and electrochemical signal amplification. Next, a few examples of the different configurations are described.

Tang et al. (Tang et al., 2010) doped MWCNTs with nanosilica and HRP, and used them as labels of the anti-staphylococcal enterotoxin B (SEB) antibodies in a sandwich-type immunosensor. Thionine was electropolymerised on graphite electrodes to mediate the HRP-catalysed reaction, and Au nanoparticles (NPs) were added to the system to enhance the electrochemical reduction of H_2O_2. Combining the catalytic properties of CNTs and the bioelectrocatalytic signal amplification, they obtained a dynamic range of 0.05 – 15 ng/mLand an LOD of 10 pg/mL. Results from the analysis of spiked watermelon and apple juices, soy milk and pork food showed excellent correlation with the values obtained using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. Competition-type immunoassays and sensors have also been developed using carbon nanomaterials as bioreceptor/label immobilisation supports. Tian et al. (Tian et al., 2014) conjugated peroxidase-mimicking DNAzyme and MC-LR to SWCNTs. This multi-labelled MC-LR competed with free MC-LR in solution for their binding to capture antibodies immobilised on SWCNTs previously adsorbed on the electrode. The electrocatalytic current decreased linearly as the MC-LR amount increased from 0.01 to 7.0 ng/mL, and the LOD was 2.31 pg/mL. This low LOD was attained because the SWCNTs allowed loading multiple label enzymes and also promoted the electron transfer between the electrolyte and the electrode. Application of the immunosensor to the MC-LR determination in spiked reservoir water provided good recoveries and agreement with LC-MS/MS results. Tang et al. (Tang et al., 2012) developed another competition-type immunoassay, using magnetic beads (MBs), for the detection of brevetoxin B (BTX-2). In this case, graphene nanoribbons (GNRs) were used as label (guanine) and antigen (BSA-BTX-2) carriers. Free BTX-2 and labelled BSA-BTX-2 competed in solution for their binding to anti-BTX-2 antibodies immobilised on MBs. Once the immunocomplexes were formed, they were magnetically captured and transferred to an electrochemical cell containing the redox catalyst $Ru(bpy)_3^{2+}$, which mediates the oxidation of guanine. Oxidation current decreased with the increase of BTX-2 in the sample, providing a linear range from 1 pg/mL to 10 ng/mL and an LOD of 1 pg/mL. Carbon nanospheres (CNSs) have also been integrated into biosensor configurations as enzyme/antibody carriers for signal amplification. In the work described by Zhao et al. (Zhao et al., 2013), anti-MC-LR antibodies conjugated to HRP-labelled CNSs competed for their binding to MC-LR free in solution and MC-LR covalently attached to GO/chitosan, previously immobilised on glassy carbon electrodes by adsorption. The immunosensor exhibited a wide linear range (0.05–15 ng/mL) and an LOD of 16 pg/mL, well below the World Health Organisation (WHO) provisional guideline limit of 1 μg L^{-1}.

3.2.3.2 Metal nanomaterials and semiconductor quantum dots

Metal nanoparticles (NPs) and nanorods (NRs) have been incorporated in electrochemical biosensor configurations with two main purposes: the fabrication of nanostructured supports and their use as signal enhancers. They possess high electrocatalytic activity, stability and biocompatibility, and they can be easily functionalised. They favour electron transfer and, as a result, higher sensitivities and lower LODs are attained by metal nanomaterial-modified electrochemical biosensors.

3.2.3.2.1 Metal nanomaterials-modified electrodes

As previously shown for carbon nanomaterials, metal nanomaterials immobilisation has also been pursued by several strategies addressed to incorporate them on the electrode surface: adsorption, entrapment into polymers, self-assembly, and electrochemical methods. Examples are described below.

3.2.3.2.1.1 Adsorption

As shown before, adsorption is the simplest approach. Castillo's group developed competitive indirect (Bone et al., 2010) and direct (Vidal et al., 2011) immunosensors for the detection of OTA. To compete with OTA in solution for the binding to anti-OTA antibodies, Au NP-modified OTA-BSA conjugates were adsorbed on screen-printed carbon electrodes. Au NPs provided a higher number of effective immobilised antigen sites and enhanced the charge-transfer kinetics of the voltammetric measurements. As a result, the immunosensors with NPs showed lower LODs compared to those without NPs: 0.20 ng/mL *vs.* 0.86 ng/mL, and 0.10 ng/mL *vs.* 0.30 ng/mL, for the indirect and direct approaches, respectively. The immunosensors were successfully applied to the analysis of OTA-spiked and certified wheat samples.

Metal NPs have also been adsorbed onto modified electrode surfaces. Such is the case of the label-free impedimetric aptasensor for OTA, developed by Rivas et al. (Rivas et al., 2015), which incorporates IrO_2 NPs onto the electrode. Herein, IrO_2 NPs were adsorbed on the thionine film electrodeposited on a screen-printed carbon electrode. An amino-terminated DNA aptamer, specific for OTA, was electrostatically immobilised on the IrO_2 NPs. The impedimetric biosensor allowed the determination of OTA in the range of 4 pg/mL to 40 ng/mL and with an LOD of 5.65 pg/mL (5.65 ng/kg in wine).

3.2.3.2.1.2 Entrapment

In some cases, metal NPs are embedded into polymers and the resulting nanobiocomposites are then adsorbed on electrodes. Some examples are the immunosensors for OTA developed by Kaushik et al. (Kaushik et al., 2009a; Kaushik et al., 2009b). Nanocomposites, prepared by dispersion of CeO_2 NPs in sol-gel (Kaushik et al., 2009a) or chitosan (Kaushik et al., 2009b), were adsorbed on ITO electrodes. Afterwards, rabbit IgG and BSA were co-immobilised on the nanocomposites via electrostatic interactions. The NPs increased the number of antigen sites and the electroactive surface area, enhancing the electron transport. As a result, an LOD of 2.5 pg/mL was achieved. The authors followed the same approach but using ZnO NPs instead of CeO_2 NPs, obtaining similar results for OTA detection (Ansari et al., 2010).

Metal NPs have also been encapsulated into dendrimers: the large surface area of dendrimers has been combined with the good conductivity of metal NPs to create 3D metal-dendrimer nanocomposites to be used as nanostructured supports. Tang et al. (Tang et al., 2011) used this approach to develop an electrochemical immunosensor for the direct detection of brevetoxin (BTX-2). Au NPs were synthesised and simultaneously encapsulated into amino-terminated dendrimers, which afterwards were adsorbed on ester-modified gold electrodes. BTX-2-BSA conjugate was then immobilised on the nanostructured surface. HRP-labelled anti-BTX-2 antibody competed with free and immobilised BTX-2. The immunosensor response was measured using *o*-phenylendiamine and H_2O_2. As expected, the 3D-network provided the highest sensitivity, wider linear range and lower LOD, the latter being 0.01 ng/mL, compared to 0.5 or 0.1 ng/mL for immunosensors without Au NPs or dendrimers, respectively.

3.2.3.2.1.3 Self-assembly

Taking advantage of the high affinity of thiol groups for the surfaces of some metals, self-assembly has been exploited to immobilise NPs on thiol-modified electrodes. Loyprasert et al. (Loyprasert et al., 2008) immobilised Ag NPs on thiourea-modified electrodes. Anti-MC-LR antibodies were adsorbed on the Ag NP-modified surface. Label-free capacitance measurements provided linearity between 10 fg/mL and 1 ng/mL and an LOD of 7 fg/mL (80 fg/mL using the same approach without NPs). The immunosensor was used to analyse spiked drinking and raw water, showing a good correlation with the results obtained by HPLC coupled to photodiode array detection (HPLC-DAD). The low LOD allowed the dilution of samples to be analysed, reducing interference effects from the matrix. A similar approach was used by Liu et al. (Liu et al., 2009) for the development of an immunosensor for OTA. In this case, 1,6-hexanedithiol was assembled on gold electrodes and Au

NPs were assembled on it. OTA-OVA conjugate was adsorbed on the Au NPs and a competitive indirect assay was performed. The working range was linear between 10 pg/mL and 100 ng/mL with an LOD of 8.2 pg/mL. Moreover, matrix effects were negligible and good recoveries were obtained for the analysis of corn samples.

Self-assembly of metal NPs on amine groups has also been reported. Such are the cases of the immunosensors developed by Liu et al. (Liu et al., 2006) for AFB_1, and Tong et al. (Tong et al., 2011) and Lebogang et al. (Lebogang et al., 2014) for MC-LR. In these works, Au NPs were self-assembled on amino-modified electrodes, where the specific antibodies were later adsorbed. Liu et al. (Liu et al., 2006) also immobilised HRP on the Au NPs, which enabled the direct electrochemical measurement of HRP. The mycotoxin presence induced local changes of conductivity, resulting in a linear range from 0.5 to 10 ng/mL with an LOD of 0.1 ng/mL. Tong et al. (Tong et al., 2011) performed hydroquinone-mediated differential pulse voltammetry measurements, obtaining an LOD of 20 pg/mL for MC-LR. Finally, Lebogang et al. (Lebogang et al., 2014) measured capacitance changes in the presence of MC-LR, without the need of redox mediators or enzymes. This was possible thanks to the high surface area provided by the NPs that enabled the immobilisation of more antibodies than on an unmodified electrode. They obtained an LOD of 10 fg/mL and good correlation with ELISA results.

3.2.3.2.1.4 Electrochemical methods

Electrophoretical deposition (EPD) has been used to immobilise Ni NPs on ITO electrodes (Kalita et al., 2012). Anti-AFB_1 antibodies were covalently bound to the Ni NP-modified electrodes. The electrochemical biosensor revealed linearity between 0.05 and 1 ng/mL and an LOD of 0.32 ng/mL. EPD has also been used to immobilise Sm_2O_3 NRs on ITO surfaces (Singh et al., 2013). The films were then modified with anti-AFB_1 antibodies. Cyclic voltammetry and impedance spectroscopy studies showed that the NRs increased the active area, enhanced the electron transport and improved the electrocatalytic behaviour. This was reflected in the broad linear range (10 – 700 pg/mL) and the low LOD (58 pg/mL) achieved. In some works, NPs are synthesised *in situ* by electroplating, process that also implies their immobilisation on electrodes. Electroplating of gold salts onto bare gold electrodes has been used to fabricate nanostructured gold electrodes. These electrodes were modified with a peptide specific for the detection of Anthrax protective antigen (Farrow et al., 2013). In this case, the combination of the nanostructuration of the electrode and the immobilisation of the peptide through a DNA monolayer to favour electron transfer provided an LOD of 170 pg/mL.

Nanoporous anodised aluminium oxide has also been used as electrode substrate for the development of electrochemical immunosensors for SEB (Chai and Takhistov, 2010) and ricin (Chai et al., 2010). Nanoporous alumina was nano-patterned via a three-step process: annealing, electropolishing and anodisation. Upon silanisation with 3-aminopropyltriethoxysilane, glutaraldehyde was used as a linker to immobilise the corresponding antibody. Impedance spectroscopy measurements allowed the detection of SEB and ricin at concentrations as low as 10 pg/mL (Chai and Takhistov, 2010) and 0.5 pg/mL (Chai et al., 2010), respectively.

In a different approach, highly ordered and vertically aligned TiO_2 NTs were used as support to synthesise MIPs for MC-LR (Chen et al., 2012). The PPy-based MIP for MC-LR was used to develop a photoelectrochemical sensor, which attained a linear range from 0.5 to 100 ng/mL and an LOD of 0.1 ng/mL. Although MIPs usually suffer from lower affinities than antibodies, the sensor exhibited appropriate sensitivity probably because of the high specific surface area and the photoelectric activity of TiO_2.

3.2.3.3 Metal nanomaterials as label supports and/or signal enhancers

As previously mentioned, metal NPs have also been conjugated to the bioreceptor to perform as label supports and/or signal enhancers. Vig et al. (Vig et al., 2009) conjugated Au NPs to anti-AFM_1 antibodies to compete for their binding between free AFM_1 in solution and immobilised BSA-AFM_1 in an impedimetric direct competitive immunosensor. Silver was then electrodeposited using the catalytic effect of the immobilised Au NPs. Impedance measurements resulted in a wide working range (15 – 1000 pg/mL) and a low LOD (12 pg/mL) due to the silver enhancement.

Metal NPs have also been used to enhance the transduction signals arising from redox labels. Kuang et al. (Kuang et al., 2010) developed a signal-off aptamer-based sensor for the detection of OTA using two oligonucleotides complementary to the DNA aptamer. The first complementary oligonucleotide was used to immobilise the aptamer on the electrode through hybridisation. The second complementary oligonucleotide, an Au NP-functionalised DNA, was hybridised to the aptamer to amplify the redox signal from the Methylene Blue intercalator, used as electrochemical probe. OTA binding forces the release of both the aptamer and Au NP-functionalised DNA and thus decreases the amount of MB to be detected. The effective sensing range was from 0.1 to 20 ng/mL with an LOD of 30 pg/mL. The method was used to analyse red wine samples showing good correlation with ELISA results.

The sandwich immunosensor developed by Liu and co-workers (Liu et al., 2014) for the detection of botulinum neurotoxin type A (BoNT) harnesses the use of NPs to modify the electrode surface and to perform as label supports and signal enhancers. On the one hand, the authors electrografted diazonium salt-functionalised Au NPs on glassy carbon electrodes; capture anti-BoNT antibodies were then covalently linked to the immobilised Au NPs (Fig. 3.2.2). On the other hand, Au NPs were used to co-immobilise detection antibody and HRP and to amplify the signal. The biosensor exhibited a working range of 4 – 35 pg/mL with an LOD of 1 pg/mL. The analysis of milk samples showed good recoveries without sample dilution, indicating negligible interference effects.

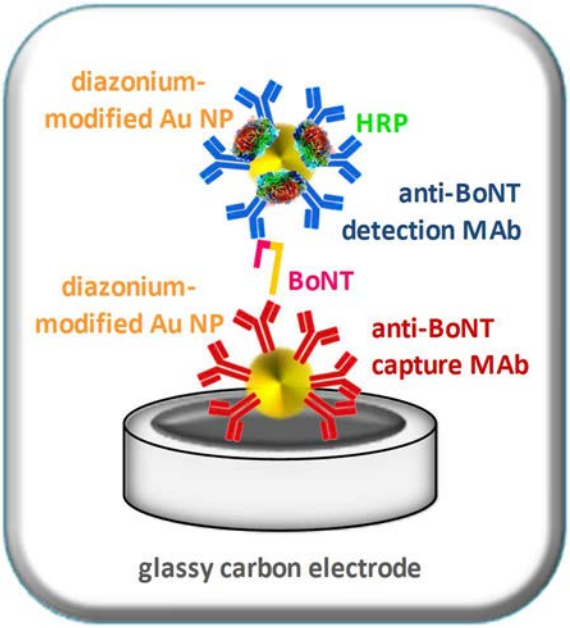


Fig. 3.2.2 Example of biosensing strategy based on Au NPs as antibody immobilisation support and label carriers.

A particular case of NPs are quantum dots (QDs), which are semiconductor nanocrystals with unique intrinsic properties. Although QDs have been usually exploited as optical (fluorescent) labels, their redox or electrocatalytic behaviour has favoured their application as electrochemical labels. An example is the immunosensor for the detection of MC-LR developed by Yu et al. (Yu et al., 2009). CdSe QDs, with a CdSe core encapsulated in the shell of ZnS and a polymer, were conjugated to anti-MC-LR antibodies and used in a competitive direct immunoassay. The immunocomplexes formed on the electrode surface were then dissolved to release cadmium ions measured by square wave stripping voltammetry. A dynamic range of 0.227 to 50 ng/mL and an LOD of 99 pg/mL were obtained. The biosensor was successfully applied to the analysis of

Microcystis aeruginosa cultures. Another example of the use of QDs as electrochemical labels is the aptamer-based sensor for the detection of OTA developed by Tong et al. (Tong et al., 2012). MBs were modified with a capture DNA that was subsequently hybridised to an OTA aptamer and a padlock DNA. OTA-aptamer binding induced aptamer release and padlock DNA-capture DNA hybridisation. Next, capture DNA was isothermally amplified via linear rolling circle amplification reaction, generating a long DNA sequence. CdS QD-labelled DNA probes were then added, which hybridised with the long sequence, and the electrochemical measurement was performed. The biosensor detected OTA down to 0.2 pg/mL with a dynamic range of more than 4 orders of magnitude. In the analysis of red wines, good correlation with ELISA results was obtained. The exploitation of QDs and nanoclusters (NCs) in biosensors for multiplexed detection is shown in detail in section 3.2.4.3.

3.2.3.4 Combination of carbon and metal nanomaterials

There are a few biosensors combining both carbon and metal nanomaterials with synergistic or unrelated purposes. In the work developed by Xu and co-workers (Xu et al., 2014), MWCNTs and Au NPs were immobilised on a glassy carbon electrode with a common purpose. A silica/thionine nanocomposite was electrodeposited on the electrode previously modified with adsorbed MWCNTs. Au NPs were then linked to thionine by electrostatic adsorption and covalent binding. After immobilisation of anti-*Clostridium difficile* toxin B antibodies on the nanocomposite, the assay was performed. This label-free biosensor was able to detect *Clostridium difficile* toxin B from 1 to 80 ng/mL with an LOD of 0.3 ng/mL. Chen (Chen, 2008) also combined MWCNTs and Au NPs to modify interdigitated microelectrodes. A MWCNT-chitosan composite, adsorbed on the microelectrodes, was modified with Au NPs which performed as immobilisation support for anti-SEB antibodies conjugated to HRP. The presence of the analyte in the sample induced local conductivity variations, providing a linear range between 0.5 and 83.5 ng/mL and an LOD of 0.5 ng/mL. More sophisticated is the combination of GO and Au NPs proposed by Srivastava et al. (Srivastava et al., 2015) and Sunday et al. (Sunday et al., 2015), where the NPs were synthesised *in situ* simultaneously to the reduction or functionalisation of GO, respectively. Srivastava et al. (Srivastava et al., 2015) immobilised then anti-AFB_1 antibodies on the Au NP-rGO nanocomposite to develop a label-free immunosensor able to detect AFB_1 between 0.1 and 12 ng/mL with an LOD of 0.1 ng/mL. Sunday et al. (Sunday et al., 2015) developed a label-free immunosensor for deoxynivalenol (DON) based on the modification of the electrode surface with a Nafion membrane containing Au NP-modified graphene. Anti-DON antibodies were immobilised on the functionalised

graphene. The immunosensor was able to detect the mycotoxin in the range of 6 – 30 ng/mL with an LOD of 0.3 ng/mL. Results for the analysis of certified corn, wheat and roasted coffee samples using the immunosensor were in agreement with those obtained by an ELISA kit.

The next two examples describe the use of carbon and metal nanomaterials for different purposes. The first work is a field-effect transistor (FET) aptamer-based sensor developed for the detection of Anthrax protective antigen (Kim et al., 2013). Comparison between a GO-based direct assay and a GO/Au NP-based sandwich assay showed slightly better results for the latter. Although the direct assay, based on capture aptamer-modified GO nanosheets immobilised on the electrode, already provided an LOD of 1 fg/mL, the sandwich assay, also incorporating detection aptamer-conjugated Au NPs, resulted in one order of magnitude lower LOD (0.1 fg/mL). Similarly, Wei et al. (Wei et al., 2011) developed an immunosensor for MC-LR that combines capture antibody-modified GO sheets adsorbed on a glassy carbon electrode and mesoporous PtRu alloy-labelled detection antibody. The alloy allowed the H_2O_2 electrochemical detection, providing a MC-LR detection range from 0.01 to 28 ng/mL and an LOD of 9.6 pg/mL. The biosensor provided appropriate quantification of the cyanotoxin in polluted water.

3.2.4 Use of magnetic beads as supports and/or carriers

Magnetic beads (MBs) have been recently exploited as supports and/or carriers. MBs have particular characteristics that make them useful in biosensor configurations: they are biocompatible, they have large surface area, they can be easily functionalised, and their location and transport can be controlled by a magnetic field.

3.2.4.1 Magnetic beads in batch-mode biosensors

Sandwich-type configurations incorporating MBs have been reported. As an example, Ge et al. (Ge et al., 2013) co-immobilised anti-MC antibody and HRP on silica-coated ferroferric oxide (Fe_3O_4) NPs, and used them as carriers. Once the sandwich immunoreaction was completed, electrochemical measurements were recorded according to the oxidation of the thionine mediator, which was catalysed by both the HRP and Fe_3O_4 NPs (peroxidase mimetics). This immunosensor provided a linear working range from 0.01 to 200 μg/mL and an LOD of 4 ng/mL. In this case, the magnetic properties of the particles were not exploited for their immobilisation on electrodes, but for both the washing steps in the conjugate synthesis and the electrochemical signal enhancement.

The use of MBs as antibody immobilisation support is described in several magneto-controlled competitive immunoassays and immunosensors. In the simplest approach, the immunocomplexes are first formed and later captured on magnetised electrodes for the electrochemical measurement. In the work described by Paniel et al. (Paniel et al., 2010), free and HRP-labelled AFM_1 competed for an anti-AFM_1 antibody conjugated to MBs (via protein G affinity) in solution. The immunocomplexes were then captured on screen-printed carbon electrodes by external magnets, and the 1-methoxy-phenazinemethosulfate (MPMS)-mediated electrochemical detection of the HRP enzyme used as label was performed. The LOD attained was 10 pg/mL. A similar approach was followed by Reverté et al. (Reverte et al., 2013), who immobilised anti-MC-LR antibodies on protein G-modified MBs for the subsequent competition between free MC-LR and MC-LR-HRP tracer (**Figure 3.2.3**). In this case, the HRP label was mediated by 3,3'5,5'-tetramethylbenzidine (TMB) in solution, resulting in an LOD of 0.4 ng/mL. Vidal et al. (Vidal et al., 2012) developed an immunosensor for the detection of OTA, also based on the competition between free and HRP-labelled toxin for their binding to biotinylated anti-OTA antibodies immobilised on streptavidin-coated MBs. Hydroquinone in solution was used as electrochemical mediator and an LOD of 0.12 ng/mL was obtained. With the aim of simplifying the experimental procedure, immobilised redox mediators have been used. Esteban-Fernández de Ávila et al. (de Avila et al., 2012) developed a direct competitive magnetoimmunosensor for the detection of Staphylococcal Protein A (Prot A). In this case, HRP-labelled immunocomplexes were captured on tetrathiafulvalene (TTF)-modified screen-printed gold electrodes, providing an LOD as low as 3.9 fg/mL. As mentioned in section 3.2.3.1.2, epoxy-modified MBs have also been used as anti-BTX-2 antibody immobilisation support, the antigen and the guanidine label being linked to GNRs (Tang et al., 2012). A step further is the work described by Hervás and co-workers (Hervas et al., 2010) in which both the competition between Zearalenone (ZEA) and ZEA-HRP for their binding to mAb-coated MBs and the electrochemical measurement were performed on the magnetised screen-printed electrodes. This format allowed lowering the LOD from 11 to 7 pg/mL, compared to the assay with separated competition and measurement steps (Hervas et al., 2009a). Antigens have also been conjugated to MBs. As an example, Hayat et al. (Hayat et al., 2011) conjugated biotinylated OA to streptavidin-modified MBs for their magnetic immobilisation on electrodes. The indirect competitive immunosensor, with detection and ALP-labelled secondary antibodies and 1-naphthyl phosphate as enzyme substrate, provided an LOD of 0.38 ng/mL.

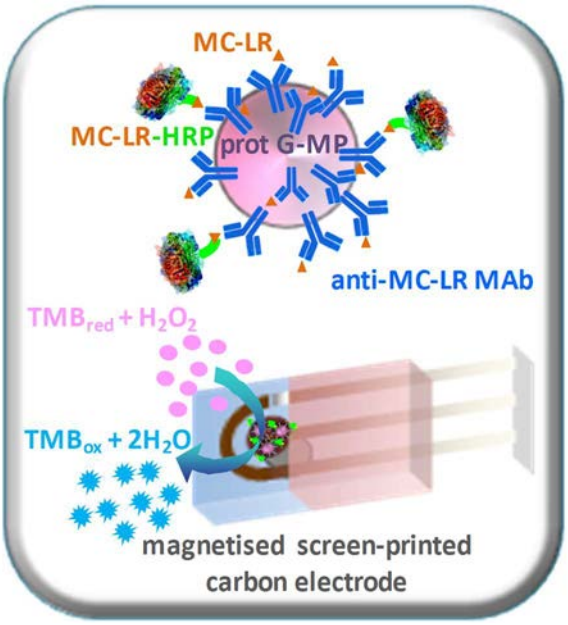


Fig. 3.2.3 Example of biosensing strategy based on magnetic particles as antibody supports.

A special configuration has been proposed by Hayat et al. (Hayat et al., 2012), who developed a non-competitive label-free immunosensor for the detection of OA, conjugating anti-OA antibodies to protein G-MBs. The label-free detection was possible because of the hindered charge transfer from the redox couple $[Fe(CN)_6]^{4-/3-}$ in solution in the presence of OA/anti-OA antibody complex on the electrode. This immunosensor attained an LOD of 0.5 ng/mL.

MBs have also been used in aptamer-based configurations. As when conjugating MBs to antibodies, some authors performed the competition step in solution to form the immunocomplexes that are later captured on the electrode surface, by using external magnets, for the electrochemical measurement. Bonel et al. (Bonel et al., 2011) conjugated streptavidin-coated MBs to a biotinylated OTA aptamer, which competed for its binding to free OTA and OTA-HRP tracer. Immunocomplexes where finally captured on magnetic screen-printed carbon electrodes and the hydroquinone-mediated HRP electrochemical detection provided an LOD of 70 pg/mL. A more sophisticated approach is that described by Barthelmebs and co-workers (Barthelmebs et al., 2011), in which indirect and direct competitive strategies for the detection of OTA were compared, both being entirely performed on screen-printed electrodes. In the indirect approach, biotinylated OTA was immobilised on streptavidin-coated MBs and compete with free OTA for their binding to HRP-labelled aptamer. MPMS redox mediator was added in solution. In the direct approach, OTA

aptamer was covalently linked to the MBs via carbodiimide reaction and, after immobilisation on the electrode, free OTA and OTA-ALP competed for the aptamer binding. 1-naphthyl phosphate was used as enzyme substrate for the electrochemical measurement. The direct competitive approach lowered the LOD from 1.1 to 0.11 ng/mL. OTA was successfully analysed in wheat and wine samples using the immunosensors developed by Bonel et al. (Bonel et al., 2011) and Barthelmebs et al. (Barthelmebs et al., 2011), respectively.

3.2.4.2 Magnetic beads in flow systems and microfluidics devices

With the aim to provide compact analysis devices for environmental surveillance and food control as well as other applications, MBs combined with immunoassays/sensors have been integrated in flow systems and microfluidics devices, providing advantages such as short analysis times and low sample and reagents volumes. Some of the immunosensors developed in the section 3.2.4.1 have been incorporated in flow systems. As an example, Dominguez et al. (Dominguez et al., 2012) injected MB-OA conjugates in a flow system and captured them on the electrode surface. Then, the competition took place and the electrochemical measurement was performed, both on the magnetised electrode. The magnetic field was enough to keep the MBs on the electrode, even despite the continuous flow. The authors achieved an LOD of 0.15 ng/mL, lower than in batch-mode (0.38 ng/mL) (Hayat et al., 2011), probably due to the signal enhancement caused by the short diffusion distance in the continuous/stopped flow system.

The first step in the development of miniaturised compact systems was taken by Hervás et al. (Hervas et al., 2009b), who electrokinetically injected the enzymatic product from the MB-Ab-ZEA-HRP immunocomplexes into a microfluidics platform where the amperometric detection took place, providing an LOD of 0.83 ng/mL. In the following work (Hervas et al., 2011), the whole system was integrated into the microfluidics chip, configuration that provided a lower LOD (0.4 ng/mL). A similar microfluidics immunosensor for ZEA but using 4-*tert*-butylcatechol as a redox mediator has also been reported (Panini et al., 2011). Similarly, Bunyakul et al. (Bunyakul et al., 2009) used MBs as immobilisation support for anti-cholera toxin subunit B (CTB) antibodies to develop a microfluidics immunosensor (**Figure 3.2.4**). For the detection, they synthesised a tracer consisting of CTB receptor ganglioside GM_1 linked to liposomes, which encapsulated $[Fe(CN)_6]^{4-/3-}$and were used as signal amplifiers. Reagents were first mixed and then injected into the magneto-controlled microfluidics device. Once immunocomplexes were formed and captured by the magnet, *n*-octyl-

β-D-glucopyranoside detergent was added to lyse the liposomes and release the $[Fe(CN)_6]^{4-/3-}$ on an interdigitated ultramicroelectrode array, providing an LOD of 1 ng/mL.

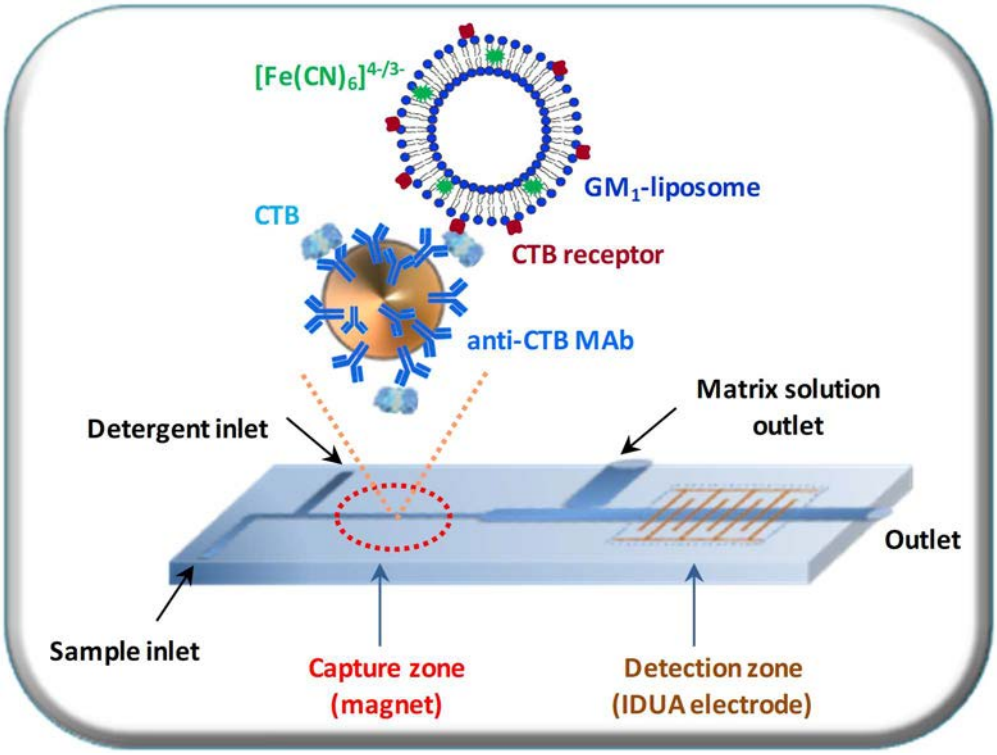


Fig. 3.2.4. Example of biosensing strategy based on magnetic particles as antibody supports in a microfluidics device.

3.2.4.3 Magnetic beads in multiplexed systems

Moving towards multiplexed electrochemical detection for simultaneous analysis of samples, Romanazzo et al. (Romanazzo et al., 2010) developed an immunoassay for DON on a strip with 8 magnetised screen-printed carbon electrodes. Biotinylated anti-DON antibodies were used in the competition between free DON and human serum albumin (HSA)-DON immobilised on tosyl-modified MBs. Then, avidin and biotinylated HRP were added. Once immunocomplexes were formed, TMB in solution mediated the HRP reduction, and the electrochemical measurements provided an LOD of 63 ng/mL. The simultaneous analysis on the strip allowed processing a high number of samples in short time. Regarding multiplexed detection with discrimination of different analytes, a direct competitive immunoassay for the simultaneous detection of BTX-2 and dinophysistoxin-1 (DTX-1) has been developed (Zhang et al., 2012). Antibodies against BTX-2 and

DTX-1 were co-immobilised on MBs and distinguishable metal nanoclusters (NCs) (cadmium and copper) were employed as both antigen-BSA conjugate carriers and labels. Both competition step and electrochemical measurements were performed in a flow-through detection cell with a sequential injection and using external magnets. The stripping voltammetry of the dissolved labels provided a wide linear range for both analytes (0.005 – 5ng/mL), and LODs of 1.8 pg/mL and 2.2 pg/mL for BTX-2 and DTX-1, respectively. Additionally, the analysis of spiked samples provided toxin contents in good agreement with those obtained by ELISA.

3.2.5 Other flow systems and microfluidics devices

The integration of electrochemical immunoassays and immunosensors in flow and microfluidics systems has also been achieved without the use of MBs. Panini et al. (Panini et al., 2010) developed an immunosensor for the determination of ZEA using a continuous-flow/stopped-flow system. Anti-ZEA antibodies were immobilised on a rotating disk, where the competition between ZEA and the tracer ZEA-HRP took place. HRP catalysed the oxidation of the redox mediator 4-*tert*-butyl-catechol to 4-*tert*-butyl-*o*-benzoquinone, which was subsequently detected on a MWCNT-modified gold electrode, placed in front of the disk. This strategy provided a fast (15 min) and sensitive detection of ZEA, with an LOD of 0.8 ng/mL, and was successfully applied to the analysis of corn silage samples. The same group developed a microfluidics system for the detection of CIT (Javier Arevalo et al., 2011). CIT-OVA conjugates were immobilised on a cysteamine-modified gold disk electrode and competed with free CIT for antibody binding. Indirect detection using IgG-HRP and catechol was performed on an adjacent electrode. The system attained an LOD of 0.1 ng/mL with a total assay time of 45 min. Olcer et al. (Olcer et al., 2014) have recently developed an immunosensing microfluidics platform for the detection of DON. Anti-DON antibodies were directly immobilised on a Prot A-modified working electrode to obtain a proper antibody orientation. Upon competition between free DON and DON-HRP, electrochemical measurements were performed using TMB as redox mediator, providing an LOD of 6.25 ng/mL. DON was successfully detected in wheat samples with an analysis time of 15 min. Finally, aptamers have also been included in microfluidics systems, in this case for the detection of BoNT (Lillehoj et al., 2010). The device had a zigzag-shaped channel to mix the reagents and a serpentine channel over the electrode to pull liquids through the fluidics network. Streptavidin-labelled dendrimer NPs located on the electrode were used to immobilise the biotin-labelled anti-BoNT aptamer, which was also labelled with fluorescein. In the presence of the neurotoxin, the aptamer conformation allowed the interaction between the fluorescein and an HRP-labelled anti-fluorescein IgG, providing the corresponding electrochemical signal in the

presence of TMB. The system detected 1 ng/mL of BoNT in less than 15 min, with a high degree of autonomy and without the requirement of external pumps.

3.2.6 Conclusions

Over the last few years, researchers working on biosensors have widened their interest towards new fields of application. The environmental and agro-food sectors need to be modernised in terms of routine analysis methods. The recent advances in electrochemical biosensors, incorporating nanotechnological concepts, and their integration into compact analysis devices, may contribute to guarantee food safety, to facilitate environmental surveillance and to protect human and animal health.

When reviewing the biosensors with nanomaterial-based supports, all works claim advantages in terms of larger electroactive surface area, promoted electron transfer, enhanced electrochemical signals and lower LODs. Proper evaluation of the improvement achieved with these novel biosensors is not always possible, since comparison of equivalent systems is often difficult to perform. To a certain extent, the integration of nanomaterials could only add sophistication to the configuration. To guarantee the benefits from their incorporation, other parameters such as biosensor lifetime should also be considered. The global evaluation of the system will help to conclude if the sophistication is worthwhile. Nevertheless, a general statement is accepted attributing significantly better performance to biosensors based on bionanotechnological approaches. What is more, the advantages of one type of nanomaterial over another are also difficult to clear up, although one can figure out that the composition, structure and dimension may play significant roles. As an example, graphene is gaining ground to carbon nanotubes due to the better adhesion provided by its flat two-dimensional configuration (Perez-Lopez and Merkoci, 2012) and the larger availability of active sites (Kumar et al., 2015).

The role of nanomaterials in electrochemical biosensors can be focused on nanostructuring of electrode supports and/or on performing as labels, signal enhancers and label supports. The method used for the immobilisation of the nanomaterial on the electrode certainly plays an important role in the biosensor performance, as it also does the function of the nanomaterial in the electrochemical transduction system (e.g. label, electrocatalysts and/or label carrier).

Adsorption is a simple technique to incorporate carbon nanomaterials in biosensors, but sometimes suffers from leakage and always from random immobilisation. Although biorecognition molecules can be attached to nanomaterials by covalent bonds that favour the stability and allow oriented immobilisation, the initial nanomaterial adsorption process compromises the biosensor performance. When combined with polymers, adsorbed bionanocomposite matrixes may increase the immobilisation stability but diffusion barriers are created, which increase the LODs. The use of carbon nanomaterial pastes, although still limiting the diffusion processes, increases the stability and additionally allows the simultaneous immobilisation of biorecognition molecules and redox mediators. Electrochemical techniques allow addressed immobilisations and are compatible with the subsequent detection methods. Depending on the electrochemical technique (e.g. electrophoretic deposition, electropolymerisation and electrografting), bonds of different nature and different immobilisation conformations are created. Metal nanomaterials have also been immobilised on electrodes for subsequent biomolecule immobilisation. Again, adsorption and entrapment into polymers, as well as electrochemical techniques, have been proposed, but self-assembly on thiolated surfaces certainly is the preferred choice because of the easy and effective immobilisation process.

Regarding the incorporation of nanomaterials in electrochemical transduction schemes, metal nanomaterials are more commonly found. Metals can directly act as electroactive labels, and they can also enhance electrochemical signals arising from redox probes because of their electrocatalytic activity. Another advantage of metal nanomaterials is the possibility to use them in multiplexed array systems. Although to a minor extent, carbon nanomaterials have also been incorporated to improve the electrochemical transduction. In this case, their most common role is to act as support for the simultaneous immobilisation of the biorecognition element (antigen or antibody/aptamer, depending on the assay format) and the label. The electrocatalytic behaviour of carbon nanomaterials together with the high number of immobilised labels result in electrochemical signal amplification and lower LODs.

As the described works demonstrate (a considerable number of them having been developed for MC-LR and OTA), nanomaterials provide advantages in terms of lower LODs and wider working ranges, rendering widely applicable biosensors. However, to really foster the use of biosensors in the agro-food and environmental sectors, compact analysis devices, sensitive, robust, reliable and easy to handle even by non-trained people, are desired. The integration of electrochemical biosensors into microfluidics is a challenge that is being achieved, but the implementation of such

devices in daily life still requires more effort. Nevertheless, the progress in this area is so fast that, provided the scientific community focuses not only on the development of the bioanalytical systems but also on their validation, it will not take a long time to implement them in routine analysis.

Table 3.2.1. Electrochemical biosensors based on nanomaterials and/or magnetic beads for the detection of mycotoxins

Analyte	Biorecognition element	Nano/magnetic material/flow	Detection technique	Linear range	LOD	Applicability	Ref.
Sterigmatocystin (ST)	Aflatoxin–Detoxifizyme (ADTZ)	MWCNTs	Differential Pulse Voltammetry (DPV)	0.04 – 1.33 µg/mL	42 ng/mL	-	(Yao et al., 2006)
ST	Aflatoxin–ADTZ	MWCNTs	DPV	0.08 – 0.66 µg/mL	0.08 µg/mL	-	(Yao et al., 2004)
Aflatoxin B1 (AFB1)	Aflatoxin Oxidase (AFO)	MWCNTs	Amperometry	1 – 225 ng/mL	0.5 ng/mL	-	(Li et al., 2011)
AFB1	Anti-AFB1 mAb	GO	Electrochemical Impedance Spectroscopy (EIS)	0.5 – 5 ng/mL	0.23 ng/mL	-	(Srivastava et al., 2014)
MC-LR	Anti-MC-LR PAb	SWCNHs	DPV	0.05 – 20 ng/mL	30 pg/mL	Lake water	(Zhang et al., 2010)
ST	AFO	SWCNTs	Amperometry	0.01 – 1.48 µg/mL	3 ng/mL	-	(Chen et al., 2010)
Ochratoxin A (OTA)	IgG	MWCNTs	DPV	25 – 600 pg/mL	25 pg/mL	-	(Kaushik et al., 2010)
AFB1	Anti-AFB1mAb	GO	EIS	10 fg/mL – 10 pg/mL	10 fg/mL	Corn	(Wang et al., 2015)
AFB1	Anti-FAB1 mAb	MWCNTs	EIS	0.1 – 10 ng/mL	0.03 ng/mL	Olive oil	(Yu et al., 2015)
Citrinin (CIT)	Horseradish Peroxidase (HRP)	MWCNTs	Amperometry	-	63 pg/mL	Rice	(Zachetti et al., 2013)
AFB1	Anti-AFB1 mAb	GO	Cyclic Voltammetry (CV)	0.125 – 1.5 ng/mL	0.15 ng/mL	-	(Srivastava et al., 2013)
OTA	Anti-OTA PAb	Au NPs	DPV	0.3 – 8.5 ng/mL	0.20 ng/mL	Wheat	(Bone et al., 2010)
OTA	Anti-OTA mAb	Au NPs	DPV	0.15 – 9.9 ng/mL	0.10 ng/mL	Wheat	(Vidal et al., 2011)
OTA	Anti-OTA aptamer	IrO2NPs	EIS	4 pg/mL – 40 ng/mL	5.65 pg/mL	White wine	(Rivas et al., 2015)
OTA	IgG	CeO2 NPs	DPV	2.5 – 60 pg/mL	2.5 pg/mL	-	(Kaushik et al., 2009a; Kaushik et al., 2009b)
OTA	IgG	ZnO NPs	EIS	2.5 – 4.2 pg/mL	2.5 pg/mL	-	(Ansari et al., 2010)

OTA	Anti-OTA mAb	Au NPs	DPV	0.01 – 100 ng/mL	8.2 pg/mL	Corn	(Liu et al., 2009)
AFB1	Anti-AFB1 mAb	Au NPs	Conductometry	0.5 – 10 ng/mL	0.1 ng/mL	Serum	(Liu et al., 2006)
AFB1	Anti-AFB1 mAb	Ni NPs	CV	0.05 – 1 ng/mL	0.32 ng/mL	-	(Kalita et al., 2012)
AFB1	Anti-AFB1 mAb	Sm2O3 NRs	CV	10 – 700 pg/mL	58 pg/mL	-	(Singh et al., 2013)
Aflatoxin M1 (AFM1)	Anti-AFM1 PAb	Au NPs	EIS	15 – 1000 pg/mL	12 pg/mL	-	(Vig et al., 2009)
OTA	Anti-OTA aptamer	Au NPs	CV	0.1 – 20 ng/mL	30 pg/mL	Red wine	(Kuang et al., 2010)
OTA	Anti-OTA aptamer	MBs CdS QDs	Square Wave Stripping Voltammetry (SWSV)	0.5 pg/mL – 10 ng/mL	0.2 pg/mL	Red wine	(Tong et al., 2012)
AFB1	Anti-AFB1 mAb	GO Au NPs	CV	0.1 – 12 ng/mL	0.1 ng/mL	-	(Srivastava et al., 2015)
Deoxynivalenol (DON)	Anti-DON Mab	GO Au NPs	EIS	6 – 30 ng/mL	0.3 ng/mL	Corn, wheat & roasted coffee	(Sunday et al., 2015)
AFM1	Anti-AFM1 mAb	MBs	Chronoamperometry (CA)	0.01 – 0.25 ng/mL	10 pg/mL	Milk	(Paniel et al., 2010)
OTA	Anti-OTA mAb	MBs	DPV	0.26 – 8.87 ng/mL	0.12 ng/mL	Red wine	(Vidal et al., 2012)
Zearalenone (ZEA)	Anti-ZEA mAb	MBs	Amperometry	-	11 pg/mL	Maize, powdered baby food & cereal milkshakes	(Hervas et al., 2009a)
ZEA	Anti-ZEA mAb	MBs	DPV	-	7 pg/mL	Maize, powdered baby food & cereal milkshakes	(Hervas et al., 2010)
OTA	Anti-OTA aptamer	MBs	DPV	0.78 – 8.74 ng/mL	70 pg/mL	Wheat	(Bonel et al., 2011)
OTA	Anti-OTA aptamer	MBs	DPV	0.11 – 15 ng/mL	0.11 ng/mL	Wine	(Barthelmebs et al., 2011)
ZEA	Anti-ZEA mAb	MBs Microfluidics	Amperometry	-	0.83 ng/mL	Maize, powdered baby food & cereal milkshakes	(Hervas et al., 2009b)
ZEA	Anti-ZEA mAb	MBs Microfluidics	Amperometry	-	0.4 ng/mL	Maize, powdered baby food & cereal milkshakes	(Hervas et al., 2011)
ZEA	Anti-ZEA mAb	MBs Microfluidics	CV	20 – 500 pg/mL	20 pg/mL	Feedstuffs	(Panini et al., 2011)
DON	Anti-DON Fab fragment	MBs Multiplexed	CA	0.1 – 4.5 µg/mL	63 ng/mL	Breakfast cereals &baby food	(Romanazzo et al., 2010)

ZEA	Anti-ZEA mAb	MWCNTs Flow system	Amperometry	-	0.77 ng/mL	Cornsilage	(Panini et al., 2010)
CIT	Anti-CIT mAb	Microfluidics	Amperometry	0.5 – 50 ng/mL	0.1 ng/mL	Rice	(Javier Arevalo et al., 2011)
DON	Anti-DON Mab	Microfluidics	Amperometry	6.25 – 250 ng/mL	6.25 ng/mL	Wheat	(Olcer et al., 2014)

Table 3.2.2. Electrochemical biosensors based on nanomaterials and/or magnetic beads for the detection of aquatic toxins.

Analyte	Biorecognition element	Nano/magnetic material/flow	Detection technique	Linear range	LOD	Applicability	Ref.
Microcystin-LR (MC-LR)	Anti-MC-LR PAb	SWCNTs	Amperometry	≤ 10 ng/mL	0.6 ng/mL	Lake water	(Wang et al., 2009)
Microcystins (MCs)	Anti-MC-LR MIP	MWCNTs	Potentiometry	0.74 – 1.2 ng/mL	0.66 ng/mL	Artesian well water	(Queiros et al., 2013)
Okadaic acid (OA)	Anti-OA mAb	Graphene	Square Wave Voltammetry (SWV)	≤5 ng/mL	19 pg/mL	Mussels	(Eissa and Zourob, 2012)
MC-LR	Anti-MC-LR mAb	MWCNTs	EIS	0.05 – 20 ng/mL	40 pg/mL	-	(Han et al., 2013)
MC-LR	Anti-MC-LR mAb	SWCNTs	DPV	0.01 – 7.0 ng/mL	2.31 pg/mL	Reservoir water	(Tian et al., 2014)
Brevetoxin B (BTX-2)	Anti-BTX-2 mAb	GNRs MBs	SWV	1 pg/mL – 10 ng/mL	1 pg/mL	Mussels, razor clams & cockles	(Tang et al., 2012)
MC-LR	Anti-MC-LR mAb	GO CNSs	DPV	0.05 – 15 ng/mL	16 pg/mL	Reservoir, tap & river water	(Zhao et al., 2013)
BTX-2	Anti-BTX-2 mAb	Au NPs	DPV	0.03 – 8 ng/mL BTX-2	10 pg/mL	Mussels, razor clams & cockles	(Tang et al., 2011)
MC-LR	Anti-MC-LR mAb	Ag NPs	Capacitance	10 fg/mL – 1 ng/mL	7 fg/mL	Drinking, tap & raw water	(Loyprasert et al., 2008)
MC-LR	Anti-MC-LR mAb	Au NPs	DPV	0.05 – 15 ng/mL	20 pg/mL	Crude algae	(Tong et al., 2011)
MC-LR	Anti-MC-LR mAb	Au NPs	Capacitance	0.1 pg/mL – 0.1 ng/mL	10 fg/mL	Microcystis aeruginosa culture	(Lebogang et al., 2014)
MC-LR	Anti-MC-LR MIP	TiO2 NTs	Photoelectrochemistry (PEC)	0.5 – 100 ng/mL	0.1 ng/mL	-	(Chen et al., 2012)

MC-LR	Anti-MC-LR mAb	CdSe QDs	SWSV	0.227 – 50 ng/mL	99 pg/mL	Microcystis aeruginosa cultures	(Yu et al., 2009)
MC-LR	Anti-MC-LR Ab	GO PtRu alloy	Amperometry	0.01 – 28 ng/mL	9.63 pg/mL	Water	(Wei et al., 2011)
MC-LR	Anti-MC-LR Ab	MBs	DPV	0.01 – 200 µg/mL	4 ng/mL	-	(Ge et al., 2013)
MC-LR	Anti-MC-LR mAb	MBs	CA	0.4 – 20 ng/mL	0.4 ng/mL	Microcystis aeruginosa culture&natural bloom	(Reverte et al., 2013)
OA	Anti-OA mAb	MBs	DPV	-	0.38 ng/mL	Mussels	(Hayat et al., 2011)
OA	Anti-OA mAb	MBs	DPV	0.78 – 500 ng/mL	0.5 ng/mL	Mussels	(Hayat et al., 2012)
OA	Anti-OA mAb	MBs Flow system	Amperometry	0.19 – 25 ng/mL	0.15 ng/mL	Mussels	(Dominguez et al., 2012)
BTX-2 Dinophysistoxin-1 (DTX-1)	Anti-BTX-2 mAb Anti-DTX-1 mAb	MBs Cd NCs Cu NCs Multiplexed Flow system	Square Wave AnodicStripping Voltammetry (SWASV)	5 pg/mL– 5 ng/mL	1.8 pg/mL (BTX-2) 2.2 pg/mL (DTX-1)	Mussels, razor clams & cockles	(Zhang et al., 2012)

Table 3.2.3. Electrochemical biosensors based on nanomaterials and/or magnetic beads for the detection of other analytes.

Analyte	Biorecognition element	Nano/magnetic material/flow	Detection technique	Linear range	LOD	Applicability	Ref.
Clostridium difficile Toxin B (Tcd B)	Anti-Tcd B mAb	MWCNTs GO	DPV	3 pg/mL– 320 ng/mL	0.7 pg/mL	Human stool	(Fang et al., 2014)
Cholera Toxin (CT)	Anti-CT mAb	MWCNTs	Adsorptive Square Wave Stripping Voltammetry (AdSWSV)	10 fg/mL – 100 ng/mL	1 fg/mL	Tap water & domestic waste water	(Viswanathan et al., 2006)
Anthrax Protective Antigen (Anthrax PA)	Anti-Anthrax PA peptide	MWCNTs	SWV	-	33 pg/mL	Blood plasma	(Huan et al., 2011)
Ricin	Anti-ricin PAbs	MWCNTs	Amperometry	0.625 – 25 ng/mL	0.56 ng/mL	-	(Suresh et al., 2010)
Staphylococcal Enterotoxin B (SEB)	Anti-SEB PAb	Au NPs MWCNTs	DPV	0.05 – 15 ng/mL	10 pg/mL	Watermelon juice, apple juice, soy milk & pork food	(Tang et al., 2010)
Anthrax PA	Anti-Anthrax PA mAb	Au NPs	CV	8.1 ng/mL – 81 µg/mL	0.17 fg/mL	Human serum	(Farrow et al., 2013)
SEB	Anti-SEB Mab	Nanoporous aluminium	EIS	-	10 pg/mL	-	(Chai and Takhistov, 2010)
Ricin	Anti-ricin PAb	Nanoporous aluminium	EIS	-	0.5 pg/mL	Milk, vegetable soup & tomato juice	(Chai et al., 2010)
Botulinum Neurotoxin Type A (BoNT)	Anti-BoNT mAb	Au NPs	CV	4 – 35 pg/mL	1 pg/mL	Full & skin milk	(Liu et al., 2014)
Tcd B	Anti-Tcd B Ab	MWCNTs Au NPs	DPV	1 – 80 ng/mL	0.3 ng/mL	-	(Xu et al., 2014)
SEB	Anti-SEB PAb	MWCNTs Au NPs	Conductometry	0.5 – 83.5 ng/mL	0.5 ng/mL	Milk	(Chen, 2008)
Anthrax PA	Anti-Anthrax PA aptamer	GO	Field-effect transistor (FET)	1 fg/mL – 10 pg/mL	1 fg/mL	-	(Kim et al., 2013)
Anthrax PA	Anti-Anthrax PA aptamer	GO Au NPs	FET	-	0.1 fg/mL	-	(Kim et al., 2013)
Staphylococcal protein A (Prot A)	Anti-prot A PAb	MBs	Amperometry	17 fg/mL– 2.6 pg/mL	3.9 fg/mL	Rawmilk	(de Avila et al., 2012)
Cholera toxin B (CTB)	Anti-CTB PAb	MBs	Amperometry	-	1 ng/mL	-	(Bunyakul et al., 2009)
BoNT	Anti-BoNT aptamer	Microfluidics	Amperometry	1 ng/mL – 1 µg/mL	1 ng/mL	-	(Lillehoj et al., 2010)

3.2.7 References

Ansari, A.A., Kaushik, A., Solanki, P.R., Malhotra, B.D., 2010. Nanostructured zinc oxide platform for mycotoxin detection. Bioelectrochemistry 77, 75-81.
Barsan, M.M., Ghica, M.E., Brett, C.M.A., 2015. Electrochemical sensors and biosensors based on redox polymer/carbon nanotube modified electrodes: A review. Anal. Chim. Acta 881, 1-23.
Bone, L., Vidal, J.C., Duato, P., Castillo, J.R., 2010. Ochratoxin A nanostructured electrochemical immunosensors based on polyclonal antibodies and gold nanoparticles coupled to the antigen. Anal. Methods 2, 335-341.
Campas, M., Garibo, D., Prieto-Simon, B., 2012. Novel nanobiotechnological concepts in electrochemical biosensors for the analysis of toxins. Analyst 137, 1055-1067.
Chai, C., Lee, J., Takhistov, P., 2010. Direct Detection of the Biological Toxin in Acidic Environment by Electrochemical Impedimetric Immunosensor. Sensors 10, 11414-11427.
Chai, C., Takhistov, P., 2010. Label-Free Toxin Detection by Means of Time-Resolved Electrochemical Impedance Spectroscopy. Sensors 10, 655-669.
Chen, J.H., Liu, D.L., Li, S.C., Yao, D.S., 2010. Development of an amperometric enzyme electrode biosensor for sterigmatocystin detection. Enzyme Microb. Technol. 47, 119-126.
Chen, K., Liu, M.C., Zhao, G.H., Shi, H.J., Fan, L.F., Zhao, S.C., 2012. Fabrication of a Novel and Simple Microcystin-LR Photoelectrochemical Sensor with High Sensitivity and Selectivity. Environ. Sci. Technol. 46, 11955-11961.
Chen, Z.G., 2008. Conductometric immunosensors for the detection of staphylococcal enterotoxin B based bio-electrocalytic reaction on micro-comb electrodes. Bioprocess. Biosyst. Eng. 31, 345-350.
Dai, Z.H., Ju, H.X., 2012. Bioanalysis based on nanoporous materials. Trac-Trends Anal. Chem. 39, 149-162.
Eissa, S., Zourob, M., 2012. A graphene-based electrochemical competitive immunosensor for the sensitive detection of okadaic acid in shellfish. Nanoscale 4, 7593-7599.
European Commission Recommendation of 18 October 2011 on the definition of nanomaterial (L 275/38), 2011/696/EU. Off. J. Eur. Union. Brussels.
Fang, Y.-S., Chen, S.-Y., Huang, X.-J., Wang, L.-S., Wang, H.-Y., Wang, J.-F., 2014. Simple approach for ultrasensitive electrochemical immunoassay of Clostridium difficile toxin B detection. Biosens. Bioelectron. 53, 238-244.
Farrow, B., Hong, S.A., Romero, E.C., Lai, B., Coppock, M.B., Deyle, K.M., Finch, A.S., Stratis-Cullum, D.N., Agnew, H.D., Yang, S., Heath, J.R., 2013. A Chemically Synthesized Capture Agent Enables the Selective, Sensitive, and Robust Electrochemical Detection of Anthrax Protective Antigen. ACS Nano 7, 9452-9460.
Han, C., Doepke, A., Cho, W., Likodimos, V., de la Cruz, A.A., Back, T., Heineman, W.R., Halsall, H.B., Shanov, V.N., Schulz, M.J., Falaras, P., Dionysiou, D.D., 2013. A Multiwalled-Carbon-Nanotube-Based Biosensor for Monitoring Microcystin-LR in Sources of Drinking Water Supplies. Adv. Funct. Mat. 23, 1807-1816.
Handford, C.E., Dean, M., Henchion, M., Spence, M., Elliott, C.T., Campbell, K., 2014. Implications of nanotechnology for the agri-food industry: Opportunities, benefits and risks. Trends Food Sci. Technol. 40, 226-241.
Huan, T.N., Ganesh, T., Han, S.H., Yoon, M.Y., Chung, H., 2011. Sensitive detection of an Anthrax biomarker using a glassy carbon electrode with a consecutively immobilized layer of polyaniline/carbon nanotube/peptide. Biosens. Bioelectron. 26, 4227-4230.
Huan, T.N., Ha, V.T.T., Hung, L.Q., Yoon, M.Y., Han, S.H., Chung, H., 2009. Square wave voltammetric detection of Anthrax utilizing a peptide for selective recognition of a protein biomarker. Biosens. Bioelectron. 25, 469-474.

Justino, C.I.L., Rocha-Santos, T.A.P., Cardoso, S., Duarte, A.C., 2013. Strategies for enhancing the analytical performance of nanomaterial-based sensors. Trac-Trends Anal. Chem. 47, 27-36.
Kalita, P., Singh, J., Singh, M.K., Solanki, P.R., Sumana, G., Malhotra, B.D., 2012. Ring like self assembled Ni nanoparticles based biosensor for food toxin detection. Appl. Phys. Lett. 100, 093702.
Kaushik, A., Solanki, P.R., Ansari, A.A., Ahmad, S., Malhotra, B.D., 2009a. A nanostructured cerium oxide film-based immunosensor for mycotoxin detection. Nanotechnology 20.
Kaushik, A., Solanki, P.R., Pandey, M.K., Ahmad, S., Malhotra, B.D., 2009b. Cerium oxide-chitosan based nanobiocomposite for food borne mycotoxin detection. Appl. Phys. Lett. 95.
Kaushik, A., Solanki, P.R., Pandey, M.K., Kaneto, K., Ahmad, S., Malhotra, B.D., 2010. Carbon nanotubes chitosan nanobiocomposite for immunosensor. Thin Solid Films 519, 1160-1166.
Kim, D.-J., Park, H.-C., Sohn, I.Y., Jung, J.-H., Yoon, O.J., Park, J.-S., Yoon, M.-Y., Lee, N.-E., 2013. Electrical Graphene Aptasensor for Ultra-Sensitive Detection of Anthrax Toxin with Amplified Signal Transduction. Small 9, 3352-3360.
Kuang, H., Chen, W., Xu, D.H., Xu, L.G., Zhu, Y.Y., Liu, L.Q., Chu, H.Q., Peng, C.F., Xu, C.L., Zhu, S.F., 2010. Fabricated aptamer-based electrochemical "signal-off" sensor of ochratoxin A. Biosens. Bioelectron. 26, 710-716.
Lebogang, L., Mattiasson, B., Hedstrom, M., 2014. Capacitive sensing of microcystin variants of Microcystis aeruginosa using a gold immunoelectrode modified with antibodies, gold nanoparticles and polytyramine. Microchim. Acta 181, 1009-1017.
Li, S.C., Chen, J.H., Cao, H., Yao, D.S., Liu, D.L., 2011. Amperometric biosensor for aflatoxin B-1 based on aflatoxin-oxidase immobilized on multiwalled carbon nanotubes. Food Control 22, 43-49.
Liu, G.Z., Zhang, Y., Guo, W.Q., 2014. Covalent functionalization of gold nanoparticles as electronic bridges and signal amplifiers towards an electrochemical immunosensor for botulinum neurotoxin type A. Biosens. Bioelectron. 61, 547-553.
Liu, X.P., Deng, Y.J., Jin, X.Y., Chen, L.G., Jiang, J.H., Shen, G.L., Yu, R.Q., 2009. Ultrasensitive electrochemical immunosensor for ochratoxin A using gold colloid-mediated hapten immobilization. Anal. Biochem. 389, 63-68.
Liu, Y., Qin, Z.H., Wu, X.F., Jiang, H., 2006. Immune-biosensor for aflatoxin B-1 based bio-electrocatalytic reaction on micro-comb electrode. Biochem. Eng. J. 32, 211-217.
Loyprasert, S., Thavarungkul, P., Asawatreratanakul, P., Wongkittisuksa, B., Limsakul, C., Kanatharana, P., 2008. Label-free capacitive immunosensor for microcystin-LR using self-assembled thiourea monolayer incorporated with Ag nanoparticles on gold electrode. Biosens. Bioelectron. 24, 78-86.
Malhotra, B.D., Srivastava, S., Ali, M.A., Singh, C., 2014. Nanomaterial-Based Biosensors for Food Toxin Detection. Appl. Biochem. Biotechnol. 174, 880-896.
Marin, S., Merkoci, A., 2012. Nanomaterials Based Electrochemical Sensing Applications for Safety and Security. Electroanalysis 24, 459-469.
Pedrero, M., Campuzano, S., Pingarron, J.M., 2012. Magnetic Beads-Based Electrochemical Sensors Applied to the Detection and Quantification of Bioterrorism/Biohazard Agents. Electroanalysis 24, 470-482.
Queiros, R.B., Guedes, A., Marques, P.V.S., Noronha, J.P., Sales, M.G.F., 2013. Recycling old screen-printed electrodes with newly designed plastic antibodies on the wall of carbon nanotubes as sensory element for in situ detection of bacterial toxins in water. Sens. Actuator B-Chem. 189, 21-29.
Rivas, L., Mayorga-Martinez, C.C., Quesada-Gonzalez, D., Zamora-Galvez, A., de la Escosura-Muniz, A., Merkoci, A., 2015. Label-Free Impedimetric Aptasensor for Ochratoxin-A Detection Using Iridium Oxide Nanoparticles. Anal. Chem. 87, 5167-5172.
Singh, J., Roychoudhury, A., Srivastava, M., Solanki, P.R., Lee, D.W., Lee, S.H., Malhotra, B.D., 2013. A highly efficient rare earth metal oxide nanorods based platform for aflatoxin detection. J. Mater. Chem. B 1, 4493-4503.

Srivastava, S., Abraham, S., Singh, C., Ali, M.A., Srivastava, A., Sumana, G., Malhotra, B.D., 2015. Protein conjugated carboxylated gold@reduced graphene oxide for aflatoxin B-1 detection. RSC Adv. 5, 5406-5414.
Srivastava, S., Ali, M.A., Umrao, S., Parashar, U.K., Srivastava, A., Sumana, G., Malhotra, B.D., Pandey, S.S., Hayase, S., 2014. Graphene Oxide-Based Biosensor for Food Toxin Detection. Appl. Biochem. Biotechnol. 174, 960-970.
Srivastava, S., Kumar, V., Ali, M.A., Solanki, P.R., Srivastava, A., Sumana, G., Saxena, P.S., Joshi, A.G., Malhotra, B.D., 2013. Electrophoretically deposited reduced graphene oxide platform for food toxin detection. Nanoscale 5, 3043-3051.
Sunday, C.E., Masikini, M., Wilson, L., Rassie, C., Waryo, T., Baker, P.G.L., Iwuoha, E.I., 2015. Application on Gold Nanoparticles-Dotted 4-Nitrophenylazo Graphene in a Label-Free Impedimetric Deoxynivalenol Immunosensor. Sensors 15, 3854-3871.
Sundramoorthy, A.K., Gunasekaran, S., 2014. Applications of graphene in quality assurance and safety of food. Trac-Trends Anal. Chem. 60, 36-53.
Suresh, S., Gupta, A.K., Rao, V.K., Kumar, O., Vijayaraghavan, R., 2010. Amperometric immunosensor for ricin by using on graphite and carbon nanotube paste electrodes. Talanta 81, 703-708.
Tang, D.A.P., Tang, J.A., Su, B., Chen, G.N., 2011. Gold nanoparticles-decorated amine-terminated poly(amidoamine) dendrimer for sensitive electrochemical immunoassay of brevetoxins in food samples. Biosens. Bioelectron. 26, 2090-2096.
Tang, D.P., Tang, J.A., Su, B.L., Chen, G.N., 2010. Ultrasensitive Electrochemical Immunoassay of Staphylococcal Enterotoxin B in Food Using Enzyme-Nanosilica-Doped Carbon Nanotubes for Signal Amplification. J. Agric. Food Chem. 58, 10824-10830.
Tang, J., Hou, L., Tang, D.P., Zhou, J., Wang, Z.P., Li, J.R., Chen, G.N., 2012. Magneto-controlled electrochemical immunoassay of brevetoxin B in seafood based on guanine-functionalized graphene nanoribbons. Biosens. Bioelectron. 38, 86-93.
Tian, J.P., Zhao, H.M., Yuan, F., Quan, X., Chen, S., 2014. Ultrasensitive immunoassay of microcystins-LR using G-quadruplex DNAzyme as an electrocatalyst. Int. J. Environ. Anal. Chem. 94, 988-1000.
Tong, P., Tang, S.R., He, Y., Shao, Y.H., Zhang, L., Chen, G.N., 2011. Label-free immunosensing of microcystin-LR using a gold electrode modified with gold nanoparticles. Microchim. Acta 173, 299-305.
Tong, P., Zhao, W.-W., Zhang, L., Xu, J.-J., Chen, H.-Y., 2012. Double-probe signal enhancing strategy for toxin aptasensing based on rolling circle amplification. Biosens. Bioelectron. 33, 146-151.
Tothill, I.E., 2011. Biosensors and nanomaterials and their application for mycotoxin determination. World Mycotoxin Journal 4, 361-374.
Vidal, J.C., Bonel, L., Duato, P., Castillo, J.R., 2011. Improved electrochemical competitive immunosensor for ochratoxin A with a biotinylated monoclonal antibody capture probe and colloidal gold nanostructuring. Anal. Methods 3, 977-984.
Vig, A., Munoz-Berbel, X., Radoi, A., Cortina-Puig, M., Marty, J.-L., 2009. Characterization of the gold-catalyzed deposition of silver on graphite screen-printed electrodes and their application to the development of impedimetric immunosensors. Talanta 80, 942-946.
Viswanathan, S., Wu, L.C., Huang, M.R., Ho, J.A.A., 2006. Electrochemical immunosensor for cholera toxin using liposomes and poly(3,4-ethylenedioxythiophene)-coated carbon nanotubes. Anal. Chem. 78, 1115-1121.
Wang, C., Yu, C., 2013. Detection of chemical pollutants in water using gold nanoparticles as sensors: a review. Rev. Anal. Chem. 32, 1-14.
Wang, D., Hu, W.H., Xiong, Y.H., Xu, Y., Li, C.M., 2015. Multifunctionalized reduced graphene oxide-doped polypyrrole/pyrrolepropylic acid nanocomposite impedimetric immunosensor to ultra-sensitively detect small molecular aflatoxin B-1. Biosens. Bioelectron. 63, 185-189.

Wang, L.B., Chen, W., Xu, D.H., Shim, B.S., Zhu, Y.Y., Sun, F.X., Liu, L.Q., Peng, C.F., Jin, Z.Y., Xu, C.L., Kotov, N.A., 2009. Simple, Rapid, Sensitive, and Versatile SWNT-Paper Sensor for Environmental Toxin Detection Competitive with ELISA. Nano Lett. 9, 4147-4152.
Wang, L.B., Ma, W., Xu, L.G., Chen, W., Zhu, Y.Y., Xu, C.L., Kotov, N.A., 2010. Nanoparticle-based environmental sensors. Mater. Sci. Eng. R-Rep. 70, 265-274.
Wei, Q., Zhao, Y., Du, B., Wu, D., Cai, Y., Mao, K., Li, H., Xu, C., 2011. Nanoporous PtRu Alloy Enhanced Nonenzymatic Immunosensor for Ultrasensitive Detection of Microcystin-LR. Advanc. Funct. Mat. 21, 4193-4198.
Xu, X.Q., Fang, Y.S., Wang, L.S., 2014. A label-free electrochemical immunosensor for clostridium difficile toxin B based on one-step immobilization thionine in a silica matrix. Anal. Lett. 47, 2255-2265.
Yao, D.-S., Cao, H., Wen, S., Liu, D.-L., Bai, Y., Zheng, W.-J., 2006. A novel biosensor for sterigmatocystin constructed by multi-walled carbon nanotubes (MWNT) modified with aflatoxin-detoxifizyme (ADTZ). Bioelectrochemistry 68, 126-133.
Yao, D.-S., Wen, S.-M., Liu, D.-L., Xie, C.-F., Bai, Y., Ran, Y.-H., 2004. [The primary study on the detection of sterigmatocystin by biologic enzyme electrode modified with the multiwall carbon nanotubes]. Chinese J. Biotechnol. 20, 601-606.
Yu, H.-W., Lee, J., Kim, S., Nguyen, G.H., Kim, I.S., 2009. Electrochemical immunoassay using quantum dot/antibody probe for identification of cyanobacterial hepatotoxin microcystin-LR. Anal. Bioanal. Chem. 394, 2173-2181.
Yu, L., Zhang, Y., Hu, C., Wu, H., Yang, Y., Huang, C., Jia, N., 2015. Highly sensitive electrochemical impedance spectroscopy immunosensor for the detection of AFB(1) in olive oil. Food Chem. 176, 22-26.
Zachetti, V.G.L., Granero, A.M., Robledo, S.N., Zon, M.A., Fernandez, H., 2013. Development of an amperometric biosensor based on peroxidases to quantify citrinin in rice samples. Bioelectrochemistry 91, 37-43.
Zhang, J., Lei, J.P., Xu, C.L., Ding, L., Ju, H.X., 2010. Carbon Nanohorn Sensitized Electrochemical Immunosensor for Rapid Detection of Microcystin-LR. Anal. Chem. 82, 1117-1122.
Zhao, H., Tian, J., Quan, X., 2013. A graphene and multienzyme functionalized carbon nanosphere-based electrochemical immunosensor for microcystin-LR detection. Colloids Surfaces B 103, 38-44.

Chapter 4

Tetrodotoxin: the puffer fish toxin

DOI: 10.1021/acs.analchem.5b02158
Anal. Chem. 2015, 87, 10839−10847

analytical chemistry

Article

pubs.acs.org/ac

Detection of Tetrodotoxins in Puffer Fish by a Self-Assembled Monolayer-Based Immunoassay and Comparison with Surface Plasmon Resonance, LC-MS/MS, and Mouse Bioassay

Laia Reverté,[†] Pablo de la Iglesia,[†] Vanessa del Río,[†] Katrina Campbell,[‡] Christopher T. Elliott,[‡] Kentaro Kawatsu,[§] Panagiota Katikou,[∥] Jorge Diogène,[†] and Mònica Campàs*[,†]

[†]IRTA, Carretera Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

[‡]Institute for Global Food Security, School of Biological Sciences, Queen's University, Stranmillis Road, Belfast BT9 5AG, Northern Ireland

[§]Division of Bacteriology, Osaka Prefectural Institute of Public Health, 3-69, Nakamichi 1-chome, Higashinari-ku, Osaka 537-0025, Japan

[∥]National Reference Laboratory on Marine Biotoxins, Ministry of Rural Development and Food, 54627 Thessaloniki, Greece

4.1 Detection of tetrodotoxins in pufferfish by a self-assembled monolayer-based immunoassay and comparison with SPR, LC-MS/MS and MBA

4.1.1 Abstract

The increasing occurrence of puffer fish containing tetrodotoxin (TTX) in the Mediterranean couldrepresent a major food safety risk for European consumers and threaten the fishing industry. The work presented herein describes the development of a new enzyme linked immunosorbent assay (mELISA) based on the immobilization of TTX through dithiol monolayers self-assembled on maleimide plates, which provides an ordered and oriented antigen immobilization and favors the antigen-antibody affinity interaction. The mELISA was found to have a limit of detection (LOD) of TTX of 0.23 mg/kg of puffer fish matrix. The mELISA and a surface plasmon resonance (SPR) immunosensor previously developed were employed to establish the cross-reactivity factors (CRFs) of 5,6,11-trideoxy TTX, 5-deoxy-TTX and 11-deoxy-TTX, norTTX-6-ol, and 4,9-anhydro-TTX, as well as to determine TTX equivalent contents in puffer fish samples. Results obtained by both immunochemical tools were correlated (R^2= 0.977).The puffer fish samples were also analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), and the corresponding CRFs were applied to the individual TTX contents. Results provided by the immunochemical tools, when compared with those obtained by LC-MS/MS, showeda good degree of correlation (R^2 = 0.991 and 0.979 for mELISA and SPR, respectively). The mouse bioassay (MBA) slightly overestimated the CRF adjusted TTX content of samples when compared with the data obtained from the other techniques. The mELISA has been demonstrated to be fit for the purpose for screening samples in monitoring programs and in research activities.

4.1.2 Introduction

Tetrodotoxin (TTX) is a potent, low molecular weight (319 Da) marine neurotoxin. It is a cage-like polar molecule that contains a cyclic guanidinium moiety connected to a dioxy-adamantane skeleton decorated with six hydroxyl groups (Goto et al., 1965; Tsuda et al., 1964; Woodward and Gougouta, 1964). Although TTX was originally discovered in puffer fish (Tahara, 1909), its presence has been reported in many other organisms, including starfish, xanthid crabs, blue-ring octopus, worms, gastropods, frogs, gobies, and newtsof the genus Taricha (Noguchi et al.,

2006). To date, 29 analogues of TTX have been described, with varying degrees of toxicity. They have been classified into three groups depending on the structure: hemylactal, lactone, and 4,9-anhydro types (Bane et al., 2014). The biosyntheticpathway through which TTXsare produced still remains unclear, but they are believed to be produced by marine endosymbiotic bacteria, including *Vibrio* and *Alteromonas* spp. Once these bacteriaare excreted into the environment, they are consumed by zooplankton, worms, or starfish and transferred to other species such as puffer fish or gastropods whereby the toxin production continues (Pratheepa and Vasconcelos, 2013).

Like saxitoxin (STX), a paralytic toxin, TTX has the ability to selectively block voltage-sensitive sodium-gated ion channels (VSGCs) (French et al., 2010; Lee and Ruben, 2008). This effect blocks the influx of sodium ions into the cell, affecting neural transmission. In humans, this effectcan result in numbness, respiratory paralysis, gastrointestinal effects, and even death. Most episodes of TTX poisoning are due to consumption of Japanese puffer fish (*fugu)* (Noguchi and Arakawa, 2008). Consequently, the bioaccumulation of TTX in seafood and subsequent entry into the food chain poses a real risk to human safety.

The Japanese government has established a regulatory limit of 2 mg/kg of TTX equivalents in food (Kawabata, 1978). In contrast, no regulatory limits have been set in Europe. However, in October 2007, the first toxic European episode was reported in Málaga (Spain), caused by the ingestion of a trumpet shell of the species *Charonia lampas lampas* (Rodriguez et al., 2008). Moreover, in 2005 and 2008, puffer fish (*Lagocephalus sceleratus*) with low levels of TTX were found migrating from the Red Sea to the Mediterranean Sea due to the Lessepsian phenomenon (Bentur et al., 2008). Very recently, the presence of TTXs has been reported in bivalve mollusk shellfish grown at the south coast of England (Turner et al., 2015) and along the Greek coast (Mediterranean Sea) (Vlamis et al., 2015). The increasing occurrence of TTX in European waters suggests that seafood may be at risk, and this highlights the need to have regulatory limits set to support specific, cost-effective, and unambiguous methods to quantify TTXs, in order to protect human health. Due to the lack of an official method for TTX measurement in Europe and with the aim of developing alternative methods to the mouse bioassay (MBA), its detection has been achieved using different methodological approaches, such as immunochemical assays and chemical analysis. The use of antibodies as biorecognition elements has been extensively reported in colorimetric (Kawatsu et al., 1997; Neagu et al., 2006; Raybould et al., 1992; Stokes et al., 2012; Tao et al., 2010; Wang et al., 2014; Zhou et al., 2009) and optical (Yakes et al., 2010) immunoassays, as well as optical surface plasmon resonance (SPR) (Campbell et al., 2013; Taylor

et al., 2008; Taylor et al., 2011; Vaisocherova et al., 2011; Yakes et al., 2011; Yakes et al., 2014) and electrochemical (Kreuzer et al., 2002; Neagu et al., 2006) immunosensors. However, a limited number of these studies have been applied to the analysis of toxin content in puffer fish (Kawatsu et al., 1997; Raybould et al., 1992; Tao et al., 2010; Taylor et al., 2008; Taylor et al., 2011; Vaisocherova et al., 2011). The application of cross-reactivity factors (CRFs) of TTX analogues is important when developing methods based on recognition of toxins by antibodies. Little is known about CRFs for TTXs determined by immunoassays and immunosensors apart from the study performed by Kawatsu and co-workers (Kawatsu et al., 1997). In this study, the affinity of a monoclonal antibody toward TTXs was investigated and showed 8.3% of cross-reactivity toward a structurally related TTX (anhydro-TTX), but did not cross-react with tetrodonic acid (<0.017%), which has quite a different chemical structure to TTX itself. Only a small number of physicochemical-based methods have been applied to the determination of TTXs. Of these, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most powerful toolin terms of its capability of delivering selective characterization of the different TTX analogues present in samples (Asakawa et al., 2012; Bane et al., 2014).

Analytical techniques based on different measurement principles provide different, often complementary, information. Whereas immunochemical-based methods can provide information about structural recognition, physicochemical procedures provide unambiguous identification of the analyte. Moreover, in the development of new measurement tools, the comparison of results obtained against those determined by well-established physicochemicalmethods is necessary in order to demonstrate the performance of new approaches. In the case of immunochemical systems, the establishment of CRFs will play a major contribution to determining how fit-for-purposenew techniques are. Thus, it is proposed that a combination of a screening tool and a confirmatory instrumental method should be the strategy employed to achieve fast and reliable quantification of TTXs present in samples. With this aim, a new enzyme linked immunosorbent assay (mELISA) configuration was developed on the basis of the immobilization of TTX through dithiol-carboxylate monolayers self-assembled on maleimide-activated plates (**Figure 4.1.1**). This immobilization strategy avoids the need to conjugate TTX to protein binders such as bovine serum albumin (BSA) or ovalbumine (OVA) (Kawatsu et al., 1997; Raybould et al., 1992; Stokes et al., 2012), which sometimes results in high nonspecific adsorption and random antigen immobilization. The self-assembling of dithiolated scaffolds on the maleimide-activated surface provides a stable, organized, and spaced monolayer, which favors the oriented antigen immobilization as well as the antigen-antibody affinity interaction, and reduces or avoids nonspecific binding (Fragoso et al., 2008). Both the

mELISA and a SPR immunosensor previously developed (Campbell et al., 2013) were used to establish the corresponding CRFs of four TTX analogues and to determine total TTX contents in puffer fish samples. These samples were then analyzed by LC-MS/MS, and the determined CRFs were applied to the measurements made in order to compare the results with those obtained by the immunochemical tools and MBA (Katikou et al., 2009). The application of the CRFs of the TTX analogues to the individual contents determined by LC-MS/MS has been crucial to compare the results between immunochemical and physicochemical approaches.

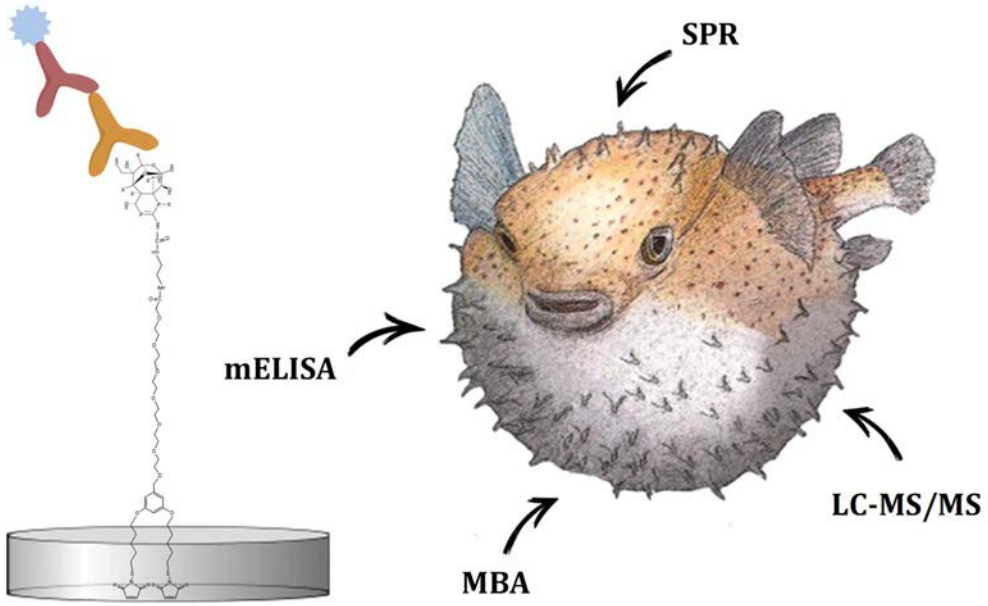


Fig. 4.1.1. Scheme of the mELISA configuration for the detection of TTXs and the analysis of puffer fish by complementary techniques.

4.1.3 Materials and methods

4.1.3.1 Reagents and materials

The TTX standard used for the functionalization of the Biacore chip was obtained from Biomol/Affiniti Research Products (Exeter, UK) whereas the TTX standard from Tocris Bioscience (Bristol, UK)was used for the SPR calibration curve and for mELISA and LC-MS/MS analysis. Both TTX standard solutions were prepared at 1 mg/mL in 10 mM acetic acid. The anti-TTX monoclonal antibody (mAb) TX-7F was produced as described in Kawatsu et al (Kawatsu, et al., 1997) and prepared as described in Campbell et al (Campbell et al., 2013). Briefly, the mAb was raised to TTX–BSA conjugate. It was produced by a cell line, concentrated in cell culture media

using Viva spin centrifuge tubes (30,000 MWCO)and purified via affinity chromatography usinga protein G-sepharose gel column (mAb Trap Kit). Afterward, a dialysis was performed over 24 h in saline solution with threebuffer changes. For mELISA experiments, Pierce maleimide-activated plates were obtained from ThermoFisher Scientific (Madrid, Spain), dithiolalkanearomaticPEG6-COOH (dithiol-carboxylate) was purchased from Sensopath Technologies (Bozeman, USA), and anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP) was supplied by Sigma-Aldrich (Tres Cantos, Spain).

4.1.3.2 Equipment

For preparative LC, a 600-MS LC pump system controller (Waters Corp. Mildford, MA, USA) with photodiode array detector (PDA) 996 (Waters Corp.) and a fraction collector FRAC-100 (Pharmacia Biotech., Uppsala, Sweden) was used. Empower software (Waters Corp.) was used for the instrument control.For LC-MS/MS analyses, a 1200 LC system connected toa binary pump (G1312B), a four-channel degasser (G1379B), a thermostated autosampler (G1367C + G1330B), and a column oven (G1316B) from Agilent Technologies (Palo Alto, CA, USA) was used. The chromatograph was coupled to a 3200 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a TurboV electrospray ion source (AB/Sciex).A nitrogen generator NM20Z (Peak Scientific, Renfrewshire, Scotland, UK) supplied all operation gases. Instrument control, acquisition, and data analysis were powered by Analyst software (version 1.4.2).

Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA). An SPR device (Biacore Q) with Control Software (Version 3.0.1), BIAevaluation software version 4.1, and CM5 sensor chips were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden).

4.1.3.3 Puffer fish samples processing and toxin extraction

Blunthead puffer (*Sphoeroidespachygaster*, Müller and Troschel, 1848) caught off the coast of Palamós, Spain (NW Mediterranean Sea) was used to obtain blank matrices of a range of pufferfish tissues. Samples of silver-cheeked toadfishes (*Lagocephalus sceleratus*, Gmelin, 1789) from Greece (E Mediterranean Sea) and La Réunion Island (Indian Ocean) naturally-containing TTXs were used as positive samples and as a source of TTX analogues.

Extracts of silver-cheeked toadfishes from Greece (number 2 (male) and 4 (female)) were obtained from the study performed by Katikou et al (Katikou et al., 2009). Blunthead puffer and

silver-cheeked toadfish from La Réunion Island were dissected and separated intointestinal tract, muscle, skin, liver, and gonads (if female). Each part was then homogenized using an Ultraturrax blender and stored at -20°C until required. Extraction of TTXswas performed according to Katikou et al.(Katikou et al., 2009) with slight modifications: a portion of 10 g from each organwas extracted individually with 25 mL of 0.1% acetic acid by heating for 10 min in a boiling water bathwith occasional stirring.The mixture was cooled down and, instead of filtration, two consecutive centrifugation steps were performed at 4,500 rpm for 5 min. In the case of liver samples, an additional liquid-liquid partition of the crude extract with hexane (1:1) was required in order to remove fats. After extraction, the final volume of crude extracts were made up to 50 mL with 0.1% acetic acid, passed through nylon syringe filters of 0.45 µm cutoff and centrifuged at 12,000 rpm using 3,000 MW molecular sieve filters. Extracts were frozen at -20ºC until needed. For LC-MS/MS analysis, solvent was evaporated and extracts were redissolved in methanol and filtered through 0.2 µm nylon filters.

4.1.3.4 Isolation and purification of TTX analogues

Tissue extracts from several specimens of silver-cheeked toadfishes (*L. sceleratus*) were pooled, evaporated to dryness under N_2 stream, and redissolved in 1 mL acetonitrile/water (90/10, v/v). Hydrophilic interaction chromatography (HILIC) fractionation was conducted at room temperature and 10 mL/min on a prep-LC column Luna HILIC AXIA (250 mm x 21.2 mm, 5 µm particle size) purchased from Phenomenex (Torrance, CA, USA). Binary gradient elution was performed with water (mobile phase A) and acetonitrile/water (90/10, v/v, mobile phase B), both containing 30 mMammonium acetate at pH 5.8. The gradient program started at 100% B, and it was kept isocratic for 5 min; then, it decreased downto 95% B at min 35. Afterward, it decreased down to 82.5% B at min 80, was held at isocratic for 5 min, and was returned to initial conditions at min 90. The pooled sample was manually loaded on a 1-mL loop, and once the chromatographic run started, fractions were automatically collected every minute. After fractionation, a 1-mL subsample was collected from each tube, evaporated to dryness, redissolved in 100 µL MeOH, and analyzed by LC-MS/MS to assess the fraction purity. Only those fractions containing exclusively one single TTX analogue were evaporated, redissolved in MeOH, and stored frozen below -18 ºC until further use. The final concentrations of TTXs analogues available for immunochemical studies were determined by LC-MS/MS.

4.1.3.5 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Analytical separations were performed at 30 °C and 500 µL/min flow rate on a HILIC XBridge Amide column (100 mm × 2.1 mm, 3.5 µm particle size) from Waters Corp. (Milford, MA,USA). As in preparative scale, a binary gradient elution was programmed with water (mobile phase A) and acetonitrile/water (90/10, v/v, mobile phase B), both containing 30 mM ammonium acetate at pH 5.8. The gradient started with 100% B for 1 min, then decreased down to 50% B for 5 min, was held at 50% B until min 8, and then came back to initial conditions at min 9. Finally, initial conditions were heldat the starting conditions for column equilibration until a total run cycleof 12 min.Mobile phase eluting from the column was divertedto waste for the first 1 min of analysis. Methanol/water (50/50, v/v) was used assolvent to wash the autosampler needle. Injection volume was 50 µL, and the autosampler was cooled at 4 °C. Extracts were analyzed with the mass spectrometer operating in positive polarity, using the parameters previously optimized with the standard of TTX: 30 psi curtain gas, 50 V declustering potential, 5500 V ion spray voltage, 600 °C nebulizer gas temperature, 50 psi nebulizer and heater gases, level 4 (arbitrary units) collision-activated dissociation gas, and 2600 V electron multiplier. Multiple reaction monitoring (MRM) transitions were monitored for the following TTX analogues (precursor ion > product ion 1 (collision energy, CE)/ product ion 2 (CE)): 320.1 > 302.1 (29 V)/ 162.2 (53 V) for TTX and 4-epiTTX, 302.1 > 256.1 (53 V)/ 162.2 (53 V) for 4,9-anhydroTTX, 304.1 > 286.1 (53 V)/ 162.2 (53 V) for 5-deoxyTTX and 11-deoxyTTX, 272.1 > 254.1 (29 V)/ 162.2 (53 V) for 5,6,11-trideoxyTTX, and 290.1 > 272.1 (29 V)/ 162.2 (53 V) for 11-norTTX-6(S)-ol and 11-norTTX-6(R)-ol. Dwell time was 90 ms for all transitions.Due to thelack of proper analytical standards for all TTX analogues, identification was supported by acquisition of the full product ion spectra (data not shown), and quantification had to be performed against TTX assuming equimolar responses for other analogues, given the equivalent structural fragmentation in MRM transitions.

4.1.3.6 Colorimetric maleimide-based immunoassay (mELISA)

The protocol used for the immobilization of TTX through dithiols self-assembled on maleimide plates was as follows: (1) maleimide-activated plates were rinsed three times with 200 µL of washing buffer (0.1 M potassium phosphate, 0.15M NaCl, 0.05% Tween-20, pH 7.2) to activate maleimide groups. (2) After washing, 100 µL of 0.1 mM dithiol-carboxylate in absolute ethanol was self-assembled for 3 h. (3) Carboxylic groups ofdithiols were activated by the addition of 100 µL of 0.1 M NHS and 0.4 M EDC (1/1, v/v) in 25 mM MES, pH 5.5, for 30 min. (4) Activated carboxylic groups reacted with primary amines of ethylenediaminefor 30 min, forming amide bonds (100 µL of 0.1 M ethylenediamine in 0.1 M sodium carbonate buffer, pH 9.6). (5) The remaining carboxylic groupswere deactivated by adding 200 µL of 1 M ethanolamine in

phosphate buffer (0.1 M potassium phosphate, 0.15 M sodium chloride, 10 mM EDTA, pH 7.2) for 30 min.(6) TTX was then immobilized on ethylenediamine through formaldehyde cross-linking following an amino-amino reaction, adding 95.6 µL of TTX at different concentrations in phosphate buffer and 3.4 µL of formaldehyde (37%) for 15 h. 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 at room temperature.

Colorimetric checkerboards were performed in order to choose the TTX concentrations as well as the mAb and IgG-HRP dilutions to use in the subsequent competitive mELISAs. Thus, after TTX immobilization, (7) 100 µL of different mAb dilutions in 1% BSA-phosphate buffer was incubated for 30 min. (8) A blocking step was then performed using 200 µL of 1% BSA-phosphate buffer for 30 min. (9) 100 µL of different IgG-HRP dilutions in 1% BSA-phosphate buffer was added to wells for 30 min. (10) Finally, 100 µL of TMB liquid substrate was incubated and after 10 min, absorbance was read at 620 nm. Microtiter plates were placed on a plate shaker at room temperature during incubations. After each step, wells were rinsed three times with 200 µL of washing buffer.

In the competitive mELISAs, the protocol differed from the checkerboard only instep 7, where the competition was performed by mixing 50 µL of mAb dilution with 50 µL of TTX standard solution or “sample” (TTX analogues, blank puffer fish matrix, TTX-spiked puffer fish matrix, or naturally-contaminated puffer fish), both diluted in phosphate buffer. Several competitive mELISAs were performeddiffering in the sample used in the competition step. A TTX standard calibration curve was constructed starting at 1,280 µg/L. Pure TTX analogues were also tested at specific concentrations, starting at 100,000 µg/L for 5,6,11-trideoxy TTX, 100 µg/L for 5-deoxy-TTX and 11-deoxy-TTX, 2,000 µg/L for norTTX-6-ol and 20,000 µg/L for 4,9-anhydro-TTX. Matrix effects of different parts of a blank puffer fish sample (intestinal tract, muscle, skin, liver and gonads, with no indication of TTXs as determined by LC-MS/MS analysis (limit of quantification (LOQ) = 1.00 mg/kg, i.e. 10 µg/L)) were evaluated using an initial matrix concentration of 160 mg/mL. Afterward, each puffer fish matrix (at 10 mg/mL) was spiked with the same concentrations of TTX as for the standard calibration curve. Finally, extracts of puffer fish 2 and 4 were analyzed starting at 10 mg/mL of matrix except muscle extract of puffer fish 2, which was started at 80 mg/mL. All toxin standard solutions and samples were prepared in phosphate buffer, assayed in triplicate and diluted by half. Microtiter plates were placed on a plate shaker at room temperature during incubations. After each step, wells were rinsed three times with 200 µL of washing buffer.

4.1.3.7 SPR immunosensor

The immobilization of TTX on the CM5 Biacore chip surface was performed according to the protocol described by Campbell and co-workers (Campbell et al., 2013). Briefly, 0.4 M EDC and 0.1 M NHS (1:1, v/v) from the amino coupling kit were mixed and added for 30 min in order to activate the carboxymethylated surface of the chip. After removing the excess solution, 0.1 M ethylendiamine in borate buffer pH 8.5 was added to the chip surface for 1 h. In order to deactivate excess reactive groups, 1 M ethanolamine was added to the chip surface for 30 min and then the chip was rinsed with water. Afterward, TTX (in 1 M sodium acetate buffer, pH 5.0) was immobilized through the amino group on the chip surface via formaldehyde coupling chemistry. The chip was then rinsed with water, dried with a stream of nitrogen gas, and stored until use at 4ºC.

The competition assays were also performed according to the protocol described by Campbell and co-workers (Campbell et al., 2013). In short: mAb was used at 1:300 dilution in HBS-EP buffer and mixed with equal volumes of either TTX standard solution or “sample” (TTX analogs, blank puffer fish matrix, TTX-spiked puffer fish matrix, or naturally-contaminated puffer fish). The mixture was then injected over the Biacore chip at a flow rate of 12 µL/min for 2 min. The difference in response units (RU) taken 10 s before and 30 s after each injection was measured. Report points were recorded 5 s before and 30 s after each injection, and relative RU was determined. A solution of 10 mM hydrochloric acid and 1% SDS solution (1:1 v/v), pH 1.75, was applied to regenerate the chip surface. The calibration curve for TTX was constructed starting at 1,280 µg/L. TTX analogues were analyzed at the same concentrations as in mELISA, except analogues 5-deoxy-TTX and 11-deoxy-TTX and 4,9-anhydro-TTX which were tested starting at 100 µg/L and 10,000 µg/L, respectively. Matrix studies were carried out starting at 80 mg/mL of each puffer fish matrix.Spiking experiments were performed at 10 mg/mL of each puffer fish matrix and using an initial TTX concentration of 80 µg/L. Puffer fish 2 and 4 extracts were quantified starting at 10 mg/mL of matrix except muscle extract of puffer fish 2, which was started at 40 mg/mL. All samples, mAb and toxin standard solutions were prepared in HBS-EP buffer, run in triplicate, and diluted by half.

4.1.4 Results and discussion

4.1.4.1 mELISA and SPR immunosensorcalibration curves

The new ELISA configuration (mELISA) was first performed in checkerboard format to choose the concentrations of TTX, mAb and IgG-HRP and then in competitive format. The initial checkerboard was performed using TTX at 125, 25, and 5 ng/mL, mAb at 1:100, 1:200, and 1:400 dilutions, and IgG-HRP at 1:500, 1:1,000, and 1:2,000 dilutions, as well as the corresponding controls without mAb, which were used as an indication of the nonspecific adsorption from the IgG-HRP on the system. All the conditions tested showed an acceptable level of nonspecific adsorption. Three competitive immunoassays were then performed using different mAb/TTX ratios; however, competition between free and immobilized TTX for mAb binding was not achievedin any of the tested conditions. Nevertheless, a 50% reduction in mAb binding was observed due to 1,000 µg/L free TTX, when using the highest TTX concentration (125 ng/mL) for immobilization and the lowest amount of antibody (1:800 dilution) in the competition step. Generally, lower limits of detection (LODs) are attained at lower immobilized antigen and antibody concentrations. Therefore, our competitive assay could be improved by using a lower mAb concentration. However, due to the small amount of immobilized TTX, the use of such low mAb concentration would result in low absorbance values that, together with the higher nonspecific adsorption, would result in low signal-to-noise ratio and thus low reliability. Our results indicated that the mAb concentration had a more pronounced effect on the competition than the amount of immobilized TTX. Accordingly, the amount of TTX immobilized was increased while decreasing the concentration of mAb. Another checkerboard was then performed using 500, 250, and 125 ng/mL of TTX, 1:400, 1:800, 1:1,600, and 1:3,200 mAb dilutions, and 1:500, 1:1,000 and 1:2,000 IgG-HRP dilutions. As expected, higher responses were achieved when using the highest amounts of TTX, mAb and IgG-HRP. Regarding the secondary antibody, the 1:500 and 1:2,000 dilutions were discarded because of the high nonspecific adsorption and the low absorbance values, respectively. With respect to the TTX concentration, when using 250 and 125 ng/mL, absorbance values from the whole system decreased while nonspecific adsorption increased, regardless the mAb and IgG-HRP concentrations. Since lower mAb concentrations could provide lower EC_{50} values, different mAb dilutions were tested. Consequently, the conditions selected for the following competitive mELISAs were: TTX at 500 ng/mL, mAb at 1:800, 1:1,600, and 1:3,200 dilutions, and IgG-HRP at 1:1,000 dilution. Under these conditions, all three assays resulted in competition between free and immobilized TTX for mAb binding sites. The calibration curves were background-corrected with respect to the controls with no mAb and fitted to sigmoidal logistic four-parameter equations. The competition performed at the lowest mAb concentration showed a 50% effective coefficient (EC_{50}) of 11.55 µg/L, an LOD (EC_{20}) of 2.28

µg/L, and a working range (EC_{20}-EC_{80}) between 2.28 and 95.19 µg/L. As expected, and also observed in other studies(Herranz et al., 2010), lower EC_{50} values were obtained when using lower mAb concentrations (35.25 and 137.10 µg/L for 1:1,600 and 1:800 mAb dilutions, respectively). The lowest LOD provided by mELISA is in accordance with other reported competitive immunoassays (2 µg/L) (Kawatsu et al., 1997; Neagu et al., 2006; Raybould et al., 1992), or slightly better (5 µg/L(Tao et al., 2010) and 10 µg/L (Stokes et al., 2012)). The good sensitivity attained by mELISA can be attributed to the novel strategy used for the immobilization of TTX. The stable, ordered, and spaced dithiol-carboxylate self-assembled monolayer (SAM) favors the oriented antigen immobilization and the subsequent antigen-antibody affinity interaction and reduces or avoids nonspecific binding or matrix effects. Moreover, thanks to the similar affinity of maleimide and gold for thiol groups, this SAM-based strategy could be easily implemented in the development of immunosensors for TTX.

Compared to mELISA, the SPR immunosensor provided very similar EC_{50} and LOD values (10.18 and 4.27 µg/L, respectively), and a narrower working range (4.27-23.88 µg/L) because of the higher sensitivity. Calibration curves for TTX obtained by mELISA and SPR immunosensor are represented by black dots in **Figure 4.1.2** a,b, respectively.

4.1.4.2 Establishment of cross-reactivity factors for TTX analogues

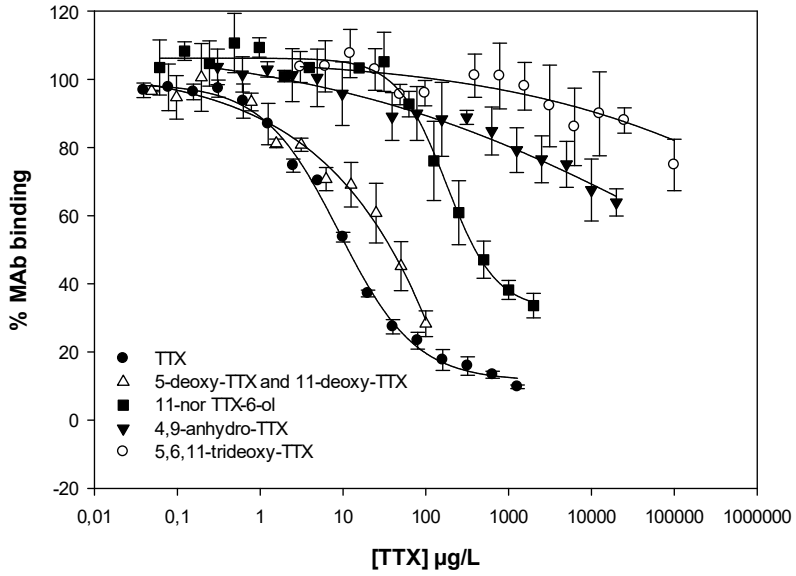
As previously mentioned, a crucial part of the current study was to assess the mAb affinity toward different TTX analogues in order to establish the corresponding CRFs. These CRFswould help tobetter understand the comparison between the quantifications provided by the immunochemical tools and those obtained by LC-MS/MS analysis in the determination of TTX equivalent contents in naturally-contaminated samples. Calibration curves for 5,6,11-trideoxy TTX, 5-deoxy-TTX and 11-deoxy-TTX, nor TTX-6-ol, and 4,9-anhydro-TTX were constructed by competitive mELISA (**Figure 4.1.2 a**) and SPR immunosensor (**Figure 4.1.2 b**) and fitted to sigmoidal logistic four-parameter equations. The CRFs of each analogue toward the mAb were calculated as the ratio of the EC_{50} value of TTX standard (µg/L) to the EC_{50} value of each analogue (µg/L) by both mELISA and SPR immunosensor, and expressed in percentage (**Table 4.1.1**). Those analogues that did not reach 50 % of binding in mELISA (for some analogues, higher concentrations could not be tested because of the lack of sufficient standards) were fitted to standard logistic four-parameter equations. The CRFs were determined as the ratio of the EC_{50} value of TTX standard to the EC_{50} value extrapolated from the corresponding analogue standard curve. Those analogues that did not reach 50 % of binding in SPR were fitted to polynomial linear

equations. However, as they did not reduce binding by any more than 10%, it was not possible to calculate the corresponding CRFs and they were deemed as being zero (i.e., very low or no recognition by the mAb).

The CRFs followed the same trend in both techniques, the analogues decreasing in cross-reactivity as follows: 5,11-deoxy, 11-nor TTX-6-ol, 5,6,11-trideoxy-4-anydroTTX and 5,6,11-trideoxy TTX. Importantly, the lowest cross-reactivity of 5,6,11-trideoxy TTX coincides with the lowest toxicity of this analogue on rat brain membranes observed by Yotsu-Yashamita and co-workers (Yotsu-Yamashita et al., 1999). This analogue together with 5,6,11-trideoxy-4-anydroTTX were clearly less reactive than 5,11-deoxy and 11-nor TTX-6-ol. Since their structures lack one of the two intramolecular epoxy groups present in TTX, 11-deoxy and 11-nor TTX-6-ol, one could hypothesize that the chemical structure affects the recognition by the antibody, and that the intramolecular epoxy group has a more pronounced effect on the cross-reactivity than other radicals.

In spite of the common trend, CRFs were quantitatively different depending on the immunological approach. The antibody showed less affinity for all of the TTX analogues compared to TTX, except for 5-deoxy-TTX and 11-deoxy-TTX in SPR, which bound more strongly to the antibody. It is also important to note that 5,6,11-trideoxy TTX and 5,6,11-trideoxy-4-anhydro TTX did not cross-react at all with the antibody as measured by SPR, but were detected at a low level by mELISA. In general, the different affinity of the antibody to the analogues could be attributed to the fact that while in mELISA end-point measurements are performed, SPR immunosensor is performed in a continuous-flow mode and measures the mAb biorecognition event in real-time. Apart from the different detection principle of the methods, the incubation time of the competition step is also different (3 min in SPR and 30 min in mELISA), and thus differences may appear depending on if an equilibrium has been reached or not. In addition, it is known that cross-reactivity decreases with incubation time and is minimal when equilibrium is reached (Karlsson and Wild).

(a)



(b)

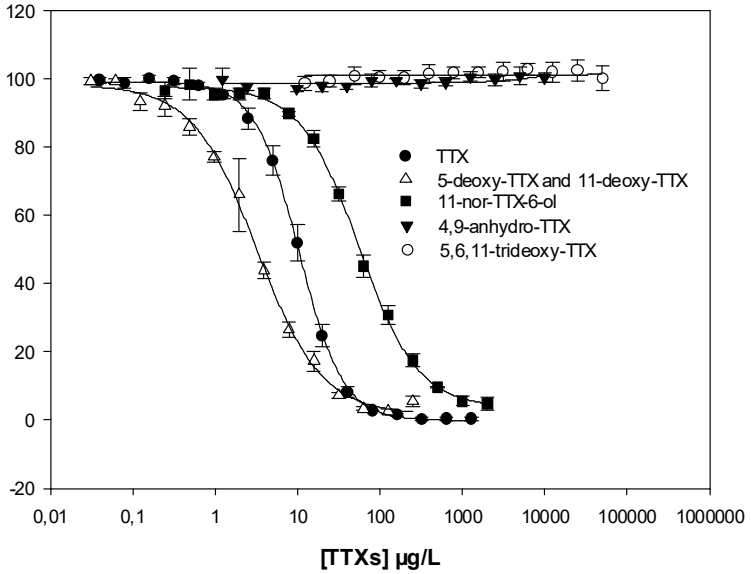


Figure 4.1.2. mAb binding percentage of TTX and analogues obtained by (a) mELISA and (b) SPR immunosensor. mAb binding percentage is expressed as the percentage of the control (without toxin); x values refer to initial toxin concentrations.

Table 4.1.1. CRFs (in percentage) for TTX analogues.

	TTX analogue			
Immunochemical approach	**5,6,11-trideoxy TTX**	**4,9-anhydro-TTX**	**11-nor TTX-6-ol**	**5-deoxy-TTX and 11-deoxy-TTX**
mELISA	$<10^{-4}$	$<10^{-2}$	2.9	29.9
SPR immunosensor	0	0	17.9	318.8

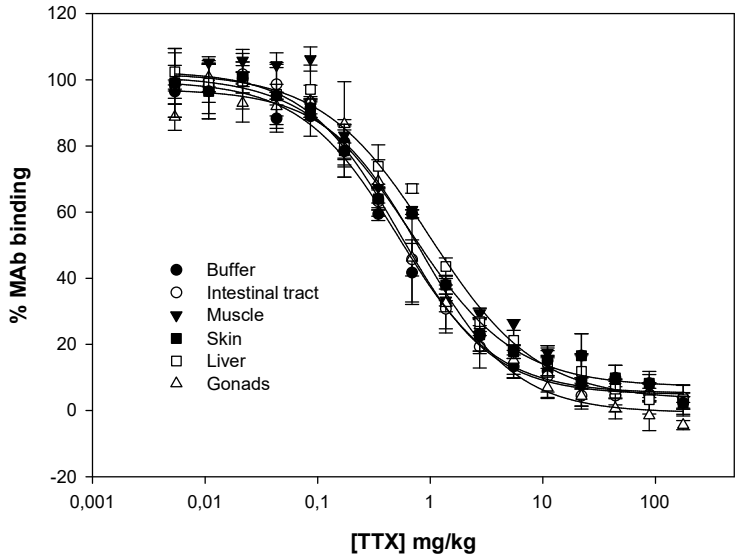
4.1.4.3 Puffer fish matrix effects in mELISA and SPRimmunosensor

In order to study the applicability of the two immunological tools to the analysis of natural samples, the effect of different puffer fish matrices was evaluated. The study of the matrix effects is crucial in the development of biochemical assays, where the functionality of biorecognition molecules (in this case, antibodies) may be strongly affected by the presence of matrix compounds. The matrix effects were quantified as the percentage of mAb binding inhibition when no free toxin is present in the configuration. For example, mAb binding is 100% with no matrix effect. In the case of them ELISA, the strongest matrix effects were observed when testing the highest matrix concentration (160 mg/mL): these were 46%, 17%, 44%, 22%, and 11% for intestinal tract, muscle, skin, liver, and gonads, respectively. Minor matrix effects (10-20%) were observed when analyzing intestinal tract, skin, and liver matrices at 80 mg/mL, and no significant effects (<10%) were observed at lower concentrations. On the other hand, a 10% matrix effect was observed for intestinal tract, muscle, and gonads matrices at 80 mg/mL in the SPR immunosensor. These were not observed at lower concentrations, and no effects were observed for skin and liver matrices. Taking into account that no matrix interferences were observed below 40 mg/mL in either mELISA or SPR immunosensor and, with the objective of using as little sample as possible, 10 mg/mL of matrix was chosen for subsequent spiking experiments and puffer fish analysis. At a matrix concentration of 10 mg/mL and using LODs established from the TTX calibration curves (2.28 and 4.27 µg TTX/L), the effective LOD for TTX in puffer fish matrix was determined to be 0.23 and 0.43 mg/kg, respectively, for mELISA and SPR. Both values are well below the Japanese regulation for food safety (2 mg/kg TTX equivalents).

4.1.4.4 Determination of TTX contents in spiked puffer fish samples

Intestinal tract, muscle, skin, liver, and gonads samples of puffer fish with no TTXs (as determined by LC-MS/MS analysis) were spiked with known concentrations of TTX to evaluate the ability of both immunochemical tools to determine TTX contents in natural samples. Therefore, these matrices were spiked with an initial concentration of TTX of 1,280 µg/L in mELISA and 80 µg/L in the SPR immunosensor. The TTX calibration curves in the presence of the different matrices were fitted to sigmoidal logistic four-parameter equations and compared to those obtained for TTX in buffer (**Figure 4.1.3**a,b).

(a)



(b)

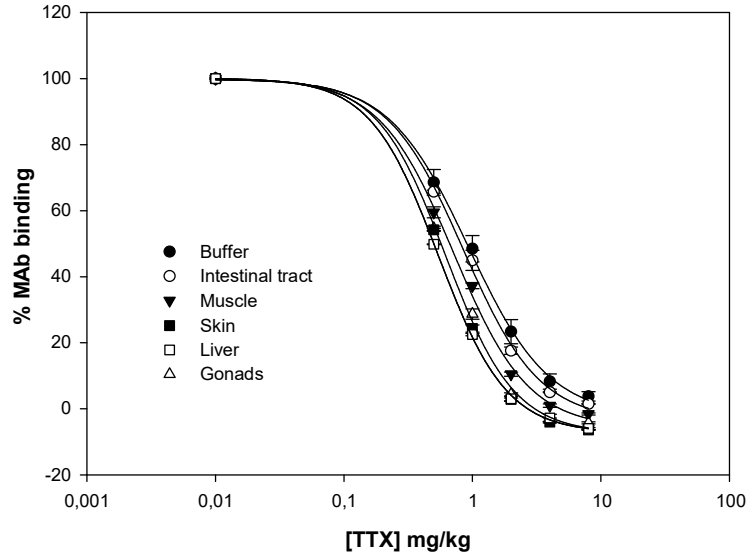


Fig. 4.1.3. mAb binding percentage of TTX in buffer and in liver, muscle, gonads, intestinal tract, and skin puffer fish matrices obtained by (a) mELISA and (b) SPR immunosensor. mAb binding percentage is expressed as the percentage of the control (without toxin); x values refer to initial toxin concentrations in 10 mg/mL matrix (mg of TTX/kg of matrix).

As can be observed in Figure 4.1.3a,b, the application of the immunochemical tools to the analysis of TTX-spiked puffer fish matrices slightly shifted the curves toward higher TTX contents in mELISAbut towards lower TTX contents in SPR immunosensor. As a consequence, correction factors (CFs) were established as the ratio of EC_{50} value ofTTX standard in each matrix to the EC_{50} valueof TTX standard in buffer (**Table 4.1.2**). Opposite trends were observed: in mELISA, TTX-spiked matrices provided higher EC_{50} values than the calibration curves in buffer (CFs >1) and the SPR immunosensor resulted in lower EC_{50} values (CFs <1). It is interesting to note that liver

presented the most pronounced shifting effect in both approaches, despite the additional liquid-liquid partition to remove fats in those extracts. Otherwise, the intestinal tract resulted in TTX curves quite similar to those constructed when using only buffer, also in both cases. From a food safety point of view, muscle,which presents an intermediate effect, would be the matrix of more interest, although consumption of liver and gonads by mistake or negligence cannot be discarded (Arakawa et al., 2010; Bentur et al., 2008). Nevertheless, the established CFs contribute to provide reliable TTX quantifications in all tissues.

Table 4.1.2. CFs determined for different puffer fish matrices.

	Puffer fish matrix				
Immunochemical approach	**Intestinal tract**	**Muscle**	**Skin**	**Liver**	**Gonads**
mELISA	1.06	1.59	1.39	1.92	1.34
SPR immunosensor	0.90	0.73	0.61	0.53	0.63

4.1.4.5 Determination of TTX equivalent contents in naturally-contaminated puffer fish samples

After the establishment of CRFs and CFs by both techniques, two pufferfish samples (referred to as puffer fish 2 and 4) were analyzed by mELISA, SPR immunosensor, and LC-MS/MS. For the determination of TTX equivalent contents (mg of TTX equiv./kg matrix), the EC_{50} value of each matrix sample analyzed and the EC_{50} value of the TTX calibration curve were taken into account, if possible. In those samples that did not attain the sigmoidal shapenor 50% binding values, TTX equivalent contents were determined using the lowest % mAb binding and interpolating to the corresponding percentage of the TTX calibration curve. **Table 4.1.3** shows the TTX equivalent contents provided by mELISA and SPR, without and with matrix correction (multiplication of the noncorrected value by the corresponding CF), the LC-MS/MS quantification of the different TTXs and the total sum, the values resulting after application of the CRFs to the individual LC-MS/MS quantifications (multiplication of the LC-MS/MS values by the corresponding CRFs), and the contents provided by MBA (Katikou et al., 2009).

As observed in other works, female puffer fish (number 4) was more toxic than male puffer fish (number 2), probably because females accumulate TTXs in the ovaries and eggs during the spawning period (Noguchi et al., 2011). Regarding the distribution of TTXs in tissues, intestinal tract was the most toxic tissue and muscle was markedly less toxic. In fact, the presence of TTXs

mainly in liver, ovaries, intestine and skin has been previously reported (Jang and Yotsu-Yamashita, 2006; Noguchi and Arakawa, 2008). Nevertheless, the number of samples of this study is too limited to be representative and draw conclusions about the TTX occurrence by fish gender or the distribution of TTXs in different tissues.

In the analysis of puffer fish 4 by mELISA and SPR, non-corrected TTX equivalent contents did not correlate (y = 1.28x + 8.92;R^2 = 0.613). By contrast, the correction of the quantification by applying the CFs, significantly improved the correlation, providing with very similar data (y = 1.26x + 1.39; R^2 = 0.977), SPR values being only slightly higher. The trend from higher to lower TTX equivalent contents was: intestinal tract > liver > gonads > skin > muscle. TTX, 5,6,11-trideoxy TTX and 11-nor TTX-6-ol were detected by LC-MS/MS in all samples except muscle (LOQ = 1.00mg/kg). In the comparison between the values provided by mELISA (CF-corrected) with the values obtained by LC-MS/MS after applying the CRFs obtained by mELISA, data highly correlate (y =1.14x + 0.42; R^2 = 0.991). Similarly, comparing SPR (CF-corrected) with the quantification provided by LC-MS/MS after applying the CRFs obtained by SPR, appropriate correlation values were obtained (y = 1.19x + 2.61; R^2 = 0.979). Comparing the quantifications provided by MBA with mELISA and SPR, those obtained by MBA are always higher (by a factor of 2.27 and 1.78, respectively), thus, worse correlations were obtained. Nevertheless, the correlation of MBA with LC-MS/MS is better than with mELISA and SPR (y =1.00x– 3.70; R^2 = 0.832). It is important to note that in the manner of the CRFs in the immunological approaches, toxicity factors for the TTX analogues should be established in order to better compare MBA and LC-MS/MS.

In the quantification of TTX contents in puffer fish 2, although correlations between mELISA and SPR were not as good as those in fish 4, this can be explained by the very low concentrations of TTX present in this sample. It is important to mention that when analyzing the muscle at 10 mg/mL by mELISA and SPR, no TTX contents were detected. Nevertheless, as higher concentrations of muscle samples were available and since this matrix showed little effects, this puffer fish sample was analyzed by mELISA and SPR immunosensor starting at 80 and 40 mg/mL, corresponding to LODs of 0.03 and 0.11 mg/kg, respectively. The use of higher matrix concentrations allowed the detection of such low TTX contents. In this case, the trend from higher to lower TTX contents was: skin > liver ≈ intestinal tract > muscle by mELISA and skin > intestinal tract > liver > muscle by SPR. Although mELISA and SPR did not provide comparable values and those trends could be nonsignificant, what is important in the analysis of puffer fish

2 is that the immunochemical approaches allowed determination of trace TTX contents, thus being useful for research purposes.

In general, both immunochemical approaches have been proved to be useful as screening tools and also for research purposes, mELISA providing a slightly lower LOD and slightly better correlations with LC-MS/MS analysis. Although both techniques providevery similar data, they present advantages and disadvantages that may be taken into consideration when using one or another. While mELISA is cheaper, allows the analysis of more samples per assay, and does not require sophisticated equipment, SPR is label free, uses lower sample volumes, chips are very stable and reusable and, moreover, the analysis is automated and at real time.

Table 4.1.3. TTX equivalent contents (mg TTX equiv./kg matrix) in puffer fish 4 (a) and 2 (b) by mELISA, SPR immunosensor, LC-MS/MS, and MBA.

(a) Puffer fish 4

Puffer fish matrix	mELISA	mELISA (CF-corrected)	SPR	SPR (CF-corrected)	LC-MS/MS						MBA
					TTX	5,6,11-trideoxy TT	11-nor TTX-6-o	Σ	Σ (mELISA CRF-corrected	Σ (SPR CRF-corrected	
Intestinal tract	23.03	24.40	34.77	31.13	20.60	11.09	22.73	54.42	21.26	24.66	56.78
Muscle	0.68	1.08	1.51	1.11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.52
Skin	1.80	2.50	5.32	3.24	1.80	3.43	2.51	7.71	1.85	2.23	2.42
Liver	5.88	11.31	31.28	16.70	8.07	11.62	13.06	32.75	8.45	10.40	16.12
Gonads	3.84	5.13	16.74	10.52	4.81	2.37	5.16	12.34	4.96	5.73	17.05

(b) Puffer fish 2

Puffer fish matrix	mELISA	mELISA (CF-corrected)	SPR	SPR (CF-corrected)	LC-MS/MS				MBA
					TTX	5,6,11-trideoxy TTX	11-nor TTX-6-ol	Σ	
Intestinal tract	0.51	0.54	0.43	0.38	n.d	n.d.	n.d.	n.d.	n.d.
Muscle	0.14	0.22	0.28	0.21	n.d.	n.d.	n.d.	n.d.	n.d.
Skin	0.42	0.59	0.88	0.54	n.d.	n.d.	n.d.	n.d.	n.d.
Liver	0.28	0.55	0.43	0.23	n.d.	n.d.	n.d.	n.d.	n.d.

4.1.5 Conclusions

The purpose of the work was to develop a new ELISA (with the corresponding study of the matrix effects and the establishment of CFs) and to prove that it is useful as a TTX screening method in the analysis of puffer fish (by comparison with LC-MS/MS, which requires the establishment of CRFs). The application of the CFs to the quantification of TTXs in puffer fish by mELISA and SPR immunosensor, together with the comparison of these results with those provided by LC-MS/MS analysis and the corresponding application of the established CRFs, allowed for a reliable determination of TTXs contents in puffer fish samples. The mELISA immunoassay, a new ELISA configuration using SAMs,has been demonstrated for the first time, as well as the utility of the application of CFs and CRFs in the quantification of TTXs in natural samples for a better comparison between data generated across a range of different methodologies. Whereas the immunochemical tools highly correlated with LC-MS/MS analysis, MBA slightly overestimated the TTX equivalent contents compared to the immunological approaches.

This study has provided evidence that, when analyzing naturally contaminated samples, complementarity between techniques providing different levels of information is very useful, especially for toxins which lack an established official method and/or regulatory legislation. The immunological tools developed in this work are suitable for screening purposes, being able to analyze a number of samples in a short time and meet the requirements in terms of sensitivity of the Japanese regulation levels. Moreover, they have been shown to be capable of detecting amounts of TTXs as low as 0.23 mg/kg, which makes them highly appropriate for research studies. It is believed that, in the future, CRFs should also be established for other described TTX analogues in order to obtain accurate, risk-based quantification in samples with different multitoxin profiles. Additionally, the evaluation of the toxicity of TTX analogues and the comparison of the toxicity degrees with the CRFs will contribute to confidently assess food safety in order to protect human health.

4.1.6 References

Arakawa, O., Hwang, D.F., Taniyama, S., Takatani, T., 2010. Toxins of pufferfish that cause human intoxications, in: Ishimatsu, A., Lie, H.-J. (Eds.), Coastal Environmental and Ecosystem Issues of the East China Sea. TERRAPUB/Nagasaki University, Tokyo, Japan, pp. 227-244.
Asakawa, M., Miyazawa, K., Shida, Y., Noguchi, T., 2012. Instrumental Analysis of Tetrodotoxin; Intech: Rijeka, Croatia.

Bane, V., Lehane, M., Dikshit, M., O'Riordan, A., Furey, A., 2014. Tetrodotoxin: Chemistry, Toxicity, Source, Distribution and Detection. Toxins 6, 693-755.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z.S., Litmanovich, M., Golik, M., Gurevych, B., Golani, D., Eisenman, A., 2008. Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. Toxicon 52, 964-968.

Campbell, K., Barnes, P., Haughey, S.A., Higgins, C., Kawatsu, K., Vasconcelos, V., Elliott, C.T., 2013. Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. Anal. Bioanal. Chem. 405, 7753-7763.

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

French, R.J., Yoshikami, D., Sheets, M.F., Olivera, B.M., 2010. The Tetrodotoxin Receptor of Voltage-Gated Sodium Channels-Perspectives from Interactions with mu-Conotoxins. Mar. Drugs 8, 2153-2161.

Goto, T., Kishi, Y., Takahashi, S., Hirata, Y., 1965. Tetrodotoxin. Tetrahedron 21, 2059-2088.

Herranz, S., Bockova, M., Marazuela, M.D., Homola, J., Moreno-Bondi, M.C., 2010. An SPR biosensor for the detection of microcystins in drinking water. Anal. Bioanal. Chem. 398, 2625-2634.

Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish Fugu pardalis. Toxicon 48, 980-987.

Karlsson, R., Wild, D., In: The Immunoassay Handbook. Fourth ed.; Elsevier: Oxford, 2005; 209–221.

Katikou, P., Georgantelis, D., Sinouris, N., Petsi, A., Fotaras, T., 2009. First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). Toxicon 54, 50-55.

Kawabata, T., 1978. The manual for the methods of food sanitation tests. Bureau of Environmental Health. Ministry of Health and Welfare; Japan Food Hygienic Association: Tokyo, Japan, Vol. II, 232.

Kawatsu, K., Hamano, Y., Yoda, T., Terano, Y., Shibata, T., 1997. Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. Jpn. J. Med. Sci. Biol. 50, 133-150.

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

Lee, C.H., Ruben, P.C., 2008. Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. Channels 2, 407-412.

Neagu, D., Micheli, L., Palleschi, G., 2006. Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. Anal. Bioanal. Chem. 385, 1068-1074.

Noguchi, T., Arakawa, O., 2008. Tetrodotoxin - Distribution and accumulation in aquatic organisms, and cases of human intoxication. Mar. Drugs 6, 220-242.

Noguchi, T., Arakawa, O., Takatani, T., 2006. TTX accumulation in pufferfish. Comp. Biochem. Physiol., Part D: Genomics Proteomics 1, 145-152.

Noguchi, T., Onuki, K., Arakawa, O., 2011. Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism. ISRN toxicol., 276939.

Pratheepa, V., Vasconcelos, V., 2013. Microbial diversity associated with tetrodotoxin production in marine organisms. Environ. Toxicol. Pharmacol. 36, 1046-1054.

Raybould, T.J.G., Bignami, G.S., Inouye, L.K., Simpson, S.B., Byrnes, J.B., Grothaus, P.G., Vann, D.C., 1992. A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. J. Clin. Lab. Anal. 6, 65-72.

Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., Botana, L.M., 2008. First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell Charonia lampas lampas in Europe. Anal. Chem. 80, 5622-5629.

Stokes, A.N., Williams, B.L., French, S.S., 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. Biol. Proced. Online 14, 3.

Tahara, Y., Hirata, Y., 1909. Studies on puffer fish toxin. *J. Pharm. Soc. Jpn.* 29, 587-625.

Tao, J., Wei, W.J., Nan, L., Lei, L.H., Hui, H.C., Fen, G.X., Jun, L.Y., Jing, Z., Rong, J., 2010. Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China. Food Addit. Contam., Part A. 27, 1589-1597.

Taylor, A.D., Ladd, J., Etheridge, S., Deeds, J., Hall, S., Jiang, S.Y., 2008. Quantitative detection of tetrodotoxin (TTX) by a surface plasmon resonance (SPR) sensor. Sens. Actuators, B. 130, 120-128.

Taylor, A.D., Vaisocherova, H., Deeds, J., DeGrasse, S., Jiang, S.Y., 2011. Tetrodotoxin Detection by a Surface Plasmon Resonance Sensor in Pufferfish Matrices and Urine. J. Sens., 10, 601704.

Tsuda, K., Tachikawa, R., Tamura, C., Ikuma, S., Kawamura, M., Sakai, K., Amakasu, O., 1964. On structure of tetrodotoxin. Chem. Pharm. Bull. 12, 642-644.

Turner, A.D., Powell, A., Schofield, A., Lees, D.N., Baker-Austin, C., 2015. Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance 20, 21009.

Vaisocherova, H., Taylor, A.D., Jiang, S.Y., Hegnerova, K., Vala, M., Homola, J., Yakes, B.J., Deeds, J., DeGrasse, S., 2011. Surface Plasmon Resonance Biosensor for Determination of Tetrodotoxin: Prevalidation Study. J. AOAC Int. 94, 596-604.

Vlamis, A., Katikou, P., Rodriguez, I., Rey, V., Alfonso, A., Papazachariou, A., Zacharaki, T., Botana, A., Botana, L., 2015. First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate Prorocentrum minimum. Toxins 7, 1779-1807.

Wang, R.Z., Huang, A.L., Liu, L.C., Xiang, S.S., Li, X.F., Ling, S.M., Wang, L., Lu, T., Wang, S.H., 2014. Construction of a single chain variable fragment antibody (scFv) against tetrodotoxin (TTX) and its interaction with TTX. Toxicon 83, 22-34.

Woodward, R.B., Gougoutas, J.Z., 1964. Structure of tetrodotoxin. J. Am. Chem. Soc. 86, 5030-5030.

Yakes, B.J., Deeds, J., White, K., DeGrasse, S.L., 2011. Evaluation of Surface Plasmon Resonance Biosensors for Detection of Tetrodotoxin in Food Matrices and Comparison to Analytical Methods. J. Agric. Food Chem. 59, 839-846.

Yakes, B.J., Etheridge, S.M., Mulvaney, S.P., Tamanaha, C.R., 2010. Fluidic Force Discrimination Assays: A New Technology for Tetrodotoxin Detection. Mar. Drugs 8, 565-576.

Yakes, B.J., Kanyuck, K.M., DeGrasse, S.L., 2014. First Report of a Direct Surface Flasmon Resonance Immunosensor for a Small Molecule Seafood Toxin. Anal. Chem. 86, 9251-9255.

Yotsu-Yamashita, M., Sugimoto, A., Takai, A., Yasumoto, T., 1999. Effects of specific modifications of several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. J. Pharmacol. Exp. Ther. 289, 1688-1696.
Zhou, Y., Li, Y.S., Pan, F.G., Liu, Z.S., Wang, Z., 2009. Identification of tetrodotoxin antigens and a monoclonal antibody. Food Chem. 112, 582-586.

4.2 Development and validation of a maleimide-based enzyme-linked immunosorbent assay for the detection of tetrodotoxins in oysters and mussels

Laia Reverté, Maria Rambla-Alegre, Sandra Leonardo, Carlos Bellés, Katrina Campbell, Christopher T. Elliott, Arjen Gerssen, Mirjam D. Klijnstra, Jorge Diogène and Mònica Campàs*

Submitted to Talanta in 29th May 2017

4.2.1 Abstract

The recent detection of tetrodotoxins (TTXs) in puffer fish and shellfish in Europe highlights the necessity to monitor the levels of TTXs in seafood by rapid, specific, sensitive and reliable methods in order to protect human consumers. A previous immunoassay for TTX detection in puffer fish, based on the use of self-assembled monolayers (SAMs) for the immobilization of TTX on maleimide plates (mELISA), has been modified and adapted to the analysis of oyster and mussel samples. Changing dithiol for cysteamine-based SAMs enabled reductions in the assay time and cost, while maintaining the sensitivity of the assay. The mELISA showed high selectivity for TTX since the antibody did not cross-react with co-occurring paralytic shellfish poisoning (PSP) toxins and no interferences were observed from arginine (Arg). Moreover, TTX-coated maleimide plates stored for 3 months at -20 °C and 4 °C were stable, thus when pre-prepared, the time to perform the assay is reduced. When analyzing shellfish samples, matrix effects and toxin recovery values strongly depended on the shellfish type and the sample treatment. Blank oyster extracts could be directly analyzed without solid-phase extraction (SPE) clean-up, whereas blank mussel extracts showed strong matrix effects and SPE and subsequent solvent evaporation were required for removal. However, the SPE clean-up and evaporation resulted in toxin loss. Toxin recovery values were taken as correction factors (CFs) and were applied to the quantification of TTX contents in the analysis of naturally-contaminated shellfish samples by mELISA. The lowest effective limits of detection (eLODs) were about 20 and 50 µg/kg for oyster extracts without and with SPE clean-up, respectively, and about 30 µg/kg for mussel extracts with both protocols, all of them substantially below the eLOD attained in the previous mELISA for puffer fish (230 µg/kg). Analysis of naturally-contaminated samples by mELISA and comparison with LC-MS/MS quantifications demonstrated the viability of the approach. This mELISA is a selective and sensitive tool for the rapid detection of

TTXs in oyster and mussel samples showing promise to be implemented in routine monitoring programs to protect human health.

4.2.2 Introduction

Tetrodotoxin (TTX) is a potent low-molecular-weight (319 Da) marine neurotoxin, whose name derives from the family of fish *Tetraodontidae*. Tetrodotoxin possesses a unique structure, consisting of a positively charged guanidine group connected to a highly oxygenated carbon backbone (Tsuda et al. 1964, Woodward et al. 1964 and Goto et al. 1965). Although TTX was originally found in the ovaries of puffer fish (Tahara et al. 1909), several marine organisms have been shown to contain the toxin such as blue-ring octopus, ribbon worms, starfish and xanthid crabs (Noguchi et al. 2006), as well as terrestrial animals such as frogs and newts (Bane et al. 2014).

Unlike many other marine toxins, which are of microalgal origin, TTX production is thought to be produced by bacteria of the species *Pseudomonas, Shewanella, Alteromonas* or *Vibrio* (Pratheepa et al. 2013), in symbiosis with certain animals (Noguchi et al. 2008). Recently, the marine dinoflagellate *Prorocentrum minimum* has been described to produce TTXs in cultures, with possible implication of endosymbiotic bacteria (Rodriguez et al. 2017). Tetrodotoxins have the ability to selectively bind to voltage-gated sodium channels (VGSCs), blocking the influx of sodium ions into the nerve cells, affecting neuromuscular transmission (Narahashi et al. 2008). The consumption of puffer fish contaminated with TTX may result in mild gastrointestinal effects, numbness, respiratory failure, and even in death (Noguchi et al. 2011). Human intoxications have been reported worldwide, mainly caused by the ingestion of contaminated puffer fish, served in Japan as a delicacy known as “fugu” (Noguchi et al. 2008 and Noguchi et al. 2011).

A toxic species of puffer fish, *Lagocephalus sceleratus*, recently reached the Mediterranean through the Suez channel (Bentur et al. 2008), resulting in new reports of food poisoning in the Western Mediterranean and further migration towards eastern waters (Kheifets et al. 2012, Acar et al. 2017 and Katikou et al. 2009). In Europe, the first toxicity episode related with TTX-contaminated shellfish occurred in Spain in 2007 and it was caused by the ingestion of contaminated trumpet shells, although the shellfish was bought in Portugal (Rodriguez et al. 2008). Since then, TTXs have been detected in bivalve shellfish in different parts of Europe, including England Turner et al. 2015), Greece (Vlamis et al. 2015) and the Netherlands (EFSA 2017). In humans, according to case studies, between 0.18 and 0.2 mg of TTX have been reported to cause severe symptoms, and a fatality was

reported after an ingestion of around 2 mg of TTX (Noguchi et al. 2001). Despite the fact that TTX is a toxin with a high fatality rate and whose distribution is spreading worldwide, neither a reference method nor regulatory limits have been specifically set for TTX. Nevertheless, in Japan a value of 2 mg TTX equiv./kg edible portion has been used as the acceptance criterion to consider puffer fish safe for consumption (Mahmud et al. 1999). Moreover, in Europe, the Regulation (EC) no. 854/2004 stipulates that "fishery products derived from poisonous fish of the following families must not be placed on the market: Tetraodontidae, Molidae, Diodontidae and Canthigasteridae". Concern for TTX in Europe has been increasing, and just recently, the European Food Safety Authority has concluded that a concentration below 44 µg TTX equiv./kg shellfish meat, based on a large portion size of 400 g, is considered not to result in adverse effects in humans (EFSA 2017).

Given the occurrence of TTXs in European shellfish and the threat that this hazardous toxin poses to human health, the development of rapid, specific, sensitive, reliable and easy-to-use methods for their detection is a matter of utmost importance. Accordingly, several methods have been reported for the detection of TTX, including immunoassays [Neagu et al. 2006, Raybould et al. 1992, Kawatsu et al. 1997, Reverté et al. 2015, Stokes et al. 2012, Wang et al. 2014 and Zhou et al. 2009) and immunosensors (Neagu et al. 2006, Kreuzer et al. 2002, Campbell et al. 2013, Taylor et al. 2008, Taylor et al. 2011, Yakes et al. 2011 and Yakes et al. 2014). However, most of them have not been applied to the analysis of natural samples or have only been used to analyze puffer fish, but not shellfish. Taking as a starting point an immunoassay previously developed for the determination of TTXs in puffer fish samples (Reverté et al. 2015), the aim of this research was to illustrate the development of an improved bioanalytical tool for the analysis of oyster and mussel samples (**Fig. 4.2.1**).

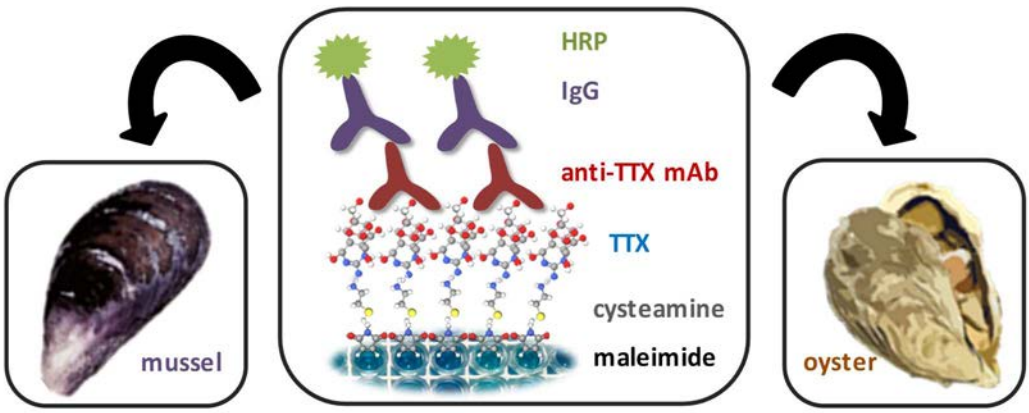


Fig. 4.2.1 Scheme of the mELISA strategy developed for the detection of TTXs in oyster and mussels

4.2.3 Materials and methods

4.2.3.1 Reagents and solutions

For mELISA, TTX standard was purchased from Tocris Bioscience (Bristol, UK) and standard solutions were prepared at 1 mg/mL in 10 mM acetic acid (AA). For LC-MS/MS analysis, TTX standard was purchased from Latoxan (Valence, France) and standard solutions were prepared at 1 mg/mL in 350 mM AA. Certified reference materials (CRMs), specifically gonyautoxin 1&4 (GTX1&4), gonyautoxin 2&3 (GTX2&3), decarbamoyl gonyautoxin 2&3 (dcGTX2&3), gonyautoxin 5 (GTX5), neosaxitoxin (NEO), decarbamoyl neosaxitoxin (dcNEO), saxitoxin (STX), decarbamoyl saxitoxin (dcSTX) and N-sulfocarbamoyl gonyautoxin 2&3 (C1&2), were obtained from the National Research Council of Canada (NRC, Halifax, NS, Canada). The anti-TTX monoclonal antibody (mAb) TX-7F was produced as described in the literature (Kawatsu et al. 1997). Pierce maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain). Ammonium hydroxide solution (NH_4OH, 25%), amorphous graphitized polymer carbon Supelco ENVI-Carb 250 mg/3 mL cartridges, anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), L-arginine (Arg), bovine serum albumin (BSA), cysteamine hydrochloride, ethylenediaminetetraacetic acid (EDTA), formaldehyde solution, 4-morpholineethanesulfonic acid (MES) hydrate, potassium phosphate dibasic, potassium phosphate monobasic and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were all supplied by Sigma-Aldrich (Tres Cantos, Spain). HPLC-grade acetonitrile (ACN), glacial AA and methanol (MeOH) were obtained from Chem-lab (Zedelgem, Belgium). Formic acid (FA) (98-100%) was purchased from Merck (Darmstadt, Germany). Ultra LC-MS ACN, Ultra LC-MS MeOH and Ultra LC-MS H_2O were purchased from Actu-All (Oss, The Netherlands). Ultrapure Milli-Q water (18.2 MΩ/cm^2) was used for the preparation of solutions (Millipore, Bedford, MA, USA).

4.2.3.2 Instrumentation

For toxin extraction, a water bath (model 6000138 600 W) purchased from J. P. Selecta S. A. (Barcelona, Spain), an Alegra X-15R centrifuge provided by Beckman Coulter (Barcelona, Spain) and a DVX-2500 multi-tube vortex mixer acquired at VWR International Eurolab S. L. (Barcelona, Spain) were used.

Extraction clean-up was performed with a Rapid Trace SPE workstation supplied by Caliper Life Sciences (Waltham, MA, USA).

Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. with GEN 2.09 software (Winooski, VT, USA).

For LC-MS/MS analysis, the separation was performed on a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) and mass spectrometric analysis on a Waters Xevo TQ-S (Waters, Milford, MA, USA).

4.2.3.3 Shellfish samples

For the evaluation of matrix effects and toxin recovery, Pacific oyster (*Crassostrea gigas*) and mussel (*Mytilus galloprovincialis)* samples from the Ebro Delta (Alfacs Bay, NW Mediterranean Sea) were used. These shellfish samples were determined as non-containing TTXs by LC-MS/MS analysis. For the analysis of naturally-contaminated shellfish, three oyster (*Crassostrea gigas*) and three mussel (*Mytilus edulis*) samples were obtained from production sites at the Oosterschelde in The Netherlands.

4.2.3.4 Improved mELISA protocol

The protocol was similar to that previously described by our group (Reverté et al. 2015), with some modifications regarding the TTX immobilization. The first step was the self-assembling of 1 mM cysteamine in phosphate buffer for 3h, followed by the direct immobilization of TTX (2 µg/mL) with formaldehyde (3.4 %) in the same buffer overnight. A competitive assay was then performed by incubating 50 µL of free TTX/shellfish extract and 50 µL of 1:3,200 anti-TTX mAb dilution in 1% BSA-phosphate buffer for 30 min. A blocking step was then performed with 200 µL of 1% BSA-phosphate buffer for 30 min and, finally, 100 µL of IgG-HRP at 1:1,000 dilution in 1% BSA-phosphate buffer was incubated for 30 min. Colorimetric response was measured at 620 nm after 10 min of TMB liquid substrate incubation.

4.2.3.5 Storage stability of TTX-coated maleimide plates

Tetrodotoxin was immobilized through cysteamine self-assembled on maleimide-activated plates as described in the section above, and TTX-coated maleimide plates were kept at 4 °C and -20 °C. Absorbance values of wells with mAb (maximum response) and without mAb (background) in the absence of free TTX were measured in triplicate at day 0 (reference value) and during several weeks up to 3 months.

4.2.3.6 Interference study

The selectivity of the mELISA was assessed by the analysis of the following Paralytic Shellfish Poisoning (PSP) toxins: GTX1&4, GTX2&3, dcGTX2&3, GTX5, NEO, dcNEO, STX, dcSTX and C1&2, as well as L-arginine (Arg). A concentration of 100 ng/mL was chosen taking into account that TTX almost completely inhibits the mAb binding. The protocol was the same as that explained in the improved mELISA protocol section, but replacing free TTX by PSP toxins or Arg prepared in 1% BSA-phosphate buffer in the competition step. Percentage of mAb binding was calculated with respect to the response obtained without toxin (maximum response).

4.2.3.7 Toxin extraction and SPE clean-up

For the analysis of toxins in shellfish by mELISA, extracts were obtained following the single dispersive procedure described in the literature (Boundy et al 2015) for other paralytic shellfish toxins, adapted to the amount of sample available. In brief: 1 g ± 0.1 g of shucked shellfish homogenate was weighed into a 50-mL tube and 1 mL of AA/H_2O (1:100, v:v) was added. After shaking the tube for 90 s on a multi-tube vortex mixer, samples were boiled in a water bath for 5 min at 100 °C. Tubes were then cooled and shaken again with the multi-vortex mixer for 90 s. Finally, samples were centrifuged at 4,500 rpm for 10 min, and the supernatants were filtered through 0.2-µm nylon filters and kept at -20 °C until analysis. The resulting extracts contained fresh shellfish matrix at a concentration of 1,000 mg equiv./mL.

For the clean-up of shellfish sample extracts, SPE was performed using graphitized polymer carbon ENVI-carb cartridges by adapting the automated protocol described in the literatura (Boundy et al. 2015). Briefly, a 0.5-mL aliquot of the AA extract was transferred to a polypropylene tube and 1.25 µL of NH_4OH solution was added. The cartridges were conditioned with 3 mL of ACN/H_2O/AA (20:80:1, v:v:v), followed by 3 mL of H_2O/NH_4OH (1000:1, v:v). Then, 400 µL of sample extracts were

loaded onto the conditioned cartridges and were washed with 700 µL of deionized H_2O. Finally, the retained TTXs were eluted with 2 mL of ACN/H_2O/AA (20:80:1, v:v:v) and stored at -20 °C until analysis. Resulting extracts were at a shellfish matrix concentration of 200 mg equiv./mL. Further dilutions for mELISA experiments were performed in phosphate buffer. When required, shellfish sample extracts were evaporated for solvent exchange (from ACN/H_2O/AA to phosphate buffer).
Blank, TTX-spiked and naturally-contaminated shellfish sample extracts were analyzed by mELISA at 3 different stages: (1) after SPE clean-up, (2) after SPE clean-up, evaporation and solvent exchange, and (3) directly after toxin extraction (without SPE clean-up nor evaporation and solvent exchange). The matrix effects of blank samples were evaluated and the TTX contents obtained in spiked and naturally-contaminated samples at each stage were determined.

4.2.3.8 LC-MS/MS analysis

For the analysis by LC-MS/MS, naturally-contaminated shellfish sample extracts were obtained as follows: 1 g of shellfish homogenate was accurately weighed, and 2 mL of H_2O/MeOH (50:50, v:v) containing 15 mM AA solution was added. First, TTXs were extracted by a 15-min head-over-head extraction. The sample was then centrifuged for 10 min at 5,200 g and the supernatant was transferred to a volumetric tube. A second extraction was performed by adding 1.5 mL of extraction solvent and vortex mixing during 1 min. After centrifugation, the total volume was brought to 4 mL with the same extraction solvent. The extract was diluted 1:9 with ACN/H_2O (70:20, v:v) containing 6.7 mM AA. Subsequently, the diluted extract was centrifuged at 16,200 g during 5 min and the supernatant was transferred to a vial. For the construction of calibration curves, matrix-matched standards were prepared by spiking blank shellfish material with known concentrations of TTX, respectively 0, 20, 50, 75, 150 µg/kg.

Chromatographic separation was achieved using a UPLC system. The system consisted of a binary solvent manager, a sample manager and a column manager. The column temperature was at room temperature and the temperature of the sample manager was kept at 10 °C. For the analysis of TTX, a 10-µL injection volume was used. Mobile phase A was H_2O and B was ACN, both containing 50 mM FA. The analytical column used was a Tosoh Bioscience TSKgel Amide-80 column (250x2 mm, 5-µm particles). A flow rate of 0.2 mL/min was used. A gradient started at 30% A and after 1 min it was linearly increased to 95% A in 7.5 min. This composition was kept for 5 min and returned to 30% A in 0.5 min. An equilibration time of 6 min was allowed prior to the next injection. The effluent was directly interfaced in the electrospray ionization (ESI) source of the triple quadrupole mass

spectrometer. The mass spectrometer operated in ESI positive ionization mode and two transitions for TTX were measured, *m/z* 320.1 > 162.1 and *m/z* 320.1 > 302.1.

4.2.3.9 Data analyses and statistics

Measurements were performed in triplicate for the mELISA experiments and singular in LC-MS/MS analysis. In the mELISA, calibration curves were background-corrected with respect to the controls with no mAb and adjusted to sigmoidal logistic four-parameter equations using SigmaPlot software 12.0 (Systat Software Inc., San José, CA, USA). From the equations, inhibitory concentrations (ICs) were calculated. Specifically, the midpoint (IC_{50}), the limit of detection (LOD) established as the IC_{20}, and the working range (IC_{20}-IC_{80}), were determined. In this work, the limit of quantification (LOQ) has been considered equal to the LOD.

To evaluate differences in the quantifications provided by the three approaches (mELISA with SPE clean-up/evaporated samples, mELISA with no SPE clean-up/not evaporated samples and LC-MS/MS analysis), a one-way analysis of variance was conducted using SigmaStat 3.1 software (Systat Software Inc., San José, CA, USA). Prior to the analysis, a normality and equal variance test was performed. Differences in the results were considered statistically significant at the 0.05 level.

4.2.4 Results and discussion

4.2.4.1 Improvement of the mELISA protocol

Rapidity, low-cost and simplicity are key parameters for the success of immunoassays. To this purpose, the mELISA described previously elsewhere (Reverté et al. 2015) was improved by using reagents readily available, reducing the protocol time and cost. The improvement was achieved by replacing the carboxylate-dithiol used for microtiter plate TTX-coating by cysteamine, which simplifies the protocol by eliminating 3 steps and shortens the analysis time by 90 min. Cysteamine can be readily purchased from different companies and it costs about 1,000-fold less than carboxylate-dithiol.

To enable the substitution of carboxylate-dithiol by cysteamine, the amount of cysteamine required for the SAM formation had to be determined, and according to the solubility of cysteamine, the solvent was changed from ethanol into an aqueous (phosphate) buffer. Thus, concentrations of 1, 10, 50 and 100 mM of cysteamine were tested in competition assays, using 2 µg/mL of TTX, 1:3,200

mAb dilution and 1:1,000 IgG-HRP dilution. All competitive assays showed appropriate trends according to the free TTX concentrations and provided similar IC_{50} values. Moreover, similar absorbance values were obtained for the positive (without free TTX) and negative (without mAb) controls, altogether indicating that 1 mM of cysteamine is a saturated concentration. Therefore, subsequent experiments were performed with 1mM cysteamine. Under the selected conditions, an IC_{50} of 8 ng/mL, an LOD, established as the IC_{20}, of 2 ng/mL and a working range (IC_{20}-IC_{80}) between 2 and 43 ng/mL were attained (**Fig. 4.2.2**), with an *R* from the sigmoidal adjustment of 0.996. The standard deviation (SD) values for the calibration points were lower than 8% of the mAb binding signal. The LOD attained with the improved mELISA described herein was similar to that obtained with the previous mELISA (using carboxylate-dithiol). In contrast, a narrower working range was obtained in the mELISA using cysteamine with respect to the previous mELISA using dithiol (2-43 *vs.* 2-95 ng/mL, respectively). This difference is attributed to the higher sensitivity of the cysteamine-based mELISA. The LOD provided by the mELISA described herein was in accordance with other immunoassays reported for TTX (~2 ng/mL (Neagu et al. 2006, Kawatsu et al. 1997 and Reverté et al. 2015)) and lower than others (5 ng/mL (Tao et al. 2010) and 10 ng/mL (Stokes et al. 2012)).

Additionally, to investigate the possibility of further shortening the time of the mELISA protocol, the storage stability of TTX-coated maleimide plates at 4 and -20 °C was evaluated. The mAb binding signal was constant up to 3 months at both 4 °C and -20 °C, demonstrating the stability of the maleimide plates with immobilized TTX (**Fig. 4.2.3**). The great stability of the TTX-coated plates significantly reduces the assay time, as multiple plates can be prepared on the same day and stored until use. Consequently, provided that TTX-coated plates are ready-to-use, the analysis of samples can be performed in less than 2 h on the same day, making a substantial improvement with respect to the previously reported mELISA (Reverté et al. 2015). Moreover, the preparation of multiple plates using the same solutions reduced the variability between assays, making the system more reproducible, particularly for a commercialization.

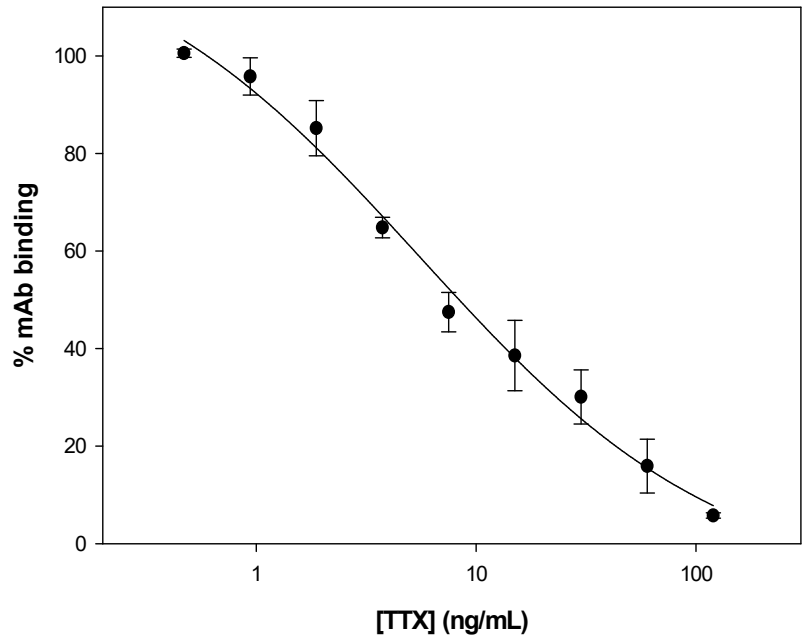


Fig. 4.2.2. TTX calibration curve obtained by the improved mELISA. Response is expressed as mAb binding percentage, normalized to mAb signal when no TTX is present. Error bars represent the standard deviation values for 3 replicates.

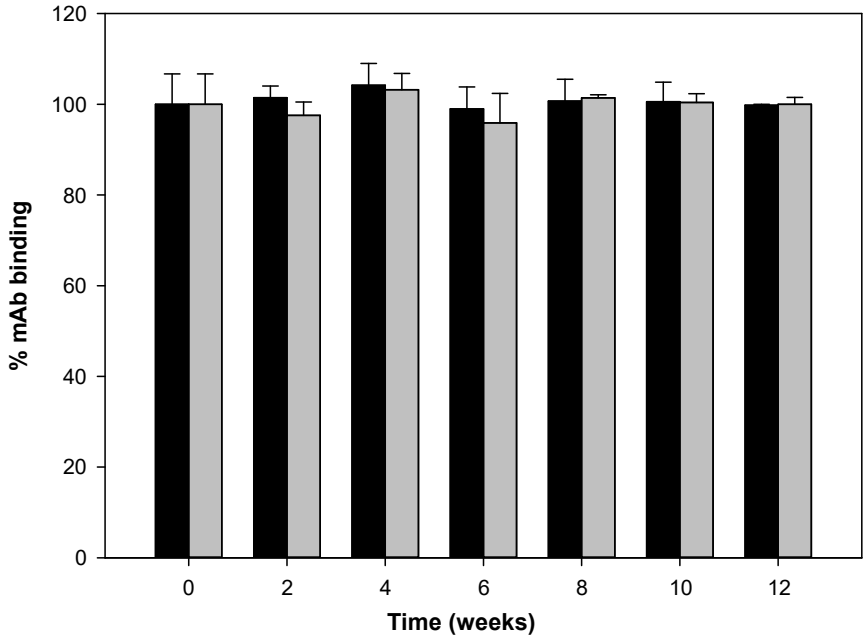


Figure 4.2.3. Storage stability of TTX-coated maleimide plates. Bars represent the % mAb binding obtained with plates stored at 4 °C (black bars) and at -20 °C (grey bars). Error bars represent the standard deviation values for 3 replicates.

4.2.4.2 Interference studies

The protocol applied for TTX extraction from shellfish is also adequate to extract PSP toxins. Therefore, the TTXs extraction process may extract PSP toxins, if present. As a consequence, prior to the analysis of naturally-contaminated samples, it is necessary to ensure that potential PSP toxins will not interfere in the TTX immunoassay performance. With this aim, the possible recognition of several PSP toxins (GTX1&4, GTX2&3, dcGTX2&3, GTX5, NEO, dcNEO, STX, dcSTX and C1&2) by the anti-TTX antibody was evaluated. As the mAb binding obtained for all toxins analyzed was close to 100% (**Fig. 4.2.4**), the cross-reactivity of the PSP toxins at a concentration of 100 ng/mL can be neglected, demonstrating the high specificity and selectivity of the mELISA. As a positive control, TTX tested at the same concentration resulted in a mAb binding decrease of more than 90% (Figure 4.2.1). This study illustrates that those PSP toxins, for which standards are available, that may co-exist with TTXs in shellfish extracts will not interfere with the immunorecognition of TTXs in the assay.

Additionally, since multiple reaction monitoring (MRM) transitions for Arg have been found to suppress TTX response in the mass spectrometer source (Turner et al 2017), the possible interference of this amino acid in the mELISA was also evaluated. As can be seen in Fig. 4.2.4, no significant effects of Arg on the mAb binding were observed.

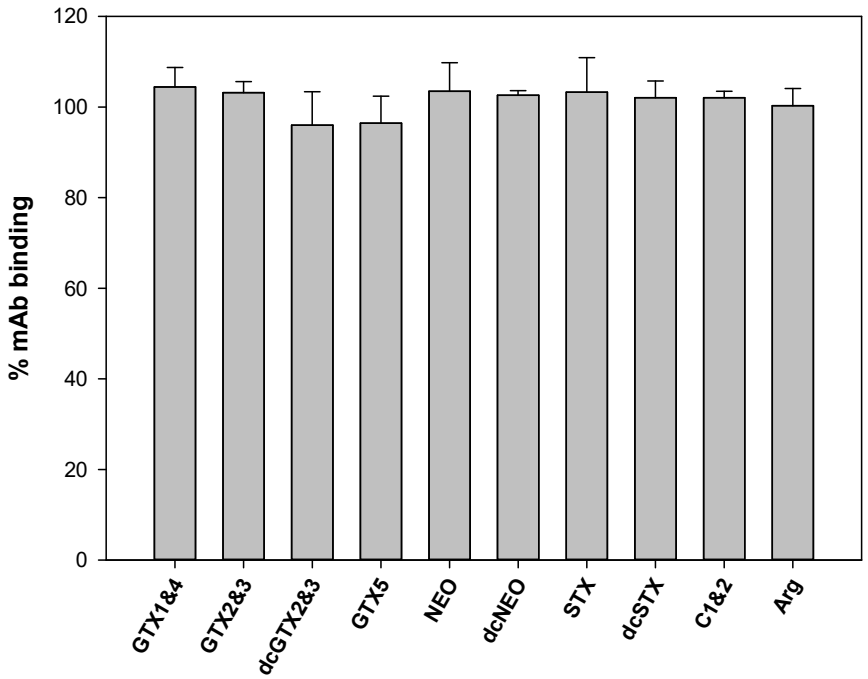


Figure 4.2.4. Percentage of mAb binding obtained by mELISA with 100 ng/mL of PSP toxins and Arg. Error bars represent the standard deviation values for 3 replicates.

4.2.4.3 Evaluation of matrix effects and establishment of matrix correction factors

Mussel and oyster matrix effects were studied by the analysis of blank shellfish extracts at different matrix concentrations (50, 75, 100 and/or 150 mg equiv./mL) at 3 different stages of the extraction protocol.

1) After SPE clean-up without solvent evaporation: Although SPE clean-up should reduce matrix effects, the highly acidic nature of the ACN/H_2O/AA solvent mixture used in the SPE protocol had a negative impact on the system, since mAb binding percentages obtained were not consistent with the shellfish matrix concentration used. The detrimental effects observed in the mELISA were assumed to be due to the solvent composition rather than to the shellfish matrix. Even decreasing the solvent mixture percentage by 4 times respect to the extract arising from the SPE column (i.e. 12.5% of the total volume of the well), the solvent effect could not be avoided.
2) After SPE clean-up, evaporation and solvent exchange: SPE cleaned-up extracts were evaporated until dryness, dissolved in phosphate buffer and analyzed by mELISA at the same matrix concentrations. mAb binding percentages were between 92 and 108% in either oyster or mussel extracts, regardless of the matrix concentration used (**Table 4.2.1**). Taking into consideration that the highest RSD value obtained was of 10%, negligible matrix effects are assumed when mAb binding percentages are between 90 and 110%. Thus, results obtained with this protocol indicate that evaporation completely removes the previously observed undesirable effects. These results also reaffirm that when no evaporation was performed, the inhibition of the mAb binding was not due to the shellfish matrix, but to the solvent mixture presence, which could be harming the cysteamine and/or TTX immobilization. Therefore, mELISA enables loading up to 150 mg equiv./mL of shellfish matrix after SPE clean-up and evaporation.

3) Directly after toxin extraction (without SPE clean-up nor evaporation and solvent exchange): In order to evaluate if the SPE clean-up can be avoided to simplify the protocol or if, on the contrary, is a crucial step in the analysis of oyster and mussel extracts, extracts without SPE clean-up and not evaporated were tested. Under these conditions, differences between oyster and mussel extracts were observed (Table 4.2.1). In the case of oyster, no matrix effects were observed (i.e. mAb binding

percentages were between 103 and 108%), indicating that the mELISA tolerates up to 150 mg equiv./mL of oyster matrix without SPE clean-up and with no need of solvent exchange. These results evidence that whereas solvent evaporation is crucial for the analysis of SPE cleaned-up oyster extracts, oyster extracts without SPE clean-up containing up to 7.5 % of the extraction solvent do not to interfere with the assay performance and, therefore, solvent evaporation is not required for the analysis of these extracts. However, in the analysis of mussel, pronounced matrix effects were obtained for all matrix concentrations tested. Although loading a lower matrix concentration could in principle remove remaining matrix effects, this would compromise the effective LOD (eLOD) of the assay. While the reason for the different behavior between mussel and oyster extracts remains unclear, it is evident that in the analysis of mussel extracts, SPE clean-up and subsequent solvent exchange are recommended to avoid non-desired matrix interferences.

Table 4.2.1. Percentages of mAb binding obtained in the analysis of blank oyster and mussel tissues extracted under different conditions (n=4 replicates).

Shellfish tissue	Protocol	[Matrix] (mg equiv./mL)			
		150	**100**	**75**	**50**
Oyster	SPE clean-up / not evaporated	X	X	X	X
	SPE clean-up / evaporated	106	92	97	-
	no SPE clean-up / not evaporated	103	110	108	108
Mussel	SPE clean-up / not evaporated	X	X	X	X
	SPE clean-up / evaporated	105	96	108	-
	no SPE clean-up / not evaporated	57	63	71	74

X: not consistent; -: not tested

4.2.4.4 Toxin recovery in oysters and mussel samples

From the experiments performed with blank oyster extracts, it was concluded that up to 150 mg equiv./mL of oyster extract can be loaded on the immunoassay without requiring a clean-up step nor solvent exchange. Nonetheless, solvent evaporation is required if the oyster extract undergoes a SPE clean-up step. Mussel extract after SPE clean-up also requires solvent evaporation prior to analysis to avoid solvent interference in the immunoassay. However, mussel extracts without SPE clean-up and with no evaporation suffer from undesirable matrix effects even at 50 mg equiv./mL of mussel matrix. Consequently, oyster and mussel tissue homogenates were spiked and extracted, and toxin recovery was evaluated without SPE clean-up/not evaporated and after SPE clean-

up/evaporated. Taking into account the matrix concentrations of the resulting extracts and to fit into the TTX calibration curves, extracts were spiked at 2 different levels of TTX in order to evaluate the toxin recovery.

1) After SPE clean-up, evaporation and solvent exchange: As SPE cleaned-up and evaporated extracts can be analyzed at 150 mg equiv./mL of shellfish matrix concentration, shellfish tissues were spiked at 75 µg TTX/kg (concentration that should provide about 50% of mAb binding inhibition when extract is analyzed at 150 mg equiv./mL of matrix concentration). Under these conditions, low toxin recovery values were obtained for both oyster and mussel tissue extracts (**Table 4.2.2**). These low recovery values can be attributed to toxin loss during the SPE clean-up and solvent exchange and/or interference of the shellfish matrix on the free toxin/antibody recognition. Nevertheless, these toxin recovery values can be taken as correction factors (CFs) and will be applied in the quantifications obtained in the subsequent analysis of naturally-contaminated samples extracted and treated under these conditions.

2) Directly after toxin extraction (without SPE clean-up nor evaporation and solvent exchange): Mussel extracts with no SPE clean-up showed higher matrix effects and, therefore, samples had to be analyzed at lower matrix concentrations. Consequently, to test these conditions, a higher TTX concentration (250 µg TTX/kg) was spiked in mussels (also for oysters, although it was not a requirement). Under these conditions, toxin recovery values were higher than those obtained for SPE cleaned-up extracts. Regarding oyster samples, the lower the matrix concentration, the higher the toxin recovery. The excellent toxin recovery at 75 mg equiv./mL (96%) indicates that these extracts do not suffer from toxin loss during SPE or solvent exchange. Consequently, the inhibition of mAb binding at higher matrix concentrations can only be due to the effect of the matrix on the free toxin/antibody recognition, which obviously decreases as the matrix concentration decreases. Again, these toxin recovery values can be taken as CFs and will be applied in the quantifications obtained in the subsequent analysis of naturally-contaminated oyster samples extracted and treated under these conditions. Regarding mussel samples, only one matrix concentration (50 mg equiv./mL) was analyzed (matrix effects at higher matrix concentrations were considered too high to conduct spiking trials), and the toxin recovery obtained was very high (166%). In this case, it is evident that the mussel matrix inhibits the response (as observed in the previous experiment where no TTX was present), causing an overestimation of the TTX content. Taking into account the toxin recovery value (166%) and the percentage of mAb binding obtained in the analysis of blank mussel tissue at 50 mg equiv./mL (74%), a CF of 123% is obtained, which will be used in the quantifications

obtained in the subsequent analysis of naturally-contaminated mussel samples extracted and treated under these conditions.

Table 4.2.2. Percentages of toxin recovery obtained in the analysis of TTX-spiked oyster and mussel tissues extracted under different conditions (n=4 replicates).

Shellfish tissue	Protocol	TTX level (µg/kg)	[Matrix] (mg equiv./mL)			
			150	100	75	50
Oyster	SPE clean-up / evaporated	75	29	-	-	-
	no SPE clean-up / not evaporated	250	71	83	96	-
Mussel	SPE clean-up / evaporated	75	50	-	-	-
	no SPE clean-up / not evaporated	250	-	-	-	166

-: not tested

Once obtained all toxin recovery values, eLODs in µg TTX/kg shellfish were calculated for each shellfish matrix and for both protocols. These eLODS were calculated as the ratio of the LOD obtained in buffer (2 ng/mL ± SD) to the shellfish matrix concentration used and applying the corresponding CF (**Table 4.2.3**).

Table 4.2.3. eLODs (µg TTX/kg shellfish) determined for SPE cleaned-up evaporated and no SPE cleaned-up not evaporated oyster and mussel extracts at different matrix concentrations.

Shellfish tissue	Protocol	[Matrix] (mg equiv./mL)			
		150	100	75	50
Oyster	SPE clean-up / evaporated	47 ± 17	-	-	-
	no SPE clean-up / not evaporated	19 ± 7	25 ± 9	28 ± 10	-
Mussel	SPE clean-up / evaporated	27 ± 10	-	-	-
	no SPE clean-up / not evaporated	-	-	-	33 ± 12

-: not tested

Regarding oysters, eLOD obtained for extracts without SPE clean-up and not evaporated were lower than for SPE cleaned-up and evaporated extracts. Consequently, SPE clean-up is certainly not worth conducting as this additional step increases the analysis time. Regarding mussels, similar eLODs were obtained after SPE clean-up at 150 mg equiv./mL of matrix and without SPE clean-up at 50 mg equiv./mL. These results reaffirm that SPE clean-up is not a requirement for the analysis of oyster and mussel extract samples.

The eLOD values obtained for both shellfish matrices are higher than those previously reported for TTX by LC-MS/MS (5 µg/kg in this work, 3 µg/kg (Turner et al. 2015), 7.2 µg/kg (Vlamis et al. 2015) and 15 µg/kg (Boundy et al. 2015)), but still very acceptable. Notably, they are substantially below from the eLOD obtained for puffer fish with the previous mELISA (230 µg/kg) (Reverté et al. 2015). Moreover, the eLODS values obtained herein proved the capability of the mELISA of detecting TTX below or only slightly above the concentration of 44 µg TTX equiv./kg shellfish meat, level that is considered not to result in adverse effects in humans (EFSA 2017). Therefore, the improved mELISA is absolutely appropriate for the screening as well as for the quantification of TTX contents in natural shellfish samples.

4.2.4.5 Analysis of oyster and mussel samples and comparison with LC-MS/MS analysis

After evaluating the matrix effects and establishing the CFs according to the toxin recovery values obtained, the mELISA was applied to the analysis of 3 oyster and 3 mussel samples from the Oosterschelde, The Netherlands. Oyster and mussel extracts were analyzed using the two different protocols (without SPE clean-up not evaporated and with SPE clean-up and evaporated). The corresponding CF values were applied to the TTX content determined in each sample, and resulting quantifications were compared to those obtained by LC-MS/MS analysis (**Table 4.2.4**).

Although the number of samples was too low for statistical treatment (due to the limited availability of natural samples of shellfish containing TTX), no significant differences (P=0.702) were observed in the quantifications provided by the three approaches (mELISA with SPE clean-up/evaporated samples, mELISA with no SPE clean-up/not evaporated samples and LC-MS/MS analysis). In the analysis of samples by LC-MS/MS, only TTX was detected (other TTX analogues were not found at detectable levels). This toxin profile, with only TTX, contributes to the similarity between techniques, even though they are based on different recognition principles.

The TTX content in the shellfish samples ranged from slightly below 44 µg/kg up to 4- or 5-fold higher. Thus, we provide the first immunoassay capable of screening and quantifying TTX in shellfish samples at levels that may be considered of concern for human health.

Table 4.2.4. TTX content of 3 oyster and 3 mussel samples from the sanitary monitoring program of The Netherlands by the cysteamine-based mELISA under two different extraction protocols and comparison with LC-MS/MS analysis.

Method	Protocol	Oyster 1	Oyster 2	Oyster 3	Mussel 1	Mussel 2	Mussel 3
mELISA (µg TTX equiv./kg shellfish)	SPE clean-up evaporated	82 ± 2	36 ± 6	67 ± 21	70 ± 11	146 ± 14	16 ± 4
	no SPE clean-up not evaporated	61 ± 7	116 ± 16	86 ± 13	55 ± 1	227 ± 7	24 ± 5
LC-MS/MS* (µg TTX/kg shellfish)	-	113	51	79	93	172	41

*Samples were analyzed singular; during the intra laboratory validation of this method the repeatability error at 20 µg/kg was 15.7%.

4.2.5 Conclusions

A modified SAM-based immunoassay has been applied to the determination of TTXs in mussel and oyster samples. The replacement of dithiols by cysteamine for the SAM formation allowed decreasing the required time and cost, while maintaining the sensitivity of the previously reported mELISA (LOD of 2 ng/mL). Storage at -20 and 4 °C of the TTX immobilization up to at least 3 months, provided ready-to-use microtiter plates, enabling a user to perform the assay in less than 2 h. Additionally, as proven by the absence of interferences from PSP toxins and Arg, the mELISA is highly selective for TTXs.

In the analysis of blank shellfish, oyster extracts did not show matrix effects even without the SPE clean-up step. The SPE clean-up of mussel extracts removed the strong matrix effects observed when no SPE was used. However, the SPE clean-up and the required solvent evaporation resulted in low toxin recovery percentages when analyzing TTX-spiked samples, probably because of toxin loses in the column and during the evaporation step. Toxin recovery values were obtained for all protocols and shellfish types, and can be used as CFs to be applied to the quantification of TTX contents in naturally-contaminated samples. Taking them into account, the lowest eLOD values obtained were about 20 and 50 µg TTX/kg for oyster extracts without and with SPE clean-up, respectively, and about 30 µg TTX/kg for mussel extracts with both protocols, substantially below the eLOD obtained by the previous mELISA for puffer fish (230 µg TTX/kg). This is in relatively good agreement with the level of 44 µg TTX equiv./kg shellfish meat, which is considered not to result in adverse effects in humans by the EFSA. Highly analogous results were determined on the comparison of the analysis of naturally-contaminated shellfish by mELISA with LC-MS/MS analysis. Overall, the improved mELISA developed herein meets the requirements in terms of selectivity and sensitivity. Although toxin recovery values, and thus CFs, were obtained for all protocols, shellfish samples can be rapidly processed and analyzed by mELISA without SPE clean-up, which is a clear

advantage over LC-MS/MS methodologies, where SPE is required. Therefore, the implementation of the mELISA for the screening of TTXs in shellfish samples in routine monitoring programs could be straightforward, providing a complementary analytical technique suitable for ensuring food safety and consumer protection.

4.2.6 References

C. Acar, S. Ishizaki, Y. Nagashima, Toxicity of the Lessepsian pufferfish *Lagocephalus sceleratus* from eastern Mediterranean coasts of Turkey and species identification by rapid PCR amplification, Eur. Food Res. Technol., 243 (2017) 49-57.

V. Bane, M. Lehane, M. Dikshit, A. O'Riordan, and A. Furey, Tetrodotoxin: Chemistry, toxicity, source, distribution and detection, Toxins (2014), 6(2) 693-755.

Y. Bentur, J. Ashkar, Y. Lurie, Y. Levy, Z.S. Azzam, M. Litmanovich, M. Golik, B. Gurevych, D. Golani, M.J. Boundy, A.I. Selwood, D.T. Harwood, P.S. McNabb, A.D. Turner, Development of a sensitive and selective liquid chromatography-mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction, J. Chromatogr. A, 1387 (2015) 1-12.

K. Campbell, P. Barnes, S.A. Haughey, C. Higgins, K. Kawatsu, V. Vasconcelos, C.T. Elliott, Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood, Anal. Bioanal. Chem., 405 (2013) 7753-7763.

EFSA (European Food Safety Authority), Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods, EFSA J., 15:4 (2017): 4752.

A. Eisenman, Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean, Toxicon, 52 (2008) 964-968.

T. Goto, Y. Kishi, Takahash.S, Y. Hirata, Tetrodotoxin, Tetrahedron, 21 (1965) 2059-2088.

P. Katikou, D. Georgantelis, N. Sinouris, A. Petsi, T. Fotaras, First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece), Toxicon, 54 (2009) 50-55.

K. Kawatsu, Y. Hamano, T. Yoda, Y. Terano, T. Shibata, Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin, Jpn. J. Med. Sci. Biol., 50 (1997) 133-150.

J. Kheifets, B. Rozhavsky, Z. Girsh Solomonovich, R. Marianna, A. Soroksky, Severe tetrodotoxin poisoning after consumption of *Lagocephalus sceleratus* (pufferfish, fugu) fished in Mediterranean Sea, treated with cholinesterase inhibitor, Case Reports in Critical Care, 2012 (2012) 782507.

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

Y. Mahmud, K. Yamamori, T. Noguchi, Occurrence of TTX in a brackish water puffer "midorifugu", *Tetraodon nigroviridis*, collected from Thailand, J. Food Hyg. Soc. Jpn., 40 (1999) 363-367.

T. Narahashi, Tetrodotoxin - A brief history, Proc. Jpn. Acad. B-Phys. Biol. Sci., 84 (2008) 147-154.

D. Neagu, L. Micheli, G. Palleschi, Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination, Anal. Bioanal. Chem., 385 (2006) 1068-1074.

T. Noguchi, J.S.M. Ebesu, Puffer poisoning: Epidemiology and treatment, J. Toxicol.-Toxin Rev., 20 (2001) 1-10.

T. Noguchi, O. Arakawa, T. Takatani, TTX accumulation in pufferfish, Comp. Biochem. Phys. D., 1 (2006) 145-152.

T. Noguchi, O. Arakawa, Tetrodotoxin - Distribution and accumulation in aquatic organisms, and cases of human intoxication, Mar. Drugs, 6 (2008) 220-242.
T. Noguchi, K. Onuki, O. Arakawa, Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism, ISRN Toxicol., (2011) 276939-276939.
V. Pratheepa, V. Vasconcelos, Microbial diversity associated with tetrodotoxin production in marine organisms, Environ. Toxicol. Pharm., 36 (2013) 1046-1054.
T.J.G. Raybould, G.S. Bignami, L.K. Inouye, S.B. Simpson, J.B. Byrnes, P.G. Grothaus, D.C. Vann, A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples, J. Clin. Lab. Anal., 6 (1992) 65-72.
L. Reverté, P. de la Iglesia, V. del Río, K. Campbell, C.T. Elliott, K. Kawatsu, P. Katikou, J. Diogène, M. Campàs, Detection of tetrodotoxins in puffer fish by a self-assembled monolayer-based immunoassay and comparison with surface Plasmon resonance, LC-MS/MS, and mouse bioassay, Anal. Chem., 87 (2015) 10839−10847.
P. Rodriguez, A. Alfonso, C. Vale, C. Alfonso, P. Vale, A. Tellez, L.M. Botana, First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe, Anal. Chem., 80 (2008) 5622-5629.
I. Rodriguez, et al., The association of bacterial C-9-based TTX-like compounds with *Prorocentrum minimum* opens new uncertainties about shellfish seafood safety, Sci. Rep. (2017), 7.
A.N. Stokes, B.L. Williams, S.S. French, An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification, Biol. Proced. Online 14:3 (2012).
Y. Tahara, Hirata, Y., Studies on the puffer fish toxin, J. Pharm. Soc. Jpn., 29 (1909) 587-625.
J. Tao, W.J. Wei, L. Nan, L.H. Lei, H.C. Hui, G.X. Fen, L.Y. Jun, Z. Jing, J. Rong, Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China, Food Addit. Contam. A, 27 (2010) 1589-1597.
A.D. Taylor, J. Ladd, S. Etheridge, J. Deeds, S. Hall, S.Y. Jiang, Quantitative detection of tetrodotoxin (TTX) by a surface plasmon resonance (SPR) sensor, Sensor. Actuat. B-Chem., 130 (2008) 120-128.
A.D. Taylor, H. Vaisocherova, J. Deeds, S. DeGrasse, S. Jiang, Tetrodotoxin Detection by a Surface Plasmon Resonance Sensor in Pufferfish Matrices and Urine, J. Sensors, (2011) Article ID 601704, 10 pages.
K. Tsuda, R. Tachikawa, C. Tamura, S. Ikuma, M. Kawamura, K. Sakai, O. Amakasu, On the structure of tetrodotoxin, Chem.Pharm. Bull., 12 (1964) 642-645.
A.D. Turner, A. Powell, A. Schofield, D.N. Lees, C. Baker-Austin, Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014, Euro surveill., 20 (2015) 2-8.
A.D. Turner, M.J. Boundy, M.D. Rapkova, Development and single-laboratory validation of a liquid chromatography tandem mass spectrometry method for quantitation of tetrodotoxin in mussels and oysters, J. AOAC Int., 100 (2017) doi: 10.5740/jaoacint.17-0017.
A. Vlamis, P. Katikou, I. Rodriguez, V. Rey, A. Alfonso, A. Papazachariou, T. Zacharaki, A.M. Botana, L.M. Botana, First detection of tetrodotoxin in Greek shellfish by UPLC-MS/MS potentially linked to the presence of the dinoflagellate *Prorocentrum minimum*, Toxins, 7 (2015) 1779-1807.
R. Wang, A. Huang, L. Liu, S. Xiang, X. Li, S. Ling, L. Wang, T. Lu, S. Wang, Construction of a single chain variable fragment antibody (scFv) against tetrodotoxin (TTX) and its interaction with TTX, Toxicon, 83 (2014) 22-34.
R.B. Woodward, J.Z. Gougouta, Structure of tetrodotoxin, J. Am. Chem. Soc., 86 (1964) 5030-5030.
B.J. Yakes, J. Deeds, K. White, S.L. DeGrasse, Evaluation of surface Plasmon resonance biosensors for detection of tetrodotoxin in food matrices and comparison to analytical methods, J. Agric. Food Chem., 59 (2011) 839-846.
B.J. Yakes, K.M. Kanyuck, S.L. DeGrasse, First report of a direct surface Plasmon resonance immunosensor for a small molecule seafood toxin, Anal. Chem., 86 (2014) 9251-9255.

Y. Zhou, Y.S. Li, F.G. Pan, Z.S. Liu, Z. Wang, Identification of tetrodotoxin antigens and a monoclonal antibody, Food Chem., 112 (2009) 582-586.

4.3 Immunosensor array platforms based on self-assembled dithiols for the electrochemical detection of tetrodotoxin in puffer fish

Laia Reverté, Katrina Campbell, Maria Rambla-Alegre, Christopher T. Elliott, Jorge Diogène, and Mònica Campàs*

Submitted to Analytica Chimica Acta in 31st May 2017

4.3.1 Abstract

The recent detection of tetrodotoxins (TTXs) in European fish and shellfish has emphasized the urgent need to develop specific, selective, rapid and easy-to-use methods for their detection to assess the potential risk posed to human health. For this purpose, a dithiol self-assembled monolayer (SAM)-based immunoassay previously performed on maleimide plates (mELISA) has been adapted to gold electrode arrays for the development of an electrochemical immunosensor for TTX. The electrochemical SAM-based immunosensor designed herein, provided an oriented, stable and spaced sensing platform for the determination of TTX, attaining a limit of detection of 2.6 ng mL^{-1}. The applicability of the biosensor array was demonstrated by the accurate quantifications obtained in the analysis of different tissues of several puffer fish species (*Lagocephalus lagocephalus, L. sceleratus and Sphoeroides pachygaster*) caught along the Mediterranean coast of Spain. The good agreements found between the TTX concentrations determined by the immunosensor array platforms and those determined by mELISA, surface Plasmon resonance (SPR) immunosensor and liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis, proved the feasibility of the approach. The electrochemical immunosensor enables the determination of TTXs at levels as low as 0.07 mg TTX equiv. kg^{-1} tissue, thus, well below the Japanese value of 2 mg TTX equiv. kg^{-1} tissue used as a criterion to consider puffer fish safe for consumption. Compared to the colorimetric SAM-based approach, the immunosensor array described herein shows promise towards the development of disposable, portable and compact analysis tools applicable in monitoring programs for the surveillance of fishery products.

4.3.2 Introduction

Tetrodotoxin (TTX) is a low-molecular-weight potent marine neurotoxin believed to be primarily produced by certain marine endosymbiotic bacteria (Moczydlowski, 2013). Once exogenously

produced, it may accumulate through the food webs and enter into other organisms (Pratheepa et al., 2013), eventually reaching humans. Whilst this toxin was originally discovered in the organs of fish from the *Tetraodontidae* family, especially puffer fish (Tahara et al., 1909), its distribution has expanded to a wide range of marine organisms including amphibians, echinoderms, cephalopods and bivalve mollusks (Noguchi et al., 2008). Structurally, TTX and several TTX analogues have been described as heterocyclic, heat-stable and hydrophilic compounds (Tsuda et al., 1964, Woodward et al., 1964). They are well-known selective sodium channel blockers (Lee et al., 2008), which obstruct neural and muscular transmission with a similar mechanism of action to saxitoxin (STX), a potent Paralytic Shellfish Poisoning (PSP) toxin.

Tetrodotoxin is responsible for numerous human intoxications worldwide in most cases with poisoning occurring following the ingestion of contaminated seafood. Typical poisoning symptoms include mild gastrointestinal effects, respiratory paralysis, numbness and even death (Noguchi et al., 2011). Food poisoning incidents were first restricted to warm water regions, particularly in the Pacific and Indian Oceans, affecting mainly Japan and China (You et al., 2015), where puffer fish (*fugu)* is legally consumed. Although fatalities and intoxications do still occur in Japan, this risk was reduced by the value of 2 mg TTX equiv. kg^{-1} of edible portion used as a criterion to consider puffer fish safe for consumption (Mahmud et al., 1999). Although there is no regulatory limit for TTX in Europe, EU regulation establishes that fishery products derived from poisonous fish of the families *Tetraodontidae*, *Molidae*, *Diodontidae* and *Canthigasteridae* must not be placed in the market. However, given that *fugu* is sometimes offered in some private events, the risk may not be completely discarded (Peck 2011). TTX poisoning episodes have also been reported following the consumption of mislabeled fish products (Cohen et al., 2009) and, additionally, may be consumed by accident due to their similarity to other non-poisonous fish.

Recently, puffer fish from the species *Lagocephalus sceleratus* have been found migrating from the Red Sea to the Mediterranean Sea through the Suez Channel (Lessepsian migration) (Bentur et al., 2008). Additionally, several reports confirm the increasing occurrence of TTX in European seafood. A first toxic episode was described in 2007 in Málaga (Spain), caused by the consumption of trumpet shells caught in Portugal (Rodriguez et al., 2008). Afterwards, two episodes were reported along the Mediterranean coast, following the ingestion of *L. sceleratus* (Katikou et al., 2009, Kheifets et al., 2012). In the last two years (2014-2015), TTXs have been found in bivalve mollusk shellfish grown in the south of England (Turner et al., 2015), along the Greek coast (Vlamis et al., 2015) and in the Netherlands.

Currently, there is no official method of analysis for TTXs in Europe. Nevertheless, different methodologies have been developed for their detection, being LC-MS/MS analysis (Asakawa, 2012) and the mouse bioassay (MBA) (Kawabata, 1978b) the most widely employed. Although proven useful techniques, the need of skilled personnel, expensive equipment, standards of TTXs and the establishment of toxicity equivalency factors (TEFs) for LC-MS/MS have hampered their appropriate performance. Additionally, the lack of specificity of MBA in discriminating between other coexisting PSP toxins and the non-availability of certain TTX standards, have restricted its use. Due to these limiting factors and given the increasing occurrence of TTX in Europe, the development of specific, rapid and cost-effective methods as support tools in monitoring programs to ensure human safety is highly required. To date, alternative methods based on antibodies that have been developed for TTX include several colorimetric immunoassays (Kawatsu et al., 1997, Raybould et al., 1992, Reverté et al., 2015, Stokes et al., 2012, Tao et al., 2010, Zhong et al., 2011, Zhou et al., 2009), two electrochemical immunosensors (Kreuzer et al., 2002, Neagu et al., 2006) and optical surface Plasmon resonance (SPR) immunosensors (Campbell et al., 2013, Taylor et al., 2008, Taylor et al., 2011, Vaisocherova et al., 2011).

Recently, a special configuration of a colorimetric immunoassay was used by our group for the development of an ELISA for TTX (Reverté et al., 2015). This assay was based on the immobilization of TTX through dithiols self-assembled on maleimide plates (mELISA) and provided an ordered and oriented antigen immobilization, which proved to be an efficient, reliable and powerful tool for the precise quantification of TTXs. With the aim of moving towards miniaturized and compact devices, this self-assembled monolayer (SAM)-based strategy for the development of an electrochemical immunosensor for TTX detection was exploited herein. The conversion of the colorimetric immunoassay to the electrochemical immunosensor was achieved by transferring the SAM-based strategy from microtiter plates to arrays of gold electrodes, and by recording the electrochemical signal after selecting the appropriate redox mediator for the horseradish peroxidase (HRP) label (**Fig. 4.3.1**). Compared to the previous reports of electrochemical immunosensors (Kreuzer et al., 2002, Neagu et al., 2006), the dithiol-based SAM strategy provides not only an oriented and stable antigen-modified sensing platform, but also decreases the non-specific adsorption. Additionally, whereas the previous reports were proofs of concept, the present work goes a step further, as demonstrated by the application of the immunosensor array platform to the analysis of natural puffer fish samples. Different tissues of several puffer fish species (*L. lagocephalus, L. sceleratus and Sphoeroides pachygaster*) were analysed for the accurate determination of TTX equivalent contents and the results compared to those provided by the previously developed mELISA, by the

SPR immunosensor and by liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis (Rambla-Alegre et al. 2017).

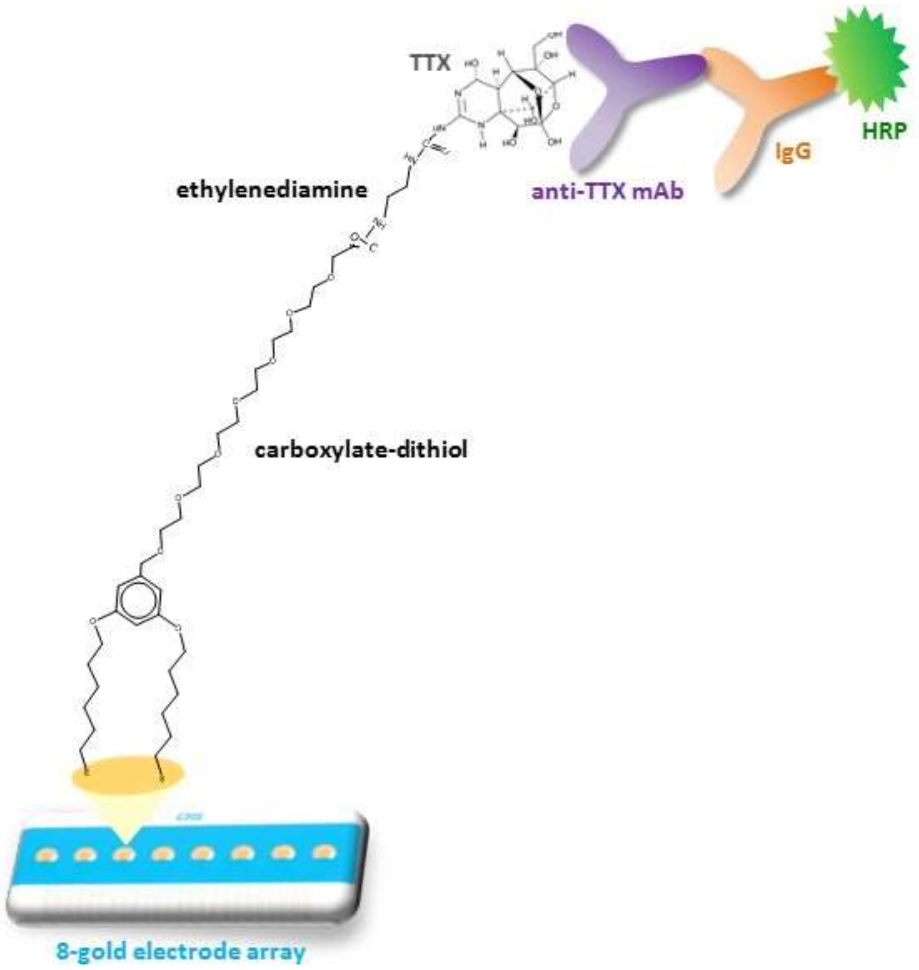


Fig. 4.3.1. Schematic representation of the electrochemical immunosensor array platform for TTX detection.

4.3.3 Materials and methods

4.3.3.1 Reagents and materials

The TTX standard was obtained from Tocris Bioscience (Bristol, UK). TTX standard solution was prepared at 1 mg mL^{-1} in 10 mM acetic acid. The anti-TTX monoclonal antibody (mAb) TX-7F was produced as previously described (Campbell et al., 2013, Kawatsu et al., 1997). DithiolalkanearomaticPEG6-COOH (carboxylate-dithiol) was purchased from Sensopath Technologies (Bozeman, USA). Anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), bovine serum albumin (BSA), ethanolamine, ethylendiamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), ethylenediaminetetraacetic acid

(EDTA), formaldehyde solution (37%), 2-(N-morpholino) ethanesulfonic acid hydrate (MES), *N*-hydroxysuccinimide (NHS), potassium chloride, potassium hexacyanoferrate II, potassium hexacyanoferrate III, potassium phosphate dibasic, potassium phosphate monobasic, thioctic acid, TMB liquid substrate, TMB enhanced one component HRP membrane substrate and Tween-20 were all supplied by Sigma-Aldrich (Tres Cantos, Spain). HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20, pH 7.4) was provided by GE Healthcare (Little Chalfont, UK). Maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain).

4.3.3.2 Equipment, electrodes and software

Cyclic voltammetry (CV) measurements were performed with a PGSTAT128N potentiostat purchased from AUTOLAB (Massó Analitica S.A., Barcelona, Spain). Data was collected and evaluated by General Purpose Electrochemical System (GPES) software version 4.9. Disposable screen-printed gold electrodes (SPGE), with gold as working and counter electrodes and silver as a reference electrode (220AT), were purchased from Dropsens S.L. (Oviedo, Spain).

Chronoamperometries (CA) were recorded with a Palmsens multiplexer potentiostat (Palmsens BV, The Netherlands) and a DRP-CAC8x connector from Dropsens S.L. (Oviedo, Spain). Data were collected and evaluated by PSTrace version 4.7.2 software. The 8x screen-printed gold electrode arrays, with gold as working and counter electrodes and silver as reference electrode (8X220AT), were purchased from Dropsens S.L. (Oviedo, Spain).

Colorimetric measurements for mELISA experiments were performed with a Microplate Reader, GEN5 2.09 software from BIO-TEK Instruments, Inc. (Vermont, USA).

An SPR device (Biacore Q) with Control Software (Version 3.0.1), BIAevaluation software version 4.1, and CM5 sensor chips were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden).
LC-HRMS analysis was carried out with an Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) and data was processed with Xcalibur 3.1 software (ThermoFisher Scientific, Bremen, Germany).

The adjustment of calibration curves to sigmoidal logistic 4-parameter equations was performed using SigmaPlot 12.0 (Systat Software Inc., California, USA).

4.3.3.3 Puffer fish samples and toxin extraction

A total of 6 female puffer fish from NW Mediterranean Sea were used in this study, 3 of them belonging to the species *L. lagocephalus* (1 from Blanes, 1 from Altafulla and 1 from Denia, Spain), 1 to the species *L. sceleratus* (from Denia, Spain) and 2 to the species *S. pachygaster* (1 from LLançà and 1 from Denia, Spain). One of the *L. lagocephalus* (determined as non-containing TTX by LC-MS/MS; "blank puffer fish sample") was used for the evaluation of matrix effects and spiking experiments.

Toxin extraction from puffer fish tissues (muscle, skin, liver and gonads) was performed as described previously [27]. Briefly, a double extraction was performed with 0.1% acetic acid for each tissue. In the case of liver, an additional liquid-liquid partition with hexane was required. The analysis by the mELISA, SPR immunosensor and electrochemical immunosensor was performed with the aqueous extracts, but for the LC-HRMS analyses, extracts were evaporated, re-dissolved in methanol and filtered through 0.2-µm nylon filters.

4.3.3.4 Electrochemical immunosensor array platforms

The titration assay protocol used for the immobilization of TTX through carboxylate-dithiol self-assembled on SPGEs was similar to that used for the mELISA described previously (Reverté et al., 2015), with some modifications of volumes and immobilization supports. The steps taken were as follows: (1) 15 µL of 0.1 and 1 mM carboxylate-dithiol in 0.1 M potassium phosphate, 10 mM EDTA, pH 7.2 (PBS) were placed on the SPGEs of the array (8x220AT) and self-assembled for 3 h. (2) Carboxylic groups of dithiols were activated by the addition of 15 µL of 0.1 M NHS and 0.4 M EDC (1/1, v/v) in 25 mM MES, pH 5.5 for 30 min. (3) Activated carboxylic groups reacted with primary amines of ethylenediamine (15 µL of 0.1 M ethylenediamine in PBS) for 30 min, forming amide bonds (4) The remaining carboxylic groups were deactivated by adding 15 µL of 1 M ethanolamine in PBS for 30 min. (5) TTX was then immobilized on ethylenediamine through formaldehyde cross-linking following an amino−amino reaction, adding 14.5 µL of 0.2 and 2 µg mL^{-1} of TTX in PBS and 0.5 µL of formaldehyde (37%) for 15 h. Following TTX immobilization, (6) 15 µL of 1/800, 1/1,600 and 1/3,200 mAb dilutions in 1% BSA-PBS were incubated for 30 min. (7) Remaining sites were blocked with 1% BSA-PBS for 30 min. (8) 15 µL of IgG-HRP at 1/1,000 dilution were incubated onto the electrodes for 30 min. (9) Finally, TMB enhanced liquid substrate was allowed to react for 10 min and chronoamperometries (CA) were recorded at -0.11 V for 5 s (current intensities were taken

at 0.5 s). After each step, electrodes were rinsed with washing buffer (0.1 M potassium phosphate, 0.15 M NaCl, 0.05% Tween-20, pH 7.2) and air-dried.

Once the optimum concentrations of carboxylate-dithiol and TTX were selected, three competition assays were performed, the protocol differing from the checkerboard only in step (6). Thus, 7.5 µL of 1:800, 1/1,600 and 1/3,200 mAb dilutions were mixed with 7.5 µL of TTX standard solution or "sample" (blank puffer fish tissue extract, TTX-spiked puffer fish tissue extract or naturally-contaminated puffer fish tissue extract) and pre-incubated in tubes for 15 min before placing the mixture on the electrodes for 30 min. Several competitive electrochemical assays were performed differing in the sample used in the competition step: 1) A TTX standard calibration curve was constructed from 0.625 to 80 ng mL^{-1} in PBS. The background was corrected with respect to the controls without mAb and the curve was fitted to a sigmoidal logistic 4-parameter equation. From the equation, inhibitory concentrations (ICs) were calculated. Specifically, the midpoint (IC_{50}), the limit of detection (LOD) established as the IC_{20}, and the working range, considering the linear interval of the curve (IC_{20}-IC_{60}), were determined. 2) Puffer fish matrix effects were evaluated using different tissues of a blank puffer fish sample (muscle, skin, liver, and gonads) with no presence of TTXs as determined by LC-MS/MS analysis, using a matrix concentration of 40 mg mL^{-1}. 3) Different puffer fish tissue extracts at a matrix concentration of 40 mg mL^{-1} were spiked with TTX at 2.5, 5 and 10 ng mL^{-1} to determine the percentage of toxin observed in spiked-puffer fish tissue extracts with respect to the spiked TTX levels. 4) Finally, extracts of different tissues from 5 puffer fish individuals were analyzed. Toxin standard solution and samples were prepared in PBS, diluted by half and assayed in quadruplicate.

4.3.3.5 SAM characterization by reductive desorption

The electrochemical reduction of the thiol-gold bond between the carboxylate-dithiol/thioctic acid SAMs and the SPGEs was evaluated through CVs. Bare, 1 mM carboxylate-dithiol and 100 mM thioctic acid SAM-modified individual SPGEs (220AT) were immersed in thoroughly degassed PBS and two consecutive CVs were recorded from -0.55 to -0.90 V at a scan rate of 10 mV s^{-1}.

4.3.3.6 TTX immobilization characterization

The protocol used for the immobilization of TTX on carboxylate-dithiol SAM and the subsequent characterization was the same as for the development of the electrochemical immunosensor from

steps 1 to 5, but using individual SPGEs (220 AT) and 40 µL of working volume instead of 15 µL. For the immobilization of TTX on thioctic acid, step 1 was performed using 40 µL of 100 mM thioctic acid in MeOH:H_20 (1:1).

The immobilization of TTX on carboxylate-dithiol and thioctic acid SAMs was characterized by CV using 40 µL of 2.5 mM potassium hexacyanoferrate redox couple (1:1, 5 mM potassium hexacyanoferrate (II):5 mM potassium hexacyanoferrate (III)) in 0.1 M phosphate buffer solution with 0.1 M KCl, pH 7.2. CVs were recorded from -0.25 to +0.45 V at scan rate of 10 mV s^{-1} on bare SPGEs, 1 mM carboxylate-dithiol and 100 mM thioctic acid SAM-modified electrodes, after ethylendiamine addition, and after TTX immobilization (5,000 ng/mL). All steps were carried out using the same electrode, rinsing thoroughly with wash buffer between steps.

4.3.3.7 mELISA

Briefly, the 5 puffer fish samples analyzed by the electrochemical immunosensor developed herein were also analyzed by the mELISA as previously reported (Reverté et al., 2015). All puffer fish tissue extracts were analyzed at a matrix concentration of 40 mg mL^{-1}. Puffer fish extracts of *L. sceleratus,* containing high levels of TTX, were further diluted to allow for quantification within the working range (IC_{20}-IC_{80}). All samples, mAb and toxin standard solutions were prepared in PBS, diluted by half and assayed in triplicate. Quantifications were corrected by applying the mELISA correction factors (CFs) established for each tissue (Reverté et al., 2015).

4.3.3.8 SPR immunosensor

The protocol used for the analysis of puffer fish samples was carried out as described in previous works (Campbell et al., 2013, Reverté et al., 2015). A TTX standard calibration curve was constructed from 0.06 to 1,000 ng mL^{-1}. As for mELISA, all puffer fish extracts were analyzed at a matrix concentration of 40 mg mL^{-1}. Puffer fish tissue extracts of *L. sceleratus,* containing high levels of TTX, were further diluted to allow for quantification within the working range (IC_{20}-IC_{60}). All samples, mAb and toxin standard solutions were prepared in HBS-EP buffer, diluted by half and assayed in duplicate. As for mELISA quantifications, TTX contents obtained by the SPR immunosensor were corrected by applying the corresponding SPR CFs established for each tissue (Reverté et al., 2015).

4.3.3.9 LC-HRMS analysis

The quantification of TTXs contents in five samples of puffer fish extracts of the species *L. lagocephalus, L. sceleratus and S. pachygaster* was performed following the protocol reported in the literature (Rambla-Alegre et al. 2017). The sum of total TTX as well as the individual TTX analogues concentrations were calculated for muscle, skin, liver and gonads tissues of five samples. To facilitate the comparison of the results with the other techniques used herein, only the sums of TTX contents are shown.

4.3.3.10 Statistical analysis

Correlations between TTX contents obtained by the electrochemical immunosensor and those obtained by mELISA, SPR immunosensor and LC-HRMS analysis were evaluated using linear regression. To evaluate if differences were significant between techniques, data were tested for normality using the Shapiro-Wilk test. For data following a normal distribution, t-test was performed; otherwise, Mann-Whitney Rank Sum Test was performed. The level of significance was set at $p<0.05$. All statistics were performed using Sigmastat 3.1 software (Systat Software Inc. California, US).

4.3.4 Results and discussion

Due to the similar affinity of maleimide and gold for thiol groups, the SAM-based strategy used for the development of the mELISA reported previously (Reverté et al., 2015) was followed for the development of the present electrochemical immunosensor, whereby maleimide plates were replaced by SPGEs and the electrochemical signal was recorded instead of the colorimetric one. Prior to the development of the electrochemical immunosensor, the SAM formation as well as the different steps for the immobilization of TTX on SAMs were characterized.

4.3.4.1 SAM characterization by reductive desorption

Since thiolated compounds have been proven to experience desorption from gold surfaces at negative potentials under highly alkaline or neutral pH solutions, reductive desorption experiments were carried out to characterize the self-assembling of carboxylate-dithiol and thioctic acid on individual SPGEs (corresponding structures are shown in **Figure 4.3.2**). **Figure 4.3.3** shows the

forward scans of the two consecutive CVs carried out on bare SPGEs and carboxylate-dithiol SAM-modified SPGEs. As expected, no reduction peaks were observed when using bare SPGEs (dotted line) in any of the scans. On the contrary, a peak at -0.8 V was obtained in the first scan on SAM-modified electrodes (solid line), which corresponds to the reduction of thiol groups (Sun et al., 2011, Tencer et al., 2008). In the second scan, no peak was obtained, suggesting that the negative potential applied to the electrodes caused the complete thiol bond break in the first scan, and demonstrating the previous presence of SAM on the modified electrode. Although being destructive, this technique has been useful to characterize the carboxylate-dithiol SAM formation. Unfortunately, thioctic acid SAM-modified SPGEs did not provide a suitable response (no reduction peak was observed), but the strategy was retained for further evaluation.

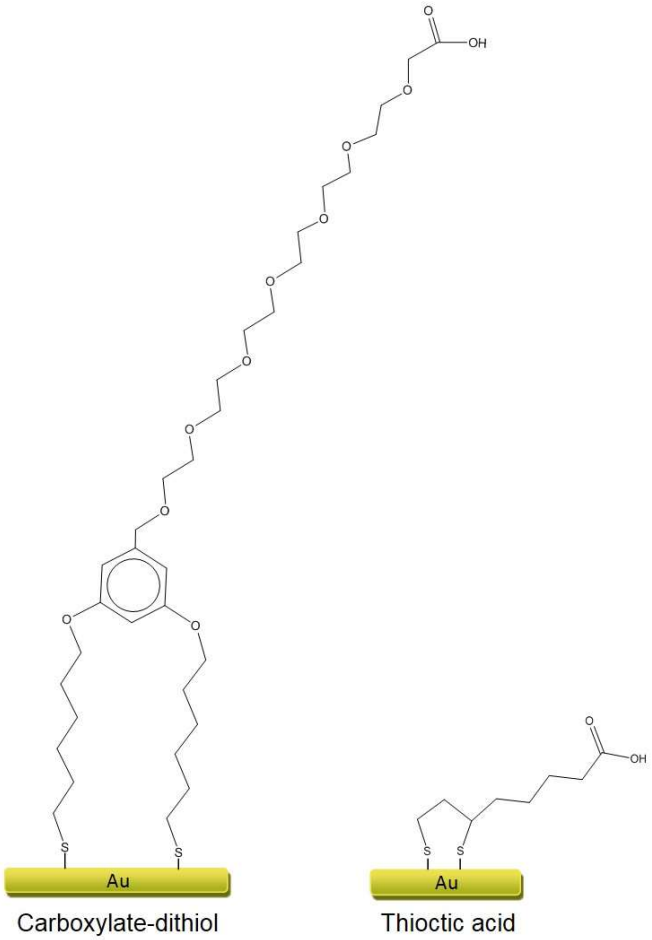


Fig. 4.3.2. Carboxylate-dithiol and thioctic acid molecules self-assembled on SPGEs.

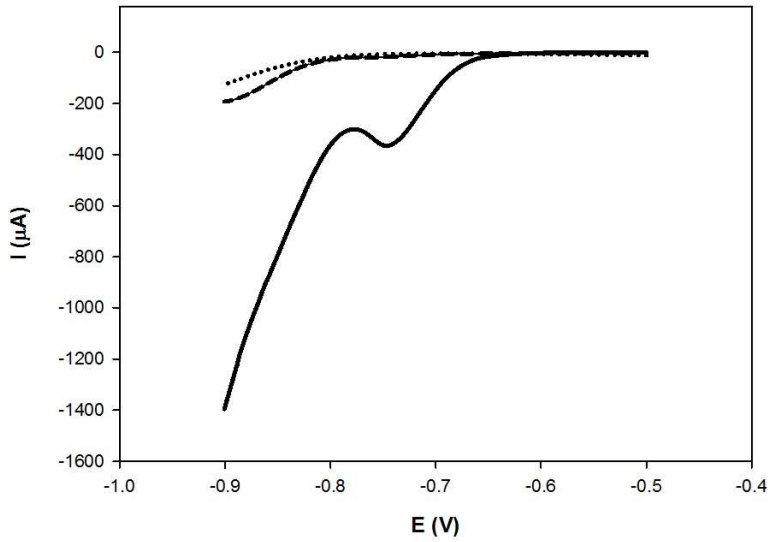


Fig. 4.3.3. Forward scan voltammograms obtained for bare SPGE (dashed line), scan 1 (solid line) and scan 2 (dotted line) of carboxylate-dithiol SAM-modified SPGE.

4.3.4.2 Characterization of TTX immobilization on SAMs

The different steps for the immobilization of TTX on carboxylate-dithiol and thioctic acid SAMs were monitored by CV. The CVs of the $[Fe(CN)_6]^{3-/4-}$ at bare, SAM, ethylendiamine and TTX-immobilized SPGEs were recorded (**Fig. 4.3.4**).

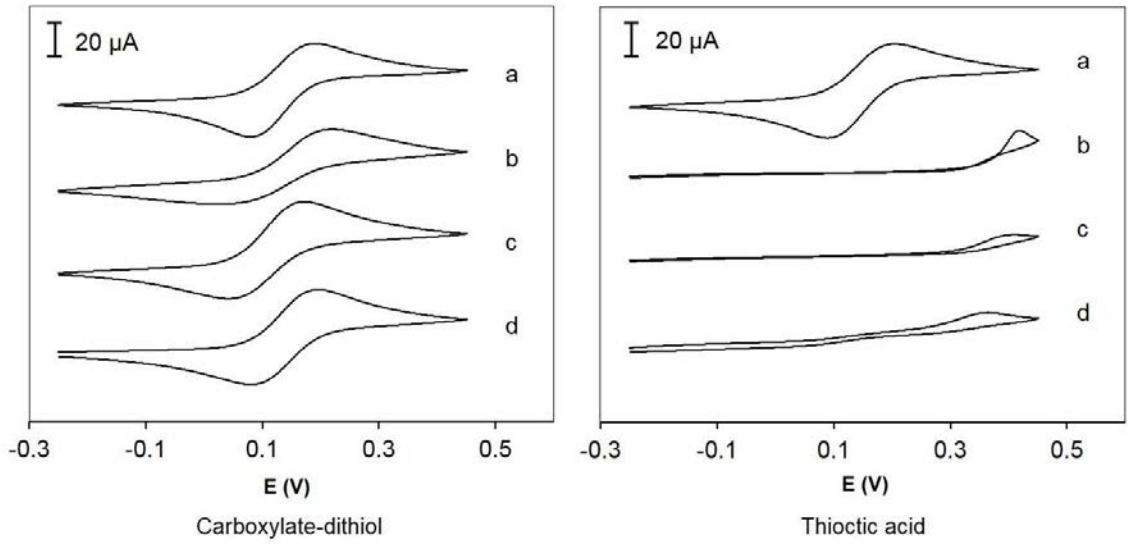


Fig. 4.3.4. Cyclic voltammograms in 2.5 mM $[Fe(CN)_6]^{3-/4-}$ obtained at **(a)** bare, **(b)** SAM, **(c)** ethylendiamine and **(d)** TTX-immobilised SPGEs for carboxylate-dithiol and thioctic acid approaches.

Both SAMs resulted in a decrease of the electrochemical signal due to the lower permeability of the electrode, being drastic in the case of thioctic acid and only slight in the case of carboxylate-dithiol. As described in the work performed by Fragoso and co-workers (Fragoso et al., 2008), the length and structure of thiolated molecules as well as the corresponding orientation and SAM packaging could influence the charge transfer in the ferrocyanide solution. In this case, despite being longer, carboxylate-dithiol provides a monolayer with the long chains of polyethylene glycol and spaced slightly titled from the perpendicular to the electrode surface, which favors the electron transfer. On the contrary, the shorter thioctic acid-based SAM was more packed and the short alkyl chain obliquely oriented to the electrode, resulting in a higher electron transfer blocking. When the positively charged ethylendiamine was added to the thioctic acid SAM-based SPGEs, oxidation and reduction peaks did not substantially change, probably due to the high blocking effect from the thioctic acid. On the contrary, the ethylendiamine addition significantly counteracted the blocking effect caused by the carboxylate-dithiol, as it is demonstrated by the reappearance of the well-defined oxidation and reduction peaks. Finally, an appropriate CV response after TTX immobilization was obtained for carboxylate-dithiol SAM-modified SPGEs (only a mild improvement was observed when using thioctic acid). Indeed, this result together with the unsatisfactory results obtained in the SAM reductive experiment for thioctic acid SAM reinforce the hypothesis that carboxylate-dithiol SAMs was the only choice for the immunosensor development. The orientation provided by carboxylate-dithiol probably favors the TTX immobilization and, consequently, the subsequent antigen-antibody affinity interaction. Furthermore, the spacing effect of carboxylate-dithiol SAM may not only favor the antigen-antibody affinity interaction, but also eases the electrochemical redox mediator to reach the electrode surface, thus, promoting the electron transfer. Given the improved orientation and permeability displayed by the carboxylate-dithiol SAM strategy, thioctic acid was no longer used in the development of the electrochemical biosensor.

4.3.4.3 Electrochemical immunosensor development

In order to select the optimal concentrations of carboxylate-dithiol, TTX and mAb, electrochemical titrations were performed using conventional TMB as redox mediator. On the evaluation of the carboxylate-dithiol concentration although 0.1 mM of carboxylate-dithiol provided a proper layer in the mELISA configuration, this concentration did not provide sufficient current intensities (<1 μA) in the electrochemical approach, probably due to the different surface properties of the SPGEs. Therefore, the following experiments were performed using 1 mM of carboxylate-dithiol, which

provided higher current intensities (>2 µA). Afterwards, immobilization of TTX on SAMs was assessed using the concentration of TTX required for the mELISA configuration of 0.5 µg mL^{-1} (Reverté et al., 2015). This TTX concentration was examined with different mAb dilutions: 1/800, 1/1,600 and 1//3,200. Controls without mAb were included in the checkerboard to have an indication of the non-specific binding of the HRP-labelled secondary antibody on the system. At this concentration, similar reduction current intensities were attained regardless of the antibody concentration (around 2 µA), suggesting that at 0.5 µg mL^{-1} of TTX, the SAM was completely saturated of mAb even when using the 1/3,200 dilution. As the high non-specific adsorption values (between 60 and 70%) seem to indicate, this amount of TTX would not be enough to fully cover the specific binding sites of the SAM and whereby these sites could be non-specifically occupied by the secondary antibody. In order to obtain higher current intensities, a higher TTX concentration (2 µg mL^{-1}) was used in the immobilization step. In this case, higher current intensities were attained and a trend was observed according to the different mAb dilutions (4.2, 3.4 and 2.8 µA for 1/800, 1/1,600 and 1/3,200 dilutions, respectively). As expected, the non-specific binding from the secondary antibody followed the opposite trend (35, 44 and 52%, respectively). Therefore, the use of a higher TTX concentration for the immobilization step increased the specific response and lowered the non-specific binding.

In order to evaluate the sensitivity of the immunosensor, electrochemical competition assays were carried out with 1 mM of carboxylate-dithiol, 2 µg mL^{-1} of TTX and several free TTX concentrations to choose between the three mAb dilutions. While the signal provided by the highest antibody dilution (1/3,200) was not sufficient to discriminate between different TTX concentrations, significant differences in the current intensity were observed when using 1/800 and 1/1,1600 mAb dilutions. As expected, slightly better sensitivity was achieved with 1/1,600 than with 1/800 mAb dilutions (50% of mAb binding corresponding to 40 and 80 ng mL^{-1} of TTX, respectively). Therefore, conditions selected to perform further competitive assays were: 1 mM of carboxylate-dithiol, 2 µg mL^{-1} of TTX in the immobilization step and 1/1,600 mAb dilution. The shift from the colorimetric to the electrochemical approach required using 10-fold higher concentration of carboxylate-dithiol, 40-fold higher of TTX, and double mAb concentration, due to the different surface characteristics of maleimide-coated microtiter wells and SPGEs.

With the aim of improving the electron transfer, thus, increasing further the current intensities, a different redox mediator (enhanced TMB) was used at the same conditions. Compared to conventional TMB, enhanced TMB provided current intensities 4-fold higher, as well as 5% less of

non-specific binding. These differences are probably due to the different nature of the final product resulting from the enzyme reaction. While the product of the reaction with conventional TMB is in liquid form, an insoluble precipitate is produced when the enzyme reacts with enhanced TMB. This solid precipitate concentrates at the electrode surface, enhancing the electrochemical response. Given the higher intensities and lower non-specific binding values obtained with enhanced TMB, the following experiments were performed with this mediator.

A calibration curve in buffer was then constructed using enhanced TMB (**Fig. 4.3.5**) and, from the adjustment, a regression factor (R) of 0.986 was obtained. Standard deviation (SD) for 4 replicates (n=4) was ≤15% for all concentrations (not shown in the graph for clarity of the results). Overall, the use of this mediator allows the improvement in sensitivity of the immunosensor, by lowering the IC_{50} from 40 to 7 ng mL^{-1}. The LOD was found to be 2.6 ng mL^{-1}, and the working range (IC_{20}-IC_{60}) was 2.6-10.2 ng mL^{-1}.

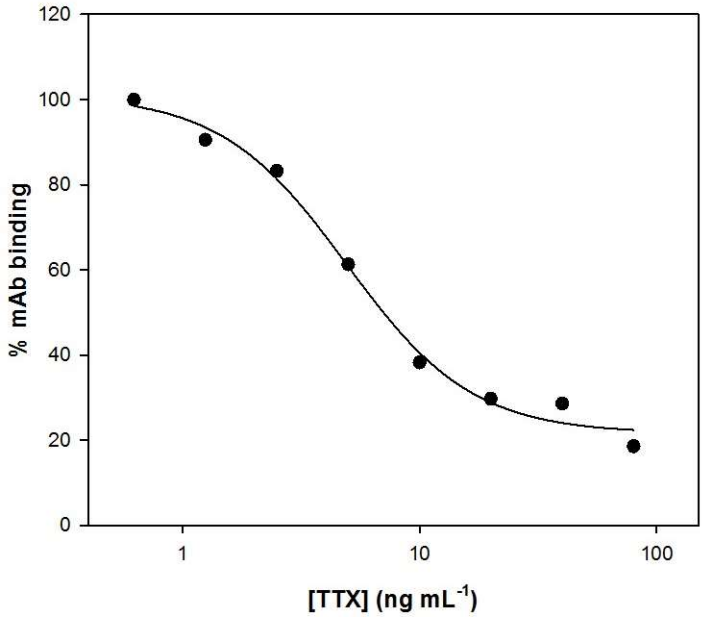


Fig. 4.3.5. Calibration curve obtained by the electrochemical immunosensor. mAb binding is expressed as percentage of the control (without free toxin). X values refer to initial toxin concentrations.

The sensitivity achieved by the present electrochemical immunosensor was in good agreement with the two electrochemical immunosensors reported for TTX (Kreuzer et al., 2002, Neagu et al., 2006). In comparison with the previous works, this electrochemical immunosensor enables for the first time the detection of TTX by immobilizing the toxin on specific locations of a SAM through stable chemical bonds. This SAM-based approach provides an oriented, specific and spaced TTX sensing

platform, avoiding the need to conjugate the toxin to protein carriers (e.g. BSA), which may block the electron transfer, and decreasing the non-specific binding thanks to the carboxylate-dithiol chemical structure. In addition, the new electrochemical platform has been characterized in detail, demonstrating the toxin immobilization as well as the enhanced electron transfer favored by the carboxylate-dithiol SAM. The shift from the colorimetric to the electrochemical SAM-based approach represents an advance towards disposable, portable and compact devices for the detection of TTX. In addition to these benefits, the multiplexed configuration permits reducing the reagent volumes (only 15 µL per electrode) as well as the consecutive measurement of 8 samples, decreasing the cost and analysis time.

4.3.4.4 Evaluation of puffer fish matrix effects

The applicability of the biosensor was first studied by evaluating matrix effects of blank puffer fish tissue extracts at a matrix concentration of 40 mg mL^{-1} (from a sample determined as non-containing TTX by LC-MS/MS). The analysis of negative puffer fish extracts at 40 mg mL^{-1} of tissue resulted in mAb binding percentages of 91, 106, 95 and 103% for muscle, skin, liver, and gonads, respectively. Considering that the highest SD in the calibration curve was of 15%, a response between 85 and 115% of mAb binding indicates no effect from the extract. Thus, matrix effects can be considered negligible at 40 mg mL^{-1} of puffer fish matrix.

Further matrix effects were then evaluated through the spiking of 2.5, 5 and 10 ng mL^{-1} of TTX into blank puffer fish tissue extracts at a matrix concentration of 40 mg mL^{-1} (corresponding to 0.06, 0.13 and 0.25 mg TTX kg^{-1} of tissue, respectively). Percentages of toxin were calculated with reference to the concentrations of TTX spiked (**Table 4.3.1**). Again, taking into account the 15% of SD, similar TTX concentrations were determined in buffer and in spiked puffer fish tissue extracts, reaffirming that matrix does not interfere with the immunosensor performance.

Table 4.3.1. Toxin percentages measured by the electrochemical immunosensor array platforms in spiked-muscle, skin, liver and gonads tissue extracts of *L. lagocephalus* with reference to the TTX spiked levels (0.06, 0.13 and 0.25 mg TTX kg^{-1} of tissue).

	TTX spiked level (mg TTX kg-1 tissue)		
Puffer fish tissue	**0.06**	**0.13**	**0.25**
Muscle	104%	112%	113%
Skin	89%	87%	97%
Liver	93%	89%	106%
Gonads	96%	114%	87%

Given the negligible matrix effects and the good toxin percentages observed in spiked-extracts, the analysis of puffer fish tissue extracts can be performed using 40 mg mL^{-1} of matrix, which means that the immunosensor should be able to detect as low as 0.07 mg TTX equiv. kg^{-1} tissue. These results proven the reliability and feasibility of this biosensor to be applied to the analysis of samples with complex matrices such as puffer fish extracts.

4.3.4.5 Analysis of naturally contaminated puffer fish samples and comparison with mELISA, SPR immunosensor and LC-HRMS analysis

In order to demonstrate the applicability of the electrochemical immunosensor, *L. lagocephalus, L. sceleratus and S. pachygaster* samples were analyzed. Quantifications obtained by this immunosensor were compared with those determined by mELISA, SPR immunosensor and LC-HRMS. Whereas no TTX was found in either *L. lagocephalus* or *S. pachygaster* puffer fish samples (mAb binding percentages between 85 and 115%), high TTX contents were determined in *L. sceleratus* tissues by all techniques. **Table 4.3.2** shows the TTX equivalent contents determined in *L. sceleratus* extracts by the electrochemical immunosensor, mELISA and SPR immunosensor, in addition to the sum of the total TTX content obtained by LC-HRMS (the individual content of TTX and each analogue provided by LC-HRMS (Rambla-Alegre et al. 2017).

Table 4.3.2. TTX equivalent contents (mg TTX equiv. kg^{-1} tissue) in *L. sceleratus* extracts obtained by the electrochemical immunosensor, mELISA and SPR immunosensor, and the sum of total TTX determined by LC-HRMS analysis. LODs were 0.23, 0.43 and 0.05 mg kg^{-1} for mELISA, SPR immunosensor and LC-HRMS, respectively.

Puffer fish tissue	Electrochemical immunosensor	mELISA (Rambla-Alegre et al. 2017)	SPR immunosensor	Σ LC-HRMS (Rambla-Alegre et al. 2017)
Muscle	1.45	2.53	3.51	0.98
Skin	2.11	3.50	4.42	2.08
Liver	16.67	25.30	24.82	5.36
Gonads	33.90	33.55	30.50	25.22

Among the species of puffer fish analyzed in this work, *L. sceleratus* is considered one of the most toxic Lessepsian invasive species of the Mediterranean (Streftaris et al., 2006). *L. lagocephalus* is a native species of the Mediterranean for which there are no known specific toxin threats and, consequently, has been listed as least concern by a European Regional Assessment

(Papakonstantinou et al., 2015)**.** Finally, *S. pachygaster* is a well-known alien species of the Mediterranean (Golani et al.), considered weakly toxic by Noguchi et al. (Noguchi et al., 2008), but non-toxic in the Mediterranean by Ragonese and co-workers (Ragonese et al., 2012). Therefore, the toxin contents found in this work for different puffer fish species were in accordance with the levels of TTX described in the literature for these species of puffer fish caught along the Mediterranean coast.

With regard to the distribution of TTX in *L. sceleratus* tissues, the same trend was obtained with all the techniques used in this study, whereby the highest to the lowest TTX content was found in: gonads>liver>skin>muscle. Distribution of TTX into tissues was in accordance with that described for other female *L. sceleratus* specimens captured along the Mediterranean (Katikou et al., 2009, Kosker et al., 2016, Reverté et al., 2015, Rodriguez et al., 2012). Notably, levels of TTX found in gonads and liver tissues were up to 10-fold above the Japanese value of 2 mg TTX eq. kg^{-1} used as criterion to judge the acceptability of puffer fish as safe for human consumption (Mahmud et al., 1999).

When comparing the techniques used for the analysis of *L. sceleratus* extracts, a good correlation is obtained between the TTX contents determined by the electrochemical immunosensor and those provided by mELISA (R^2=0.933), as well as equivalent quantifications (y=0.95x-1.81; t=0.245, P=0.815). The similarity between results obtained with both techniques is due to the fact that both approaches are based on the antigen-antibody affinity principle and, additionally, TTX immobilization was achieved using the same strategy. Even though the transducer and the detection method are different, the recognition of antibody antigen binding and the competition step are similar.

When comparing the TTX contents determined by the electrochemical method with those provided by the SPR immunosensor, results correlate well (R^2=0.907), and equivalent TTX contents are reported by SPR immunosensor (y=1.05x-3.05; t=0.221, P=0.833). Despite the different approach used in the TTX immobilization (oriented through dithiols self-assembled on gold electrodes *vs.* randomly on dextran chips), the different detection principles of these techniques (electrochemical *vs.* optical) and the measurement of the biorecognition event (end-point *vs.* real-time), comparable toxin contents were obtained, with no significant differences.

Finally, in order to compare the quantifications obtained by this immunosensor with those provided by LC-HRMS, the known cross-reactivity factors (CRFs) (Reverté et al. 2015) were applied to the individual contents of TTX and analogues. As the similar recognition event occurs between immunosensor and immunoassay, and due to the lack of available TTX analogues, CRFs established by mELISA (Reverté et al. 2015) were assumed for this immunosensor. Good correlation was obtained between both techniques (R^2=0.902) and, as observed in the previous study (Reverté et al. 2015), slightly lower contents were determined by LC-HRMS, although differences were statistically not significant (y=1.28x+2.78; t=0.538, P=0.610). As mentioned in the previous work (Rambla-Alegre et al. 2017), within the different tissues analyzed, the greatest disparity between the quantifications provided by mELISA and LC-HRMS was observed in case of the liver. The lack of CRFs for some analogues determined by LC-HRMS, the possible presence of unknown analogues as well as other matrix components could be responsible for this disagreement between techniques. Overall, TTX contents determined by the electrochemical immunosensor were in good agreement with those obtained by mELISA, SPR immunosensor and LC-HRMS. **Table 4.3.3** reviews and compares the main analytical parameters of each technique. The good agreements found between the quantifications provided by this immunosensor and those provided by the other techniques proved the reliability and usefulness of the biosensor in the analysis of puffer fish samples. Certainly, this biosensor enabled the screening of TTX in several puffer fish species and tissues, from which *S. pachygaster* and *L. lagocephalus* individuals were considered safe for human consumption, whereas *L. sceleratus* was determined risky for human safety because of the high TTX contents determined, according to the Japanese criterion.

Table 4.3.3. Comparison of the performance parameters: LOD, high throughput sample analysis, ease of sample preparation, ease of use, cost and portability provided by the different analytical techniques used in this work.

Technique	LOD (mg TTX kg^{-1} puffer fish)	High-throughput sample analysis	Ease of sample preparation*	Ease of use	Cost	Portability
Electrochemical immunosensor	0.07	medium	yes	medium	medium	high
mELISA	0.23	high	no	high	medium	medium
SPR immunosensor	0.43	high	no	medium	high	low
LC-HRMS	0.05	high	no	low	high	low

4.3.5 Conclusions

Encouraged by the necessity to develop alternative methods to MBA and LC-MS/MS analysis for the detection of the emerging TTX in European fish and shellfish, an immunosensing platform based on the TTX immobilization through dithiols self-assembled on 8-gold electrode arrays is described. The spaced and oriented TTX immobilization on the carboxylate-dithiol SAM as well as the choice of a precipitating redox mediator favored the electron transfer. Good sensitivity was attained by the immunosensor, with an LOD of 2.6 ng mL^{-1}.

The immunosensor allowed working with the high loading tissue concentration of 40 mg mL^{-1} and showed no matrix effects under these conditions. Therefore, taking into account the LOD in buffer and the tolerable matrix concentration, the immunosensor was able to detect as low as 0.07 mg equiv. TTX kg^{-1} tissue. TTX contents determined in puffer fish samples by the electrochemical immunosensor were in good agreement with those obtained by mELISA, SPR immunosensor and LC-HRMS analysis. Thus, the analytical performance of the SAM-based electrochemical immunosensor shows feasibility for its implementation in food safety programs, since it enables the determination of TTX in puffer fish well below the Japanese value of 2 mg equiv. TTX kg^{-1} tissue and provides TTX quantifications comparable to other techniques. The format of the immunosensor described in this work makes it promising as a screening tool, with advantages such as the possible integration in miniaturized devices, the use of low reagent volumes, and the short time required for the consecutive analysis of 8 samples. In conclusion, given the improved sensitivity, the high accuracy and the good agreement of the immunosensor with other methods, this work constitutes a breakthrough in the development of rapid, compact, robust, reliable and easy-to-use analysis devices for the detection of the emerging TTX in puffer fish samples.

4.3.6 References

Asakawa, M., Shida, Y., Miyazawa, K., Noguchi, T. (2012). Chromatography - The Most Versatile Method of Chemical Analysis. Instrumental Analysis of Tetrodotoxin. Rijeka, Croatia, InTech Open Science: 245.

Bentur, Y., J. Ashkar, Y. Lurie, Y. Levy, et al. (2008). Lessepsian migration and tetrodotoxin poisoning due to Lagocephalus sceleratus in the eastern Mediterranean. Toxicon 52(8): 964-968.

Campbell, K., P. Barnes, S. A. Haughey, C. Higgins, et al. (2013). Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. Anal. Bioanal. Chem. 405(24): 7753-7763.

Cohen, N. J., J. R. Deeds, E. S. Wong, R. H. Hanner, et al. (2009). Public Health Response to Puffer Fish (Tetrodotoxin) Poisoning from Mislabeled Product. Journal of Food Protection 72(4): 810-817.

European Comission. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption Off. J. Eur. Union 2004, L226, 83-127.
European Comission. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin Off. J. Eur. Union 2004, L139, 22-82.
Fragoso, A., N. Laboria, D. Latta and C. K. O'Sullivan (2008). Electron permeable self-assembled monolayers of dithiolated aromatic scaffolds on gold for biosensor applications. Anal. Chem. 80(7): 2556-2563.
Golani, D., L. Orsi Relini, E. Massuti and J. P. Quignard 2010. CIESM atlas of exotic species in the Mediterranean. Vol. 1 Fishes. CIESM publisher, Monaco. World Wide Web electronic publication, accessible at: http://www.ciesm.org/online/atlas/index.htm
Katikou, P., D. Georgantelis, N. Sinouris, A. Petsi, et al. (2009). First report on toxicity assessment of the Lessepsian migrant pufferfish Lagocephalus sceleratus (Gmelin, 1789) from European waters (Aegean Sea, Greece). Toxicon 54(1): 50-55.
Kawabata, T. (1978b). Tetrodotoxin. In: Veterinary Division, Environmental Health Bureau, Ministry of Health and Welfare (Ed.), Food Hygiene Examination Manual (Shokuhin Eisei Kensa Shishin) II. Japan Food Hygiene Association, Tokyo, pp. 223-241
Kawatsu, K., Y. Hamano, T. Yoda, Y. Terano, et al. (1997). Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. Jpn. J. Med. Sci. Biol. 50(3): 133-150.
Kheifets, J., B. Rozhavsky, Z. Girsh Solomonovich, R. Marianna, et al. (2012). Severe Tetrodotoxin Poisoning after Consumption of Lagocephalus sceleratus (Pufferfish, Fugu) Fished in Mediterranean Sea, Treated with Cholinesterase Inhibitor. Case Rep. Crit. Care: 782507-782507.
Kosker, A. R., F. Ozogul, M. Durmus, Y. Ucar, et al. (2016). Tetrodotoxin levels in pufferfish (Lagocephalus sceleratus) caught in the Northeastern Mediterranean Sea. Food Chem. 210: 332-337.
Kreuzer, M. P., M. Pravda, C. K. O'Sullivan and G. G. Guilbault (2002). Novel electrochemical immunosensors for seafood toxin analysis. Toxicon 40(9): 1267-1274.
Lee, C. H. and P. C. Ruben (2008). Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. Channels 2(6): 407-412.
Mahmud, Y., K. Yamamori and T. Noguchi (1999). Occurrence of TTX in a brackish water puffer "Midorifugu", Tetraodon nigroviridis, collected from Thailand. Shokuhin Eiseigaku Zasshi 40(5): 363-367.
Moczydlowski, E. G. (2013). The molecular mystique of tetrodotoxin. Toxicon 63: 165-183.
Neagu, D., L. Micheli and G. Palleschi (2006a). Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. Anal. Bioanal. Chem. 385(6): 1068-1074.
Neagu, D., L. Micheli and G. Palleschi (2006). Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. Analytical and Bioanalytical Chemistry 385(6): 1068-1074.
Noguchi, T. and O. Arakawa (2008). Tetrodotoxin - Distribution and accumulation in aquatic organisms, and cases of human intoxication. Mar. Drugs 6(2): 220-242.

Noguchi, T., K. Onuki and O. Arakawa (2011). Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism. ISRN toxicology 2011: 276939-276939.
Papakonstantinou, C. and A. D. Rijnsdorp. (2015). *Lagocephalus lagocephalus.* The IUCN Red List of Threatened Species 2015: e.T190464A45883323.
http://www.iucnredlist.org/details/summary/190464/1. Accessed by december 2016.
Peck, T. Want to Eat the World's Most Deadly Dish? Join the Club (2011). Available online: http://www.independent.co.uk/life-style/food-and-drink/news/want-to-eat-the-worlds-most-deadly-dish-join-the-club-2292393.html. Accessed by December 2016.
Pratheepa, V. and V. Vasconcelos (2013). Microbial diversity associated with tetrodotoxin production in marine organisms. Environmental Toxicology and Pharmacology 36(3): 1046-1054.
Ragonese, S. and U. Morara (2012). Evidence of short-term discard mortality of trawled Sphoeroides pachygaster (Osteichthyes, Tetraodontidae) off the southern coast of Sicily (Central Mediterranean Sea). Panam. J. Aquat. Sci. 7(2): 73-76.
Rambla-Alegre, M. et al. (2017). Evaluation of tetrodotoxins in puffer fish caught along the Mediterranean coast of Spain. Toxin profile of *Lagocephalus sceleratus.* Environ. Res. 158, 1-6.
RASFF Consumers Portal. Notification details - 2016.0845. Possible presence of tetrodotoxin in live oysters from the Netherlands. https://webgate.ec.europa.eu/rasff-window/consumers/?event=notificationDetail&NOTIF_REFERENCE=2016.0845&country=NL. Accessed by December 2016.
Raybould, T. J. G., G. S. Bignami, L. K. Inouye, S. B. Simpson, et al. (1992). A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. J. Clin. Lab. Anal. 6(2): 65-72.
Reverté, L., P. de la Iglesia, V. del Río, K. Campbell, et al. (2015). Detection of Tetrodotoxins in Puffer Fish by a Self-Assembled Monolayer-Based Immunoassay and Comparison with Surface Plasmon Resonance, LC-MS/MS, and Mouse Bioassay (vol 87, pg 10839, 2015). Anal. Chem. 88(4): 2511-2511.
Rodriguez, P., A. Alfonso, P. Otero, P. Katikou, et al. (2012). Liquid chromatography-mass spectrometry method to detect Tetrodotoxin and Its analogues in the puffer fish Lagocephalus sceleratus (Gmelin, 1789) from European waters. Food Chem. 132(2): 1103-1111.
Rodriguez, P., A. Alfonso, C. Vale, C. Alfonso, et al. (2008). First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell Charonia lampas lampas in Europe. Analytical Chemistry 80(14): 5622-5629.
Stokes, A. N., B. L. Williams and S. S. French (2012). An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. Biological Procedures Online 14: 5.
Streftaris, N. and A. Zenetos (2006). Alien Marine Species in the Mediterranean - the 100 'Worst Invasives' and their Impact. Mediterr. Mar. Sci. 7(1): 87-117.
Sun, K., B. Jiang and X. Y. Jiang (2011). Electrochemical desorption of self-assembled monolayers and its applications in surface chemistry and cell biology. J. Electroanal. Chem. 656(1-2): 223-230.
Tahara, Y. and Y. Hirata (1909). Studies on the puffer fish toxin. J. Pharm. Soc. Jpn 29: 587-625.
Tao, J., W. J. Wei, L. Nan, L. H. Lei, et al. (2010). Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China. Food Addit. Contam. Part A 27(11): 1589-1597.
Taylor, A. D., J. Ladd, S. Etheridge, J. Deeds, et al. (2008). Quantitative detection of tetrodotoxin (TTX) by a surface plasmon resonance (SPR) sensor. Sensors and Actuators B-Chemical 130(1): 120-128.

Taylor, A. D., H. Vaisocherova, J. Deeds, S. DeGrasse, et al. (2011). Tetrodotoxin Detection by a Surface Plasmon Resonance Sensor in Pufferfish Matrices and Urine. J. Sens.
Tencer, M. and P. Berini (2008). Toposelective Electrochemical Desorption of Thiol SAMs from Neighboring Polycrystalline Gold Surfaces. Langmuir 24(21): 12097-12101.
Tsuda, K., R. Tachikawa, C. Tamura, S. Ikuma, et al. (1964). On structure of tetrodotoxin. Chem. Pharm. Bull. 12(5): 642-+.
Turner, A. D., A. Powell, A. Schofield, D. N. Lees, et al. (2015). Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Euro surveill. 20(2): 2-8.
Vaisocherova, H., A. D. Taylor, S. Jiang, K. Hegnerova, et al. (2011). Surface Plasmon Resonance Biosensor for Determination of Tetrodotoxin: Prevalidation Study. J. of AOAC Int. 94(2): 596-604.
Vlamis, A., P. Katikou, I. Rodriguez, V. Rey, et al. (2015). First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate Prorocentrum minimum. Toxins 7(5): 1779-1807.
Woodward, R. B. and J. Z. Gougouta (1964). Structure of tetrodotoxin J. Am. Chem. Soc. 86(22): 5030-&.
You, J., Y. Yue, F. Xing, W. Xia, et al. (2015). Tetrodotoxin poisoning caused by Goby fish consumption in southeast China: a retrospective case series analysis. Clinics 70(1): 24-29.
Zhong, Q., A. Huang, B. Wang and X. Dong (2011). Development of direct competitive ELISA kit for the detection of tetrodotoxin using HRP labeled antigen. Application of Chemical Engineering, Pts 1-3. Z. Cao, Y. H. He, L. Sun and X. Q. Cao. 236-238: 2820-2824.
Zhou, Y., Y. S. Li, F. G. Pan, Z. S. Liu, et al. (2009). Identification of tetrodotoxin antigens and a monoclonal antibody. Food Chemistry 112(3): 582-586.

4.4 Tetrodotoxin detection in puffer fish by a sensitive planar waveguide immunosensor

Laia Reverté, Mònica Campàs, Betsy Jean Yakes, Jonathan R. Deeds, Panagiota Katikou, Kentaro Kawatsu, Michael Lochhead, Christopher T. Elliott and Katrina Campbell*

Resubmitted to Sensors and Actuators B chemical on 30th April 2017

4.4.1 Abstract

A nanoarray planar waveguide biosensor was developed for the detection of tetrodotoxin (TTX). This technique, specifically designed for the first time for TTX, provided a compact versatile device, with printed toxin-conjugate arrays, for the analysis of puffer fish samples under high flow conditions and through a simple user friendly 10 minute protocol. The applicability to natural samples was investigated through the study of matrix effects and toxin recovery. The biosensor enabled the detection of TTX from 0.4 to 3.29 µg g-1 puffer fish tissue. The sensitivity attained demonstrates this assay as a rapid and sensitive screening method to detect TTX in different species of puffer fish, well below the Japanese maximum permitted level (2 µg g-1) and the estimated safety level used in the EU and the US (0.8 µg g-1). Assay repeatability and reproducibility were assessed at 0.4 and 0.8 µg g-1, showing relative standard deviation (RSD) values below 15% and toxin recovery within 85-115%. The appropriate correlation of data obtained from the biosensor compared to that reported by ELISA, RBA, SPR biosensor and LC-MS/MS for the analysis of 12 puffer fish samples, proved the feasibility and reliability of this immunosensor to support monitoring programs and research activities

4.4.2 Introduction

Tetrodotoxin (TTX) is one of the most hazardous low-molecular-weight marine neurotoxins (Moczydlowski, 2013), whereby poisoning following consumption has a notorious lethal incidence. TTX is commonly found in the organs of select puffer fish species, typically the liver, ovaries, and gastrointestinal (GI) tract but also occasionally in the meat. TTX is also found in other organisms, and this toxin is believed to be exogenously produced, starting from bacteria which accumulate up through the food chain into fish, bivalve shellfish, amphibians and octopus (Pratheepa and Vasconcelos, 2013). Human fatalities from Puffer Fish Poisoning (PFP) are mostly caused by the

ingestion of puffer fish ("fugu") in Japan (Noguchi and Arakawa, 2008), but the presence of TTX has also been reported in bivalve shellfish, harvested in several parts of Europe (Rodriguez et al., 2008; Turner et al., 2015a; Vlamis et al., 2015) and other countries (Bentur et al., 2008; McNabb et al., 2014). The main symptoms of PFP range from neuromuscular (lips and tongue paraesthesia, tingling and numbness) to gastrointestinal (nausea, abdominal pain, vomiting and diarrhea) (Noguchi et al., 2011). More severe symptoms consist of uncoordinated movements, muscle spasms, and progressive muscle paralysis that can result in respiratory failure in severe intoxication cases. TTX toxicity (LD50) is 2-10 µg kg-1 intravenously and 10-14 µg kg-1 subcutaneously in mammals as well as 334 µg kg-1 orally in mice (Alcaraz et al., 1999).

TTX and its related analogues are hydrophilic, heat-stable, heterocyclic compounds (Tsuda et al., 1964; Woodward and Gougouta, 1964), able to selectively block sodium channels (French et al., 2010; Lee and Ruben, 2008). To date, 29 analogues of TTX have been described, which when classified according to their structure are: hemylactal, lactone and 4,9 anhydro types. To the best of our knowledge, little is known about the role that these analogues may play in terms of toxicity, with the exception of the study performed by Yotsu-Yamashita and co-workers in rat membranes where 5,6,1-trideoxy TTX showed less toxicity than other analogues (Yotsu-Yamashita et al., 1999). To protect consumers against PFP in Japan, the regulatory limit for TTX in food is 2 µg TTX equivalents g-1 (Kawabata, 1978). In the US, no guidance level has been established, and TTX intoxications from puffer fish are predominantly limited through importation restrictions which only allow the puffer fish species Takifugu rubripes from Japan to be brought into the country with additional strict processing guidelines (FDA, 1989). All other imported puffer fish products are subject to automatic detention without physical examination by the US Food and Drug Administration (FDA) (FDA, 2015). Despite these strict import restrictions, several cases of PFP have been reported in recent years in the US due to the unapproved importation of puffer species (Cohen et al., 2009; Cole et al., 2015). Additionally, no regulatory limits have been specifically set for TTX in Europe. However, in both Europe and the US, safety limits have been set at 0.8 µg saxitoxin (STX) equivalents g-1 shellfish meat for the pharmacologically similar paralytic shellfish poisoning (PSP) toxins. This concentration can be used as a guide in determining the role of TTX in incidents of illness. The increasing incidence of seafood TTX poisoning episodes in Europe and in other non-regulated regions might have relevant consequences to food safety and economic fisheries. Therefore, there is a real need for the development of methods suitable to regulate this toxin in food by means of routine monitoring, thus, providing sufficient protection to human health and fishery products (Turner et al., 2015b).

In Europe, the shift for PSP toxins screening in bivalves and seafood from the mouse bioassay (MBA) which inadvertently detected TTX toxins, to the method of Lawrence using high-performance liquid chromatography with fluorescence detector (HPLC-FLD) (Lawrence et al., 2005), created a gap in the detection for other important paralytic toxins such as TTX that are relevant to food safety and that are not detected by the latter technique. Current methods for TTX detection are mainly those based on analytical chemistry, such as HPLC and liquid chromatography tandem-mass spectrometry (LC-MS/MS) (Asakawa, 2012). Although useful techniques, their performance is occasionally hampered by the lack of commercially available analytical standards for some TTX analogues and by the requirement of skilled personnel and expensive equipment. TTX detection has also been achieved by biochemical methods, including immunoassays (Kawatsu et al., 1997; Neagu et al., 2006; Raybould et al., 1992; Reverté et al., 2015; Stokes et al., 2012; Wang et al., 2014; Zhou et al., 2009) and electrochemical (Kreuzer et al., 2002; Neagu et al., 2006) and optical immunosensors (Campbell et al., 2013; Taylor et al., 2008; Taylor et al., 2011; Vaisocherova et al., 2011; Yakes et al., 2011a; Yakes et al., 2014), some of these having shown promise not only as research activities but also as screening tools in monitoring programs. Biosensors are increasingly being investigated due to the advantages they offer in terms of sensitivity, portability, ease of use and robustness. To date, surface plasmon resonance (SPR) immunoassay has been the most widely applied biosensor-based method for TTX, though the cost of instrumentation has prevented this method's broader uptake. More recently, another optical biosensor, based on planar waveguide technology, is gaining attention, and it has been successfully developed for other marine toxins, such as okadaic acid (OA), domoic acid (DA) and saxitoxin (STX) (McNamee et al., 2014), but not yet explored for TTX detection. Accordingly, an immunosensor based on the planar waveguide technology with fluorescence detection for the determination of TTX is described herein. In this technique, waveguide cartridges are previously spotted with picoliter drops of the desired toxin-conjugate, and the extent of the molecular interaction upon addition of the antibody is determined through a fluorescence reader. In the assay, when toxin is not present in a sample, nearly all the primary antibody is bound to the toxin conjugate attached on the surface, and a high fluorescence signal is obtained from the secondary antibody. When toxin is present in the sample, the antibody binds to this toxin in solution, thereby decreasing the amount of primary antibody available to bind to the surface and thus the fluorescence signal decreases proportionally to the toxin concentration. The signal itself is generated from light that is transmitted from the bottom of the waveguide cartridges, leading to the excitation of the fluorophores on the labeled-secondary antibody, thus enabling the sensitive recording of binding events on the surface (**Fig. 4.4.1**). The aim of this work was to investigate this planar waveguide biosensor for the rapid detection of TTX in puffer fish samples

using a simple extraction method. After development, this biosensor was applied to the analysis of samples, and the results were compared to other methods of analysis including the MBA, receptor binding assay (RBA), ELISA, SPR biosensor and LC-MS/MS analysis.

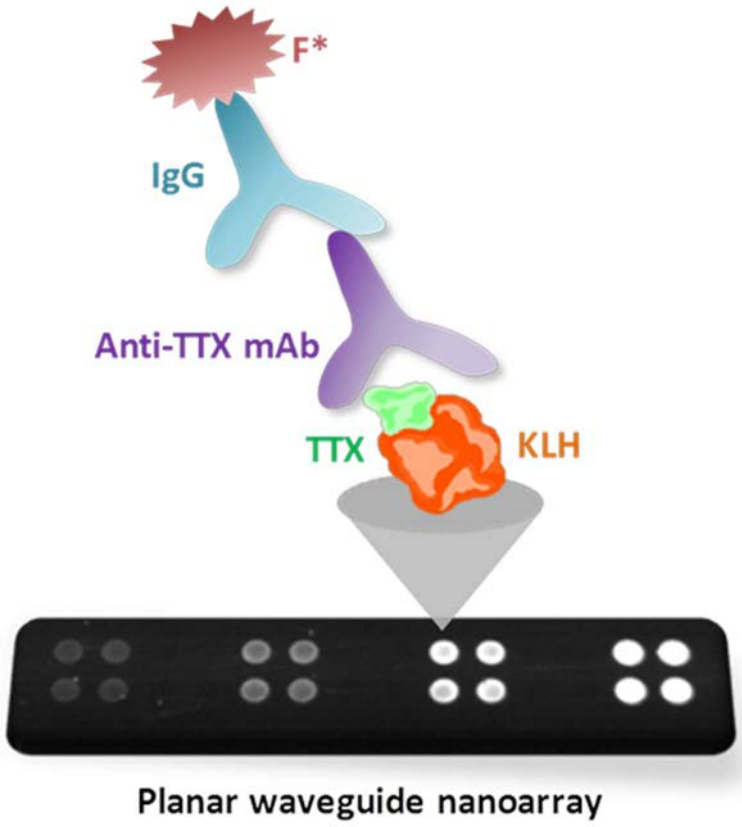


Fig. 4.4.1. Schematic representation of the planar waveguide immunosensor for TTX.

4.4.3 Materials and methods

4.4.3.1 Reagents

Bovine serum albumin (BSA), casein from bovine milk, phosphate buffered saline (PBS) tablets, sodium acetate anhydrous, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic and Tween 20 were purchased from Sigma-Aldrich (Dorset, UK). Alexa Flour 647 goat anti-mouse IgG (GaM) antibody and BSA-Alexa Fluor 647 conjugate were obtained from Invitrogen Ltd. (Paisley, Scotland). TTX standard was procured from Biomol/Affiniti Research Products (Exeter, UK).

4.4.3.2 Sample collection

Seven puffer fish samples (Lessepsian migrant puffer fish Lagocephalus sceleratus) were obtained from the National Reference Laboratory for Marine Biotoxins (NRLMB) of Greece. Five puffer fish muscle samples (Lagocephalus lunaris), which were previously associated with several outbreaks of illness in the US in 2007 (Cohen et al., 2009), were from collaborators at the FDA. TTX-free puffer fish muscle from Takifugu rubripes was also provided by FDA and used as negative tissue for matrix effects and toxin recovery experiments.

4.4.3.3 Sample extraction method

The extraction method used was previously described by Bates and co-workers (Bates et al., 1978) for the SPR analysis of PSP toxins in shellfish (Campbell et al., 2010). Briefly, 1 g portions of puffer fish homogenate were weighed into 20-mL disposable Sterilin centrifuge tubes, and 4 mL of sodium acetate buffer pH 5.0 was added. Each tube was vigorously vortex mixed for 10 s followed by roller mixing (STUART SRT9, Bibby Scientific Ltd, UK) at 33 rpm for 30 min. After mixing, samples were centrifuged at 3,600×g (SORVALL Legend RT, DJB Labcare Ltd, UK) for 10 min at room temperature. The supernatant was decanted, collected, and 40-fold diluted in MBio assay buffer at pH 7.4 (25 µL extract to 975 µL buffer). MBio assay buffer consisted of 1% BSA, 0.05% Tween 20, one PBS tablet and 200 mL of distilled water.

4.4.3.4 Synthesis of immunological reagent

TTX conjugate, TTX-jeffamine-keyhole limpet hemocyanin (TTX-jeff-KLH), was produced by combining different methods and with some modifications (Campbell et al., 2007; Huot et al., 1989; Johnson et al., 1964; Kawatsu et al., 1997; Tao et al., 2010). In brief, jeffamine was conjugated initially to KLH via amine coupling in the same manner as that previously described for BSA (Campbell et al., 2007). One mg of TTX and 50 µL of 37% formaldehyde were then added to 5 mg of jeff-KLH protein. This mixture was allowed to react for 72 h at room temperature followed by dialysis in 0.15 M saline solution (3 x 4 L) over 24 h. The production of monoclonal antibody against TTX (mAb) was previously described (Kawatsu et al., 1997). Moreover, the mAb has been further characterized and used in published works (Campbell et al., 2010; Reverté et al., 2015).

4.4.3.5 Cartridge spotting and standardized building process

Microarrays were printed using a sciFLEXARRAYER S5 spotter (Scienion, Germany) equipped with a Bio-Jet print head capable of dispensing 25-nL droplets with spot diameters of approximately 0.5 mm on the slides in a 2×22-array format. TTX conjugate diluted in sterile filtered printing buffer

(100 mM sodium phosphate, 50 mM sodium chloride, 100 µg mL-1 BSA, 0.005% Tween 20, pH 8.0) was spotted at four concentrations (10, 25, 50, and 100 µg mL-1) in replicates of two. Upon optimization, 50 and 100 µg mL-1 TTX conjugate concentrations were selected and spotted in replicates of four per cartridge for the following experiments. In addition, fluorescently labeled BSA conjugate was spotted at 4 µg mL-1 at each of the four corners of the array to enable the proper alignment within the SnapESi reader. Printing buffer drops were spotted as negative controls between BSA and TTX conjugate spots. All printing procedures were performed at room temperature and 65% humidity. Printed waveguide arrays were then left at 25 °C and 35% humidity overnight. After this, the waveguide arrays were blocked with casein solution for 5 min, rinsed with deionized water and dried by 1,000 rpm centrifugation (SORVALL Legend RT, DJB Labcare Ltd, UK) for 5 min. To fit the waveguides on the MBio reader, waveguides were inserted into cartridge housing that provided a flow channel of 5-mm-wide, 50-mm-long and 0.145-mm-high. The building process was completed with the addition of an adsorbent pad and a top cover. Cartridge components and the SnapEsi LS system were supplied by MBio Diagnostics Inc. (Boulder, Colorado, US).

4.4.3.6 Immunosensor protocol

MBio cartridges were placed on a cartridge rack at a special angle to enhance fluidic flow. The protocol was similar to that described by McGrath and co-workers (McGrath et al., 2015). Total assay time was 10 min. First, 150 µL of 1/500 mAb dilution:standard TTX or sample (1:1, v:v) were applied to the cartridge for 5 min, followed by 150 µL of 10 µg mL-1 Alexa Fluor 647 GaM IgG solution for 5 min. Each cartridge was then read on the MBio assay reader. Initially, different time exposures were collected, ranging from 25 to 100 ms of exposure. After this evaluation, readings were recorded at the fixed time point 75 ms after sample addition and were exported into Microsoft Excel for data analysis.

A stock solution of 1,000 µg L-1 was used to prepare TTX calibrants at concentrations of 0, 0.5, 1, 2.5, 5, 10 and 100 µg L-1 in MBio assay buffer. These working standards were equivalent to 0, 0.08, 0.16, 0.4, 0.8, 1.6 and 16 µg TTX g-1 puffer fish. A calibration curve was prepared for each conjugate concentration. Two cartridges (n=2) were used for each TTX working standard. Each cartridge consisted of a total of 16 spots, 8 spots per conjugate concentration. Curves were normalized with respect to the controls without TTX (0 µg L-1) and fitted to a sigmoidal logistic four-parameter equation using SigmaPlot software 12.0 (Systat Software Inc., California, US). The concentrations of TTX in the samples were then interpolated from these curves. In order to compare the response obtained with both TTX conjugate concentrations, data was first tested for normality using the Shapiro-Wilk test. For data following a normal distribution, t-test was performed; otherwise, Mann-Whitney Rank Sum Test was done. The level of significance was set at $p<0.05$. Finally, correlation between both TTX conjugate concentrations was evaluated using the linear regression model. All statistics were carried out using Sigmastat 3.1 software (Systat Software Inc., California, US).

4.4.3.7 Evaluation of puffer fish matrix effects and toxin recovery

For puffer fish matrix calibration curves, aliquots of 1 g of negative puffer fish muscle tissue homogenate were fortified with 20 µL of several concentrations of TTX and then extracted. Additionally, negative puffer tissue was extracted and then fortified with TTX (50 µL of TTX solution to 950 µL MBio assay buffer). With this procedure, puffer fish tissue and puffer fish extracts were fortified with TTX at 0, 0.08, 0.16, 0.4, 0.8, 1.6 and 16 µg TTX g-1 puffer fish. The spiking protocol was designed for the regulatory limit for PSP of 0.8 µg STX equiv. g-1 tissue to fall within the middle of the calibration curves pre and post-extraction, thus ensuring the capability of the present method to screen TTX effectively at this concentration. Both, TTX fortified-puffer fish tissue and

extracts were 40-fold diluted in MBio assay buffer and then run on the immunosensor for subsequent analysis. Curves were analyzed and compared to that prepared in buffer in order to evaluate the puffer fish matrix effects and to measure the TTX recovery under experimental conditions. Sensitivity (IC50) and dynamic range (IC20-IC80) of the assay for the three curves were obtained by fitting the data to sigmoidal, logistic, four-parameter equations. The LOD for the present work was defined as the 20% inhibitory concentration (IC20), which corresponds to the concentration of toxin required to decrease the response by 20% compared to the response when no toxin is present in the system (100% mAb binding). This was calculated from an average of two replicates of calibration curves. In order to calculate the toxin recovery, mAb binding percentages obtained at TTX fortification levels of 0.4 and 0.8 µg g-1 of puffer fish tissue were compared to the fortification levels.

4.4.3.8 Repeatability studies

The performance of the method was assessed using the PSP toxin maximum permitted level of 0.8 µg STX equivalents (equiv.) g-1 shellfish, since no regulatory limit is currently set within EC legislation for TTX. This repeatability study was carried out at two levels of TTX fortification (0.8 and 0.4 µg g-1) and by the analysis of 5 replicates (n=5) of a single puffer fish sample. Each replicate was extracted and 40-fold diluted in MBio assay buffer for analysis. Then, toxin recoveries as well as relative standard deviation (RSD) at each fortification level were calculated using the response value obtained from each individual analysis. Additionally, the same extracts were reanalyzed the following day, and toxin recoveries and RSD values at each fortification level were calculated.

4.4.3.9 Puffer fish sample analysis and comparison with analytical, biological and biochemical assays, and biosensors

Different tissues and species of puffer fish (n=12) were analyzed (16 replicates each) by the present method using the extraction method previously detailed. Once extracted, samples were 40-fold diluted in MBio assay buffer. Specifically, 2 muscle samples, 2 liver samples, 2 skin samples and 1 GI tract from 3 individuals of L. sceleratus, and 5 muscle samples from 5 individuals of L. lunaris were analyzed, and TTX contents expressed in µg g-1 were calculated and compared with other analytical techniques. Puffer fish tissues were split into two groups, named as set 1 and set 2, according to the other techniques used to analyze the samples. Therefore, set 1 includes samples 1-5 (L. lunaris samples), which were analyzed by LC-MS/MS, RBA, ELISA and SPR biosensor. Analysis methods and data of this first set of samples was previously described in the work performed by

Cohen and co-workers (Cohen et al., 2009) and Yakes and colleagues (Yakes et al., 2011b). Set 2 comprises three different fish (L. sceleratus samples) with different tissues: 6 (muscle), 7.A (GI tract), 7.B (liver), 7.C (skin), and 8.A (liver), 8.B (skin) and8.C (muscle), previously analyzed by LC-MS/MS and MBA. Analysis methods and data of the second set were described in the work performed by Katikou and co-workers (Katikou et al., 2009) and Rodríguez and colleagues (Rodriguez et al., 2012), respectively. Statistics to compare data obtained with different methods was performed following the same procedure used to compare data from calibration curves of both TTX conjugate concentrations. TTX concentrations in these samples was achieved by taking into account the mAb binding inhibitory concentration of the sample (within the dynamic range IC20-IC80) from the associated calibration curve. Those samples that showed responses above 80% were further diluted to bring their fluorescence signals within the dynamic range of the calibration curve.

4.4.4 Results and discussion

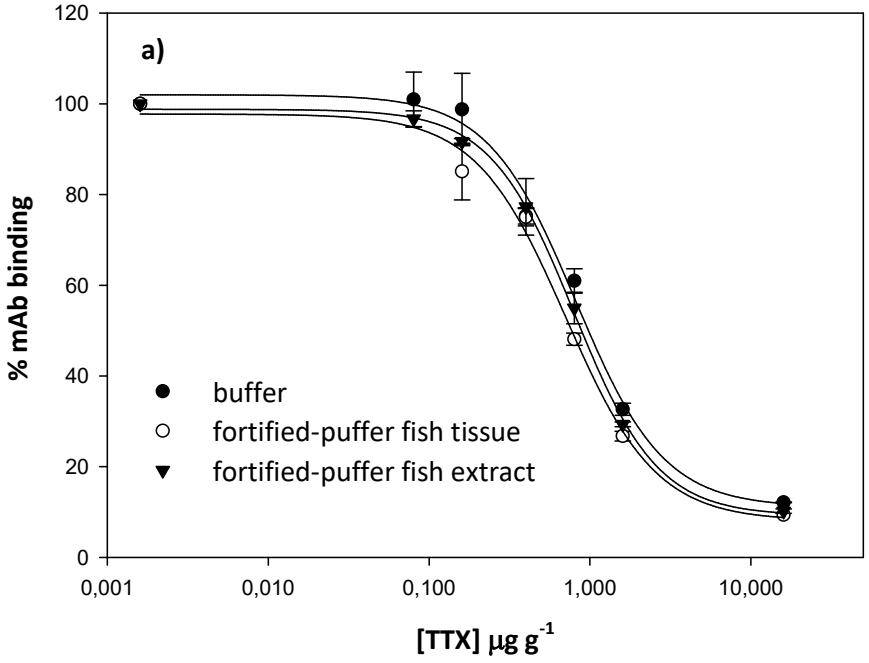
Given the increasing concern about the prevalence of TTXs in fish and shellfish products in European waters and the high potency of these toxins, increased monitoring through the development of rapid, sensitive and robust methods is needed to ensure human health protection. With the aim of replacing animal use methods such as the MBA for TTX detection, in the present work the development of a sensitive planar waveguide-based biosensor able to detect TTX in multiple puffer fish tissues is described.

4.4.4.1. Immunosensor protocol optimization and calibration curve

Following the improved MBio procedure described by McGrath and co-workers (McGrath et al., 2015), TTX-jeff-KLH conjugate concentration and mAb dilution were firstly optimized. Of the four spotted TTX conjugate concentrations (10, 25, 50 and 100 µg mL^{-1}), those that gave a fluorescence value below 150 or above 500 when no TTX was present, were discarded. Following this criteria, 10 and 25 µg mL -1 TTX conjugate concentrations were no longer used. As such, given that the spotting of TTX conjugate at 50 and 100 µg mL^{-1} provided similar responses, both concentrations were selected for comparison in subsequent experiments. The capacity of the immobilized TTX conjugates to inhibit the mAb binding response was then assessed using 1/250 and 1/500 mAb dilutions at 100 µg L-1 of TTX standard. At both antibody dilutions, this TTX concentration was able to completely inhibit the mAb binding. However, as a lower antibody concentration generally results in a higher sensitivity, the 1/500 mAb dilution was selected for the assay. With the experimental parameters set at TTX conjugate spotting concentrations of 50 and 100 µg mL -1, mAb

at 1/500 dilution and fluorescently-labeled GaM IgG antibody at 10 µg mL-1 (concentration set through checkerboard titration in the work performed by Meneely and colleagues (Meneely et al., 2013)), the whole calibration curves were performed in buffer.

Calibration curves obtained for both TTX conjugate spotting concentrations in MBio assay buffer are shown in **Fig. 4.4.2** a and b, and, the midpoint (IC50), limit of detection (LOD) and dynamic range determined are shown in **Table 4.4.1**. The IC50 and LOD values were fairly similar at both spotting concentrations (P=0.797; T=0.275) with a correlation of y=1.45x-1.72. A narrower dynamic range was obtained with 100 µg mL-1 of TTX conjugate, likely due to the binding kinetics effects of reduced competition in binding with additional coating antigen on the arrayed surface. IC50 and LOD values provided by this new immunosensor (6.0 and 2.5 µg L-1, respectively), compared well with a previous indirect SPR immunosensor that used the same antibody (6.6 and 2.4 µg L-1, respectively) (Campbell et al., 2013), as well as with an SPR biosensor that employed a commercial antibody (approximately 6 and 0.3 µg L-1, respectively) (Taylor et al., 2008). Further, the IC50 values of the sensor developed herein were better than those recently reported for other indirect SPR immunosensors for TTX on a commercial instrument ((IC50 of 28.9 and IC20 of 7.8 µg L-1) (Yakes et al., 2011b) and from the average of three laboratories (IC50 of 74 and IC20 of 14 µg L-1) (Vaisocherova et al., 2011)). As such, the sensitivity achieved in buffer was determined to be suitable for further assay development and the evaluation of matrix effects.



[TTX conjugate]	Matrix	TTX fortification level	
		0.8 µg g^{-1}	**0.4 µg g^{-1}**
50 µg mL^{-1}	fortified-puffer fish tissue	0.82 ± 0.03 (102.5%)	0.35 ± 0.02 (87.5%)
	fortified-puffer fish extract	0.78 ± 0.06 (97.5%)	0.40 ± 0.09 (100.0%)
100 µg/mL^{-1}	fortified-puffer fish tissue	0.81 ± 0.03 (101.3%)	0.35 ± 0.05 (87.5%)
	fortified-puffer fish extract	0.77 ± 0.06 (96.3%)	0.38 ± 0.01 (95.0%)

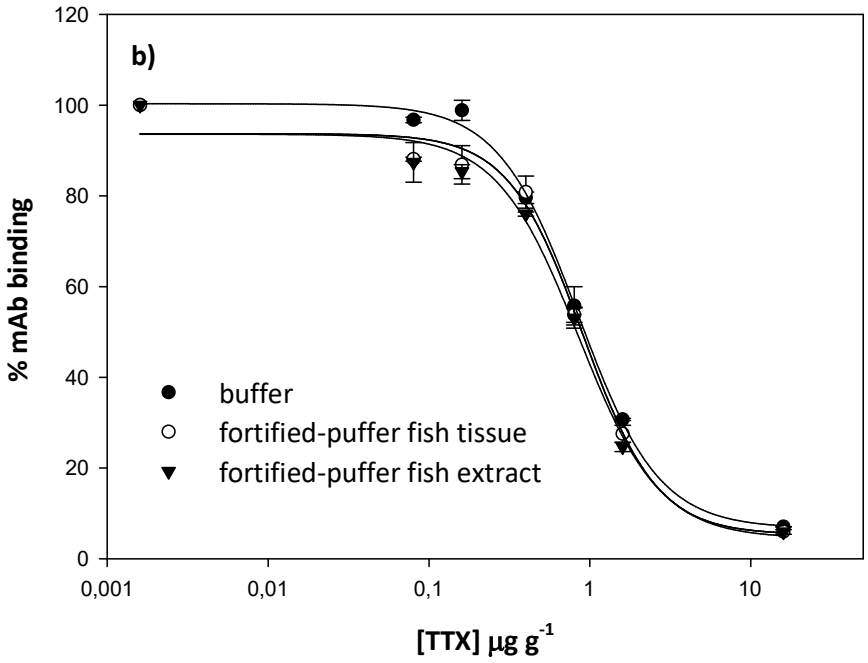


Fig. 4.4.2. Calibration curves in buffer and fortified-puffer fish tissues and extracts obtained for a) 50 µg mL^{-1} and b) 100 µg mL^{-1} TTX-conjugate. Curves normalized to mAb signal when no TTX is present. Standard deviations for 16 replicates are represented by the error bars.

Table 4.4.1. Midpoint of the curve (IC_{50}), LOD (IC_{20}) and dynamic range (IC_{20}–IC_{80}) values ± SD provided by the planar waveguide biosensor in buffer for both TTX conjugate concentrations and for 16 replicates.

4.4.4.2. Evaluation of puffer fish matrix effects and toxin recovery

In order to demonstrate the applicability of the method to the analysis of real samples, matrix effects of puffer fish and of the extraction protocol procedure were investigated through spiking TTX into puffer fish extracts and into puffer fish fish tissue homogenates, respectively. Calibration curves obtained for both TTX conjugate spotting concentrations in buffer and in puffer fish matrix fortified pre and post-extraction are shown in figure 2. As the curves essentially overlap in the dynamic range of the assay, it can be concluded that there were little matrix effects from the puffer fish tissue and extraction procedure when these are diluted and run on the immunosensor. Furthermore, TTX concentrations and toxin recoveries obtained pre and post-extraction are shown in **Table 4.4.2**.

Table 4.4.2. TTX concentration ± standard deviation for 16 replicates in fortified-puffer fish tissue and extracts determined by the planar waveguide biosensor. Toxin recovery is expressed in percentages (%) and calculated with reference to the TTX fortification levels of 0.4 and 0.8 µg TTX g^{-1} puffer fish.

TTX concentrations determined in fortified-puffer fish extracts were similar to those obtained in buffer (P=1.000; T=5.000). Statistically, no significant differences were found between the 50 or 100 µg mL-1 TTX conjugate surfaces for both fortification levels (P=0.667; T=6.000; y=0.98x+0.01). These similar results demonstrate that there are negligible puffer fish matrix effects on the system.

With regard to TTX concentrations determined when spiking TTX in puffer fish tissues, appropriate recovery values were obtained, and these concentrations were similar to the TTX fortification levels (P=1.000; T=5.000). These accurate recovery values demonstrated the good efficiency of the extraction protocol and that there were limited toxin losses during the extraction process. Again, no significant differences were found between using 50 or 100 µg mL-1 of TTX conjugate (P=0.667; T=5.500; y=1.03x-0.03). TTX concentrations obtained in fortified-puffer fish tissues were also similar to that in fortified-puffer fish extracts (P=1.000; T=5.000), reaffirming the negligible matrix effects on the biosensor and that accurate results from natural samples could be obtained.

[TTX conjugate]	Midpoint (IC_{50}) µg L^{-1}	LOD (IC_{20}) µg L^{-1}	Dynamic range (IC_{20}-IC_{80}) µg L^{-1}
50 µg mL^{-1}	6.1 ± 0.5	2.5 ± 0.1	2.5 (± 0.1)-20.5 (± 2.2)
100 µg mL^{-1}	5.8 ± 0.3	2.6 ± 0.1	2.6 (± 0.1)-15.2 (± 1.7)

4.4.4.3 Repeatability studies

The five homogenate-tissue samples fortified at 0.8 as well as five at 0.4 μg g-1 extracted and analyzed over two days, showed acceptable toxin recovery values. Specifically, results expressed in percent values with respect to the TTX-fortified concentration (0.8 or 0.4 μg g-1) and determined from the calibration curve in buffer were 89.1 and 78.5% for TTX-conjugate spotted at 50 μg mL-1 and 106.3 and 106.8% for 100 μg mL-1 of conjugate, respectively, on day 1. Similarly, the same extracts reanalyzed on day 2 provided toxin recoveries of 81.5 and 97.6% for 50 μg mL-1 of conjugate and 96.6 and 107.3% for 100 μg mL-1 of conjugate. To assess the repeatability of the present sensor, precision was expressed as the RSD of the results , according to the 2012 AOAC International Guidelines (AOAC, 2012). Hence, on day 1, RSD values with respect to 50 μg mL-1 of TTX conjugate were 14.6% for 0.8 μg g-1 and 10.8% for 0.4 μg g-1, while values were 9.9% for the 0.8 μg g-1 and 2.1% for the 0.4 μg g-1 for TTX conjugate 100 μg mL-1. On day 2, RSD values were 12.5 and 8.3% for 50 μg mL-1 of TTX conjugate and, 10.4 and 13.1 for 100 μg mL-1 of TTX conjugate at 0.8 and 0.4 μg g -1, respectively. Considering the expected precision as a function of analyte concentration, RSD values for 0.8 and 0.4 μg toxin g-1 puffer fish tissue should be lower than 15%, and all the obtained RSD values for this assay were below this value. It should be noted that this was a comparison of extracts between days and not re-extraction of the fish due to limitations in toxin availability. Nevertheless, these results illustrate the degree of repeatability in analysis. Additionally, the toxin recovery values obtained in same extracts on day 2, demonstrate that samples containing TTX were stable at least for 1 day. All these results demonstrated that the new planar waveguide biosensor allowed for suitable precision under repeatability conditions within independent tests and that already extracted samples could be reanalyzed on the following day with the same precision. Additionally, differences between values obtained with both TTX conjugate concentrations were statistically not significant. The good agreement of the results provided by this repeatability study at 0.8 and 0.4 μg TTX g-1 fish tissue confirmed the feasibility and reliability of the planar waveguide biosensor for being applied to the analysis of real samples.

4.4.4.4 Puffer fish samples analyses and comparison with other methods

Table 4.4.3 shows the TTX concentrations determined per g of puffer fish tissue by the nanoarray-based biosensor as well as by other analytical and biochemical methods. In general, TTX contents determined in the samples provided by the present biosensor correlated well with the data reported by the other methods.

Among the 12 puffer fish samples analyzed, 4 samples (1 and 5 muscle tissues from L. lunaris, and 8.B (skin) and 8.C (muscle) from *L. sceleratus*) were found to contain TTX contents below the regulated Japanese level of 2 µg TTX g-1 fish tissue by all the techniques. With respect to the other samples, within the L. lunaris samples (Set 1, Table 4.4.3a), the trend from the most to the lowest toxic muscle tissue was: 4 > 3 > 2 > 1 > 5 for all the techniques employed. The high concentrations of TTX found in muscle tissue extracts 3 and 4 from L. lunaris individuals were in accordance with the high contents of TTX found in the meat of this species of puffer fish, which is a causative source of food poisonings worldwide (Yang et al., 1996). On the contrary, the distribution of TTX found among the L. sceleratus tissues from the most to the lowest toxicity was: GI-tract (7.A) > liver (7.B, 8.A) > skin (7.C, 8.B) ≥ muscle (6, 8.C). Again, the toxin distribution found within tissues agrees well with that reported for L. sceleratus species (Jang and Yotsu-Yamashita, 2006; Noguchi and Arakawa, 2008), as the gastrointestinal tract is expected to be the most toxic, followed by the liver and finally by the skin and muscle.

In the analysis of the first set of samples (1-5), excellent correlation was obtained between the TTX contents determined by the planar-waveguide biosensor at both conjugate concentrations ($y=1.06x-0.11$; $R^2=0.996$; P=1.000; T=27.500). Because of this similarity (differences statistically not significant) and to simplify the results, the comparison with other methods was only performed with reference to the 100 µg mL-1 conjugate surface. When comparing the quantifications provided by the planar waveguide biosensor with those LC-MS/MS values reported previously in Cohen and co-workers (Cohen et al., 2009) and Yakes and colleagues (Yakes et al., 2011b), higher TTX contents were reported by the biosensor with a correlation of $y=1.70x-0.08$ ($R^2=0.987$) obtained, although the differences were statistically not significant (P=0.517; T=0.677). The higher TTX concentrations in the L. lunaris muscle samples determined by the biosensor could be attributed to the fact that the previous LC-MS/MS quantifications for these samples were determined solely for TTX while the mAb used herein may react with multiple TTX analogues potentially present in the puffer fish samples. Rodriguez and co-workers (Rodriguez et al., 2012) found multiple TTX analogs in the closely related species L. sceleratus, several of which were in higher concentration than TTX itself. Most of these analogs are less potent than TTX, as evidenced by the poor correlation to MBA data in that study. It is not currently known if these same TTX analogs are also present in L. lunaris and what their contributions are to total toxicity. This possibility was supported by the correlation obtained by RBA and the planar-waveguide biosensor ($y=1.32x-0.36$; $R^2=0.994$; P=0.757; T=0.320). The high TTX concentration achieved by the biosensor in this case could also be due to the different

affinity between the two biorecognition molecules (mAb for the waveguide and sodium-channel receptor for the RBA) as well as the final reporting element (fluorescently labeled secondary antibody for the waveguide and H3-STX for the RBA).

The same trend was observed when comparing the waveguide assay data to that obtained by ELISA ($y=1.12x+0.36$; $R^2=0.933$; $P=0.489$; $T=0.725$) and SPR immunosensor ($y=1.70x+0.02$; $R^2=0.986$; $P=0.510$; $T=0.690$). Although data obtained by the biosensor was similar to that obtained by ELISA and SPR immunosensor, small differences in determining TTX content can similarly be attributed to differences in biorecognition elements and transduction techniques. Specifically, in the SPR biosensor the TTX was chemically immobilized on dextran chips, and the signal was measured in real time. In ELISA and the waveguide sensor, TTX-conjugate was coated on wells and on nanoarrays, respectively, which likely resulted in the slightly different responses. Furthermore, although both ELISA and the biosensor are indirect strategies that use a secondary labeled antibody, the final output is given by the absorbance of an enzyme in ELISA and by fluorescence in the biosensor. Although TTX contents determined by the planar waveguide biosensor were slightly higher than those reported by the other techniques, they were still in good agreement. Within this set of samples, the trend from higher to lower TTX concentrations was the same by all techniques.

With regard to the second set of samples, excellent correlation was obtained between data provided by the present biosensor for both conjugate concentrations ($y=1.07x-0.39$; $R^2=0.998$), with no significant statistical difference ($P=0.902$; $T=51.00$). As for the first set of samples, data provided by the biosensor using 100 µg mL-1 of conjugate was used for further comparison. As observed in other works (Reverté et al., 2015), TTX concentrations determined by the planar waveguide biosensor were generally slightly lower than those reported by MBA, but the data were still in good agreement ($y=0.74x+0.42$; $R^2=0.987$), with the differences statistically not significant ($P=1.000$; $T=27.000$). The overestimation of the TTX contents provided by MBA is attributed to the different principle of the techniques; specifically, MBA reports global toxicity using animal models and could detect co-existing toxins other than TTXs. Moreover, since no cross-reactivity data of the TTX analogues is available for this biosensor and antibody and toxin equivalency factors (TEFs) are not well-established, the biosensor response was not necessarily expected to fully match the MBA data.

Different scenarios were assumed when comparing the TTX contents obtained with the current biosensor to those determined by LC-MS/MS analysis. If only individual TTX is evaluated, the data

correlated quite well (R2=0.945) but much higher TTX contents were determined by the biosensor (y=3.16x+0.76). These differences in the concentrations, although statistically not significant (P=0.310; T=1.109), seem to indicate that some of the TTX analogues detected in the LC-MS/MS were also recognized by the antibody. In the second scenario, TTX analogues were considered to have the same mAb affinity as TTX, thus with a cross-reactivity factor (CRF) of 1. Unfortunately, worse correlation (R2=0.702) and higher overestimation (y=8.83x+28.91) were obtained compared to LC-MS/MS analysis using this scenario. This underestimation of LC-MS/MS analysis with respect to the planar waveguide biosensor suggests that not all the TTX analogues were recognized by the antibody in the MBio biosensor, or they are recognized with low affinity. Looking at the LC-MS/MS individual concentrations, it seems evident that the analogues 5,6,11-trideoxy-TTX (1 and 2) cannot be recognized by the antibody at the same extent as TTX (i.e., much higher quantifications would have been obtained with the planar waveguide biosensor for 7.A (GI tract) and 7.B (liver) samples). Moreover, this analogue has been considered almost non-toxic by other authors (Jang and Yotsu-Yamashita, 2006).

As an intermediate approach, only 4-epiTTX, 4,9-anhydroTTX, 5-deoxyTTX and 11-deoxyTTX were theorized to be recognized by the antibody with same affinity than TTX. In this case, excellent correlations (R2=0.954) and similar quantifications (y=1.07x+0.68) were obtained between MBio biosensor and LC-MS/MS analysis (P=0.690; T=30.000). Of course, this criterion was used only to demonstrate that results can change to a high extent depending on the analogues considered. Ideally, and according to what has been previously published (Reverté et al., 2015), to better compare the concentrations obtained by the biosensor to that provided by LC-MS/MS, CRFs of the antibody towards co-existing TTX analogues should be established and applied to the individual measurements determined by LC-MS/MS. Unfortunately, TTX analogue standards were not available to investigate this issue in detail. As for the general results, the biosensor, MBA and LC-MS/MS were in agreement in determining the gastrointestinal tract as the most toxic tissue, followed by the liver tissue of L. sceleratus species. Due to the low TTX content, it was more difficult to observe a trend in muscle and skin tissues by the different methods used herein.

Notably, the present biosensor was proven to be useful as screening tool and was also capable of rapidly (in 10 min) detecting trace TTX contents, thereby meeting the requirements in terms of sensitivity not only of the Japanese regulation but also of the similar mode-of-action PSP toxins legislation.

Table 4.4.3. TTX concentration (µg TTX g^{-1} puffer fish tissue) determined in **a)** 5 sample tissues from *L. lunaris* and **b)** 7 sample tissues from *L. sceleratus* by the MBio biosensor and comparison with other methods (Katikou et al., 2009; Rodriguez et al., 2012; Yakes et al., 2011)

a)

µg TTX g^{-1} puffer fish tissue						
Sample code	MBio biosensor		LC-MS/MS (TTX) (Yakes et al., 2011)	RBA (Yakes et al., 2011)	ELISA (Yakes et al., 2011)	SPR (Yakes et al., 2011)
	TTX conjugate 50 µg mL^{-1}	TTX conjugate 100 µg mL^{-1}				
1	0.27 ± 0.07	0.31 ± 0.04	0.10	0.18	0.04	0.07
2	3.38 ± 0.08	3.23 ± 0.15	2.14	3.52	2.68	2.47
3	13.26 ± 0.64	13.49 ± 0.64	8.76	10.50	14.12	7.09
4	19.02 ± 5.19	17.50 ± 5.03	9.61	13.24	12.48	10.74
5	0.24 ± 0.03	0.24 ± 0.07	0.10	0.16	0.02	0.07

b)

µg TTX g^{-1} puffer fish tissue													
Sample code	MBio biosensor		MBA (Katikou et al., 2009)	LC-MS/MS (Rodriguez et al., 2012)									
	TTX conjugate 50 µg mL^{-1}	TTX conjugate 100 µg mL^{-1}		TTX	4-epiTTX	4,9-anhydroTTX	5-deoxyTTX	11-deoxyTTX	11-norTTX-6(R)-ol	11-norTTX-6(S)-ol	5,6,11-trideoxyTTX (1)	5.6.11-trideoxyTTX (2)
6	2.71 ± 0.79	2.73 ± 0.54	1.69	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	1.20	2.30
7.A	41.07 ± 8.49	43.72 ± 4.88	56.78	12.05	2.96	1.97	3.67	17.45	3.53	16.50	109.75	220.75
7.B	10.18 ± 1.64	9.08 ± 0.99	16.12	2.39	0.47	0.52	0.76	2.55	0.36	4.38	94.00	192.25
7.C	2.99 ± 0.49	3.07 ± 0.33	2.42	0.65	<LOD	<LOQ	<LOQ	0.56	1.08	0.83	23.55	56.50
8.A	6.64 ± 0.72	6.73 ± 0.84	10.84	4.20	0.46	0.63	1.62	5.50	3.16	8.70	0.71	4.07
8.B	1.34 ± 0.45	1.34 ± 0.24	<1.10	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
8.C	1.41 ± 0.23	1.41 ± 0.07	<1.10	<LOQ	n.d.	<LOD	<LOQ	0.54	0.47	0.48	<LOQ	<LOQ

LOQ: 0.32 µg g^{-1}; LOD: 0.08 µg g^{-1}; n.d.: not detected

4.4.5 Conclusions

The present work reported the development of a rapid and specific nanoarray planar waveguide biosensor for the determination of TTX concentration in puffer fish samples. The biosensor attained good sensitivity (LOD of 2.5 μg L^{-1}), thus being capable of detecting TTXs as low as 0.4 μg g^{-1} puffer fish. This concentration is below the EU and US regulatory limit of 0.8 μg STX equiv. g^{-1} shellfish meat for the similar PSP toxins and can be used as a guide. Additionally, the negligible effects of puffer fish matrix on the biosensor and the excellent toxin recoveries obtained reinforce the reliability and applicability of the innovative method. This is further evidenced by the general agreement found between the TTX concentrations determined by the biosensor and the other methods (LC-MS/MS, RBA, ELISA, MBA and SPR biosensor) in the analysis of naturally-contaminated puffer fish samples of different species.

This biosensor configuration presents multiple benefits, including simplicity and rapidity of the assay, design versatility, small reagent volumes, good reproducibility, and accurate and precise toxin quantifications. In addition to these advantages, this nanoarrayed configuration could be used together with other related toxins for simultaneous multi-toxin detection, which represents a breakthrough in the development of compact, multiplexed devices. Despite the planar waveguide technology not being new per se, this is the first immunosensor using this technique that has been specifically developed for TTX. Thus, this new approach can be considered as a proof of concept, being not only applicable to TTX but also to other emerging toxins of seafood safety and environmental surveillance fields.

On the whole, the biosensor described herein has been proven to be a promising high-throughput sample screening tool for implementation in research and monitoring programs, being able to analyze multiple samples with toxin contents below the permitted levels of concern. Furthermore, taking into consideration the presence of TTX recently reported in European shellfish mollusks of England, Greece and Netherlands, the biosensor developed herein could have suitable sensitivity, at lower proposed action levels, to be applicable to shellfish by only slightly modifying the sample preparation.

4.4.6 References

Alcaraz, A., Whipple, R.E., Gregg, H.R., Andresen, B.D., Grant, P.M., 1999. Analysis of tetrodotoxin. Forensic Sci. Int. 99, 35-45.

AOAC, 2012. Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals. http://www.aoac.org/Official_Methods/slv_guidelines.pdf. Accessed by October 2016.

Asakawa, M., Shida, Y., Miyazawa, K., Noguchi, T., 2012. Chromatography - The Most Versatile Method of Chemical Analysis. Instrumental Analysis of Tetrodotoxin. InTech Open Science, Rijeka, Croatia, p. 245.

Bates, H.A., Kostriken, R., Rapoport, H., 1978. Chemical assay for saxitoxin - improvements and modifications. J. Agric. Food Chem. 26, 252-254.

Bentur, Y., Ashkar, J., Lurie, Y., Levy, Y., Azzam, Z.S., Litmanovich, M., Golik, M., Gurevych, B., Golani, D., Eisenman, A., 2008. Lessepsian migration and tetrodotoxin poisoning due to Lagocephalus sceleratus in the eastern Mediterranean. Toxicon 52, 964-968.

Campbell, K., Barnes, P., Haughey, S.A., Higgins, C., Kawatsu, K., Vasconcelos, V., Elliott, C.T., 2013. Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. Anal. Bioanal. Chem. 405, 7753-7763.

Campbell, K., Haughey, S.A., van den Top, H., van Egmond, H., Vilarino, N., Botana, L.M., Elliott, C.T., 2010. Single Laboratory Validation of a Surface Plasmon Resonance Biosensor Screening method for Paralytic Shellfish Poisoning Toxins. Anal. Chem. 82, 2977-2988.

Campbell, K., Stewart, L.D., Doucette, G.J., Fodey, T.L., Haughey, S.A., Vilarino, N., Kawatsu, K., Elliott, C.T., 2007. Assessment of specific binding proteins suitable for the detection of paralytic shellfish poisons using optical biosensor technology. Anal. Chem. 79, 5906-5914.

Cohen, N.J., Deeds, J.R., Wong, E.S., Hanner, R.H., Yancy, H.F., White, K.D., Thompson, T.M., Wahl, M., Pham, T.D., Guichard, F.M., Huh, I., Austin, C., Dizikes, G., Gerber, S.I., 2009. Public Health Response to Puffer Fish (Tetrodotoxin) Poisoning from Mislabeled Product. Journal of Food Protection 72, 810-817.

Cole, J.B., Heegaard, W.G., Deeds, J.R., McGrath, S.C., Handy, S.M., 2015. Tetrodotoxin Poisoning Outbreak from Imported Dried Puffer - Fish Minneapolis, Minnesota, 2014. Mmwr-Morbidity and Mortality Weekly Report 63, 1222-1225.

FDA, 2015. Import Alert #16-20: Detention without physical examination of puffer fish and foods that contain puffer fish. http://www.accessdata.fda.gov/cms_ia/importalert_37.html Accessed by October 2016.

FDA, U., 1989. Exchange of letters between Japan and US Food and Drug Administration regarding puffer fish. *In* International cooperative agreements manual. p. 57-73. Available at: http://www.fda.gov/InternationalPrograms/Agreements/MemorandaofUnderstanding/ucm107601.htm. Accessed 9 October 2016.

French, R.J., Yoshikami, D., Sheets, M.F., Olivera, B.M., 2010. The Tetrodotoxin Receptor of Voltage-Gated Sodium Channels-Perspectives from Interactions with mu-Conotoxins. Marine Drugs 8, 2153-2161.

Huot, R.I., Armstrong, D.L., Chanh, T.C., 1989. Protection against nerve toxicity by monoclonal-antibodies to the sodium-channel blocker. J. Clin. Invest. 83, 1821-1826.

Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish Fugu pardalis. Toxicon 48, 980-987.

Johnson, H.M., Campbell, J.E., Angelotti, R., Lewis, K.H., Frey, P.A., 1964. Haptenic properties of paralytic shellifsh poison conjugated to proteins by formaldehyde treatment. Proc. Soc. Exp. Biol. Med. 117, 425-&.

Katikou, P., Georgantelis, D., Sinouris, N., Petsi, A., Fotaras, T., 2009. First report on toxicity assessment of the Lessepsian migrant pufferfish Lagocephalus sceleratus (Gmelin, 1789) from European waters (Aegean Sea, Greece). Toxicon 54, 50-55.

Kawabata, T., 1978. The manual for the methods of food sanitation tests . Bureau of Environmental Health, Ministry of Health and Welfare, Japan Food Hygienic Association, Tokyo, Japan, pp. 232-240.

Kawatsu, K., Hamano, Y., Yoda, T., Terano, Y., Shibata, T., 1997. Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. Jpn. J. Med. Sci. Biol. 50, 133-150.

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

Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: Collaborative study. J. AOAC Int. 88, 1714-1732.

Lee, C.H., Ruben, P.C., 2008. Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. Channels 2, 407-412.

McGrath, T.F., McClintock, L., Dunn, J.S., Husar, G.M., Lochhead, M.J., Sarver, R.W., Klein, F.E., Rice, J.A., Campbell, K., Elliott, C.T., 2015. Development of a rapid multiplexed assay for the direct screening of antimicrobial residues in raw milk. Anal. Bioanal. Chem. 407, 4459-4472.

McNabb, P.S., Taylor, D.I., Ogilvie, S.C., Wilkinson, L., Anderson, A., Hamon, D., Wood, S.A., Peaice, B.M., 2014. First Detection of Tetrodotoxin in the Bivalve Paphies australis by Liquid Chromatography Coupled to Triple Quadrupole Mass Spectrometry With and Without Precolumn Reaction. J. AOAC Int. 97, 325-333.

McNamee, S.E., Elliott, C.T., Greer, B., Lochhead, M., Campbell, K., 2014. Development of a Planar Waveguide Microarray for the Monitoring and Early Detection of Five Harmful Algal Toxins in Water and Cultures. Environ. Sci. Technol. 48, 13340-13349.

Meneely, J.P., Campbell, K., Greef, C., Lochhead, M.J., Elliott, C.T., 2013. Development and validation of an ultrasensitive fluorescence planar waveguide biosensor for the detection of paralytic shellfish toxins in marine algae. Biosens. Bioelectron. 41, 691-697.

Moczydlowski, E.G., 2013. The molecular mystique of tetrodotoxin. Toxicon 63, 165-183.

Neagu, D., Micheli, L., Palleschi, G., 2006. Study of a toxin-alkaline phosphatase conjugate for the development of an immunosensor for tetrodotoxin determination. Anal. Bioanal. Chem. 385, 1068-1074.

Noguchi, T., Arakawa, O., 2008. Tetrodotoxin - Distribution and accumulation in aquatic organisms, and cases of human intoxication. Marine Drugs 6, 220-242.

Noguchi, T., Onuki, K., Arakawa, O., 2011. Tetrodotoxin poisoning due to pufferfish and gastropods, and their intoxication mechanism. ISRN toxicology 2011, 276939-276939.

Pratheepa, V., Vasconcelos, V., 2013. Microbial diversity associated with tetrodotoxin production in marine organisms. Environmental Toxicology and Pharmacology 36, 1046-1054.

Raybould, T.J.G., Bignami, G.S., Inouye, L.K., Simpson, S.B., Byrnes, J.B., Grothaus, P.G., Vann, D.C., 1992. A monoclonal antibody-based immunoassay for detecting tetrodotoxin in biological samples. J. Clin. Lab. Anal. 6, 65-72.

Reverté, L., de la Iglesia, P., del Río, V., Campbell, K., Elliott, C.T., Kawatsu, K., Katikou, P., Diogène, J., Campàs, M., 2015. Detection of Tetrodotoxins in Puffer Fish by a Self-Assembled Monolayer-Based Immunoassay and Comparison with Surface Plasmon Resonance, LC-MS/MS, and Mouse Bioassay (vol 87, pg 10839, 2015). Anal. Chem. 88, 2511-2511.

Rodriguez, P., Alfonso, A., Otero, P., Katikou, P., Georgantelis, D., Botana, L.M., 2012. Liquid chromatography-mass spectrometry method to detect Tetrodotoxin and Its analogues in the puffer fish Lagocephalus sceleratus (Gmelin, 1789) from European waters. Food Chemistry 132, 1103-1111.

Rodriguez, P., Alfonso, A., Vale, C., Alfonso, C., Vale, P., Tellez, A., Botana, L.M., 2008. First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell Charonia lampas lampas in Europe. Anal. Chem. 80, 5622-5629.

Stokes, A.N., Williams, B.L., French, S.S., 2012. An improved competitive inhibition enzymatic immunoassay method for tetrodotoxin quantification. Biol. Proced. Online 14, 3.

Tao, J., Wei, W.J., Nan, L., Lei, L.H., Hui, H.C., Fen, G.X., Jun, L.Y., Jing, Z., Rong, J., 2010. Development of competitive indirect ELISA for the detection of tetrodotoxin and a survey of the distribution of tetrodotoxin in the tissues of wild puffer fish in the waters of south-east China. Food Addit. Contam. Part A 27, 1589-1597.

Taylor, A.D., Ladd, J., Etheridge, S., Deeds, J., Hall, S., Jiang, S.Y., 2008. Quantitative detection of tetrodotoxin (TTX) by a surface plasmon resonance (SPR) sensor. Sens. Actuator B-Chem. 130, 120-128.

Taylor, A.D., Vaisocherova, H., Deeds, J., DeGrasse, S., Jiang, S., 2011. Tetrodotoxin Detection by a Surface Plasmon Resonance Sensor in Pufferfish Matrices and Urine. Journal of Sensors.

Tsuda, K., Tachikawa, R., Tamura, C., Ikuma, S., Kawamura, M., Sakai, K., Amakasu, O., 1964. On structure of tetrodotoxin. Chemical & Pharmaceutical Bulletin 12, 1357-1374.

Turner, A.D., Higgins, C., Higman, W., Hungerford, J., 2015b. Potential Threats Posed by Tetrodotoxins in UK Waters: Examination of Detection Methodology Used in Their Control. Marine Drugs 13, 7357-7376.

Turner, A.D., Powell, A., Schofield, A., Lees, D.N., Baker-Austin, C., 2015a. Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance 20, 2-8.

Vaisocherova, H., Taylor, A.D., Jiang, S., Hegnerova, K., Vala, M., Homola, J., Yakes, B.J., Deeds, J., DeGrasse, S., 2011. Surface Plasmon Resonance Biosensor for Determination of Tetrodotoxin: Prevalidation Study. J. AOAC Int. 94, 596-604.

Vlamis, A., Katikou, P., Rodriguez, I., Rey, V., Alfonso, A., Papazachariou, A., Zacharaki, T., Botana, A.M., Botana, L.M., 2015. First Detection of Tetrodotoxin in Greek Shellfish by UPLC-MS/MS Potentially Linked to the Presence of the Dinoflagellate Prorocentrum minimum. Toxins 7, 1779-1807.

Wang, R., Huang, A., Liu, L., Xiang, S., Li, X., Ling, S., Wang, L., Lu, T., Wang, S., 2014. Construction of a single chain variable fragment antibody (scFv) against tetrodotoxin (TTX) and its interaction with TTX. Toxicon 83, 22-34.

Woodward, R.B., Gougouta, J.Z., 1964. Structure of tetrodotoxin. Journal of the American Chemical Society 86, 5030-&.

Yakes, B.J., Deeds, J., White, K., DeGrasse, S.L., 2011a. Evaluation of Surface Plasmon Resonance Biosensors for Detection of Tetrodotoxin in Food Matrices and Comparison to Analytical Methods. Journal of Agricultural and Food Chemistry 59, 839-846.

Yakes, B.J., Deeds, J., White, K., DeGrasse, S.L., 2011b. Evaluation of Surface Plasmon Resonance Biosensors for Detection of Tetrodotoxin in Food Matrices and Comparison to Analytical Methods. J. Agric. Food Chem. 59, 839-846.
Yakes, B.J., Kanyuck, K.M., DeGrasse, S.L., 2014. First Report of a Direct Surface Flasmon Resonance Immunosensor for a Small Molecule Seafood Toxin. Anal. Chem. 86, 9251-9255.
Yang, C.C., Liao, S.C., Deng, J.F., 1996. Tetrodotoxin poisoning in Taiwan: An analysis of poison center data. Vet. Human Toxicol. 38, 282-286.
Yotsu-Yamashita, M., Sugimoto, A., Takai, A., Yasumoto, T., 1999. Effects of specific modifications of several hydroxyls of tetrodotoxin on its affinity to rat brain membrane. Journal of Pharmacology and Experimental Therapeutics 289, 1688-1696.
Zhou, Y., Li, Y.S., Pan, F.G., Liu, Z.S., Wang, Z., 2009. Identification of tetrodotoxin antigens and a monoclonal antibody. Food Chem. 112, 582-586.

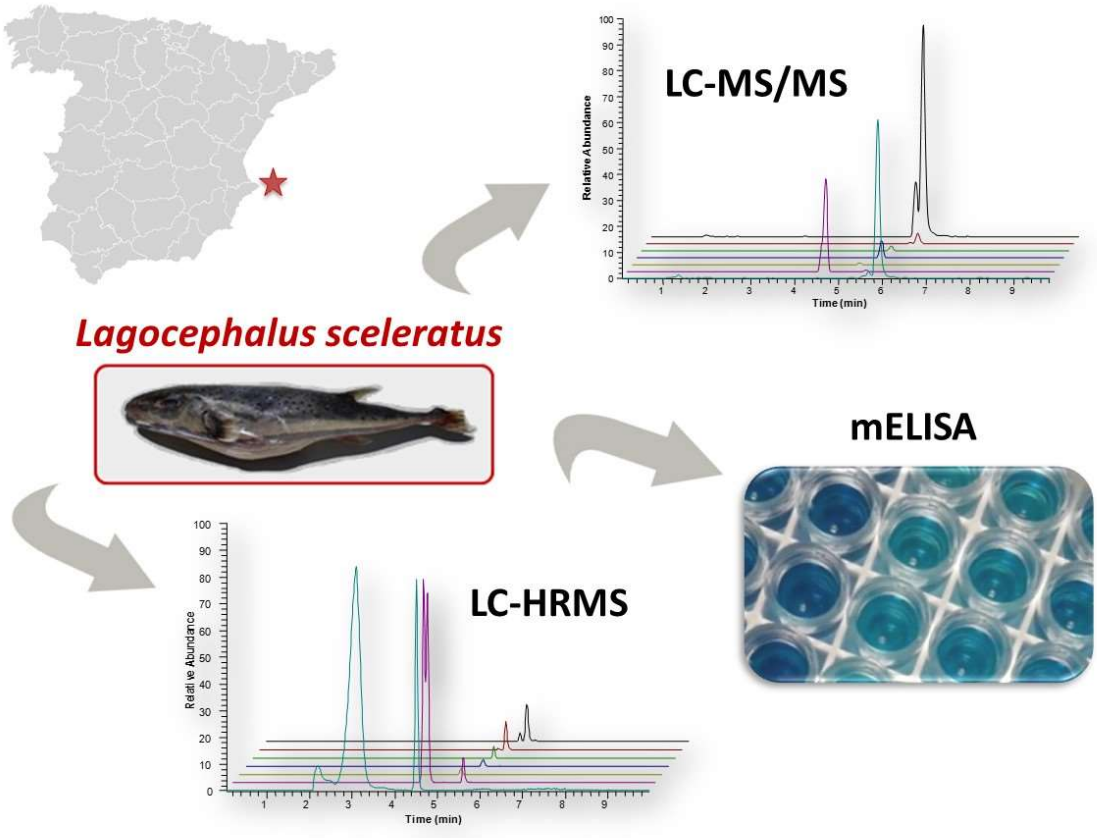
Environmental Research

Volume 158, October 2017, Pages 1–6

Evaluation of tetrodotoxins in puffer fish caught along the Mediterranean coast of Spain. Toxin profile of *Lagocephalus sceleratus*

Maria Rambla-Alegre[a], Laia Reverté[a], Vanessa del Río[a], Pablo de la Iglesia[a], Oscar Palacios[b], Cintia Flores[b], Josep Caixach[b], Katrina Campbell[c], Christopher T. Elliott[c], Andrés Izquierdo-Muñoz[d], Mònica Campàs[a], Jorge Diogène[a]

Received 6 April 2017, Revised 16 May 2017, Accepted 24 May 2017, Available online 3 June 2017



4.5 Evaluation of tetrodotoxins in puffer fish caught along the Mediterranean coast of Spain. Toxin profile of *Lagocephalus sceleratus*

Maria Rambla-Alegre*, Laia Reverté, Vanessa del Río, Pablo de la Iglesia, Oscar Palacios, Cintia Flores, Josep Caixach, Katrina Campbell, Christopher T. Elliott, Andrés Izquierdo-Muñoz, Mònica Campàs and Jorge Diogène

4.5.1 Abstract

Although consumption of Tetraodontidae species is prohibited in the EU, intoxications are still reported. The evaluation of tetrodotoxins (TTXs) by mass spectrometry (LC-MS/MS) and a screening immunoassay (mELISA) in tetraodontid fishes caught along the Western Mediterranean Sea revealed high concentrations of TTXs in *Lagocephalus sceleratus* while no TTXs were identified in *L. lagocephalus* and Sphoeroides pachygaster individuals. The high TTXs content found in the *L. sceleratus* analysed herein demonstrate the occurrence of highly toxic puffer fish in the Western Mediterranean Sea. Being *L. sceleratus* a recent invasive species in the Mediterranean, surveillance, risk assessment and risk management measures are necessary. The strategy used within this research work could be a valuable tool for future food safety monitoring.

4.5.2 Introduction

Tetraodontidae is a family of marine and estuarine fish that includes 130 species grouped in 19 genera that are potential carriers of tetrodotoxins (TTXs) (Smith, 1986). Most puffer fish are from tropical waters but some have been found in temperate waters. *Sphoeroides pachygaster* is an expanding species that most probably reached the Mediterranean from the Atlantic Ocean and *Lagocephalus lagocephalus* is a circumglobal species, considered as native in the Mediterranean (Froese et al., 2016). However, *L. sceleratus* only recently entered the Mediterranean Sea through the Suez Chanel (Lessepsian migration) and is considered an invasive species. *L. sceleratus* was first reported in the Mediterranean Sea in February 2003 at Gökova Bay (southern Aegean Sea, Turkey) (Akyol et al., 2005), in November 2004 at Jaffa along the Israeli coast (Golani and Levy, 2005), and in July 2005 in the Cretan Sea, Greece (Kasapidis et al., 2007). Since then, *L. sceleratus* has been recorded with increasing frequency in Greece, Cyprus, Malta, Algeria, and Turkey and is considered

as one of the fastest expanding Lessepsian invaders (Acar et al., 2017; Deidun et al., 2015; Kara et al., 2015; Katikou et al., 2009; Kosker et al., 2016; Rodriguez et al., 2012; Rousou et al., 2014). In Spain, one individual of *L. sceleratus* was captured in July 2014 in Denia (Alicante, Western Mediterranean) (Katsanevakis et al., 2014) and is the object of the present publication.

Tetrodotoxin is a neurotoxin responsible for human intoxications and fatalities, commonly following the consumption of puffer fish (Bane et al., 2014). Structurally, TTX consists of a guanidinium moiety connected to a highly oxygenated carbon skeleton that possesses a 2,4-dioxaadamantane portion containing five hydroxyl groups (Isbister and Kiernan, 2005). TTX co-exists with its naturally occurring analogues whose structures have already been described for at least 30 of them (Bane et al., 2014). In Japanese waters, the presence of puffer fish belonging to the Tetraodontidae family is very common. In fact, Japanese people are well-known consumers of *fugu*, which is considered a gastronomic delicacy. In humans, according to case studies, between 0.18 and 0.2 mg of TTX have been reported to cause severe symptoms, and a fatality was reported after an ingestion of around 2 mg of TTX (Noguchi and Ebesu, 2001).

Additionally, the Ministry of Health, Labour and Welfare published a guide with the edible parts and species of puffer fish that are allowed for consumption (HP of Ministry of Health) where *L. sceleratus* is not included among the edible species. However, TTX poisoning cases still occur in this and other Asian countries (Noguchi and Arakawa, 2008; Yotsu-Yamashita et al., 2011). In Europe, the current legislative requirements (European Commission, 2004a, European Commission, 2004b) establish that poisonous fish of the family Tetraodontidae and products derived from them must not be placed on the European markets. Despite this fact, the possibility for accidental consumption of these species is possible. Since 2007, when the first toxic European episode was reported in Málaga (Spain) by the consumption of trumpet shells of the species *Charonia lampas lampas* containing TTXs (Rodriguez et al., 2008), several episodes along the Mediterranean coastal countries have been reported due to the consumption of puffer fish (Bentur et al., 2008; Kheifets et al., 2012). Very recently the presence of TTXs has been reported in gastropods from Portugal (Silva et al., 2012) as well as in bivalve mollusk shellfish grown at the south coast of England (Turner et al., 2015), along the Greek coast (Vlamis et al., 2015) and in the Netherlands (RASFF, 2016). Following these events, the European Food Safety Authority (EFSA) has recently published an opinion on the risks for public health related to the presence of TTX and TTX analogues in marine bivalves and gastropods (EFSA, 2017).

The aim of this work was to characterise the toxin profile of different Tetraodontidaespecies including the *L. sceleratus* caught in Denia (Alicante, Western Mediterranean). To this purpose, different puffer fish tissues were analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS), and a self-assembled monolayer-based enzyme-linked immunosorbent assay (mELISA).

4.5.3 Materials and methods

4.5.3.1 Standards and reagents

TTX standard was from Tocris Bioscience (Bristol, UK). The TTX standard solution was prepared at 1 mg/mL in 10 mM acetic acid.For LC-MS/MS analyses, hypergrade acetronitrile (ACN) was used for separation and gradient. HPLC methanol (MeOH) was used for washing the injection needle. Both ACN and MeOH were purchased from Fisher Scientific (Loughborough, UK). Ultrapure water (resistivity >18 MΩ·cm) was obtained from a Milli-Q water purification system (Millipore Iberica Ltd., Madrid, Spain). Ammonium acetate, acetic acid and formic acidwere purchased from Sigma-Aldrich (Tres Cantos, Spain). For mELISA experiments: the anti-TTX monoclonal antibody was produced as previously described (Kawatsu et al., 1997) and prepared as in the literature (Campbell et al., 2013); Pierce maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain); dithiolalkanearomaticPEG6-COOH (dithiol-carboxylate) was purchased from Sensopath Technologies (Bozeman, USA), and anti-mouse IgG (whole molecule)-horseradish peroxidase antibody (IgG-HRP), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), and 3,3',5,5'-tetramethylbenzidine (TMB) wereall supplied by Sigma-Aldrich (Tres Cantos, Spain).

4.5.3.2 Sample preparation

Fourteen oceanic puffer fish (*L. lagocephalus*, Linnaeus, 1758), one silver-cheeked toadfish (*L. sceleratus*, Gmelin, 1789) and five blunthead puffer fish (*S. pachygaster*, Müller and Troschel, 1848) were caught from 2014 to 2016 over the Spanish coast along the Mediterranean Sea (**Figure 4.5.1**). All puffer fishes were dissected and gonads (only in females), liver, skin and muscle were retrieved. Extraction of TTXs was performed as previously described (Reverté et al., 2015). Briefly, a double extraction of each organ with 0.1% acetic acid was performed. In the case of liver, an additional liquid-liquid partition with hexane was required. The analysis by the immunoassay was performed

with the aqueous extracts, but for the LC-MS/MS and LC-HRMS analyses, extracts were evaporated, re-dissolved in methanol and filtered through 0.2-µm nylon filters.

Fig. 4.5.1. Map of Spain including locations where puffer fishes have been caught and received at IRTA from 2014 to 2016. ● *L. lagocephalus*, ▲ *S. pachygaster*, ★ *L. sceleratus* (left: 2014, center: 2015, and right: 2016).

4.5.3.3 Sample analysis

Fourteen *L. lagocephalus*, one *L. sceleratus* and five *S. pachygaster* were first analysed for the presence of TTXs by LC-MS/MS. LC-HRMS was subsequently used for confirmatory purposes on samples containing TTXs. Additionally, the mELISA was applied to the analysis of *L. sceleratus* since this technique provides complementary information. TTX standard was used for LC-MS/MS, LC-HRMS and mELISA analysis. For the other analogues, for which no reference standards are available, identification was done according to the transitions previously published (Bane et al., 2014, Reverté et al. 2015). In addition, quantification was carried out through external calibration using TTX standard as a reference.

LC-MS/MS analysis was performed by a TSQ Quantum system (Thermo Fisher Scientific, Bremen, Germany) as previously described (Reverté et al., 2015). Briefly, analytical separation was performed on a HILIC XBridge Amide column; a binary gradient elution was programmed with water (mobile phase A) and acetonitrile/water (mobile phase B), both containing ammonium acetate; extracts were analysed with the mass spectrometer operating in positive mode, [M+H]+; two multiple reaction monitoring (MRM) transitions were monitored; identification was supported by

toxin retention time and MRM ion ratios. ESI parameters and voltages were optimised to: spray voltage of 3.5 kV, capillary temperature of 300°C, sheath gas flow rate of 40 (arbitrary units) and auxiliary gas flow rate of 10 (arbitrary units), capillary voltage of 30.0 V, tube lens voltage of 130 V and skimmer voltage of 28 V were used. Data was processed with Xcalibur 2.0.7 SP1 software (ThermoFisher Scientific, Bremen, Germany). Six level calibration curves between 1-250 ng/mL (0.1 - 25 mg/kg) showed good intra-batch performance and linear adjustment (r2) ≥ 0.9992.

LC-HRMS analysis was carried out with an Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany). The chromatography and ESI source parameters used were the same as LC-MS/MS methodology. The working mass range was m/z 100-1,200 in full scan acquisition mode. The resolution was 50,000 (m/z 200, FWHM) at a scan rate of 2 Hz. The automatic gain control (AGC) was set as "balanced (1e6)" with a maximum injection time of 250ms. Peaks were identified by retention time, exact mass (mass window ± 5 ppm) and isotope pattern ratio. Data was processed with Xcalibur 3.1 software (ThermoFisher Scientific, Bremen, Germany).

The recently developed mELISA, based on the immobilisation of TTX through dithiol-carboxylate self-assembled monolayers (Reverté et al., 2015) was used to determine the TTX equivalent contents in the *L. Sceleratus* tissues. Briefly, carboxylate–dithiol was self-assembled on maleimide-activated plates; carboxylic groups of dithiols were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) for subsequent reaction with ethylenediamine; remaining carboxylic groups were deactivated with ethanolamine; TTX was immobilised through formaldehyde cross-linking (amino-amino reaction); competition between free (standard or extract) and immobilised TTX for the anti-TTX monoclonal antibody took place; a blocking step with BSA was performed; immunoglobulin G-horseradish peroxidase (IgG-HRP) was added; TMB liquid substrate was incubated; and absorbance was read at 620 nm.

4.5.4 Results and discussion

The twenty puffer fishes were first analysed by LC-MS/MS. Whereas TTX presence was not detected in any of the *L. lagocephalus* and *S. pachygaster* tissues (limit of detection (LOD) and quantification (LOQ) were 0.05 mg/kg and 0.1 mg/kg, respectively), TTXs were found in all of the *L. sceleratus* tissues (results are summarised in **Table 4.5.1**). **Figure 4.5.2** shows the toxin profile of the *L. sceleratus* gonads obtained by LC-MS/MS, as an example. TTX was co-existing with various analogues such as 4-epiTTX and 4,9-anhydroTTX, which are the chemical equilibrium TTX analogues, in all four tissues (gonads, liver, skin and muscle). In addition, four deoxy analogues (5-deoxyTTX, 11-deoxyTTX, 5,11-dideoxy/6,11-dideoxy and 5,6,11-trideoxyTTX) and two 11-nor analogues (11-

norTTX-6(R)-ol and 11-norTTX-(S)-ol) were also identified (Bane et al., 2014; Yotsu-Yamashita et al., 2011; Yotsu-Yamashita et al., 2013).

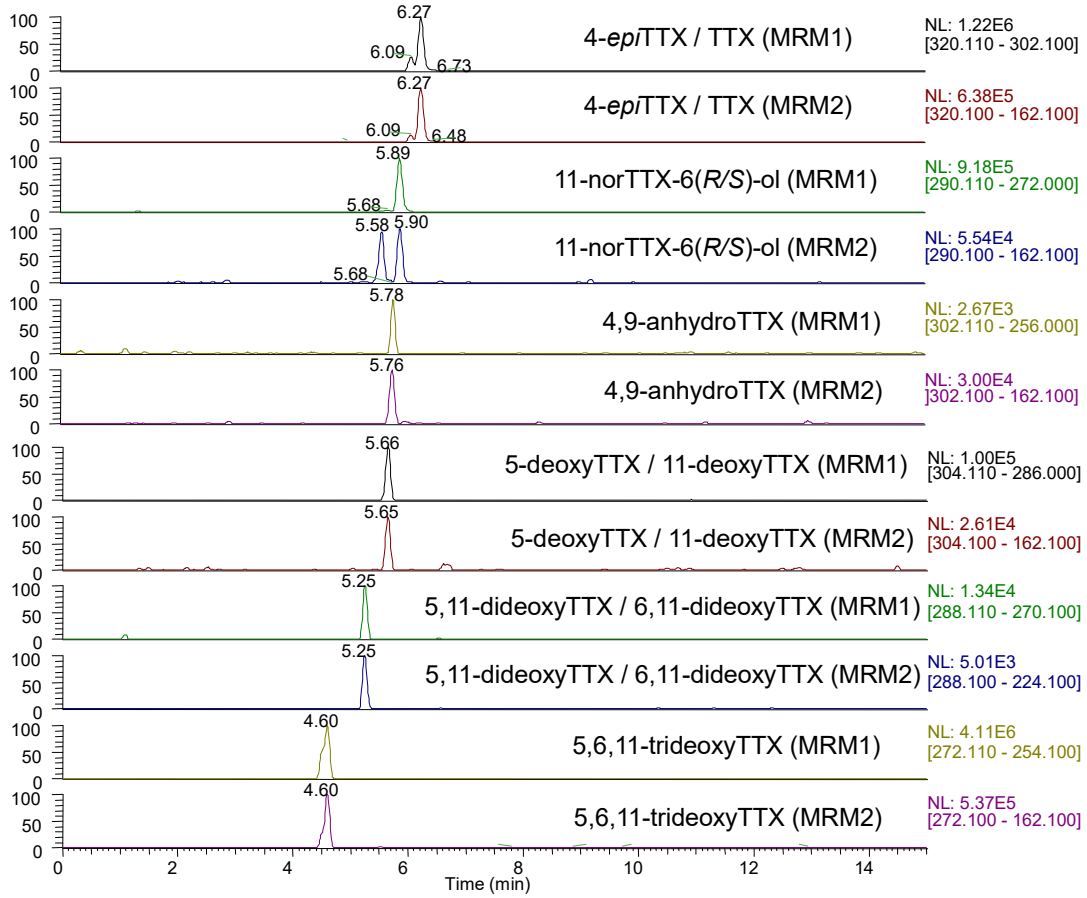


Fig. 4.5.2. Multiple reaction monitoring chromatogram of transition monitored obtained following the analysis of TTX and its analogues in the *L.sceleratus* gonads by LC-MS/MS.

A multi-toxin LC-HRMS analysis method was developed to confirm and quantify the TTX and analogues described in the *L. sceleratus* by LC-MS/MS, as well as to identify other possible analogues described in the literature (Bane et al., 2014). **Figure 4.5.3** shows the toxin profile of the *L. sceleratus* gonads by LC-HRMS, as an example. In general, quantifications by LC-HRMS (LOD = 0.05 mg/kg and LOQ = 0.1 mg/kg) agreed with those previously obtained by LC-MS/MS (Table 4.5.1). It is important to mention that LC-HRMS analysis allowed the identification and quantification of some analogues (4,9-anhydro-5,6,11-trideoxyTTX and 4,4a-anhydro-5,6,11-trideoxyTTX) not included in the LC-MS/MS analysis method. These two compounds have been already identified in puffer fish and newt from Japan and Solomon Islands (Kudo et al., 2012; Puilingi et al., 2015).

Table 4.5.1. TTX and analogues contents (mg TTX or analogue/kg tissue) in *L. sceleratus* by LC-MS/MS and LC-HRMS. Ion composition, theoretical accurate and measured m/z and mass accuracy of main signals obtained from ESI-Orbitrap fragmentation spectra.

	TTX	**4-e*pi*TTX**	**11-norTTX-6(*R*)-ol**	**11-norTTX-6(*S*)-ol**	**4,9-anhydroTTX**	**5-deoxyTTX**	**11-deoxyTTX**	**5,11-dideoxyTTX/ 6,11-dideoxyTTX**	**5,6,11-trideoxyTTX**	**4,9-anhydro-5,6,11-trideoxyTTX**	**4,4a-anhydro-5,6,11-trideoxyTTX**
LC-MS/MS											
MRM1	320.1>302.1	320.1>302.1	290.1>272.1	290.1>272.1	302.1>256.1	304.1>286.1	304.1>286.1	288.1>270.1	272.1>254.0	-	-
MRM2	320.1>162.2	320.1>162.2	290.1>162.2	290.1>162.2	302.1>162.2	304.1>162.2	304.1>162.2	288.1>224.0	272.1>162.2		
Gonads	21.8	4.3	1.1	16.3	0.5	0.9	1.1	0.4	94.3	-	-
Liver	2.3	0.7	0.3	1.3	0.2	ND	0.2	0.2	12.4	-	-
Skin	1.2	0.3	0.1	1.1	ND	ND	0.1	ND	1.8	-	-
Muscle	0.7	0.3	0.2	0.6	0.1	ND	0.1	0.1	1.2	-	-
LC-HRMS											
Ion composition $[M+H]^+$	$[C_{11}H_{18}N_3O_8]^+$	$[C_{11}H_{18}N_3O_8]^+$	$[C_{10}H_{16}N_3O_7]^+$	$[C_{10}H_{16}N_3O_7]^+$	$[C_{11}H_{16}N_3O_7]^+$	$[C_{11}H_{18}N_3O_7]^+$	$[C_{11}H_{18}N_3O_7]^+$	$[C_{11}H_{18}N_3O_6]^+$	$[C_{11}H_{18}N_3O_5]^+$	$[C_{11}H_{16}N_3O_4]^+$	$[C_{11}H_{16}N_3O_4]^+$
Theoretical accurate m/z	320.1088	320.1088	290.0983	290.0983	302.0983	304.1139	304.1139	288.1190	272.1241	254.1135	254.1135
Measured m/z	320.1086	320.1088	290.0982	290.0981	302.0980	304.1137	304.1138	288.1191	272.1240	254.1134	254.1134
Mass Accuracy (ppm)	-0.63	-0.25	-0.33	-0.57	-0.75	-0.61	-0.51	0.24	-0.17	-0.28	-0.32
Gonads	20.0	3.5	0.7	13.1	4.7	0.1	4.2	2.9	167.2	427.7	94.0
Liver	4.6	0.5	ND	1.4	1.0	0.1	0.4	0.3	43.0	78.2	35.3
Skin	1.8	0.2	ND	0.5	0.4	ND	0.1	ND	3.0	15.4	1.3
Muscle	0.9	0.1	ND	0.3	0.2	ND	0.1	ND	1.4	7.4	0.9

ND: not detected; -: not searched. The LOD of LC-MS/MS and LC-HRMS was 0.05 mg/kg using 20 µL and 10 µL injection volume, respectively.

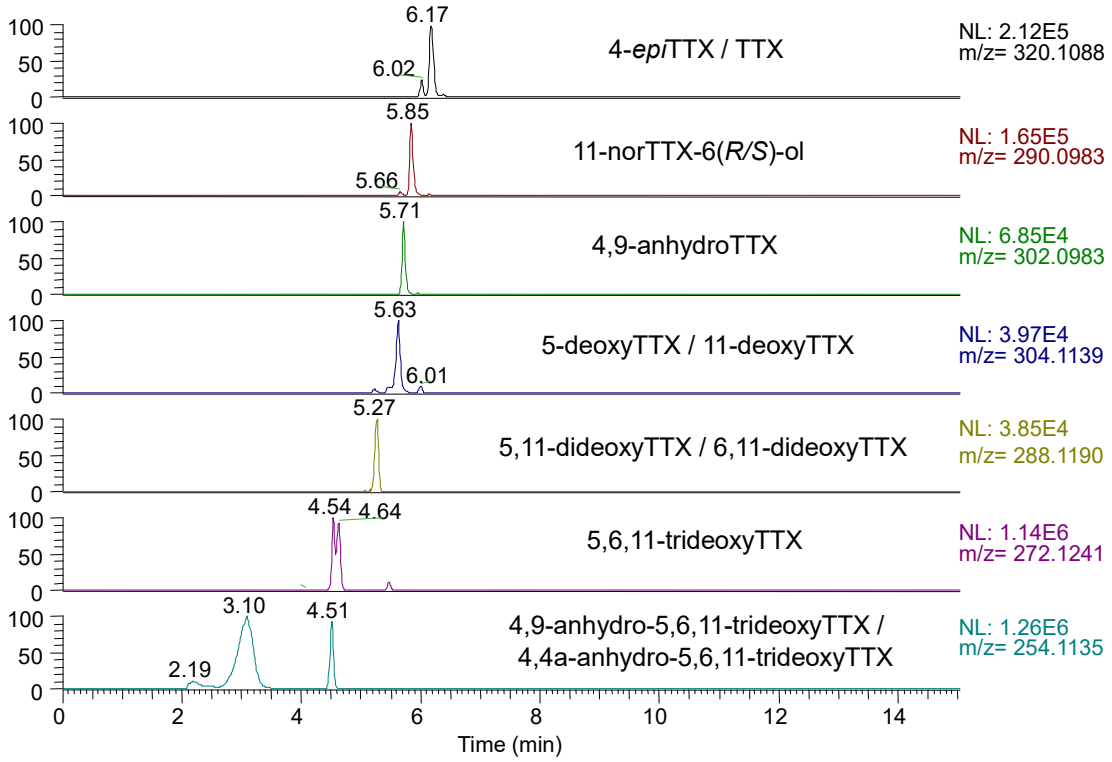


Fig. 4.5.3. Accurate mass extracted chromatogram of TTX ($[TTX+H]^+$) and its analogues in the *L. sceleratus* gonads by LC-HRMS (mass window: ± 5 ppm).

Lagocephalus sceleratus was also analysed by mELISA (LOQ = 0.23 mg/kg). As in the LC-MS/MS and LC-HRMS analysis, TTX was detected in all tissues. Whereas LC-MS/MS and LC-HRMS analyses determine individual TTX and TTX analogues contents, mELISA provides a global response from all TTX and TTX analogues that cross-react with the TTX antibody. **Table 4.5.2** shows the TTX equivalent contents provided by mELISA and the sums of the LC-MS/MS and LC-HRMS quantifications, values resulting after application of the known cross-reactivity factors (CRFs) of the different TTX analogues previously determined (Reverté et al., 2015) to the individual LC-MS/MS and LC-HRMS quantifications. Cross-reactivity factors (in percentage) are 29.9 for 5-deoxyTTX and 11-deoxyTTX, 2.9 for 11-norTTX-6(*R*)-ol and 11-norTTX-6(*S*)-ol, $<10^{-2}$ for 4,9-anhydroTTX, and $<10^{-4}$ for 5,6,11-trideoxyTTX; CRF for 4-*epi*TTX was assumed to be 1; CRFs for 5,11-dideoxyTTX/6,11-dideoxy and the two new 5,6,11-trideoxyTTX analogues were not available and thus these analogues were not included in the sum of TTXs. Tetrodotoxin equivalent contents determined by mELISA in the gonads, skin and muscle were in the range of the quantifications obtained by LC-MS/MS and LC-HRMS, but a considerable disparity was observed in the liver analyses between the quantifications by the mELISA method in relation to those provided by the chromatographic methods. The presence of four analogues with no available CRF may justify this difference between the mELISA and the

instrumental analysis methods. However, 5,11-dideoxyTTX/6,11-dideoxy was found in small amounts, and the two 5,6,11-trideoxyTTX analogues can be supposed to have a very low CRF in relation to TTX because of their similar structure to the 5,6,11-trideoxyTTX. For that reason, it may be hypothesised that their presence may not significantly change the TTX quantification, but it cannot be discarded that there is a possible antigen-antibody affinity interaction. Other unknown TTX analogues or liver matrix compounds could also be responsible for the disagreement between the mELISA technique and the LC-MS/MS and LC-HRMS approaches.

Table 4.5.2. TTX equivalent contents (mg TTX equiv./kg tissue) in *L. sceleratus* by LC-MS/MS, LC-HRMS and mELISA.

	Σ LC-MS/MS	**Σ LC-HRMS**	**mELISA**
Gonads	25.95	25.22	33.55
Liver	3.08	5.36	28.30
Skin	1.65	2.08	3.50
Muscle	1.01	0.98	2.53

When comparing TTX content in the different tissues of *L. sceleratus*, all techniques showed the same trend from higher to lower TTX content: gonads > liver > skin > muscle. The higher TTXs content in gonads and liver compared to skin and muscle had also been observed in silver-cheeked toadfishes from Greece (Acar et al., 2017; Katikou et al., 2009; Kosker et al., 2016; Reverté et al., 2015; Rodriguez et al., 2012). Tetrodotoxin level in gonads was above 10-fold the Japanese acceptability criterion value of 2 mg TTX equiv./kg fish tissue for human consumption (HP of Ministry of Health; Mahmud et al., 1999; Noguchi and Ebesu, 2001). In fact, TTX levels were in the range of those found in previous studies: 0.17-239.32 mg/kg, 0.19-87.53 mg/kg, 0.07-10.16 mg/kg and 0.15-6.63 mg/kg in gonads, liver, muscle and skin tissues, respectively (Acar et al., 2017; Katikou et al., 2009; Kosker et al., 2016; Reverté et al., 2015; Rodriguez et al., 2012). This *L. sceleratus* was caught in November. These high TTX values may be in agreement with the results described by Kosker and co-workers (Kosker et al., 2016), who reported that TTX levels in *L. sceleratus* species caught from the Mediterranean Sea were higher in autumn and winter seasons.

In accordance with Rodriguez and collaborators (Rodriguez et al., 2012), 5,6,11-trideoxyTTX was the major analogue detected by LC-MS/MS. In our case, this analogue was followed by TTX and the isomer 11-norTTX-6(*S*)-ol in all four tissues. Regarding LC-HRMS results, 4,9-anydro-5,6,11-trideoxyTTX was the major analogue followed by 5,6,11-trideoxyTTX and 4,4a-anhydro-5,6,11-trideoxyTTX, which were found in higher amounts than TTX. Although the 5,6,11-trideoxyTTX

analogues were not included in the LC-MS/MS method, they were detected by LC-HRMS and in quite high amounts. These analogues should be included in future analysis of *L. sceleratus* in the LC-MS/MS method, regardless that they may be considered of low toxicity and may not significantly change the TTX equivalent content. A consensus on the LC-MS/MS method between the number of analogues included in the analysis and sensitivity of the method is necessary.

This work constitutes the first report that describes the toxin profile of *L. sceleratus* reaching Spanish waters and complements previous information on TTX present in puffer fish. The LC-MS/MS approach is a suitable technique to identify and quantify TTXs in puffer fish. The LC-HRMS method, which provides a high mass accuracy, has allowed the identification of new TTX analogues. Up to 11 TTX analogues were identified in *L. sceleratus*. Additionally, the mELISA has demonstrated to be a useful method to quantify the total TTX equivalent content in puffer fish. This method does not require expensive instrumentation, provides results in short time and can be used as a TTX screening tool.

Present regulation in the EU is scarce for TTXs, and no maximum permitted levels are established by the legislation. In the recently published EFSA opinion, a concentration below 44 µg TTX equivalents/kg shellfish meat, based on large portion size of 400g, was considered not to result in adverse effects in human; however, more data is needed to provide a more reliable exposure assessment (EFSA, 2017). The LOD for TTX of the LC-MS/MS and LC-HRMS methods on this work (0.05 mg/kg) is in the same order of magnitude than other LODs previously reported 0.08 mg/kg (Rodriguez et al., 2012) and 0.01 mg/kg (Kosker et al., 2016) and slightly higher than 0.007 mg/kg described by Acar et al. 2017). Nevertheless, thisLOD (and also the LOQ for mELISA, 0.23 mg/kg) is well below the Japanese acceptance criterion to consider puffer fish safe for consumption (2 mg TTX equiv/kg).The protocols for the mass spectrometry analysis and mELISA described in this work can be adjusted to fit for more restrictive criteria.

This work provides additional data of TTX in *L. sceleratus* that may contribute to better assess the risk for TTXs in puffer fish. Fish belonging to this group, although banned for consumption, may not be well-known by fishermen and the risk of using these fish in soups or other formats may exist.

4.5.5 Conclusions

Herein we provide a new evidence for the presence of puffer fish containing high amounts of TTXs in the Western Mediterranean Sea, thereby demonstrating the potential hazard for TTX in this area. Whereas *L. lagocephalus* and *S. pachygaster* individuals were determined as non-containing TTX, *L. sceleratus* caught in Denia was determined to be highly toxic and a detailed toxin profile is provided. Tetrodotoxin levels in gonads tissues were up to 10-fold above the Japanese criterion to judge the acceptability of puffer fish for human consumption. The use of an immunoassay as a screening tool for TTX (mELISA) and a confirmatory analysis by liquid chromatography coupled to mass spectrometry is a useful strategy to quantify TTXs in puffer fish and assess the risk they may represent for consumers.

4.5.6 References

Acar, C., Ishizaki, S., Nagashima, Y., 2017. Toxicity of the Lessepsian pufferfish *Lagocephalus sceleratus* from eastern Mediterranean coasts of Turkey and species identification by rapid PCR amplification. European Food Research and Technology. 243**,** 49-57.

Akyol, O., Unal, V., Ceyhan, T., Bilecenoglu, M., 2005. First confirmed record of *Lagocephalus sceleratus* (Gmelin, 1789) in the Mediterranean Sea. Journal of Fish Biology. 66**,** 1183-1186.

Bane, V., et al., 2014. Tetrodotoxin: Chemistry, Toxicity, Source, Distribution and Detection. Toxins. 6**,** 693-755.

Bentur, Y., et al., 2008. Lessepsian migration and tetrodotoxin poisoning due to *Lagocephalus sceleratus* in the eastern Mediterranean. Toxicon. 52**,** 964-968.

Campbell, K., et al. Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood. Analytical andBioanalytical Chemistry. 2013, 405, 7753−7763.

Deidun, A., et al., 2015. First record of the silver-cheeked toadfish *Lagocephalus sceleratus* (Gmelin, 1789) from Malta. BioInvasions Records. 4**,** 139-142.

EFSA, 2017, Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. EFSA J. 15:4, 4752.

European Commission, 2004a. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Official Journal of the European Union. L226**,** 83-127.

European Commission, 2004b. Regulation (EC) No. 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Official Journal of the European Union. L139**,** 22-82.

Froese, R., Pauly, D., In:Fishbase. (Froese, R. & Pauly D. eds). Worldwide Web Electronic Publication, Version 06, 2016. Available at www.fishbase.org. Accessed 5 April 2017.

Golani, D., Levy, Y., 2005. New records and rare occurrences of fish species from the Mediterranean coast of Israel. Zoology in the Middle East. 36**,** 27-32.

HP of Ministry of Health, Labour and Welfare of Japan. Available at: http://www.mhlw.go.jp/topics/syokuchu/poison/animal_01.htmlAccessed on 11May 2017 (in Japanese).

Isbister, G. K., Kiernan, M. C., 2005. Neurotoxic marine poisoning. Lancet Neurology. 4**,** 219-228.

Kara, M. H., Ben Lamine, E., Francour, P., 2015. Range expansion of an invasive pufferfish, *Lagocephalus sceleratus* (Actinopterygii: Tetraodontiformes: Tetraodontidae), to the south-western Mediterranean. Acta Ichthyologica Et Piscatoria. 45**,** 103-108.
Kasapidis, P., Peristeraki, P., Tserpes, G., Magoulas, A., 2007. First record of the Lessepsian migrant *Lagocephalus sceleratus* (Gmelin, 1789) (Osteichthyes: Tetraodontidae) in the Cretan Sea (Aegean, Greece). Aquatic Invasions. 2**,** 71-73.
Katikou, P., et al., 2009. First report on toxicity assessment of the Lessepsian migrant pufferfish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters (Aegean Sea, Greece). Toxicon. 54**,** 50-55.
Katsanevakis, S., et al., 2014. New Mediterranean Biodiversity Records (October, 2014). Mediterranean Marine Science. 15**,** 675-695.
Kawatsu, K., et al. Rapid and highly sensitive enzyme immunoassay for quantitative determination of tetrodotoxin. JapaneseJournal of Medical Science and Biology. 50 (3), 133-150.
Kheifets, J., et al., 2012. Severe Tetrodotoxin Poisoning after Consumption of *Lagocephalus sceleratus* (Pufferfish, Fugu) Fished in Mediterranean Sea, Treated with Cholinesterase Inhibitor. Case reports in critical care. 2012**,** 782507-782507.
Kosker, A. R., et al., 2016. Tetrodotoxin levels in pufferfish (*Lagocephalus sceleratus*) caught in the Northeastern Mediterranean Sea. Food Chemistry. 210**,** 332-337.
Kudo, Y., et al., 2012. Isolation and Structural Determination of the First 8-*epi*-type Tetrodotoxin Analogs from the Newt, *Cynops ensicauda popei*, and Comparison of Tetrodotoxin Analogs Profiles of This Newt and the Puffer Fish, Fugu *poecilonotus*. Marine Drugs. 10**,** 655-667.
Mahmud, Y., Yamamori, K., Noguchi T, 1999. Occurrence of TTX in a brackish water puffer "midorifugu", Tetraodon nigroviridis, collected from Thailand. Journal of the Food Hygienic Society of Japan. 40**,** 363-367.
Noguchi, T.,Arakawa, O., 2008. Tetrodotoxin - Distribution and accumulation in aquatic organisms, and cases of human intoxication. Marine Drugs. 6**,** 220-242.
Noguchi, T., Ebesu, J. S. M., 2001. Puffer poisoning: Epidemiology and treatment. Journal of Toxicology-Toxin Reviews. 20**,** 1-10.
Puilingi, C. G., et al., 2015. Tetrodotoxin and its analogues in the pufferfish *arothron hispidus* and *A-nigropunctatus* from the Solomon Islands: A comparison of their toxin profiles with the same species from Okinawa, Japan. Toxins. 7**,** 3436-3454.
RASFF, Consumers Portal. Notification details - 2016.0845. Possible presence of tetrodotoxin in live oysters from the Netherlands. 2016.
Reverté, L., et al., 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.
Rodriguez, P., et al., 2012. Liquid chromatography-mass spectrometry method to detect tetrodotoxin and its analogues in the puffer fish *Lagocephalus sceleratus* (Gmelin, 1789) from European waters. Food Chemistry. 132**,** 1103-1111.
Rodriguez, P., et al., 2008. First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas lampas* in Europe. Analytical Chemistry. 80**,** 5622-5629.
Rousou, M., et al., 2014. Maturity of the pufferfish *Lagocephalus sceleratus* in the southeastern Mediterranean Sea. Sexuality and Early Development in Aquatic Organisms. 1**,** 35-44.
Silva, M., et al., 2012. New gastropod vectors and tetrodotoxin potential expansion in temperate waters of the Atlantic Ocean. Marine Drugs. 10**,** 712-726.
Smith, M. M., Heemstra, P. C., 1986. Tetraodontidae. Springer-Verlag, Berlin.
Turner, A. D., et al., 2015. Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. Eurosurveillance. 20**,** 2-8.
Vlamis, A., et al., 2015. First Detection of tetrodotoxin in Greek shellfish by UPLC-MS/MS potentially linked to the presence of the Dinoflagellate *Prorocentrum minimum*. Toxins. 7, 1779-1807.

Yotsu-Yamashita, M.,Jang, J.H., Cho, Y., Konoki, K., 2011. Optimization of simultaneous analysis of tetrodotoxin, 4-epitetrodotoxin, 4,9-anhydrotetrodotoxin, and 5,6,11-trideoxytetrodotoxin by hydrophilic interaction liquid chromatography-tandem mass spectrometry. Forensic Toxicology. 29, 61-64.

Yotsu-Yamashita, M., et al., 2013. First identification of 5,11-dideoxytetrodotoxin in marine animals, and characterisation of major fragment ions of tetrodotoxin and its analogs by high resolution ESI-MS/MS. Mar. Drugs. 11, 2799-2813.

Chapter 5

Ciguatoxins and *Gambierdiscus* sp.

5.1. Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean

Jorge Diògene*, Laia Reverté, Maria Rambla-Alegre, Vanessa del Río, Pablo de la Iglesia, Mònica Campàs, Oscar Palacios, Cintia Flores, Josep Caixach, Christian Ralijaona, Iony Razanajatovo, Agathe Pirog, Hélène Magalon, Nathalie Arnich and Jean Turquet

Resumbitted to the journal Scientific Reports on 3rd May 2017

5.1.1 Abstract

Severe food poisoning events after the consumption of sharks have been reported since the 1940s; however, there has been no clear understanding of their cause. Herein, we report for the first time the presence of ciguatoxins (CTXs) in sharks. The tentative identification by mass spectrometry of CTXs, including two new analogues, in a bull shark (*Carcharhinus leucas*) that was consumed by humans, causing the poisoning and death of 11 people in Madagascar in 2013 is described. Typical neurotoxic ciguatera symptoms were recorded in patients, and toxicological assays on extracts of the shark demonstrated CTX-like activity. These results confirm this episode as a ciguatera poisoning event and expand the range of pelagic fish species that are involved in ciguatera in the Indian Ocean. Additionally, gambieric acid D, a molecule originally described in CTX-producing microalgae, was identified for the first time in fish. This finding can contribute to a better understanding of trophic relations within food webs. The present work confirms that consumption of sharks from the Indian Ocean should be considered a ciguatera risk, and actions should be taken to evaluate its magnitude and risk in order to manage shark fisheries.

5.1.2 Introduction

Ciguatera is a well-known food poisoning that occurs when fish containing ciguatoxins (CTXs) are consumed. These potent neurotoxins are produced by microalgae of the genus *Gambierdiscus*[1,2,3] and *Fukuyoa*[4,5]. Ciguatoxins produced by these microalgae may be transferred along the food web, eventually reaching carnivorous fish like barracuda or amberjack. Ciguatera is the main cause of seafood poisoning due to the consumption of fish, and estimations point out around 50,000-

500,000 people are affected by ciguatera each year[6], although these should be re-evaluated for a better assessment of the present impact of ciguatera.

Numerous incidences of human poisoning after the consumption of several species of shark have been reported since the 1940s. These cases have been proposed to be ciguatera events according to the toxicity in animal assays or due to the symptoms in patients[7]. However, the presence of CTXs has never been confirmed in sharks. In Madagascar, a first possible event of ciguatera was described in 1993, after the consumption of shark in Manakara (south-east coast) and was noted for its unprecedented severity. Several hundred people (between 200 and 500 depending on the different authors) were poisoned due to the consumption of a shark, either a bull shark (*Carcharhinus leucas*) or a pigeye shark (*C. amboinensis*), two species that are difficult to distinguish. This event resulted in the death of between 60 and 98 people, depending on the different authors, a fatality rate of 20 to 30%[8,9]. In this particular event, patients presented almost exclusively neurological symptoms. Boisier *et al.* identified two toxic extracts from the liver of the shark, which were proposed to be the causative agent of the poisoning, and tentatively named the new toxins as carchatoxin-A and carchatoxin-B[9]. However, the toxicity levels of the shark flesh did not match that of the liver extracts, and thus, remained unexplained. No further information regarding the chemical behaviour, structure, toxicity or mechanism of action of carchatoxins has been published since then. Another event, occurring between November 14 and 19, 2013 at Fenerive-Est in Madagascar, caused the poisoning of 97 people that presented ciguatera symptoms after eating the flesh, the liver or the head of a bull shark, 11 of whom died. Our preliminary laboratory results of this particular event were communicated to the French “Agence nationale de sécurité sanitaire de l’alimentation, de l’environnement et du travail” (ANSES) in order to quickly manage the potential risk of food poisoning by shark consumption in the Madagascar area[10]. Further data regarding the epidemiology of this event, described that the major symptoms were neurological and digestive [11].

We report herein the presumptive confirmation of ciguatera, caused by consumption of this bull shark (*C. leucas*) in Madagascar in November 2013. This was based on the evidence of symptoms in patients and in mice, cellular toxicity, and unequivocal identification of CTXs by liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS). To the best of our knowledge, this is the first identification of CTXs in sharks.

5.1.3 Materials and methods

5.1.3.1 Samples

Shark samples were collected and analyzed in the framework of a research and development agreement funded by ANSES. Five samples were recovered by the health authorities of Madagascar "Agence de Contrôle Sanitaire et de la Qualité des Denrées Alimentaires de Madagascar" (ACSQDA) and were transferred to the laboratory by the WHO and the "Pasteur Institute of Madagascar". The salted stomach, three dried fins and partially cooked flesh samples were stored at -25 °C until extraction. Samples of flesh and stomach were crushed and homogenized before starting the analyses. Until extraction, they were stored at -25 °C.

5.1.3.2 Shark species identification through microsatellite genotyping and Dloop sequencing

The five samples (flesh, stomach and fins 1 to 3) from elasmobranch tissues, suspected to be from bull shark (*C. leucas*), were used for identification. Total genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The mitochondrial control region (D-loop; 832 bp) was also sequenced using primers designed previously[25], but failed to amplify due to DNA degradation before collection. Therefore, genotyping was performed using a total of 22 microsatellite loci [20 loci developed for *C. leucas*[26], one locus (Cli106) for *C. Limbatus*[27] and one locus (Gc01) for *Galeocerdo cuvier*[28]]. All amplifications were performed as described in the literature[26]. Then, identical multi-locus genotypes (MLGs) were identified using the software GenClone v. 2.0 [29] and, using the software STRUCTURE v. 2.3.1[30], assignment tests were performed together with MLGs obtained from other carcharhinid shark species (*C. leucas, C. obscurus* and *C. plumbeus*) that are known to amplify with the same loci.

5.1.3.3 Toxin standards

Pacific type 1 CTX (P-CTX-1 or P-CTX-1), P-CTX-2 and P-CTX-3 standard solutions were provided by Pr. Richard J. Lewis (The Queensland University, Australia). P-CTX-1 standard was used for CBA and LC-ESI-HRMS analysis. P-CTX-2, P-CTX-3 standards were used only for LC-ESI-HRMS analysis.

5.1.3.4 Flesh, stomach and fins extraction

Samples were extracted and purified according to the protocol described in the literature[31] with minor modifications provided by ANSES. In brief, 10 g ± 0.1 g of flesh, stomach or fins homogenates were placed in 50-mL tubes. Samples were extracted in 20 mL of acetone and homogenized with an Ultraturrax blender. Samples were heated in the sealed tube at 70 °C for 10 min in a water bath. The supernatant was recovered by centrifugation at 3,000 g for 10 min at 4 °C and filtered using 0.45-µm nylon filters. The sample pellets were re-extracted with acetone and supernatants were pooled and evaporated until dry. Liquid/liquid partition was then performed twice in the tubes with 20 mL of water/diethyl ether (DEE) (1:4, v:v). The DEE upper phase was recovered and pooled with the second DEE partition. Both DEE phases were evaporated until dry. The dried extracts were then dissolved in 4 mL of *n*-hexane and 2 mL of methanol/water (4:1, v:v). The hexane upper phase was removed. This liquid/liquid partition was repeated three times and the methanol phases were pooled and evaporated until dry. Finally, the resulting residues were re-dissolved in 4 mL of HPLC-grade methanol and preserved at -20 °C until analyzed.

5.1.3.5 Stomach extract fractionation

A total of 2,750 µL of stomach extract were evaporated until dry using $N_{2(g)}$, and re-dissolved in 1,000 µL of HPLC-grade methanol. The analytical fractionation of this extract was performed as described before for the chromatographic separation of CTXs[3]. Once the chromatographic run started, fractions were collected every 30 seconds (n=28). After fractionation, the volume of each tube was evaporated to until dry, re-dissolved in 500 µL of HPLC-grade methanol and preserved at -20 °C until analyzed. Stomach fractions were analyzed by LC-ESI-HRMS and CBA, but not by MBA.

5.1.3.6 Mouse bioassay

The protocol used at HYDROREUNION was validated beforehand by the ethics committee (Protocol agreement n° EU0450 - GIP CYROI - APAFiS - Autor. APAFiS #2641-2015110916009490) and was in accordance with the regulations in force. This protocol is based on a standard method developed by ANSES (CATNAT-10). The extracts of shark were solubilized in Tween-60 1-5% saline solution, and then injected into three mice (male, OF1; 20 ±2 g) by intraperitoneal (i.p.) route. The mice were observed continuously during the first 2 h, and then monitored regularly up to 24 h after injection. The interpretation of the results was based on the symptoms observed and the time-to-death of

the mice. The typical symptoms of the presence of CTXs include profuse diarrhea, piloerection, respiratory disorders, dyspnoea and, when using male mice, transient pre-erectional cyanosis of the penis (which can become priapism). With the Indian Ocean toxins, this last symptom is observed only very rarely. It therefore does not appear in the classical description for this region. The death of 1 or 2 mice within 24 h was deemed a positive result indicating the presence of CTXs (sample therefore non-edible).

5.1.3.7 Assessment of ciguatoxin-like activity by Neuro-2a cell-based assay

Neuroblastoma mice (Neuro-2a cell line: CCL-131) were purchased from the American Type Culture Collection (ATCC) (LGC standards S.L.U, Barcelona, Spain). The presence of CTX-like activity in shark tissues extracts was evaluated on Neuro-2a cells according to the method based on the use of ouabain and veratridine published by Caillaud and co-workers[32]. Briefly, cells were exposed to the P-CTX-1 standard and to shark extracts for 24 h, and the CTX-like activity was measured in the presence of ouabain and veratridine with the MTT colorimetric assay[33]. Previous to the analysis of flesh, stomach and fins crude extracts or stomach fractions by CBA, methanol was removed from the extracts/fractions and P-CTX-1 standard solution by evaporation under $N_{2(g)}$ and re-dissolved in RPMI medium. CTX-like activity was estimated with respect to P-CTX-1.

5.1.3.8 Liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS)

An Orbitrap-Exactive HCD (Thermo Fisher Scientific, Bremen, Germany) mass spectrometer equipped with heated electrospray source (H-ESI II), a Surveyor MS Plus pump and an Accela Open AS auto-sampler kept isothermal at 15 °C (Thermo Fisher Scientific, San Jose, California) were used for the analysis by LC-ESI-HRMS.

The chromatographic separation was performed on a reversed-phase Hypersil Gold C_{18} (50 mm x 2.1 mm, 1.9 µm) (Thermo Fisher, Scientific, Bremen, Germany) at a flow rate of 250 µL/min. Mobile phase A was water and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 0.1% formic acid. The gradient elution program for the analysis was: 30 % B 1 min, 30-40 % B 2 min, 40-50 % B 1 min, 50-90 %B 5 min, 90% B 3 min and return to initial conditions for re-equilibrate (11 min 30 % B). A 5-µL injection volume was used. The total duration of the method was 25 min.
The analyses were carried out in positive electrospray ionization (ESI+) mode, and the instrument was calibrated daily. P-CTX-1 was used to optimize the source, transmission and HRMS conditions

in positive mode. The final parameters were: spray voltage of 4.0 kV, capillary temperature of 275 °C, heater temperature of 300 °C, sheath gas flow rate of 35 psi and auxiliary gas flow rate of 10 (arbitrary units). In addition, capillary voltage of 47.5 V, tube lens voltage of 186 V and skimmer voltage of 18 V were used. Nitrogen (purity > 99.999%) was employed as sheath gas, auxiliary gas and collision gas. The mass range was *m/z* 400-1,500 in full scan acquisition mode. The resolution was 50,000 (*m/z* 200, FWHM) at a scan rate 2Hz, the automatic gain control (AGC) was set as "balanced" ($1e^6$) with a maximum injection time of 250 ms. The data was processed with Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, Bremen, Germany).

Automatic identification/quantification were performed. The peaks were extracted from the chromatogram using the exact mass of both $[M+NH_4]^+$ and $[M+Na]^+$ diagnostic ions, the mass accuracy (±10 ppm extraction window), and the retention time window. In addition to HRMS and accuracy parameters for identification, in the present study, to be confident of the identification and the proposed elemental formulae, the following restrictive criteria were applied: elements considered were restricted in accordance with CTXs molecular formulae and adduct signals [C 55 to 70, H 64 to 110, O 11 to 25, N 0 to 1, and cations (Na) 0 to 1]; the isotopic pattern was matched and the charge, the ring double bond equivalents (RDBEs) and nitrogen rule were taken into account. Additionally, the monoisotopic pattern (M+1 ion) of these signals was used to assist in the further confirmation of the toxin's identity. Therefore, in total four diagnostic signals were used for toxin identification. The relative ion intensities between $[M+NH_4]^+$, $[M+Na]^+$ and their M+1 ions were calculated and matched taking into account a tolerance according to the EU Decision 2002/657/EC. The toxins in study are characterised by a vast amount of carbon atoms, leading to relative abundances of the ^{13}C isotopic ion higher than 65%. This high sensitivity of M+1 ion render these signals a high identification potential. The combination of high resolution, AMM and restrictive criteria was crucial for identification of both targeted and unknown compounds, as well as precise quantification of analytes. An external standard calibration was carried out from 12.5 to 100 ng/mL using P-CTX-1 and showed good linearity (R^2=0.996), with the LOD being 1.25 ng/mL. Due to the lack of proper analytical standards for all CTX congeners, in order to calculate concentrations of CTX analogues it was assumed that related analogues would give a similar response to that obtained with the P-CTX-1 standard.

5.1.4 Results

5.1.4.1 Poisoning event in Madagascar

In November 2013, an outbreak of fish poisoning following the consumption of shark was reported in the district of Fenoarivo Atsinanana (Fenerive-Est, Madagascar). According to the information transmitted to the ANSES by the "French Institute for Public Health Surveillance," dated 22 April 2014, 124 people, 11 of whom died, were poisoned after consuming the flesh, liver, head and part of the viscera of a shark[11]. The patients developed symptoms between 2 and 12 h following ingestion of their meal, and the predominant neurological signs were paraesthesia of the extremities, dysesthesia, and reversing sensitivity of hot and cold. These symptoms were accompanied by headache, dizziness, and arthralgia. The digestive symptoms were moderate and inconsistent. The clinical profile was similar to that of patients that had previously been poisoned after consumption of shark in Madagascar[9]. A detailed epidemiological report is presented by Rabenjarison *et al.*[11], and additional investigations conducted by agents of the "Institut Halieutique et des Sciences Marines" (Tulear, Madagascar) concluded that the shark in question was a female of about 1.5 m in length. Samples of the fish implicated in the episode consisted of salted stomach, three dried fins, and partially cooked flesh. The genetic analyses performed using 22 microsatellite loci on the five samples demonstrated that they all belong to the species *C. leucas* and surely to the same individual (identical multilocus genotypes; Supplementary **Table S5.1.1** and **Fig. 5.1.1**).

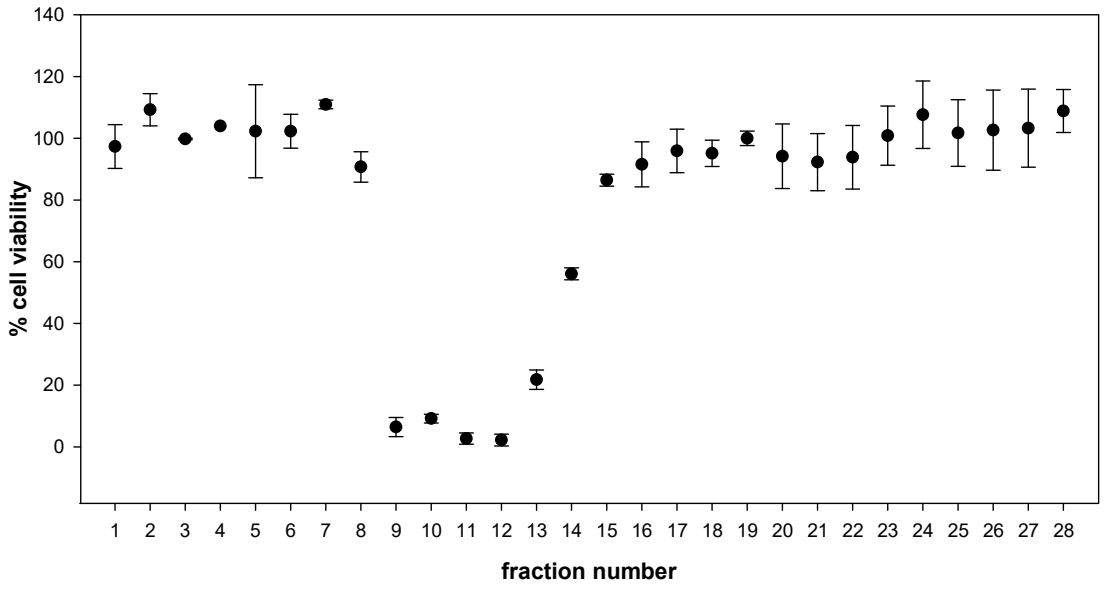


Figure 5.1.1. Toxicity of stomach fractions by cell-based assay (CBA). Toxicity was estimated according to the cell viability obtained after exposing cells to 0.27 µL of each fraction/mL. Error bars represent standard deviation (SD) values for 3 replicates (n=3).

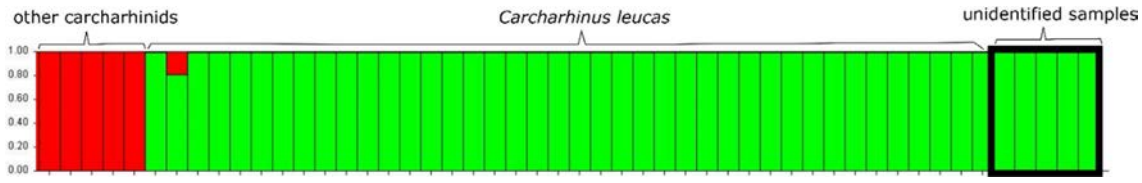


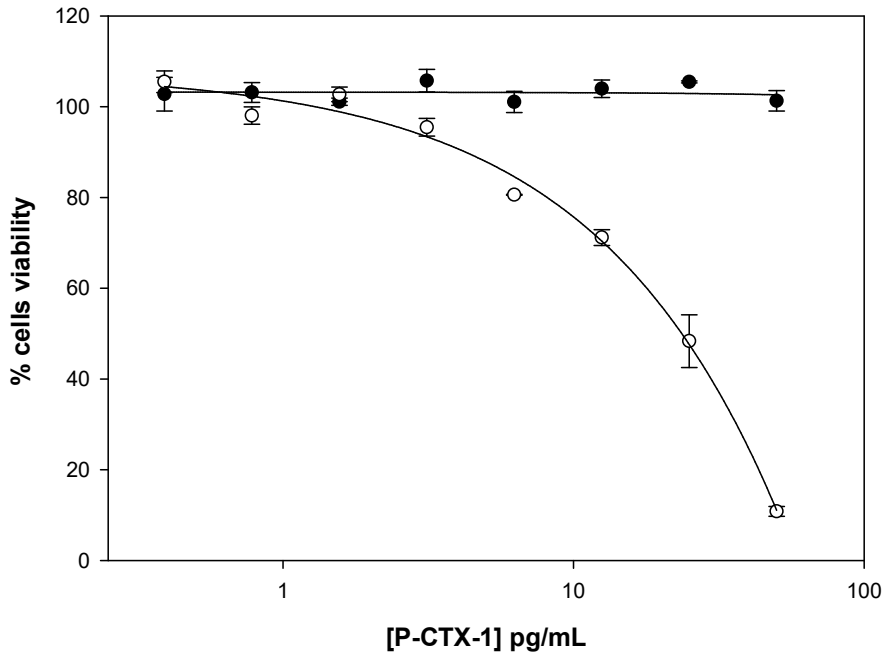
Fig. S5.1.1. Assignment test (STRUCTURE software) for shark species identification. Each bar on the *x*-axis represents one individual, and the *y*-axis represents the probability to belong to one or another cluster. The multilocus genotypes of the unidentified shark samples (presumed to be bull shark) were used in assignment with other bull shark individuals[26] and other carcharinid species that successfully amplified the loci used (*Carcharinus obscurus* or *C. plumbeus*). Two clusters were found: the red one corresponds to other shark species and the green one corresponds to bull shark individuals. The unidentified samples clustered with the bull shark cluster.

5.1.4.2 Toxicity evaluation of shark samples by mouse bioassay (MBA)

The MBA performed on the flesh samples showed toxicity, although quantification was not possible due to the limited amount of samples. The single dose injected corresponds to an amount of 50 g equiv. flesh /mouse. The symptoms observed in the mice included: paralysis of limbs, dyspnoea, convulsions, mild diarrhea, and mortality from respiratory failure between 3 and 4 hours after injection of the extract. Toxicity and symptoms in mice were similar to those previously described in the study on shark toxins in Madagascar[9]. The MBA performed on the stomach sample showed a very high toxicity. Even at low doses, when mortalities were observed these were always rapid (survival time less than 1.5 h) and otherwise the mice recovered. The symptoms observed were dominated by neurological problems, including difficulty with breathing, followed by a severe respiratory arrest. In some mice, hyper-salivation was also observed. The lowest dose tested that resulted in the death of mice was estimated at 72 mg equiv. stomach per mouse of 20-22 g. Fin samples were not tested by MBA.

5.1.4.3 Evaluation of ciguatoxin-like activity by Neuro-2a cell-based assay (CBA)

Neuro-2a cells exposed to Pacific ciguatoxin P-CTX-1 (CTX1B) standard presented the expected dose-response curve with a regression factor (R^2) of 0.996. The concentration of P-CTX-1 that caused a 50% cell mortality (IC_{50}) was 19.9 pg/mL. The limit of detection (LOD), defined as the concentration of P-CTX-1 that causes a 20% of cell mortality (IC_{20}), was 8.4 pg/mL (Supplementary **Fig. S5.2.2**). Considering a maximum exposure concentration of 200 mg/mL for flesh, 100 mg/mL for stomach and fins 1 and 3, and 50 mg/mL for fin 2 (Supplementary Figure S5.1.3), the effective LODs (eLODs) for P-CTX-1 in shark samples were 0.04 µg P-CTX-1 equiv./kg for flesh, 0.08 µg P-CTX-1 equiv./kg for stomach and fins 1 and 3, and 0.17 µg P-CTX-1 equiv./kg for fin 2. Flesh, stomach and fins 1 to 3 crude extracts of *C. leucas* contained 0.06, 92.78, 0.12, 0.79 and 0.17 µg P-CTX-1 equiv./kg matrix, respectively (**Table 5.1.1**).



Supplementary Figure S5.2.2. Cytotoxicity of P-CTX-1 on Neuro-2a cells with (white dots) and without (black dots) ouabain/veratridine treatment. Error bars represent standard deviation (SD) values for 3 replicates (n=3).

With the aim of separating the different compounds to better identify the toxin profile of the most toxic sample, the stomach extract was fractionated by high-performance liquid chromatography (HPLC). In order to identify the distribution of the toxin within the 28 fractions recovered, cells were first exposed to 2.17 µL of each fraction/mL CTX-like activity was observed in fractions F8 to F22. Fractions F8 and F17 to F22 fell within the working range (IC_{20}-IC_{80}), and CTX-like content was able to be quantified. The use of lower fraction volume (0.27 µL of each fraction/mL) was required to

quantify fractions F13 and F14 (Figure 1). Further dilution was required to quantify fractions F9 to F12. Distribution of ciguatoxins after fractionation of the stomach crude extract is shown in **Table 5.1.2**.

A liquid chromatography electrospray ionization high-resolution mass spectrometry (LC-ESI-HRMS) method was developed for the analysis of CTXs in extracts of *C. leucas*, based on previous LC-MS/MS methods[3,12,13]. The spectra of I-CTXs were dominated by $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ in accordance with Hamilton et al.[13] The adduct ions giving higher signals, specifically $[M+NH_4]^+$ and $[M+Na]^+$, were chosen for confirmation and quantification purposes.

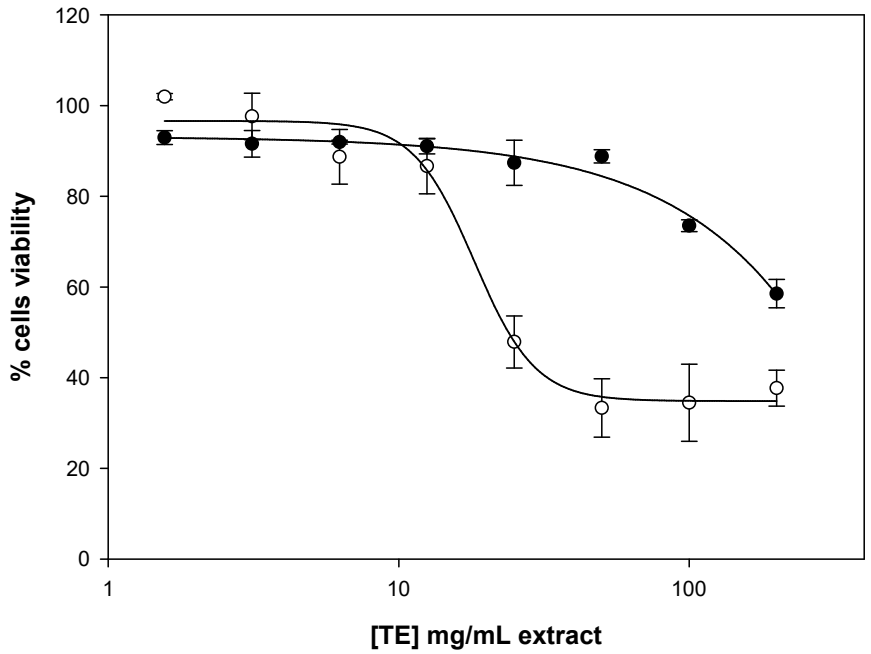
Table 5.1.1. Concentration of P-CTX-1 equiv./kg tissue in crude stomach, flesh and fin extracts as determined by mouse bio-assay (MBA), Neuro-2a cell-based assay (CBA) and liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS).

Table 5.1.2. Distribution of ciguatoxins (CTXs) after fractionation of the stomach crude extract.

Fractions	CBA (% P-CTX-1 equiv.)	LC-ESI-HRMS (% P-CTX-1 equiv.)		
		I-CTX-1&2	I-CTX-3&4	Σ I-CTXs
F8	0.23	n.d.	n.d.	n.d.
F9	5.15	n.d.	5.67	5.67
F10	5.80	n.d.	29.66	29.66
F11	9.37	13.87	15.75	29.62
F12	8.05	16.12	3.86	19.98
F13	1.79	n.d.	n.d.	n.d.
F14	1.44	n.d.	n.d.	n.d.
F15	2.30	n.d.	n.d.	n.d.
F16	0.62	n.d.	n.d.	n.d.
F17	0.29	n.d.	n.d.	n.d.
F18	0.27	n.d.	n.d.	n.d.
F19	0.33	n.d.	n.d.	n.d.
F20	0.23	n.d.	n.d.	n.d.
F21	0.19	n.d.	n.d.	n.d.
F22	0.12	n.d.	n.d.	n.d.

Percentages of P-CTX-1 equiv. recovered in each fraction in relation to the P-CTX-1 equiv. injected, estimated by the Neuro-2a cell-based assay (CBA) and liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS).

Crude extract	MBA (µg P-CTX-1 equiv./kg tissue)	CBA (µg P-CTX-1 equiv./kg tissue)	LC-ESI-HRMS (µg P-CTX-1 equiv./kg tissue)		
			I-CTX-1&2	I-CTX-3&4	Σ I-CTXs
flesh	n.q.	0.06	n.d.	n.d.	n.d.
stomach	83	92.78	6.54	9.74	16.28
fin 1	-	0.12	-	-	-
fin 2	-	0.79	n.d.	n.d.	n.d.
fin 3	-	0.17	-	-	-



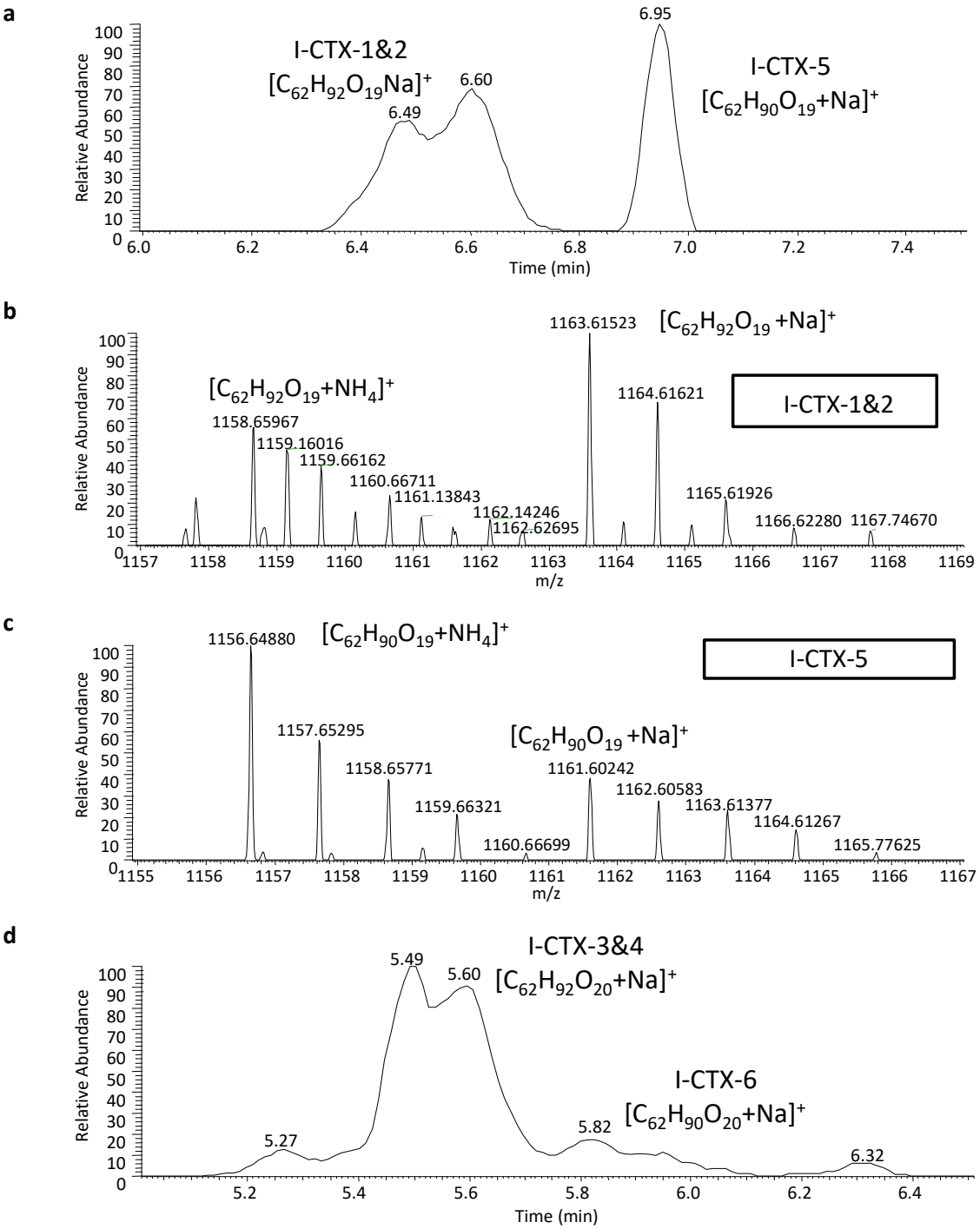
Supplementary Figure S5.1.3. Cytotoxicity of fin extract 2 on Neuro-2a cells with (white) and without (black) ouabain/veratridine treatment. In the absence of ouabain/veratridine, a matrix effect leading to cell toxicity was observed at concentrations of 100 mg TE/mL or higher. In the presence of ouabain/veratridine, a CTX-like effect was observed at lower concentrations. (TE: tissue equivalents). Error bars represent standard deviation (SD) values for 3 replicates (n=3).

5.1.4.4 Confirmation of ciguatoxins (CTXs) by liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS)

Crude extracts of flesh, stomach and fin 2 were analyzed by LC-ESI-HRMS. The presence of CTX analogues was not observed in the flesh nor in the fin 2 crude extracts. A possible explanation is that the LOD attained by CBA (0.04 µg P-CTX-1 equiv./kg flesh tissue and 0.17 µg P-CTX-1 equiv./kg fin 2 tissue) is lower than the LOD attained by LC-ESI-HRMS (0.5 µg P-CTX-1 equiv./kg tissue). In the stomach crude extract, the CTX analogues I-CTX-1&2 and I-CTX-3&4 were detected and quantified (Table 5.1.1).

All analogues were confirmed using their theoretical accurate mass (*m/z*), measured *m/z,* and mass accuracy (ppm): i) I-CTX-1&2 ($[C_{62}H_{92}O_{19}NH_4]^+$ and $[C_{62}H_{92}O_{19}Na]^+$): 1158.6571, 1158.6606, <5.49 ppm and 1163.6125, 1163.6148, <4.93 ppm, respectively; and ii) I-CTX-3&4 ($[C_{62}H_{92}O_{20}NH_4]^+$ and $[C_{62}H_{92}O_{20}Na]^+$): 1174.6540, 1174.6565, <6.31 ppm and 1179.6084, 1179.6146, <5.89 ppm, respectively. From a qualitative point of view, both LC-ESI-HRMS and CBA showed the presence of P-CTX-1 equiv. in the stomach crude extract. However, lower contents of P-CTX-1 equiv. were estimated by LC-ESI-HRMS in relation to the CBA. This difference in the quantification could be attributed to the different principles of the techniques: while LC-ESI-HRMS is based on structural identification of specific CTX analogues, and may neglect some non-described CTX analogues, CBA measures a composite toxicity, which is a global response indicative of the toxic effect of several CTX analogues on cells.

Having identified the toxic stomach fractions using the CBA, fractions F8 to F22 were analyzed by LC-ESI-HRMS for toxin identification. Fractionation of the stomach crude extract reduced matrix interferences and confirmed the presence of I-CTX-1&2 and/or I-CTX-3&4 in fractions F9 to F12, the most toxic ones by CBA (Table 2). Extracted ion chromatograms for I-CTX-1&2 and I-CTX-3&4 found in fraction F12 from stomach are shown in **Fig. 5.1.2a** and **2d**, respectively. Full HRMS exact mass spectra of I-CTX-1&2 and I-CTX-3&4 (Figure 5.1.2b and 2e, respectively) confirmed the presence of these toxins. The isotopic pattern of each signal was taken into consideration in assigning their molecular formula. In addition, these toxins showed a profile similar to P-CTX-1 according to $[M+NH_4]^+$ and $[M+Na]^+$. As for the analysis of crude extracts, the percentages of P-CTX-1 equiv. in fractions F9 to F12 determined by LC-ESI-HRMS were higher than those obtained by CBA. Nevertheless, both techniques concluded that fractions F9 to F12 contained the highest CTX content among all fractions.


a
I-CTX-1&2
[C62H92O19Na]+
6.49
6.60
6.95
I-CTX-5
[C62H90O19+Na]+
Relative Abundance
Time (min)
b
[C62H92O19+NH4]+
1158.65967
1159.16016
1159.66162
1160.66711
1161.13843
1162.14246
1162.62695
1163.61523
[C62H92O19 +Na]+
1164.61621
1165.61926
1166.62280
1167.74670
I-CTX-1&2
Relative Abundance
m/z
c
1156.64880
[C62H90O19+NH4]+
I-CTX-5
1157.65295
1158.65771
1159.66321
1160.66699
[C62H90O19 +Na]+
1161.60242
1162.60583
1163.61377
1164.61267
1165.77625
Relative Abundance
m/z
d
I-CTX-3&4
[C62H92O20+Na]+
5.49
5.60
5.27
5.82
6.32
I-CTX-6
[C62H90O20+Na]+
Relative Abundance
Time (min)

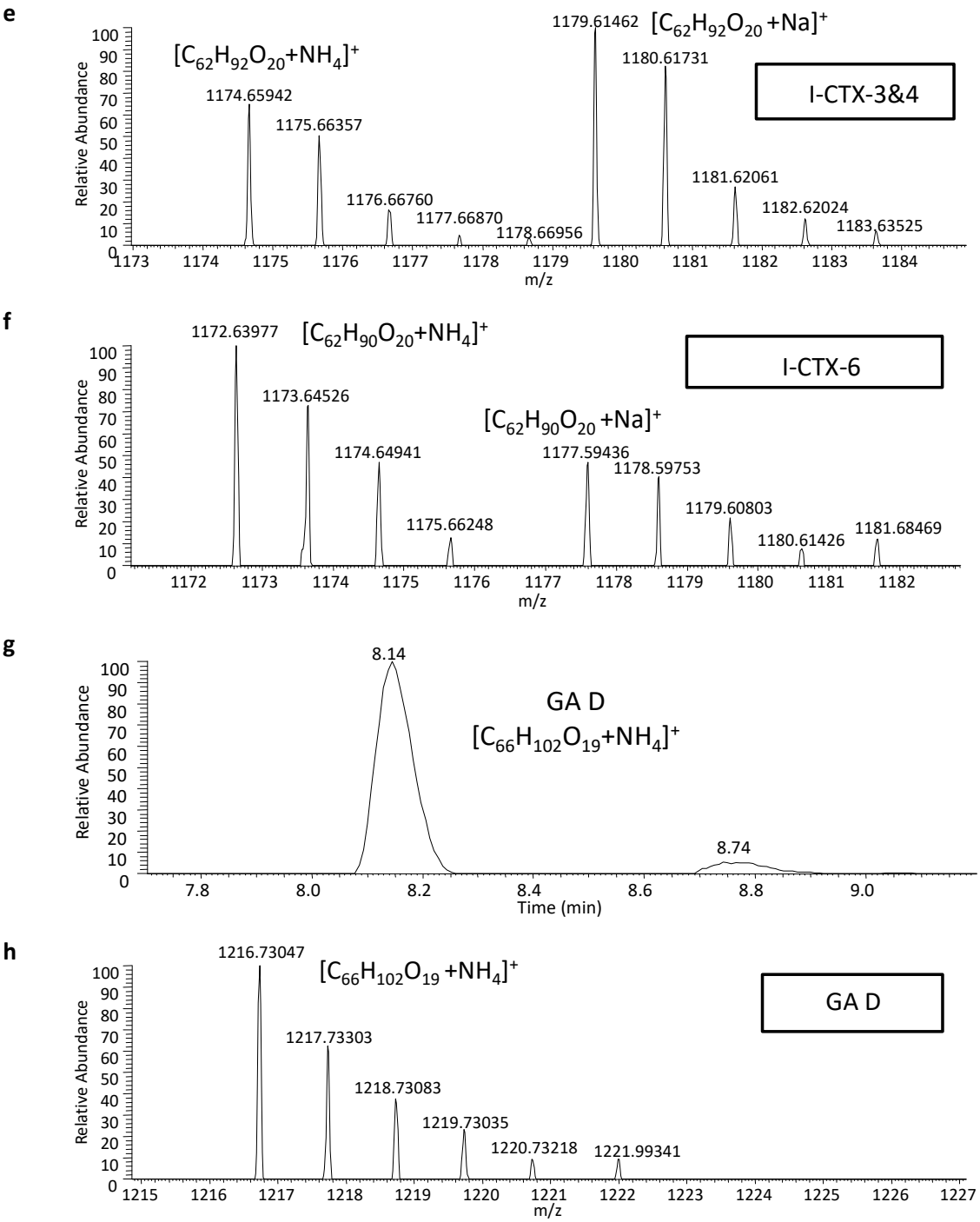


Figure 5.1.2. Evidence for the presence of of ciguatoxins (CTXs) and gambieric acid D (GA D) in shark tissues. **a,** Extracted ion chromatogram of I-CTX-1&2 $[M+Na]^+$ at 1163.6125 and HRMS exact mass spectra of **b,** I-CTX-1&2 $[M+Na]^+$ at 1163.6125 and **c,** I-CTX-5 ($C_{62}H_{90}O_{19}$) $[M+Na]^+$ at 1161.6070, in fraction F12 from stomach; **d,** extracted ion chromatogram of I-CTX-3&4 ($[M+Na]^+$ at 1179.6084 and HRMS exact mass spectra of **e,** I-CTX-3&4 $[M+Na]^+$ at 1179.6084 and **f,** I-CTX-6 ($C_{62}H_{90}O_{20}$) $[M+Na]^+$ at 1177.5910, in stomach crude; **g,** extracted ion chromatogram of GA D

$[M+NH_4]^+$ at 1216.7354 and **h,** HRMS exact mass spectra of GA D $[M+NH_4]^+$ at 1216.7354, in flesh crude extract, respectively.

Two new CTX analogues (I-CTX-5 and I-CTX-6) were detected by LC-ESI-HRMS (Figure 5.1.2c and 2f). A tentative identification of these new CTX analogues related to I-CTXs in the stomach crude extract and fractions was given using the restrictive criteria to propose elemental formulae. Ion assignment indicated that the new CTX analogues had nearly the same molecular formula as I-CTX-1&2 and I-CTX-3&4 with only 2H less, which corresponds to the formation of a double bond. The ring double bond equivalents (RDBEs) for both unknown compounds was 16.5, which corresponds to the 15.5 RDBE value from CTX analogues (I-CTX-1&2 and I-CTX3&4). Analogues were tentatively confirmed according to their theoretical accurate *m/z*, measured *m/z,* and mass accuracy (ppm): i) I-CTX-5 ($[C_{62}H_{90}O_{19}NH_4]^+$ and $[C_{62}H_{90}O_{19}Na]^+$): 1156.6414, 1156.6479, 5.57 ppm and 1161.5968, 1161.6030, 5.29 ppm, respectively; and ii) I-CTX-6 ($[C_{62}H_{90}O_{20}NH_4]^+$ and $[C_{62}H_{90}O_{20}Na]^+$): 1172.6496, 1172.6381, -1.44 ppm and 1177.5918, 1177.5953, 3.03 ppm in fraction F12 from stomach, respectively. Full HRMS exact mass spectra of both new I-CTXs in Figure 5.1.2c and 2f confirm the presence of these toxins.

5.1.4.5 Identification of gambieric acid D (GA D) by liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS)

Gambieric acid D ($C_{66}H_{102}O_{19}$) (GA D), which is also produced by *Gambierdiscus* spp., was identified in the flesh crude extract of the shark using the theoretical accurate *m/z*, measured *m/z,* and mass accuracy (ppm) of $[C_{66}H_{102}O_{19}NH_4]^+$: 1216.7354, 1216.7304, -4.02 ppm at 8.14 min, respectively. Quantification by LC-ESI-HRMS was not carried out due to the lack of GA D standard solutions. Extracted ion chromatograms (Figure 5.1.2g) and HRMS exact mass spectra (Figure 5.1.2h) confirm the presence of GA D by the *m/z* of most abundant ion peaks $[M+NH_4]^+$. No GA D was identified in the stomach or in the fins.

5.1.5 Discussion

The methodology presented in this paper, which combines a multi-disciplinary approach focused on epidemiology, toxicology and instrumental analysis, has proved to be effective in the identification of CTXs in seafood and contributes to a better characterization of the present incidence of ciguatera.

This is the first evidence of the presence of CTXs in sharks. The tentative identification of CTXs in the shark responsible for a food poisoning event in Madagascar presented herein, along with additional observations, confirm the episode as a ciguatera event and the suspected implication of sharks in ciguatera[7]. The evidences that support this confirmation are: i) the symptoms observed in patients matched those of ciguatera, ii) injection of flesh and stomach crude extracts to mice resulted in symptoms characteristic of CTXs, which were comparable to those reported for I-CTX in the bony fish *Lutjanus sebae* from the Indian Ocean[13], iii) neuroblastoma cells exposed to flesh, stomach and fin crude extracts showed the characteristic toxicity of CTXs through activation of voltage-gated sodium channels, and iv) LC-ESI-HRMS provided the identification of I-CTX- 1&2 and I-CTX-3&4 in the stomach extracts. The stomach was extremely toxic with an estimation of 92.78 µg P-CTX-1 equiv./kg by cell-based assay (CBA), a concentration approximately 10,000 times the guidance level concentration of P-CTX-1 causing ciguatera in humans, established at 0.01 µg P-CTX-1/kg by the FDA[14], and considered by EFSA[15] as the level expected not to exert effects in sensitive individuals. The estimation of the stomach levels by LC-ESI-HRMS was lower than determined by CBA, 16.28 P-CTX-1 equiv./kg; however, this level is well above the FDA guidance level. Flesh and fins presented toxicity with an estimation by CBA of 0.06 µg P-CTX-1 equiv./kg in the flesh and 0.12, 0.79 and 0.17 µg P-CTX-1 equiv./kg in fins 1 to 3, respectively, which are also above the FDA guidance level. Identifying CTXs in viscera is significant, since local food habits from the Indian Ocean include eating liver and viscera[11], some of which are dried and salted. Since the liver of the shark was not available, the possible presence of carchatoxins previously described in other shark poisoning events[16] could not be studied.

Currently, only four I-CTX analogues (I-CTX-1&2 and I-CTX-3&4) have been described in the literature[13]. As for the already known CTXs, I-CTX-1&2 and I-CTX -3&4, our results obtained in the stomach of shark revealed higher amount of I-CTX-3&4 in relation to I-CTX-1&2 (60% vs 40% of the total amount of I-CTXs estimated by LC-ESI-HRMS). Contrarily, in that previous study on Indian CTXs in one fish (*Lutjanus sebae*), a lower amount of I-CTX-3&4 in relation to I-CTX-1&2 was described[13]. This difference may be explained by the tissue evaluated, since in their work the whole fish was analysed[13]. Additionally, Hamilton and collaborators postulated that I-CTXs-1&2 might originate from dinoflagellates and that I-CTX-3&4 would be metabolites produced in fish[13]. Since sharks are higher in the trophic webs than *L. sebae,* this may explain the higher amounts of I-CTX3&4 in relation to I-CTX-1&2 obtained in shark stomach. Herein, two new I-CTX analogues have been tentatively identified by LC-ESI-HRMS in the stomach extract and fractions, I-CTX-5 showing 2H less

than I-CTX-1&2 and I-CTX-6 showing 2H less than I-CTX-3&4, which corresponds to the formation of a double bond. This result widens the number of CTXs analogues possibly present in nature[3,12,13] and this will impact our understanding of ciguatera. First, the identification of new CTX analogues may indicate that the metabolism of CTXs may be more complex than previously foreseen. Second, these new CTX analogues possibly present in seafood will need to be taken into account for a better evaluation of ciguatera risks.

Gambieric acid D (GA D)[17] was tentatively identified in shark flesh. To the best of our knowledge, this work is the first report of GA in any organism other than *Gambierdiscus* spp.[16]. The identification of GA D in the flesh of the shark evidences, additionally to the presence of CTXs, the link between *Gambierdiscus* spp. and this particular food poisoning event. Identifying GA D in sharks that, as carnivorous pelagic fish are situated at the highest levels of the marine food webs, demonstrates how stable these molecules may be throughout their transfer and metabolic transformations along the food webs. The identification of molecules produced by microorganisms in animals situated at higher trophic levels, such as GA D would constitute, as for the analysis of fatty acids or stable isotopes, a good strategy to understand trophic relations in the ecosystems. Gambieric acid A has been demonstrated no toxicity in mice, while a mixture of GA C and GA D was moderately toxic to mouse lymphoma cells L5178Y[18,19]. Additionally, GA A has been demonstrated to bind to the voltage gated sodium channels in synaptosomes isolated from rat brains[20] in the same manner as CTXs, but with much less affinity to these than CTXs, possibly explaining its low toxicity. To better understand the potential harmful effects of GAs, and more specifically of GA D, their toxicity should be further characterized; however, at this moment reference material for GAs is not commercially available. Urgent need exists for the availability of certified standards for CTXs and GAs.

About 100 million sharks are caught each year world-wide[21], and the global shark fin trade is estimated to be worth US $ 400-500 million a year[22]. The identification of CTXs in shark from the Indian Ocean may favour the re-consideration of local food safety measures that could affect the shark fisheries industry, which is of special relevance in areas such Madagascar[22]. The present work confirms that shark consumption, in this example, a bull shark (*C. leucas*), from the Indian Ocean should be considered a ciguatera risk, and actions should be taken to evaluate its magnitude and risk in order to manage shark fisheries. As for the numerous suspicious cases of ciguatera involving sharks[7], it may be postulated that sharks with CTXs will not be restricted to the species *C. leucas* and to the Indian Ocean. Consequently, other species of shark and other oceans should also be

considered for ciguatera evaluation, especially to account for the migration of sharks and current changes in the geographical distribution of sharks due to fishing pressure and global warming[23,24].

5.1.6 References

1 Adachi, R. & Fukuyo, Y. Thecal structure of a marine toxic dinoflagellate *gambierdiscus-toxicus* gen et sp-nov collected in a ciguatera-endemic area. *Bull. Japn. Soc. Sci. Fish.* 45, 67-71 (1979).

2 Chinain, M. *et al.* Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. *Toxicon* 56, 739-750 (2010).

3 Caillaud, A. *et al.* Monitoring of dissolved ciguatoxin and maitotoxin using solid-phase adsorption toxin tracking devices: Application to *Gambierdiscus pacificus* in culture. *Harmful Algae* 10, 433-446 (2011).

4 Holmes, M. J. *Gambierdiscus yasumotoi* sp. nov. (Dinophyceae), a toxic benthic dinoflagellate from southeastern Asia. *J. Phycol.* 34, 661-668 (1998).

5 Lewis, R. J. *et al.* Rapid extraction and identification of maitotoxin and ciguatoxin-Like toxins from Caribbean and Pacific *Gambierdiscus* using a new functional bioassay. *Plos One* 11 (2016).

6 Friedman, M. A., *et al*. An updated review of ciguatera fish poisoning: clinical, epidemiological, environmental, and public health management. *Mar. Drugs* 15(3),72 (2017).

7 Meyer, L., Capper, A., Carter, S. & Simpfendorfer, C. An investigation into ciguatoxin bioaccumulation in sharks. *Toxicon* 119, 234-243 (2016).

8 Habermehl, G. G., Krebs, H. C., Rasoanaivo, P. & Ramialiharisoa, A. Severe ciguatera poisoning in madagascar - a case - report. *Toxicon* 32, 1539-1542 (1994).

9 Boisier, P. *et al.* Fatal mass poisoning in madagascar following ingestion of a shark (*Carcharhinus-leucas*) - Clinical and epidemiologic aspects and isolation of toxins. *Toxicon* 33, 1359-1364 (1995).

10 Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES): Contamination des requins, notamment tigre et bouledogue, par des ciguatoxines : occurrence, méthodes analytiques, cas humains rapportés et éléments d'éthologie. Avis de l'Anses Rapport d'expertise collective. Avis de l'Anses Saisine n° 2013-SA-0198. (2015).11 Rabenjarison, F., Ramarolahy, A. R. N., Velomora, A., Rahajaniaina, M. P., & Raveloson, N. E. Intoxication à la ciguatoxine après consommation de requin à Fenerive-Est : profil épidémio-clinique et résultats de laboratoire. Ciguatera poisoning after consumption of shark in Fenerive-Est : epidemiological and clinical aspects and laboratory results. *Revue d'Anesthésie-réanimation, Médecine d'Urgence et Toxicologie* 8 (1), 9-12 (2016). ISSN 2225-5257. Accessible online at http://rarmu.org/publications/8(1)/full_text/8(1)9-12.pdf.

12 Yogi, K., Oshiro, N., Inafuku, Y., Hirama, M. & Yasumoto, T. Detailed LC-MS/MS analysis of ciguatoxins revealing distinct regional and species characteristics in fish and causative alga from the Pacific. *Anal. Chem.* 83, 8886-8891 (2011).

13 Hamilton, B., Hurbungs, M., Jones, A. & Lewis, R. J. Multiple ciguatoxins present in Indian Ocean reef fish. *Toxicon* 40, 1347-1353 (2002).

14 Dickey, R. W. & Plakas, S. M. Ciguatera: A public health perspective. *Toxicon* 56, 123-136 (2010).

15 EFSA. Panel on Contaminants in the Food Chain; Scientific Opinion on marine biotoxins in shellfish-Emerging toxins: Ciguatoxin group. *EFSA J.* 8, 38 (2010).

16 Yasumoto, T. Fish poisoning due to toxins of microalgal origins in the Pacific. *Toxicon* 36, 1515-1518 (1998).

17 Fuwa, H., Ishigai, K., Hashizume, K. & Sasaki, M. Total Synthesis and Complete Stereostructure of Gambieric Acid A. *J. Am. Chem. Soc.* 134, 11984-11987 (2012).

18 Nagai, H., Murata, M., Torigoe, K., Satake, M. & Yasumoto, T. Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus-toxicus*. *J. Org. Chem.* 57, 5448-5453 (1992).

19 Nagai, H. *et al.* Gambieric acids - unprecedent potent antifungal substances isolated from cultures of a marine dinoflagellate *Gambierdiscus-toxicus*. *J. Am. Chem. Soc.* 114, 1102-1103, doi:10.1021/ja00029a057 (1992).

20 Inoue, M., Hirama, M., Satake, M., Sugiyama, K. & Yasumoto, T. Inhibition of brevetoxin binding to the voltage-gated sodium channel by gambierol and gambieric acid-A. *Toxicon* 41, 469-474 (2003).

21 Worm, B. *et al.* Global catches, exploitation rates, and rebuilding options for sharks. *Marine Policy* 40, 194-204 (2013).

22 Indian Ocean Commission (IOC). A preliminary value chain analysis of shark fisheries in Madagascar IOTC-2015-WPEB-11-17, SF/ 2015/ 34. 43 (2015).

23 Sguotti, C., Lynam, C. P., Garcia-Carreras, B., Ellis, J. R. & Engelhard, G. H. Distribution of skates and sharks in the North Sea: 112 years of change. *Glob. Chang. Biol.* 22, 2729-2743 (2016).

24 Lezama-Ochoa, N. *et al.* Present and future potential habitat distribution of *Carcharhinus falciformis* and *Canthidermis maculata* by-catch species in the tropical tuna purse-seine fishery under climate change. *Front. Mar. Sci* . 3 (2016).

25 Tillett, B. J., Meekan, M. G., Field, I. C., Thorburn, D. C. & Ovenden, J. R. Evidence for reproductive philopatry in the bull shark *Carcharhinus leucas*. *J. Fish Biol.* 80, 2140-2158 (2012).

26 Pirog, A., Blaison, A., Jaquemet, S., Soria, M. & Magalon, H. Isolation and characterization of 20 microsatellite markers from *Carcharhinus leucas* (bull shark) and cross-amplification in *Galeocerdo cuvier* (tiger shark), *Carcharhinus obscurus* (dusky shark) and *Carcharhinus plumbeus* (sandbar shark). *Conserv. Genet. Resour.* 7, 121-124 (2015).

27 Keeney, D. B. & Heist, E. J. Characterization of microsatellite loci isolated from the blacktip shark and their utility in requiem and hammerhead sharks. *Mol. Ecol. Notes* 3 (2003).

28 Pirog, A., Jaquemet, S., Blaison, A., Soria, M. & Magalon, H. Isolation and characterization of eight microsatellite loci from *Galeocerdo cuvier* (tiger shark) and cross-amplification in *Carcharhinus leucas, Carcharhinus brevipinna, Carcharhinus plumbeus* and *Sphyrna lewini*. *PeerJ* 4, 9 (2016).

29 Arnaud-Haond, S. & Belkhir, K. GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Mol. Ecol. Notes* 7, 15-17 (2007).

30 Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* 155, 945-959 (2000).

31 Lewis, R. J. in *Manual on Harmful Marine Microalgae* eds G. M Hallegraeff, D.M Anderson, & A. D Cembella) 267 (UNESCO, 2003).

32 Caillaud, A. *et al.* Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. *Food Addit. & Contam.: Part A* 29, 1000-1010 (2012).

33 Manger, R. L., Leja, L. S., Lee, S. Y., Hungerford, J. M. & Wekell, M. M. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. *Anal. Biochem.* 214, 190-194 (1993).

5.2. Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesian Islands by Neuro-2a cell-based assays

Laia Reverté, Anna Toldrà, Karl B. Andree, Santi Fraga, Giada de Falco, Mònica Campàs and Jorge Diogène*

Sumbitted to the journal Aquatic Toxicology on 10th April 2017

5.2.1 Abstract

The involvement of ciguatoxins (CTXs) in causing ciguatera food poisoning (CFP) worldwide highlights the necessity to assess the risk that this food borne represents for human health and fisheries in a particular area. Within the genus *Gambierdiscus*, several species have been identified as producers of CTXs and maitotoxins (MTXs), which are potent marine toxins that accumulate through the food chain, contributing to cause ciguatera when fish contaminated with CTXs is consumed. Thus, the purpose of this work was to evaluate the production of CTX and MTX in culture of 10 strains of *G. australes* isolated from two Islands located in the Atlantic Ocean: Selvagem Grande Island (Madeira, Portugal) and the Hierro Island (Canary Islands, Spain). The 10 strains were first characterised by microscopy and molecular biology, being identified as *G. australes*. Following the species identification, the toxicity of *G. australes* extracts was evaluated using a cytotoxicity assay on mammalian cell cultures. Neuro-2a cells were exposed to *G. australes* extracts and CTX and MTX production was evaluated at the exponential growth phase. Additionally, the production of MTX was also investigated in two of the G. *australes* strains collected at the stationary growth phase. Interestingly, 9 out of 10 strains were found to produce CTX-like compounds, ranging from 200 to 697 fg equiv. CTX1B/cell. None of the *G. australes* strains showed MTX-like activity at the exponential phase, but MTX production was observed in two strains collected at the stationary growth phase (227 and 275 pg equiv. MTX/cell). Therefore, the presence of *G. australes* strains potentially producing CTX and MTX in these two Macaronesian Islands is confirmed herein.

5.2.2 Introduction

Gambierdiscus spp. (Dinophyceae) are marine unicellular algae that reside in epiphytic, benthic and planktonic habitats of tropical and subtropical areas. They are commonly found in shallow and warm waters associated with macroalgae or coral reefs, with low light intensities, elevated salinities

and preferably high nutrient contents (Dickey and Plakas, 2010; Kibler et al., 2015; Parsons et al., 2012).

The taxonomy of *Gambierdiscus* was initiated by the description of *G. toxicus* in 1979 in the Gambier Islands, French Polynesia (Pacific Ocean) by Adachi and Fukuyo (Adachi and Fukuyo, 1979). It was not until 16 years later that another species of *Gambierdiscus* was described from Belize, Central America (Caribbean Sea), named *G. belizeanus* (Faust, 1995). Since then, several species have been added to the genus: *G. yasumotoi* from Singapore, Asia (Holmes, 1998), *G. australes, G. pacificus* and *G. polynensiensis* from the Pacific Ocean (Chinain et al., 1999). In 2009, the genus was revised and 4 new species were discovered: *G. carolinianus* from North Carolina (USA), *G. caribaeus*, *G. ruetzleri* and *G. carpenteri* from Belize (Caribbean Sea) (Litaker et al., 2009). Two years later, Fraga et al. identified *G. excentricus* in the Canary Islands, Spain (Atlantic Ocean) (Fraga et al., 2011) and, in 2014, discovered in the same place the species *G. silave* nov. (Fraga and Rodriguez, 2014). Also in 2014, *G. scabrosus* was described in Japan and, very recently, *G. balechii* from the Celebes Sea (Pacific Ocean) (Fraga et al., 2016). Finally, in 2016, 2 more species have been described: *G. cheloniae*, isolated from the Cook Islands, Pacific Ocean (Smith et al., 2016) and *G. lapillus* from Australia (Kretzschmar et al., 2016). Interestingly, the species *G. yasumotoi* and *G. ruetzleri*, originally described as having a shape distinct from other *Gambierdiscus* (globular rather than lenticular-shaped and smaller in size) (Holmes, 1998; Litaker et al., 2009), have been recently assigned to a new genus of the *Gambierdiscus* lineage, designated as *Fukuyoa* gen. nov. (Gomez et al., 2015). The new genus currently contains *F. paulensis* gen. et sp. nov., *F. yasumotoi* com. nov. and *F. ruetzleri* com. nov.

With reference to toxin production, significant variation has been described within the genus depending on the origin, growth conditions and species. Despite few works which address this issue in detail, toxicity has been mainly attributed to be species-specific (Caillaud et al., 2010b; Chinain et al., 1999; Fraga et al., 2011; Holland et al., 2013; Lewis et al., 2016; Litaker et al., 2009; Parsons et al., 2012). Benthic dinoflagellates of the genus *Gambierdiscus* are well-known producers of ciguatoxins (CTXs) and maitotoxins (MTXs) (Holmes et al., 1991; Murata et al., 1990; Murata and Yasumoto, 2000). CTXs are lipid-soluble polyether compounds with polycyclic backbones, for which numerous congeners have been described differing in toxicity and structure, found in the Pacific Ocean (P-CTX), in the Indian Ocean (I-CTX) and in the Caribbean Sea (C-CTX) (Caillaud et al., 2010a). MTXs are potent water-soluble polyether toxins that increase intracellular calcium concentration (Murata and Yasumoto, 2000). Certain *Gambierdiscus* species also produce other related

compounds, such as gambierol (Cuypers et al., 2008; Satake et al., 1993), gambieric acids (Nagai et al., 1993; Nagai et al., 1992) and gambierone (Rodriguez et al., 2015). Up to now, among the different species described within the genus *Gambierdiscus*, *G. polynesiensis* has shown to produce remarkably higher CTX amounts than *G. toxicus, G. pacificus and G. australes* (Chinain et al., 2010). *G. excentricus* was also found to produce much higher CTX equivalents than *G.australes*, *G.carolinianus*, *G. carpenteri*, *G. balechi*, *G. caribaeus*, *G. silvae* and *G. pacificus* (Pisapia et al., 2017).

In the mid-1970s, these toxic microalgae were associated for the first time with ciguatera (Yasumoto et al., 1977), the most common seafood poisoning spread worldwide (Bagnis et al., 1980). Being at the bottom of the food chain, when *Gambierdiscus* species produce CTXs, these are bioaccumulated and undergo biotransformation through the food webs, starting with planktivorous organisms, reaching predator fishes, and ultimately, humans (Lewis and Holmes, 1993; Parsons et al., 2012). Ciguatera is a seafood-borne illness primarily associated with the consumption of fish containing CTXs that globally affects between 25,000 and 500,000 people per year (Dickey and Plakas, 2010). Although little is known about the global distribution, the toxicity and the role that each *Gambierdiscus* species plays in causing ciguatera events, according to well-documented reports bloom events and toxin production seem to be enhanced mostly in association to elevated water temperatures and variable environmental conditions (Litaker et al., 2010). Out of all the different toxins produced by *Gambierdiscus* spp.*,* CTXs have shown to be the main agent involved in ciguatera because of their mechanism of action, lipophilic nature and the likelihood to accumulate through the food chain. In fact, the 3 different MTXs produced by *Gambierdiscus* have not been shown to accumulate to significant levels in fish flesh (Lewis and Holmes, 1993). Therefore, investigations are warranted to determine whether additional species or strains of Gambierdiscus occur in temperate to subtropical water regions.

The present work aims at defining the potential production of CTXs by 10 strains of *G. australes* obtained from a sampling carried out in October 2013 in the Canary Islands (Spain) and in November 2013 in the Selvagem Grande Island (Portugal), both belonging to the Macaronesian Islands (Atlantic Ocean), in order to better characterise the risk of ciguatera in this area. In addition, presence of MTXs was also evaluated. To achieve this goal, single cell isolates were grown under specific conditions, cultures were harvested at early exponential phase and finally toxins were extracted for quantification purposes. Following the identification of the species by light microscopy and further confirmation by molecular biology, cytotoxicity was evaluated for all 10

G. australes strains. Specifically, the production of CTX and MTX was assessed by two different approaches previously described using a neuroblastoma (Neuro-2a) cell-based assay (CBA) to determine CTX and MTX-like toxicity.

5.2.3 Materials and methods

5.2.3.1 Reagents and equipment

Neuroblastoma murine cells (Neuro-2a) were obtained from de CIC cellular bank of University of Granada. CTX1B standard was provided by R. Lewis, obtained as described in the literature (Lewis et al., 1991), and stored in absolute methanol at -20 °C. MTX was purchased from Wako Pure Chemical Industries GmbH (Germany), reconstituted in methanol:H_20 (50:50; v:v) at 20 µg/mL and stored at -20°C. Absolute methanol, biotin, boric acid, cobalt (II) chloride hexahydrate, dimethyl sulfoxide (DMSO), disodium ethylenediaminetetraacetate (EDTA) dehydrate, fetal bovine serum (FBS), fluorescent Brightener 28, iron(III) chloride hexahydrate, L-glutamine solution, manganese (II) chloride tetrahydrate, ouabain, PBS, penicillin/streptomycin, RPMI-1640 medium, SKF-96365, sodium nitrate, sodium phosphate dibasic, sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), thiamine hydrochloride, trypsin-EDTA, veratridine, vitamin B12 and zinc chloride were all supplied by Sigma Aldrich (Tres Cantos, Spain).

Taq Polymerase was purchased from Invitrogen (Barcelona, Spain). QIAquick PCR Purification Kit was obtained from Qiagen (Hilden, Germany). 175-cm^2 cell culture flasks (vented cap) NUNCLON and surface 96 MicroWell NUNC plates were purchased from VWR International S.L. (Barcelona, Spain).

Neuro-2a cells were maintained at 37 °C and 5% CO_2 in an incubator (Binder, Tuttlingen, Germany). Absorbance was measured with a Microplate reader KC4 from BIO-TEK Instruments, Inc. with the GEN 2.09 software (Vermont, USA).

5.2.3.2. Sampling strategy and *Gambierdiscus* sp. culture

Sampling sites were located along the Macaronesian Islands, specifically, along El Hierro (Canary Islands, Spain) and the Selvagem Grande Island (Madeira, Portugal) in October and November 2013, respectively. Sampling procedure was carried out as described in the work reported by Carnicer and co-workers (Carnicer et al., 2015). Briefly, macroalgae were collected between 1.5 and 0.5 m depth and placed in plastic bottles containing 200 mL of 0.2-µm filtered seawater. With the aim of releasing the epiphyte community from macroalgae, bottles were vigorously shaken for 1 min and

then filtered through a 200-µm mesh to remove detritus and larger grazers (Reguera, 2011). The remaining 100 mL of each sample were fixed in 3% of lugol solution at 4 °C in order to evaluate cell abundance. Seawater was taken from the same location where macroalgae were collected and later used for initiation of cultures of *Gambierdiscus* sp. Additionally, water temperature and salinity were measured *in situ* using a portable multi-sensor probe CTD (YSI556 MPS).

In the laboratory, single *Gambierdiscus* sp. cells were isolated under an inverted microscope (Leica, DMIL) using a glass pipette following the capillary method (Hoshaw, 1973) and transferred to 12-well microtiter plates (NUNC) containing filtered and autoclaved seawater from the original location: modified ES medium (Provasoli, 1968) at 24 °C (50:50; v:v) at a salinity of 36. Once culture densities reached approximately 20-35 cells/mL, isolates were inoculated in 25-cm^2 glass Erlenmeyer flasks (vented cap) filled with 50 mL of ES medium and maintained at 24 °C, with a 12:12 light:dark regime and a photon flux rate of 80 µmol photons/m^2 s. Upon reaching exponential growth phase (after approximately 15 days), cultures were reinoculated in 1.8-L glass fernbach culture flask filled with 450 mL of acclimated medium. Finally, cultures were reinoculated in 3 L of acclimated medium and transferred to sterile glass bottles under mild aeration (air pump 275R). Cell counts for each culture were obtained every 2 days by removing a 10-mL aliquot. Culture densities (cells/mL) were determined per triplicate for each strain removing an aliquot of 10 mL and by settling 3-mL samples for 3 h in Utermöhl Hydrobios chambers for plankton (Tecnicas Ecologicas Indalo S. L., Madrid, Spain). A total of 3 L per strain of *Gambierdiscus* sp. cultures were harvested in the early exponential growth phase and collected in sterile 50-mL tubes by centrifugation at 4,500 rpm for 20 min (Alegra X-15R, Beckman Coulter). Supernatants were discarded and pellets were then pooled, reconstituted in absolute methanol and stored at -20 °C in sterile glass bottles until toxin extraction.

Considering that *Gambierdiscus* isolates were cultured for a period of more than 1 year before being tested for toxin production and according to what was claimed by Bomber and co-workers (Bomber et al., 1990), acclimation of the cultures is ensured in the present study.

5.2.3.3 Molecular analyses

5.2.3.3.1. DNA extraction

Prior to DNA extraction, *Gambierdiscus* cultures were collected at the early exponential phase by centrifugation as described in the literature (Andree et al., 2010). Cell pellets were resuspended in 200 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6) and transferred to 2-mL screw-up cryotubes containing ~50 µg of 0.5-mm diameter zirconium glass beads (Biospec). Then, 25 µL of 10% DTAB and 200 µL of chloroform were added, and the mixture disrupted using a BeadBeater-8 (BioSpec) pulsed for 30 s at full speed. DNA was extracted from the aqueous phase using standard phenol-chloroform procedures (Sambrook, 1989). Precipitation of the DNA from the final aqueous solution obtained was achieved by the addition of 2 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH=8). The DNA pellet was then rinsed with 70% ethanol and dissolved in 50 µL of molecular biology-grade water. Genomic DNA was quantified and checked for its purity in a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and stored at -20 °C for later analysis.

5.2.3.3.2. PCR amplification and DNA sequencing

The divergent domain D1-D3 of the LSUrRNA gene was amplified from all strains using the pair of primers D1R/LSUB (5-ACCCGCTGAATTTAAGCATA-3/5-ACGAACGATTTGCACGTCAG-3) (Litaker et al., 2003; Scholin et al., 1994). Each 25-µL reaction mixture contained 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq Polymerase, 5% DMSO and 10-50 ng of template DNA. Amplifications were carried out in an Eppendorf Mastercycler nexus gradient as follows: an initial denaturation step of 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 45 s at 52 °C and 30 s at 72 °C, and a final extension step of 10 min at 72 °C. Each PCR reaction was checked by agarose gel electrophoresis and visualized with ethidium bromide stain. PCR products were purified with QIAquick PCR Purification Kit and bidirectionally sequenced using the same primers as those applied in the amplification by an external company (Sistemas Genómicos, LLC, Valencia, Spain). Consensus sequences obtained from both reads for each strain were manually edited using BioEdit v7.0.5.2 (Hall, 1999) to remove primer sequences and terminal artefacts. The D1-D3 sequences obtained in this study were deposited in GenBank under accession numbers: KY564320-KY564329.

5.2.3.3.3 Phylogenetic analyses

Gambierdiscus consensus sequences were aligned using the ClustalW algorithm in BioEdit v7.0.5.2 with publically available *Gambierdiscus* sequences from GenBank (NCBI). Evolutionary analyses were conducted in MEGA v5. All ambiguous positions were removed for each sequence pair, using a final dataset of 884 positions. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (Felsenstein, 1985) (1000 replicates) was calculated. The evolutionary distances were computed using the Jukes-Cantor method (Jukes, 1969) and are in the units of the number of base substitutions per site.

5.2.3.4. Light microscopy characterisation

Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with epifluorescence, an UV lamp and UV excitation filters. Cultured cells were observed fixed with formalin. For plate pattern identification, cells were stained with Fluorescent Brightener 28 following a modified technique (Fritz and Triemer, 1985). Cells were dissected, squashing them by gently pressing the cover slip over them, occasionally with the aid of sodium hypochlorite. Microphotographs were taken with an Axiocam HRc (Carl Zeiss, Jena, Germany) digital camera. When the depth of field was insufficient to obtain clear focus for the whole object, several pictures were taken at a series of different foci and were automatically merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA).

5.2.3.5. Toxin extraction

Cell pellets were resuspended in methanol and sonicated for 30 min at 38% amplitude (Sonics Vibracell, Newton, USA) keeping temperature low (ice below the bottle). After the first sonication, extracts were transferred to 50-mL falcon tubes and centrifuged for 5 min at 4 °C and 600 g. Sonication of the cell pellet was repeated twice in methanol:H_2O (50:50; v/v) using the same volume as for the first sonication. Extracts were centrifuged at 600 g and 4 °C for 5 min, supernatants were pooled and evaporated until dryness at 40 °C (Büchi Syncore, Flawil, Switzerland). Once evaporated, pellets were redissolved in absolute methanol, centrifuged at same conditions and filtered through 0.45-µm nylon filters. Methanolic extracts were stored at -20 °C until analysis.

5.2.3.6. Toxin analyses

Prior to expose Neuro-2a cells to microalgal extracts, an aliquot of *G. australes* extract was evaporated under nitrogen stream and resuspended in RPMI medium containing 5% FBS.

5.2.3.6.1 CTX-like toxicity

CTX-like toxicity was determined in *Gambierdiscus* sp. crude extracts using the CBA reported by Caillaud and co-workers (Caillaud et al., 2009). Briefly, Neuro-2a cells were exposed for 24 h to several concentrations of CTX1B standard/*G. australes* extracts with and without ouabain/veratridine (0.1 mM/0.01 mM) per triplicate. Absorbance values were recorded following the MTT method described elsewhere (Manger et al., 1993) and expressed as percentage (%) of viable cells with respect to the controls with and without ouabain/veratridine treatment. Toxin contents were quantified as the ratio between the concentration of CTX1B causing 50% of cell viability inhibition (IC_{50} CTX1B) and the equivalent concentration of *G. australes* cells resulting in 50 % inhibition of cell viability (IC_{50} *G. australes* extract), and expressed in pg equiv. of CTX1B/cell of *G. australes.* A calibration curve for CTX1B standard was performed ranging from 0.39 to 50 pg/mL per well each day of the experiment, and dose-response curve was fitted to sigmoidal logistic 4-parameter equation using SigmaPlot software 12.0 (Systat Software Inc., California, US). From this equation, the limit of detection (LOD) was calculated, corresponding to the concentration of CTX1B necessary to inhibit the cell viability by 20% (IC_{20}). Similarly, the IC_{50} and the working range (IC_{20}-IC_{80}) were determined from the equation.

5.2.3.6.2 MTX-like toxicity

MTX-like toxicity was determined using the CBA described elsewhere (Caillaud et al., 2010b). Briefly, before exposing Neuro-2a cells to MTX standard or *G. australes* extracts, cells were treated with and without SKF (30 µM per well), a molecule that acts as inhibitor of MTX cytotoxicity. For each experimental day, a calibration curve was set for the MTX standard, ranging from 0.23 to 29.2 nM per well (corresponding to 0.79 and 99.9 pg/mL per well). As for CTX1B dose-response curve, responses obtained with and without SKF treatment were fitted to sigmoidal logistic 4-parameter equations and IC_{50} values and LODs were calculated. In order to determine whether *G. australes* produce MTX or not, dose-ratio (DR) was first calculated as the ratio between the IC_{50} provided by *G. australes* extract treated with SKF and the IC_{50} obtained for the extract without SKF treatment. Afterwards, as proposed by Pöch and colleagues (Poch et al., 1995), slope DR was corrected (DR_{corr}) as $\log(DR_{obs})slope_{obs}$, where DR_{obs} is the DR measured from the curve and $slope_{obs}$ is the absolute

value of the Hill slope coefficient of the curve with SKF treatment provided by the SigmaPlot software from the adjustment of the curve. Therefore, while DR values above 1 imply the presence of MTX-like compounds, DR values below 1 indicate the absence of these compounds. If DR_{corr} are >1, the MTX equiv. contents determined in *G. australes* extracts are expressed in nmols per 10^6 cell and calculated according to the following formula:

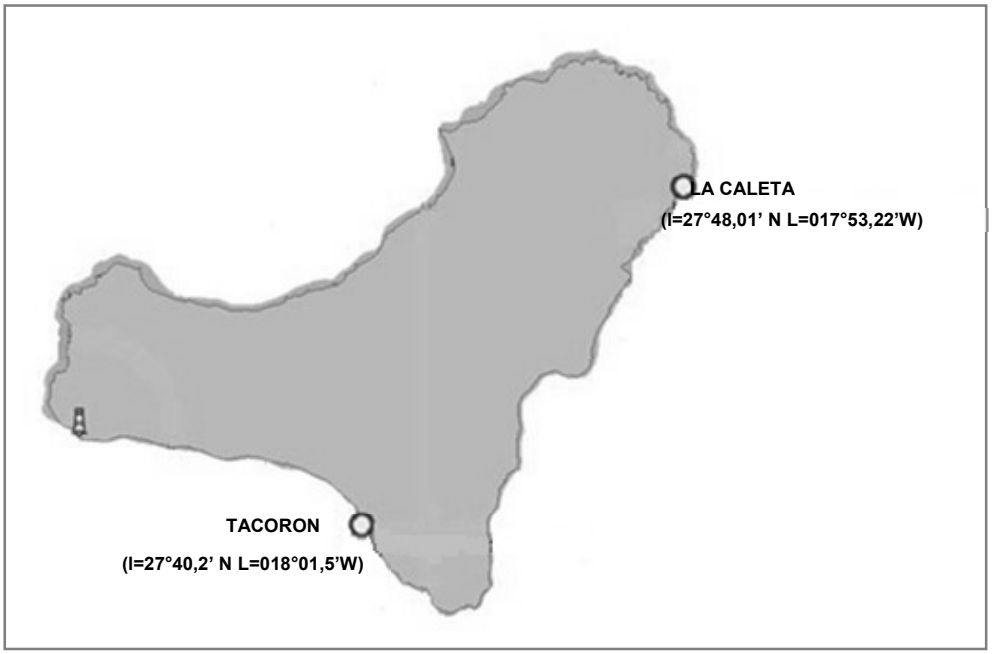
[MTX-like]=((IC_{50} SKF^+ of MTX (nM)/IC_{50} SKF^+ of *Gambierdiscus* extract (cells /mL)) x 10^3

To better compare the amounts of MTX produced by *G. australes* with the estimated CTX equiv. concentrations, MTX equiv. contents were also expressed in pg equiv. MTX/cell.

5.2.4 Results and discussion

Gambierdiscus asutrales was first described in the Pacific Ocean in 1999 (Chinain et al., 1999) and its presence in Hawaii and Japan was further confirmed (Litaker et al., 2010; Parsons et al., 2012). In 2014, *G. australes* was observed for the first time in Tenerife and Gran Canaria (Canary Islands, Spain), Atlantic Ocean (Fraga and Rodriguez, 2014). The present work reports the presence of G. *australes* in El Hierro (Canary Islands, Spain) and in the Selvagem Grande Island (Madeira, Portugal), both in the Atlantic Ocean. Exact locations from where isolates were obtained are shown in **fig. 5.2.1**.

A



B

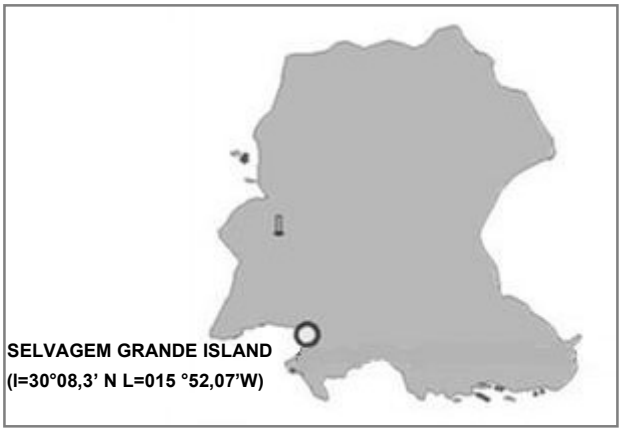


Fig. 5.2.1. A) Map of El Hierro (Canary Islands, Spain). B) Map of the Selvagem Grande Island (Madeira, Portugal). Black circles indicate specific locations from where *G. australes* isolates were obtained and coordinates are shown in brackets.

5.2.4.1. Species identification

Partial LSU rRNA sequences (D1-D3 and D8-D10 region) and partial SSU rDNA sequences are commonly used to delineate *Gambierdiscus* species (e.g. (Chinain et al., 1999; Fraga et al., 2016; Litaker et al., 2009; Nishimura et al., 2013; Smith et al., 2016) and more sequences of this region are publicly available in GenBank. In this study, the D1-D3 domain of the LSUrRNA gene was chosen as a molecular marker. The results of the analysis of the D1-D3 region demonstrate that the sequences of the 10 strains of *Gambierdiscus* were nearly identical. BLAST (Basic Local Alignment

Search Tool) analyses showed that the D1-D3 sequences had high identity with existing *Gambierdiscus* species previously registered in GenBank, with *G. australes* isolates showing the highest similarity. The optimal D1-D3 tree including 38 taxa with the sum of branch length=1.73422842 is shown in **fig. 5.2.2**. The phylogenetic results confirm that the 10 isolated *Gambierdiscus* strains are clustered into a well-supported group, which correspond to *G. australes*.

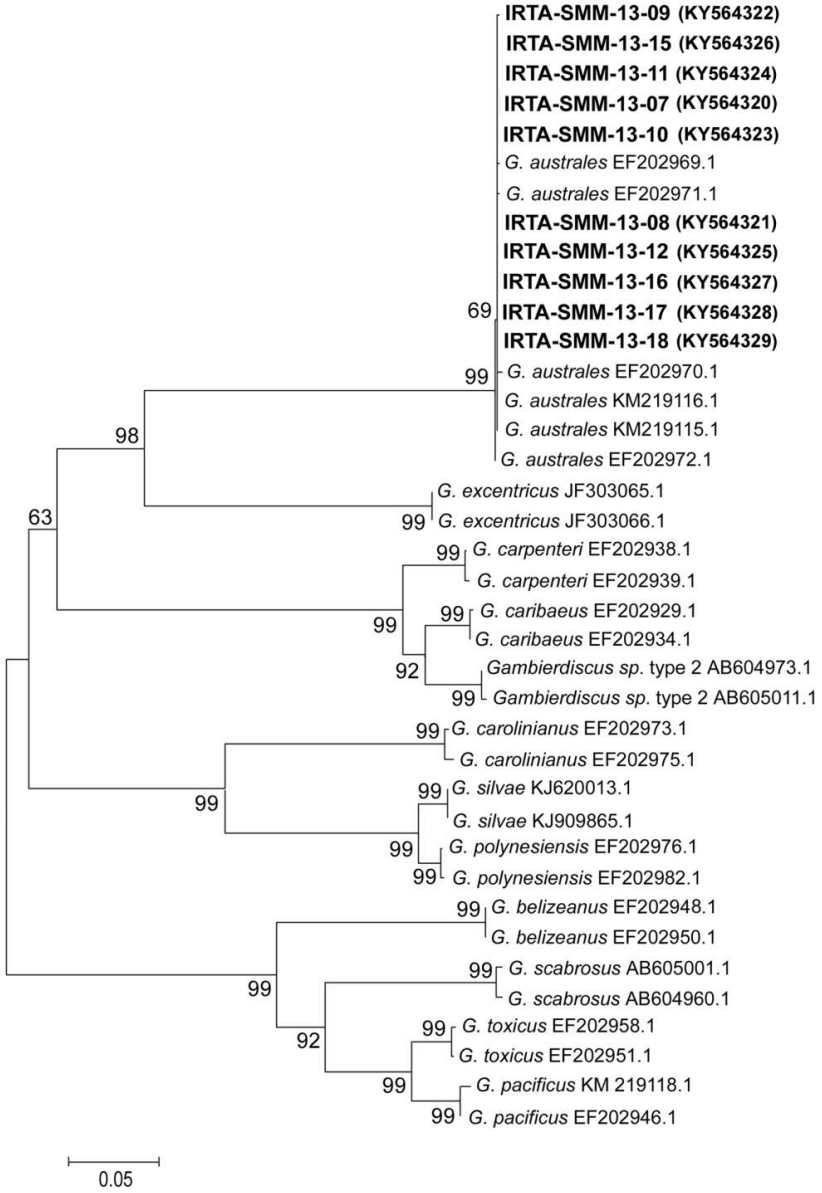


Fig. 5.2.2. Phylogenetic analysis of LSU rDNA gene (D1-D3 region) showing the relationship between *Gambierdiscus* strains from this study (in bold) and other *Gambierdiscus* strains. Values at nodes are bootstrap values obtained by the Neighbor-Joining method. Bootstrap values less than 30% are not shown. Scale bar represents substitutions per site.

The 10 strains of this work identified as *G. australes* by molecular biology were morphologically characterised by calcofluor stain method (**fig. 5.2.3**). A very high morphological variability was found within each studied strain, mainly in the shape of plate 4' which is used to differentiate species of genus *Gambierdiscus* making two groups: one with a hatched-shaped plate and another with a rectangular one (Litaker et al., 2009). *G. australes* (Chinain et al., 1999; Litaker et al., 2009) was described as belonging to the group with a rectangular plate 4', but in the examined strains this was found to be variable character as many cells showed a hatchet-shaped plate as shown in Fig. 5.2.3A.

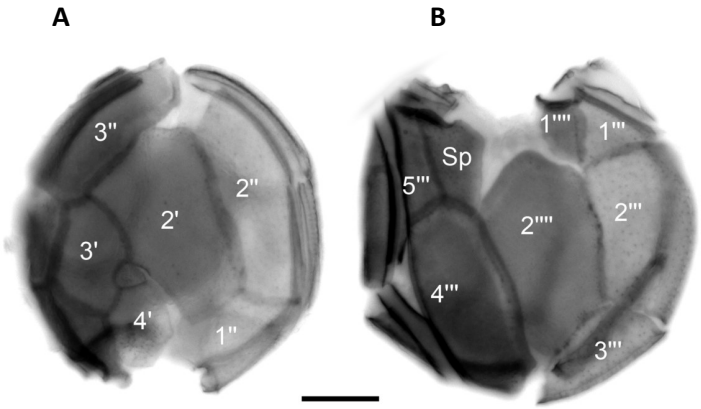


Fig. 5.2.3. Fluorescence microscopy images of calcofluor-stained thecae of *Gambierdiscus australes* (strain IRTA-SMM-13-11-12). **A)** Epitheca, **B)** Hypotheca. Scale bar 20 µm.

5.2.4.2. Determination of CTX-like activity by the Neuro-2a assay

The calibration curve obtained from the exposure of CTX1B to Neuro-2a cells resulted in an IC_{50} of 8.1 pg/mL, an LOD, set at IC_{20}, of 3.2 pg/mL and a working range (IC_{20}-IC_{80}) between 3.2 and 17.9 pg/mL (**fig. 5.2.4a**). Since a good sensitivity was attained by the Neuro-2a assay, reliable quantifications could be obtained by the exposure of Neuro-2a cells to *G. australes* extracts with and without the ouabain/veratridine (o/v) treatment. For all the toxic *G. australes* strains, the maximum concentration of extracts at which the Neuro-2a cells were exposed was 136 cells/mL without any observation of matrix effect, and quantification of CTX was assessed according to the IC_{50}. The limit of quantification (LOQ) was calculated as the ratio of the LOD obtained with CTX1B to the maximum concentration of *G. australes* extracts used in the assay, which was of 23.5 fg equiv. CTX1B/cell. Among the 10 strains of *G. australes* analysed, 9 strains showed CTX-like toxicity

between 200 and 697 fg equiv. CTX1B/cell, and IRTA-SMM-13-16 strain was not toxic (**Table 5.2.1** and **fig. 5.2.4b**). To confirm the absence of CTX production by IRTA-SMM-13-16 strain, the Neuro-2a cells were exposed up to 1,135 cells/mL, and no matrix effect or CTX production was observed. The LOQ for this specific strain at the extract concentration of 1,135 cells/mL was of 2.8 fg equiv. CTX1B/cell.

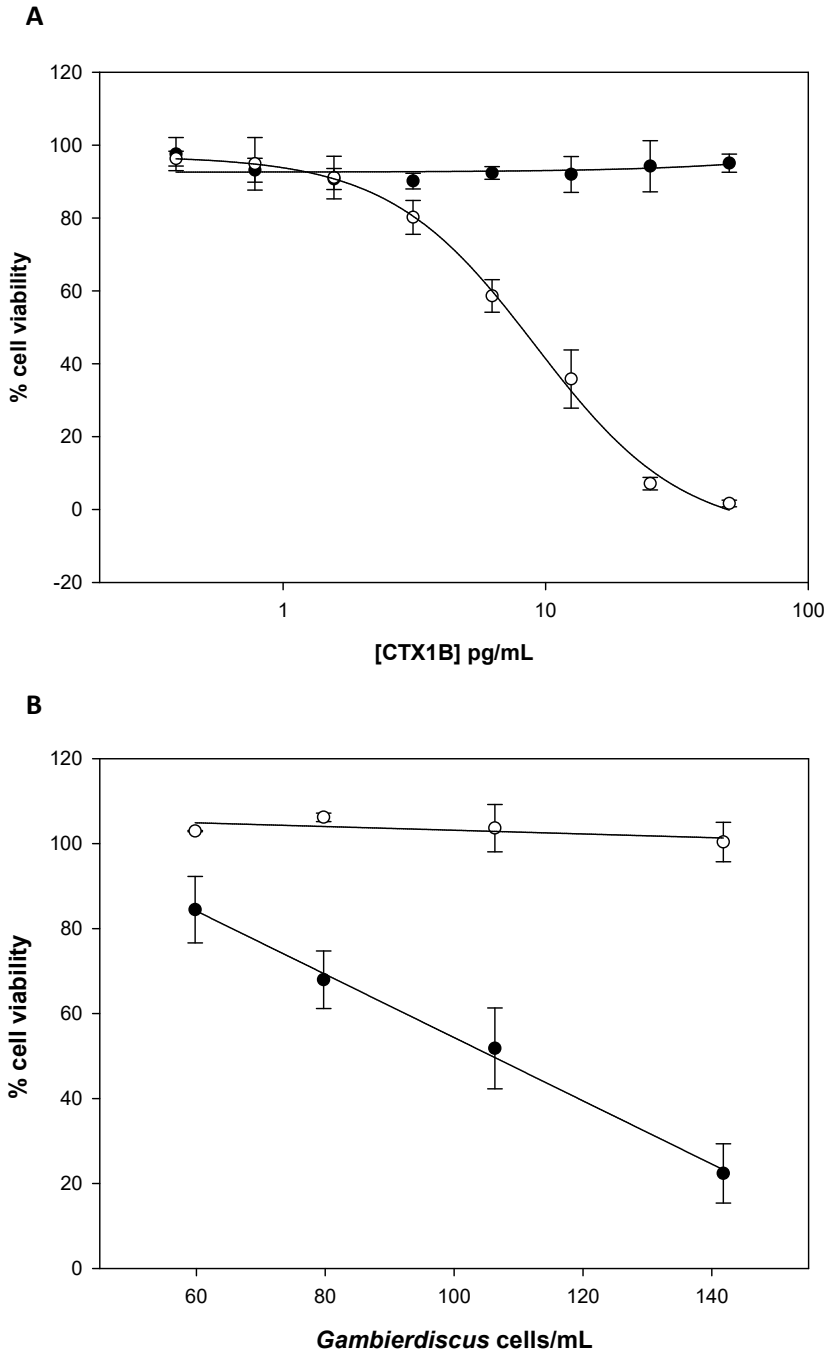


Fig 5.2.4. Dose-response curves of Neuro-2a cells exposed to **A)** CTX1B standard and **B)** *G. australes* IRTA-SMM-13-10 extract with (o) and without (●) o/v treatment.

Although some disparity has been observed for *G. australes* in the literature with reference to toxicity depending on the origin, culture conditions, harvesting growth phase and analysis method, results are consistent with data published regarding the toxicity of *G. australes* in exponential phase. In a first attempt to describe the toxin production by three different species of *Gambierdiscus* harvested in the late exponential phase, low/moderate toxicity was reported for *G. australes* strains from the Raivavae Island (Australes Archipelago) measured by the mouse bioassay (MBA), which was described as the least toxic species in both CTX and MTX production, compared to other species such as *G. polynesiensis* ($4 \cdot 10^{-4}$ *vs.* $800 \cdot 10^{-4}$ MU/1,000 cells of CTX) (Chinain et al., 1999). Later, the same group tested the toxicity of several *G. australes* strains by receptor binding assay (RBA) with respect to P-CTX-3C under the same harvesting conditions and, again, a low CTX-like activity was found, specifically at the fg level (between 17 and 30 fg equiv. P-CTX-3C/cell) (Chinain et al., 2010). Similarly, Roeder and colleagues (Roeder et al., 2010) showed the presence of the CTX analogue 2,3-dihydroxy P-CTX-3C by LC-MS/MS in *G. australes* from Hawaii (Pacific Ocean) harvested at the exponential growth phase. Despite the identification of this analogue, quantification was not possible due to the lack of the corresponding standard. Another work, probably more comparable to our work since it was performed with the Neuro-2a assay, also found low cytotoxicity (Rhodes et al., 2010) at the sub-pg range (40 fg CTX1B equiv./cell) for *G. australes* isolated from the Cook Islands. Screening of toxicity in *G. australes* strains from the Atlantic and the Pacific Ocean also showed low toxicity by the Neuro2A assay (0.6 and 2.7 fg P-CTX-3C equiv./cell respectively) (Pisapia et al., 2017). It is important to mention that although CTX levels are being compared with results given by different methods and using different CTX standards, the data obtained herein seem to indicate that *G. australes* coming from three different locations in Macaronesia (Atlantic Ocean) produce similar amounts of CTXs, with the exception of one strain that did not produce CTX-like compounds. Ciguatoxin equiv. contents determined in this work are 10-fold (Chinain et al., 2010; Rhodes et al., 2010) and 100-fold higher (Pisapia, 2017) compared to the quantities reported in other works. Nevertheless, those works gave the quantifications with reference to another CTX standard (e.g. P-CTX-3C). Therefore, different sensitivity could be achieved depending on the analytical method and toxin standard used.

Table 5.2.1. Identification code, origin, sampling point, culture cell density at sampling (exponential growth phase) and CTX equivalent concentration in 10 strains of *Gambierdiscus australes.*

ID	Origin	Sampling point	Culture cell density (cells/mL)	CTX concentration (fg equiv. CTX1B/cell)
IRTA-SMM-13-07	SGI, Portugal	30°08.3'N, 015°52.07'W	2,475	404
IRTA-SMM-13-11	SGI, Portugal	30°08.3'N, 015°52.07'W	1,310	515
IRTA-SMM-13-15	SGI, Portugal	30°08.3'N, 015°52.07'W	4,470	388
IRTA-SMM-13-16	SGI, Portugal	30°08.3'N, 015°52.07'W	1,362	n.d.*
IRTA-SMM-13-17	SGI, Portugal	30°08.3'N, 015°52.07'W	1,896	200
IRTA-SMM-13-18	SGI, Portugal	30°08.3'N, 015°52.07'W	2,630	338
IRTA-SMM-13-08	HI, Spain	27°40.2'N, 018°01.5'W	3,887	697
IRTA-SMM-13-09	HI, Spain	27°40.2'N, 018°01.5'W	3,434	625
IRTA-SMM-13-10	HI, Spain	27°40.2'N, 018°01.5'W	2,488	656
IRTA-SMM-13-12	HI, Spain	27°48.01'N, 017°53.22'W	3,425	280

*n.d.: not detected; the LOQ was 23.5 fg equiv. CTX1B/cell for all strains except for strain IRTA-SMM-13-16 that was 2.8 fg equiv. CTX1B/cell.

5.2.4.3. Determination of MTX-like activity by Neuro-2a assay

The MTX calibration curve provided an IC_{50} value of 9.54 nM in cells treated with SKF and an IC_{50} value of 3.17 nM in cells without SKF treatment. Moreover, IC_{20} values (LODs) of 6.76 nM with SKF treatment and 2.54 nM without treatment were obtained. These values were in accordance with the sensitivity reported in the literature (Caillaud et al., 2010b), demonstrating the antagonistic effect rendered by the MTX inhibitor. As can be observed in **fig. 5.2.5,** the pre-treatment of cells with SKF induced a rescue effect on cells: higher percentage of viable cells at higher MTX concentrations compared to the percentage of viable cells without SKF treatment.

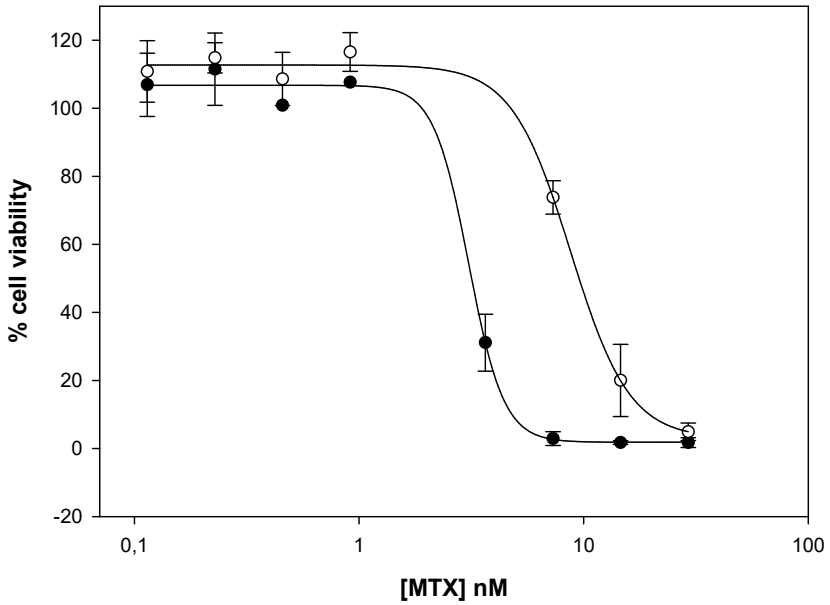


Fig 5.2.5. Dose-response curves obtained for Neuro-2a cells exposed to MTX standard with (o) and without (●) SKF treatment.

Exponential phase

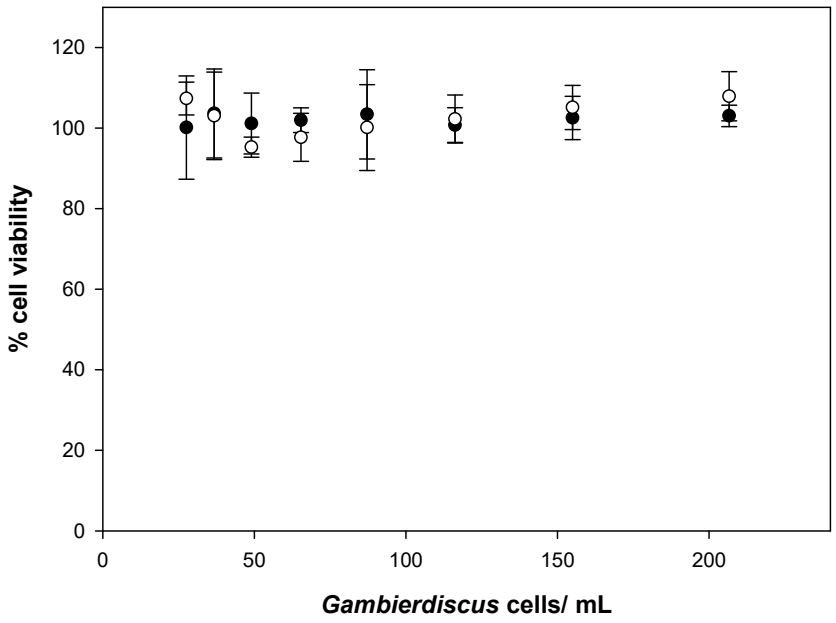
Neuro-2a cells were exposed to all microalgal extracts with and without SKF, at a maximum concentration of extract of 136 cells/mL per well. All 10 strains were non-toxic to Neuro-2a cells, as indicated by the 100% of viable cells obtained with and without SKF treatment. Since none of the *G. australes* extracts concentrations showed a toxic effect to Neuro-2a cells under these conditions (~100% cell viability), neither DR nor DR_{corr} values could be calculated in exponential phase. The LOQ, calculated as the ratio between the IC_{20} obtained with MTX standard with SKF treatment and the maximum concentration of extract of *G. australes* cells/mL, was of 88.91 pg equiv. MTX/cell. **Figure 5.2.6a** shows an example of the response obtained upon exposure of Neuro-2a cells to an extract of *G. australes* with and without SKF treatment. These results indicated the lack of

production of MTX-like compounds by *G. australes* extracts at the exponential phase regardless the origin. This observation is supported by the statement that *Gambierdiscus* sp. produces huge amounts of MTX-like compounds in the stationary growth phase, but it is likely to produce CTX-like compounds in the exponential phase (Holland et al., 2013). Due to the different culture conditions and origin despite belonging to the same species, there is some controversy about this issue in the literature (Parsons et al., 2012). While Chinain and co-workers (Chinain et al., 1999) reported higher MTX-like toxicity than CTX-like toxicity of *G. australes* from Hawaii and Japan (Pacific Ocean) in the late exponential growth phase by MBA, the production of 2-3-dihidroxy P-CTX-3C was described for *G. australes* from Hawaii by liquid chromatography tandem mass-spectrometry (LC-MS/MS) collected at the same growth phase (Roeder et al., 2010). Additionally, different results were obtained in other works (Rhodes et al., 2014; Rhodes et al., 2010), which reported the production of MTX but not CTX of *G. australes* isolated from the Cook Islands by MBA. Nevertheless, the comparison with the later works is difficult since no details are given regarding if these strains were collected in the exponential or in the stationary growth phase. A more complete work has been published by Holland and co-workers (Holland et al., 2013), in which further comparison was made between the production of MTX and CTX in different growth phases, species, origins and culture conditions. Although little was done for *G. australes* isolated from Hawaii, the authors proved that haemolytic activity triggered by MTX significantly increased from 7 to 40% from exponential to late exponential-early stationary growth phase. Contrarily, in another work, the presence of MTX (4.7 and 5 pg MTX equiv./cell) in *G. australes* strains from the Atlantic Ocean and Pacific, respectively, collected at the exponential phase was determined using the erythrocyte lysis assay (Pisapia, 2017).

Stationary phase

Although the study of MTXs was beyond the scope of this work, having observed that *G. australes* strains were not MTX producers in the exponential phase but CTX producers, two *G. australes* strains (IRTA-SMM-13-09 and IRTA-SMM-13-10) were additionally tested for MTX production in the stationary phase. As shown for one of the strains (**fig. 5.2.6b**), MTX-like compounds were detected in *G. australes* strains collected in the stationary growth phase. Estimated MTX equiv. concentrations were 8.04 and 6.62 nmols/10^6 cells for IRTA-SMM-13-09 and IRTA-SMM-13-10 strains, respectively (equivalent to 275 and 227 pg equiv. MTX/cell, respectively). These results were in agreement with the results reported in the literature (Holland et al., 2013), pointing out the likelihood of MTX production in the stationary phase.

A



B

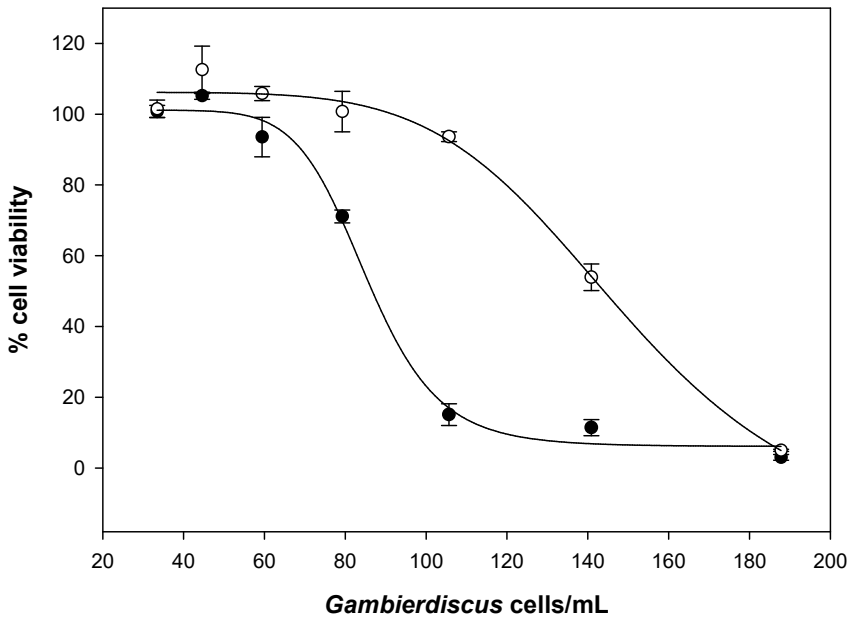


Fig 5.2.6. Dose-response curves of Neuro-2a cells exposed to **A)** *G. australes* IRTA-SMM-13-17 extract at exponential phase and **B)** *G. australes* IRTA-SMM-13-10 extract at stationary phase with (o) and without (●) SKF treatment.

Despite the great strides that have recently been made in this field, further work should be done to provide insight into the relation between toxin production and environmental parameters, to better establish toxin patterns according to the growth phase within specific species and therefore, to predict harmful blooms and/or ciguatera incidence in endemic and non-endemic geographical

areas. Hence, the present work contributes to broaden the knowledge about the toxin production by *G. australes* strains isolated from different sampling sites of the Macaronesian Islands.

5.2.5 Conclusions

The present study reports the unequivocal identification of ten strains of *G. australes* in El Hierro (Canary Islands, Spain) and in the Selvagem Grande Island (Madeira, Portugal). The species has been identified by electron microscopy and confirmed by molecular biology. *G. australes* cultures were established and acclimated for one year, and toxin production was assessed at the exponential growth phase. Furthermore, MTX-like activity was tested in two strains collected at the stationary phase. Production of CTX-like compounds was confirmed in nine out of ten strains in the exponential phase, containing from 200 to 697 fg equiv. CTX1B/cell. None of the screened strains were found to produce MTX-like compounds at the exponential phase, but two of the *G. australes* extracts analysed at the stationary phase produced MTX (227 and 275 pg equiv. MTX/cell). The findings obtained herein evidence that in these Macaronesian Islands most of the *G. australes* strains were CTX producers at the exponential phase and prove the MTX production at the stationary phase.

5.2.6 References

Adachi, R., Fukuyo, Y., 1979. Thecal structure of a marine toxic dinoflagellate *gambierdiscus-toxicus* gen et sp-nov collected in a ciguatera-endemic area. B. Jpn. Soc. Fish. 45, 67-71.

Andree, K.B., Fernandez-Tejedor, M., Elandaloussi, L.M., Quijano-Scheggia, S., Sampedro, N., Garces, E., Camp, J., Diogene, J., 2010. Quantitative PCR coupled with melt curve analysis for detection of selected *Pseudo-nitzschia* spp. (Bacillariophyceae) from the northwestern Mediterranean Sea. Appl. Environ. Microb. 77, 1651-1659.

Bagnis, R., Chanteau, S., Chungue, E., Hurtel, J.M., Yasumoto, T., Inoue, A., 1980. Origins of ciguatera fish poisoning-a new dinoflagellate, *Gambierdiscus-toxicus* Adachi and Fukuyo, definitively involved as a causal agent. Toxicon 18, 199-208.

Bomber, J.W., Tindall, D.R., Venable, C.W., Miller, D.M., 1990. Pigment composition and low-light response of 14 clones of *Gambierdiscus-toxicus*. Elsevier, New York.

Caillaud, A., Canete, E., de la Iglesia, P., Gimenez, G., Diogene, J., 2009. Cell-based assay coupled with chromatographic fractioning: A strategy for marine toxins detection in natural samples. Toxicol. in Vitro 23, 1591-1596.

Caillaud, A., de la Iglesia, P., Darius, H.T., Pauillac, S., Aligizaki, K., Fraga, S., Chinain, M., Diogene, J., 2010a. Update on methodologies available for ciguatoxin determination: perspectives to confront the onset of ciguatera fish poisoning in Europe. Mar. Drugs 8, 1838-1907.

Caillaud, A., Yasumoto, T., Diogene, J., 2010b. Detection and quantification of maitotoxin-like compounds using a neuroblastoma (Neuro-2a) cell based assay. Application to the screening of maitotoxin-like compounds in *Gambierdiscus* spp. Toxicon 56, 36-44.

Carnicer, O., Tunin-Ley, A., Andree, K.B., Turquet, J., Diogene, J., Fernandez-Tejedor, M., 2015. Contribution to the genus *Ostreopsis* in Reunion Island (Indian Ocean): molecular, morphologic and toxicity characterization. Cryptogamie Algol. 36, 101-119.

Chinain, M., Darius, H.T., Ung, A., Cruchet, P., Wang, Z., Ponton, D., Laurent, D., Pauillac, S., 2010. Growth and toxin production in the ciguatera-causing dinoflagellate *Gambierdiscus polynesiensis* (Dinophyceae) in culture. Toxicon 56, 739-750.

Chinain, M., Faust, M.A., Pauillac, S., 1999. Morphology and molecular analyses of three toxic species of *Gambierdiscus* (Dinophyceae): *G-pacificus*, sp nov., *G-australes*, sp nov., and *G-polynesiensis*, sp nov. J. Phycol. 35, 1282-1296.

Cuypers, E., Abdel-Mottaleb, Y., Kopljar, I., Rainier, J.D., Raes, A.L., Snyders, D.J., Tytgat, J., 2008. Gambierol, a toxin produced by the dinoflagellate *Gambierdiscus toxicus*, is a potent blocker of voltage-gated potassium channels. Toxicon 51, 974-983.

Dickey, R.W., Plakas, S.M., 2010. Ciguatera: A public health perspective. Toxicon 56, 123-136.

Faust, M.A., 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. J. Phycol. 31, 996-1003.

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

Fraga, S., Rodriguez, F., 2014. Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with description of *Gambierdiscus silvae* sp nov., a new potentially toxic epiphytic benthic dinoflagellate. Protist 165, 839-853.

Fraga, S., Rodriguez, F., Caillaud, A., Diogene, J., Raho, N., Zapata, M., 2011. *Gambierdiscus excentricus* sp nov (Dinophyceae), a benthic toxic dinoflagellate from the Canary Islands (NE Atlantic Ocean). Harmful Algae 11, 10-22.

Fraga, S., Rodriguez, F., Riobo, P., Bravo, I., 2016. *Gambierdiscus balechii* sp nov (Dinophyceae), a new benthic toxic dinoflagellate from the Celebes Sea (SW Pacific Ocean). Harmful Algae 58, 93-105.

Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing calcofluor withe M2R for the visualization of dinoflagellate thecal plates. J. Phycol. 21, 662-664.

Gomez, F., Qiu, D., Lopes, R.M., Lin, S., 2015. *Fukuyoa paulensis* gen. et sp nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). Plos One 10.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucl. Acid. S. 41, 95-98.

Holland, W.C., Litaker, R.W., Tomas, C.R., Kibler, S.R., Place, A.R., Davenport, E.D., Tester, P.A., 2013. Differences in the toxicity of six *Gambierdiscus* (Dinophyceae) species measured using an in vitro human erythrocyte lysis assay. Toxicon 65, 15-33.

Holmes, M.J., 1998. *Gambierdiscus yasumotoi* sp. nov. (Dinophyceae), a toxic benthic dinoflagellate from southeastern Asia. J. Phycol. 34, 661-668.

Holmes, M.J., Lewis, R.J., Poli, M.A., Gillespie, N.C., 1991. Strain dependent production of ciguatoxin precursors (gambiertoxins) by *Gambierdiscus-toxicus* (Dinophyceae) in culture Toxicon 29, 761-775.

Hoshaw, R.W., Rosowski J. R., 1973. Methods for microscopic algae, in: Stein, J.R. (Ed.), Handbook of phycological methods, culture methods and growth measurements. Cambridge University Press, Cambridge, pp. 53-67.

Jukes, T.H., Cantor, C. R., 1969. Evolution of protein molecules, mammalian protein metabolism (ed. Munro, H. N.). Academic Press, New York, pp. 21-123.

Kibler, S.R., Tester, P.A., Kunkel, K.E., Moore, S.K., Litaker, R.W., 2015. Effects of ocean warming on growth and distribution of dinoflagellates associated with ciguatera fish poisoning in the Caribbean. Ecol. Model. 316, 194-210.

Kretzschmar, A.L., Verma, A., Harwood, D.T., Hoppenrath, M., Murray, S., 2016. Characterization of *Gambierdiscus lapillus* sp. nov. (Gonyaulacales, Dinophyceae): a new toxic dinoflagellate from the Great Barrier Reef (Australia). J. Phycol. doi:10.1111/jpy.12496

Lewis, R.J., Holmes, M.J., 1993. Origin and transfer of toxins involved in ciguatera. Comp. Biochem. Phys. C 106, 615-628.

Lewis, R.J., Inserra, M., Vetter, I., Holland, W.C., Hardison, D.R., Tester, P.A., Litaker, R.W., 2016. Rapid extraction and identification of maitotoxin and ciguatoxin-like toxins from Caribbean and Pacific *Gambierdiscus* using a new functional bioassay. Plos One 11.

Lewis, R.J., Sellin, M., Poli, M.A., Norton, R.S., Macleod, J.K., Sheil, M.M., 1991. Purification and characterization of ciguatoxins from moray eel (*Lycodontis-javanicus*, *Muraenidae*) Toxicon 29, 1115-1127.

Litaker, R.W., Vandersea, M.W., Faust, M.A., Kibler, S.R., Chinain, M., Holmes, M.J., Holland, W.C., Tester, P.A., 2009. Taxonomy of *Gambierdiscus* including four new species, *Gambierdiscus caribaeus, Gambierdiscus carolinianus, Gambierdiscus carpenteri* and *Gambierdiscus ruetzleri* (Gonyaulacales, Dinophyceae). Phycologia 48, 344-390.

Litaker, R.W., Vandersea, M.W., Faust, M.A., Kibler, S.R., Nau, A.W., Holland, W.C., Chinain, M., Holmes, M.J., Tester, P.A., 2010. Global distribution of ciguatera causing dinoflagellates in the genus *Gambierdiscus*. Toxicon 56, 711-730.

Litaker, R.W., Vandersea, M.W., Kibler, S.R., Reece, K.S., Stokes, N.A., Steidinger, K.A., Millie, D.F., Bendis, B.J., Pigg, R.J., Tester, P.A., 2003. Identification of *Pfiesteria piscicida* (Dinophyceae) and Pfiesteria-like organisms using internal transcribed spacer-specific PCR assays. J. Phycol. 39, 754-761.

Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium-channels - semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Anal. Biochem. 214, 190-194.

Murata, M., Legrand, A.M., Ishibashi, Y., Fukui, M., Yasumoto, T., 1990. Structures and configurations of ciguatoxin from the moray eel *gymnothorax-javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus-toxicus*. J. Am. Chem. Soc. 112, 4380-4386.

Murata, M., Yasumoto, T., 2000. The structure elucidation and biological activities of high molecular weight algal toxins: maitotoxin, prymnesins and zooxanthellatoxins. Nat. Prod. Rep. 17, 293-314.

Nagai, H., Mikami, Y., Yazawa, K., Gonoi, T., Yasumoto, T., 1993. Biological-activities of novel polyether antifungals, gambieric acids A and B from a marine dinoflagellate *Gambierdiscus-toxicus.* J. Antibiot. 46, 520-522.

Nagai, H., Murata, M., Torigoe, K., Satake, M., Yasumoto, T., 1992. Gambieric acids, new potent antifungal substances with unprecedented polyether structures from a marine dinoflagellate *Gambierdiscus-toxicus*. J. Org. Chem. 57, 5448-5453.

NCBI, National Center for Biotechnology Information Search database, U.S. National Library of Medicine. Available online at: https://www.ncbi.nlm.nih.gov/. Accessed by 11th April 2017.

Nishimura, T., Sato, S., Tawong, W., Sakanari, H., Uehara, K., Shah, M.M.R., Suda, S., Yasumoto, T., Taira, Y., Yamaguchi, H., Adachi, M., 2013. Genetic diversity and distribution of the ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Dinophyceae) in coastal areas of Japan. Plos One 8.

Parsons, M.L., Aligizaki, K., Bottein, M.-Y.D., Fraga, S., Morton, S.L., Penna, A., Rhodes, L., 2012. *Gambierdiscus* and *Ostreopsis*: Reassessment of the state of knowledge of their taxonomy, geography, ecophysiology, and toxicology. Harmful Algae 14, 107-129.

Pisapia, F., Holland, W. C., Hardison, D. R., Litaker, R. W., Fraga, S., Nishimura, T., Adachi, M., Nguyen-Ngoc, L., Sechet, V., Amzil, Z., Herrenknecht, C., Hess, P., 2017. Toxicity screening of 13 *Gambierdiscus* strains using neuro-2a and erythrocyte lysis bioassays. Harmful Algae 63, 173–183.

Poch, G., Reiffenstein, R.J., Baer, H.P., 1995. Quantitative estimation of potentiation and antagonism by dose ratios corrected for slopes of dose-response curves deviating from one. J. Pharmacol. Toxicol. 33, 197-204.

Provasoli, L., 1968. Media and prospects for the cultivation of marine algae, in: Watanabe, A., Hattori, A. (Ed.), Cultures and Collection of Algae, Proceedings of the US-Japanese Conference, Hakone, pp. 63-75.

Reguera, B., Alonso, R., Moreira, A., Méndez, S. , 2011. Guía para el diseño y puesta en marcha de un plan de seguimiento de microalgas productoras de toxinas (in Spanish only). COI de UNESCO y OIEA, Paris y Viena. Available online at: http://unesdoc.unesco.org/images/0021/002145/214510s.pdf. Accessed by 11th April 2017.

Rhodes, L., Harwood, T., Smith, K., Argyle, P., Munday, R., 2014. Production of ciguatoxin and maitotoxin by strains of *Gambierdiscus australes*, *G. pacificus* and *G. polynesiensis* (Dinophyceae) isolated from Rarotonga, Cook Islands. Harmful Algae 39, 185-190.

Rhodes, L.L., Smith, K.F., Munday, R., Selwood, A.I., McNabb, P.S., Holland, P.T., Bottein, M.-Y., 2010. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. Toxicon 56, 751-758.

Rodriguez, I., Genta-Jouve, G., Alfonso, C., Calabro, K., Alonso, E., Sanchez, J.A., Alfonso, A., Thomas, O.P., Botana, L.M., 2015. Gambierone, a ladder-shaped polyether from the dinoflagellate *Gambierdiscus belizeanus*. Org. Lett. 17, 2392-2395.

Roeder, K., Erler, K., Kibler, S., Tester, P., Ho, V.T., Lam, N.N., Gerdts, G., Luckas, B., 2010. Characteristic profiles of ciguatera toxins in different strains of *Gambierdiscus* spp. Toxicon 56, 731-738.

Saitou, N., Nei, M., 1987. The neighbor-joining method - a new method for reconstructing pyhlogenetic trees. Mol. Biol.Evol. 4, 406-425.

Sambrook, J., Fritsch, E. F., Maniatis, T. , 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Satake, A., Murata, M., Yasumoto, T., 1993. Gambierol: A new toxic polyether compound isolated from the marine dinoflagellate *Gambierdiscus toxicus*. J. Am. Chem. Soc. 115, 361-362.

Scholin, C.A., Herzog, M., Sogin, M., Anderson, D.M., 1994. Identification of group and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae) II. Sequence-analysis of a fragment of the LSU Ribosomal rRNA gene. J. Phycol. 30, 999-1011.

Smith, K.F., Rhodes, L., Verma, A., Curley, B.G., Harwood, D.T., Kohli, G.S., Solomona, D., Rongo, T., Munday, R., Murray, S.A., 2016. A new *Gambierdiscus* species (Dinophyceae) from Rarotonga, Cook Islands: *Gambierdiscus cheloniae* sp nov. Harmful Algae 60, 45-56.

Yasumoto, T., Nakajima, I., Bagnis, R., Adachi, R., 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. B. Jpn. Soc. Sci. Fish. 43, 1021-1026.

Chapter 6

Conclusions and future work

6.1. Conclusions

The findings achieved in this PhD thesis lead to draw the following conclusions:

6.1.1 Tetrodotoxin (TTX)

- The use of dithiols-based self-assembled monolayers (SAMs) for the immobilisation of TTX on maleimide plates and subsequent development of a new immunoassay configuration (mELISA) has been proved useful for the sensitive detection and quantification of TTXs in puffer fish samples.

- The establishment of the cross-reactivity factors (CRFs) for the TTX analogues 5,6,11-trideoxy TTX, 4,9-anhydro-TTX, 11-nor TTX-6-ol and 5-deoxy-TTX and 11-TTX-deoxy-TTX by mELISA and the SPR immunosensor and subsequent application to the individual contents determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in puffer fish samples, resulted in good agreements obtained between the immunochemical and the instrumental analysis techniques.

- The replacement of dithiols by cysteamine for the SAM formation in the mELISA allowed decreasing the time and cost of the assay, while maintaining the sensitivity attained with the dithiols SAM-based strategy.

- The cysteamine SAM-based mELISA was successfully applied to the determination of TTX contents in oyster and mussel samples, attaining a sensitivity below the proposed levels of concern for human consumption.

- The determination and application of the correction factors (CFs) to the TTX contents determined by the mELISAs and by the SPR immunosensor allowed overcoming the matrix effects observed in the analysis of puffer fish and shellfish samples.

- The orientation and spaced effect provided by the dithiols-based SAMs used in the development of the electrochemical immunosensor favour the electron transfer and reduced the non-specific adsorption arising from puffer fish samples.

- The immobilisation strategy consisting of nanoarrayed scaffolds of TTX-conjugates leads to the development of a planar waveguide biosensor for the accurate and rapid determination of TTX contents in several species of puffer fish.

- The combination of the mELISAs and immunosensors as screening tools with LC-MS/MS analysis as confirmatory technique is a useful strategy to quantify TTXs in puffer fish and shellfish.

- The high TTX levels found in the specimen of *Lagocephalus sceleratus* evidence the risk that the presence of this toxic fish in the Mediterranean Sea may represent for seafood safety.

6.1.2 Ciguatoxin (CTX)

- A multi-disciplinary approach providing epidemiologic, toxicologic (cell-based assay (CBA) and mouse bioassay (MBA)) and instrumental (high-resolution mass spectrometry (HRMS)) information was used to confirm CTX as responsible for the fatal food poisoning following the consumption of shark in Madagascar.

- Two new analogues tentatively designated as I-CTX-5 & I-CTX-6 were identified by HRMS in the stomach of a bull shark. The presence of gambieric acid was also confirmed in the flesh of the same shark, although further confirmatory studies are still required.

- Ten strains of *Gambierdiscus australes* were obtained from two Islands of the Macaronesian Islands. In the exponential growth phase, the Neuro-2a CBA determined the production of CTX-like compounds in 9 of the strains, but any of them were found to produce maitotoxin (MTX). Additionally, MTX-like activity was observed in two of the strains collected at the stationary growth phase.

6.2 Future work

6.2.1 Tetrodotoxin

- CRFs should be established by the immunological tools developed herein for the known TTX analogues in order to obtain better correlations in the quantifications of samples with different multi-toxin profiles between the immunological and the instrumental analysis techniques. Moreover, the evaluation of the toxicity of TTX analogues and the correlation of the toxicity equivalency factors (TEFs) with the CRFs will contribute to improve risk-based mitigation approaches.

- The applicability of the immunoassays and immunosensors should be extended to other seafood matrices to further validate the new approaches.

- The use of the multi-disciplinary approach combining the mELISA and the immunosensors together with LC-MS/MS analysis for the analysis of TTX contents in puffer fish and shellfish should be further exploited in research programs through the analysis of a higher number of samples.

- The set-up of the developed biosensors in a multiplexed format for the simultaneous detection of paralytic shellfish poisoning (PSP) toxins that can co-exist with TTX in natural samples would be an interesting point to address. Similarly, automation of the systems is highly desired for the straightforward integration of the immunosensors within electronic devices for routine use in monitoring programs.

6.2.2 Ciguatoxin

- The study in depth of the structure of the new CTX analogues described in the stomach of a shark using RMN methods is required. To improve the surveillance program in ciguateric areas such as Madagascar by implementing preventive measures is crucial to evaluate the magnitude of the risk and protect human health.

- To broaden the knowledge of the presence of toxic *Gambierdsicus* and/or *Fukuyoa* strains in order to assess the risk and determine if the levels of toxins are of concern. The toxin profile of *G.*

australes depending on the growth phased collected as well as the characterization of the toxin profiles of these and other species of *Gambierdiscus* in the Macaronesian Islands are also necessary.

List of publications

Published

1) Reverté, L., Soliño, L., Carnicer, O., Diogène, J., & Campàs, M. (2014). Alternative methods for the detection of emerging marine toxins: Biosensors, biochemical assays and cell-based assays. *Marine Drugs 2014, 12*(12), 5719-5763. Doi: 10.3390/md12125719.

2) Reverté, L., de la Iglesia, P., del Río, V.,Campbell, K., Elliott, C.T.; Kawatsu, K.; Katikou, P.; Diogène, J. & Campàs, M. (2015). Detection of tetrodotoxins in puffer fish by a self-assembled monolayer-based immunoassay and comparison with surface plasmon resonance, LC-MS/MS, and mouse bioassay. *Analytical Chemistry 2015,* 87 (21), 10839–10847. Doi:10.1021/acs.analchem.5b02158.

3) 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 2016,* 908*, 8-21.* Doi: 10.1016/j.aca.2015.11.050

4) Rambla-Alegre, M, Reverté, L., del Río, V., de la Iglesia, P., Palacios, O., Flores, C., Caixach, J., Campbell, K., Elliott, C. T., Izquierdo-Muñoz, A., Campàs, M., and Diogène, J. (2017). Evaluation of tetrodotoxins in puffer fish caught along the Mediterranean coast of Spain. Toxin profile of *Lagocephalus sceleratus. Environmental Research 2017, 158, 1-6 (in press).*

Submitted

1) Tetrodotoxin detection in puffer fish by a sensitive planar waveguide immunosensor (*Sensors and Actuators*)

2) Identification of ciguatoxins in a shark involved in a fatal food poisoning in the Indian Ocean (*Scientific Reports*)

3) Assessment of cytotoxicity in ten strains of *Gambierdiscus australes* from Macaronesia Islands by Neuro-2a cell-based assays (*Harmful Algae*)

4) Development and validation of a maleimide-based enzyme-linked immunosorbent assay for the detection of tetrodotoxins in oysters and mussels (*Talanta*)

5) Immunosensor array platforms based on self-assembled dithiols for the electrochemical detection of tetrodotoxin in puffer fish (*Analytica Chimica Acta*)